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A STUDY OF SOME ENDEMIC VIRUSES OF CATTLE, WITH PARTICULAR REFERENCE TO ENTERIC VIRUSES.

A thesis presented in partial fulfilment of the requirements for the degree of Master of Veterinary Science at Massey University, New Zealand.

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ABSTRACT

This investigation was undertaken to isolate and characterise a number of viruses from diarrhoeic cattle faeces, as a preliminary step in the systematic investigation of viruses as possible causes of diarrhoea in cattle, and more particularly calves. A further and important aim was to gain further experience in a number of virological procedures.

Using 3 passages of inocula in each of secondary foetal bovine kidney and lung cells, and monkey kidney (Vero) cells, 7 viruses were isolated from 56 faeces, 2 intestinal samples, and 1 spleen, all from scouring animals. Five of the isolates were found to produce a rapid and complete cytopathic effect in a variety of cell cultures, and their biological and physicochemical properties were subsequently studied in some depth. One of these isolates was also studied with the immunofluorescent technique, and its buoyant density was determined in a caesium chloride gradient. These isolates were concluded to be bovine enteroviruses, and were found in further studies to be separable into 2 types on the basis of cross-neutralisation tests, fluorescent antibody tests, and behaviour in the presence of low concentrations of hydroxybenzyl benzimidazole. These 2 serotypes on further cross-neutralisation tests were found to be serologically distinct from the 7 U.S. standard serotypes that were available.

Another of the isolates was found to cause a slow growing and relatively nonprogressive type of cytopathic effect in only Vero cells, and was consequently harder to study. On the basis of limited studies of this isolate, it was concluded to be probably a member of the diplornavirus group, possibly having some affinity with the "reo-like" viruses. Further more precise studies will be needed to confirm or refute this relationship.

The remaining virus, which was isolated from the spleen, was identified as being bovine viral diarrhoea (BVD) virus on the basis of its cytopathic effect and neutralisation by standard BVD antiserum.

A limited survey for neutralising antibodies to infectious bovine rhinotracheitis and BVD viruses, and haemgglutinating-inhibiting antibodies to parainfluenza 3 virus and reoviruses 1, 2, and 3 was undertaken. It showed that antibody to all the viruses was present in a considerable proportion of the North Island cattle population.

This work can only be regarded as a preliminary study, as it is probable on the basis of overseas work that a number of other viruses remain to be isolated from diarrhoeic cattle faeces. It is hoped to continue this investigation and to eventually establish a better understanding of the relationships between viruses and bovine gastrointestinal disease, and more particularly to establish their possible economic significance.

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GENERAL INTRODUCTION.

One important aim of this study was to gain further experience in a number of virological procedures. Consequently, a considerable range of techniques was utilised in order to achieve this objective.

In recent years, a considerable number of newly discovered viruses have been incriminated overseas as causes of disease in livestock, particularly in reference to the gastro-intestinal tract. In New Zealand, past lack of virological facilities and trained personnel has resulted in little progress being made in the systematic investigation of diseases of possible viral aetiology. Hence, other important objectives of this study were to investigate the role of viruses in causing gastrointestinal disease in New Zealand cattle, and also the prevalence of a number of viral agents in a representative sample of these animals.

Whilst most of the thesis is concerned with the isolation and characterisation of a number of viruses isolated from diarrhoeic cattle faeces, this should only be regarded as a preliminary investigation. Considerable further work will be required to isolate and characterise a number of other viruses which are probably present in New Zealand, and more particularly to establish their economic significance to the livestock industry.

LITERATURE REVIEW

A considerable number of viruses have been associated with gastrointestinal disease syndromes in calves and adult cattle. These include bovine viral diarrhoea (BVD) virus, infectious bovine rhinotracheitis (IBR) virus, reoviruses, "reo-like" viruses, adenoviruses, coronaviruses, parvoviruses, enteroviruses, and also rinderpest and foot and mouth disease viruses. It is not intended to comment on the last 2 viruses, as it is considered they are beyond the scope of this review, which is restricted to those agents thought likely to be endemic in New Zealand cattle and which have not been subjected to any detailed local investigations relative to enteric disease.

The literature on BVD viral infections is voluminous, and has been adequately reviewed by Pritchard (1963), hence a further general review will not be attempted here. In New Zealand, the virus was first isolated by Jolly et al., (1967), and has since been isolated on a number of occasions from calves and adult cattle with diarrhoea or ulcerative disease of the gastrointestinal tract (Durham, unpublished data). Serologically, the virus appears widespread in New Zealand, with neutralising titres being reported in 35% of 178 cattle (Fastier and Hansen, 1966), 66% of 60 cattle (Robinson, 1971) and 34% of 922 cattle diagnostic sera (Durham and Forbes-Faulkner, 1975). The clinical disease has been described in local adult cattle by Salisbury et al., (1961) and Horner and Buddle (1970), but to date there have been no reports of the virus affecting neonatal calves.

The literature on IBR virus is also extensive, and mostly relates to diseases of systems other than the alimentary tract. It has been reviewed by McKercher (1959), and more recently with particular reference to bovine abortion, by Durham (1974). A few reports however do incriminate the virus as a cause of enteric disease. Diarrhoea was reported to occasionally occur in cattle with IBR infection by Chow et al., (1955), Jensen (1955), and Miller (1955). Necrotic lesions have been found to occur in the mouth, oesophagus and

forestomachs of IBR infected calves by Baker et al., (1960) and Thomson and Savan (1963). More recently, Gratzek (1966) isolated both IBR and BVD viruses from a calf with clinical evidence of BVD infection, and found that the IBR virus isolate was capable of inducing diarrhoea upon intravenous inoculation into 6 to 8 month old calves. Chennekatu (1966), working with the same IBR virus isolate, also found it to induce diarrhoea experimentally in calves, and on autopsy found ulcers in the abomasum and rumen, and a catarrhal enteritis. Microscopically, focal necrosis and inflammation was found to be widespread in the gastrointestinal tract, lymphoid tissues, adrenal glands, and to a lesser degree, the lung. Virus was also shown to be replicating in these tissues. There has only been a single report of IBR virus being isolated from faeces (Crandell, 1974), and this was not associated with enteric disease. However, survival of the virus in faeces is probably limited by the action of bile salts on the viral envelope, as this has been shown to be sensitive to the action of sodium deoxycholate (Durham, unpublished data).

Though IBR virus has been isolated from New Zealand cattle on a number of occasions (Webster and Manktelow, 1959; Manktelow and Hansen, 1961; Fastier and Smith, 1962; Durham, unpublished data), it has not yet been locally associated with gastrointestinal disease.

Reovirus infections of cattle have been briefly reviewed by Lamont (1968), who commented on the difficulties experienced in isolating the virus, due to the rather indistinct and nonprogressive nature of the cytopathic effect produced in cell culture systems. The report by Wallis et al., (1966) that pancreatin treatment of reoviruses enhances viral yields and plaque formation may however assist in isolation procedures. All mammalian reoviruses isolated to date have been shown to possess haemagglutinins (Eggers et al., 1962; Lerner et al., 1963; Kurogi et al., 1974), and this has proved to be a very useful tool in studies on these agents. Reovirus strains all possess a common complement-fixing antigen (Sabin, 1959), 3 basic serotypes

being described by Rosen (1960), using the haemagglutination-inhibition test.

The relationship of reoviruses to enteric disease is still vague, though some involvement in respiratory disease syndromes appears to be widely accepted. Reoviruses of all 3 main serotypes have also been isolated from the faeces of cattle (Rosen and Abinanti, 1960; Rosen et al., 1963). However, these isolates were not associated with enteric disease. Trainor et al., (1966) found that inoculation of reovirus 1 into calves by the respiratory route induced a mild respiratory disease syndrome only. More recently, Kurogi et al., (1974) isolated 2 new serotypes of reoviruses in Japan, 1 of these being from the faeces of calves showing diarrhoea. However, the pathological significance of these isolates is yet to be established.

A number of strains of adenoviruses have been isolated from cattle, using a variety of types of cell cultures and requiring from 1 up to several passages before cytopathic effect was evident (Cole, 1970; Mohanty, 1971). Though the majority of bovine adenovirus serotypes have been associated with respiratory disease (Mohanty, 1971), a number of reports also incriminate adenoviruses as causes of pneumoenteric disease. Thus adenovirus serotypes 4 and 5 have been isolated from calves with pneumoenteric disease in Hungary (Bartha and Aldasy, 1966) and were believed by Bartha (1967) to be a major cause of the disease syndrome. An adenovirus was also isolated from a bull with pyrexia and soft faeces by Tanaka et al., (1968), and was shown by the same authors to induce a similar syndrome in calves experimentally. This isolate was subsequently identified as serotype 4 by Matumoto et al., (1969). Another strain was isolated from a cow with pneumoenteric disease by Inaba et al., (1968), this being identified as serotype 7 by Matumoto et al., (1970). Adenovirus serotype 3 was consistently isolated over a number of years from 1 to 4 week old calves at a property in the U.S.A. by Mattson (1973a and b), where it was associated with respiratory and enteric disease. This author showed that the disease was reproducible in

calves inoculated with the virus by a number of routes. Adenovirus-like particles were also demonstrated by Bulmer et al., (1975) in the intestinal epithelium of scouring calves. In the U.K., Darbyshire et al., (1966) has also described the production of diarrhoea in calves inoculated experimentally with serotype 3. In New Zealand, there have as yet been no reports of adenoviruses being isolated from cattle.

A haemadsorbing virus was first isolated from the gastro-intestinal tract of calves by Abinanti and Warfield (1961), who termed it a haemadsorbing enteric (HADEN) virus, as it haemadsorbed guinea pig erythrocytes onto infected bovine embryonic kidney cell cultures. A number of the physicochemical properties of the virus were reported by Abinanti and Warfield (1961) and these were confirmed and extended by Spahn et al., (1966a) and Bachman (1971). Though the virus was thought to behave like a RNA virus by Spahn et al., (1966a) who classified it as an enterovirus, this was not corroborated by Storz and Warren, (1970) and Bachman (1971), who concluded that it was a DNA virus and classified it as bovine parvovirus 1. The virus was shown to be serologically unrelated to parvoviruses of other species of animals by Bachman (1971) and Storz et al., (1972). Parvovirus infections of calves have been well reviewed by Storz and Bates (1973).

Isolates of bovine parvoviruses have also been reported from Japan by Inaba et al.,(1973), who cited further isolates by Yago et al., (unpublished data) and Sugimara et al., (unpublished data). These isolates were found to possess similar physicochemical properties to bovine parvovirus 1, and were all serologically similar to this strain, except for that isolated by Yago et al., (unpublished data), which appeared to be of a different serotype.

Though foetal bovine kidney cells have been used to isolate bovine parvoviruses by most workers, it was found by Bates et al., (1972) that foetal bovine lung cells were more sensitive for isolation

purposes. The virus was found to cause rounding-up of cells with the formation of intranuclear inclusions, resulting in the eventual destruction of the entire cell sheet, and also appears to have a selective affinity for actively dividing cells (Storz and Warren, 1970).

Serologically, the virus appears to be widespread in the cattle population of the U.S.A., with significant haemagglutination-inhibiting antibody titres to the virus being reported in 86% of the cattle population by Abinanti and Warfield (1961), 83% by Spahn et al., (1966b) and 65% by Storz et al., (1972). Antibody has also been demonstrated in foetal bovine serum by the latter authors.

The relationship of the virus to bovine disease has not been fully investigated. Though most isolates have been obtained from calves with enteric disease (Bates et al., 1972; Inaba et al., 1973; Yago et al., unpublished data) the isolate obtained by Sugimura et al., (unpublished data) was from an aborted bovine foetus. Experimentally, Spahn et al., (1966b) found that inoculation of the virus into 2 to 8 month old calves per os resulted in the production of diarrhoea whilst virus given intranasally induced both diarrhoea and mild respiratory disease. Virus was isolated from faeces after inoculation by both routes and from nasal swabs of the intranasally inoculated calves. All calves showed a serological response to the infection, as measured by the haemagglutination-inhibition and serum neutralisation tests, though titres were higher in the intranasally inoculated group. Parvoviruses have not yet been isolated from cattle in New Zealand.

A coronavirus-like agent was first demonstrated in calf faeces by electron microscopy during experiments with "reo-like" viruses, and was subsequently found to be present in diarrhoeic faeces from several cattle herds (Mebus et al., 1972). The virus was found to produce diarrhoea experimentally following inoculation into gnoto-biotic, colostrum-deprived and colostrum-fed calves using unpurified

virus (Stair et al., 1972), typcial viral particles being demonstrated in the faeces of these animals. The same authors purified and concentrated the virus and seubsequently measured its size and density. They concluded it was a primary pathogen, as it was capable of producing diarrhoea in the absence of a bacterial microflora. Coronavirus-like agents have also been seen in the faeces or scouring calves by Woode et al., (1974) and Woode and Bridger (1975) in the U.K. and by Morin et al., (1973) in Canada, in conjunction with "reo-like" viruses. It was suggested that the 2 viruses may act in combination in the production of enteric disease (Morin et al., 1973).

The coronavirus was first propagated in primary and secondary foetal bovine kidney cells derived from the same foetus by Mebus et al., (1973a), who reported that a slight CPE was seen in the third passage of the virus. This CPE consisted of small numbers of rounded floating cells which fluoresced in the presence of a fluorescein-conjugated antiserum to the virus. The antiserum was derived in rabbits from an antigen produced from infected faeces by ultracentrifugation. Immunofluorescent stained cells showed fluorescent cytoplasmic granules initially, this later developing to whole cytoplasmic fluorescence at the stage when the cells detached. Syncytia were also seen after 24 viral passages. Recently, Inaba (unpublished data) demonstrated the production of an obvious CPE in a BEK 1 cell line (Burgess, pers. com.).

Further studies by Mebus et al., (1973a, b) in neonatal calves confirmed the disease-producing potential of the agent and showed by immunofluorescent tests that the virus localised in the intestinal epithelium, causing a change in the epithelial morphology from columnar to cuboidal shape, and cell lysis. These changes were seen in large and small intestine, and in the small intestine resulted in villous atrophy. Mebus et al., (1973b) noted that colostrum did provide some protective effect, but did not prevent the disease, whilst serum antibody appeared ineffective in protecting against the disease. Cell culture attenuated virus was shown by Mebus et al., (1973a) to provide protection against challenge with

virulent virus, when given by the oral route.

A coronavirus-like agent has been demonstrated in the faeces of scouring adult cattle in New Zealand (Horner, pers. com.), but its relation to the disease syndrome is not clear.

A "reo-like" virus was discovered in the faeces of neonatal scouring calves by Mebus et al., (1969a) and was thought by these authors to be a possible cause of neonatal calf diarrhoea in the U.S.A. They showed that it localised in the small intestinal epithelium by means of immunofluorescence techniques. The antiserum was produced in rabbits from an antigen derived from infective faeces by ultra-centrifugation. The virus was subsequently demonstrated to be present in the faeces of scouring calves in several states of the U.S.A. by Mebus et al., (1969a, b; 1970) and White et al., (1970), whilst similar particles were later demonstrated in diarrhoeic calf faeces in Australia (Turner et al., 1973), Canada (Morin et al., 1974) and the U.K. (Woode et al., 1974). Recently, a similar virus was demonstrated in diarrhoeic calf faeces in New Zealand by Burgess (pers. com.) who showed it to be serologically related to the U.K. isolate of Woode et al., (1974).

A number of physicochemical properties of this "reo-like" virus were determined by Welch (1971), Fernelius et al., (1972) and Welch and Thompson (1973), it being suggested by these authors that the virus had some affinity with the reoviruses. However, Bishop et al., (1974) commented on the detailed morphological similarity of the "reo-like" virus to epizootic diaarrhoea virus of mice, which was shown by Much and Zajac (1972) to possess 32 capsomeres, and thus the reo-like virus appears to resemble orbiviruses more than reo-viruses. It was noted by Welch and Thompson (1973) that the virus was thermobile in the presence of molar $MgCl_2$, which was not consistent with the properties of known reoviruses, and that it was acid resistant, in contrast to the orbivirus group. Fernelius et al., (1972) noted that the agent appeared serologically unrelated to reo-

viruses and bluetongue virus, and Welch and Twiehaus (1973) also reported lack of relationship between the "reo-like" virus and reovirus serotypes 1 and 3. Though Woode (1974) initially though the virus was probably an orbivirus, the lack of antigenic relationship with this group, the slight morphological differences and the acid resistent nature of the reo-like virus led Flewett et al., (1974) to suggest the name rotavirus for this "reo-like" virus and for a similar virus causing diarrhoea in children.

Though earlier studies of the disease were done using immunofluorescence techniques, the first report of cultivation of the virus was that of Mebus et al., (1971a) who demonstrated viral multiplication in primary cell cultures of bovine lung, thyroid, kidney and choroid plexus, using immunofluorescence to demonstrate the virus. These authors concluded that the lung cells were the best for culture of 1 isolate of the virus, whilst kidney cells proved best for another isolate. Cytopathic effect was only very slight, affected cells becoming "granular" in appearance, and eventually detaching after a period of semi-detachment in which they were only held by a single cell process. The CPE was noted to decline after several passages of the virus. Fernelius et al., (1972) also reported the production of a CPE in a pig kidney cell line, this effect developing at the third passage level and continuing up till the 95th passage. CPE was also shown to occur in a mouse L cell line and in primary lamb kidney cells. Affected cells were seen to swell and "round-up" in the pig and lamb kidney cells, with eventual detachment, whilst in the mouse L cells affected cells were noted to assume a "leathery" appearance. Welch and Twiehaus (1973) described the production of a CPE in bovine embryonic kidney cells which was similar to that described by Mebus et al., (1971a) but commented that the CPE did not progress to affect more than 50% of the cells, even on prolonged culture. They also noted the presence of vacuolation and inclusions in the cytoplasm of affected cells, the nuclei being noted to become pyknotic. Cytoplasmic granules were also seen by Mebus et al., (1971a) using the immunofluorescence technique.

The only reported attempt at plaquing with "reo-like" viruses is that by Welch and Twiehaus 1973), who commented that plaques grown under ag r were generally of microscopic size and required several days cultivation before they were seen.

The disease produced by the virus was generally seen in calves less than 7 days old, and was manifested by a profuse yellow watery diarrhoea and dehydration (Mebus et al., 1971b) with a morbidity rate of up to 100% and a mortality rate from near zero up to 50%. The severity of the clinical disease experimentally was greatly increased in the presence of Escherichia coli (Mebus et al., 1969a, 1973b). Working with calves resistant to Providencia stuartii, Waldhalm et al., (1974) found that diarrhoea was only produced in neonatal calves when inoculated with a mixture of P. stuartii and "reo-like" virus. Those calves receiving virus alone remaining clinically normal, though virus was demonstrated by immunofluorescence in the intestinal epithelium. These authors thought that colostrum fed to calves provided sufficient protection to prevent diarrhoea from the virus alone. From these results, it would appear that much of the damage is due to the action of secondary invaders.

Immunofluorescence and histological studies by Mebus et al., (1971b) indicated that the virus infected the small intestinal epithelial cells. Infected cells migrated up to the intestinal villi into the intestinal lumen, and were replaced by cuboidal epithelial cells. These latter cells were shown to be resistant to challenge with virulent virus. Electron microscopic studies by Stair et al., (1973) generally agreed with the above findings and further demonstrated the presence of virus within membrane bound intracytoplasmic structures of infected cells. The replacement epithelium was noted to be immature, and consequently not fully functional, this probably contributed to fluid loss.

Oral vaccination of new born calves was shown by Mebus et al., (1972) to provide effective control of the disease, using a cell culture attentuated vaccine.

Enteroviruses have been reported on numerous occasions to be isolated from calves and adult cattle, these being too numerous for all to be individually listed. Whilst many of the reports refer to isolation from faeces of normal calves (Klein and Earley, 1957); Kunin and Minuse, 1964; Moscovici et al., 1961; McFerran, 1962a; Spradbrow, 1963, 1964; Mattson and Reed, 1974), other isolates have been obtained from calves with respiratory disease (Moll and Finlayson, 1957; Moll and Davis, 1959) or diarrhoea (Moll and Davis, 1959; McFerran, 1962a; Schiott and Hyldegaard-Jensen, 1966; Storz et al., 1969). In the latter report, the virus was isolated in conjunction with chlamydia. Enteroviruses have also been isolated from aborted bovine foestuses by Moll and Finlayson (1957) Moll and Davis (1959) and Mattson and Reed (1974). These and other reports originate from a number of countries, including Australia, Ireland, U.S.A., Japan and several countries in Europe.

Most isolates of bovine enteroviruses have been made in foetal bovine kidney cells where a focal CPE was produced in 1 or 2 passages, this progressing rapidly to result in the destruction of the entire cell sheet (McFerran, 1962a; Spradbrow, 1963, 1964). Affected cells became rounded, the nuclei eccentric in position, and sometimes developed eosinophilic inclusions in the cytoplasm (Spradbrow, 1963, 1964; Rovozzo et al., 1965), similar to those described for human enteroviruses by Barski (1962) and Wenner (1962). Though similar changes were described by other authors, they do not comment on the presence of inclusions in the cytoplasm, and 1 report specifically states that they were not present (Schiott and Hyldegaard-Jensen, 1966). Some bovine enteroviruses have been shown to be capable of infecting a variety of host cell types (Moll and Davis, 1959; Moscovici et al., 1961; McFerran, 1962a) similar to the wide host range exhibited by some human enteroviruses (Melnick, 1962). Many of the bovine enteroviruses have been shown to possess haemagglutinins for guinea pig and primate erythrocytes, especially for those of the monkey (Moscovici et al., 1961; La Placa et al., 1965; Schiott and Hyldegaard-Jensen, 1966; Mattson and Reed, 1974).

The plaque forming behaviour of a number of bovine enteroviruses was studied by Moscovici et al., (1961), McFerran (1962a), Moll and Ulrich (1963), Gratzek et al., (1967), Van Der Maaten and Packer (1967) and Mattson and Reed (1974). Though the effect of various chemical and enzymatic additivies on plaque formation has been studied with human enteroviruses, (Wallis et al., 1966a; Wallis and Melnick, 1968) there appear to have been no equivalent studies with bovine enteroviruses.

The physicochemical properties of bovine enteroviruses have been studied by a number of authors (McFerran 1962a; Moll and Ulrich 1963; Spradbrow 1963, 1964; Rovozzo et al., 1965; Van Der Maaten and Packer 1967; Mattson and Reed 1974) who found the properties to be similar to those described by Melnick (1962) for human enteroviruses. The properties of the bovine virus are best summarised by Dunne et al., (1974). More recently, it was found possible to subdivide bovine enteroviruses into 2 subgroups, depending on their susceptibility to hydroxybenzyl benzimidazole (HBB) (Portolani et al., 1968).

In excess of 63 serotypes of bovine enterovirus have been described by various authors, mostly by using cross neutralisation tests upon limited numbers of local isolates (Barya et al.. 1967). These studies have been of limited value because of their restricted scope and because the antisera were usually produced in the rabbit, which has been shown by La Placa et al., (1965) to be relatively unsuitable for the purpose. Moscovici and La Placa (1961) provided the most useful study of this type, identifying 7 groups of enterovirus from a total of 15 strains, mainly based on the criteria of the cross neutralisation test. One of these groups was thought possibly to be a reovirus rather than an enterovirus. Moll and Ulrich (1963) identified 8 serotypes from a total of 10 isolates. A useful step forward was made by La Placa et al., (1965) who showed a correlation between antigenic character and ability to haemagglutinate monkey erythrocytes. These authors divided a large number of bovine enteroviruses into 2 groups, the first being relatively closely

serologically related and showing haemagglutinating activity and the second group being serologically distinct, showing a lesser degree of internal relationship, and exhibiting no haemagglutinating activity. A more precise type of cross neutralisation test was used by Barya et al., (1967) to type enteroviruses, using a measure of the dynamics of the reactions. They described 4 serotypes, and also summarised the relationships between a number of earlier studies of serotypes.

More recently, Dunne et al., (1974) extended these studies of previously established serotypes, and grouped the bovine enteroviruses into 7 serotypes. Because of quarantine restrictions, these were based on U.S. isolates only. However, serotype 1 had previously been shown by McFerran (1962a) to be closely related to a strain isolated in Ireland.

Apart from the serotyping of isolates, little serological work has been done with bovine enteroviruses, possibly because of the presence in bovine sera of nonspecific inhibitors of bovine enteroviruses (McFerran 1962b; Klein et al., 1964). These were found by these authors to be widely distributed in the cattle population. On the basis of gel filtration behaviour, McFerran et al., (1968) concluded that the inhibitors were non-antibody substances of a size similar to albumin. They also demonstrated the additional presence of 7S and 19S antibody to bovine enterovirus in suckled calves and adult cattle. The behaviour of the inhibitors in the presence of neuraminidase, periodate, or other standard serum treatment does not appear yet to have been satisfactorily determined. Nevertheless, Moll and Davis (1959) and Storz et al., (1969) found a serological response in paired sera to bovine enterovirus infection during experimental studies. Dunne et al., (1973) also showed that IgM antibodies were produced in foetal calves, apparently as a response to intrauterine infection with enterovirus.

Experimental studies of the disease producing potential of bovine enteroviruses are few. Diarrhoea was found to be induced

in young calves by McFerran (1962a), Van Der Maaten and Packer (1967) and Dunne *et al.*, (1974). In the latter report, though diarrhoea was produced in both colostrum fed and colostrum deprived calves, it was more severe in the animals not fed colostrum. Paralysis and death was found to occur in suckling mice by Kunin and Minuse (1958), following intraperitoneal inoculation. Moll and Davis (1959) reported the production of weak and stillborn offspring from inoculated pregnant guinea pigs, and of myocardial necrosis in cortisone treated weaned mice, following intraperitoneal inoculation. Experimentally induced abortion was also reported in pregnant guinea pigs by Moll (1964) and Van Der Maaten and Packer (1967). Studies by Dunne *et al.*, (1973, 1974) have also suggested the possible role of enteroviruses in causing bovine abortion.

The disease producing potential of bovine enteroviruses still remains obscure, but it appears conceivable that they may have some role in the production of calf diarrhoea, and possibly bovine abortion.

GENERAL MATERIALS AND METHODS

1. Glasswashing And Sterilizing Procedures.

Glassware was routinely soaked overnight in 2% Decon[1] 75 or 0.5% Pyroneg[2], brushed, and then washed and rinsed in a Heinicke Typhoon glasswasher[3], being finally rinsed twice in distilled deionized water. The glassware was then sterilized in an autoclave at 15 lbs pressure for 15 minutes.

Contaminated glassware was either soaked overnight in 0.5% iodophor solution[4], or was autoclaved as above, prior to washing.

2. Preparation And Maintenance Of Primary Cell Cultures.

Primary cell cultures were prepared from foetal bovine kidney, lung, testes and thyroid.

Foetuses approximately 5 - 7 months old were obtained from a local freezing works and transported to the laboratory inside an unopened uterus. The foetus was then removed from its membranes and opened aseptically to remove the desired organs. Prior to opening both uterus and body cavity, the incision sites were swabbed with 70% ethanol.

Using aseptic technique, the kidney cortex, lung and thyroid tissues were finely minced using crossed scalpels. Testes were cut in half, the pulp expressed by pressure, and this then minced similarly.

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1 Decon Laboratories Ltd., Brighton, U.K.

2 Diversey Wallace Ltd., Papatoetoe, N.Z.

3 Heinicke Co., Hollywood, Florida, U.S.A.

4 "Redene", Nicholas Products Ltd., Auckland, N.Z.

Cell fragments and residual connective tissue were removed from the trypsinised cells by passing the suspension through a sterile stainless steel sieve (40 mesh to the inch). The suspension was then centrifuged at 1000 G for 10 minutes, and the total packed cell volume determined for each cell type.

The foetal bovine kidney, lung and thyroid cells were suspended at a concentration of 0.5% by volume, and testes cells at 0.3% by volume, in HLA medium (Appendix 2) containing 10% bovine serum. These suspensions were then dispensed into culture vessels using about 100 ml per Roux bottle, or 50 ml per 500 ml flat bottle. Bottles were sealed with screw caps, or with silicone rubber stoppers.

Cultures were then incubated at 37°C, and the medium was changed at 2 days. Once confluent, monolayers were maintained in ELA medium (Appendix 2) with 2% bovine serum, medium changing at weekly intervals.

3. Production And Maintenance Of Secondary Cells And Cell Lines.

Stocks of primary cells were subcultured once or twice only to provide secondary cells for test purposes. Two cell lines were also maintained, Madin Darby bovine kidney (MDBK) (Madin and Darby, 1958) and African Green Monkey kidney (Vero) (Yasamura and Kawakita, 1963).

To subculture the above cells, medium was removed by aspiration or decanting, the monolayers washed twice in a small volume of PBS (Appendix 2), and the cells then treated with 2 - 3 ml of ATV (Appendix 2), rocking gently to distribute the fluid over the cells. The bottles of cells were then incubated at 37°C, agitating at intervals until all cells were detached from the glass. The resulting cell suspension was brought to 10 ml using growth medium, and an aliquot was diluted 1 in 10 in 0.1% trypan blue solution (Appendix 2). This was mixed and

then examined in a haemocytometer to determine the average viable (unstained) cell count per ml of suspended cells (Hathaway *et al*., 1964). The average count per large square was determined, each cell of this count then representing 1×10^5 cells per ml of cell suspension.

The cells were then diluted in further growth medium to give a concentration of 1×10^5 cells per ml, except for Vero cells which were diluted to give 3×10^4 cells per ml. For microtitre tests, these concentrations were tripled.

With secondary cells, medium 199[1] with 10% serum was used for growth purposes, reducing to 2% serum for maintenance. MDBK cells were grown in MEM[1] or medium 199, with similar serum concentrations. Medium 199 was used for Vero cells, but serum concentrations were reduced to 5% for growth, and 2% for maintenance. Where cells were to be used for test purposes with bovine specimens or isolates, equine serum was routinely used in preference to bovine serum.

Secondary cells and cell lines were dispensed as required at the following rates:

Container	Volume
Roux bottle	100 ml
500 ml flat bottle	50 ml
50 ml plastic flask[2]	6 ml
microtitre plate (flat bottomed)[3]	0.05 ml per well
Linbro 24 well plate[4]	1 ml per well
tubes (pyrex, screw cap, 16 x 150 mm)[5]	1 ml per well
tubes, with flying cover slip	2 ml per well

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1 Wellcome Reagents Ltd., Beckenham, England

2 Falcon flask. Becton Dickenson Co., California, U.S.A.

3 Cooke Engineering Co., Alexandria, Virg., U.S.A.

4 Linbro DispocoTray. Linbro Chemical Co., Newhaven, U.S.A.

5 Kimax. Kimble Products Div., Owens-Illinois Inc., Ohio, U.S.A.

Frozen Storage of Cell Cultures

Surplus cells to current needs were preserved in frozen storage. Primary cells were suspended at 10% concentration in a freezing medium consisting of medium 199 with 10% dimethyl sulphoxide and 20% bovine serum. Secondary cells and cell lines were suspended at 1 to 5 x 10^6 cells per ml concentration in the same medium.

Suspensions were dispensed in 4 ml volumes into small screw cap containers, or into 1 ml ampoules which were then sealed. These were then cooled slowly and stored at -80°C in an ultra- deep freeze, or in the case of ampoules, were also stored in liquid nitrogen. Retarded freezing was achieved by wrapping the cell containers in cotton wool prior to placing in the ultra-deep freeze.

