The isolation and characterization of some bovine enteric viruses

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THE ISOLATION AND CHARACTERIZATION OF
SOME BOVINE ENTERIC VIRUSES

by

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INTRODUCTION

Prior to the 1950's it was generally accepted that all viruses were pathogenic for their natural hosts. With the development of tissue culture techniques and their application to the field of virology it became apparent that this was not the case. The isolation of viral agents from apparently healthy individuals led to the establishment of an "orphan virus" group consisting of viral agents which could not be associated with clinical disease.

In subsequent studies many of these "orphan viruses" were isolated from the intestinal tract of man. These agents became known as the Enteric Cytopathogenic Human Orphan (ECHO) viruses. It was originally proposed that as these agents were shown to be pathogenic they would be removed from the ECHO group but this has not been done.

Later studies indicated that the ECHO viruses were very similar to the Coxsackie and polio viruses in physical, chemical and biological properties. With an increased tendency to classify viruses on the basis of structure and chemical composition it was proposed that all three of the above groups be included in a single category known as the enteroviruses. This classification has been widely accepted at the present time.

It was, of course, natural that the discovery of the ECHO viruses would arouse curiosity concerning the presence
of similar agents in animals. Workers engaged in poliomyelitis research were particularly interested in bovine enteroviruses because cattle sera frequently contained substances capable of neutralizing the polio viruses. The agents which were subsequently isolated from cattle appeared to be analogous to the ECHO viruses and became known as the Enteric Cytopathogenic Bovine Orphan (ECBO) viruses. With the subsequent change in nomenclature of the human viruses it has become common to refer to the bovine agents as either ECBO viruses or bovine enteroviruses.

The availability of tissue culture techniques and the isolation of numerous viral agents from cases of bovine mucosal disease—viral diarrhea led to an increased interest in bovine enteric viruses among veterinary research workers. It was during the course of studies of the etiologic significance of viral agents isolated from cattle with mucosal disease that the present research was conceived. The differential diagnosis of the various enteric diseases is in itself a formidable task and the additional problem of differentiating pathogens from nonpathogens makes it even more difficult. It was thought that additional studies of the ECBO virus group would contribute to the solution of some of these problems.

As information regarding these agents accumulates it becomes apparent that all members of the ECBO group will not remain as true "orphan viruses". Some of these agents will
undoubtedly be incriminated as the causes of enteric diseases which are of great economic importance among young calves. The fact that ECBO viruses have been isolated from the genital tract of cattle and may be capable of causing abortions also indicates the need for further studies in these areas.

In addition, studies of the basic physical and biological properties of the bovine enteroviruses are necessary so that they may be properly classified on the basis of their own characteristics rather than on assumed analogies to the ECHO viruses.
REVIEW OF LITERATURE

In 1958, Kunin and Minuse (29) reported on attempts to isolate viral agents from cattle feces using calf kidney cell cultures, HeLa cell cultures, embryonating chicken eggs and suckling mice. Eight viral agents were isolated. All of these agents were detected by their ability to produce cytopathic changes in calf kidney cell cultures. It was subsequently shown that they grew and produced cytopathic changes in monkey kidney cell cultures as well. The isolates were designated as Enteric Cytopathogenic Bovine Orphan (ECBO) viruses because of their similarity to the Enteric Cytopathogenic Human Orphan (ECHO) viruses of man. One of the strains isolated, designated as LCR4, was selected for further study. It failed to produce cytopathic changes in HeLa cell cultures and was not pathogenic for three week old mice or for cynomolgus monkeys. Serial passage by the intraperitoneal route of injection caused paralysis and death of suckling mice. Tissue culture fluids and the original specimens from calf feces both successfully infected embryonating chicken eggs when inoculated into the amniotic cavity. Dwarfing of the embryo, denuding of feathers and the release of melanin granules into the amniotic fluid were commonly observed.

Neutralization tests in tissue culture and embryonating eggs as well as complement-fixation tests, using immune serum
prepared in rabbits against virus LCR⁴, indicated that the eight isolates were antigenically similar.

Serum from a high percentage (88.5%) of the animals in the herd from which the LCR⁴ agent was isolated contained antibodies against this virus in relatively high titer (1:8 to 1:512) while serum from only 50% of the cattle in an uninfected herd contained antibodies and then in relatively low titers (1:8 to 1:32). Cross-neutralization studies showed that the agent was not related to polio virus types I, II or III, ECHO virus types 1 to 14, vesicular stomatitis virus types New Jersey and Indiana, calf pneumonia-enteritis virus, Sabin calf 25 enteric cytopathogenic virus, bovine mucosal disease virus or bovine rhinotracheitis virus. Antibodies against the LCR⁴ agent were not found in human and porcine gamma globulin.

The LCR⁴ virus was found to be ether resistant. Ultra-centrifugation studies indicated the presence of a relatively small particle with a sedimentation constant in the range of 150 to 200 Svedberg units.

In the above report the authors also mentioned that Sabin isolated a virus from the feces of a normal calf in 1953 but further reference to this virus, known as the Sabin calf 25 virus, does not appear in the literature. However, it would constitute the first report of the isolation in tissue culture of an agent from the intestinal tract of a normal calf.
Soliman (52), in 1958, studied the host range of an ECBO virus obtained from Kunin and Minuse. Although the specific strain is not identified, various statements in his thesis indicate that he was probably working with the LCR4 strain. He found that the virus multiplied in the amniotic cavity of embryonating chicken eggs and after several passages could be adapted to the allantoic sac. Suckling mice and hamsters developed paralysis and died following inoculation. Intracerebral inoculation into adult mice, hamsters, white rats, cotton rats, guinea pigs, cats, dogs, chicks and chickens failed to produce evidence of infection. Two calves were exposed to the virus, the first by the oral route and the second intravenously. The first calf died four days after inoculation from apparently unrelated causes. The second calf showed no clinical evidence of infection but the virus could be isolated from the feces of the animal for thirteen days.

Studies of preinoculation and postinoculation serum samples from experimentally infected cats, dogs, chickens and the one calf which survived, indicated that only the calf developed antibodies against this agent. He also reported that antiserum from calves experimentally infected with virus diarrhea virus (New York) did not neutralize the virus obtained from Kunin and Minuse.

Moll and Finlayson (43) isolated a cytopathogenic viral agent from the feces of cattle with respiratory disease in
1957. The same viral agent was recovered from an aborted fetus from one of the affected cows. The agent produced cytopathic changes in bovine kidney cell cultures characterized by rounding and shrinking of cells within 12 to 24 hours after inoculation and subsequent loosening from the glass surface. The virus was found to be ether resistant and did not produce illness or pathological changes in suckling mice but would cause illness and extensive myocardial necrosis in weaned mice which had been pretreated with cortisone acetate.

In 1958, McFerran (36) reported the isolation of 112 cytopathogenic agents from cattle in North Ireland. He stated that these agents appeared to fall into three serologically distinct groups with one of the isolates (VG(5)27) being serologically indistinguishable from the LCR4 virus isolated by Kunin and Minuse.

Moscovici and Maisel (47) isolated eleven ECBO strains in 1958 using rhesus monkey kidney cell cultures and reported on hemagglutination studies with these viruses. Testing these agents with human "0", sheep, chicken, cow, guinea pig and horse erythrocytes at room temperature and at 5 to 8 C they found that five of the strains caused hemagglutination of bovine erythrocytes in the cold and three different strains were capable of causing hemagglutination of guinea pig erythrocytes at this temperature. Results with cells of the other species tested were negative when incubated at 5 to 8 C. All
tests incubated at room temperature yielded negative results.

In 1957 and 1960 Klein and Earley (28) and Klein (27) reported on the isolation of a virus from the feces of an apparently normal cow but further studies indicated that this agent was a member of the adenovirus group and not a true enterovirus. They did mention, however, that they obtained some seventy viral isolates from healthy cattle using calf kidney cell cultures but no further information regarding these isolates was presented.

In a continuation of earlier studies Moll and Davis (40) reported in 1959 that they had recovered viral agents from cattle in six herds with histories of respiratory disease. The agents were isolated from fecal samples using bovine kidney cell cultures. Nasal washings collected from these animals at the same time yielded negative results. Cross-neutralization studies with these agents using immune serum produced in rabbits indicated that the BE-1, BE-122, BE-165 and BE-167 viruses belonged to one serological group with BE-160 and BE-180 forming a second unrelated group. All of these agents produced a similar cytopathic effect in bovine kidney cell cultures. The BE-1 virus also caused cytopathic changes in swine kidney, human kidney, HeLa, rhesus monkey kidney and patas monkey kidney cell cultures. These agents passed through a membrane filter with an average pore diameter of 50 to 80 μ but not a membrane with an average pore diameter
of 10 to 50 μm. The viruses were all ether resistant and survived at 4 C to 22 C for at least two months, 37 C for at least 120 hours and at 56 C for at least 30 minutes. The BE-1 virus did not cause signs of illness when inoculated into suckling mice. Virus BE-165 caused abortion when inoculated intracardially into guinea pigs in advanced pregnancy but the virus could not be reisolated from the aborted fetuses. No evidence of infection was observed when BE-1 virus was inoculated into two colostrum fed calves nor could the virus be reisolated from the feces of these animals.

Moll and Davis (41) reported in 1960 that BE-1 and BE-180 both appeared to be immunologically related to infectious bovine rhinotracheitis (IBR) virus. They reported that the IBR antiserum would neutralize the two enteric viruses but that the reciprocal test i.e. the immune sera prepared against the enteric viruses showed no neutralizing activity against the IBR virus. In view of the present knowledge concerning normal inhibition of bovine enteric viruses by rabbit sera and no indication that preinoculation IBR antiserum was checked against the bovine enteric viruses these results have been questioned.

The spread of bovine enteric viruses among healthy cattle brought onto infected premises was studied by Moll and Davis (42) in 1961. They found that most animals became infected with the BE-1 virus within three and one-half months after
arriving on the premises. Some of these infected animals became virus carriers for periods of more than six months. Most animals developed significant antibody titers against the BE-1 virus with maximum titers occurring between one to eight months and persisting for two to four months. However, it was also noted that several animals in one group did not show an increased antibody level following infection with the BE-1 virus.

In 1960, Rosen and Abinanti (51) reported the isolation of viral agents from rectal swabs collected from five calves. These agents, which were cytopathogenic for rhesus monkey kidney cell cultures, were subsequently shown by cross-neutralization studies to be related to members of the human reovirus group.

In 1960, Bögel and Mussgay (7) reported on the isolation of an agent (H786) in Germany which appeared to be a member of the bovine enterovirus group. This agent was obtained from a herd of calves with respiratory and gastrointestinal disturbances which were only occasionally accompanied by diarrhea. The agent was found to produce a cytopathic effect in mouse and calf kidney cell cultures but not in swine kidney or HeLa cell cultures. The agent multiplied in chick fibroblast cell cultures without producing any cytopathic effect. Low virus titers were demonstrated in embryonating chicken eggs following inoculation into the amniotic sac or onto the
chorioallantoic membrane. Inoculation of the virus into mice of various ages did not cause any visible signs of illness. By alternating passages the virus could be adapted to suckling mice, often one retropassage to tissue culture being sufficient to bring about adaptation. When pregnant guinea pigs were inoculated intracardially they aborted 7 to 15 days later. Nonpregnant guinea pigs and rabbits showed no clinical signs of illness following inoculation. It was found that virus H786 was stable at 4 C and -20 C for six weeks with the titer decreasing only slightly upon exposure to these temperatures for 15 weeks. The virus was found to be chloroform resistant and approximately 26 μm in diameter.

Virus H786 was not capable of causing hemagglutination of calf or guinea pig erythrocytes. Cross-neutralization tests showed that the virus was not related to the LCR4 virus of Kunin and Minuse. Serological studies using sera collected from calves in the infected herd during the acute phase of the disease and again four months later disclosed a general rise in titer against the H786 agent. The following year when the disease reappeared in the herd no rise in serum antibody titer could be determined and in many cases a decrease in titer against this agent occurred during the course of the disease outbreak. Attempts to produce experimental infection were successful when colostrum deprived calves were used. Symptoms observed were extremely mild and difficult to
correlate with virus isolations from the test animals. One calf was reported to have undergone a mild febrile response, slight leukopenia and diarrhea between the second and the ninth day after inoculation. A pair of twin calves were inoculated and one subsequently died from septicemia while the other remained depressed and weak in the hindquarters for two days before returning to normal. A fourth calf which had received colostrum prior to inoculation showed no clinical evidence of illness.

In 1961, McFerran (37) reported upon further investigations with the agents he had isolated. The VG(5)27 virus was found to cause a cytopathic effect in cell cultures of bovine, monkey, lamb, rabbit and chicken embryo origin but was incapable of causing observable changes in swine, dog, kitten and guinea pig cell cultures. The virus produced a mild and transient cytopathic effect in HeLa cells but could not be transferred by serial passage. The pathogenicity of the virus was tested in monkeys, guinea pigs, rabbits and mice of various ages with negative results. Determination of particle size by the use of gradacol membrane filtration indicated that the infectious particle had a diameter of approximately 23 mμ. The virus appeared quite heat stable with a 3.0 log loss in titer after twelve weeks storage at room temperature and a 2.5 log decrease in titer after 120 hours exposure to 37 C. At temperatures above 45 C there was a rapid loss in
titer and complete inactivation occurred within thirty minutes at 58 C. Lyophilization reduced the titer markedly indicating that the virus was not resistant to drying. Repeated freezing and thawing had no effect on the virus titer and it was also found that hydrogen ion concentrations ranging from pH 2.2 to pH 10.0 had little effect on infectivity. The virus was found to be ether resistant and resistant to chloroxylenol disinfectants but was inactivated by formalin and chlorine. The virus failed to hemagglutinate cattle, sheep, swine, guinea pig, fowl, rhesus monkey or human erythrocytes at 4 C, 18 C or 37 C.

In 1951, Mayr and Bögel (34) reported on the development of a chloroform resistance test for use in characterizing enteroviruses. They also stated that chloroform treatment of rectal swabs prior to attempts to isolate viruses from them not only destroyed viruses other than those of the enterovirus group but also was beneficial in decreasing bacterial contamination and removing proteins or lipids which had a cytotoxic effect.

Further studies of respiratory and enteric diseases of calves were reported by Bögel (5) in 1961. At this time he reported the isolation of an agent from rectal swabs which he designated as the R-1 agent. This agent was subsequently shown to be a virus having a particle diameter of about 25 to 30 mµ. The virus did not agglutinate erythrocytes of calves
or guinea pigs and failed to cause any indication of illness following intracerebral or intraperitoneal inoculation into four-day-old or three-week-old mice. Cross-neutralization tests using the R-1 agent, the previously isolated H786 virus and the LCR4 agent of Kunin and Minuse demonstrated that the R-1 virus was antigenically related to both of the other viruses even though they were apparently unrelated to each other. Antiserum against the R-1 virus showed no neutralizing activity when tested against the other two agents indicating a one way antigenic relationship.

Utilizing the chloroform resistance test, Bögel and Mayr (6) studied several bovine enteric viruses in 1961 including the H786 and R-1 viruses, the LCR4 virus and five isolates obtained from Luginbuhl. The studies showed that all of these agents were chloroform resistant. In addition they reported that sedimentation constants found for these agents were such that they would indicate a particle size between 20 μm and 30 μm.

Abinanti (1), in 1961, pointed out some of the similarities in viruses isolated from animals and man and indicated in his paper that he had isolated three types of reoviruses from apparently healthy cattle.

Moscovici et al. (46), in 1961, reported the isolation of 11 viral agents from a total of 272 rectal swabs obtained from 91 calves. The eleven isolates included nine enteroviruses, one reovirus and one agent which produced
intranuclear inclusion bodies but did not appear to be serologically related to any of the other isolates. The nine enterovirus isolates were divided into two serological groups. One of these groups was found to be related to the BE-1 and BE-180 viruses isolated by Moll as well as to the LCR/4 strain of Kunin and Minuse. The second group was not related to any other bovine enteroviruses included in their study. All nine enteroviruses grew in monkey kidney and bovine kidney cell cultures to about the same titer. Several of these agents multiplied and produced visible changes in guinea pig kidney and HeLa cells but not in rabbit kidney cells. Suckling mice, cortisone treated weaned mice and adult mice were refractory to infection with these agents. Several of the viruses hemagglutinated guinea pig and monkey erythrocytes but only one caused hemagglutination of mouse erythrocytes and then only when grown in monkey kidney cell cultures. All viruses were negative for human "O" and bovine erythrocytes. Comparisons with other viral agents failed to show any serological relationship between the enteroviruses isolated and vesicular stomatitis or calf pneumonia enteritis viruses. Neutralizing substances against these enteroviruses were found in a high percentage of human and bovine serum samples and some monkey sera. Results were negative with horse, swine and sheep sera. The authors also found that a previously plaque purified strain could be further divided into small and large plaque
producing substrains by using a N, N-diethyl-aminoethyl-cellulose column.

Luginbuhl and Black (33) isolated 26 agents from 111 cattle tested in 1961. They found that 25 of these agents grew equally well in bovine kidney or monkey kidney cell cultures with 84 per cent of the isolates being capable of producing more than one type of plaque. The agents could be divided into three groups on a serological basis with one of these groups showing a relationship to agents isolated by Moll but not to the LCR4 agent isolated by Kunin and Minuse. One of the prototype viruses chosen to represent each serological group was less than 32 µm in diameter and the other two were somewhat larger as determined by gradacol membrane filtration. Studies with antisera against viruses isolated from man showed that the cattle viruses were not related to ECHO virus types 1 to 14, Coxsackie B1 to B5 or A9. Sera from some of the calves from which the agents were isolated contained neutralizing activity against polio viruses, Coxsackie viruses or ECHO viruses but there was no apparent correlation between the type of bovine agent isolated and the human agent neutralized.

In 1961, Abinanti and Warfield (2) described a hemadsorbing virus which they isolated from the intestinal tract of calves. They named this virus a HADEN (hemadsorbing enteric) virus. This agent did not produce a marked cytopathic
effect in bovine embryonic kidney cell cultures and did not grow in monkey kidney tissue cultures. The virus was found to be ether resistant and had a size of approximately 30 m\( \mu \) as determined by grada col membrane filtration. The outstanding characteristic of this virus was its ability to hemadsorb and hemagglutinate human and guinea pig erythrocytes. This agent failed to hemadsorb or hemagglutinate bovine, chicken or rhesus monkey erythrocytes. Hemagglutination-inhibition tests indicated that antibodies against this virus were widespread among adult cattle and that aerosol infection of experimental calves caused high levels of neutralizing antibody to develop but evoked no obvious clinical signs of disease. Hemadsorption-inhibition tests indicated that the virus was not related to vaccinia, influenza A, B or C, Newcastle disease virus, or parainfluenza 1, 2 or 3 viruses. The virus caused no evidence of disease when injected into suckling or adult mice, guinea pigs, rabbits, or into the allantoic cavity of embryonating chicken eggs. The authors proposed that this was a new group of viruses possessing unique biological qualities.

Falk (21) reported in 1961 on attempts to isolate cytopathic agents from three herds in Germany. In the first herd no agents were isolated from the ten cattle tested, in the second herd 15 isolates were obtained from 20 animals and in the third herd 1 isolate was obtained from a total of 20
animals. These agents caused no clinical evidence of disease and no gross lesions when inoculated into guinea pigs, rabbits, suckling mice, adult mice, or a calf. The virus could not be reisolated from the tissues of the calf subsequent to inoculation but was easily isolated from the feces.

McFerran (38), in 1962, studied substances in the sera of man and animals capable of neutralizing a bovine enteric virus, (VG(5)27), which he had isolated. As a result of this study he reported the occurrence of neutralizing substances in the sera of man, monkeys, swine, rabbits, guinea pigs, horses, cattle and some sheep and fowl against this agent. No antiviral substances were found in the sera of pigeons. Additional studies with human sera showed that the neutralizing substance existed in the sera of newborn babies (fetal cord blood), infants, children, adolescents and adults. This activity was not associated with the gamma globulin fraction of human serum, however, neither was it destroyed by any of the usual methods for removing nonspecific inhibitory substances from serum.

In 1962, Moscovici and La Placa (45) reported on further studies with a number of bovine enterovirus isolates. Working with strains which were plaque purified separately on monkey kidney and bovine kidney cells they proposed a classification scheme which included seven antigenically distinct groups. One group contained the LCR4 virus of Kunin and
Minuse, the BE-1 and BE-180 strains of Moll and four of their isolates. The six other groups contained one or more of their isolates. They indicated that although there is some diversity in biological properties within these groups, a system employing antigenic relationships utilizing plaque purified materials would still be the most satisfactory and practical method of classification.

In 1962, Liess and Hölken (32) reported on their work with bovine enteric viruses in Germany. They isolated 24 viruses from a total of 317 cattle. These agents produced a cytopathic effect in monkey kidney, calf kidney and calf testicle cell cultures. All agents isolated were found to be ether and chloroform resistant. Further studies with 13 of these agents indicated that they fell into two serologic groups. The plaque morphology was of no value in differentiating these groups. No attempts to determine antigenic relationships to other viruses were reported.

In a review of bovine enteroviruses, McFerran (35) proposed in 1962 that foot-and-mouth disease virus be included in the enterovirus group because of properties similar to the bovine enteric viruses. He reported the isolation of prime and double prime strains of VG(5)27 as well as strains showing a broadening of antigenicity and suggested that bovine enteric viruses are capable of showing a greater antigenic variation than enteric viruses of other species. In attempts
to infect calves with the VG(5):7 virus he reported that there was no thermal response but that some animals developed a marked diarrhea but showed no other clinical signs of illness. It was suggested, however, that it might be unwise to assume that this virus is not a pathogen. In view of its variation in antigenicity it may also show considerable variation in pathogenicity. The formation of double precipitin lines in agar gel using enterovirus and immune sera were reported but appeared to be of limited value because rather high concentrations of antigen and antibody were required.

In 1962 Bärki (10) published the first of a series of articles dealing with bovine enteric viruses isolated in the vicinity of Bern, Switzerland. A total of 56 isolates were obtained from 311 calves. These agents could be divided into two groups on the basis of their cytopathic effect on bovine kidney cell cultures. One group killed the epithelial cells present but left a network of undamaged fibroblasts, while the other group rapidly destroyed all the cells. A third group was later included which appeared intermediate to the other two. The authors admitted, however, that the type of cytopathic effect produced was quite dependent upon the quality of the cell sheet. Seventeen of the 56 isolates were investigated as to their plaque-producing properties. All of these agents produced plaques in calf kidney cell cultures and on the basis of plaque morphology could be divided into
four groups. The authors indicated that many physical and chemical factors had a considerable effect on plaque morphology and subsequent serological studies proved that one serological type could produce more than one type of plaque.

Bürki's second article (11) deals with the serological classification of the 56 enteroviruses he isolated. In the course of these studies he was able to divide 50 of these agents into four groups. One of the groups (group 1) showed some diversity with evidence indicating the existence of prime strains and strains which were only neutralized with considerable difficulty even by the homologous antiserum. Members of his group 1 were isolated most frequently, accounting for 28 of the 50 strains typed, ten belonged to group 2, five to group 3, and seven to group 4. Serological studies of the original unpurified isolates indicated that excretion of only one type of virus was the rule in these cattle.

Bürki, in his third article (12) discussed the epizootiology of enterovirus infections in three herds. He found that age, season and environmental conditions were important in the transmission of enteroviruses. Twenty three per cent of the calves being fed milk and 17.7 per cent of the animals between seven and thirty months of age were found to be enterovirus carriers. He found that quite often animals which were stabled next to each other would be excreting the same virus type. A seasonal incidence was also noted with more animals
sheddimg virus in the summer and fall than at other times of the year. This was assumed to be due to increased animal contact when the animals were on pasture. It was not uncommon to have different animals in the same herd excreting different virus types but no cases of dual infection within one animal were found.

The fourth article by Bürki (13) deals with cross-neutralization studies using his viruses plus those obtained from other workers. In this report he states that his prototype 1, which was the type he isolated most frequently, was not found to be related to any of the other strains tested. His prototype 2 was found to be related to the LCR4 strain of Kunin and Minuse, as well as Moll's BE-1 strain and strain number 48 of Luginbuhl. The prototype 3 strain of Bürki was apparently not related to any of the other viruses tested. His prototype 4 strain showed a unilateral ability to neutralize his prototype 2 and 3 strains as well as the BFI strain isolated by Inaba et al. (23, 25). No relationship to the H786 or the R-1 strain of Bögel could be determined with the antiserum used.

In Bürki's most recent article (14) he reported on the biological properties of his isolates. Using his four prototypes he showed that they were all chloroform resistant, cytopathogenic for calf kidney cell cultures and by filtration studies appeared to have a particle size of 35 μ or less. His prototypes 2, 3 and 4 were cytopathogenic for HeLa
cells and prototypes 2 and 3 were also cytopathogenic for KB cells. Prototype 1 hemagglutinated chicken erythrocytes at 4 C and at room temperature. The remaining prototypes showed no hemagglutinating activity when tested against chicken, sheep and bovine erythrocytes.

Cliver (16) in 1960 and Cliver and Bohl (17, 18) in 1962 reported on the isolation of viral agents from cattle in Ohio. They isolated their agents using bovine kidney cell cultures. Two serologic types were found and it was determined that these agents differed in their ability to form plaques. In the course of their studies a group of 12 calves were tested weekly for 25 weeks to determine whether they were excreting virus. It was found that 11 of the calves excreted virus at some time during the sampling period. Some calves were found to be excreting virus of both serologic types at the same time. When the two strains of virus were tested on kidney cell cultures from various species it was found that the serologic type 1 produced plaques readily in monkey and bovine cell cultures but was considerably less efficient in producing plaques on porcine kidney cultures and produced no plaques on human kidney cultures. Serologic type 2 also produced plaques readily on bovine and monkey kidney cell cultures, produced some plaques on human kidney cell cultures and was found to be incapable of producing plaques on porcine kidney cells.