Prior to routine use of stored frozen cells, a sample of each batch was thawed rapidly in a 37°C water bath, and then diluted 1 in 10 in growth medium, using a pipette. The drop of cell suspension remaining in the pipette was used to inoculate a bottle of nutrient broth, which was then incubated at 37°C to check for contamination. A viable cell count was also carried out on thawed secondary cells and cell lines, and the remainder was diluted to the appropriate concentration of viable cells in growth medium and dispensed. Thawed primary cells were not routinely counted, but diluted 1 in 10 in growth medium and dispensed. Rate of growth of cells was checked, and used to guide the future use of the cells.

Storage Of Viral Isolates

Following first isolation of a virus and successful repassage to confirm the CPE, further stocks of virus were prepared, and these frozen in ampoules at either -80°C in an

ultra-deep freeze, or in liquid nitrogen. Some stocks were also held frozen at -20°C for current use. Samples of deep frozen stocks were subsequently titrated for activity, following rapid thawing in a 37°C water bath.

Viral Titration Procedures

Serial tenfold dilutions of virus were prepared by diluting 0.2 ml of virus suspension in 1.8 ml of maintenance medium, using a separate sterile pipette for each step. Up to seven dilution steps were used routinely.

A 0.1 ml volume of each dilution was inoculated into duplicate tube cell cultures. With microtitre titrations, 0.05 ml of each dilution was added to duplicate wells, and these were then seeded with cell suspension. With either procedure, several cultures were left as uninoculated cell controls. The cultures were then sealed and incubated at 37°C until the CPE was cell developed. Following observation of the end-points, titres were determined using the method of Reed and Muench (1938), the results being expressed as median tissue culture infective dose ($TCID_{50}$) per inoculum volume.

With plaque titrations, 0.1 ml of each dilution was added to duplicate monolayers in Linbro plates, left to adsorb at 37°C for one hour, and then overlaid with agar medium. Some cells were left as uninoculated cell controls. Following further incubation until plaques were adequately developed, the average number of plaques was determined at the limiting dilution and the result expressed as plaque forming units (PFU) per 0.1 ml.

Serum Neutralisation Test

This was carried out in duplicate by the microtitre procedure using a similar method to that described by Rossi and Kiesel (1971).

Sera were heat inactivated at $56^{o}C$ for 30 minutes prior to test. Doubling dilutions of serum were prepared using 0.05 ml "tulip"[1] diluters. A serum control well was included except with bovine serum, where previous experience indicated it was not necessary. A 0.05 ml volume of viral suspension calculated to contain 100 $TCID_{50}$ was added to all wells except serum control and cell control wells. A viral check titration was also carried out using serial tenfold dilutions over 5 dilution steps.

Following incubation of the virus-serum mixture at $37^{o}C$ for one hour, 0.05 ml of cell suspension was added to all wells. Following sealing, the plates were incubated at $37^{o}C$ until the viral titration showed a well developed CPE. Subject to the viral titre proving satisfactory, the serum end points were then observed and the titre of antiserum determined using the method of Reed and Muench (1938). Titre was expressed as the reciprocal of the highest dilution providing protection against viral CPE.

Viral Haemagglutination (HA) Test

Blood was collected into modified Alsever's solution (Appendix 2), and stored at $4^{o}C$ until use within 2 weeks. Prior to use the erythrocytes were washed three times in PBS and a 0.4% by volume suspension prepared in PBS.

The test was carried out in duplicate in V bottomed microtitre[1] trays, using 0.05 ml volumes. The diluent used was PBS pH7.2, as recommended by Hierholzer et al., (1969). Doubling dilutions of virus were prepared, commencing from a 1 in 10 dilution. A 0.05 ml volume of diluent was added to all wells (to make the final volume the same as that used in the haemagglutination inhibition test).

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1 Cooke Engineering Co., Alexandria, Virg., U.S.A.

A further volume of 0.05 ml of erythrocyte suspension was now added to all wells; several erthrocyte control wells were also prepared, using two volumes of diluent and one volume of cell suspension. The plate was then agitated and incubated at the required temperature until the erythrocyte controls had settled to a distinct "button", usually within 1 to 2 hours.

The titre of the test was taken following Reed and Muench (1938) analysis, as the highest dilution of virus which gave complete agglutination of the erythrocytes, this containing one haemagglutinating unit (HAU) of virus per 0.05 ml.

Haemagglutination Inhibition (HI) Test

Erythrocyte suspensions were prepared as for the HA test. The test was carried out in duplicate in V bottomed microtitre plates using PBS as a diluent and 0.05 ml volumes.

Sera were subjected to pretest treatments as required for the individual virus concerned, and then serial doubling dilutions were made commencing from a 1 in 10 dilution. Control wells were left for each serum. A 0.05 ml volume of haemagglutinin containing 4 HAU was added to all wells except serum controls, and the haemagglutinin was further doubly diluted over a 5 step range using some spare wells, to make a check titration. The mixtures were then incubated for one hour at room temperature.

A 0.05 ml volume of erythrocyte suspension was then added to wells, and to some further cell control wells. The test was then incubated at the required temperature for one to two hours, until the control cells had settled to a distinct button. Subject to a satisfactory check titration of the haemagglutinin, the highest dilutions of sera which completely or almost completely suppressed haemagglutination were then determined. The titre was determined using Reed and Muench (1938) nalysis, and was expressed as a reciprocal of the highest dilution.

Negative Staining Procedure For Electron Microscopy

Infected cell cultures were harvested when the CPE was well advanced, and concentrated viral suspensions were prepared by ultracentrifugation, chemical precipitation, or more usually by lysis of infected cells as described by Spradbrow and Francis, (1969). The method briefly is as follows:

Cells were scraped off the walls of the vessel and the resulting suspension of cells centrifuged at about 1000 G for 10 minutes. The sedimented cells were drained free of fluid, suspended in a few drops of distilled water, and then frozen and thawed once.

A drop of lysate was placed on a carboned, formvar coated electron microscope grid, left for up to a minute, and then the excess was removed with filter paper. A drop of 2% potassium phosphotungstate pH 7.0 was then placed on the grid for about 30 seconds, and then also removed with filter paper.

The specimen was then examined in a Phillips EM 200 electron microscope.

Sectioning Procedures For Electron Microscopy

Infected cells were harvested when the CPE was well advanced by scraping the cells into suspension and then recovering them by centrifugation at 1000 G for 10 minutes.

Subsequent procedures were similar to those described by Doane et al., (1974). The cells were resuspended in a few drops of medium, formed into a pellet in a capillary tube by microhaematocrit centrifugation, and then fixed in 2.5% glutaraldehyde followed by 1.0% osmium tetroxide, with appropriate washes in phosphate buffer. Following dehydration through a graded sequence of acetone, the pellet was embedded in epon

araldite resin[1] or in Spurr's medium (Spurr, 1969) inside a gelatine capsule.

Both rapid and standard processing times as described by Doane et al., (1974), were used. Subsequently the epon aradite resin was polymerised at 60°C for 36 hours, and the Spurr's medium was polymerised at either 70°C for 18 hours, or 95°C for 1 hour.

After removal from the polymerising oven, the blocks were trimmed and then sectioned on an ultramicrotome. Pale gold sections were floated onto carboned formvar coated electron microscope grids and stained with uranyl acetate and lead citrate (Appendix 3). They were then examined in a Phillips EM 200 electron microscope which was periodically calibrated using negatively stained catalase crystals.

Photography

Unstained or haematoxylin and eosin stained monolayers were observed in an inverted microscope and photographed using a tungsten balanced Agfa 50L film.

With fluorescent stained preparations, monolayers were observed with a fluorescent microscope using a FITC 3 excitation filter, a matching barrier filter, and transmitted blue light illumination for a quartz halogen bulb. They were photographed using a daylight balanced Kodak Ektachrome 160 ASA film.

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1 Fluka A.G. Chemische Fabrik, Buchs, Switzerland.

ISOLATION PROCEDURES

Fifty nine samples were received from calves and young adult cattle with histories of diarrhoea, the animals being located in widely scattered areas of New Zealand. The samples included 56 faeces, 2 intestines, and 1 spleen.

Because of the high bacterial content and frequently toxic nature of the faecal material, some preliminary investigations were made of suitable processing regimes to minimise toxicity and contamination problems upon inoculation of cell cultures.

Materials And Methods

Faecal samples were suspended at concentrations of from 10 to 50% (w/v) in Hank's buffered salt solution (Appendix 2) containing 2,000 units/ml of penicillin and 2,000 μg/ml of streptomycin (Hank's AB). Following sonification for 1 minute at 20 kc/second in an ultrasonic disintegrator[1], the samples were centrifuged for periods of 30 and 60 minutes at 4,000 G, in an angle rotor. Various dilutions of the clarified supernatants were then inoculated onto secondary foetal bovine kidney (FBK) cell monolayers. Adsorption of the inoculum onto the cells for a period of 1 hour at 37°C followed by replacement with fresh maintenance medium, was also attempted.

Faeces in Hank's solution were also subjected to various periods of centrifugation at 4,000 G in the presence or absence of the high levels of antibiotics mentioned above. The levels of residual viable bacteria were then determined, by titration in nutrient broth of serial tenfold dilutions of aliquots of the samples taken at various stages. Following incubation at 37°C for 3 days, the nutrient broths were examined for evidence of bacterial growth.

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1. Soniprobe. Dawe Intruments Ltd., London, England.

Based on the findings of these experiments, the following procedure was adopted for the preparation and inoculation of samples onto cell cultures: Faeces were suspended at 10% concentration (w/v) in Hank's AB solution, sonified at 20 kc/second for 1 minute, and then centrifuged at 4,000 G for 30 minutes in an angle rotor. The supernatent was then inoculated into cell cultures at a final concentration of 0.25%. The samples of intestines and spleen were ground up in a TenBroeck tissue grinder, using a small volume of Hank's AB solution and then treated in a similar manner to the faeces, except that the spleen was inoculated to give a final concentration of 1% on the cells.

Each sample was inoculated into duplicate monolayer cultures of foetal bovine kidney, lung (FBL) and Vero (African green monkey kidney) cells in tubes, or into single monolayers of these cells in 50 ml plastic flasks. The occasional sample giving problems with fungal or bacterial contamination of the cell cultures was filtered through a 220 nm apd membrane filter[1], and then reinoculated. Where toxicity remained a problem, the sample was diluted further, and then reinoculated into cell cultures.

Samples were passaged 5 times at weekly intervals through each cell type, using 0.2 ml of frozen and thawed cell culture for repassaging. On final passage, coverslip cultures were also inoculated.

All cell cultures were examined daily for evidence of cytopathic effect (CPE). In addition, the final passage was tested after 7 days incubation for evidence of haemadsorption with guinea pig erythrocytes, and was further examined for cpe and viral inclusions using the coverslip cultures stained with

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1. Millipore Corp., Bedford, Mass., U.S.A.

haematoxylin and eosin (Appendix 3). The haemadsorption test was carried out using similar technique to that described by Shelakov *et al.*, (1958). The monolayer was washed with PBS after removal and storage of the medium, and was then overlaid with 1 ml of 0.4% washed guinea pig erythrocytes, prepared as for the haemagglutination test (General materials and methods). Following incubation at room temperature for 1 hour, the monolayer was washed twice with PBS and examined for haemadsorption.

Results

It was found that a 10% suspension of faeces in Hank's AB solution was adequately clarified by 30 minutes centrifugation at 4000 G. Higher concentrations took much longer to clarify.

The use of final inoculum concentrations of 0.5% or greater on cell cultures resulted in considerable problems of toxicity, characterised by rounding up and detachment of cells within 24 hours. This effect was usually generalised, but on occasion was focal, simulating a viral CPE. The effect was not however transmissible. Adsorption of the inoculum onto the cells for 1 hour, followed by replacement with fresh maintenance medium, delayed the onset of toxicity, but did not appreciably alter the final outcome. It was found that reduction of the final inoculum concentration to 0.25% reduced toxicity to negligible proportions. When it still occurred, slight further dilution alleviated the problem.

Contamination of cell cultures by the inoculum was an occasional problem, mostly due to fungi; it was readily corrected by filtration. The studies on the relative effectiveness of centrifugation and antibiotic treatment in controlling the levels of viable bacteria in the inocula showed that the majority of the

effect was due to the influence of the antibiotics; centrifugation played a minor role in this regard, but was more of use in removing suspended debris, making for easier handling.

Following examination of the 59 samples, 7 viruses were isolated. Three of these (D74/18, D74/19A, D74/19B) came from the same property, but from different animals. All were recognised by the CPE they produced. No evidence of haemadsorption with guinea pig erythrocytes was seen at third passage, and no further evidence of viral infection was seen on examination of haematoxylin and eosin stained monolayers. All isolates were confirmed by reisolation from the original material. Frozen stocks were then prepared and titrated.

The pattern of isolation of the viruses in the various cells and passages is shown in Table 1.

Table 1

Cultural histories of samples yielding cytopathic agents

Sample	1st Passage in:			2nd Passage in:			3rd Passage in:		
	FBL	FBK	Vero	FBL	FBK	Vero	FBL	FBK	Vero
D74/2 (faeces)	$-^{+}$	-	-	4	-	-	1	-	-
D74/13-49 "	-	-	-	-	-	2	-	-	2
D74/18 "	2^{++}	3	2	1	1	1	1	1	1
D74/19A "	-	-	-	-	5	-	-	1	-
D74/19B "	-	-	-	1	6	2	1	1	1
D74/22-4 "	3	-	-	1	-	-	1	-	-
D74/25A (spleen)	-	-	-	4	-	-	1	-	-

\+ Indicates no CPE was seen by 7 days post inoculation.

++ Indicates the number of days post inoculation when CPE was first seen.

Discussion

Most workers refer to the use of a 10% centrifuged faecal suspension for inoculation into cell cultures, but make no mention of the precise inoculation procedure, so that the final concentration in contact with the cells is left unspecified. Assuming that a further dilution of 1 in 10 would take place on inoculation, as is common practice, the final concentration on the cells would be about 1%. At this level, most workers make no reference to toxicity problems, though Spradbrow (1965) does mention that toxicity was a problem with some groups of calf faeces.

It was found in this series of faecal samples that toxicity was a serious problem even using a 0.5% faecal suspension on the cells, and that reduction to 0.25% was necessary for routine use to minimise the effect. Why toxicity was more of a problem with these samples is uncertain; it did not appear to be related to any particular age group of animal, though some batches of faeces were worse than others. Spradbrow (1965) suggested on the basis of his experiments that the toxic substance was a bacterial toxin. It may be that variation in toxicity is related to dietary factors influencing the intestinal bacterial flora, or toxin production.

The use of adsorption techniques for inoculation is also a frequently used laboratory procedure, particularly with toxic substances, but did not appreciably reduce the toxicity problem, only delaying the onset of the effect. It would appear that the toxin (or toxins) exerts it's effect on cell metabolism relatively quickly, and that effect is to a large extent irreversible, as replacement with fresh medium did not correct the problem.

Three cell types were utilised for isolation purposes. Bovine kidney and lung cells were chosen in accordance with the recommendations of McFerran et al., (1972), who suggested that primary cells of the same species as the test material offered the best chance of successful viral isolation. Secondary cells at early passage were however chosen for this study, rather than primary cells, as offering a more consistent type of monolayer of uniform appearance, and having some practical advantages from the point of view of production. Lung cells were included especially because of their suitability for the isolation of parvoviruses, as Bates et al., (1971, 1972) found they obtained the highest rate of isolation of these viruses with this cell type. Monkey kidney cells have been used by a number of authors (Sabin, 1959; Rosen and Abinanti, 1960; Trainor et al., 1966) for isolation of and studies on reoviruses. Consequently, African green monkey kidney cells (Vero) were chosen as the third cell type to improve the chances of detection of this group of viruses.

It was decided to limit the number of passages of samples in the above cells to 3 as being consistent with the available resources and yet still offering a reasonable chance of isolation of most of the viruses likely to be encountered. It was realised however, that coronaviruses would probably not be detected by this sytem, as the CPE is reported by Mebus et al., (1973a) to be very slight and indefinite at the third passage in bovine kidney cells.

Equine serum was chosen for serous enrichment of the maintenance medium after inoculation, as it was considered to offer the least chance of interference with viral isolation. Foetal bovine serum was considered unsuitable, as it is known to sometimes contain antibodies to enteroviruses (Dunne et al., 1973) and to bovine viral diarrhoea virus (Horner et al., 1973).

The examination of haematoxylin and eosin stained monolayers at the end of the third passage was designed as an additional check on the presence of viral CPE and more particularly on the production of intranuclear and intracytoplasmic inclusions, particularly those due to parvoviruses and reoviruses. Intranuclear inclusions have been reported with bovine parvoviruses by Spahn et al., (1966) Bates et al., (1972) and Inaba et al., (1973). With reoviruses intracytoplasmic inclusions have been reported by Sabin (1959) and Gomatos et al., (1962).

Parvoviruses are reported to cause haemadsorption of guinea pig and human type O erthrocytes (Abinanti and Warfield, 1961; Bates et al., 1972). Hence guinea pig erthrocytes were used to check for haemadsorption at the end of the third passage.

In retrospect, some improvements can be suggested in the procedure used for isolation. It would have been advisable to check the medium from the end of the third passage in Vero cells for presence of reovirus haemagglutinins with human type O red cells (Lerner et al.,1963). A further check for haemagglutinins to bovine erthrocytes in the same medium would also have been useful to check for reovirus 3, as these erthrocytes appear generally to be more readily agglutinated than human type O cells with this serotype (Eggers et al., 1962).

Coriphosphene O or acridine orange stains could have been used as an alternative to haematoxylin and eosin stain, to check for viral inclusions. Their differential staining properties with regard to single and double stranded nucleic acids would have made the detection of parvovirus and reovirus inclusions somewhat easier. If necessary, monolayers treated with these fluorescent stains could have been destained after examination, and restained with haematoxylin and eosin, as suggested by Keeble and Jay (1962).

An improvement in the rate of isolation of viruses could probably have been obtained by the use of concentration techniques on the faeces. Using differential centrifugation, a pellet of possible viral material could have been obtained and used for inoculation. Alternatively, the faeces could have been treated with trifluorotrichlorethane followed by polyethylene glycol 6000, as suggested by Much and Zajac (1972) and by Bishop et al., (1974), resulting in a purified and concentrated preparation for inoculation.

It was considered that the use of 50 ml plastic flasks for isolation offered some advantages over tube cultures, as they allowed a somewhat larger inoculation sample, and more especially allowed easier visualisation of the monolayer for the detection of CPE.

Though lung cells proved to be the most productive of the three cell types used for isolation, kidney and Vero cells nevertheless each detected a virus that was missed by the other cells, demonstrating the value of using several cell types when screening material for virus content.

Finally, it is worth noting that the use of a third passage with these samples did not result in any further isolations of viruses. For routine purposes, it is probable that 2 passages are adequate, allowing more samples to be screened. Exceptions would be where searching for slower adapting viruses such as coronaviruses, where several more passages would be necessary.

BIOLOGICAL CHARACTERISTICS OF THE ISOLATES

Of the 7 viruses isolated, one (D74/25A) was suspected on the basis of it's CPE in bovine lung cells of being bovine viral diarrhoea (BVD) virus. Upon confirmation of this identity by neutralisation test with positive antiserum to BVD virus (Serological studies of isolates), this virus was not studied in detail.

Five of the viruses isolated (D74/2, D74/18, D74/19A, D74/19B, D74/22-4) were subsequently identified as being enteroviruses. Throughout the remainder of the text, they are collectively referred to as the enterovirus isolates. The remaining virus (D74/13-49) is referred to by it's laboratory designation.

Materials and Methods

(a). Cytopathic effects of isolates

The cytopathic effects of the isolates were examined in unstained and haematoxylin and eosin stained (Appendix 3) infected cell monolayers. The enterovirus isolates were studied in MDBK cells, isolate D74/13-49 in Vero cells, and the BVD isolate (D74/25A) in foetal bovine lung cells.

(b). Acridine orange staining

This stain has been used under a variety of conditions of pH and concentration by various authors. Because of this variation, and the emphasis laid by some authors on the importance of concentration (Schummelfeder, 1958) and pH (Anderson *et al.*, 1959), it was decided to examine the effect of varying the staining over a limited pH range, using 2 concentrations of dye for varying periods of time.

Uninoculated monolayers of MDBK cells were stained with acridine orange following the procedure described in Appendix 3, but using the dye at concentrations of 0.05% and 0.01% in acetate

buffer at pH 3, 3.5, 4 and 4.5. Staining times were varied from 5 to 30 minutes.

Based on the results of the above experiments, MDBK cell monolayers infected with enterovirus isolates were examined following 15 minutes staining with 0.01% acridine orange in acetate buffer at pH 4, following the procedure described in Appendix 3. Vero cell monolayers infected with reovirus 3 and uninfected MDBK and Vero cell cultures were used as controls.

(c). Coriphosphene O staining

Monolayers of MDBK cells infected with the enterovirus isolates, and Vero cells infected with isolate D74/13-49, were stained using coriphosphene O stain as described in Appendix 3. Control preparations were the same as that used for the acridine orange stain.

(d). Host cell range of isolates

Duplicate monolayers of foetal bovine thyroid, testis, kidney, and lung cells, and of MDBK and Vero cell lines, were each inoculated with 100 $TCID_{50}$ of each of the enterovirus isolates, and incubated at 37°C. The monolayers were observed for the development of cpe, and when this affected about 75% of the cells, the affected cultures were frozen and thawed, the duplicates pooled, and the titre of virus subsequently determined in triplicate tube cultures of MDBK cells, as described in the general materials and methods. Further duplicate monolayers of the same cells were inoculated with 0.1 ml aliquots of undiluted, freeze-thawed infective culture fluid of isolate D74/13-49, and with 100 $TCID_{50}$ of isolate D74/25A, and observed for the development of CPE. In addition, 100 $TCID_{50}$ of the enterovirus isolates was inoculated into duplicate monolayers of neonatal canine kidney and lung cells, and these observed for the development of CPE.

(e). Haemagglutinating properties of isolates

The 7 isolates were tested for haemagglutinating properties according to the procedure described in the general materials and methods, using duplicate test wells, and dilutions from 1 in 10 to 1 in 1280. Each virus was tested against human type O, guinea pig and bovine erythrocytes, using incubation temperatures of 4°C, 20°C and 37°C. Tests were read when the erythrocyte control cells had settled to a distinct button.

(f). Plaque production by enterovirus isolates

(i) Confluent duplicate monolayers of MDBK cells in 50 ml plastic flasks were washed with PBS and then inoculated with 0.1 ml aliquots of tenfold dilutions of the enterovirus isolates. Following incubation at 37°C for 1 hour, the monolayers were overlaid with medium 199 containing 2% equine serum, antibiotics and 1% agar, as described in Appendix 2. Following solidification of the overlay at room temperature, the cultures were incubated at 37°C for 2 days, following which they were given a further overlay of the same medium but also containing 0.01% of neutral red dye. After a further incubation at 37°C in the dark for 18 hours, the cultures were observed for plaque production. Plaque counts in duplicate flasks at the highest dilution containing virus were averaged, and the titre expressed as plaque forming units (PFU) per 0.1 ml.

(ii) Following determination of the titres of the stocks of the enterovirus isolates, the plaque tests were repeated in duplicate flasks, using dilutions of inocula calculated to contain about 40 PFU per 0.1 ml. The procedure varied from that described above in that after the intial adsorption period of 1 hour, the monolayers were washed twice with PBS to remove unadsorbed virus so as to reduce the production of late developing plaques. One set of enterovirus infected monolayers were overlaid with the agar medium described above, and the other set was

overlaid with a similar medium in which 1% seaplaque agarose[1] replaced the agar. Cultures were held at 4°C for 10 minutes to hasten solidification of the agarose, and then incubated at 37°C for 4 days, with a second overlay incorporating 0.01% neutral red dye being added at the 3rd day. They were then observed for plaque production and photographed.

(c) The plaque tests were also repeated in 24-well Linbro plates, using replicate MDBK cell monolayers infected with approximately 40 PFU of isolate D74/18. One set of wells was overlaid with agar medium containing 0, 1, or 2% equine serum, and the other set was overlaid with medium containing 2% methyl cellulose instead of agar, and the same serum concentrations as above. The monolayers were observed microscopically for plaque development over the next 3 days.

(g). Effect of additives on enterovirus plaque production

Further replicate monolayers of MDBK cells in plastic flasks were infected with approximately 40 PFU of enterovirus isolate D74/18, and following adsorption for 1 hour and washing with PBS, were overlaid with agar or seaplaque agarose media prepared as above, but with the further incorporation of additives as follows:

diethyl aminoethyl dextran (DEAE-dextran) (25 to 400 µg/ml)
protamine sulphate (200 and 400 µg/ml)
magnesium chloride (30 nM)

One culture was used for each treatment, and further cultures were left without these additives as controls. All cultures were incubated at 37°C for 4 days, with a second overlay containing 0.01% neutral red being added at 3 days. They were then observed for plaque development.

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1 Marine Colloids Inc., Rockland, Maine, U.S.A.

(h). Attempts at plaque production with isolate D74/13-49

Replicate Vero cell monolayers in Linbro 24-well plates were infected with tenfold dilutions of isolate D74/13-49, and after adsorption for 1 hour, were overlaid without washing with medium 199 containing antibiotics, 1% seaplaque agarose, and further additives as follows:

(i) 1% equine serum.
(ii) " " " plus DEAE-dextran (50 to 400 µg/ml).
(iii) " " " " protamine sulphate (200 and 400 µg/ml).
(iv) " " " " magnesium chloride (30 mM).
(v) " " " " DEAE-dextran and magnesium chloride, as above.
(vi) no serum.

Cell controls and inoculated wells subjected to each treatment were held at 4°C for 10 minutes to solidify the agarose, incubated at 37°C for 7 days, and checked daily for plaque production by microscopic observation.

(i). Plaque purification of enterovirus isolates

MDBK cell monolayers in 50 ml plastic flasks were inoculated with approximately 20 PFU of each of the enterovirus isolates, adsorbed for about 1 hour, washed twice with PBS and then overlaid with agar medium as above, with no additives. Following solidification at room temperature, the cultures were inverted and incubated at 37°C for 4 days.

Plaques were located without staining, and a single isolated plaque at least 1 cm distant from it's nearest neighbour was removed for each isolate, by aspiration into a sterile glass tube. The plug of agar removed with the associated virus plaque was added to a small volume of medium, and then frozen and thawed, to aid diffusion of the virus from the agar. A 0.1 ml volume of this medium was then used to inoculate a further monolayer, which

was treated similarly. The virus from the second plaque was then grown up into viral stocks, which were frozen and titrated before use.

(j). One step growth curve

Isolate D74/18 was selected for use with this procedure, which is based on that described by Mattson *et al.*, (1969).

(i) A number of MDBK cell monolayers in tubes were prepared, and when confluent, the average count of cells per tube was determined by trypsinisation and counting in a haemocytometer. This was found to be 5×10^5 cells per tube.

(ii) The remaining tube cell cultures were infected with a dose of virus calculated to give a tenfold multiplicity of infection.

(iii) Following adsorption for 30 minutes at 37°C, the monolayers were washed 7 times with PBS to remove free virus, and then maintenance medium was added.

(iv) After equilibration for 10 minutes, the cultures were incubated at 37°C, this being taken as time zero for the purpose of the experiment.

(v) Cultures were harvested in duplicate at 0, $\frac{1}{2}$, 1, 2, 3, 4, 5, 6, and 8 hours.

(vi) Culture media from duplicate cell cultures were pooled together with the first 2 washings from the cell monolayers (see below), and centrifuged at 1000 G for 10 minutes. The supernatent fluid was then stored frozen, and any deposited cells washed 3 times in PBS by centrifugation.

(vii) The cell monolayers from duplicate cultures were washed 3 times with PBS for incubation times up to 2 hours, and 7 times with further incubation up to 8 hours. The first 2 washes were added to the culture medium as above.

(viii) A small volume of medium was added to each washed cell monolayer, together with recovered cells from the original medium and washings, and the cells then frozen and thawed 3 times.

(ix) Residual cell debris was washed off the tube walls with a vortex mixer, and the suspension of cell debris from duplicate tubes pooled together in a bottle, together with a further small volume of medium used to rinse the tubes.
(x) The cell debris and the culture medium from each harvest were then titrated in triplicate MDBK tube cell cultures, and the resulting titres expressed as $TCID_{50}$ per 10^6 cells.

Results

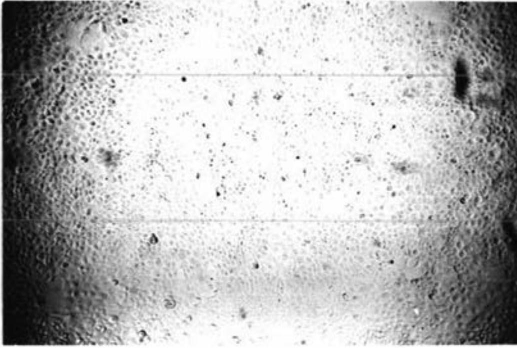
(a). Cytopathic effects of isolates

The enterovirus isolates (D74/2, D74/18, D74/19A, D74/19B, D74/22-4) were all characterised by the early development of foci of round refractile cells. Secondary foci soon developed, and the effect spread rapidly to involve the whole cell sheet.

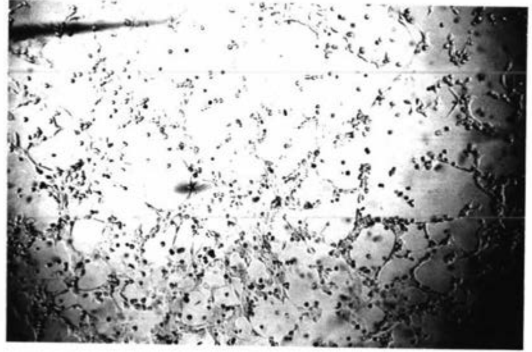
Affected cells were noted to assume a spherical shape and then to shrink, frequently leaving cytoplasmic strands attached to the glass in a dendritic fashion. The cells subsequently detached and floated free in the medium. Occasional cells were noted to develop cytoplasmic vacuolation during the degeneration process.

Haematoxylin and eosin staining revealed that in addition to the above changes, the nuclei became pyknotic, assumed an eccentric position in the cytoplasm, and eventually fragmented. The cytoplasm was frequently seen to develop a paler staining eosinophilic central area inside a more amphophilic staining peripheral margin. Frequently too, a more intensely eosinophilic spherical mass was to be seen inside a vacuolated pale central area.