It was noticed during the course of this work that viral
agents were never recovered from calves less than four weeks of age in spite of the likelihood of exposure to virus infection shortly after birth. Studies were therefore made of serum antibody levels in parturient cows, newborn calves and the passive transfer of antibody in colostrum. It was found that serum from several of the cows contained antibodies against one or both of the enterovirus strains isolated from the herd. In most cases the colostrum had a higher neutralizing antibody titer than the cow's serum. This antibody titer of colostrum was lost within four to seven days after parturition. Calf sera collected at birth had some ability to neutralize enteroviruses but the neutralizing potency was considerably higher after ingestion of colostrum, in some cases rising higher than that of the cow at the time of parturition. It was determined that if nursing was prevented during the first 12 to 24 hours after birth the ability to absorb antibody from the colostrum was greatly diminished. Studies of serum antibody levels of experimental calves revealed, however, that these levels showed little correlation with the age at which calves were naturally infected with enteroviruses. The authors proposed that the four week delay between birth and natural infection may have been due to either an immaturity of the intestinal mucosa or to antibody present in the surplus colostrum which the calves received. They postulated that this antibody neutralized enteroviruses
within the digestive tract before they could successfully enter the intestinal mucosa and initiate infection.

In 1962, Inaba et al. (23, 25) partially characterized a bovine enteric virus which they isolated from cattle in Japan. This agent, designated by them as BF1 virus, appeared to be unrelated serologically to the LCR virus of Kunin and Minuse. Other characteristics led the authors to believe that this virus was also unlike other agents described by previous workers. The BF1 virus multiplied and produced visible cytopathic effects in cultures of various bovine tissues, swine, rabbit, and guinea pig kidneys and chicken embryo cell cultures. It failed to multiply in horse, dog or cat kidney cell cultures. The virus was found to be quite heat stable at temperatures below 65°C and resistant to treatment with ethyl ether. This agent was able to multiply when inoculated intracranially into one day old suckling mice or into chicken embryos but no visible changes were produced. Experimental calves became virus carriers and showed increased antibody titers following inoculation with this agent but no clinical evidence of disease was observed. Rather high preinoculation titers against the virus may have been a factor, however, in preventing the development of clinical signs of disease.

In additional papers (24, 26) dealing with this virus the authors reported on rather extensive investigations of the hemagglutinating properties of the virus. They found
that the BF1 virus was capable of agglutinating sheep and horse erythrocytes at 4 C in Ringer's solution but was not able to agglutinate calf, pig, goat, guinea pig, rat, mouse, rabbit, pigeon or chicken erythrocytes. In the course of their investigation they discovered that hemagglutination titers were greatly increased when divalent cations were present in the suspending media with the optimum temperature for the reaction being 4 C. Since both the hemagglutinin and the infective particle were adsorbed onto and eluted from the erythrocytes in a similar manner the authors believed that they were one and the same entity. The hemagglutinating activity persisted, however, even though the infectivity of the particle was destroyed by heat or ultraviolet irradiation. They also showed that hemagglutination could be prevented by specific inhibitors in bovine sera and a hemagglutination-inhibition test was devised.

Evans (20) reported in 1962 on his investigations of nonspecific substances in human and rabbit serum capable of neutralizing bovine enteroviruses. In his studies he utilized the agents isolated by Klein and Earley. He concluded that the inhibitors in the sera of the two species were similar in most respects. He assumed that the neutralizing substance was not specific antibody because it could be absorbed by virus strains which were shown to be antigenically unrelated by virus neutralization studies. It was found that the
inhibitor was much more active when serum-virus mixtures were incubated at 37 C. The inhibitor was stable when heated to 56 C for 30 minutes but was destroyed by heating to 70 C for one hour. It was also shown that it acted against the virus particle directly and not against the tissue culture cells. The virus neutralizing substances could be removed almost completely by heated homogenates of calf kidney cortex and were partially destroyed by trypsin, crude receptor destroying enzyme and sodium periodate. The inhibiting activity was not destroyed by ether or acetone. Response to these agents caused the author to conclude that the inhibitor was a protein, probably a glycoprotein. As a result of testing sera of other species he found inhibitors in guinea pig serum to be about the same level as in the rabbit and human sera. Horse and dog sera had low activity and no neutralizing activity could be found in chicken or mouse sera. On the basis of this work the author pointed out the necessity of testing both preinoculation and postinoculation serum samples from hyperimmunized animals. He also suggested that chickens might be the most suitable animal for use in the production of typing sera.

Spradbrow (53) reported on the isolation of a bovine enterovirus in Australia in 1963. The agent produced cytopathic effects in embryonic bovine kidney cell cultures, passed a 50 μm Millipore filter, was ether and chloroform resistant and exhibited cationic stabilization to heat inactivation.
Further isolations of similar agents were mentioned and preliminary surveys indicated that these agents were widespread in Australian cattle.

Moll and Ulrich (44) reported in 1963 on the biologic characteristics of some agents they had isolated previously. Among the ten agents studied they found eight serologic groups. All ten isolates were found by filtration studies to be between 40 and 160 μm in diameter, ether resistant, sensitive to formalin and unable to agglutinate guinea pig or sheep erythrocytes. The only difference noted between members of any given serotype was a difference in heat sensitivity.

Zeleznik and Jung (58) reported the isolation of two cytopathogenic agents from cattle in Yugoslavia in 1963. These agents produced a marked cytopathic effect in bovine kidney and human amnion cell cultures but did not grow in HeLa or swine kidney cells. The type of cell destruction and resistance of the virus to chloroform led the authors to conclude that these agents were probably ECBO viruses.

Niederman et al. (48) reported on their work with bovine enteric viruses in 1963. They limited their investigations to a single isolate which could be divided into a large and a small plaque producing strain. The fact that the two plaque types were antigenically distinct strains was subsequently determined by the use of reciprocal cross-neutralization tests in tissue culture using hyperimmune sera produced in chickens.
Both strains caused hemagglutination of bovine erythrocytes when tested undiluted but not when diluted more than 1:2. Neither agent caused hemadsorption or hemagglutination of guinea pig erythrocytes. These strains could not be adapted to embryonating chicken eggs. Two calves, one to two months of age, were infected with each strain by intranasal instillation of six separate, 2.0 to 5.0 ml. doses of tissue culture material. These inoculations were given over an eleven day period. Virus was recovered from at least one calf infected with each agent. An increased neutralizing antibody titer developed after infection with the small plaque producing strain but no serological response was noted in calves infected with the large plaque forming virus. Although temperatures of the calves were slightly higher after inoculation, no visible illness was noted. There were, however, low levels of neutralizing antibodies in the serum of some of the calves prior to experimental inoculation.

In 1963, Bügel, Straub and Dinter (8) reported on the characterization of strain 6976 of a group of G-UP viruses isolated from the genital organs of cattle by Florent (22). They found that this agent was a small chloroform resistant, ribonucleic acid-containing virus which was stabilized against heat inactivation by magnesium chloride, was relatively stable at 37°C and was not inactivated upon exposure to pH 5.2. The agent also produced a typical enterovirus type cytopathic
effect in calf kidney cells. Experimental infection of 14 calves via the intranasal, intravenous, intrapreputial or intravaginal routes of inoculation resulted in frequent re-isolation of the virus from the feces and prepuce and a single isolation from nasal secretions. The agent was never recovered from the vagina or blood stream. Serological studies failed to demonstrate any relationship between this agent and the LCR4, H786 or R1 agents isolated by other workers. The virus was also found to be unrelated to foot-and-mouth disease virus types A, O or C, rhinovirus or virus diarrhea virus.

LaPlaca, Portolani and Lamieri (31) reported in 1963 on the isolation of 30 enterovirus strains from 337 stool specimens obtained from 148 healthy calves in Italy. They found that their agents fell into three serological groups containing 20, 9 and 1 strains respectively. All of the members of serological group I agglutinated rhesus monkey erythrocytes. Many of these agents also agglutinated guinea pig and human "O" erythrocytes and a few caused agglutination of mouse and chicken erythrocytes. All of the members of serological groups II and III failed to agglutinate rhesus monkey erythrocytes but did agglutinate erythrocytes from some of the other species. None of the 30 agents were capable of causing hemagglutination of calf or rabbit erythrocytes.

The authors reported results of cross-neutralization studies utilizing their agents and virus strains obtained from
Kunin, Moll, Moscovici, Bürki, Luginbuhl and Inaba. In addition to demonstrating antigenic relationships among the above viruses they also showed that the viruses found to be antigenically related to their group I all hemagglutinated rhesus monkey erythrocytes while those found to be unrelated to their group I failed to do so. They pointed out that this close correlation between antigenic type and ability to agglutinate rhesus monkey erythrocytes would undoubtedly be of considerable value in the rapid identification of new isolates.

In 1964 Straub and Böhm (54) studied the properties of a virus isolated by McKercher from a case of catarrhal bovine vaginitis. Their studies indicated that the virus might be a member of the bovine enterovirus group. The infectious agent was a small particle, ether and chloroform resistant, relatively stable to heat inactivation at 37 C, resistant to trypsin and low pH and was readily propagated in cultures of calf and bull testicles as well as chick fibroblasts and pig kidney cells. Intranasal and intravaginal inoculation of the virus resulted in recovery of the virus for a short period of time (2 to 4 days) from vaginal swabs and for a period of up to 25 days from rectal swabs. The virus was never recovered from nasal swabs. Based on this information they concluded that the agent was a member of the enterovirus group which may or may not have been responsible for the vaginitis present at the time of isolation.
MATERIALS AND METHODS

Tissue Culture

**Ion exchange water**

Distilled water was further purified by passage through a Quickpure Lab-Flow Demineralizer\(^1\). This routinely yielded water which gave a reading of less than 0.1 P.P.M. of solids expressed as sodium chloride measured by a conductivity meter. This water was used for glassware washing as well as for the preparation of media.

**Glassware washing**

Immediately after use contaminated glassware was immersed in ion exchange water containing a solution of a one per cent detergent (Microsolve\(^2\)) and boiled for thirty minutes. Boiling was omitted if the glassware was not contaminated with live virus. All glassware was then allowed to soak in the detergent solution, brushed, placed in a clean detergent solution and heated to approximately 70 C for twenty minutes. The glassware was then rinsed in three changes of ion exchange water. In the last rinse the glassware was heated to 80 C for

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\(^1\)Comroe Laboratories, Inc., 5208 S. Lakepark Avenue, Chicago 15, Illinois.

\(^2\)Microbiological Associates, Inc., 4813 Bethesda Avenue, Washington 14, D. C.
thirty minutes and subsequently allowed to cool to approximately 60°C. The rinse water was then checked with a conductivity meter and if it contained 0.2 P.P.M. total solids expressed as sodium chloride or less the glassware was removed and allowed to air dry. If the reading was not within these limits the final rinsing procedure was repeated.

After the glassware had been allowed to air dry it was placed in suitable metal containers or wrapped in heavy aluminum foil and sterilized either by autoclaving at 120°C for thirty minutes or by dry heat at 190°C for three hours.

Solutions

Hanks' balanced salt solution with lactalbumin hydrolysate (Hanks'-LH) A 10X solution was prepared as suggested by Merchant et al. (39) in the following manner:

Solution 1

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>80.0 gm.</td>
</tr>
<tr>
<td>KCl</td>
<td>4.0 gm.</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>2.0 gm.</td>
</tr>
<tr>
<td>Na₂HPO₄·2H₂O</td>
<td>0.6 gm.</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.0 gm.</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.6 gm.</td>
</tr>
<tr>
<td>Ion exchange water</td>
<td>800.0 ml.</td>
</tr>
</tbody>
</table>

Solution 2

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
<td>1.4 gm.</td>
</tr>
<tr>
<td>Ion exchange water</td>
<td>100.0 ml.</td>
</tr>
</tbody>
</table>
Solution 3
Phenol red 0.2 gm.
Ion exchange water 75.0 ml.
Final volume brought to 100 ml. with ion exchange water and sufficient N/20 NaOH to adjust to pH 7.0.

Solutions 2 and 3 were combined and then mixed with solution 1. This stock solution was then dispensed into screw capped bottles, a few drops of chloroform were added to each bottle and they were stored in the refrigerator.

A working solution was prepared from the 10X stock solution in the following manner:

10X stock solution 100.0 ml.
Ion exchange water 900.0 ml.
Lactalbumin hydrolysate 5.0 gm.

The bicarbonate solution used in the final steps of preparing Hanks' L-H was mixed and autoclaved separately. It consisted of:

NaHCO₃ 3.5 gm.
Ion exchange water 250.0 ml.

The bicarbonate and working solutions were dispensed into screw capped bottles and autoclaved at 120 C for 15 minutes. After being allowed to cool they were mixed in the following proportions:

Working solution 100.0 ml.
Bicarbonate solution 2.5 ml.
Because a considerable portion of the research involved the use of highly contaminated rectal swabs, several antibiotics were included in all solutions. The antibiotics, and quantities used, are listed below.

- **Penicillin**¹ 100 units per ml.
- **Streptomycin**² 0.1 mg. per ml.
- **Kantrex**³ 0.1 mg. per ml.
- **Fungizone**⁴ 2.0 μg. per ml.

**Earle's balanced salt solution** This was prepared as a 2X solution for use in agar overlays in plaque studies. The solution was made up as shown below.

- NaCl 6.8 gm.
- KCl 0.4 gm.
- CaCl₂·2H₂O 0.2 gm.
- MgSO₄·7H₂O 0.2 gm.
- NaH₂PO₄·H₂O 0.125 gm.
- Dextrose 1.0 gm.
- Phenol red 0.01 gm.
- Ion exchange water 1000.0 ml.

¹The Upjohn Company, Kalamazoo, Michigan.
²The Upjohn Company, Kalamazoo, Michigan.
³Bristol Laboratories, Syracuse, New York.
The solution was sterilized by autoclaving at 120°C for fifteen minutes. After being allowed to cool, sterile 7.5 per cent sodium bicarbonate was added aseptically at the rate of 6 ml. per 100 ml. of the above solution. Antibiotics were added at twice the levels previously indicated.

Trypsin solution The trypsin solution used to disperse cells for primary cell cultures was prepared as indicated below.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.0 gm.</td>
</tr>
<tr>
<td>KCl</td>
<td>0.4 gm.</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.0 gm.</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.35 gm.</td>
</tr>
<tr>
<td>Trypsin 1:250</td>
<td>3.3 gm.</td>
</tr>
<tr>
<td>Ion exchange water</td>
<td>1000.0 ml.</td>
</tr>
</tbody>
</table>

This solution was sterilized by filtration through a Selas 03 filter, dispensed into prescription bottles and stored frozen. Antibiotics were added as previously described.

Trypticase soy broth This was purchased as a dehydrated prepared medium from a commercial firm. As directed, 

1Difco Laboratories, Incorporated, Detroit 1, Michigan.
2Selas Corporation of America, Dresher, Pennsylvania.
3Baltimore Biological Laboratories, 2201 Aisquith Street, Baltimore 18, Maryland.
thirty grams of the dry powder were mixed with one liter of ion exchange water and sterilized by autoclaving at 120 °C for fifteen minutes. After the medium had cooled antibiotics were added as previously described.

**Agar overlay solution** This was prepared by mixing 2 gm. Noble agar to 100 ml. ion exchange water and sterilized by autoclaving at 120 °C for fifteen minutes. After being allowed to cool to 50 °C this was mixed with an equal quantity of 2X Earle's solution previously heated to 50 °C and used as an overlay for the plaque technique.

**Neutral red overlay solution** This was prepared by adding 0.1 per cent neutral red to a 1 per cent solution of Noble agar in ion exchange water.

**Titraver solution** The titraver solution used in transferring cell cultures was prepared using the following formula:

\[
\begin{align*}
\text{Titraver}^1 & \text{(Disodium Dihydrogen Ethylenediaminetetraacetate Dihydrate)} \\
& 1.5 \text{ gm.} \\
\text{Ion exchange water} & 98.5 \text{ ml.}
\end{align*}
\]

This solution was sterilized by autoclaving at 120 °C for 30 minutes.

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1Hach Chemical Company, Ames, Iowa.
Bicarbonate solution The bicarbonate solution used in transferring cell cultures was prepared as follows:

\[
\begin{align*}
\text{NaHCO}_3 & \quad 5 \text{ gm.} \\
\text{Ion exchange water} & \quad 100 \text{ ml.}
\end{align*}
\]

This was sterilized by autoclaving for 15 minutes at 120 °C.

Ringer's solution This solution, used in the hemagglutination studies, consisted of the following ingredients:

\[
\begin{align*}
\text{NaCl} & \quad 8.0 \text{ gm.} \\
\text{KCl} & \quad 0.2 \text{ gm.} \\
\text{CaCl}_2 & \quad 0.2 \text{ gm.} \\
\text{MgCl}_2 & \quad 0.1 \text{ gm.} \\
\text{NaH}_2\text{PO}_4 & \quad 0.1 \text{ gm.} \\
\text{NaHCO}_3 & \quad 0.4 \text{ gm.} \\
\text{Ion exchange water} & \quad 1000.0 \text{ ml.}
\end{align*}
\]

Sera Calf and lamb serum used in tissue culture media, were obtained from a commercial source\(^1\) in 100 ml. bottles and added aseptically to tissue culture growth media in quantities sufficient to give a final concentration of 10 per cent serum.

Preparation of cell cultures

Bovine kidney cell cultures Fetal calf kidneys or

\(^1\)Microbiological Associates, Inc., 4813 Bethesda Avenue, Washington 14, D. C.
kidneys from 1 to 3 month old calves were obtained shortly after the animals were killed, placed in a plastic bag and packed in crushed ice until their delivery to the laboratory, several hours later. The tissues were processed immediately upon arrival at the laboratory. Using sterile instruments the capsule was stripped from the kidney and the cortex removed and placed into a sterile beaker. The tissue was then minced with a sterile scissors so that it consisted of fragments no larger than 2 to 3 mm. in any dimension. The minced tissue was then washed two or three times with 100 ml. of serum-free Hanks'-LH solution and after thorough draining placed into a 500 ml. erlenmeyer flask containing a teflon-coated stirring bar. Approximately 200 ml. of 0.33 per cent trypsin solution was then added and the flask placed on a magnetic stirrer. Trypsinization was allowed to continue for one and one half to two hours at room temperature or overnight in the refrigerator at 4 C. The trypsin containing the dispersed cells was then strained through four thicknesses of sterile gauze, placed into 50 ml. screw cap centrifuge tubes and the cells sedimented by centrifugation for ten minutes at 1000 rpm. The supernatant fluid was withdrawn and discarded and the sedimented cells resuspended in serum-free Hanks'-LH solution. The centrifugation was then repeated twice more and the supernatant fluid removed each time in order to more completely remove the trypsin and cell debris. The sedimented
cells, following the final centrifugation, were suspended in an equal volume of serum-free Hanks'-LH solution and then mixed with Hanks'-LH solution containing 10 per cent serum at a rate of 1 ml. of concentrated cell suspension per 200 ml. of medium. This cell suspension was then dispensed into 16 x 150 mm. culture tubes at the rate of 1.5 ml. per tube, or 7 ml. was placed into 3 oz. prescription bottles. The bottles or tubes were then placed into a 37 C incubator. The medium was changed 24 hours later and every 48 hours thereafter until a complete cell sheet was formed, usually on the fifth to the seventh day.

Bovine testicle cell cultures  Calf testicles were procured in the same manner as the kidneys. Using sterile instruments the outer tunic was removed aseptically and the parenchyma exposed by cutting a small portion off the end of the testicle. A sterile forceps was then used to put pressure on the outside of the testicle and the parenchyma forced into a sterile petri dish. A sterile scalpel was used to scrape the parenchymal cells free from the mediastinum testis and they were then transferred directly into the trypsin solution. From this point on the cells were handled in exactly the same manner as described above for the kidney cells except that trypsinization was only continued for 30 minutes at room temperature and cell sheets formed much more rapidly, usually within 48 to 72 hours.
Sheep kidney cell cultures  Kidneys were obtained locally from animals approximately one year of age which were killed in the course of unrelated experiments. These kidneys were handled in exactly the same manner as described for the bovine kidney cell cultures except that they were grown in medium containing 10 per cent lamb serum.

Guinea pig kidney cell cultures  Kidneys were collected from 1 to 3 day-old guinea pigs and handled as described for bovine kidney cell cultures except that the trypsinization time was extended to two and one half to three hours at room temperature. These cells were grown in medium containing 10 per cent lamb serum.

Swine kidney cell cultures  These cells were obtained as a cell suspension in medium ready to be placed in tubes through the courtesy of Dr. W. P. Switzer. Subsequent handling of the cells was as described above.

Chick fibroblast cell cultures  Nine day-old embryonating chicken eggs were obtained through the courtesy of Dr. M. S. Hofstad. The large end of the eggs was disinfected by

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1Dr. W. P. Switzer, Veterinary Medical Research Institute, Iowa State University, Ames, Iowa.

2Dr. M. S. Hofstad, Veterinary Medical Research Institute, Iowa State University, Ames, Iowa.
painting with tincture of iodine and the shell removed with sterile forceps. A second pair of sterile forceps was then used to pierce the shell membranes and grasp the embryo. The embryo was lifted and torn free from the fetal membranes and placed into a sterile petri dish containing a small quantity of serum-free Hanks'-LH solution. Using sterile instruments the head was removed and the embryo eviscerated. The torso was then transferred to another sterile petri dish. Several torsos were then placed into the barrel of a sterile 10 cc. glass syringe. The plunger was inserted and the torsos forced into a flask containing 0.33 per cent trypsin solution and a teflon-coated stirring bar. Trypsinization was carried out at room temperature for 30 minutes and the cells were subsequently handled as described for bovine kidney cells. Complete cell sheets usually formed after 48 hours in the 37 C incubator.

HeLa cell cultures This cell line was obtained through the courtesy of Mr. M. F. Coria. The cells were obtained as monolayers in 8 oz. bottles. The medium was removed and 5 ml. of fresh medium added. Then 0.5 ml. of 1.5 per cent tritraver and 5 drops of 5 per cent sodium bicarbonate were added and the bottles placed into a 37 C incubator for approximately 15 minutes. This incubation period allowed the cells to loosen

1Mr. M. F. Coria, United States Department of Agriculture National Animal Disease Laboratory, Ames, Iowa.
from the glass surface. At this time 60 ml. of medium containing 10 per cent calf serum was added and the bottle was shaken briefly to disperse the cells. These cells were then transferred either to tubes or bottles for future use. The medium was changed 48 hours later and complete cell sheets were usually formed within 72 hours.

**Monkey kidney cell cultures** This cell line was obtained from the same source as the HeLa cells and was handled in an identical fashion.

**Secondary bovine kidney cell cultures** These cells were obtained from the primary cell cultures set up in the laboratory and transferred as described for the HeLa cell cultures. Because of their superior morphological qualities these cells were used in the studies of cytopathic changes.

**Virus Isolations from Rectal Swabs**

Rectal contents were collected using sterile cotton swabs. Immediately after collection each swab was placed into a centrifuge tube containing 4 ml. of sterile trypticase soy broth with antibiotics. This material was then taken to the laboratory, the swab removed with a sterile forceps and the fluid centrifuged at 10,000 rpm for 30 minutes in a Servall

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1Ivan Sorvall, Inc., Norwalk, Connecticut.
refrigerated centrifuge. The supernatant fluid was then removed and inoculated onto cell cultures immediately or put into 1 dram screw capped glass vials and stored at -40 F for future use.

Source of Isolates

The first herd studied consisted of 204 Hereford steers weighing about 400 pounds located in a feedlot near Stratford, Iowa. These animals were western cattle purchased through a local sales barn and had been on the farm for approximately two and one half months. Conditions of care, feeding and sanitation were about average for cattle feeding operations of this type. Shortly after their arrival on the farm a severe outbreak of respiratory and intestinal disease occurred with a resulting death loss of 24 animals. The disease subsequently subsided but a group of 18 animals were kept in a separate barn because they were apparently chronically affected. These 18 animals were considered to be good subjects for virus isolation studies due to the previous history of intestinal disease and the apparent chronicity of the condition.

The second group of cattle studied consisted of 16 dairy calves, two weeks to three months of age, located in the Dairy Nutrition Barn at Iowa State University. These calves were housed in individual pens with solid walls four feet high
separating the pens. Conditions of sanitation were excellent. No adult animals were housed in this barn. All the calves included in this study were apparently healthy, however, there had been occasional outbreaks of enteric disease in the herd during the preceding months.

The final herd studied was a dairy herd maintained by the Department of Veterinary Obstetrics, Iowa State University. Nine cows and two calves in this herd were tested.Sanitary conditions were about equal to those of an average farm dairy herd. The two calves were housed in a common pen not isolated from the milking herd.

Plaque Methods

Studies of plaque morphology and plaque purification procedures were carried out using cell cultures grown in 3 oz. prescription bottles as previously described. The medium was removed and the cell sheet washed once with serum-free Hanks'-LH solution prior to inoculation. Cell sheets were inoculated with 0.2 ml. of the virus dilutions and the virus was allowed to adsorb for 30 minutes at room temperature. Any excess fluid was then drained from the bottles and the cell sheets were covered with 7 ml. of a mixture consisting of equal parts of 2X Earle's solution and 2 per cent Noble agar. After plaques formed (approximately 72 hours) an additional overlay of 2.5 ml. of neutral red agar was added and the bottles were
returned to the incubator. Staining was usually complete at the end of 24 hours.