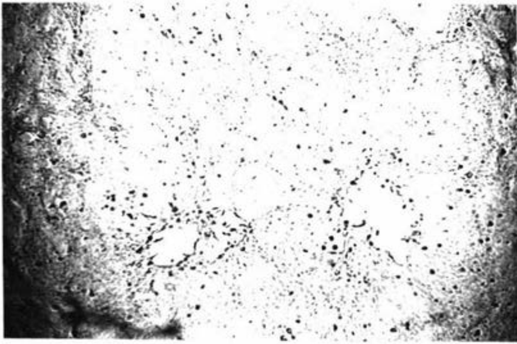
Isolate D74/13-49 differed from the above in that the early foci of cpe were somewhat slower to form, and though other



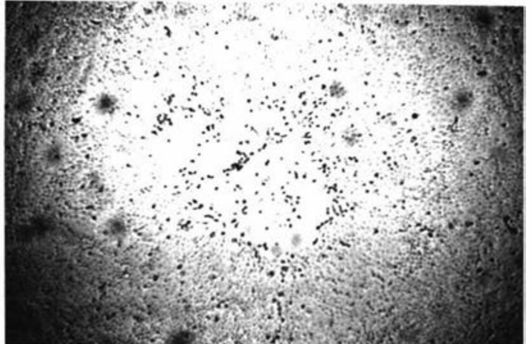
MDBK cell control



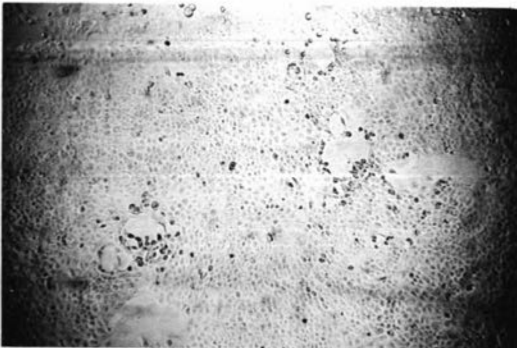
D74/2



D74/18



D74/19A

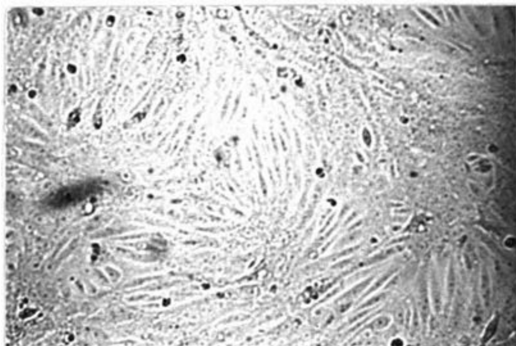


D74/19B

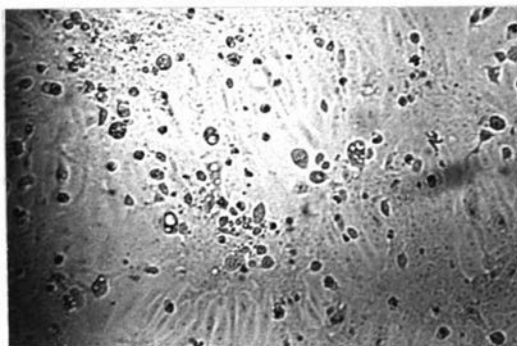
D74/22-4

FIGURE 1

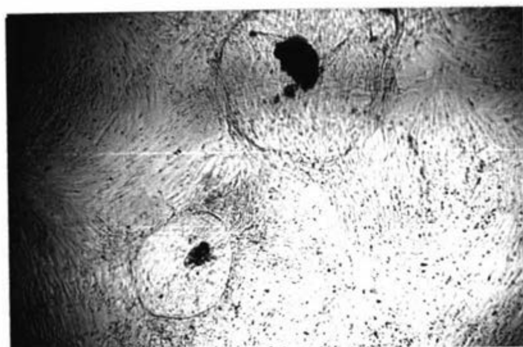
This shows the CPE induced in unstained monolayers of MDBK cells by the 5 enterovirus isolates. Early CPE is shown by isolates D74/18, D74/19A and D74/19B, and late CPE by isolates D74/2 and D74/22-4.



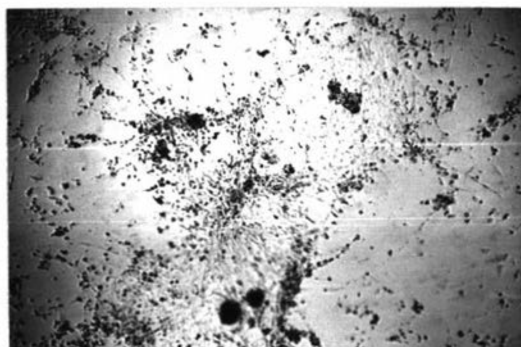
Vero cell control



Vero cells infected with isolate D74/13-49



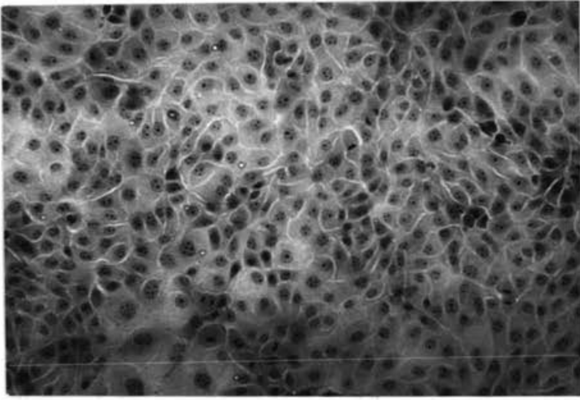
FBL cell control



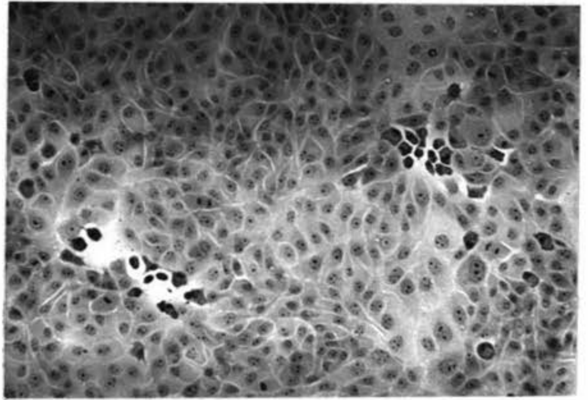
FBL cells infected with isolate D74/25A

FIGURE 2

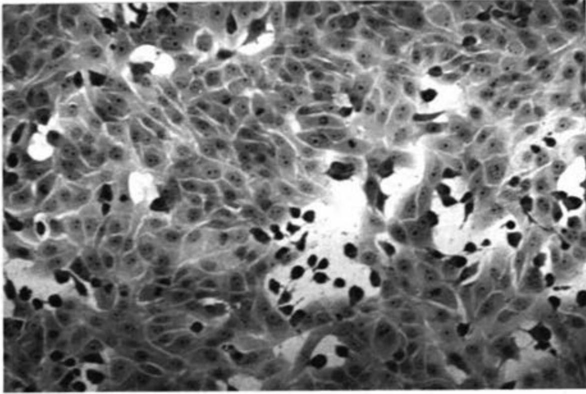
Demonstrates the CPE induced by isolates D74/13-49 and D74/25A in comparison with their respective cell controls.
Note the diffuse nature of the CPE produced by isolate D74/13-49 in Vero cells, and the selective affinity of isolate D74/25A for fibroblastic cells, leaving the epithelial cell islands relatively untouched.



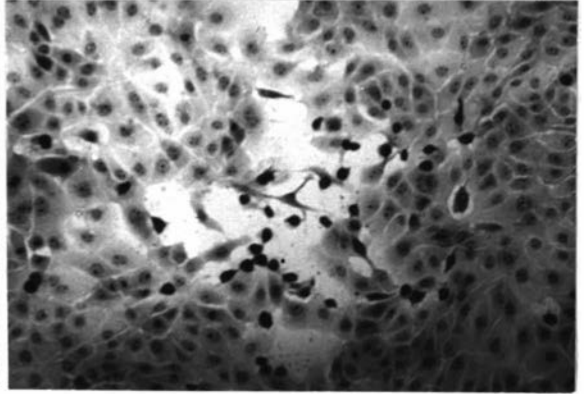
MDBK cell control



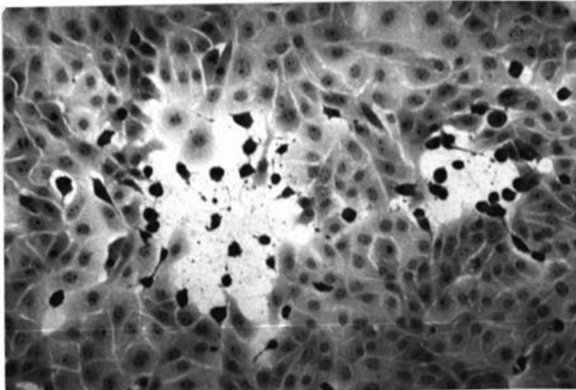
D74/2



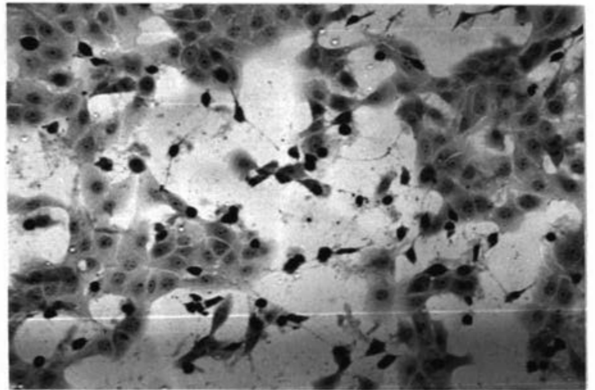
D74/18



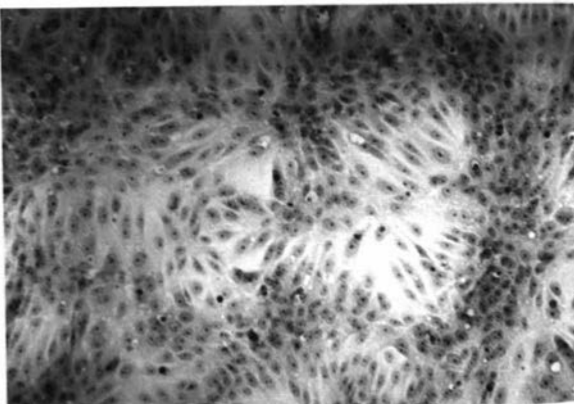
D74/19A



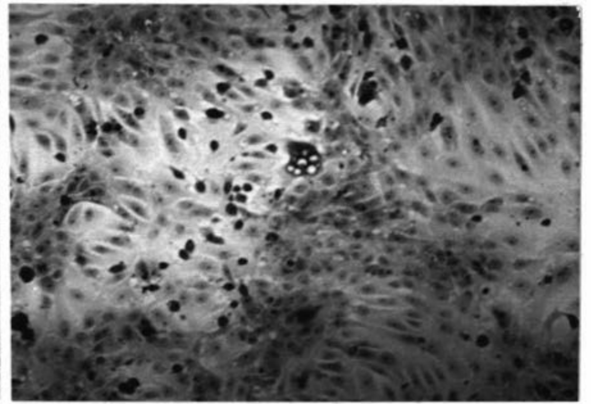
D74/19B



D74/22-4



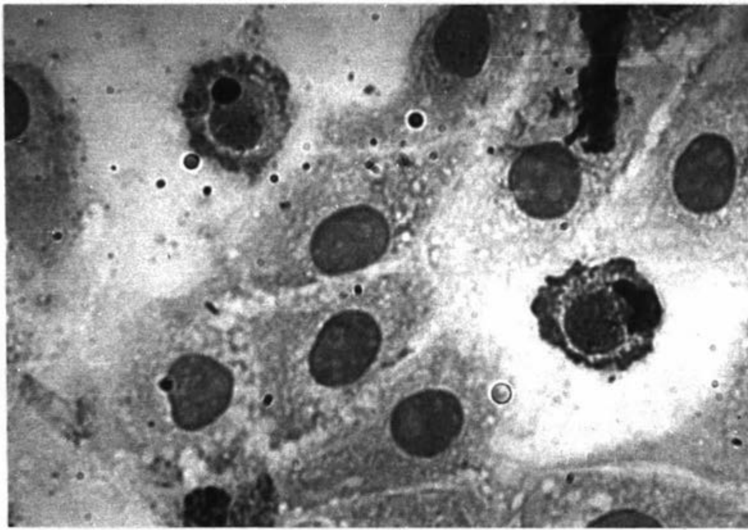
Vero cell control



D74/13-49

FIGURE 3

Haematoxylin-eosin stained cell monolayers showing the CPE induced by 6 of the viral isolates. All isolates are in MDBK cells except D74/13-49 which is in Vero cells. Note the diffuse rounding-up of cells produced by the latter isolate, and the cell showing cytoplasmic vacuolation.



D74/19B

FIGURE 4

Higher magnification of MDBK cells infected with isolate D74/19B. Note the eccentric pyknotic nuclei and the central cytoplasmic inclusion bodies.

foci later developed elsewhere on the cell sheet, there was only slow progression. At no stage were more than a small percentage of the cell population affected. Although the CPE often tended to have a focal distribution, a considerable number of affected cells were also located individually in a scattered fashion.

The cells were noted to assume a round refractile appearance and did not show evidence of shrinkage for some considerable time. No cytoplasmic processes were seen, but cytoplasmic vacuolation was occasionally present, though this was also present to a lesser degree in uninfected control cells. Degenerating cells were slow to detach from the monolayer.

Haematoxylin and eosin staining showed also that the nuclei usually stayed in a central position and showed little change initially. Eventually however they became pyknotic. The cytoplasm showed some increase in staining intensity and in a small proportion of cells contained irregularly shaped eosinophilic inclusion material, which was not seen in healthy control cells, although smaller spherical eosinophilic inclusions sometimes were.

Isolate D74/25A, subsequently identified as BVD virus, was only studied in the unstained state in foetal bovine lung cells. It was seen to cause a focal CPE which soon spread to involve considerable areas of the cell sheet, and was characterised by the production of small round refractile cells which had a tendency to cluster prior to detachment. Affected cells sometimes showed cytoplasmic vacuolation. The virus selectively affected fibroblastic cells, epithelial cells showing little change.

(b). Acridine orange staining

It was found that varying the pH over the tested range made little difference in the final stained appearance. Likewise,

the use of 0.25 and 0.01% concentrations of the dye resulted in very similar staining, though the lower concentration appeared to give a slightly better defined result. Staining for 15 minutes appeared to be adequate.

As a consequence, the procedure adopted involved a 0.01% dye concentration in acetate buffer of pH 4, using a 15 minute staining time (Appendix 3).

Enterovirus affected cells were all noted to show a patchy intensified staining of the cytoplasm. Subsequently, the peripheral cytoplasm (except for the dendritic strands) developed an even more intense staining, surrounding a paler central zone, which sometimes contained a central area of intense orange colour. The chromatin of the nucleus adopted a yellow stained appearance in contrast to the normal green colour, and eventually became a yellow pyknotic mass which finally fragmented.

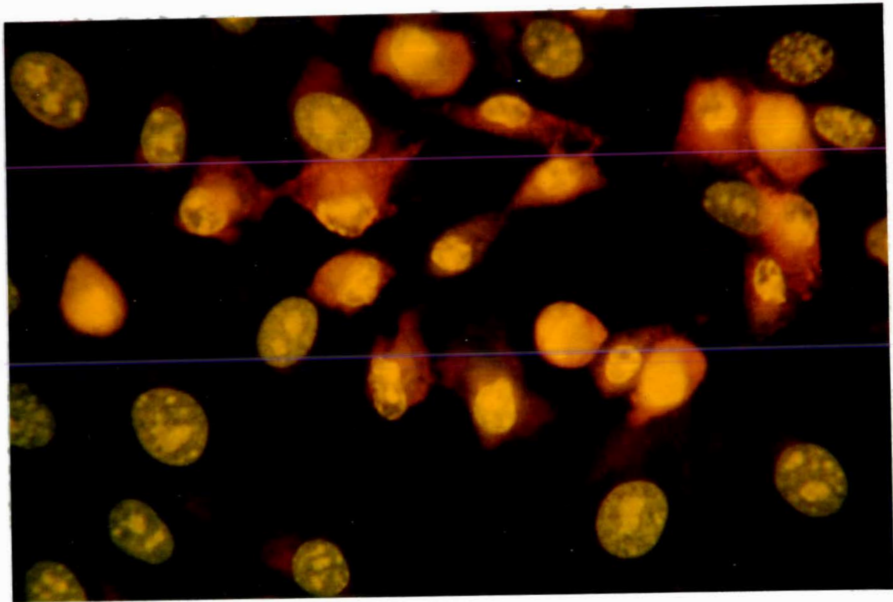
The reovirus 3 used as a further stain control showed the production of well defined green cytoplasmic inclusions located around the nucleus as described by Gomatos _et al._, (1962).

Some fading of fluorescence with prolonged excitation by blue light was noted and caused some problems with photography.

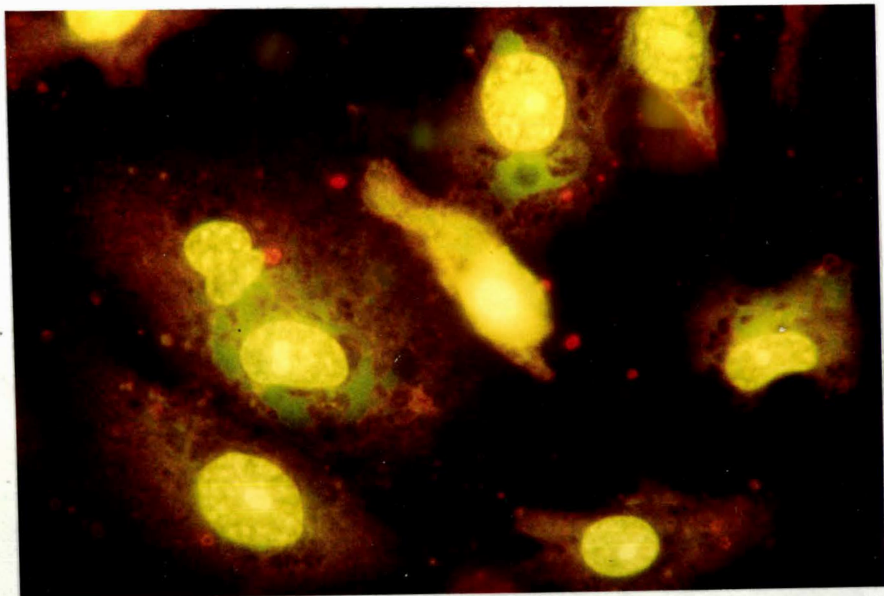
(c). Corisphosphene O staining

Enterovirus infected MDBK cells and reovirus 3 infected Vero cells all showed similar changes to those described for acridine orange, except that the cytoplasmic fluorescence with the enterovirus infected cells appeared a stronger red colour than acridine orange.

The isolate D74/13-49 was also examined with this stain. The cytoplasm of affected cells was noted to show an intensified red-brown staining, and the nucleus showed a yellow colouration of the chromatin, eventually becoming a yellow pyknotic mass.



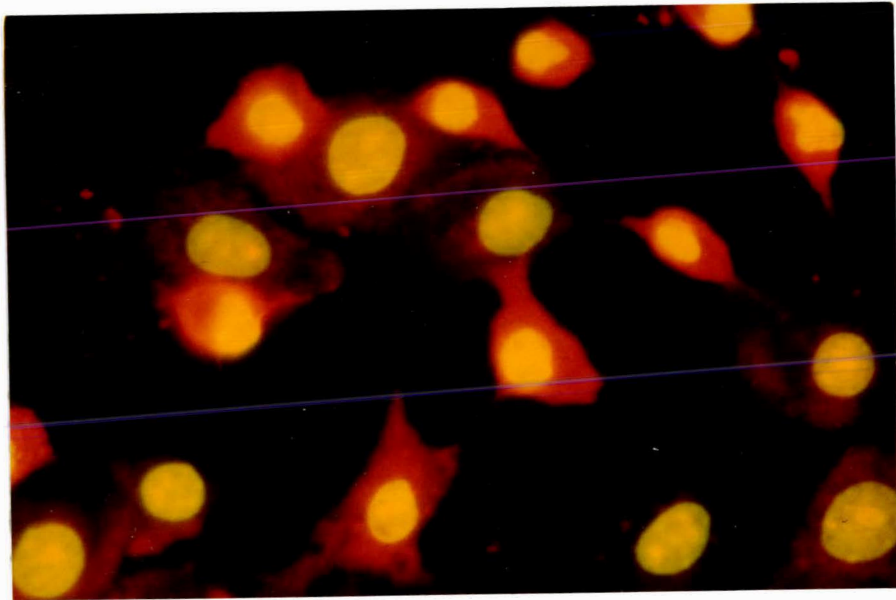
MDBK cell culture infected with enterovirus D74/18 (Acridine orange stain).



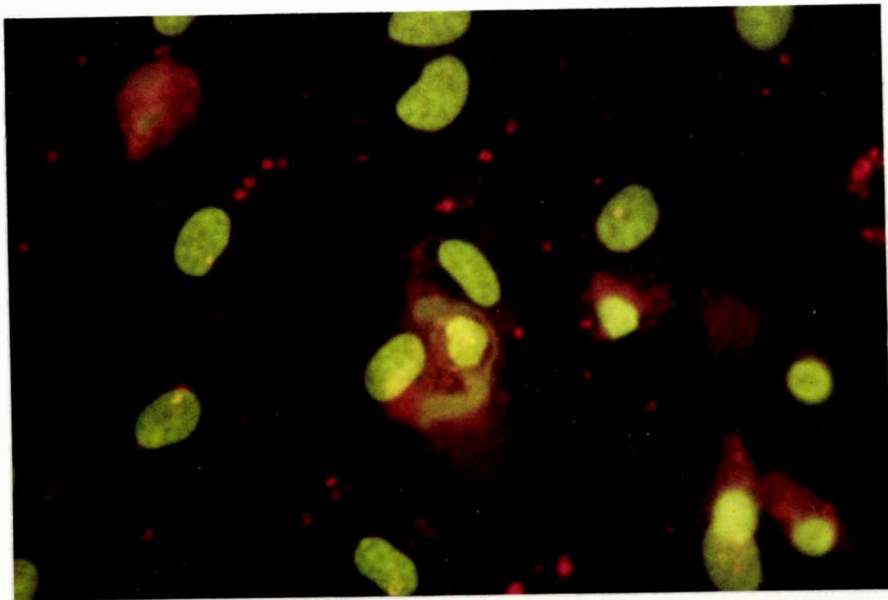
Vero cell culture infected with reovirus 3. (Acridine orange stain).

FIGURE 5

Acridine orange stained MDBK cells infected with enterovirus isolate D74/18. The nuclei of infected cells have become yellow and pyknotic (in contrast to the normal green staining nuclei nearby), and also show increased intensity of cytoplasmic staining. A reovirus 3 infected Vero cell culture is also shown for control purposes, and clearly demonstrates the irregular cytoplasmic inclusions obtained with this virus.



MDBK cell culture infected with enterovirus D74/18 (coriphosphene O stain).



Vero cell culture infected with reovirus 3 (Coriphosphene O stain).

FIGURE 6

Coriphosphene O stained MDBK cells infected with enterovirus isolate D74/18. The nuclei of infected cells have become yellow and pyknotic, (in contrast to the normal green staining nuclei nearby), and also show increased intensity of cytoplasmic staining. A reovirus 3 infected Vero cell culture is also shown for control purposes, and clearly demonstrates the irregular cytoplasmic inclusions due to this virus.

Two types of green cytoplasmic inclusions were seen. One consisted of discrete spherical objects of variable size which were seen occasionally in control Vero cells, but were much more numerous and occasionally multiple in infected cell monolayers. The other type appeared to commence as an irregular green cytoplasmic area with an indefinite margin, later developing a more discrete appearance, though remaining irregular in shape. These were not seen in control Vero cells.

Monolayers stained with this dye appeared to show little colour change with prolonged exposure to blue light.

(d). Host cell range of isolates

Table 2 shows the susceptibility of the tested cell types to the production of CPE by the various virus isolates. The enterovirus titration results in the 6 types of cells where they produced CPE are shown in Table 3.

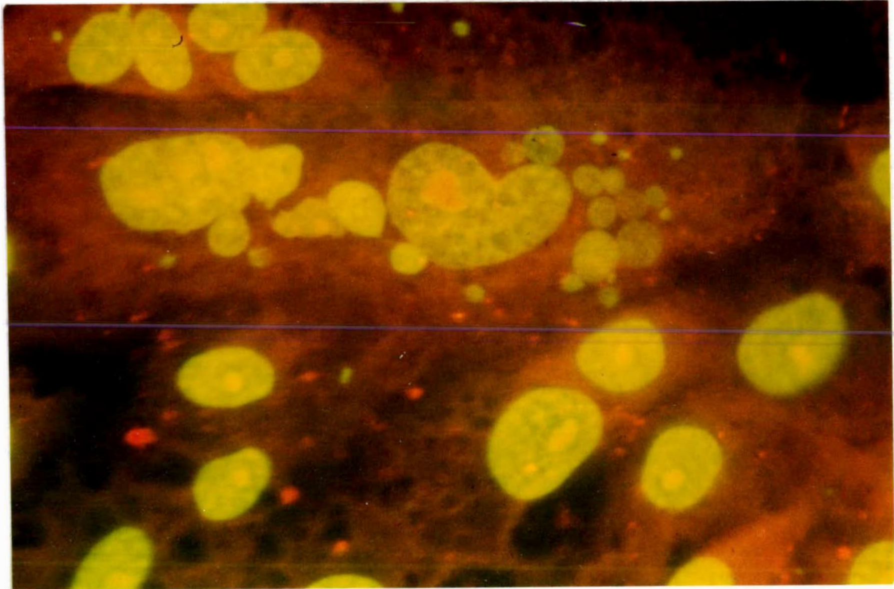
Table 2

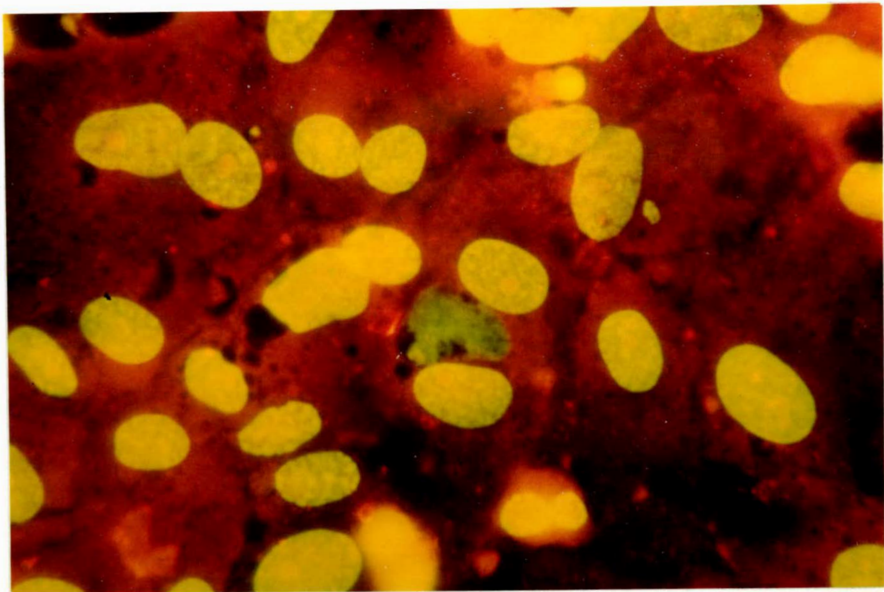
The production of CPE in various cell cultures by the 7 isolates

Cell culture	Virus isolate						
	D74/ 2	D74/ 13-49	D74/ 18	D74/ 19A	D74/ 19B	D74/ 22-4	D74/ 25A
F.B. thyroid	+	-	+	+	+	+	+
F.B. test's	+	-	+	+	+	+	+
F.B. kidney	+	-	+	+	+	+	-
F.B. lung	+	-	+	+	+	+	+
MDBK cells	+	-	+	+	+	+	-
Vero cells	+	+	+	+	+	+	-
N.C. kidney	-	N.T.	-	-	-	-	N.T.
N.C. lung	-	N.T.	-	-	-	-	N.T.

+ = CPE produced
- = no CPE produced

N.T. = not tested
N.C. = neonatal canine
F.B. = foetal bovine

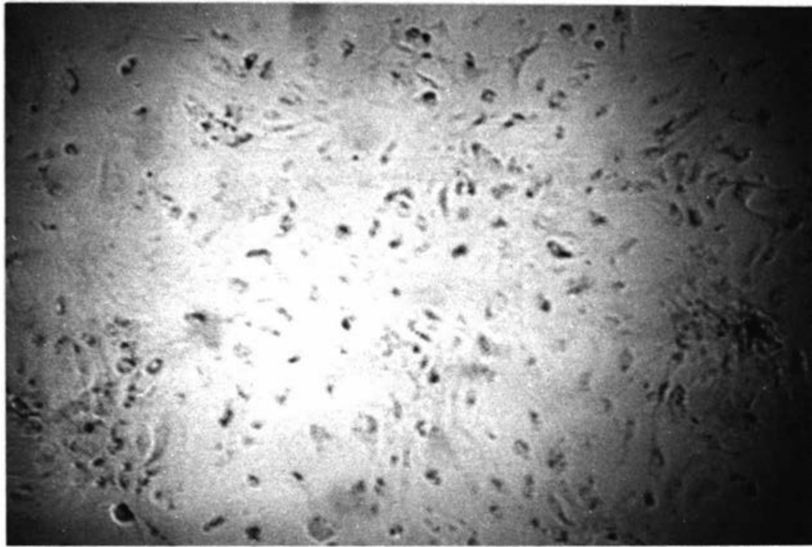




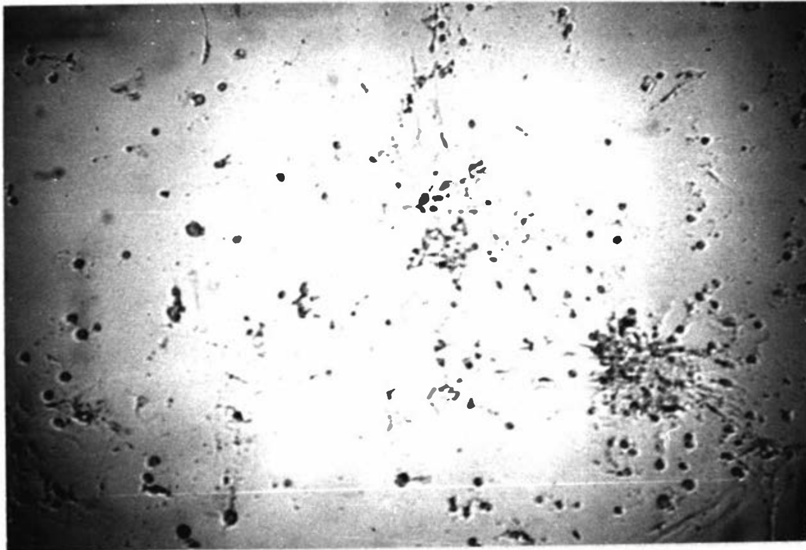
Vero cell cultures infected with isolate D74/13-49. (Coriphosphene O stain).

FIGURE 7

Coriphosphene O stained Vero cell cultures infected with isolate D74/13-49. Note the two types of cytoplasmic inclusion produced, one a discrete globular body, and the other irregular and somewhat diffuse.



Foetal bovine thyroid cell control.



Foetal bovine thyroid cell culture
infected with isolate D74/18.

FIGURE 8

Foetal bovine thyroid cell culture infected with enterovirus isolate D74/18, and uninfected cell control.

Table 3

The titres of enterovirus isolates in cell cultures in which they produced CPE

Cell culture	Titre of enterovirus isolate ($\log_{10}$ $TCID_{50}$ /0.1 ml)				
	D74/2	D74/18	D74/19A	D74/19B	D74/22-4
MDBK cells	5.75	5.25	4.75	5.75	4.75
F.B. kidney	5.25	4.75	4.5	4.75	4.5
F.B. lung	5.75	5.5	5.25	5.25	5.25
F.B. testis	5.75	5.25	5.25	4.75	4.5
F.B. thyroid	4.5	4.25	3.75	4.25	4.25
Vero cells	4.75	5.25	4.5	4.75	4.5

F.B. = foetal bovine

(e). Haemagglutinating properties of isolates

The seven isolated viruses (D74/2, D74/13-49, D74/18, D74/19A, D74/22-4, D74/25A) all appeared at 1 in 10 dilution to possess no haemagglutinins to human type O, guinea pig, or bovine erythrocytes at 4°C, 20°C, or 37°C.