Plaque Purification

For plaque purification 2 mm. I.D. glass tubing was heated and bent at right angles so that it could be inserted into the mouth of the bottle and still have the open end of the tube parallel with the agar surface. The tube could then be pushed into the agar of a selected plaque and thereby cut a small agar plug. This plug was removed, placed into 1 ml. trypticase soy broth and frozen. This material was then thawed and carried through three additional plaque passages. Virus collected from single well isolated plaques was used as the inoculum for each passage. The plaque purified strains were then inoculated into bovine kidney cell cultures in 8 oz. prescription bottles to provide a large quantity of each agent for storage and future use. In some cases one additional passage was necessary to provide sufficient material to complete all the studies. Depending upon whether or not this additional passage was made and whether the virus was carried through two or three passages prior to plaque purification all viruses used were at the 7th to the 10th passage level, all passages being on primary bovine kidney cell cultures.
Hematoxylin and eosin

Secondary bovine kidney cells grown on 9 mm. by 22 mm. glass coverslips in Leighton tubes were infected with each of the prototype virus strains and the development of the cytopathic changes was observed. Coverslips were removed during the early, middle and final stages of destruction of the cell sheet. These coverslips were fixed in 10 per cent neutral formalin solution and subsequently stained with hematoxylin and eosin. The solutions used in the staining procedure were prepared in the following manner:

**Harris's alum hematoxylin**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematoxylin crystals</td>
<td>5.0 gm.</td>
</tr>
<tr>
<td>Alcohol, 100%</td>
<td>50.0 ml.</td>
</tr>
<tr>
<td>Ammonium alum</td>
<td>100.0 gm.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000.0 ml.</td>
</tr>
<tr>
<td>Mercuric oxide</td>
<td>2.5 gm.</td>
</tr>
</tbody>
</table>

The hematoxylin was dissolved in alcohol and the alum in hot water. The two solutions were then mixed and brought to a boil as rapidly as possible, removed from the heat and the mercuric oxide added very slowly. The solution was re-heated until it became a dark purple and then promptly

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removed from the flame and cooled in a basin of cold water. After the solution had cooled it was filtered. Immediately prior to use 2.0 ml. of glacial acetic acid were added to each 100.0 ml. of staining solution to intensify nuclear staining.

**Ethyl Eosin**

- Eosin Y\(^1\) (water and alcohol soluble) 10.0 gm.
- Ethyl alcohol, 70% 1000.0 ml.
- Glacial acetic acid 2.0 ml.

The eosin solution was filtered immediately prior to use.

**Acid alcohol**

- HCl, concentrated 1.0 ml.
- Ethyl alcohol, 70% 99.0 ml.

**Saturated lithium carbonate**

- Lithium carbonate 1.0 gm.
- Distilled water 100.0 ml.

A small amount of undissolved lithium carbonate was kept in the staining dish to maintain the strength of the solution.

The following staining procedure was used:

1. Distilled water 3 minutes
2. Harris's hematoxylin 8 minutes

\(^1\)Hartman Leddon Co., Inc., 5821 Market Street, Philadelphia 39, Pennsylvania.
3. Running tap water 3 minutes
4. Acid alcohol 3 seconds
5. Lithium carbonate 1 minute
6. Tap water 3 minutes
7. Distilled water 3 minutes
8. Eosin Y 30 seconds
9. Ethyl alcohol, 100% 5 seconds
10. Ethyl alcohol, 95% 3 seconds
11. Ethyl alcohol, 95% 3 minutes
12. Xylene 3 minutes
13. Xylene 3 minutes

After the coverslips were removed from the xylene they were mounted on glass microscope slides with a commercial histological mounting medium.

**Acridine orange**

Secondary bovine kidney cells were grown on 9 mm. x 22 mm. coverslips in Leighton tubes. When a complete cell sheet had formed they were washed once with serum-free Hanks'-LH solution and infected with a sufficiently concentrated inoculum to assure the presence of 10 to 100 virus particles per cell. Coverslips were removed at hourly intervals and stained by the following procedure:

1. Acid alcohol 5 minutes
2. Rinse in 2 changes of McIlvaine's buffer 2 minutes
3. Stain with .01 per cent acridine orange in McIlvaine's buffer 4 minutes
4. Rinse in 2 changes of McIlvaine's buffer 2 minutes
5. Mount coverslip in slide using McIlvaine's buffer

McIlvaine's citric acid buffer pH 3.8 was prepared in the following manner:

\[
\begin{align*}
\text{Na}_2\text{HPO}_4 & \quad 10.081 \text{ gm.} \\
\text{Citric acid monohydrate} & \quad 13.554 \text{ gm.} \\
\text{Ion exchange water} & \quad 1000.0 \text{ ml.}
\end{align*}
\]

Immediately after staining, the cells were studied with a Leitz Ortholux microscope\(^1\) using an Osram HBO 200 mercury vapour lamp Type L 11 light source with a 2 mm. UG1-UV filter and a dark field condenser.

Production of Immune Serum

Immunization of rabbits

Young adult white rabbits were used in these studies. Preinoculation serum samples were collected by cardiac puncture the day before hyperimmunization began. Two animals were hyperimmunized with each of the virus strains. The inoculum

\(^1\)E. Leitz, Inc., 468 Park Avenue South, New York 16, New York.
consistent of undiluted tissue culture fluids collected from infected bovine kidney cell cultures. The infective material was frozen and subsequently allowed to thaw in order to break down any intact cells and centrifuged at 1000 rpm for 30 minutes to remove tissue debris. The supernatant virus-containing fluid was removed, frozen and stored at -40 C until used. Two control animals were inoculated with fluid from noninfected cell cultures.

One milliliter of undiluted tissue culture fluid was inoculated intravenously into each rabbit on the 1st, 4th and 7th day of the immunization period. On the 11th day each rabbit received 5 ml. of a mixture consisting of equal parts of infectious tissue culture fluids and Freund's adjuvant\(^1\). This material was injected subcutaneously in the cervical, thoracic and flank regions. Ten days later the rabbits were bled by cardiac puncture to obtain postinoculation serum. The blood was placed in sterile culture tubes and allowed to remain in the refrigerator at 4 C for 24 hours. The tubes were centrifuged at 1000 rpm for 1 hour and the supernatant serum removed with a sterile pipette. The serum was inactivated at 56 C for 30 minutes in a waterbath, dispensed in 1.5 ml. amounts into one half dram screw capped vials and stored at -10 C for future use.

\(^1\)Difco Laboratories, Incorporated, Detroit 1, Michigan.
Immunization of guinea pigs

Preinoculation blood samples were collected by cardiac puncture and three guinea pigs hyperimmunized with each virus prototype using the following schedule.

On the 1st day 5 ml. of infectious tissue culture fluid were inoculated subcutaneously. On the 14th and 28th day each guinea pig was inoculated subcutaneously with 10 ml. of a mixture consisting of equal parts of infectious tissue culture fluid and Freund's adjuvant. Three animals were also inoculated with noninfectious tissue culture fluids. Blood was collected by cardiac puncture seven days after the last injection and the serum separated and handled as described above.

Immunization of mice

Adult white mice were used in attempts to produce ascitic fluid containing specific antibodies against the bovine enteroviruses. Groups of 10 mice were inoculated with each virus type or with uninfected tissue culture fluid. The animals received 0.5 ml. of either virus or control material intramuscularly on the 1st and 3rd day of immunization. On the 9th, 13th and 17th day 0.3 ml. of a mixture consisting of equal parts of Freund's adjuvant and tissue culture fluid was inoculated intraperitoneally. On the 23rd day 0.5 ml. of Freund's adjuvant was inoculated intraperitoneally. Ascitic fluid was collected on the 30th day and handled in a manner
similar to that described for serum.

**Immunization of chickens**

Two white leghorn cockerels were immunized with each virus prototype. Preinoculation as well as postinoculation serum samples from the two birds were pooled. On the 1st day of hyperimmunization each bird was given 2.0 ml. of virus-containing tissue culture fluid intravenously. On the 7th, 14th and 21st day each bird received the same quantity intravenously plus 2.0 ml. of a mixture consisting of equal parts of virus-containing tissue culture fluid and Freund's adjuvant injected intramuscularly into the breast muscles. On the 28th day blood was collected from the chickens by cardiac puncture and handled as previously described.

**Neutralization Tests**

The decreasing virus-constant serum method was employed to determine the neutralizing activity of rabbit, guinea pig, chicken and calf sera. All tests were conducted using primary bovine kidney cell cultures. An initial 1 to 5 dilution of the virus was made and from this seven serial 10-fold dilutions were prepared. Rabbit, calf and chicken sera were diluted 1 to 5 and guinea pig serum 1 to 10 prior to use. The neutralization test was then set up by mixing 0.5 ml. of each virus dilution with 0.5 ml. of the diluted serum to be tested. This gave final virus dilutions of $10^{-1}$ through $10^{-8}$
and a 1 to 10 dilution of rabbit, calf or chicken serum. The final dilution of guinea pig serum was 1 to 20.

The virus and serum were mixed thoroughly and allowed to stand at room temperature for one hour. Subsequently, three cell cultures, previously washed once with serum-free Hanks' LH solution, were each inoculated with 0.1 ml. of the virus-serum mixture and left at room temperature for 30 minutes to allow the virus to adsorb to the cell sheet. At this time 1.5 ml. of serum-free Hanks' LH solution was replaced and the cells were returned to the 37 C incubator. Infected cell cultures were observed daily for four days and results recorded. Fifty per cent end points were calculated using the method of Reed and Muench (50). Neutralization indexes were determined by comparing preinoculation and postinoculation serum samples when determining the antigenic relationships of the viruses. In studies of reisolated virus strains neutralization indexes were calculated by comparing neutralization by specific immune sera with titers of virus plus diluent controls.

Titration of the antiviral activity of colostrum was carried out as described above. Colostrum was diluted 1 to 5 before use thereby giving a final dilution of 1 to 10. Controls consisted of virus dilutions mixed with equal parts of a 1 to 5 dilution of homogenized pasteurized whole milk. Procedures were exactly the same as for the serum samples except
that after the virus-colostrum mixtures were allowed to adsorb to the bovine kidney cell cultures the tubes were washed twice with 1.5 ml. of serum-free Hanks'-LH solution to avoid the cytotoxic effect of the milk or colostrum. Fifty per cent end points were calculated and neutralization indexes determined by comparing the neutralizing activity of the colostrum with the titer of virus plus pasteurized homogenized milk.

Studies of neutralizing activity of the mouse ascitic fluid were carried out using the constant virus-serum dilution technique. Virus titers were determined by preliminary titrations and appropriate dilutions were made to yield 200 tissue culture infective doses (TCID$_{50}$) per 1.0 ml. The virus was mixed with an equal volume of each ascitic fluid dilution and allowed to remain at room temperature for one hour. This material was used to infect cell cultures as described above. The neutralization titer of the serum was recorded as that dilution which completely neutralized virus activity. Controls in this study consisted of ascitic fluid from mice inoculated with noninfectious tissue culture fluid.

**Ether Sensitivity**

Ether sensitivity of the virus strains was tested as suggested by Andrewes and Horstmann (3). Eight ml. of undiluted infectious tissue culture fluid was mixed with 2 ml. of ethyl ether to give a final concentration of 20 per cent
ether by volume. This mixture was put into a screw-cap test tube, shaken by hand briefly and placed in a refrigerator at 4°C for 24 hours. At this time the mixture was poured into a sterile 100 mm. petri dish with the cover tilted and left for 30 minutes to allow the ether to evaporate. The remaining fluid was titrated to determine the quantity of surviving virus. Controls were handled in the same way except that the ether was omitted.

Chloroform Resistance

The chloroform resistance of the viruses was tested by the method of Mayr and Bögel (34). The viruses were grown in calf kidney cell cultures in 3 oz. prescription bottles in a medium consisting of Earle's solution plus 10 per cent of a 5 per cent solution of lactalbumin hydrolysate in Hanks'-LH solution. After the cytopathic changes had progressed to the point that approximately 90 per cent of the cells were destroyed the cultures were frozen at -10°C for a short time, allowed to thaw at room temperature and centrifuged at 1000 rpm for 30 minutes. Eighteen milliliters of the supernatant fluid was placed into a sterile 3 oz. prescription bottle and 2 ml. chloroform added to give a final concentration of 10 per cent chloroform. The 3 oz. bottles thus contained only 20 ml. of fluid thereby allowing ample space for the fluids to mix when the bottles were agitated. These bottles
containing the virus plus chloroform were placed in an Eberbach shaker (Model 55) and the speed adjusted so that the shaker would complete two cycles per second. The entire apparatus was placed in a cold room at 4 C and the mixtures were shaken for one hour. The mixtures were subsequently allowed to stand 23 hours at 4 C in a refrigerator and then centrifuged at 1500 rpm for 15 minutes to separate the aqueous and chloroform phases. The aqueous phase was removed and titrated to determine its virus content. Control virus suspensions were handled in a similar manner except that the addition of chloroform was omitted.

Animal Inoculations

Suckling mouse inoculations

Suckling mice were inoculated before they were 24 hours old. Entire litters, except for two controls, were inoculated with infectious material. The control animals received non-infectious tissue culture fluids. The suckling mice were inoculated either intracerebrally with 0.05 ml. or intraperitoneally with 0.1 ml. of infectious material using one fourth ml. tuberculin syringes and one fourth inch 28 gauge hypodermic needles. The animals were observed twice daily for five days. On the fifth day two control mice and two inoculated mice were removed from each cage and euthanatized with chloroform. The entire animal was placed into 5 ml. of
trypticase soy broth containing antibiotics and ground with a Ten Broek tissue grinder. The resulting tissue suspension was frozen and stored at -40 F. Upon thawing this material was centrifuged for 15 minutes at 1000 rpm to sediment tissue debris before being used for additional passages in suckling mice. Both prototype strains were carried through three passages by the intracerebral and the intraperitoneal routes. In attempts to reisolate the virus using cell cultures, it was necessary to make a 1 to 2 dilution of the tissue suspension in trypticase soy broth and centrifuge for 30 minutes at 10,000 rpm in order to avoid the cytotoxic action of the cell debris. Methods of inoculation of cell cultures were identical to those described for isolation techniques from rectal swabs.

**Pregnant guinea pig inoculations**

No breeding dates were available for the pregnant guinea pigs but only those animals in which well formed fetuses could be detected upon abdominal palpation were used. Subsequent observation confirmed that these animals were all within the last 20 days of their gestation period at the time of inoculation. The animals were inoculated intracardially with 1.0 ml. of undiluted infectious tissue culture fluid. Animals were observed twice daily until they either aborted or delivered normal young. Aborted fetuses were necropsied and portions of the spleen, liver, kidney, skeletal muscle,
small intestine, large intestine, heart, lung and brain were collected. These tissues were pooled in 20 ml. of trypticase soy broth, ground with a Ten Broek tissue grinder and the resulting tissue suspension frozen at -40 F until cell cultures were available for virus isolation studies. Prior to inoculation onto cell sheets the tissue suspension was centrifuged for 30 minutes at 10,000 rpm in a refrigerated centrifuge. The supernatant fluid was used as an inoculum, the procedures being identical to those already described for virus isolations from rectal swabs. From one animal which aborted the placenta was also collected and the guinea pig sow was euthanatized and the uterus removed for virus isolation studies. The placenta and uterus were handled as described above.

**Calf inoculations**

All calves were obtained from cows in the dairy herd maintained by the Department of Veterinary Obstetrics. When it was evident that parturition was approaching the cow's rectum was manually emptied of feces and the perineal area, tail and hindquarters of the cow were scrubbed with a 1:5000 solution of Roccal\(^1\). A specially constructed canvas bag 34 inches by 60 inches with a plastic lining and a drawstring around the open end was used to catch the calf at birth. This bag, a

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\(^1\)Winthrop Laboratories Inc., 1450 Broadway, New York 18, New York.
40 gallon galvanized can and a 5 by 6 foot piece of canvas were all disinfected with Roccal 1:5000 immediately prior to use. As the delivery of the calf progressed care was exercised to keep the fetal membranes intact thereby encasing the calf and preventing, in so far as possible, direct contact between the calf and the walls of the uterus and vagina. As the head and forequarters of the calf were exposed they were immediately inserted into the canvas bag and by grasping the forelegs through the bag traction was exerted to bring about a rapid delivery of the calf into the disinfected bag. The bag containing the calf was immediately dropped into the sanitized 40 gallon can and the lid placed on it to prevent contamination of the exterior of the bag. The galvanized can was then immediately carried to a waiting vehicle and the bag containing the calf transferred to the disinfected canvas. The calf was then transported, in the canvas bag, to a previously disinfected and formaldehyde fumigated isolation unit. Upon arrival at the isolation unit the canvas bag containing the calf was carried to the door of the unit and the calf was allowed to fall from the bag into the unit, at the same time being careful to prevent contact of the bag or personnel with the inside of the isolation unit. The door to the unit was closed and personnel changed clothing and boots and scrubbed their hands with 1:5000 Roccal solution. The unit was then entered and the calf was washed with warm water and Roccal
disinfectant, dried with sterile towels and placed into the holding pen within the unit.

All the calves were reared in a similar manner except that the first calf received no colostrum. When the calves were an hour or two old they were given a pint of colostrum from the first milking of the dam using a commercial plastic calf bottle and nipple. This was repeated at approximately six hour intervals for the first two days. The quantity of colostrum was then increased to one and one half pints per feeding and the calves were fed only three times a day. Starting on the third day pasteurized homogenized milk was mixed with the colostrum and the proportions of milk gradually increased so that by the fifth day the calves were receiving only pasteurized homogenized milk and no colostrum. Starting on the sixth day the calves were fed only twice a day and the amount of milk per feeding increased to three pints. On the eighth day a portion of the pasteurized milk was replaced with commercial calf milk replacer mixed as recommended by the manufacturer. The proportion of the total diet made up by the milk replacer was gradually increased until the eleventh or twelfth day when the calves received only milk replacer. Quantities were also increased so that by the time the calf was two weeks old it was receiving two quarts per feeding. When the calf was about one week old water was also made available and the calves were force fed a small amount of
commercial pelleted calf starter with an added amount made available free choice. The quantity of calf starter made available was increased as the calves grew older. During the third week a small amount of alfalfa hay was fed and this quantity was also increased in time. During the second month of life the calf starter was gradually replaced with a calf supplement and the milk was limited to two quarts per day given at the time of the morning feeding.

The calves were placed on experiment when approximately one month of age with the exception of the colostrum deprived calf which was infected at one week of age. For three days prior to inoculation the calves' temperature, total leucocyte count and hematocrit were checked morning and evening to determine normal values. Rectal swabs were also collected every morning and virus isolations attempted to determine whether any pre-existing virus infections were present. The calves were inoculated intravenously with 10.0 ml. of undiluted infectious tissue culture fluid and an additional 10 ml. was given orally. During the next 10 days the calves were observed twice a day, temperatures recorded and blood samples collected for laboratory studies. Rectal swabs were collected daily for attempted reisolation of the viruses and handled as previously described in the section on primary isolation of viruses. Total leucocytes were counted as outlined by Benjamin (4) and hematocrit values determined using a
microhematocrit method.

Approximately 200 ml. of blood was collected from the jugular vein prior to inoculation in order to obtain pre-inoculation reference serum. About three weeks after the initial inoculation, blood was again collected in order to obtain postinoculation serum samples and the calf was then inoculated a second time using the other prototype virus strain. Procedures were identical to those described for the initial inoculation. Two weeks after the second inoculation the calves were bled again to obtain a final serum sample after the calves had been infected with both prototype strains.

Filtration Studies

Filtration studies were carried out using Millipore filter membranes and a standard Millipore filtration apparatus. The infectious material utilized was undiluted tissue culture fluid. This material was first filtered through a 220 μm filter to remove tissue particles. The titer of the resulting fluid was determined using the dilution and tissue culture assay system described above. Subsequently a portion of the above fluid was filtered through a 100 μm filter and a second portion through a 50 μm filter. Each filtrate was

1Millipore Filter Corporation, Bedford, Massachusetts.
then titrated to determine virus activity.

Cationic Stabilization

Cationic stabilization studies were performed as described by Wallis and Melnick (55). Virus samples consisting of undiluted infectious tissue culture fluids were mixed with an equal quantity of 2 molar magnesium chloride, dispensed into one half dram screw capped glass vials and placed into a waterbath at 50 °C. Control material was handled in the same manner except that it was mixed with an equal quantity of serum-free Hanks' LH solution. At various time intervals a control tube and one containing 2 molar magnesium chloride were removed, cooled immediately in ice water and subsequently titrated to determine the quantity of viable virus present.

Electron Microscopy

Viruses were examined using the negative staining technique of Brenner and Horne (9). Undiluted infectious tissue culture fluids were placed into plastic centrifuge tubes and centrifuged for 30 minutes at 10,000 rpm in a Servall refrigerated centrifuge to sediment cellular debris. The supernatant fluid was withdrawn with a sterile pipette and placed into a metal centrifuge tube and the virus sedimented by centrifugation for three hours at 40,000 rpm at 0 °C using the number 40 rotor in a Spinco model L ultracentrifuge. The
supernatant fluid was withdrawn and the virus pellet resuspended in a small quantity of 1 per cent ammonium acetate. This virus suspension was then mixed with an equal quantity of 2 per cent phosphotungstic acid previously adjusted to a pH of 7.2 using 1 N potassium hydroxide. A small drop of the virus suspension was then allowed to dry in the center of a carbon coated parlodion membrane supported by a 200 mesh perforated production grid. Specimens were examined immediately using a Hitachi\textsuperscript{1} model HU 11A electron microscope. Control material consisting of tissue culture fluid from noninfected cell cultures was examined in a similar manner.

\textsuperscript{1}Hitachi, Ltd., Tokyo, Japan.
RESULTS

Viral agents were isolated from 7, or 15.5 per cent, of the 45 animals tested in the three herds studied. These results are shown in more detail in Table 1. From this table it is obvious that agents were most commonly isolated from the younger animals. Table 2 shows the origin and numerical designation of the seven isolates.

All of the agents isolated were obtained from the initial inoculation of rectal swab material into bovine kidney cell cultures. No attempt was made to carry out additional passages when initial isolation attempts were negative. Following inoculation of rectal swab material a cytopathic effect was usually not detected until 48 to 72 hours later. In subsequent passages using an undiluted inoculum the cytopathic effect was readily apparent within 24 hours or less.

It was subsequently found that all seven strains would produce plaques in bovine kidney cell cultures and that some isolates produced plaques of more than one size. Using this difference in plaque size the seven isolates could be further divided as shown in Table 3. Four of the isolates gave rise to both small and large plaques while the three remaining isolates formed only large plaques. This subdivision of the original seven isolates on the basis of plaque size gave a total of eleven strains for use in this study. It was found that strains 1 and 2 would also produce plaques in primary
Table 1. The isolation of enteric viruses from three herds of cattle

<table>
<thead>
<tr>
<th>Name and location of herd</th>
<th>Type of herd</th>
<th>Age</th>
<th>Number tested</th>
<th>Number positive</th>
<th>Per cent positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Westrum, Stratford, Iowa</td>
<td>Feeder cattle</td>
<td>5-6 mo.</td>
<td>18</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>Dairy Nutrition, I. S. U.</td>
<td>Dairy calves</td>
<td>1/2-3 mo.</td>
<td>16</td>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td>Veterinary Obstetrics, I. S. U.</td>
<td>Dairy cows and calves</td>
<td>Adult 1-2 mo.</td>
<td>9</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>TOTAL</strong></td>
<td></td>
<td>45</td>
<td>7</td>
<td>15.5</td>
</tr>
</tbody>
</table>

Table 2. Herd origin of seven bovine enteric virus isolates

<table>
<thead>
<tr>
<th>Name and location of herd</th>
<th>Numerical designation of strains isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Westrum, Stratford, Iowa</td>
<td>1, 7</td>
</tr>
<tr>
<td>Dairy Nutrition, I. S. U.</td>
<td>2, 3, 4, 5</td>
</tr>
<tr>
<td>Veterinary Obstetrics, I. S. U.</td>
<td>6</td>
</tr>
</tbody>
</table>
Table 3. The origin and designation of small (S) and large (L) plaque producing strains among seven bovine enteric virus isolates

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>Plaque type designation</th>
<th>Small plaques 2.0-4.0 mm.</th>
<th>Large plaques 7.0-12.0 mm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1S</td>
<td></td>
<td>1L</td>
</tr>
<tr>
<td>2</td>
<td>none</td>
<td></td>
<td>2L</td>
</tr>
<tr>
<td>3</td>
<td>3S</td>
<td></td>
<td>3L</td>
</tr>
<tr>
<td>4</td>
<td>4S</td>
<td></td>
<td>4L</td>
</tr>
<tr>
<td>5</td>
<td>none</td>
<td></td>
<td>5L</td>
</tr>
<tr>
<td>6</td>
<td>none</td>
<td></td>
<td>6L</td>
</tr>
<tr>
<td>7</td>
<td>7S</td>
<td></td>
<td>7L</td>
</tr>
</tbody>
</table>
bovine testicle cell cultures but that all plaques were much smaller than in bovine kidney cells. Figures 1, 2 and 3 illustrate the plaques produced by these agents in the two types of cell cultures.

A study of the cytopathic effects produced by the eleven virus strains failed to reveal any further differences among strains. When high titer material was inoculated all isolates produced a rapid cytopathic effect. Widespread early changes frequently developed within eight hours after inoculation and complete destruction of the cell sheet resulted within 24 hours. If a less concentrated inoculum was used changes were often delayed as much as 48 to 72 hours with early changes consisting of focal lesions which usually spread rapidly and resulted in complete destruction of the cell sheet within the next 24 to 36 hours. Figures 4, 5 and 6 show cytopathic changes typical of those produced by all virus strains studied. Infected cells apparently became detached from the glass surface at their periphery thereby allowing them to become more spherical in shape. These cells therefore appear smaller and are highly refractile when viewed in unstained preparations. The cells illustrated were infected with a high titer inoculum so changes were widespread, but in a few cases, as shown in Figure 6, some idea can be gained of the appearance of focal lesions when a less concentrated inoculum was used.