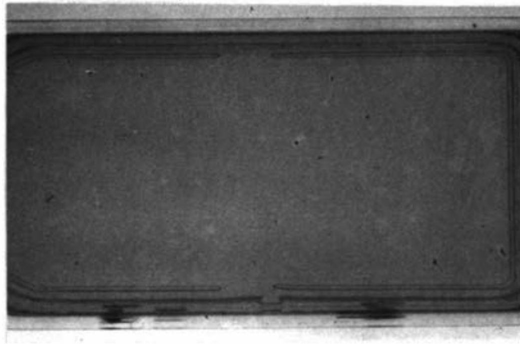
(f). Plaque production by enterovirus isolates

The results of plaquing the 5 enteroviruses isolates under agar and seaplaque agarose media are shown in Table 4. Average plaque sizes were obtained by estimation.

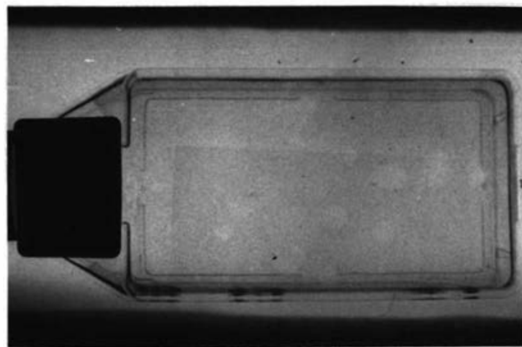
Table 4

Plaque development at 4 days under agar and seaplaque agarose media

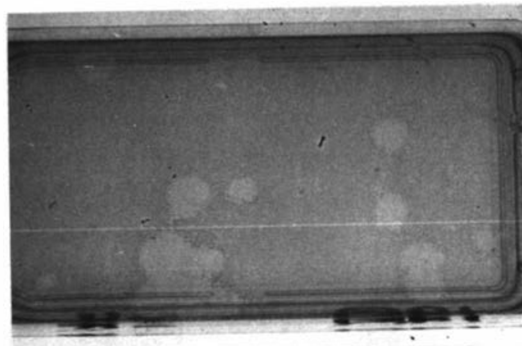
Virus isolate	Sizes of plaques under agar medium (mm)		Sizes of plaques under seaplaque agarose medium (mm)	
	range	average	range	average
D74/2	1-3	2	1-3	2
D74/18	3-5	4	2-7	4
D74/19A	2-8	5	2-6	5
D74/19B	0.5-2	1	0.5-2.5	1
D74/22-4	2-8	5	3-9	7



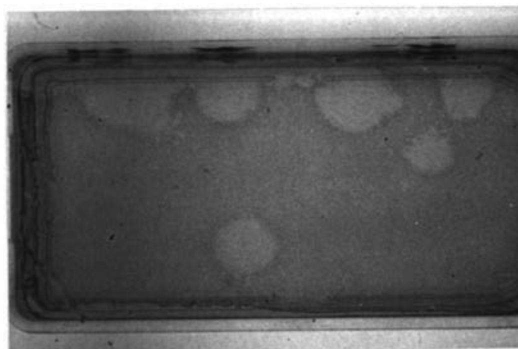
D74/2



D74/18



D74/19A



D74/22-4

FIGURE 9

Plaque development of 4 of the enterovirus isolates at 4 days under agarose. Note the very small size of plaques with isolate D74/2, and the large plaques with isolates D74/19A and D74/22-4.

Considerable variation was noted in plaque sizes within each viral isolate. Nevertheless, differences in average plaque size between the viral isolates were easily recognisable. Isolates D74/19A and D74/22-4 both produced large plaques, in contrast to the small plaques of D74/2 and D74/19B. The plaques of D74/18 were of an intermediate size. Plaques were all of a circular type, with sharp edges, and clear unstained centres. The margins of the plaques showed some increased affinity for the stain.

Generally, the use of agar and seaplaque agarose resulted in the production of similar sized plaques, with the exception of D74/22-4, where larger plaques were produced under the agarose overlay. Comparison of plaque numbers under either overlay was not attempted, as the monolayers were washed after the period of adsorption, to make studies of plaque morphology and growth easier.

The use of methyl cellulose in the overlay with isolate D74/18 resulted in a much slower development of plaques, being only about hald the size of plaques grown under agar. Varying the concentration of serum from 0% to 2% in either agar of methyl cellulose overlays made no difference in rate of plaque growth. Because of the poorer results, methyl cellulose was not examined further as an overlay.

(g). <u>Effect of additives on enterovirus plaque production</u>

The results of using various additives in the overlay medium are shown in Table 5. Average plaque sizes were obtained by estimation.

Table 5

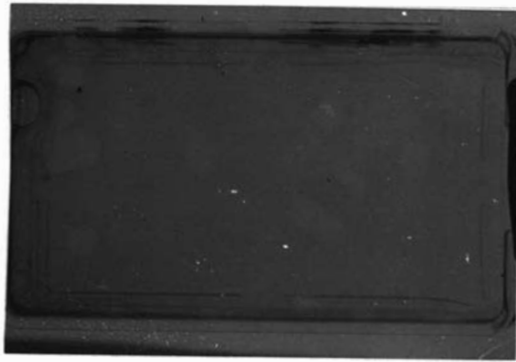
Effect of additives on enterovirus D74/18 plaque production at 4 days, using agar and agarose overlay media

Additive concentration	Plaque size under agar		Plaque size under agarose	
	range (mm)	average (mm)	range (mm)	average (mm)
none	3-5	4	2-6	4
DEAE-dextran				
25 µg/ml	NT	NT	4-6	5
50 µg/ml	NT	NT	4-6 (T)	5 (T)
100 µg/ml	2-7	4	(T)	(T)
250 µg/ml	2-		NT	NT
400 µg/ml	2-7	5	NT	NT
protamine sulphate				
200 µg/ml	NT	NT	2-5	4
400 µg/ml	2-6	4	(T)	(T)
$MgCl_2$ 30 mM	2-10	7	6-9	8

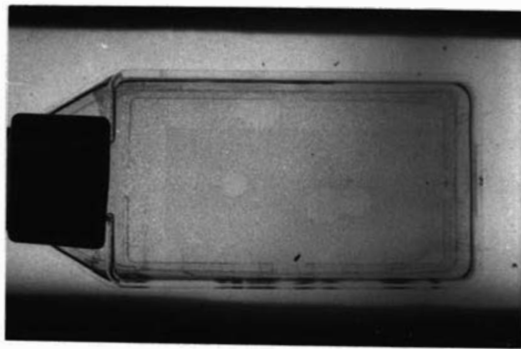
NT = not tested (T)= toxic overlay

DEAE-dextran caused a slight increase in plaque size in both agar and agarose overlays. This effect was seen at low doses of DEAE-dextran in agarose, but required much higher dosage in agar before the effect was seen. Concentrations of the polymer of 50 µg/ml and above were toxic to the cells under agarose overlay.

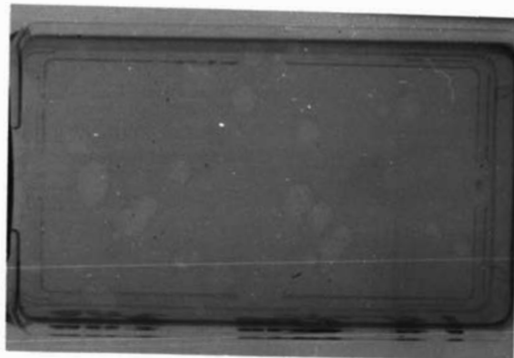
Protamine sulphate caused no effect on plaque size at the concentrations used, though toxicity was noted at the higher dose rate in agarose. In contrast, magnesium chloride at the concentration used, caused considerable enhancement of plaque growth, this effect being most evident in the agarose overlay.



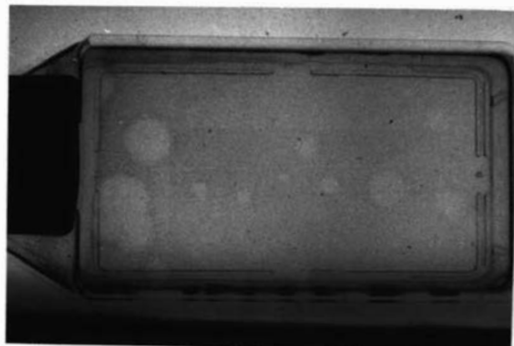
No additives



400 µg/ml DEAE dextran



400 µg/ml Protamine sulphate



30 mM $MgCl_2$

FIGURE 10

Plaque development at 4 days of isolate D74/18 under agar, in the presence of the designated additives. Note the larger plaques developed in the presence of $MgCl_2$.

(h). Attempts at plaque production with isolate D74/13-49

Small numbers of scattered round refractile cells were noted for form at 10^{-2} dilution of virus beneath overlays containing no serum, serum, and serum plus protamine sulphate. These did not appear to progress. Vero cells overlaid with DEAE-dextran at 50 µg/ml still developed some toxic changes after 2 days, no CPE being recognisable.

The incorporation of magnesium chloride in the overlay resulted in the development of foci of round refractile cells at a dilution of 10^{-2} and 10^{-3} of virus. This effect was even more evident in the overlay containing a mixture of magnesium-chloride and DEAE-dextran. However, although some progression of the foci was evident, it was too slow to result in macroscopic plaque formation.

One interesting finding related to the use of protamine sulphate in the overlay. It was found that Vero cells persisted in a much healthier state when this chemical was in the overlay medium. A small side experiment was made using tube cultures of Vero cells in maintenance medium containing 400 µg/ml of protamine sulphate and no solidifying agent. Vero cells were still found to persist in a relatively healthy state without medium change for up to 18 days, whereas control cells without the chemical commenced to degenerate at about 7 days.

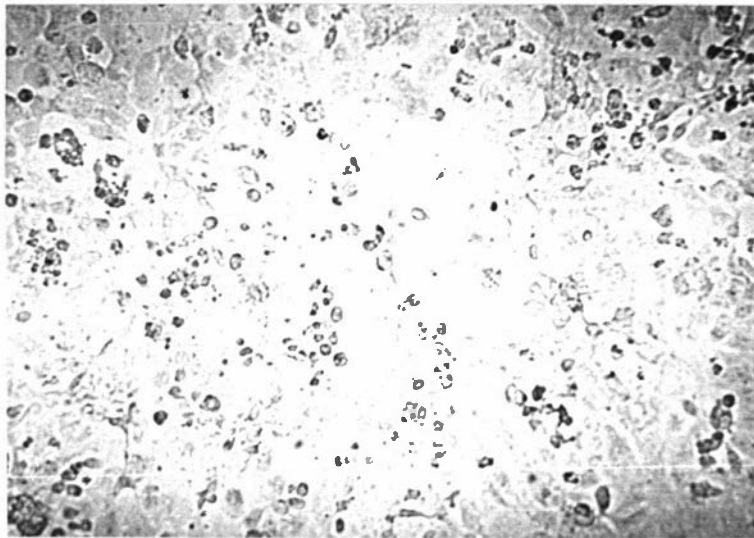
(i). Plaque purification of enterovirus isolates

The method adopted for double plaque purification of the enterovirus isolates posed no particular problems, though the second purification step had to be repeated at slight dilution for 2 viruses, as the plaques were too numerous for selection of well spaced plaques.

(j). One step growth curve

The results of the one step growth curve with isolate D74/18 are shown graphically in Figure 13 More detailed

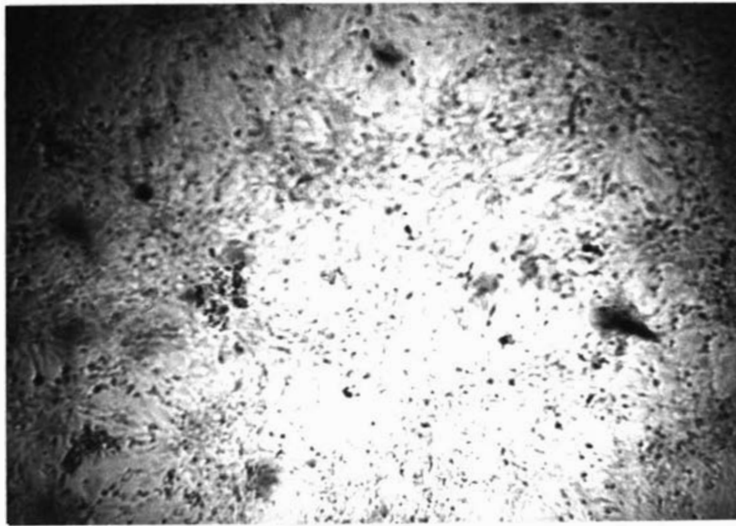
Vero cell control.



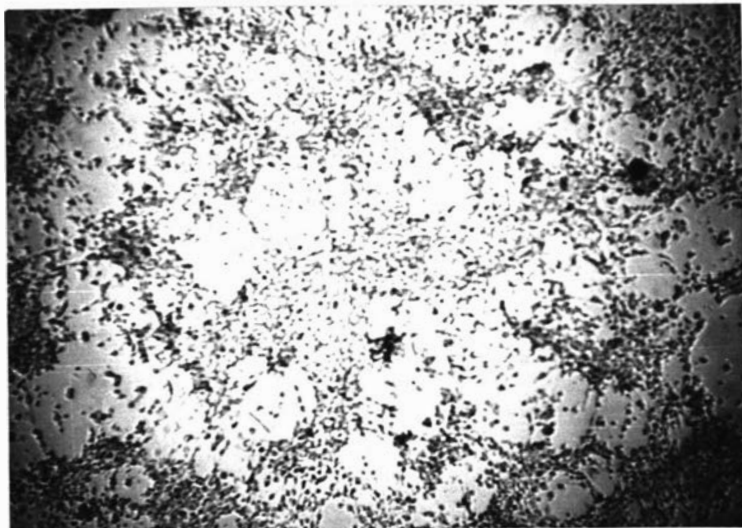
Vero cells with D74/13-49
microplaques.

FIGURE 11

Vero cell culture infected with isolate D74/13-49, and non-infected control cells, both overlaid with agarose medium containing supplemental $MgCl_2$ and DEAE dextran. Note the small microplaques forming in the infected culture.



Vero cell culture at 15 days, with protamine sulphate.



Vero cell culture at 15 days without protamine sulphate.

FIGURE 12

Vero cell cultures at 15 days, with and without protamine sulphate in the nutrient medium. Note the much healthier state of cells maintained in the presence of protamine sulphate.

data are presented in Appendix 4 (Table A).

The eclipse phase lasted about 1 hour, and was followed by a rapid increase in cell associated virus. The average maturation time was found to be approximately $3\frac{1}{2}$ hours with maximal production being reached at about $4\frac{1}{2}$ hours.

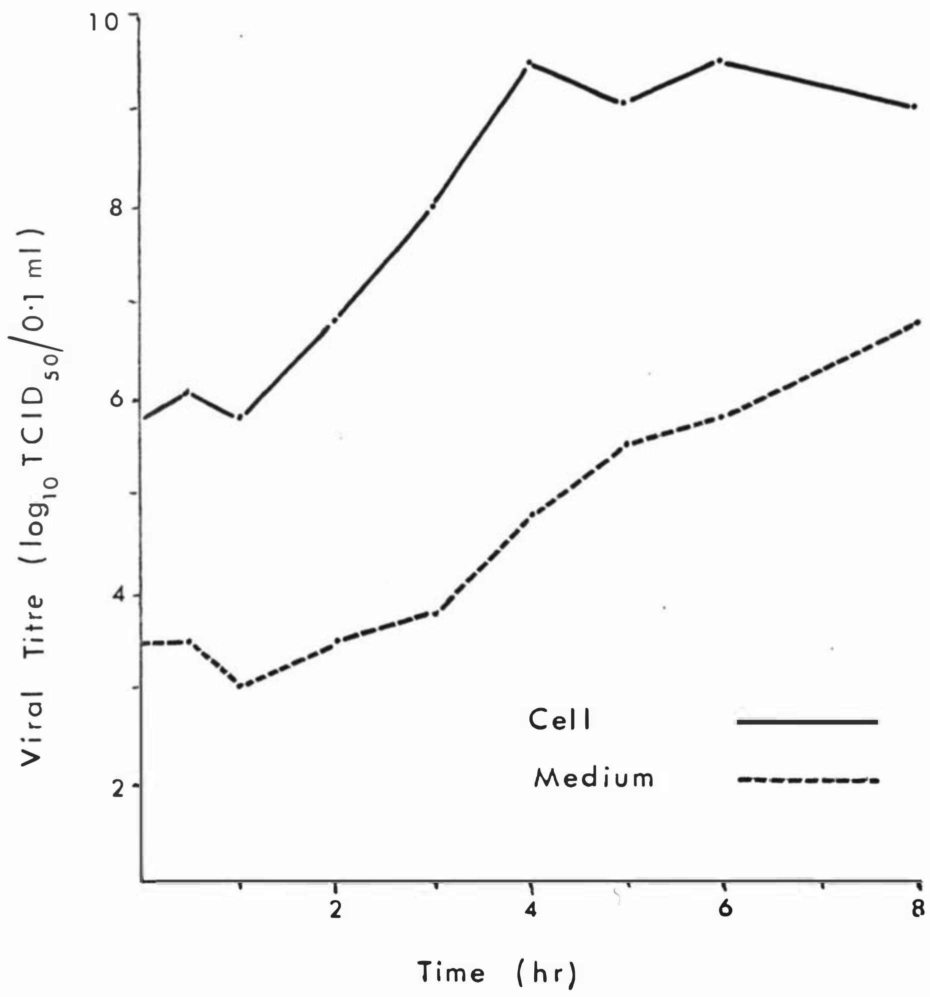
Increase in extracellular virus was detected at about 3 hours, being somewhat slower in it rate of development than cell associated virus. Viral release was less than 1% complete at 8 hours after time zero.

Despite a tenfold multiplicity of dose rate, the titres of cell associated virus indicate that slightly less than 100% of the cells appear to have been infected during the 30 minute adsorption period, though the exact percentage was not able to be determined, due to the limitations of the triplicate tube titration technique. Nevertheless, theproduction of virus was quite high, being in the vicinity of 2000 $TCID_{50}$ per cell.

Discussion

The cytopathic effect produced by the enterovirus isolates in MDBK cells is similar to that described by several authors studying bovine enteroviruses (Moll and Davis, 1959: Spradbrow, 1963, 1964; Rovozzo et al., 1965) and human enteroviruses (Wenner, 1962; Melnick and Wenner, 1969).

Similar eosinophilic intracytoplasmic inclusion bodies have been commented on by Barski (1962) and Melnick and Wenner (1969) with human poliovirus. Rifkind et al., (1961) confirmed the presence of these bodies electron microscopically and found them to be due to the localised production of ultra-microscopic vesicles in the juxtnuclear region. Intracytoplasmic inclusion bodies have also been described with bovine enteroviruses by Spradbrow (1963, 1964) and Rovozzo et al., (1965), though the latter authors found them only with 2 out of their 3 isolates, and then only inconstantly.



10
8
6
4
2
Viral Titre (log$_{10}$ TCID$_{50}$/0·1 ml)
Cell
Medium
2
4
6
8
Time (hr)

FIGURE 13

One step curve with isolate D74/18. Viral replication is seen to occur in the cell in approximately 1.5 hours, but release of virus into the medium does not commence till about 3 hours.

The acridine series of dyes, which includes both dyes used in this study, have been reported to differentially stain double stranded and single stranded nucleic acids (Jawetz et al., 1974), possibly due to distortion of the dye molecule during it's incorporation between the 2 strands of the double stranded nucleic acid (Lerman, 1964). Using these dyes on enterovirus infected cells showed some early localised increase in cytoplasmic staining, but did not differentially stain the inclusion material, suggesting that no double stranded nucleic acids were present in the cytoplasm. The greater stability of corisphosphene O under blue light excitation compared with acridine orange as seen in this study, was also commented upon by Keeble and Jay (1962).

The rapid production and spread of CPE with the enterovirus isolates, together with their fairly wide host cell spectrum, is in accordance with previously published reports (Moll and Davis, 1959; Moscovici et al., 1961) on bovine enteroviruses. Though no CPE was produced in canine cells, it was not determined whether viral multiplication had taken place.

The lack of haemagglutination with the enterovirus isolates was not unexpected. Bovine enteroviruses have been tested against a wide range of species of erythrocytes at various temperatures, frequently with no success, (Moll and Ulrich, 1963; Rovozzo et al.,1965). However, Moscovici et al., (1961) and Mattson and Reed (1974) reported haemagglutination with guinea pig and monkey erythrocytes at 4°C with some of their isolates. La Placa et al., (1963) and Schiott and Hyldgaard-Jensen (1966) also confirmed haemagglutination of some isolates with monkey erythrocytes. Occasional reactions to human type O and bovine erythrocytes have also been demonstrated by Moscovici et al., (1961). It was not readily possible to determine whether the viruses isolated in this study were capable of haemagglutinating monkey erythrocytes, though it would appear that these are the red cells of choice. La Placa et al., (1965) used haemagglutination with these cells to subdivide bovine enteroviruses into

2 classes, these showing close correlation with their antigenic characters.

The production of plaques by bovine enteroviruses has been recorded by several authors (Moscovici _et al_., 1961; McFerran, 1962a; Gratzek _et al_., 1967). The plaques produced by these isolates were all of a circular type with sharp edges and clear centres, similar to some of those described by Moscovici _et al_., (1961).

There was considerable variation in plaque size within each isolate, though this has also been reported by McFerran (1962a) with bovine enteroviruses. Even using plaque purified enteroviruses, Moscovici _et al_., (1961) still found some variation in plaques within isolates. Despite the above variation, however, there were marked differences in plaque sizes between viral isolates, the sizes being similar to those described by Moscovici _et al_., (1961).

Substitution of seaplaque agarose for agar in the overlay resulted in increased plaque size with 1 isolate, D74/22-4. This effect has been claimed to be due to the low level of sulphated mucopolysaccharides in the purified agarose, but more recently it has been suggested by Wallis and Melnick (1968a) that it is due to the lower hydrogen ion concentration present in the agarose, as a consequence of the purification procedure.

The use of methyl cellulose in the overlay resulted in the retarded growth of plaques with isolate D74/18. Working with human poliovirus, Wallis and Melnick (1968b) also found that plaques grown under methyl cellulose overlays were diminished in size and number, and suggested this is due to the more acid nature of this medium.

The enhancement of plaque formation by DEAE-dextran was relatively small, but was in accordance with the

findings of Wallis et al., (1966a) and Wallis and Melnick (1968a), for some of their isolates. The lower level of the DEAE-dextran needed to produce this effect in agarose overlays is probably due to the low level of sulphated mucopolysaccharides mentioned previously. Wallis and Melnick (1968a, b) suggested that the sulphated mucopolysaccharides present in agar bind the major part of the DEAE-dextran, leaving little or none to enhance plaque growth. A similar mechanism probably explains the difference in toxicity in the 2 overlay media.

Protamine sulphate did not enhance plaque formation with the single virus tested, but did prove toxic at the higher concentration in the agarose overlay. The difference in toxicity in the 2 media is probably for the same reason as with the DEAE-dextran, as Wallis and Melnick (1968a) also found this level of the polymer toxic in starch gel and methyl cellulose overlays, which do not contain any sulphated mocopolysaccharides.

Considerable enhancement of plaque growth was seen in the presence of 30 mM magnesium chloride. This effect was somewhat greater when agarose was used, possibly because of the lower hydrogen ion concentration mentioned previously. Magnesium chloride was also found to enhance plaque growth with many bovine enteroviruses by Wallis et al., (1966a).

The variation in the plaque enhancing activity of the various additives appears to relate to the particular strain of enterovirus under test. Wallis et al., (1966a) found that magnesium chloride enhanced the largest number of strains of enteroviruses, whereas DEAE-dextran and protamine sulphate were much more restricted in their activity. It has been suggested by Wallis and Melnick (1968a) that the enhancing action of the cationic polymers DEAE-dextran and protamine sulphate on entero-virus plaque production is largely related to their positively charged nature, rather than being a direct chemical effect.

Plaque purification of the enteroviruses was carried out in an inverted position and without a further neutral red overlay because of the recommendations of Mosley and Enders (1961), so as to avoid contamination of the selected plaque by virus from another plaque. Two purification steps were considered adequate.

The results of the one step growth curve agree well with the results obtained for a bovine enterovirus by Mattson et al., (1969), and showed that viral replication took place very rapidly. The rate of virus release was somewhat slower than that recorded by these authors, but follows a similar pattern. The yield of about 2,000 $TCID_{50}$ of virus per cell is fairly high, compared with the 800 PFU per cell reported by Mattson et al., (1969). However, higher yields of up to 5,100 PFU per cell have been reported with human poliovirus by Schwerdt and Fogh (1957).

The CPE produced in Vero cells by isolate D74/13-49 was notable for it's slow progression, cytoplasmic inclusions, and lack of intranuclear inclusions and syncytia. It shows some similarity to the CPE produced by viruses of the diplornavirus group, though the lack of haemagglutinating activity with the erythrocytes tested makes it less likely to be a reovirus. Because of the slow nature of the CPE, it proved impossible to titrate this virus by ordinary tube titration techniques.

Attempts at plaque production with isolate D74/13-49 were relatively unsuccessful, though the enhanced focal CPE produced in the presence of magnesium chloride and DEAE-dextran suggests that further investigation may result in a workable plaque titration system. The apparently additive effect of the 2 chemicals in enhancing the development of CPE has previously been reported by Fiala and Kenny (1966) with rhinovirus plaques. It may also be of some relevance that magnesium chloride has been reported by Wallis et al., (1964) and Spendlove and Schaffer (1965) to cause activation of reoviruses following heating to $50^{\circ}C$.

Other substances which were not tried but which deserve investigation of their effect on plaque production and infectivity with this virus, are pancreatin and chymotrypsin. The former enzyme has been reported to induce plaque production with reoviruses (Wallis et al., 1966b) whilst chymotrypsin was found by Spendlove and Schaffer (1965) to enhance reovirus infectivity.

Isolate D74/25A, which was subsequently identified serologically as BVD virus, was not examined in detail. However, the rapid production of CPE in lung cells, coupled with it's non-cytopathic nature in kidney cells, suggests that the use of foetal bovine lung may be a useful and sensitive system for isolation of this virus.

The mode of action of protamine sulphate in promoting the healthy maintenance of the Vero cells is uncertain. Tytell et al., (1962) reported that monolayers of cells under agar overlays were better maintained in the presence of this chemical and attributed this to protamine sulphate overcoming the toxicity of the agar. However, this appears to be an unlikely explanation as the small side experiment mentioned earlier showed that the same effect still occurred in the absence of any solidifying agent. Protamine sulphate consists largely of arginine (Wallis and Melnick, 1968a) and it may be that the release of this substance from the protamine sulphate by cellular enzymes promotes the longer and healthier maintenance of Vero cells.

PHYSICOCHEMICAL PROPERTIES OF THE ISOLATES

Materials And Methods

(a). Filtration studies of the isolates

(i) Undiluted freeze-thawed infected cell culture fluids of each of the enterovirus isolates were filtered through a 450 nm (apd) membrane filter. Freeze-thawed cell culture fluids infected with vaccine strains of infectious bovine rhinotracheitis[1] (IBR) and bovine viral diarrhoea[2] (BVD) viruses were diluted 1 in 10, filtered similarly, and used as virus controls.

Aliquots of each virus were then filtered in sequence through membrane filters of 220, 100, and 50 nm (apd), retaining samples at each step. Attempts at filtering through a 25 nm (apd) membrane filter were not successful. Filters were all checked for correct function after use.

The 200, 100 and 50 nm filtrates were then titrated for viral content, using triplicate tube cultures of MDBK cells for the enterovirus isolates and IBR virus, and FBL cells for BVD virus. Titres were determined after Reed and Muench (1938) analysis.

(ii) Undiluted freeze-thawed Vero cell cultures harvested at 6 days following inoculation with D74/13-49 virus were similarly filtered through 220, 100, and 50 nm (apd) membrane filters. Aliquots of each filtrate together with unfiltered infective culture fluid, were inoculated into duplicate tube cell cultures of Vero cells. Because titration was not possible with this slow growing virus, infectivity of the inocula was determined by the production of CPE at 5 days.

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1. Rhinovax. Tasman Vaccine Laboratories, Upper Hutt, N.Z.
2. Bovax. Tasman Vaccine Laboratories, Upper Hutt, N.Z.

(b) . Sensitivity of the isolates to bromodeoxyuridine (BUDR)

(i) Replicate tube cell cultures, with or without the addition of 100 μg per ml (0.33 mM) of BUDR in the maintenance media, were inoculated with 100 $TCID_{50}$ of each of the enterovirus isolates, and of vaccine strains of IBR and BVD viruses. MDBK cells were used for all viruses except BVD, where FBL cells were used.

Duplicate cultures of either treatment of each virus were harvested at post inoculation intervals as follows: isolate D74/18, at 1, 5, 8, 16, and 24 hours; IBR virus at 1, 12, 24, 48, and 72 hours; BVD virus at 1, 12, 24, 48, 72, and 96 hours; and the remaining 4 enterovirus isolates at 24 hours.

Following harvesting, infected cell cultures were frozen, and subsequently titrated in triplicate tube cell cultures of corresponding type to the above, and titres determined following Reed and Muench (1938) analysis.

(ii) Because of the slow growing nature of isolate D74/13-49, a different procedure was adopted. Replicate bottles of Vero cells were prepared, in maintenance medium with or without the addition of 50 μg per ml of BUDR. (Previous work indicated that 100 μg per ml of BUDR caused some slight toxicity to the Vero cells.)

A 0.1 ml volume of undiluted stock of isolate D74/13-49 was inoculated into a set of cultures, with or without the addition of BUDR. 100 $TCID_{50}$ of reovirus 1 and vaccinia virus were inoculated similarly and used as resistant and susceptible viral controls.

All 3 viruses were serially passaged 3 times at weekly intervals through similar treatments, using 0.1 ml of freeze-thawed culture fluid for reinoculation. Results were

assessed by the presence or absence of CPE, cultures being observed every 2 to 3 days.

(c). Chloroform sensitivity of the isolates

The enterovirus isolates and isolate D74/13-49 were tested for chloroform sensitivity using the method of Feldman and Wang (1961). IBR and vaccinia viruses were used as controls.

Duplicate 1 ml volumes of each virus were prepared, using 1 in 10 dilutions of stocks of each virus except vaccinia, where undiluted stock was used. One sample of each virus was treated with 0.05 ml of chloroform, mixed well for 10 minutes at room temperature, and then centrifuged together with it's untreated control at 1,000 G for 10 minutes, to deposit the chloroform. Supernatant fluids were removed and frozen.

Chloroform treated and control samples were then tested for viral activity as follows: the enterovirus isolates and IBR virus samples were titrated in triplicate tube cell cultures of MDBK cells, the vaccinia samples in triplicate Vero cell cultures,and isolate D74/13-49 was inoculated into duplicate Vero cell cultures and assessed on the basis of development of CPE.

(d). Ether sensitivity of the isolates

The enterovirus isolates were tested for sensitivity to diethyl ether following the method of Andrewes and Horstman (1949), using IBR and vaccinia viruses as controls. Two ml volumes of 1 in 10 diluted stocks of the enterovirus isolates and of IBR virus, and 2 ml volumes of undiluted vaccinia virus stock, were treated with 0.5 ml of diethyl ether, leaving further replicate samples as untreated controls. Samples were then sealed and left for 18 hours at 4°C. The ether was subsequently removed by

evaporation and all samples were then frozen, pending titration.

Vaccinia samples were titrated in triplicate tube culutres of Vero cells, and the other samples in triplicate MDBK cell cultures. Results were assessed following Reed and Muench (1938) analysis.

(e). Sensitivity of the isolates to sodium deoxycholate

The enterovirus isolates, together with IBR and vaccinia virus controls, were tested according to the technique of Theiler (1957), as modified by Calisher and Maness (1973).

Solutions of 0.2% bovine serum albumin in PBS were prepared, with and without the addition of 1 in 1,000 dilution of sodium deoxycholate, and sterilised by filtration. Aliquots of 0.5 ml of each virus suspended in maintenance medium were mixed with an equal volume of either solution, incubated in a $37^{\circ}C$ water bath for 1 hour, and then titrated in triplicate tube cell cultures, using MDBK cells for all viruses except vaccinia, where Vero cells were used. Titres were determined following Reed and Muench (1938) analysis.

(f). Acid sensitivity of the enterovirus isolates

The enterovirus isolates were tested for acid sensitivity using a modification of the method of Mattson *et al.*, (1969). IBR virus diluted 1 in 10 from frozen stocks was used as a susceptible viral control, and enterovirus stocks were used undiluted.

Maintenance media of pH 2.5 and 9.2 were prepared by adjustment with 1N HCl and 1N NaOH respectively. A 0.5 ml volume of viral suspension was added to 2 ml of maintenance medium pH 2.5, to give a final pH of 3. A similar volume of virus was added to 2 ml of normal maintenance medium pH 7.2. Duplicates of

both treatments were incubated in a 37°C water bath, harvesting a set of each treatment at 1 and 3 hours. Acid treated viral samples were then diluted with an equal volume of alkaline maintenance medium pH 9.2, to give a final pH of 7.2. The corresponding control samples were similarly diluted in normal maintenance medium pH 7.2.

Acid treated and control samples were then titrated in triplicate MDBK tube cell cultures, and the titre determined following Reed and Muench (1938) analysis.

(g). Heat stability of the enterovirus isolates.

The enterovirus isolates were all tested for thermal stability at 56°C and 37°C. Replicate volumes of each virus were placed in bijoux bottles, and incubated, using a 56°C water bath and a 37°C incubator. Samples were harvested from the water bath at 0, $\frac{1}{2}$, 1, 2, and 4 hours, and from the incubator at 0, 2, 4, 7, and 14 days.

Following harvesting, samples were frozen rapidly in a -80°C ultradeepfreeze, and then titrated in triplicate tube cultures of MDBK cells, titres being determined following Reed and Muench (1938) analysis.

(h). Cationic stabilisation of the enterovirus isolates

The enterovirus isolates were tested for cationic stabilisation at 50°C with molar $MgCl_2$ solution using a procedure similar to that described by Wallis and Melnick (1962).

Viral stocks were diluted 1 in 10 and mixed with equal volumes of either sterile distilled water or sterile 2M $MgCl_2$ in distilled water. Following incubation in a 50°C water bath for 0, 1, and 3 hours, samples were harvested and frozen rapidly, and subsequently titrated in triplicate MDBK tube cell cultures, using Reed and Muench (1938) analysis to assess the end points.

(i). Sensitivity of isolates to hydroxybenzyl - benzimidazole (HBB)

Portolani *et al.*, (1968) showed that there was a correlation between antigenic characters of bovine enteroviruses and their ability to multiply in the presence of 5 μg per ml concentration of HBB. The enterovirus isolates were therefor tested for their ability to grow in the presence of this concentration of HBB, by inoculating 100 $TCID_{50}$ of each virus into duplicate MDBK tube cell cultures maintained with or without 5 μg per ml of HBB in the maintenance medium. Cultures were harvested when viral CPE was fully developed in the untreated samples, the duplicates pooled, and the titres determined by titration in triplicate MDBK tube cell cultures, using Reed and Muench (1938) analysis.

(j) . Enterovirus concentration by polyethylene glycol (PEG) 6,000 precipitation

Polyethylene glycol 6,000 has been used by a number of authors to purify and concentrate several viruses, using PEG 6,000 concentrations of 6% (McSharry and Benzinger, 1970), 8% (Della-Porta and Westaway, 1972) and 10% (Panina and DeSimone, 1973).

To determine the optimal concentration of PEG 6,000 for concentration of enteroviruses, a volume of isolate D74/18 was rendered cell free by centrifugation at 2,000 G for 10 minutes, and dispensed in 20 ml aliquots. To these, PEG 6,000 was then added and dissolved to give final concentrations of 0, 2, 4, 6, 7, 8, 9, and 10%, and they were left for 1 hour at 4°C for the precipitate to form. Each aliquot, including the untreated control, was then centrifuged at 3,000 G for 10 minutes. The precipitates were suspended in 0.4 ml of maintenance medium, and frozen, together with the untreated control.

The control and test samples were then titrated in triplicate MDBK tube cell cultures and the titres determined by

Reed and Muench (1938) analysis.

(k). Enterovirus concentration by protamine sulphate precipitation

Protamine sulphate has been used at 0.25% concentration by Tanaka et al., (1969) to concentrate bovine ephemeral fever virus. To determine the effectiveness of this chemical in concentrating enteroviruses, sterile solutions of 0.5, 1, 1.5, 2, and 2.5% protamine sulphate were prepared in distilled water. A volume of isolate D74/18 was rendered cell free by centrifugation at 2,000 G for 10 minutes, and dispensed in 20 ml aliquots. A 5 ml volume of each strength of protamine sulphate was added to each aliquot of virus, so as to give final concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5% protamine sulphate. Following 2 hours at 4°C, the treated samples and an untreated control were centrifuged at 3,000 G for 10 minutes. The recovered precipitates were dissolved in 1 ml of maintenance medium. The supernatent fluids, the resuspended viruses and the untreated control virus were all then titrated in triplicate MDBK tube cell cultures and the titres determined by Reed and Muench (1938) analysis.

(l). Enterovirus concentration by ammonium sulphate precipitation

Ammonium sulphate has been widely used as a protein precipitant in the purification of the globulin fraction of serum, (Hebert et al., 1973) but has had much more limited use in the purification and concentration of viral antigens and viruses. To determine the effectiveness of this chemical in concentrating enteroviruses, infective culture fluid of isolate D74/18 was rendered cell free by centrifugation at 2,000 G for 10 minutes, dispensed in 20 ml aliquots, and equal volumes of sterile ammonium sulphate solution added, to give final concentrations of the chemical of 20%, 30%, 40% and 50% saturation at room temperature. A further cell aliquot was left untreated, as a control.

Following 2 hours at 4°C, samples were all centrifuged at 3,000 G for 15 minutes and any precipitate collected and

redissolved in 0.4 ml of maintenance medium. Supernatant fluids, redissolved precipitate, and untreated control virus were then titrated in triplicate MDBK tube cell cultures and titres determined following Reed and Muench (1938) analysis.

(m). Buoyant density determination of isolate D74/18

The buoyant density of isolate D74/18 was determined by isopycnic centrifugation in a caesium chloride gradient using the method of Rowlands et al., (1971).

(i) Caesium chloride was made up to 30% and 40% concentrations (w/w) in 0.04 M phosphate buffer, pH 7.6. Using an Abbé type refractometer and pyknometer bottles, a standard curve was prepared of refractive index against density for caesium chloride solutions of several strengths between 30% and 40%, being corrected for phosphate buffer content.

(ii) A concentrated preparation of isolate D74/18 was prepared by precipitation with 8% polyethylene glycol 6,000, the precipitate being redissolved in 1 ml of 30% caesium chloride in phosphate buffer.

(iii) Three linear gradients of caesium chloride from 30% to 40% were then made using a gradient making device, and 0.1 ml of concentrated viral preparation carefully overlaid on top. The remainder of each tube was filled with liquid paraffin and then sealed, and the tubes centrifuged in a swing-out rotor at 90,000 G for 6 hours, using a Beckman model L preparative ultracentrifuge.

(iv) Following centrifugation, 10 drop fractions were collected from each gradient by puncturing the bottom of each tube, and the corresponding fractions pooled together.

(v) The density of caesium chloride in each fraction was determined from it's refractive index, and the viral content determined

by plaque titration in Linbro 24-well plates, plaque numbers being assessed by microscopic observation. Fractions were also examined in a Phillips EM 200 electron microscope, using a negative staining technique with 2% PTA as described in the general materials and methods, but also incorporating a cycle of washing in distilled water prior to staining, so as to remove the caesium chloride deposits.

(n). Ultracentrifugation of isolate D74/13-49

Triple freeze-thawed distilled water lysates of D74/13-49 infected Vero cell cultures harvested at 6 days, were rendered cell free by centrifugation at 3,000 G for 15 minutes. They were then centrifuged at 100,000 G for 2 hours in an angle rotor in a Beckman model L preparative ultracentrifuge, the pellets dissolved in a small volume of distilled water, and these then examined under a Phillips EM 200 electron microscope following negative staining with 2% PTA (general materials and methods).

(o). Electron microscopic examination of negatively stained isolates

MDBK cell cultures infected with the enterovirus isolates were harvested when about 75% of the cells showed CPE and were prepared for electron microscopic examination by the negative staining technique with 2% PTA, as described in the general materials and methods. They were then examined in a Phillips EM 200 electron microscope. Vero cell cultures infected with isolate D74/13-49 were harvested at 6 days and examined in a similar manner.

(p). Electron microscopic examination of thin sections of cell cultures infected with the enterovirus isolates and isolate D74/13-49

MDBK cell cultures infected with the enterovirus isolates were harvested when approximately 60% of the cells showed CPE, and Vero cells infected with isolate D74/13-49 were

harvested at 6 days. They were then prepared into pellets, sectioned and stained as described in the general materials and methods, and examined under a Phillips EM 200 electron microscope.

Results

(a). Filtration studies of the isolates

The results of filtration studies on the enterovirus isolates, and on IBR and BVD viral controls, are shown in Table 6.

Typical CPE was found to be produced in Vero cells inoculated with unfiltered culture fluid of isolate D74/13-49, and with 220 nm and 100 nm filtrates of this fluid. This effect was not seen in Vero cell controls, nor in cells inoculated with the 50 nm filtrate.

On the basis of these results, the enterovirus isolates would all appear to be smaller than 50 nm diameter, isolate D74/13-49 would be between 50 nm and 100 nm, and the majority of IBR viral particles would be between 100 nm and 220 nm. The size of BVD virus is harder to determine; it would appear to be between 50 nm and 100 nm diameter, but was also retained to a considerable degree by the 100 nm filter.

Table 6

Titres of filtrates of the enterovirus isolates and of viral controls

Virus	Titres of filtrates ($\log_{10}$ $TCID_{50}$ / 0.1 ml)		
	220 nm	100 nm	50 nm
D74/2	4.0	4.25	3.75
D74/18	5.75	5.75	5.5
D74/19A	5.5	5.5	5.5
D74/19B	4.75	4.75	4.75
D74/22-4	4.75	4.5	3.75
IBR	3.75	0.75	0.0
BVD	2.75	1.75	0.0

(b). Sensitivity of the isolates to bromodeoxyuridine (BUDR)

(i) The growth curve of an enterovirus isolate (D74/18) and of BVD and IBR viruses in the presence and absence of BUDR are shown in Figure 14. More detailed data are presented in Appendix 4 (Table B).

The titres of the remaining 4 enterovirus isolates grown for 24 hours in the presence and absence of BUDR are shown in Table 7.

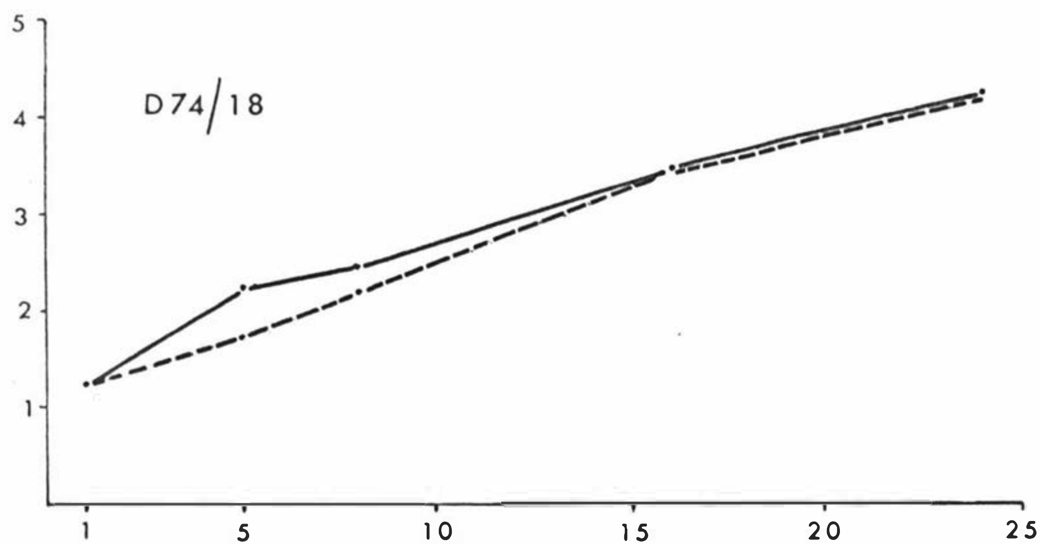
BVD virus and the enterovirus isolates all proved to be resistent to the action of BUDR, in contrast to IBR virus, which showed marked inhibition of viral multiplication.

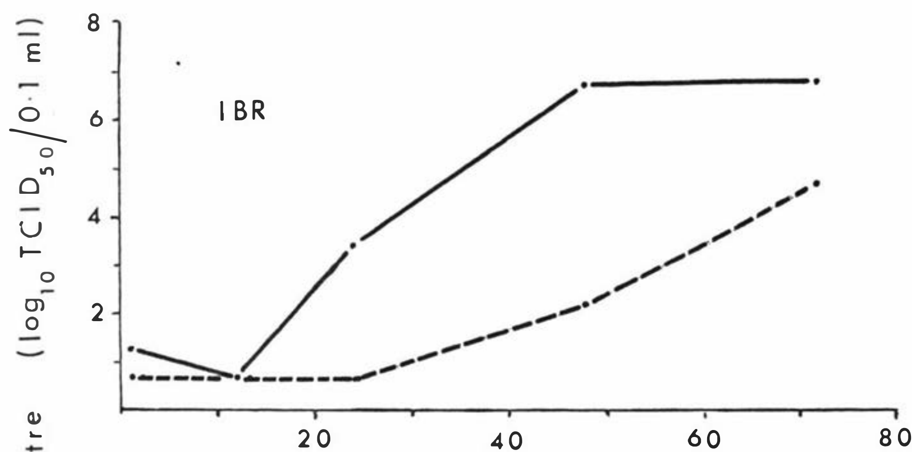
Table 7

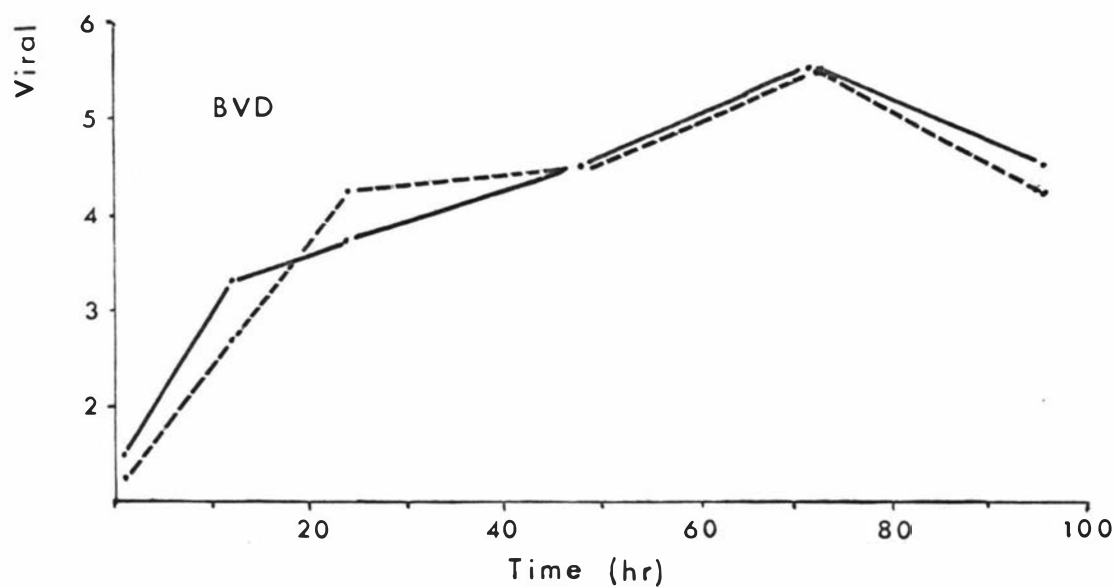
Growth of 4 enterovirus isolates in the presence and absence of BUDR

Virus	BUDR concentration ($\mu g/ml$)	Viral titre at 24 hours ($\log_{10} TCID_{50}/0.1$ ml)
D74/2	0	6.25
	100	6.25
D74/19A	0	5.5
	100	5.75
D74/19B	0	5.75
	100	5.75
D74/22-4	0	5.75
	100	6.25

(ii) Cytopathic effect developed normally in untreated control cultures inoculated with isolate D74/13-49, reovirus 1 and vaccinia viruses, with all 3 serial passages. It also developed







Control ———— BUDR - - - - - -

FIGURE 14

Growth of enterovirus D74/18, and IBR and BVD viruses in the presence and absence of BUDR. Note the retarded growth of IBR virus in the presence of BUDR.

normally in BUDR treated cultures inoculated with isolate D74/13-49, and reovirus 1 viruses, at all 3 serial passage levels. Vaccinia virus showed complete suppression of CPE in BUDR treated cultures.

(c). Chloroform sensitivity of the isolates

The results of chloroform sensitivity testing of the various isolates are shown in Table 8.

Table 8

Effect of chloroform treatment on viral infectivity

Virus	Viral infectivity	
	Untreated control virus	Chloroform treated virus
D74/2	3.75 +	3.5
D74/18	4.5	4.75
D74/19A	4.75	4.5
D74/19B	4.5	4.5
D74/22-4	4.5	4.25
D74/13-49	pos ++	pos ++
IBR	3.25	0.0
Vaccinia	3.25	0.0

+ = $\log_{10}$ $TCID_{50}$ /0.1 ml

++ = CPE present.

The enterovirus isolates and isolate D74/13-49 all proved to be resistant to the action of chloroform. Both IBR and vaccinia virus were inactivated by chloroform treatment.

(d). Ether sensitivity of the isolates

The titres of the enterovirus isolates and of IBR and vaccinia viruses treated with and without 20% diethyl ether at 4°C are shown in Table 9.

Table 9

Effect of ether treatment at 4 ^{o}C on viral infectivity

Virus	Titres of virus ($\log_{10}$ $TCID_{50}$ /0.1 ml)	
	Untreated control virus	Ether treated virus
D74/2	3.75	3.5
D74/18	4.5	4.5
D74/19A	4.25	4.5
D74/19B	4.5	3.75
D74/22-4	3.75	4.25
IBR	3.5	0.0
Vaccinia	3.25	3.25

The enterovirus isolates and vaccinia virus were all resistent to the action of diethyl ether at 4 ^{o}C, in contrast to IBR virus, which was inactivated.

(e). Sensitivity of the isolates to sodium deoxycholate

The titres of viruses treated with and without 1 in 2, 000 dilution of sodium deoxycholate (SDC) are shown in Table 10.

Table 10

Effect of sodium deoxycholate on viral infectivity

Virus	Titres of virus ($\log_{10}$ $TCID_{50}$ /0.1 ml)	
	Untreated control virus	SDC treated virus
D74/2	4.5	4.25
D74/18	5.75	5.75
D74/19A	6.0	6.5
D74/19B	5.25	4.75
D74/22-4	5.75	5.75
IBR	4.75	0.0
Vaccinia	4.25	3.75

The enterovirus isolates and vaccinia virus were all found to be resistent to the action of sodium deoxychlorate, in contrast to IBR virus which was inactivated.

(f). Acid sensitivity of the enterovirus isolates

The titres of viruses following incubation at 37°C for periods of 1 and 3 hours at pH 7.2 and pH 3 are shown in Table 11.

Table 11

Effect of acid treatment (pH 3) for 1 and 3 hours at 37°C on viral infectivity

Virus	Titres of viruses ($\log_{10}$ $TCID_{50}$ /0.1 ml)			
	Untreated control virus		Acid treated virus	
	1 hr	3 hr	1 hr	3 hr
D74/2	4.25	4.5	4.25	4.25
D74/18	4.75	4.25	4.5	4.25
D74/19A	4.25	4.25	4.5	4.25
D74/19B	4.5	4.25	4.25	4.25
D74/22-4	4.5	4.25	4.75	4.25
IBR	3.5	3.25	0.0	0.0

The enterovirus isolates all proved to be stable at pH 3 for up to 3 hours, in contrast to the IBR virus control which was inactivated within 1 hour.

(g). Heat Stability of the enterovirus isolates

The effects of incubation at 56°C and 37°C on the infectivities of the enterovirus isolates are shown graphically in Figures 15 and 16 respectively together with the corresponding half lives. More detailed results are presented in Appendix 4 (Table C and D). Because of the anomalous results obtained with isolate D74/18, the procedure was repeated for this virus, using alternative viral stocks.

All the enterovirus isolates showed a marked drop in titre, within 30 minutes at 56°C, and within 4 days at 37°C. Half lives of the isolates averaged about 3 minutes at 56°C and about 7 hours at 37°C. With the exception of isolate D74/2, all isolates showed an eventual slowing of the inactivation rate at both temperatures. Isolate D74/18 showed the longest persistence at both temperatures, and was particularly notable for the slight resurgence in infectivity between 1 and 2 hours at 56°C.

(h). Cationic stabilisation of the enterovirus isolates

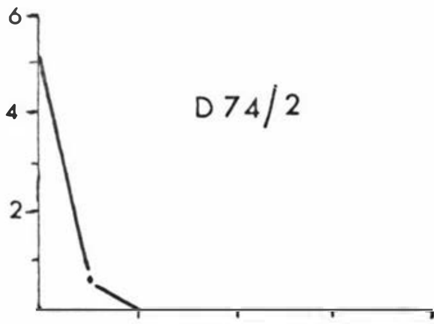
The effects of incubating the enterovirus isolates at 50°C in the presence and absence of molar magnesium chloride are shown in Table 12.

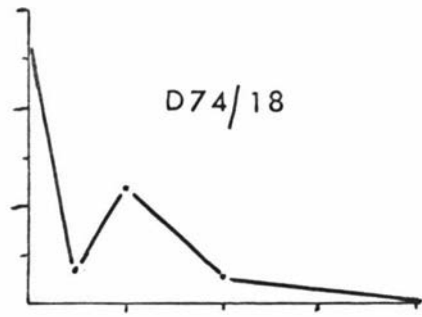
Table 12

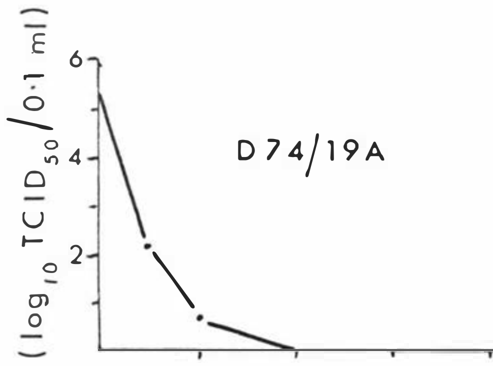
Cationic stabilisation of enterovirus isolates by molar magnesium chloride ($MgCl_2$), at 50°C.

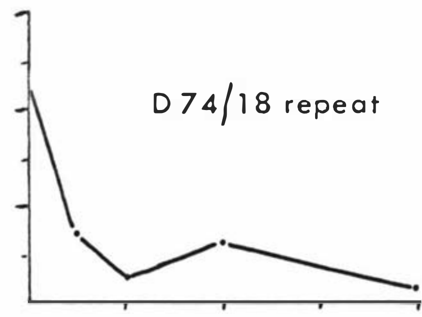
Virus	Titres of viruses ($\log_{10}$ $TCID_{50}$/ 0.1 ml at times (hrs):					
	Untreated control virus			Molar $MgCl_2$ treated virus		
	0	1	3	0	1	3
D74/2	3.5	0.25	0.0	3.75	3.5	3.25
D74/18	4.25	1.25	0.5	4.5	4.25	4.25
D74/19A	4.75	1.75	0.5	4.75	4.25	3.75
D74/19B	3.5	0.5	0.0	3.5	3.25	3.25
D74/22-4	4.5	1.25	0.75	4.25	3.75	3.75

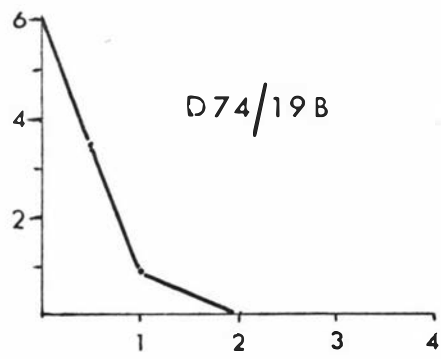
The enterovirus isolates were all stabilised at 50°C by molar magnesium chloride, in contrast to untreated control viruses.

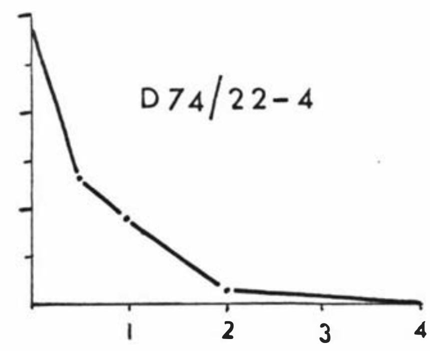












Viral Titre ($\log_{10}$ $TCID_{50}$/0·1 ml)

Time (hr)

FIGURE 15

Thermal inactivation of enterovirus isolates at 56°C. Note the relatively rapid rate of inactivation of isolate D74/2, and the apparent reactivation of isolate D74/18 on the 2 occasions it was tested.

Initial half lives of isolates were as follows:-

D74/2:	2 minutes
D74/18:	2 "
D74/18: (repeat)	3 "
D74/19A:	3 "
D74/19B:	3 "
D74/22-4:	3 "

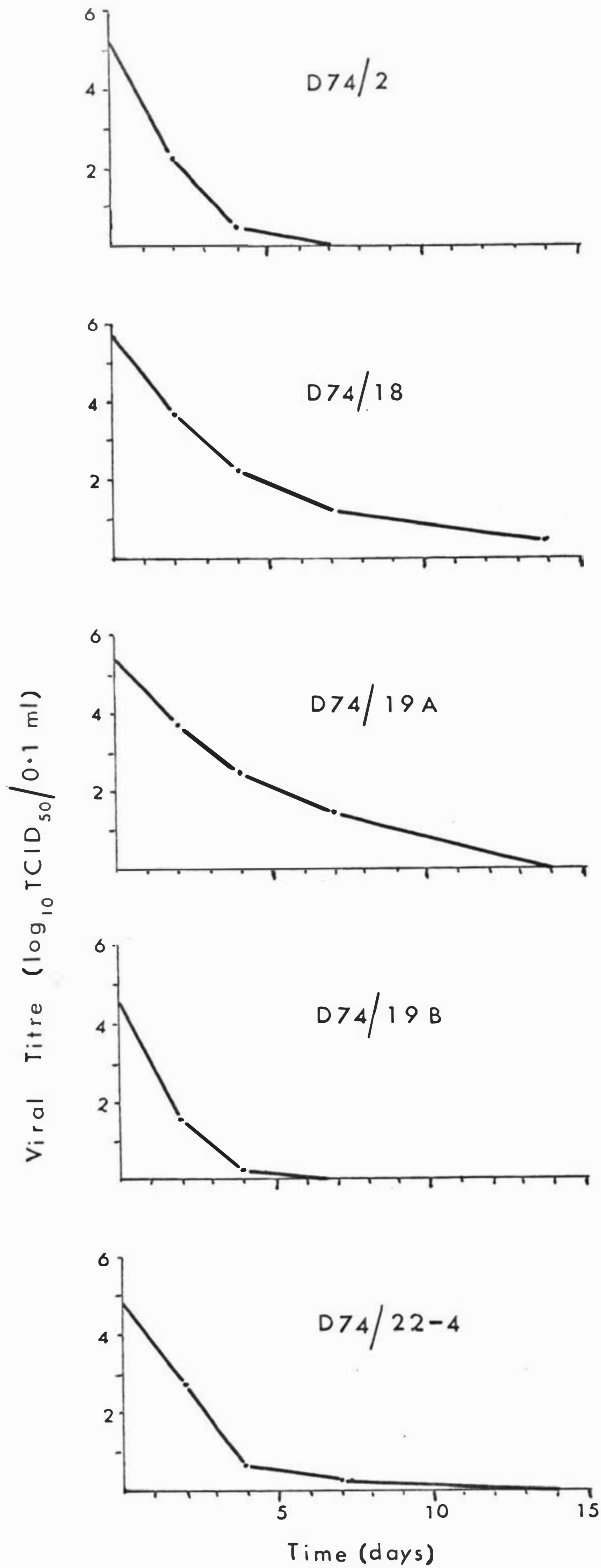

D74/2
D74/18
D74/19 A
D74/19 B
D74/22-4
6
4
2
5
10
15
Viral Titre (log_{10} $TCID_{50}$/0·1 ml)
Time (days)

FIGURE 16

Thermal inactivation of enterovirus isolates at 37°C. Note the lower stability of isolates D74/2 and D74/19B.

Initial half lives of isolates were as follows:

D74/2:	5 hours
D74/18:	8 "
D74/19A:	8 "
D74/19B:	5 "
D74/22-4:	7 "

(i). Sensitivity of isolates to hyroxybenzylbenzimidazole (HBB)

The results of growing the enterovirus isolates in the presence and absence of HBB are shown in Table 13. The growth of only a single isolate (D74/2) was found to be inhibited by the chemical.

Table 13

Growth of enterovirus isolates in the presence and absence of HBB

Virus	Titres of viruses ($\log_{10}$ $TCID_{50}$ /0.1 ml)	
	Untreated control virus	HBB treated virus
D74/2	4.75	1.75
D74/18	4.5	4.75
D74/19A	4.75	4.75
D74/19B	4.75	5.25
D74/22-4	5.25	5.5

(j). Enterovirus concentration by polyethylene glycol (PEG) 6,000 precipitation

The results of concentrating isolate D74/18 by precipitation with various strengths of PEG 6,000 are shown in Table 14. Concentrations of PEG 6,000 of between 6% and 8% appear to be adequate for precipitation and concentration of the virus, no further effect being gained by higher concentrations of the chemical.

Table 14

Infectivity of viral concentrates obtained by precipitation with various concentrations of PEG 6,000

Untreated virus titre ($\log_{10}$ $TCID_{50}$ /0.1 ml)	PEG 6 000 (%, w/v)	Titre of viral concentrate ($\log_{10}$ $TCID_{50}$ /0.1 ml)
4.75	2	4.5
	4	5.5
	6	5.75
	7	5.75
	8	6.25
	9	6.25
	10	6.25

(k). Enterovirus concentration by protamine sulphate

The results of concentrating virus D74/18 by various concentrations of protamine sulphate are shown in Table 15. All concentrations of the chemical appeared equally effective in concentrating the virus. Although it was originally intended to redissolve the precipitate in 0.4 ml of maintenance medium, this proved impossible, as the precipitates from the 0.4% and 0.5% treatments would not dissolve in this volume. Consequently, all precipitates were dissolved in 1 ml of the medium, for the sake of uniformity.