The examination of infected cells stained with hematoxylin
Figure 1. Small plaques produced by bovine enteric viruses in bovine kidney cell cultures.

Figure 2. Large plaques produced by bovine enteric viruses in bovine kidney cell cultures.
Figure 3. Small and large plaques produced by bovine enteric viruses in bovine testicle cell cultures.

Figure 4. Widespread cytopathic changes in bovine kidney cell cultures caused by bovine enteric viruses. X50. Unstained preparation.
Figure 5. Spherical refractile cells typical of cytopathic effect produced by bovine enteric viruses. X125. Unstained preparation.

Figure 6. Small foci of infected cells typical of early cytopathic changes caused by bovine enteric viruses. X125. Unstained preparation.
and eosin reveals, as shown in Figures 7, 8, 9 and 10, that when the cells begin to detach and "round up" there are concurrent changes taking place in the nuclei. The nuclei become pyknotic and later karyoschises and karyolysis occurs. No evidence of intranuclear or intracytoplasmic inclusion bodies was seen.

Preliminary studies of the antigenic and biological properties of the 11 plaque-purified strains indicated a marked dissimilarity between the 1L and 4S viruses. These two agents were therefore chosen for additional studies designed to clarify their relationship to each other and to the other 9 strains.

In initial attempts to hyperimmunize rabbits difficulties were encountered in producing satisfactory hyperimmune serum against both the 1L and 4S viruses in this species. For this reason, rabbits, guinea pigs, mice and chickens were subsequently hyperimmunized. The responses of rabbits, guinea pigs and chickens to hyperimmunization are shown in Table 4. The table shows that rabbits responded satisfactorily to hyperimmunization with the 4S virus but failed to respond to the 1L virus. Guinea pigs, on the other hand, responded to the 1L virus but not to the 4S strain. The hyperimmunization of chickens produced satisfactory antiserum against both strains.

The quantities of neutralizing substances in the preinoculation serum of rabbits, guinea pigs and chickens which were
Figure 7. Normal bovine kidney cell culture. X125. Hematoxylin and eosin stain.

Figure 8. Cytopathic changes in bovine kidney cell cultures caused by bovine enteric viruses. X125. Hematoxylin and eosin stain.
Figure 9. Infected cells with pyknotic nuclei. X400. Hematoxylin and eosin stain.

Figure 10. Infected cells showing advanced cytopathic changes with karyoschises and karyolysis. X400. Hematoxylin and eosin stain.
Table 4. The response of rabbits, guinea pigs and chickens to hyperimmunization with bovine enteric viruses

<table>
<thead>
<tr>
<th>Virus Strain</th>
<th>Neutralization index(^a) of homologous serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rabbit</td>
</tr>
<tr>
<td>IL 1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>4S</td>
<td>4.3</td>
</tr>
</tbody>
</table>

\(^a\)Expressed as Log\(_{10}\) TCID\(_{50}\).  

Table 5. The neutralization of bovine enteric viruses by substances present in preinoculation serum samples collected from rabbits, guinea pigs and chickens

<table>
<thead>
<tr>
<th>Virus Strain</th>
<th>Neutralization index(^a) of preinoculation serum samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rabbit</td>
</tr>
<tr>
<td>IL 2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>4S 1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

\(^a\)Expressed as Log\(_{10}\) TCID\(_{50}\).
hyperimmunized are shown in Table 5.

Attempts to produce antibodies in mice were unsuccessful. Not only were yields of ascitic fluid small and difficult to separate from the oily adjuvant which persisted in the peritoneal cavity but titers obtained with this material were extremely low when tested against 100 TCID\textsubscript{50} of the homologous virus. The titers in the range of 1 to 2 or 1 to 4 were considered to be too low to be useful in studying antigenic relationships between the eleven strains to be tested.

Results of neutralization studies of the eleven plaque-purified strains using antisera prepared in rabbits, guinea pigs and chickens are presented in Tables 6 through 9. A comparison of neutralization indexes of the various sera when tested against each of the eleven agents is presented in Table 10.

The 1S, 1L, 2L and 3S strains all appear to be closely related to the 1L prototype strain and show no significant relationship to the 4S prototype virus. Likewise the 4S and 7S strains seem to be closely related to each other and unrelated to the 1L prototype strain.

The 3L agent appears to be related to the 1L prototype when tested with hyperimmune serum produced in chickens but shows no relationship to either prototype strain when tested with the guinea pig and rabbit sera. The 5L agent appears related to the 4S prototype when tested with hyperimmune serum
Table 6. Neutralization of 11 enteric virus strains by rabbit anti 4S hyperimmune serum

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Virus titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Neutralization index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Virus plus preinoculation serum</td>
<td>Virus plus postinoculation serum</td>
</tr>
<tr>
<td>1S</td>
<td>8.5</td>
<td>8.2</td>
</tr>
<tr>
<td>1L</td>
<td>8.5</td>
<td>8.2</td>
</tr>
<tr>
<td>2L</td>
<td>8.2</td>
<td>8.5</td>
</tr>
<tr>
<td>3S</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>3L</td>
<td>6.2</td>
<td>5.5</td>
</tr>
<tr>
<td>4S</td>
<td>6.8</td>
<td>2.5</td>
</tr>
<tr>
<td>4L</td>
<td>7.2</td>
<td>3.8</td>
</tr>
<tr>
<td>5L</td>
<td>4.8</td>
<td>2.5</td>
</tr>
<tr>
<td>6L</td>
<td>6.2</td>
<td>6.2</td>
</tr>
<tr>
<td>7S</td>
<td>5.5</td>
<td>2.5</td>
</tr>
<tr>
<td>7L</td>
<td>5.5</td>
<td>5.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as Log<sub>10</sub> TCID<sub>50</sub> per ml.
Table 7. Neutralization of 11 enteric virus strains by guinea pig anti 1L hyperimmune serum

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Virus titera</th>
<th>Neutralization index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Virus plus preinoculation serum</td>
<td>Virus plus postinoculation serum</td>
</tr>
<tr>
<td>1s</td>
<td>7.3</td>
<td>4.5</td>
</tr>
<tr>
<td>1L</td>
<td>7.8</td>
<td>2.5</td>
</tr>
<tr>
<td>2L</td>
<td>7.3</td>
<td>3.8</td>
</tr>
<tr>
<td>3S</td>
<td>7.5</td>
<td>3.2</td>
</tr>
<tr>
<td>3L</td>
<td>5.2</td>
<td>4.5</td>
</tr>
<tr>
<td>4S</td>
<td>6.5</td>
<td>5.2</td>
</tr>
<tr>
<td>4L</td>
<td>6.3</td>
<td>6.5</td>
</tr>
<tr>
<td>5L</td>
<td>5.5</td>
<td>3.2</td>
</tr>
<tr>
<td>6L</td>
<td>7.8</td>
<td>6.5</td>
</tr>
<tr>
<td>7S</td>
<td>4.5</td>
<td>4.2</td>
</tr>
<tr>
<td>7L</td>
<td>5.0</td>
<td>3.5</td>
</tr>
</tbody>
</table>

aExpressed as Log₁₀ TCID₅₀ per ml.
Table 8. Neutralization of 11 enteric virus strains by chicken anti 1L hyperimmune serum

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Virus titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Virus plus preinoculation serum</th>
<th>Virus plus postinoculation serum</th>
<th>Neutralization index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1S</td>
<td>8.0</td>
<td>1.5</td>
<td></td>
<td>6.5</td>
</tr>
<tr>
<td>1L</td>
<td>8.2</td>
<td>1.5</td>
<td></td>
<td>6.7</td>
</tr>
<tr>
<td>2L</td>
<td>8.2</td>
<td>1.5</td>
<td></td>
<td>6.7</td>
</tr>
<tr>
<td>3S</td>
<td>7.5</td>
<td>4.2</td>
<td></td>
<td>3.3</td>
</tr>
<tr>
<td>3L</td>
<td>7.5</td>
<td>5.5</td>
<td></td>
<td>2.0</td>
</tr>
<tr>
<td>4S</td>
<td>7.5</td>
<td>5.2</td>
<td></td>
<td>2.3</td>
</tr>
<tr>
<td>4L</td>
<td>7.2</td>
<td>5.2</td>
<td></td>
<td>2.0</td>
</tr>
<tr>
<td>5L</td>
<td>6.7</td>
<td>4.8</td>
<td></td>
<td>1.9</td>
</tr>
<tr>
<td>6L</td>
<td>7.2</td>
<td>4.2</td>
<td></td>
<td>3.0</td>
</tr>
<tr>
<td>7S</td>
<td>7.5</td>
<td>6.0</td>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td>7L</td>
<td>8.2</td>
<td>5.5</td>
<td></td>
<td>2.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as Log<sub>10</sub> TCID<sub>50</sub> per ml.
Table 9. Neutralization of 11 enteric virus strains by chicken anti 4S hyperimmune serum

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Virus titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Virus plus preinoculation serum</th>
<th>Virus plus postinoculation serum</th>
<th>Neutralization index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1S</td>
<td>8.0</td>
<td>7.2</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>1L</td>
<td>7.5</td>
<td>7.5</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>2L</td>
<td>8.5</td>
<td>6.5</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>3S</td>
<td>7.5</td>
<td>6.5</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>3L</td>
<td>7.2</td>
<td>7.0</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>4S</td>
<td>7.5</td>
<td>2.8</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td>4L</td>
<td>6.5</td>
<td>5.2</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>5L</td>
<td>6.2</td>
<td>2.2</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>6L</td>
<td>7.5</td>
<td>5.5</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>7S</td>
<td>7.2</td>
<td>1.5</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td>7L</td>
<td>7.5</td>
<td>4.5</td>
<td>3.0</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as Log<sub>10</sub> TCID<sub>50</sub> per ml.
Table 10. A comparison of neutralization indices obtained by testing 11 enteric viruses with hyperimmune sera produced in rabbits, guinea pigs and chickens

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Neutralization index(^a) using 1L antiserum</th>
<th>Neutralization index(^a) using 4S antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chicken Guinea pig</td>
<td>Chicken Rabbit</td>
</tr>
<tr>
<td>1S</td>
<td>6.5</td>
<td>2.8</td>
</tr>
<tr>
<td>1L</td>
<td>6.7</td>
<td>5.3</td>
</tr>
<tr>
<td>2L</td>
<td>6.7</td>
<td>3.5</td>
</tr>
<tr>
<td>3S</td>
<td>3.3</td>
<td>4.3</td>
</tr>
<tr>
<td>3L</td>
<td>2.0</td>
<td>0.7</td>
</tr>
<tr>
<td>4S</td>
<td>2.3</td>
<td>1.3</td>
</tr>
<tr>
<td>4L</td>
<td>2.0</td>
<td>0.0</td>
</tr>
<tr>
<td>5L</td>
<td>1.9</td>
<td>2.3</td>
</tr>
<tr>
<td>6L</td>
<td>3.0</td>
<td>1.3</td>
</tr>
<tr>
<td>7S</td>
<td>1.5</td>
<td>0.3</td>
</tr>
<tr>
<td>7L</td>
<td>2.7</td>
<td>1.5</td>
</tr>
</tbody>
</table>

\(^a\)Expressed as Log\(_{10}\) TCID\(_{50}\)
produced in chickens but is not sharply differentiated by the
guinea pig and rabbit sera. This may have been due to the
fact that the 5L virus pool did not have a particularly high
titer and the presence of neutralizing substances in the pre-
inoculation rabbit serum prevented the development of a high
neutralization index. The 4L virus appeared to be related to
both prototype viruses when tested with the chicken antiserum.
When tested with the guinea pig and rabbit antiserum, however,
this virus was shown to be rather closely related to the 4S
prototype but unrelated to the 1L prototype strain.

In an attempt to clarify the relationships of the 6L
and 7L viruses, studies were carried out using antiserum pro-
duced against these agents. Results of cross neutralization
tests using these agents and their respective antisera plus
the 1L and 4S prototype viruses are presented in Tables 11 and
12. If these results are combined with the earlier results
using the guinea pig and rabbit antisera against the 1L and 4S
prototype strains they would appear as shown in Table 13.

The antiserum prepared against the 7L strain exhibits no
neutralizing activity when tested against the 1L prototype
virus but shows a rather high neutralization index when tested
against the 4S virus. It is also readily apparent that the 7L
antiserum exhibits little activity against the homologous
virus and furthermore that the 7L agent was poorly neutralized
by all of the immune sera. This gives rise to the speculation
Table 11. Neutralization of 4 enteric virus strains by rabbit anti 6L hyperimmune serum

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Virus titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Virus plus preinoculation serum</th>
<th>Virus plus postinoculation serum</th>
<th>Neutralization index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1L</td>
<td>7.5</td>
<td>7.2</td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>4S</td>
<td>6.2</td>
<td>3.8</td>
<td></td>
<td>2.4</td>
</tr>
<tr>
<td>6L</td>
<td>6.8</td>
<td>1.5</td>
<td></td>
<td>5.3</td>
</tr>
<tr>
<td>7L</td>
<td>5.2</td>
<td>4.5</td>
<td></td>
<td>0.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as Log<sub>10</sub> TCID<sub>50</sub> per ml.