Table 15

Infectivity of viral concentrate obtained by precipitation with various strengths of protamine sulphate

Untreated viral titre ($\log_{10}$ $TCID_{50}$ /0.1 ml)	Protamine SO_4 conc.(%, w/v)	Titre ($\log_{10}$ $TCID_{50}$/0.1 ml) of concentrate	Titre ($\log_{10}$ $TCID_{50}$/0.1 ml) of supernatent
5.25	0.1	6.5	3.5
	0.2	6.75	3.25
	0.3	6.25	3.5
	0.4	6.5	3.25
	0.5	6.5	3.25

(l). Enterovirus concentration by ammonium sulphate precipitation

The use of ammonium sulphate concentrations at 20% and 30% final strengths resulted in no visible precipitate being formed. The infectivities of the concentrates produced from the use of 40% and 50% concentrations of ammonium sulphate are shown in Table 16 , moderate precipitates being formed with both concentrations, though slightly more profusely at the higher rate.

Table 16

Infectivity of viral concentrates obtained by precipitation with 30% and 40% ammonium sulphate

Untreated viral titre ($\log_{10}$ $TCID_{50}$/ 0.1 ml)	$(NH_4)_2SO_4$ conc. (%, w/v)	Titre ($\log_{10}$ $TCID_{50}$/0.1 ml of:	
		concentrate	supernatent
4.75	40	5.5	2.75
	50	5.5	2.5

(m). Buoyant density determination of isolate D74/18

The results of plaque titrations of the various fractions, together with results of electron microscopic examinations, are shown graphically in Figure 17 . More detailed data are presented in Appendix 4 (Table E). Peak infectivity was found to occur at a density of approximately 1.34 g/ml of caesium chloride solution.

Two fractions, numbers 12 and 13, were found to give anomalous refractive index results, and were disregarded. It is thought that traces of water may have been in the bottles used for collecting these fractions.

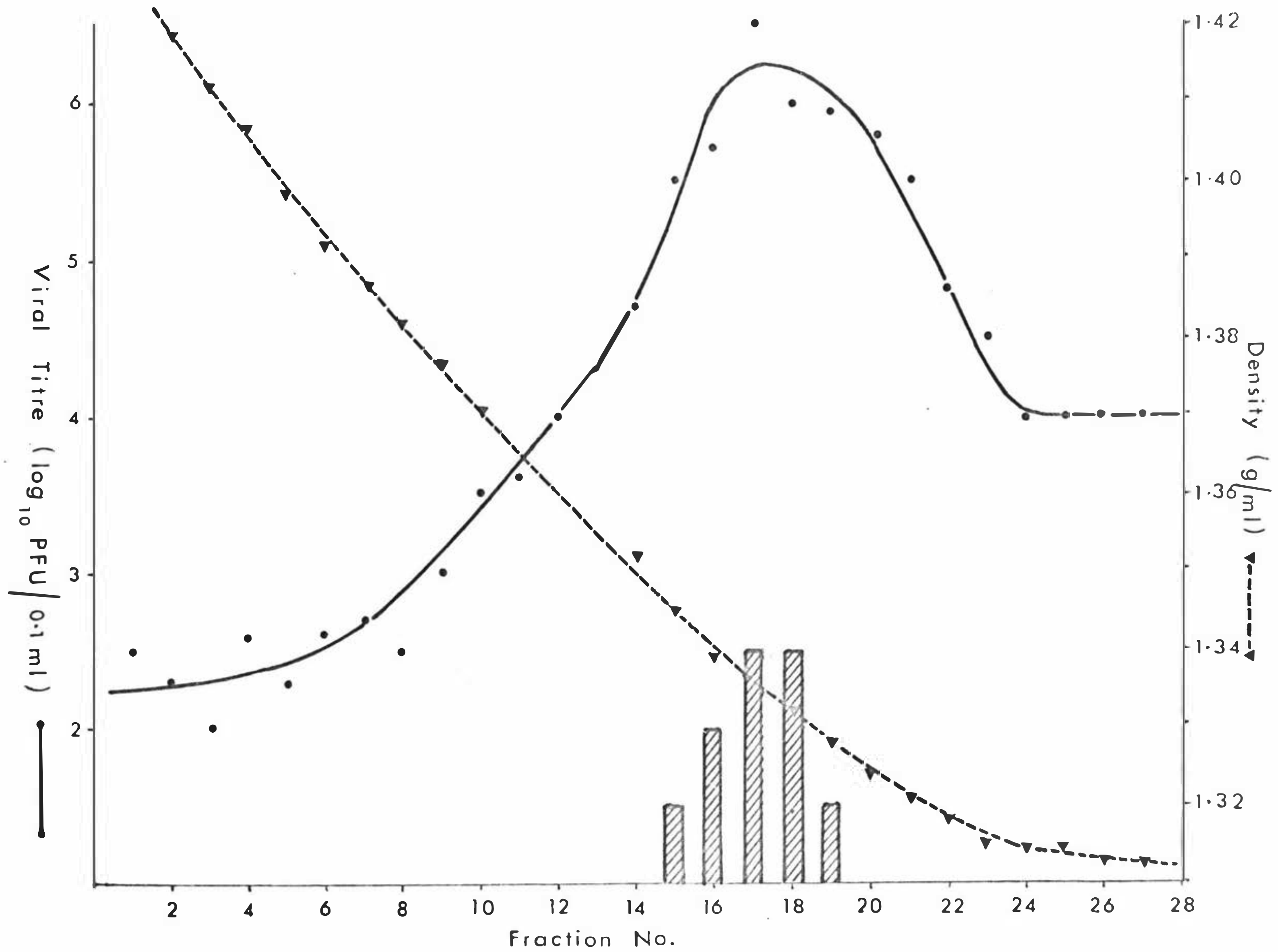

Viral Titre (log₁₀ PFU/0·1 ml)
Density (g/ml)
Fraction No.
6
5
4
3
2
1·42
1·40
1·38
1·36
1·34
1·32
2
4
6
8
10
12
14
16
18
20
22
24
26
28

FIGURE 17

Density and infectivity of collected fractions of CsCl gradient with isolate D74/18. The histogram shows the relative frequency with which viral particles were seen on electron microscopic examination of negatively stained fractions, this being seen to coincide with infectivity.

(n). Ultracentrifugation of isolate D74/13-49

Though considerable searching of grids was undertaken, few viral particles were detected upon electron microscopic examination of negatively stained preparations of the pellets obtained by ultracentrifugation.

(o). Electron microscopic examination of negatively stained isolates

Electron micrographs of the enterovirus isolates and isolate D74/13-49 are shown in Figure 18 . Though most of the enteroviruses were found as single particles, D74/ 18 was also found as an aggregate enclosed in a membrane bound vesicle. Isolate D74/13-49 posed considerable problems in locating recognisable particles, and few micrographs were taken.

The enterovirus isolates were all of a similar size, of approximately 25 nm to 28 nm, whilst isolate D74/13-49 had a diameter of approximately 80 nm. Both classes of virus possessed a spherical to hexagonal shape and appeared to show cubic symetry.

(p). Electron microscopic examination of thin sections of cell cultures infected with the enterovirus isolates and with isolate D74/13-49

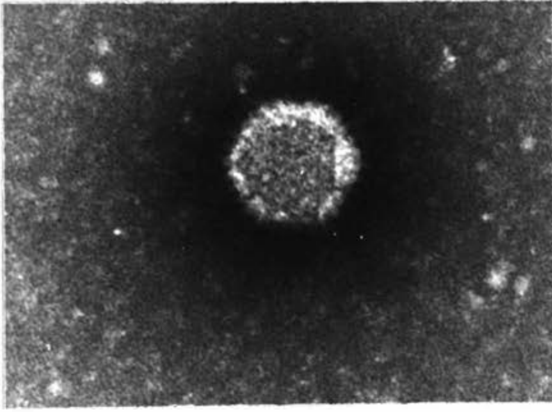
Both short and long methods of preparation (Doane et al., 1974) using epon-araldite and Spurr's resins were found to be satisfactory, though the latter embedding medium proved much more convenient to handle, on account of it's lower viscosity.

Whilst a detailed study of the ultramicroscopic changes was not made, it was noted that cells infected with the enterovirus isolates produced large numbers of smooth surfaced vesicles situated near to the nucleus and also showed apparent loss of ground substance. Numerous electron dense granules, of similar size and density to ribosomes, were to be found in the cytoplasm,

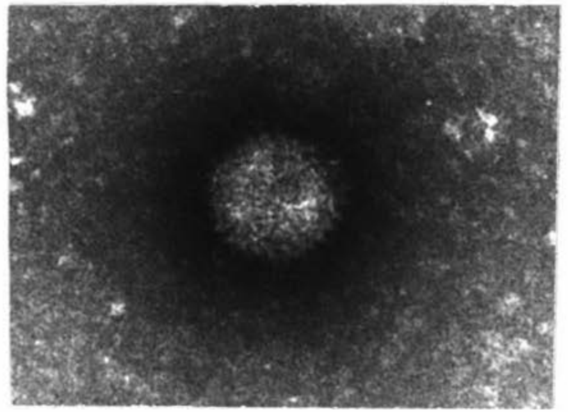
often in large aggregates. With 3 of the enteroviruses large ordered arrays of these particles were seen at 24 hours post infection, though not at 8 hours. The diameter of the dense particles was found to be between 12 nm abd 14 nm, with a centre to centre distance in close-packed aggregates of about 24 nm.

No cells infected with isolate D74/13-49 could be located, possibly because of the low proportion of infected cells in inoculated cell cultures.

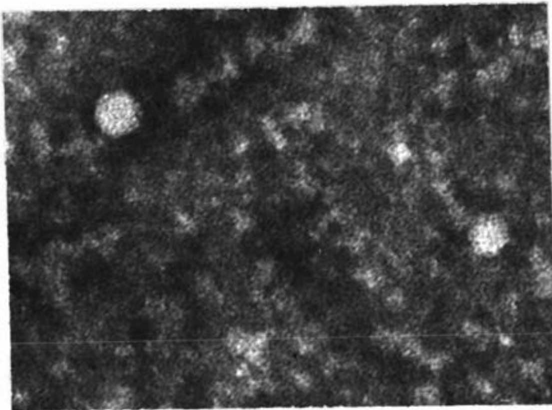
Electron micrographs of some of the sections of enterovirus infected cells are shown in Figure 19.



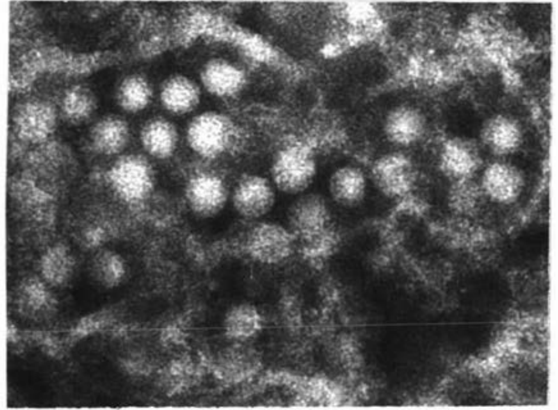
D74/13-49



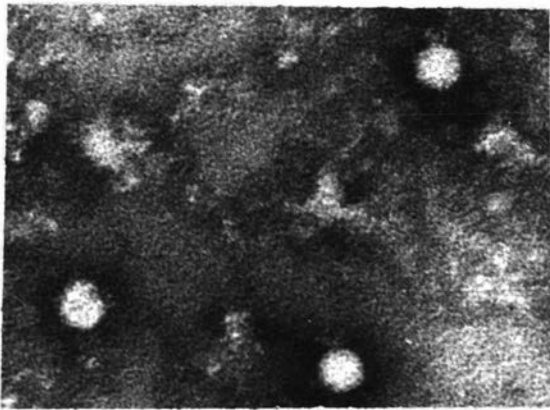
D74/13-49



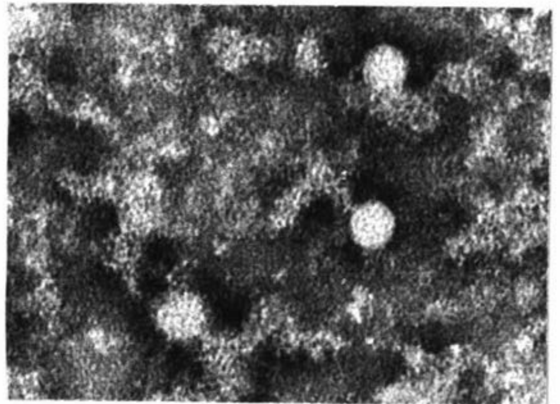
D74/2



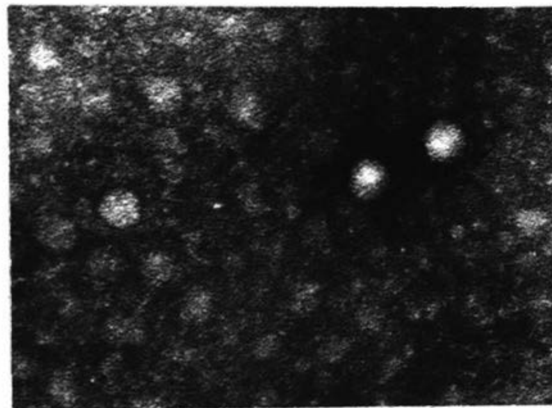
D74/18



D74/19A



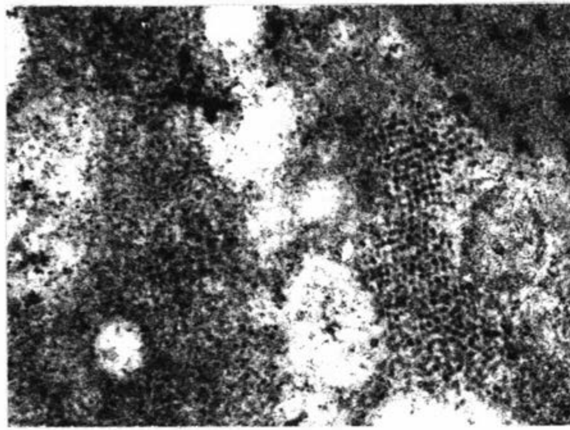
D74/19B



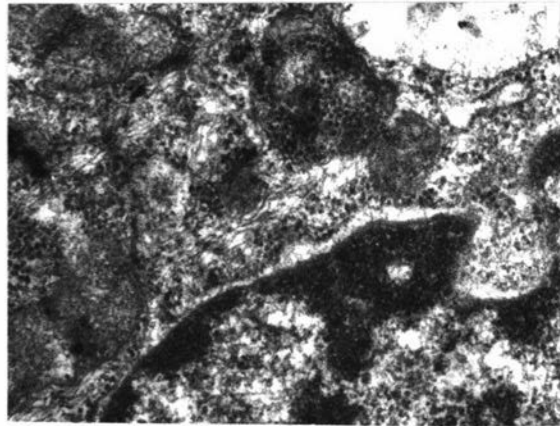
D74/22-4

FIGURE 18

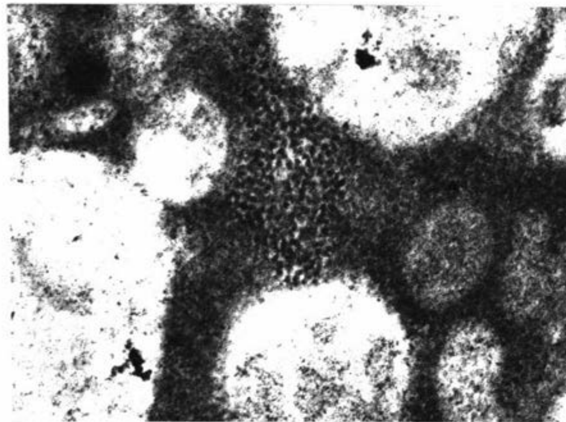
Electron micrographs of negatively stained enterovirus isolates (D74/2, A74/18, D74/19A, D74/19B, D74/22-4) and possible diplornavirus D74/13-49.
Note the membrane enclosing viral particles with isolate D74/18. EM x 200 000.



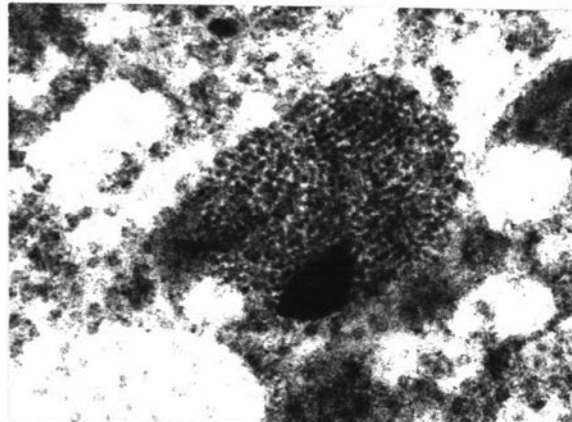
D74/2



D74/18



D74/19B



D74/22-4

FIGURE 19

Electron micrographs of thin sections of cell cultures infected with some of the enterovirus isolates. Note the large aggregates of dense viral particles and the cytoplasmic vacuolation.
Uranyl acetate - lead citrate stain. EM x 39000.

Discussion

The results of the filtration studies with the enterovirus isolates suggests they all have a size of less than 50 nm. Using the data of Elford (1933) on filtration behaviour of small particles, sizes of 25 nm or less are indicated. These results show good agreement with published reports, which suggest a size of between 23 nm and 28 nm (McFerran, 1962a; Van Der Maaten and Packer, 1967; Mattson et al., 1969) or of less than 50 nm (Rovozzo et al., 1965).

Isolate D74/13-49 would appear to have a size of between 50 nm and 100 nm, judging by it's filtration behaviour, though using the formula of Black (1958) relating filtration behaviour to particle size, a diameter of 48 nm is suggested. However, sizes obtained by filtration can only be regarded as approximate, precise measurement requiring other methods (Atoynatan and Hsiung, 1964).

The filtration behaviour of the IBR control virus is in accordance with reports by Tousimis et al., (1958) and Armstrong et al., (1961), suggesting a size of between 100 nm and 150 nm.

Considerable variation exists in reports of the size of BVD virus, varying from 22 nm to 275 nm. It has been suggested by Fernelius (1968) that BVD viruses may be heterogeneous in size, and that noncytopathic strains may show a higher degree of cell association than cytopathic strains, resulting in differences in filtration behaviour. However, most recent reports suggest a size of between 50 nm and 100 nm, based on filtration behaviour (Taylor et al., 1963; Castrucci et al., 1968; Fernelius 1968) or on electron microscopic studies (Ritchie and Fernelius 1969; Maess and Meczko, 1970). The filtration behaviour of the control BVD virus in this experiment shows good agreement with these reports.

BUDR has been used to indicate whether DNA is synthesized during viral replication. It acts by inhibiting DNA synthesis, due to it's incorporation into DNA in place of thymidine, resulting in the production of faulty nucleic acid (Smith et al., 1960). Though this method has some limitations with regard to those enteroviruses requiring DNA synthesis as part of their replication cycle, it nevertheless provides a useful indication of the nucleic acid type of virus. The results obtained here suggest that the enterovirus isolates all contain RNA, which is consistent with previous reports on the sensitivity of bovine enteroviruses to both BUDR and iododeoxyuridine (Spradbrow, 1964; Rovozzo et al., 1965; Schiott and Hyldegaard-Jensen, 1966; Mattson and Reed, 1974) and also agrees with results obtained by enzyme digestion studies by Mattson et al., (1969). Isolate D74/13-49 also appeared to contain RNA, on the basis of it's behaviour with BUDR.

The control viruses behaved according to published reports. Inhibition of IBR replication by BUDR has been reported by Stevens and Groman (1964). The replication of BVD virus is reported by Castrucci et al., (1968) to be unaffected by this chemical. Because these viral controls were used, it was considered unnecessary to further confirm the results obtained using thymidine reversal of inhibition as shown by Smith et al., (1960) and Herrmann (1961).

The results of the chloroform and ether sensitivity tests suggest that the enterovirus isolates all lacked an envelope of essential lipid material. This agrees with previous reports on bovine enteroviruses (Moll and Davis, 1959; Spradbrow, 1964; Rovozzo et al., 1965; Schiott and Hyldegaard-Jensen, 1966; Mattson and Reed, 1974). Behaviour in the presence of sodium deoxycholate was similar to that reported by McFerran (1962a), and further suggests the lack of an envelope. The original report by Theiler (1957) mentioned that several strains of Coxsackie and poliovirus were

resistant to treatment with this chemical, and in fact sometimes showed slight increase in titre in it's presence. It is of interest to note that one enterovirus isolate (D74/19A) also showed a mild increase in titre after treatment. This may be due to disaggregation of viral particles from host materials by the chemical.

The control viruses behaved with these lipid solvents in accordance with their known nature. IBR virus has been reported to be ether sensitive by McKercher (1959) and Armstrong et al., (1961). Vaccinia virus has also been reported to be chloroform sensitive (Hamperian et al., 1963), but is ether resistent at 4 °C (Andrewes and Horstman, 1949) and resistant to treatment with bile salts (Smith, 1939). The response obtained with sodium deoxycholate is in agreement with the latter behaviour.

The enterovirus isolates all proved to be acid resistant, which agrees with published reports (Spradbrow, 1964; Rovozzo et al., 1965; Schiott and Hyldegaard-Jensen, 1966; Mattson et al., 1969; Mattson and Reed, 1974). This property separates them from the rhinoviruses, which are acid sensitive (Tyrrell and Shanock, 1963). The acid sensitive nature of IBR virus has been reported by McKercher (1959).

The rapid rate of inactivation of the enterovirus isolates at 56 °C has been previously reported with bovine enteroviruses by Spradbrow (1964) and Rovozzo et al., (1965). One feature seen with 4 of the enterovirus isolates which was not reported by these authors was the longer persistence at low titre of a more heat stable fraction of the viruses. Isolate D74/18 also behaved in an anomolous manner, showing evidence of increased infectivity after 1 to 2 hours at this temperature. This phenomenon may be due to disaggregation of groups of viral particles which were previously bound by host materials.

The enterovirus isolates were much more stable at 37°C, and the half-lives obtained are similar to that reported by McFerran (1962a). In contrast, Mattson et al., (1969) reported considerably longer half-lives of about 39 hours. The variation is probably due to different strains of virus varying in their heat stability. Once again, 4 of the enterovirus isolates showed persistence at low titres of a more heat stable fraction of virus, this not being reported by either of the above two authors, though it was commented on by Barya et. al., 1967.

It is interesting to note the similarity in behaviour of the viral isolates at both temperatures. Isolate D74/2 showed a uniform rate of degradation at both temperatures, with little evidence of persistence of more heat stable fractions of virus, whilst the remaining enterovirus isolates all showed some evidence of longer survival at low titre.

The enterovirus isolates were all stabilised against thermal inactivation at 50°C by molar magnesium chloride, in accordance with published reports on bovine enteroviruses (Spradbrow, 1963, 1964; Rovozzo et al., 1965; Van Der Maaten and Packer, 1967; Mattson et al., 1969). This is regarded as a basic property of enteroviruses by Wallis and Melnick (1962).

Sensitivity to a concentration of 5 μg./ml of HBB has been shown by Portolani et al., (1968) to separate the enteroviruses into 2 groups, which correspond to the 2 serological groups established by LaPlaca et al., (1965). Though a different procedure was used in these experiments to that described by Portolani et al., (1968), the concentration of chemical was the same, and the results should be correspondingly valid. The results obtained would indicate that both groups are represented amongst the enterovirus isolates.

Precipitation of the enterovirus isolates with PEG 6 000, protamine sulphate, and ammonium sulphate resulted in increased viral titres on resuspension. PEG 6 000 at 8%

concentration gave up to a 30 fold increase in titre with the volumes used, and would no doubt have given even larger increases in titre had a larger reduction in volume been used. This agrees well with the report by McSharry and Benzinger (1970), who used this chemical to concentrate vesicular stomatitis virus.

Protamine sulphate at 0.2% concentration also gave a 30 fold increase in titre, and appeared almost as effective using a 0.1% concentration of the chemical. This contrasts with the 0.25% concentration used by Tanaka et al., (1968) to purify bovine ephemeral fever virus. Using the lower concentration of protamine sulphate may allow a larger reduction in the volume of the viral suspension and hence allow higher concentrations of virus to be obtained. At 0.4% and higher concentrations of the chemical, no further increase in titre could be expected because of solubility problems.

Ammonium sulphate gave somewhat inferior results to the above 2 chemicals, using both 40% and 50% strengths. Use of the 50% concentration of the chemical did not increase the yield.

The purification aspect of treating the virus with these chemicals was not investigated. However, the use of the lowest concentration of PEG 6 000 which gave optimal concentration of the virus was reported to result in very high efficiency of recovery of virus, and to give relatively low levels of recovery of nonviral materials. (McSharry and Benzinger, 1970). This principle may equally apply to treatment with the other 2 chemicals. Certainly, although heavier precipitates were obtained at the higher concentrations of protamine sulphate and ammonium sulphate, no further increase in viral concentration resulted, suggesting that the extra precipitate consisted of nonviral material.

The buoyant density of the isolate D74/18 was found to be 1.34 g/ml in caesium chloride, which is in agreement with the results obtained by Martin et al., (1970) and Rowlands et al.,

(1971) with other bovine enteroviruses. In excess of 99% of viral activity was concentrated in the vicinity of this density in the gradient, which was also where the highest concentration of viral particles were seen under the electron microscope.

The morphology and size of the enterovirus isolates in negatively stained preparations were all similar to the bovine enteroviruses seen by Van Der Maaten and Packer (1967) and Mattson et al., (1969), these all showing a spherical to hexagonal shape, a size of about 25 nm, and a suggestion of cubic symmetry in the capsomeres. Isolate D74/13-49 had a size and shape more suggestive of a diplornavirus or adenovirus, but because of the limited number of particles detected, detailed studies of it's morphology could not be made. The difficulty in locating particles of this virus probably relates to the apparent low titres obtained in culture.

Though not studied in detail, cells affected with the enterovirus isolates showed changes similar to those described by Mattson and Reed (1974) with bovine enteroviruses, and by Rifkind et al., (1961) with human enteroviruses. Both reports found that smooth surfaced vacuoles developed in the cytoplasm. Rifkind et al., (1961) concluded these corresponded to the eosinophilic cytoplasmic inclusions seen with light microscopy. Loss of ground substance was reported by Rifkind et al., (1961) to occur in the late stages of poliovirus infection.

Orderly arrays of viral particles were also found by Rifkind et al., (1961) and Mattson and Reed (1974), though the latter authors commented that these were only rarely observed and then only after 12 or more hours post infection. Rifkind et al., (1961) referred to the location of these orderly arrays of viral particles upon fine cytoplasmic fibrils, but these were not seen in the present study. Instead, the arrays tended to show more resemblance to the crystalline type of array reported by Mattson and Reed (1974). The size of the viral particles seen in the

present study is similar to the size reported by Rifkind *et al*., (1961) with human enterovirus type 9. It was not possible to discern the outer membrane reported by these authors.

SEROLOGICAL STUDIES OF THE ISOLATES

Materials And Methods

(a). Antiserum production

(i) Isolate D74/18 wasgrown in MDBK cells using a maintenance medium containing 0.2% bovine serum albumin instead of serum, and was harvested when 75% of the cells showed CPE. Cultures were then frozen and thawed once, and the virus was precipitated using 8% polyethylene glycol 6 000 for 2 hours at 4°C. Following centrifugation at 3,000 G for 10 minutes, the precipitate was resuspended to 2% of the original volume in medium 199.

Two rabbits were bled, and then inoculated with 1 ml volumes of the virus concentrate, using a course of 3 injections. The first injection was given intramuscularly into 2 sites, as a 50% water in oil emulsion using Freund's complete adjuvant[1], the stability of the emulsion being tested on water. The second injection was given by the intraperitoneal route 4 weeks later, using a 50% water in oil emulsion with Freund's incomplete adjuvant[1]. The final injection was given 7 days later via the intravenous route, using viral concentrate only. After a further 2 weeks, blood was taken and serum separated.

(ii) All 5 enterovirus isolates were grown in MDBK cells in a maintenance medium containing 0.2% of bovine serum albumin instead of serum. The viruses were harvested when approximately 75% of the cells showed CPE, the cultures were frozen and thawed, and then centrifuged at 3,000 G for 10 minutes to remove particulate cell debris.

Following preinoculation bleeding, chickens were inoculated intravenously with the culture fluids using the following schedule of injections, which is similar to that described by Barya *et al.*,

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1. Difco Laboratories, Detroit, Michigan, U.S.A.

(1967). The animals were then bled a fortnight later, and the serum separated.

Time	Inoculum dose
Day 1	1 ml
" 4	2 ml
" 7	3 ml
" 11	4 ml
" 14	5 ml

(b). Titration of antisera

Preinoculation and postinoculation sera were titrated in duplicate for activity against their homologous viruses, according to the previously described microtitre procedure (General materials and methods). Doubling dilutions of serum from 1 in 10 to 1 in 1280 were used and titres were assessed following Reed and Muench (1938) analysis.

In addition, a viral titration was carried out with isolate D74/18, in the presence of doubling equine serum concentrations from 1 in 2 to 1 in 256, to determine if serum concentration had any influence on the endpoint of the titration. Results were determined following Reed and Muench (1938) analysis.

(c). Cross neutralisation tests with the enterovirus isolates

Cross neutralisation tests were carried out with each enterovirus isolate, following the previously described microtitre neutralisation test method (General materials and methods). Serum dilutions used were from 1 in 10 to 1 in 1280. The challenge virus does was 100 $TCID_{50}$, and the cells used were MDBK.

The procedure adopted was similar to that described by Moscovici *et al.*, (1961) and Dunne *et al.*, (1971), except for the microtitre adaptation. Titres were determined after Reed and Muench (1938) analysis.

(d). Cross neutralisation tests with American enterovirus serotypes and local enterovirus prototypes

These were carried out in a similar manner to the above cross neutralisation tests, using 7 U.S. bovine enterovirus serotypes (BES I to VII) as described by Dunne et al., (1974) and 2 enterovirus isolates (D74/2 and D74/18) which had been selected as local prototype strains.

(e). Identification of isolate D74/25A

Following suspensions on the basis of it's CPE that isolate D74/25A was bovine viral diarrhoea (BVD) virus, the culture fluid from the second passage of the isolate in FBL cells was mixed with an equal volume of undiluted stanrd antiserum[1] against BVD and left at room temperature for 1 hour to react. A 0.3 ml volume of a vaccine strain of BVD virus containing 1 x 10^4 $TCID_{50}$ per ml was treated similarly. After incubation, 0.2 ml of each mixture was inoculated into duplicate FBL culture tubes and these incubated at 37°C for 3 days. A 0.1 ml volume of the same strengths of test virus and vaccine strain of BVD was inoculated into duplicate FBL tubes and incubated similarly, to check infectivity.

(f). Fluorescent antibody production

An antiserum against enterovirus isolate D74/18 was produced in a chicken as described earlier, and tested for suitable reactivity using the serum neutralisation test. (General materials and methods). The globulin fraction was then recovered from the antiserum by the use of a triple precipitation procedure with an equal volume of 70% saturated ammonium sulphate solution as described by Hebert (1974). The final precipitate was resuspended in distilled water and dialysed against 4 changes of PBS until free of sulphate radicals, as shown by testing the dialysing fluid with barium chloride solution.

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1. Supplied by Wallaceville Animal Health Reference Laboratory, Upper Hutt, N.Z.

The concentration of protein was then determined by measuring the optical density of a 1 in 40 dilution of the globulin preparation at 280 nm wavelength in a spectrophotometer, and comparing this result with a standard graph derived from bovine γ globulin.

The globulin was then conjugated at pH 9.5 with fluorescein isothiocynate[1] isomer 1, following the procedure described by Nairn (1968). The crude conjugate was then purified in a similar manner to that described by Goldman (1968). Unconjugated dye was removed by gel filtration in a Sephedex G25[2] column, using 0.0175 M phosphate buffer, pH 6.3. The product obtained was then fractionated in a DEAE cellulose[3] column, and 3 fractions collected using (a) the above buffer, (b) the above buffer in 0.125 M NaCl, and (c) the above buffer in 0.250 M NaCl.

Each fraction was reduced to a convenient volume by dialysing against polyethylene glycol 20 000, and then dialysed against 2 changes of PBS, pH7.2, to adjust the pH. The fractions were preserved with 1 in 5000 merthiolate, and stored frozen.

(g). Testing of conjugated antiserum

The 3 fractions obtained were tested for activity against Vero and MDBK cells infected with isolate D74/18, using conjugate dilutions of 1 in 2 to 1 in 10, and following the staining procedure described as follows:

(i) Wash monolayer with PBS.
(ii) Wash monolayer with 4 changes of acetone, and let fix for 10 to 15 minutes at room temperature. Air dry.
(iii) Stain for 30 minutes at room temperature in a moist chamber, using a suitable dilution of conjugated antiserum.
(iv) Wash for 10 minutes with PBS.
(v) Rinse the monolayer with distilled water.
(vi) Mount in 90% glycerol, pH 8.6 and examine under blue light.

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1. Schwarz and Mann, Division of Becton Dickinson Co., Orangeburg, New York, U.S.A.
2. Pharmacia Fine Chemicals AB, Uppsala, Sweden.
3. Whatman DE 52.; W.R. Balston Ltd., Maidstone, Kent, U.K.

Unstained infected and stain uninfected monolayers were also examined as controls.

(h). Fluorescent antibody tests with enterovirus isolates

Vero cell monolayers were infected with each of the enterovirus isolates and with reovirus 3, and left to incubate at 37°C until showing obvious CPE. They were then stained as above with the optimal strength of conjugated antibody against isolate D74/18 and examined for specific fluorescence. In addition, uninfected Vero cell monolayers were similarly stained and examined, to further check for nonspecific fluorescence, and uninfected unstained cell monolayers were examined to check for autofluorescence.

Results

(a). Antiserum production

One rabbit died after the third injection of virus, apparently of anaphyllactic shock. The remaining rabbit was used to provide 20 ml of antiserum. Ten ml of antiserum was taken from each chicken.

(b). Titration of antisera

The preinoculation sera obtained from the chickens showed no evidence of inhibitory activity at 1 in 10 dilution against the corresponding viruses. The preinoculation serum from the surviving rabbit showed inhibitory activity up to a dilution of 1 in 16.

Post inoculation sera from the chickens and the surviving rabbit were all found to show reactivity against their homologous viruses as shown in Table 17. The antisera produced a mild precipitation and some cell degeneration when titrated in MDBK cells. This was only mild at the 1 in 10 dilution of the chicken antisera, and did not interfere with the test, but with the rabbit antiserum,

the effect was noted up to a 1 in 40 dilution. Because of this degenerative effect and the lower level of activity against the virus, the rabbit antiserum was not utilised any further.

Table 17

Titres of antisera against their homologous viruses

	Chicken antisera					Rabbit antiserum
Identification	D74/2	D74/18	D74/19A	D74/19B	D74/22-4	D74/18
Titre	320 +	960	480	1280	320	160

\+ reciprocal of highest dilution neutralising 100 $TCID_{50}$ of homologous virus

The viral titration carried out in the presence of various concentrations of equine serum showed that the serum concentration had no influence on the endpoint of the test.

(c) Cross neutralisation tests with the enterovirus isolates

The results of the cross neutralisation tests are shown in Table 18 which expresses the reactions as percentages of the homologous titres, to aid comparison. Detailed results are shown in Appendix 4 (Table F).

Table 18

Antigenic relationships of the enterovirus isolates as revealed by cross neutralisation tests

Virus	Avian antisera				
	D74/2	D74/18	D74/19A	D74/19B	D74/22-4
D74/2	100	3.1+	4.1	6.2	-
D74/18	-++	100	66.6	37.5	100
D74/19A	-	18.7	100	18.7	25
D74/19B	-	50	100	100	66.6
D74/22-4	-	12.5	25	12.5	100

\+ = titres expressed as a per cent reaction of homologous serum titre

++ = no reaction obtained at 1 in 10 dilution of serum

Based on the results of the titration, viruses D74/2 and D74/18 were selected as local prototype enterovirus strains.

(d). Cross neutralisation tests with American enterovirus serotypes and local enterovirus prototypes

The results of cross neutralisation tests with the 2 selected local prototypes strains of enterovirus and of 7 enterovirus serotypes of U.S. origin (Bovine enterovirus serotypes I to VII) are shown in Table 19. Titres are expressed for comparative purposes as per cent reactions of the corresponding homologous serum titre. Detailed results are shown in Appendix 4 (Table G).

Table 19

Antigenic relationships of U.S. enterovirus serotypes and local enterovirus prototype strains

Virus	Antisera of U.S. origin								
	BES I	BES II	BES III	BES IV	BES V	BES VI	BES VII	D74/2	D74/18
BES I	100	$-^{+}$	-	-	-	-	-	-	2.0
BES II	-	100	4.1^{++}	-	-	-	-	8.3	6.2
BES III	-	-	100	-	-	-	16.6	12.5	3.1
BES IV	-	-	-	100	-	-	-	-	1.0
BES V	-	-	-	8.3	100	9.3	-	-	4.1
BES VI	-	-	-	-	-	100	-	-	1.0
BES VII	-	-	-	-	-	-	100	-	-
D74/2	6.2	-	12.5	6.2	-	-	8.3	100	3.1
D74/18	9.3	-	-	16.6	-	-	-	-	100

+ = no reaction obtained at 1 in 10 dilution of serum

++ = titres expressed as per cent reaction of homologous serum titre

BES = bovine enterovirus serotype

(e). Identification of isolate D74/25A

The CPE of isolate D74/25A and of the known BVD virus was completely neutralised by the BVD antiserum, while the CPE developed normally at 2 days in the FBL cells inoculated with the test virus and control virus. It was concluded that the isolated virus was a strain of bovine viral diarrhoea virus.

(f). Fluorescent antibody production

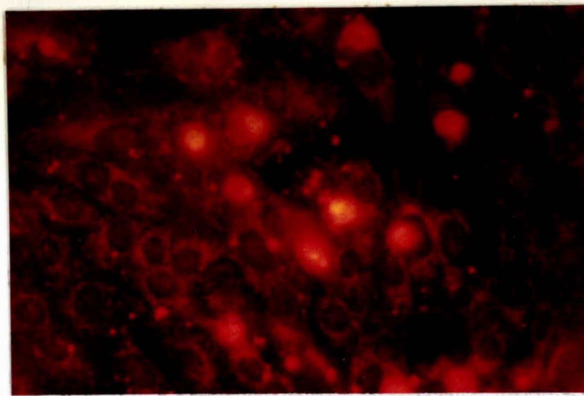
The quantity of globulin obtained by the triple ammonium sulphate procedure was somewhat lower than expected, but was sufficient for the procedures required. The second fraction obtained from the DEAE cellulose column was found to show moderate colouration with the fluorescent dye, though little was seen in the first fraction.

(g). Testing of conjugated antiserum

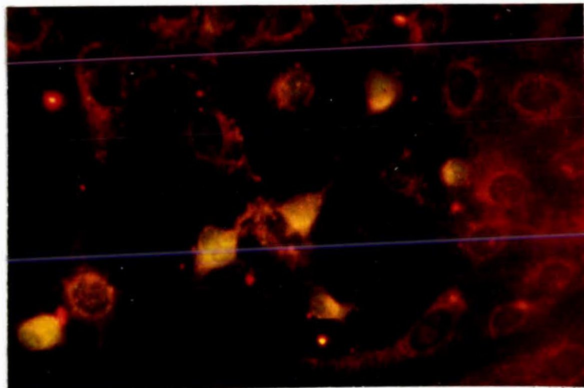
The second fraction of conjugated antiserum was found to possess optimal reactivity, using a dilution of 1 in 2 to 1 in 4. MDBK cells were found to be unsuitable because of reactivity against them by the conjugated antiserum. Vero cells proved to be satisfactory and allowed specific fluorescence to be seen. Autofluorescence was not found to be a problem.

(h). Fluorescent antibody tests with enterovirus isolates

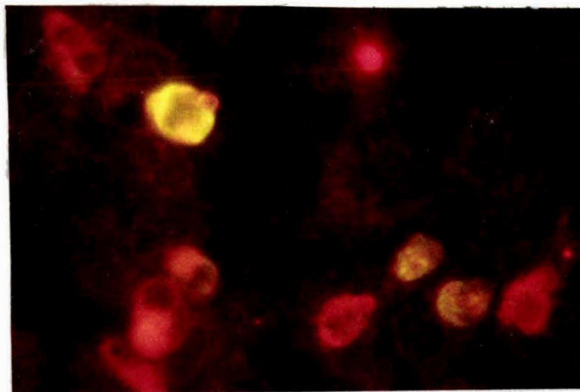
Specific fluorescence was found to be present when the conjugated antiserum against isolate D74/18 was tested against Vero cells infected with isolates D74/18, D74/19A, D74/19B, and D74/22-4. It was not seen when tested against isolate D74/2 or reovirus 3 .



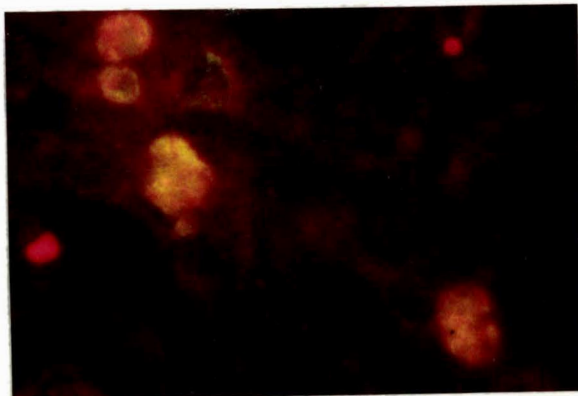
D74/2



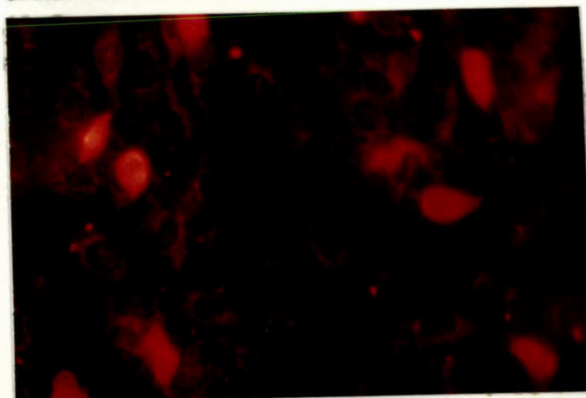
D74/18



D74/19A



D74/22-4



Reovirus 3

FIGURE 20

Vero cell cultures infected with some enterovirus isolates, and reovirus 3, and subsequently stained with fluorescein-conjugated D74/18 antiserum. Note the lack of fluorescent labelling with isolate D74/2 and reovirus 3, in contrast to the other labelled enteroviruses.

Discussion

The presence in rabbit sera of inhibitory substances to bovine enteroviruses has been previously reported by McFerran (1962), La Placa et al., (1965) and Barya et al., (1967). The inhibitor was found by Klein et al., (1964) and La Placa et al., (1964) to be a non-antibody protein. Inhibitory substances have also been identified in the sera of many animals, which are active against poliovirus (Bartell and Klein, 1955) and bovine enteroviruses (McFerran, 1962b; Klein et al., 1964). Chicken sera, on the other hand, have been reported to contain low levels of inhibitory substances against bovine enteroviruses (McFerran, 1962b; La Placa et al., 1965; Barya et al., 1967).

Because of the presence of these inhibitors, the rabbit has been found by La Placa et al., (1965) to be an inferior animal to the chicken for the production of antisera to the bovine enteroviruses. This is apparently because the inhibitory substances in the rabbit result in the rapid removal of most of the inoculated viral antigen from the blood stream (Klein and DeForest, 1964), leaving little residual virus for antigenic challenge. The serological results obtained in these experiments generally support the above findings.

The relatively high level of reactivity against MDBK cells in the rabbit antiserum probably relates to the use of a longer immunisation schedule involving the use of Freund's adjuvants. Though the level of MDBK cell derived antigens injected into the rabbit would be low as a consequence of the PEG 6000 purification step, there was still sufficient to result in considerable serological response. In contrast, the chicken antiserum produced by a short immunisation schedule of unpurified viral inoculum resulted in a much lower level of reactivity against MDBK cells. It has been stated by Herbert (1973) that the use of long immunisation schedules and of adjuvants in the production of antisera, results in the production of less specific antibodies, and more particularly, in the production of more antibodies to minor components of the antigenic mixture.

The choice of the described cross neutralisation test procedure as used by Moscovici et al., (1961), Moscovici and La Placa (1962), and Dunne et al., (1971) was based on it's simplicity. A number of other methods have been used by various authors working with bovine enteroviruses. Van Der Maaten and Packer (1967) preferred to use a constant serum-varying virus procedure, which was claimed to show greater sensitivity to minor antigenic components. The plaque reduction test was used by Mattson and Reed (1974), this method being stated by Wenner (1962) to be more sensitive than the conventional neutralisation test. A more involved procedure was used by Barya et al., (1967), who studied the kinetics of neutralisation of a number of enteroviruses. This method involved plaque titration of infectivity of virus-serum mixtures at various time intervals. From the results obtained, a rate of reaction was derived which was characteristic of each virus-antiserum reaction. This method was claimed by the authors to be the most sensitive and reliable of procedures available.

In the cross neutralisation tests with local enteroviruses, isolate D74/2 was found to behave quite differently serologically to the other isolates, showing little evidence of cross reactivity. In contrast, the other 4 enterovirus isolates showed a considerable degree of cross reactivity, and would appear to be serologically related. It was concluded therefor that 2 serotypes of enterovirus had been isolated, isolates D74/2 and D74/18 being selected as the prototype strains. Isolate D74/22-4 differed slightly from the other 3 members of it's serotype in that the reaction was predominantly of the "one way" type. It could possibly be regarded as a prime variant of the serotype, based on the definition of Melnick and Wenner (1969), who stated that prime strains are poorly neutralised by antisera to the prototype strain, but induce the production of antibody which neutralises the prime strain and prototype strain equally well. It is relevant that the 3 isolates D74/18, D74/19A, and D74/19B all came from the same property, though from different animals.

The cross neutralisation tests comparing the 2 local prototypes with the 7 enterovirus serotypes of U.S. origin showed little evidence of cross reactivity. Isolate D74/2 was found to show a low degree of relationship with BES III, whilst D74/18 appeared to have some affinity with BES I and BES IV, though the latter only showed as a "one way" reaction. It was concluded that the 2 local prototype strains are probably different from the U.S. serotypes. It may be relevent to note that the antisera to the 2 local prototype strains showed a broader type of reactivity than the antisera to the U.S. serotypes; this is possibly due to the use of unpurified virus for the production of antiserum, though plaque purified viruses were used for the actual cross neutralation tests.

Most strains of BVD virus isolated to date in New Zealand have been cytopathogenic in bovine kidney cells, though this is probably a result of the limitations of the methods used for isolation. Though procedures are now being improved to include methods capable of detecting strains noncytopathogenic in bovine kidney cells, (such as immunofluorescence and gel precipitation) the use of FBL cells may provide a sensitive alternative cell system to bovine kidney.

The fluorescein conjugated antiserum to isolate D74/18 showed considerable reactivity against MDBK cells, probably because of the unpurified nature of the antigen used for the production of the antiserum. It would be preferable to purify the antigen to avoid this problem. However, reactivity against Vero cells was minimal, and allowed satisfactory fluorescent antibody tests to be carried out.

The results of the fluorescent antibody tests confirmed that isolates D74/18, D74/19A, D74/19B and D74/22-4 were serologically related, and that isolate D74/2 was of a different serotype. The use of this procedure may have some merit for the rapid provisional serotyping of enterovirus isolates.

A SEROLOGICAL SURVEY OF SOME ENDEMIC VIRUSES

Materials And Methods

One hundred adult bovine sera were obtained from 20 properties widely distributed over the North Island of New Zealand, 5 sera being taken from each property. These sera were subjected to the serum neutralisation test (SNT) for IBR and BVD viruses, and to the haemagglutination inhibition (HI) test for parainfluenza 3 (PI_3) virus and reoviruses 1, 2, and 3.

Sera for the SNT were heat inactivated at 56°C for 30 minutes, prior to the test. Sera for the PI_3-HI test were similarly heat inactivated, with and without treatment with receptor destroying enzyme[1] (RDE). This was carried out according to the manufacturer's instructions, using a 1 in 5 dilution of serum in RDE solution, incubating at 37°C overnight, and then heat inactivating as above. Sera for the reovirus HI test were adsorbed with kaolin as described by Mann et al., (1967). If they showed evidence of sufficient natural haemagglutinins to interfere with the reading of the HI test, the test was repeated following a period of adsorption of the sera with the relevant erythrocytes, as described by Hierholzer et al., (1969).

The SNT for IBR and BVD viruses was carried out according to the previously described microtitre procedure (General materials and methods). Serum dilutions used were from 1 in 2 to 1 in 256, and no serum controls were included, as previous experience indicated that bovine sera are only rarely toxic to cells. Viral doses of 100 $TCID_{50}$ of vaccine strains of IBR and BVD viruses were used, the dose being confirmed by a check titration carried out at the time of the test.

Haemagglutinins for the HI test were prepared from freeze-thawed Vero cell cultures harvested at 6 days following infection with

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1 Wellcome Reagents Ltd., Beckenham, U.K.

the relevant virus. These were titrated for haemagglutinating activity at 37°C as described (General materials and methods), using guinea pig erythrocytes for the PI_3 test, human type "O" erythrocytes for the reovirus tests, and also, bovine erythrocytes for the reovirus 3 test. Whilst satisfactory haemagglutinins were produced with PI_3 and reovirus 2 viruses, this was not the case with the other 2 reoviruses. Consequently, these latter haemagglutinins were sonicated at 20 000 Kc/second for 2 minutes and then retitrated for haemagglutinating activity.

The HI tests were then carried out at 37° as described (General materials and methods) using a viral dose of 4 haemagglutinating units (HAU), serum dilutions of 1 in 10 to 1 in 640, and using serum controls. Guinea pig erythrocytes were used for the PI_3 tests, human type "O" erythrocytes for the tests with reoviruses 1 and 2, and bovine erythrocytes for the reovirus 3 test.

Results

Haemagglutinin titres obtained for PI_3 virus and reovirus 2 were 1 in 16 and 1 in 24, respectively per 0.05 ml. Reovirus 1 only produced a low haemagglutinin titre, but on sonication this doubled to 1 in 16. Reovirus 3 gave a 1 in 4 titre with human type "O" erythrocytes, and 1 in 8 with bovine erythrocytes. This latter figure rose to 1 in 16 on sonication.

The results of the tests are summarised in Table 20, which also shows the dilutions at which the results are read. Reactions to 1 or more serotypes of reovirus were found in 87% of the sera.

Table 20

The prevalence of antibodies to some endemic viruses

Test	IBR (SNT)	BVD (SNT)	PI_3 (HI)	Reovirus 1 (HI)	Reovirus 2 (HI)	Reovirus 3 (HI)
Serum dilution	1/2	1/8	1/20	1/10	1/10	1/10
Per cent positive	53	33	77	58	29	70

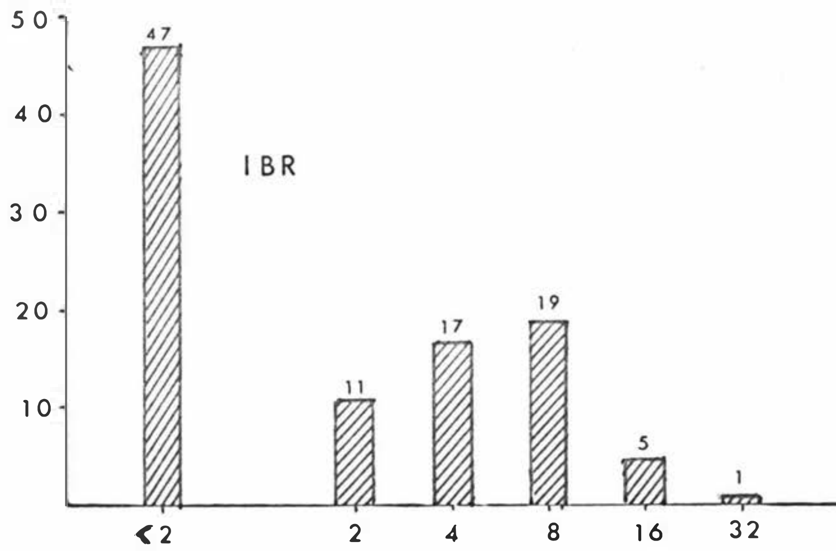
The frequency distribution of titres for each virus is shown in Figures 21 and 22.

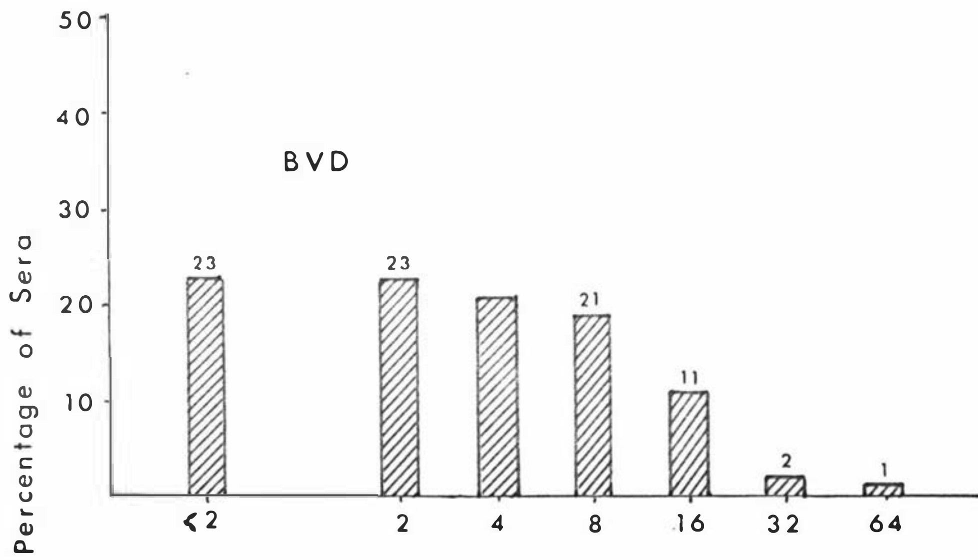
With the PI_3 test, RDE treatment made no difference to the overall reaction rate, though a few sera showed a titre increase or decrease over 1 dilution step. Only 10 sera possessed natural haemagglutinating activity against guinea pig erythrocytes, but these were all trace reactions only at the 1 in 10 dilution and did not interfere with the test results.

With the reovirus HI test, it was found that 44% of the sera possessed natural agglutinins to human type "O" erythrocytes, and 26% to bovine erythrocytes. Tests with these sera had to be repeated following adsorption with the relevant erythrocytes, except for 5 sera with the reovirus 3 test, where the serological titres were considered sufficiently great to be uninfluenced by the natural haemagglutinin titre.

Discussion

Opinions differ on the need for pretreatment of bovine sera prior to the HI test for PI_3 virus. Reisinger et al., (1959) and Horner et al.(1973) reported that some bovine sera possessed non-specific inhibitors of PI_3 virus, and that these inhibitors could be reduced or eliminated by RDE treatment. On the other hand, Ketler et al., (1961), Dawson (1963), and Rossi and Kiesel (1971) concluded that nonspecific inhibitors are either absent or infrequent


50
40
30
20
10
IBR
47
11
17
19
5
1
<2
2
4
8
16
32


Percentage of Sera
50
40
30
20
10
BVD
23
23
21
11
2
1
<2
2
4
8
16
32
64

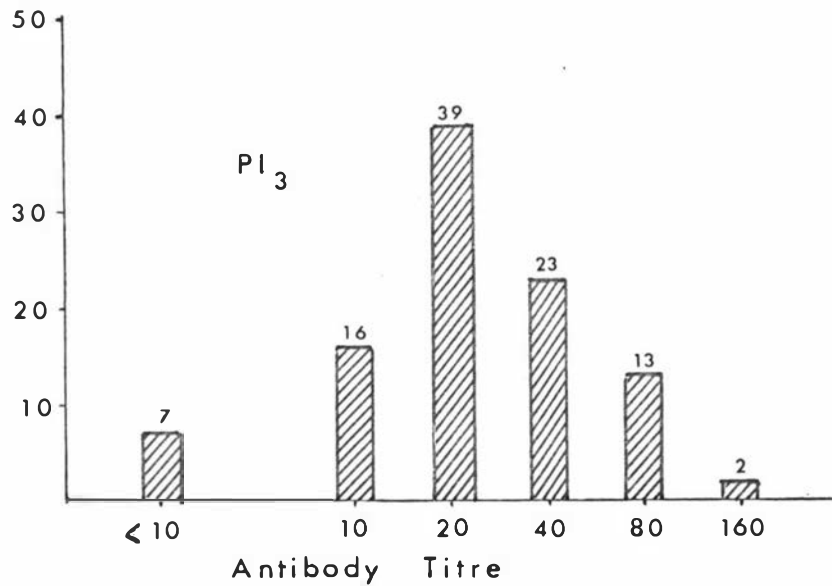
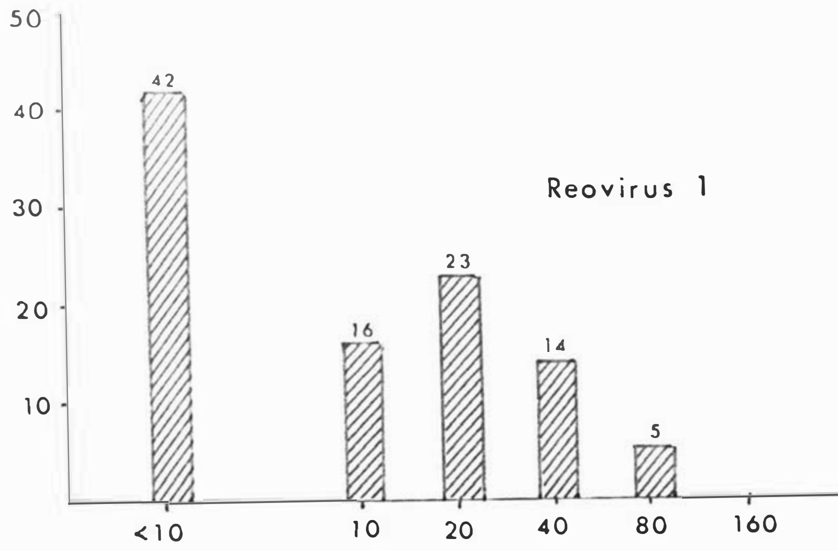
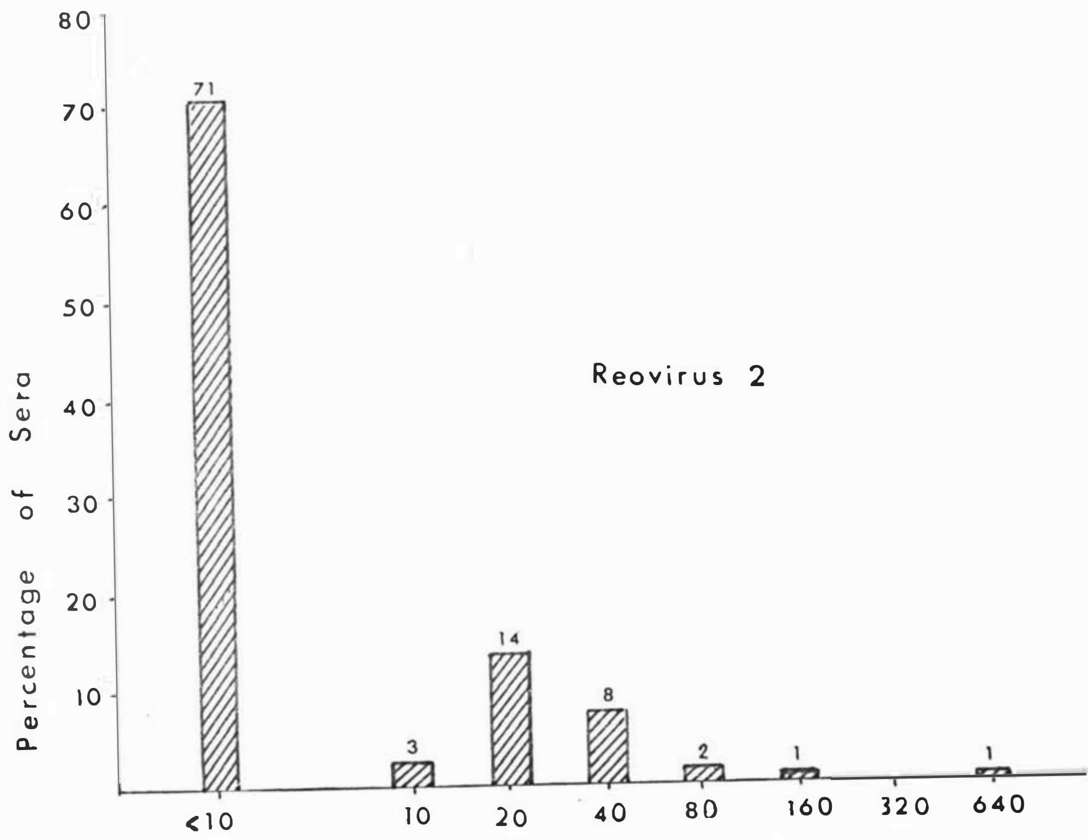

50
40
30
20
10
PI 3
7
16
39
23
13
2
<10
10
20
40
80
160
Antibody Titre

FIGURE 21

Distribution of antibody titres to IBR, BVD, and PI_3 viruses.


50
40
30
20
10
42
16
23
14
5
Reovirus 1
<10
10
20
40
80
160


80
70
60
50
40
30
20
10
Percentage of Sera
71
3
14
8
2
1
1
Reovirus 2
<10
10
20
40
80
160
320
640

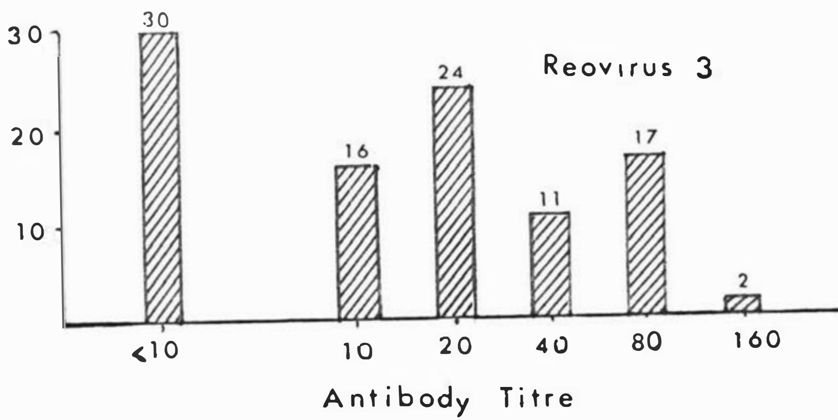

30
20
10
30
16
24
11
17
2
Reovirus 3
<10
10
20
40
80
160
Antibody Titre

FIGURE 22

Distribution of antibody titres to 3 serotypes of reovirus.

in bovine sera. The results obtained with the 100 sera would tend to support the latter conclusion, though it would be desirable that a larger number of sera be examined before RDE treatment is routinely discontinued. The results also suggest that routine adsorption of bovine sera with erythrocytes prior to the test is probably not essential, provided serum controls are used. Where sera are found to possess natural haemagglutinins, the test could be repeated following a period of adsorption with erythrocytes.

Kaolin pretreatment of sera has been routinely used with the HI test for a number of viruses, and is thought to remove lipoprotein associated nonspecific inhibitors of reovirus haemagglutination (Mann et al., 1967). Whether these inhibitors exist in New Zealand cattle sera has not been determined. However, it would appear worthwhile that New Zealand cattle sera be adsorbed with appropriate erythrocytes prior to the reovirus HI test, as the number of sera showing natural haemagglutinins to human type "O" and bovine erythrocytes was quite high.

The choice of a screening dilution at which a serological test is considered positive or negative is somewhat arbitrary. Because the SNT for IBR virus is regarded as being of low sensitivity, a positive reaction at a dilution of 1 in 2 is considered significant (York, 1968). Titres with BVD virus are generally somewhat higher than with IBR virus and slightly higher screening dilutions are commonly used. Malmquist (1968) considered that a reaction at a dilution of 1 in 4 of serum is indicative of exposure to BVD virus, and this dilution was also used by Rossi and Kiesel (1971). In New Zealand, a slightly higher serum dilution of 1 in 8 has been used (Fastier and Hansen, 1966; Robinson, 1971; Durham and Forbes-Faulkner, 1975). To aid comparison with local results, this dilution was used in Table 20.

Abinanti et al., (1961) and Rossi and Kiesel (1971) recommended that a reaction at 1 in 20 or greater serum dilution should be considered positive with the HI test for PI_3 virus. The latter authors based their opinion on comparative studies with the

SNT for PI_3. This dilution was consequently used for Table 20

A screening serum dilution of 1 in 10 was chosen for the HI test with the reoviruses, as it has been commonly used by other investigators (Rosen, 1960; Rosen and Abinanti, 1960; Rosen et al., 1962). The specifity of the results should be treated with some caution, as the reoviruses have been noted to produce a heterotypic serolcgical response in some animals.

The titres of haemagglutinin produced were generally rather low. Difficulty in producing satisfactory haemagglutinin titres has also been reported previously by Rosen (1960) and Cook (1963), particularly with reoviruses 1 and 3, which is in accordance with the results obtained in these studies. However, it was found that a brief period of sonication was effective in doubling the haemagglutinin titre with these viruses, presumably by disaggregating the viral particles, as found by Hermodsson (1960) with parainfluenza virus.

Bovine erythrocytes were used for testing with reovirus 3, as they proved to give superior haemagglutination, which is in agreement with the findings by Eggers et al., (1962), who found that bovine erythrocytes gave better and more consistent results with reovirus 3 than did human type "O" cells.

Further comment on the serological results is left to the general discussion section.

GENERAL DISCUSSION

As stated in the introduction, an important objective of these studies was to gain some expertise in the use of a number of virological procedures. The isolates obtained proved to be very suitable for this purpose in that they allowed a considerable number of techniques to be successfully attempted. A further objective was to initiate studies on the possible aetiological role of viruses in gastrointestinal disease in young cattle in New Zealand. Whilst some progress was made in this regard, it is realised that much more work remains to be done, and consequently it is hoped to extend these investigations considerably in the future, particularly in relation to diarrhoea in neonatal calves.

Five of the isolates (D74/2, D74/18, D74/19A, D74/19B, D74/22-4) were classified as enteroviruses on the basis of their characteristic CPE, small size of about 26 nm, uninhibited growth in the presence of BUDR, lack of sensitivity to lipid solvents, acid stability, and their stabilisation at 50°C by molar $MgCl_2$. The buoyant density of 1.34 g/ml in CsCl of isolate D74/18 was also consistent with this classification.

Enteroviruses have been mainly subclassified on the basis of their reactions in cross neutralisation tests, the main problems being the variation in the levels of sensitivity of the various test procedures and the existence of some enterovirus strains showing a broader antigenicity than others. A further complication has been the effect of passaging the various viruses in different types of host cells, resulting in selection for different characteristics (Moscovici et al., 1961). Nevertheless, the results of cross neutralisation tests with these 5 isolates were reasonably clear, in that isolate D74/2 was serologically distinct from the other 4 enterovirus isolates. This finding was supported by the results of the fluorescent antibody test using fluorescein-conjugated antiserum to isolate D74/18, which also showed that isolate D74/2 was of a different serological group. This latter procedure has not been previously

reported as being used for the serotyping of bovine enteroviruses, but has been used to classify human Coxsackie and polioviruses (Shaw et al., 1961) and porcine enteroviruses (Watanabe, 1971). All 5 enterovirus isolates appeared to be serologically unrelated to the 7 U.S. serotypes which were available for comparison, though some minor degrees of antigenic similarity were seen.

The separation of the enterovirus isolates into 2 serotypes is further supported by their behaviour in the presence of HBB, which indicated on the basis of the findings of Portolani et al., (1968) that isolate D74/2 probably belongs to group 1 in the classification of La Placa et al., (1965), whilst the other 4 enterovirus isolates probably belong to group 2. On this basis, although no haemagglutinins were demonstrated with bovine, guinea pig and human type O erythrocytes with any of the isolates, it would appear likely that isolate D74/2 should show haemagglutinating activity with monkey erythrocytes, this being a basic property of group 1 enteroviruses (La Placa et al., 1965).

Immunofluorescent staining with fluorescein-conjugated antiserum to isolate D74/18 also showed that viral antigens were confined to the cytoplasm during replication in the cell, and this was further supported by the results of staining with acridine orange and coriphosphene O dyes, which showed an intensification of normal cytoplasmic staining only. This latter finding is consistent with the known single-stranded nature of enteroviruses (Andrewes and Pereira, 1972). Though all of the enterovirus isolates were relatively heat stable, isolates D74/2 and D74/19B were found to have comparatively shorter half-lives at 37°C. These same 2 isolates were also notable for the small size of plaques produced at this same temperature. This correlation may be due to their lower heat stability causing a more rapid inactivation of extracellular virus, leading to a decreased rate of infection of adjacent cells and hence a slower rate of growth of viral plaques. Isolates D74/2 had the shortest half-life at 56°C, this further emphasizing it's biological distinctness from the other 4 enterovirus isolates.

The production of large plaques has been found to be associated with increased virulence in animals for a number of viruses, including human Coxsackie and polioviruses (Vogt et al., 1957) and foot and mouth disease virus (Cottral et al., 1966). In this regard it is interesting to speculate on the possible increased virulence of isolates D74/19A and D74/22-4, as these both produced considerably larger plaques.

The pathological significance of the 5 enterovirus isolates is uncertain, though all were obtained from the faeces of scouring animals. Whilst bovine enteroviruses have been shown to be capable of inducing diarrhoea in calves (McFerran, 1962a; Van Der Maaten and Packer, 1967), myocarditis and paralysis in mice (Kunin and Minuse, 1958; Moll and Davis, 1959) and abortion and stillbirth in pregnant guinea pigs (Moll and Davis, 1959; Moll, 1964; Van Der Maaten and Packer, 1967), the remaining evidence of pathogenicity is largely circumstantial. Thus, considerable levels of antibody to bovine enteroviruses have been demonstrated in the body fluids of aborted bovine foetuses, apparently as a result of active foetal infection in utero, and led Dunne et al., (1973) to suggest enteroviruses as possible important causes of bovine abortion. Enteroviruses have also been isolated from aborted bovine foetuses (Moll and Finlayson, 1957; Moll and Davis, 1959; Mattson and Reed, 1974) and from faeces and nasal swabs of adult cattle and calves with diarrhoea, respiratory disease and abortion (Moll and Davis, 1959; McFerran, 1962a; Moll and Ulrich, 1963; Schiott and Hyldegaard-Jensen, 1966; Mattson and Reed, 1974). The significance of these latter isolates is to some extent reduced by the frequent isolation of enteroviruses from normal cattle (Kunin and Minuse, 1957; McFerran, 1962a).

Nevertheless, enteroviruses of man, mice, pigs and chickens have all been well documented as causes of a variety of disease syndromes in these species (Andrewes and Pereira, 1972), and it would be rather unexpected if bovine enteroviruses were all incapable of

similar pathogenic behaviour on occasion, especially in young calves. Though colostrum deprivation may be necessary in calves before pathogenicity can be elicited, this situation may not be that uncommon, as Bailey and McLean (1972) showed in a survey that 18% of Friesian calves at 1 week of age were hypoglobulinaemic, apparently as a result of management errors.

Thus, further investigation of the role of enteroviruses in causing disease in young cattle would appear well worthwhile, particularly in relation to neonatal calf diarrhoea and bovine abortion.

Isolate D74/13-49 was found to be more difficult to study because of its slow and relatively non-progressive type of CPE, which was only evident in Vero cells. Because of this, it proved impossible to titrate the virus on the basis of it's CPE, as degenerative changes in the cells mimicked the viral CPE at low titre. Attempts at developing a plaque titration system were only partially successful, as it proved possible only to develope microplaques, and then only when supplemental magnesium chloride and DEAE-dextran were included in the overlay medium. It is possible that with further work, a practical plaque titration method may be developed, particularly as a number of other chemical additives remain to be tried in the overlay medium, such as pancreatin and chymotrypsin, these having been found useful to enhance plaque development with reoviruses (Wallis et al., 1966b). An alternative method would be to develop a titration system based on immunofluorescence as an indicator of viral activity, as has been used with a number of other viruses. It is hoped to proceed with work on this system in the near future.

The properties of isolate D74/13-49 are apparently similar to those described for viruses of the diplornavirus group (Melnick, 1973) in that it appeared to show cubic symmetry, a size of about 80 nm, and was resistant to the action of BUDR and chloroform. The development of cytoplasmic inclusions of apparently double-stranded

nucleic acid, as shown by coriphosphene O staining of infected cells, supports this provisional classification. Once suitable titration procedures have been developed the physicochemical tests should be repeated in a more precise manner to corroborate the above findings.

The further classification of this isolate into 1 of the 3 probable subgroups of the diplornaviruses will require further detailed study of the properties of the virus. In particular, a study should be made of its thermal stability at 50°C in the presence of molar $MgCl_2$ and its acid sensitivity, as these properties will serve to distinguish between reoviruses, "reo-like" viruses and orbiviruses. In addition, a much more detailed study of the viral morphology is required under the electron microscope, so as to determine the capsid structure and the number and nature of the capsomeres. However, the apparent lack of haemagglutinating activity of this isolate together with it's production of discrete cytoplasmic inclusions suggests it may have similar properties to the "reo-like" viruses (Welch and Thompson, 1973).

As isolate D74/13-49 was isolated from a scouring calf at a property where neonatal calf scours had been a problem for several years, and has shown some affinity with the "reo-like" virus group, it would appear well worthwhile for further investigations to be made of its properties, particularly in relation to it's possible pathogenic effect in neonatal calves.

The small serological survey of North Island cattle was carried out as a pilot survey, as it is intended that more extensive surveys will be carried out in the near future so as to better define the prevalence of a number of viruses in the farm animal population of New Zealand.

Fastier and Hansen (1966) reported that 81.6% of 118 North Island cattle from herds with respiratory disease problems possessed antibodies to IBR virus, in contrast to 36% of 60 cattle from herds

where respiratory disease was not a problem. More recently, Durham and Forbes-Faulkner (1975) found that 41% of 669 diagnostic sera from North Island cattle possessed IBR antibodies, a much lower figure of 19% of 472 diagnostic sera being obtained for South Island cattle. The result obtained in this pilot survey of 53% of 100 North Island cattle of unknown disease status was somewhat higher than expected, but may reflect the small size of the sample population. It nevertheless confirms the widespread nature of the virus in the cattle population.

Fastier and Hansen (1966) also reported that 41% of North Island cattle from herds with respiratory disease problems possessed BVD antibodies, in contrast to 25% of 60 apparently normal cattle. A similar figure of 31% of 507 diagnostic sera from North Island cattle, and of 39% of South Island cattle was obtained by Durham and Forbes-Faulkner (1975). The result obtained here of 33% is in close agreement with the above data.

There has only been 1 report of the prevalence of antibodies to PI_3 virus in the New Zealand cattle population, being that of Fastier and Hansen (1966), who found that 98% of 118 North Island cattle from respiratory disease problem herds possessed antibodies to the virus, this comparing with a rate of 58% of 60 apparently normal cattle. The level found in this survey of 77% of 100 North Island cattle is in reasonable agreement with the above data and emphasizes the widespread nature of the agent. The role of PI_3 virus in causing disease in cattle was briefly reviewed by Jolly (1967) who stated that there was little information to suggest PI_3 virus was an important cause of disease in New Zealand cattle. Nevertheless, the lack of information on the role of this virus in contributing to calf respiratory disease is partly due to the past lack of virological facilities in New Zealand, and it is considered therefore that the virus should not be dismissed as unimportant at this stage. Recent reports by Sattar et al., (1965, 1967), Swift and Kennedy (1972) and Dunne et al., (1973) associating the virus

with bovine abortion also suggest that a more careful appraisal of the pathological significance of PI_3 virus may be warranted.

There is no published data on the prevalence of antibodies to the 3 main serotypes of reoviruses in New Zealand cattle, and little has been reported overseas. One of the problems in interpreting such data is the development of heterologous reactions following infection with a reovirus serotype (Rosen et al., 1963), particularly with serotypes 1 and 2. Reports by Rosen et al., (1963) and Lamont (1968) indicate that reoviruses 1 and 3 are the serological types most commonly isolated from cases of respiratory disease and diarrhoea, and the higher prevalence of serotypes 1 and 3 is supported by some serological data (Rosen et al., 1963). Stanley and Leak (1963) found in a small survey in Australia that 43%, 43% and 62% of bovine sera possessed antibodies to reoviruses 1, 2 and 3 respectively, and that multiple infections appeared common. The levels of 58%, 29% and 70% found for the corresponding 3 serotypes in this survey also confirm the widespread distribution of these viruses in New Zealand.

Although some progress has recently been made in the study of viral infections of livestock in New Zealand, a considerable amount of work still remains to be attempted before the economic significance of viral infections can be properly evaluated.

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APPENDIX 1

List of abbreviations

apd	Average pore diameter
ATV	Antibiotic-trypsin-versene solution
BEK 1	Bovine embryonic kidney cell line
BUDR	5 - Bromo - 2 deoxyuridine
BVD	Bovine viral diarrhoea
CPE	Cytopathic effect
DEAE	Diethyl aminoethyl
DNA	Deoxyribonucleic acid
FBK	Foetal bovine kidney
FBL	Foetal bovine lung
FITC	Fluorescein isothiocyanate
g	Gram
G	Force of gravity
Hank's AB	Hank's buffered salt solution with 2000 units per ml of penicillin and 2000 μg/ml of streptomycin
HA	Haemagglutination
HAU	Haemagglutinating unit
HI	Haemagglutination inhibition
IBR	Infectious bovine rhinotracheitis
Kc/second	Kilocycles per second
$\log_{10}$	Logarithm to the base of 10
M	Molar
MDBK	Madin-Darby bovine kidney cell line
mg	Milligram
ml	Millilitre
mm	Millimetre
mM	Millimolar
Mouse L cell	Mouse fibroblast cell line
μ	Micron = 10^{-6} metre
μg	Microgram = 10^{-6} gram
nm	Nanometre = 10^{-9} metre
PEG	Polyethylene glycol
PBS	Phosphate buffered saline (calcium and magnesium free)
PFU	Plaque forming unit

PI_3	Parainfluenza 3
PTA	Phosphotungstic acid
RDE	Receptor destroying enzyme (Neuraminidase)
RNA	Ribonucleic acid
SDL	Sodium deoxycholate
SNT	Serum neutralisation test
$TCID_{50}$	50% tissue culture infective dose
Vero	Continuous line of African green monkey kidney cells
w/v	Weight for volume
w/w	Weight for weight

APPENDIX 2

Preparation Of Media And Solutions.

Agar overlay for cell culture monolayers

A. Prepare double strength cell culture medium 199 (Wellcome).
Store at 4°C.
Before use, warm in water bath to 43°C after addition of required final amount of sterile serum and antibiotics.

B. Noble Agar (Difco) or Seaplaque Agarose (Marine Colloids Inc).
Water (distilled deionised) 48 ml
Autoclave at 10 lbs pressure for 10 minutes.
Store at 4°C.
Before use, melt in boiling water bath, and then cool to 43°C, if using agar, or to 37°C if using agarose.
Pour contents of A into B, mix well, and dispense required amount over monolayer.
For staining, add further monolayer incorporating 0.01% neutral red solution.

Antibiotics

Make these up as a x100 concentrate in PBS.

Drug	Working Strength
Benzyl Penicillin	100 units/ ml
Streptomycin sulphate	100 μg / ml
Kanamycin sulphate	100 μg / ml
Gentamycin	200 μg / ml

Sterilise by filtration (220 nm apd)

Antibiotic-trypsin-versene (ATV)

(Reference: Madin and Darby, 1958)

Trypsin	0.5 g
Versene	0.2 g
NaCl	8.0 g
KCl	0.4 g
Dextrose	1.0 g
$NaHCO_3$	0.58 g
Penicillin	2×10^5 units
Streptomycin	100 mg
Phenol Red	0.02 g

Make up to 1,000 ml with water (distilled deionised).
Sterilise by filtration (220 nm apd).
Store at -20°C in 20 ml aliquots.

Buffers

Unless otherwise stated, all buffers were prepared according to the tables drawn up by Gomori (1955).

Celloidin (collodion) solution for electron microscopy grids

Celloidin	0.5 gm
Amyl Acetate	100 ml

Eagles minimal essential medium (MEM)

Eagles minimal essential medium (Wellcome)	10 g
Deionised water	950 ml
$NaHCO_3$ (4.4% solution)	50 ml

Mix as above and sterilise by filtration (220 nm apd) under pressure.
Store at 4°C.
Add antibiotics and serum as required.

Earles balanced salt solution (without bicarbonate)

NaCl	6.8 g
KCl	0.4 g
$MgSO_4.7H_2O$	0.2 g
$NaH_2PO_4.2H_2O$	0.163 g
Dextrose	1.0 g
Phenol Red	0.02 g
$CaCl_2$ (anhydrous)	0.2 g
Deionised water to	1000 ml

Sterilise by filtration (220 nm apd).
Store at 4^oC.

Earles lactalbumin hydrolysate solution (ELA)

Earles balanced salt solution	845 ml
Lactalbumin hydrolsate solution	100 ml
Yeast extract	5 ml
Sodium bicarbonate (4.4% solution)	50 ml

Sterlise by filtration (220 nm apd) under pressure.
Store at 4^oC.
Add antibiotics and serum as required.

Formvar solution for electron microscopy grids

Formvar (poly vinyl formol)	0.5 g
Ethylene dichloride	100 ml

Glutaraldehyde

Glutaraldehyde	2.5 g
0.1 M phosphate buffer pH 7.2	100 ml

Hank's balanced salt solution (without bicarbonate)

Solution A (x10 stock solution)

NaCl	80 g
KCl	4.0 g
$MgSO_4.7H_2O$	2.0 g
$CaCl_2$ (anhydrous)	1.4 g

Make up to 1000 ml with water.

Solution B (x10 stock solution)

Na_2HPO_4 (anhydrous)	0.6 g
KH_2PO_4	0.6 g
Dextrose	10.0 g
Phenol Red	0.2 g

Make up to 1000 ml with water.

Mix 100 ml of Solution A and 100 ml of Solution B, and make up to 1000 ml with water.

Sterilise by filtration (220 nm apd) or by autoclaving for 10 minutes at 10 lbs pressure.

Store at room temperature.

Hank's lactalbumin solution (HLA)

Hank's basic salt solution	845 ml
Lactalbumin hydrolysate solution	100 ml
Yeast extract solution	5 ml
Sodium bicarbonate (4.4% solution)	50 ml

Sterlise by filtration (220 nm apd).

Store at 4°C.

Add antibiotics and serum as required.

Lactalbumin hydrolysate solution (LAH)

Lactalbumin hydrolysate (Difco)	50.0 g
NaCl	8.0 g
KCl	0.2 g
Na_2PO_4	1.15 g
KH_2PO_4	0.2 g

Make up to 1,000 ml with water and autoclave at 10 lbs pressure for 10 minutes. Store at $-20^{\circ}C$. Discard precipitate, if any, before use.

Lead Citrate
(Reference: Venable and Coggeshall, 1965)

Lead citrate	0.02 g
Water	10 ml

Add 0.1 ml of 10 N NaOH to dissolve.
Filter through membrane filter (450 nm apd).
Store in stoppered bottle.

Medium 199

Medium 199 dried powder (Wellcome)	10 g
Deionised water	950 ml
Tricine (Calbiochem)	1.8 g
$NaHCO_3$	1.5 g

Mix as above, adjust volume to 1,000 ml with deionised water and sterilise by filtration (220 nm apd) under pressure.
Store at $4^{\circ}C$.
Add antibiotics and serum as required.
N.B. Tricine is used as a buffer and to control mycoplasma contaminants (reference: Spendlove *et al.*, 1971).

Methyl cellulose overlay for cell culture monolayers

Methyl cellulose powder (Hopkins and Williams Ltd.)	3.0 g
Water	100 ml

Suspend with vigourous mixing the methyl cellulose powder in boiling water, and autoclave at 15 lbs pressure for 15 minutes. (N.B. Emerges opaque from autoclave.)
Cool to 4°C, when liquifies and clears.
Store at -20°C.

This medium gels at 37°C, and reliquifies if returned to 4°C. For use, add to equal volume of double strength cell culture medium incorporating required amount of serum and antibiotic, at temperature of 4°C. Then overlay cells.

Neutral red solution

Neutral red powder	1 g
Phosphate Buffered Saline to	100 ml

Sterilise by filtration (220 nm apd).
Store at 4°C.
N.B. This solution has a photodynamic action on cells if exposed to light.

Osmium tetroxide

OsO_4	0.5 g
0.1 M Phosphate buffer, pH 7.2	50 ml

Phosphate buffered saline (PBS) (Ca^{++} and Mg^{++} free)

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
Water to	1000 ml

Sterilise by autoclaving (15 lbs for 15 minutes).
Final pH 7.2-7.4

Phosphotungstic acid

Potassium phosphotungstate	2 g
Water	100 ml

Adjust to pH7 with N KOH.

Trypan blue solution

Trypan blue	0.2 g
Phosphate buffered saline	100 ml

Filter through filter paper.
Dispense in 1.8 ml quantities.
Store at 4°C.

Trypsin solution

Trypsin (Difco 1:250)	2.5 g
Hanks Basic Salt Solution to	1000 ml
Penicillin	2×10^5 units
Streptomycin	100 mg
Phenol Red	0.02 g

Adjust pH to 7.6 with $NaHCO_3$ solution.
Sterilise by filtration (220 nm apd).
Store at -20°C.

Uranyl acetate

Prepare saturated solution in 50% ethanol.
Store shielded from light.

Yeast extract solution

Bacto yeast extract (Difco)	5.0
Phosphate buffered saline	250 ml

Dispense into 5.0 ml volumes and store frozen.

APPENDIX 3

Preparation And Use Of Fixitives And Stains

Acridine orange stain

Adapted from Mayer and Diwan, (1961).

1. Rinse in PBS - 1-2 seconds.
2. Wash in Carnoy's fixitive - 1-2 minutes.
3. Wash in 100% ethanol - 2-5 seconds.
4. Wash in 70% ethanol - 2-5 seconds.
5. Wash in acetate buffer pH 4.0 - 5 seconds.
6. Stain with 0.01% acridine orange (or acridine orange R)(Gurr) in pH 4 acetate buffer - 10-15 minutes.
7. Wash in two changes of acetate buffer pH 4 - 2 minutes.
8. Mount in buffer.
9. Examine under UV or blue light.

Results: Double stranded nucleic acid stains yellow/green.
Single stranded nucleic acid stains red-brown.

N.B. Monolayers may be satisfactorily fixed in Bouin's fixitive, provided they are adequately washed afterwards.

Bouin's fixitive

Picric acid (saturated aqueous solution)	75 ml
Formalin	25 ml
Glacial acetic acid	5 ml

Fix monolayers for 5-10 seconds, and tissues for 1 hour. Wash off excess picric acid with several changes of 80% ethanol or water.

Carnoy's fixitive

Good fixitive for nucleic acids.

Ethanol	60 ml
Chloroform	30 ml
Glacial acetic acid	10 ml

Fix monolayers for 1-2 minutes, and tissues for 1 hour. Wash in 100% ethanol, and store in 70% ethanol until stained.

Coriphospine O stain

Reference: Keeble and Jay (1962)

Coriphospine O stain (Gurr)	0.1 g
Acetate buffer pH 4.4	20 ml

Mix the above, and dilute to 1/10 in PBS for use.

1. Rinse in PBS - 1-2 seconds.
2. Fix in Carnoy's fixitive - 1-2 minutes.
3. Wash in 100% ethanol - 2-5 seconds.
4. Wash in 70% ethanol - 2-5 seconds.
5. Wash in PBS - 2-5 seconds.
6. Stain in Coriphosphine O stain solution - 1-5 minutes.
7. Wash in PBS till excess stain removed.
8. Mount in PBS, glycerol or liquid paraffin.
9. Examine under UV or blue light.

Results: Double stranded nucleic acid stains green.
Single stranded nucleic acid stains red-brown.

N.B. (a) Monolayers can be destained with ethanol and restained.

(b) Monolayers may be satisfactorily fixed in Bouin's fixitive, provided they are adequately washed afterwards.

(c) If liquid paraffin is used as a mountant, optical resolution is increased due to the resultant dark background.

Haematoxylin and eosin stain (Rapid)

For cell monolayers.

1. Rinse in PBS - 1-2 seconds.
2. Fix in Bouin's fixitive - 5 seconds.
3. Wash in 80% ethanol - 3 changes.
4. Stain in Mayer's Haematoxylin - 2-5 minutes.
5. Wash and "blue" in tap water containing a few drops of saturated lithium carbonate - 30 seconds.
6. Stain in 0.1% eosin (aqueous) - 30 seconds.
7. Wash in 95% ethanol - 10 seconds.
8. Wash in 100% ethanol - 10 seconds.
9. Wash in xylol - 30 seconds.
10. Mount in DPX.

Uranyl acetate-lead citrate stain for electron microscopy

A. Craig, Personal Communication.

Stain sections by immersion in, or floating on the following solutions.

Uranyl acetate in 50% ethanol	5 minutes
50% ethanol	3 washes
Distilled water	1 wash
Lead citrate solution	5 minutes
Distilled water	3 washes

Dry and examine.

APPENDIX 4

Table A

Titres of virus D74/18 in cells and medium during one step growth curve

Time (hours)	Titre in cells ($\log_{10}$ $TCID_{50}$/ 10^6 cells)	Titre in medium ($\log_{10}$ $TCID_{50}$/ 10^6 cells)
0	5.8	3.55
½	6.05	3.55
1	5.8	3.05
2	6.8	3.55
3	8.05	3.8
4	9.55	4.8
5	9.05	5.55
6	9.55	5.8
8	9.05	6.8

Table B

Growth of D74/18, IBR and BVD viruses in the presence and absence of BUDR

Virus	Concentration BUDR (μg/ml)	Viral titres ($\log_{10}$ $TCID_{50}$/0.1 ml) at times (hrs) :								
		1	5	8	12	16	24	48	72	96
D74/18	0	2.25	3.25	3.5	-	4.5	5.25	-	-	-
	100	2.25	2.75	3.25	-	4.5	5.25	-	-	-
IBR	0	1.25	-	-	0.75	-	3.5	6.75	6.75	-
	100	0.75	-	-	0.75	-	0.75	2.25	4.75	-
BVD	0	1.5	-	-	3.25	-	3.75	4.5	5.5	4.5
	100	1.25	-	-	2.75	-	4.25	4.5	5.5	4.25

- = not tested

Table C

Thermal inactivation of enterovirus isolates at 56°C

Virus	Titres of viruses ($\log_{10}$ $TCID_{50}$ /0.1 ml) at times (hrs):				
	0	½	1	2	4
D74/2	5.25	0.75	0.0	0.0	-
D74/18	5.75	0.75	2.25	0.5	-
D74/18 (repeat)	4.75	1.5	0.5	1.25	0.25
D74/19A	5.75	2.25	0.75	0.0	-
D74/19B	6.25	3.5	0.75	0.0	-
D74/22-4	5.75	2.5	1.75	0.25	-

Table D

Thermal inactivation of enterovirus isolates at 37°C

Virus	Titres of viruses ($\log_{10}$ $TCID_{50}$ /0.1 ml) at times (days):				
	0	2	4	7	14
D74/2	5.25	2.25	0.5	0.0	0
D74/18	5.75	3.75	2.25	1.25	0.5
D74/19A	5.5	3.75	2.5	1.5	0.0
D74/19B	4.75	1.5	0.25	0.0	0.0
D74/22-4	4.75	2.75	0.5	0.25	0.0

Table E

Viral content and density of the various fractions obtained by isopynic centrifugation of isolate D74/18 in a continuous caesium chloride gradient

Fraction No.	Density (g/ml)	Infectivity (PFU/0.1 ml)	Ultramicroscopic examination results
1	1.425	3×10^2	NE
2	1.419	2×10^2	NE
3	1.412	1×10^2	NE
4	1.407	4×10^2	NE
5	1.399	2×10^2	NE
6	1.392	4×10^2	NE
7	1.387	5×10^2	NE
8	1.382	3×10^2	NE
9	1.377	1×10^3	NE
10	1.371	3×10^3	NE
11	1.362	5×10^3	NE
12	deleted ǂ	9×10^3	-
13	deleted	2×10^4	-
14	1.352	5×10^4	-
15	1.345	3×10^5	+
16	1.339	5×10^5	++
17	1.337	3×10^6	+++
18	1.332	1×10^6	+++
19	1.328	9×10^5	+
20	1.324	7×10^5	-
21	1.321	4×10^5	-
22	1.318	7×10^4	NE
23	1.315	3×10^4	NE
24	1.314	1×10^4	NE
25	1.314	1×10^4	NE
26	1.313	1×10^4	NE
27	1.313	1×10^4	NE
28	1.313	1×10^4	NE
29	1.313	1×10^4	NE

+, ++, +++, = viral particles seen, graded according to frequency.
- = no viral particles seen. NE = not examined. ǂ = see text.

Table F

Antigenic relationships of the enterovirus isolates as revealed by cross neutralisation tests

Virus	Avian antisera				
	D74/2	D74/18	D74/19A	D74/19B	D74/22-4
D74/2	240+	40	20	40	-
D74/18	- ++	1280	320	240	240
D74/19A	-	240	480	120	60
D74/19B	-	640	480	640	160
D74/22-4	-	160	120	80	240

\+ = reciprocal of dilution neutralising 100 $TCID_{50}$ of virus.
++ = no neutralisation at 1 in 10 dilution of antiserum.

Table G

Antigenic relationships of U.S. enterovirus serotypes and local enterovirus prototype strains

Virus	Avian antisera								
	BES I	BES II	BES III	BES IV	BES V	BES VI	BES VII	D74/2	D74/18
BES I	320+	-	-	-	-	-	-	-	20
BES II	- ++	320	20	-	-	-	-	20	60
BES III	-	-	480	-	-	-	40	30	30
BES IV	-	-	-	240	-	-	-	-	10
BES V	-	-	-	20	30	30	-	-	40
BES VI	-	-	-	-	320	320	-	-	10
BES VII	-	-	-	-	-	-	240	-	-
D74/2	20	-	60	15	-	-	20	240	30
D74/18	30	-	-	40	-	-	-	-	960

\+ = reciprocal of dilution neutralising 100 $TCID_{50}$ of virus.
++ = no neutralisation at 1 in 10 dilution of antiserum.