Table 12. Neutralization of 4 enteric virus strains by rabbit anti 7L hyperimmune serum

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Virus titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Virus plus preinoculation serum</th>
<th>Virus plus postinoculation serum</th>
<th>Neutralization index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1L</td>
<td>7.5</td>
<td>7.5</td>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>4S</td>
<td>6.5</td>
<td>2.2</td>
<td></td>
<td>4.3</td>
</tr>
<tr>
<td>6L</td>
<td>6.8</td>
<td>4.5</td>
<td></td>
<td>2.3</td>
</tr>
<tr>
<td>7L</td>
<td>5.2</td>
<td>4.8</td>
<td></td>
<td>0.4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as Log<sub>10</sub> TCID<sub>50</sub> per ml.
that the 7L virus is a strain similar to those reported by Bürki (11) and Wigand (56) which are difficult to neutralize. If this is taken into consideration, then on the basis of the reaction between the 7L antiserum and the 1L and 4S prototype viruses the 7L virus can at least be tentatively grouped with the 4S prototype strain. There must be some reservations concerning this grouping, however, because even though all neutralization indexes were low the 1L antiserum did show more affinity for the 7L virus than did the 4S antiserum. In spite of these minor inconsistencies the 7L virus will, for the reasons given above, be included as a member of the 4S group.

Although tests using the 6L antiserum indicated some relationship of the 6L virus to both the 1L and 4S viruses no definite placement within either of these groups could be made on the basis of reciprocal cross-neutralization tests. This agent was therefore placed into a separate antigenic group.

As Table 14 shows, on the basis of serological studies the eleven strains were separated into two groups consisting of 5 viruses each with one agent remaining as the only member of a third group. It also appeared that the 1L and 4S agents would serve as suitable representatives of each of the larger groups so the remaining studies were, for the most part, limited to a comparison of these two agents.

It was considered important in the course of this work
Table 13. A comparison of neutralization indices obtained in cross neutralization tests with the 1L, 4S, 6L and 7L virus strains

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Neutralization index&lt;sup&gt;a&lt;/sup&gt;</th>
<th>anti 1L</th>
<th>anti 4S</th>
<th>anti 6L</th>
<th>anti 7L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1L</td>
<td>5.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>4S</td>
<td>1.3</td>
<td>4.3</td>
<td>2.4</td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td>6L</td>
<td>1.3</td>
<td>0.0</td>
<td>5.3</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>7L</td>
<td>1.5</td>
<td>0.5</td>
<td>0.7</td>
<td>0.4</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as Log<sub>10</sub> TCID<sub>50</sub>.

Table 14. The grouping of 11 bovine enteric viruses on the basis of antigenic relationship

<table>
<thead>
<tr>
<th>Antigenic group</th>
<th>Prototype strain</th>
<th>Other strains included in the group</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1L</td>
<td>1S, 2L, 3S, 3L</td>
</tr>
<tr>
<td>II</td>
<td>4S</td>
<td>4L, 5L, 7S, 7L</td>
</tr>
<tr>
<td>III</td>
<td>6L</td>
<td>none</td>
</tr>
</tbody>
</table>
to determine the antigenic relationship of the two prototype strains with at least one previously described ECBO virus. The agent used for this comparison was the LCR4 virus isolated by Kunin and Minuse. This was one of the first ECBO viruses isolated and was certainly the first with which any appreciable amount of research was done. It has been compared with enteroviruses isolated by workers in various parts of the world. Hyperimmune rabbit serum against this agent was obtained through the courtesy of Dr. C. M. Kunin. Unfortunately, preinoculation serum samples were not available so comparisons were made between titers of virus plus antiserum and virus plus diluent controls. Results of these neutralization tests using the LCR4 antiserum and the LCR4, 1L and 4S viruses indicated that all three viruses were neutralized to about the same extent. The neutralization indexes were extremely low, however, even when the LCR4 antiserum was tested against the homologous virus strain. The reciprocal test, on the other hand, showed a marked difference between the action of the 1L and 4S antisera against the LCR4 agent. Table 15 shows that the 1L antiserum neutralized the LCR4 virus to a high degree while the 4S antiserum shows almost no activity against the LCR4 virus.

A further characterization of the 1L and 4S viruses

1Dr. C. M. Kunin, University of Virginia Medical School, Charlottesville, Virginia.
showed that both agents were resistant to treatment with 20 per cent ethyl ether or chloroform when tested using recommended procedures. Results of these tests are shown in Tables 16 and 17.

Alterations and accumulations of intracellular ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) during viral replication were studied using the fluorochrome, acridine orange. No differences could be detected between IL and 4S infected cells. Figure 11 shows that three hours after inoculation there seemed to be a slight clearing of the diffuse DNA in the nucleus and some uneven staining of the RNA in the cytoplasm as compared to the uninfected control cells shown in Figure 12. At six hours, as shown in Figure 13, these changes had progressed to the point where there was a very evident clumping and margination of the DNA in the nucleus with definite areas in the cytoplasm showing an increased RNA content. Figure 14 shows that at 12 hours the cells had begun to detach from the glass surface and "round up", the nucleus was collapsing and intracytoplasmic RNA was still increasing. At 20 hours, as shown in Figure 15, many of the cells consisted of small spheres, their nuclei now only a bright staining clump of DNA, and there was evidence of the disappearance of cytoplasmic RNA especially in the perinuclear areas. A high power view of cells in the advanced stages of infection is shown in Figure 16. The cells have become spherical and
Table 15. Neutralization of the LCR4 virus by chicken anti 1L and 4S hyperimmune serum

<table>
<thead>
<tr>
<th>Serum</th>
<th>Virus plus preinoculation serum</th>
<th>Virus plus postinoculation serum</th>
<th>Neutralization index</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti 1L</td>
<td>7.2</td>
<td>1.8</td>
<td>5.4</td>
</tr>
<tr>
<td>anti 4S</td>
<td>7.5</td>
<td>7.2</td>
<td>0.3</td>
</tr>
</tbody>
</table>

aControl titer of LCR4 virus 7.5.
bExpressed as $\log_{10} \text{TCID}_{50}$ per ml.
Table 16. Ether susceptibility of the 1L and 4S prototype viruses

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Virus titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Decrease in titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Virus plus diluent</td>
<td>Virus plus ether</td>
</tr>
<tr>
<td>1L</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>4S</td>
<td>8.2</td>
<td>7.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as Log<sub>10</sub> TCID<sub>50</sub> per ml.

Table 17. Chloroform resistance of the 1L and 4S prototype viruses

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Virus titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Decrease in titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Virus plus diluent</td>
<td>Virus plus chloroform</td>
</tr>
<tr>
<td>1L</td>
<td>7.5</td>
<td>6.2</td>
</tr>
<tr>
<td>4S</td>
<td>7.5</td>
<td>8.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as Log<sub>10</sub> TCID<sub>50</sub> per ml.
Figure 11. Uneven staining of cytoplasmic RNA in bovine kidney cell cultures 3 hours after infection with bovine enteric virus. X450. Acridine orange stain.

Figure 12. Normal bovine kidney cell culture. X1000. Acridine orange stain.
Figure 13. Increase in cytoplasmic RNA and margination of nuclear DNA 6 hours after infection with bovine enteric virus. X1000. Acridine orange stain.

Figure 14. Further increase in cytoplasmic RNA and collapse of the nucleus 12 hours after infection. X1000. Acridine orange stain.
developed an eccentric pyknotic nucleus and the RNA in the cytoplasm stains very brilliantly. This is apparently followed by a release of RNA, presumably as mature virus particles. All that remains is an empty cell wall, with some adherent RNA, containing a collapsed nucleus as shown in Figure 17.

Results of tests to determine the heat stability of the 1L and 4S viruses in the presence of 1 molar MgCl₂ are shown in Figures 18 and 19. From these results it is obvious that the 4S prototype is protected from heat inactivation at 50°C by the presence of 1M MgCl₂. On the other hand, although there is some heat stabilization of the 1L virus by the same treatment this is not as great compared to the titer of the untreated virus controls. There is, however, a marked difference in the heat sensitivity of the two prototype strains in the absence of MgCl₂.

Studies of the hemagglutinating activity of the two prototype strains and the ability of infected cell sheets to adsorb erythrocytes were carried out using rabbit, chicken, guinea pig, swine, sheep, calf and human "O" erythrocytes. All tests were negative except those using swine erythrocytes which were hemagglutinated by undiluted infectious tissue culture fluids at 4°C but were negative when this material was diluted 1 to 4 or 1 to 8. No evidence of hemadsorption was observed.
Figure 15. Spherical cells containing large amounts of RNA and pyknotic nuclei 20 hours after infection. X450. Acridine orange stain.
Figure 16. Two spherical cells containing large quantities of RNA and eccentric pyknotic nuclei. X1000. Acridine orange stain.
Figure 17. Cell after viral release. X1000. Acridine orange stain.
Figure 18. Cationic stabilization of the IL virus at 50°C by molar magnesium chloride.

--- Virus plus magnesium chloride

--- Virus control

Figure 19. Cationic stabilization of the 4S virus at 50°C by molar magnesium chloride.

--- Virus plus magnesium chloride

--- Virus control
Fig. 18—Magnesium chloride stabilization studies with the IL virus

Fig. 19—Magnesium chloride stabilization studies with the 4S virus
Results of attempts to grow the eleven strains in various cell cultures are presented in Table 18. From this table it can be seen that all eleven isolates grew in bovine kidney, sheep kidney and chick fibroblast cells. It is also apparent that the 1S and 1L agents were able to grow in all the cell types tested while the 7S and 4S viruses were able to grow in only half or less of the types of cell cultures tested. Most of the cell cultures tested supported growth of at least half or more of the virus strains with the exception of the guinea pig kidney cells which were infected by only the 1S, 1L and 7L viruses.

Intracranial or intraperitoneal inoculation of suckling mice with the 1L or 4S virus strains failed to produce any clinical evidence of infection. Attempts to reisolate the inoculated virus from mouse tissues or to carry this material through two additional passages in mice also yielded negative results.

Inoculation of female guinea pigs in advanced pregnancy produced the results shown in Table 19. The 4S virus had no effect on the female guinea pigs but the 1L virus apparently caused abortion in two of the four inoculated. The aborted fetuses were well developed but the hair coat was not yet fully formed. It was estimated that these abortions occurred seven to ten days before the end of the normal gestation period. No gross lesions were observed at necropsy of the
Table 18. The production of cytopathic changes in various cell cultures by 11 bovine enteric viruses

<table>
<thead>
<tr>
<th>Cell culture</th>
<th>1S</th>
<th>1L</th>
<th>2L</th>
<th>3S</th>
<th>3L</th>
<th>4S</th>
<th>4L</th>
<th>5L</th>
<th>6L</th>
<th>7S</th>
<th>7L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine kidney</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bovine testicle</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sheep kidney</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Guinea pig kidney</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Monkey kidney</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chick fibroblast</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HeLa</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Swine kidney</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
aborted fetuses and virus could not be reisolated from fetal tissues. The second female to abort was killed shortly after the abortion occurred and her uterus removed for virus isolation studies. The placentas were also collected from this animal. Virus could not be reisolated from the placentas but it was possible to isolate an agent from the uterus which produced a cytopathic change in bovine kidney cell cultures typical of that produced by the bovine enterovirus strain inoculated. This agent was later tested using IL antiserum. Results of these tests are presented in Table 20. The high neutralizing index obtained with the reisolated virus leaves little doubt that this virus was the IL agent.

Results of attempts to determine the particle size of the two prototype viruses by means of filtration through Millipore filters are presented in Table 21. It becomes apparent that both the IL and 4S agents were capable of passing through the 50 μ filter membrane. There was, however, some difference in titer following filtration indicating that the 4S agent did not pass the filter membrane as readily. If we use the figures of Elford (19) regarding the comparison of particle size and pore diameter we would calculate a particle size for the two agents to be approximately 25 μ or less.

Electron micrographs of the IL and 4S viruses are shown in Figures 20 through 24. Calculation of particle size of
Table 19. Inoculation of pregnant guinea pigs with the 1L and 4S prototype viruses

<table>
<thead>
<tr>
<th>Guinea pig number</th>
<th>Virus inoculated&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1L</td>
<td>Gave birth to 5 live young 9 days later</td>
</tr>
<tr>
<td>2</td>
<td>1L</td>
<td>Gave birth to 4 live young 8 days later</td>
</tr>
<tr>
<td>3</td>
<td>1L</td>
<td>Aborted 2 fetuses 12 days later</td>
</tr>
<tr>
<td>4</td>
<td>1L</td>
<td>Aborted 2 fetuses 6 days later</td>
</tr>
<tr>
<td>5</td>
<td>4S</td>
<td>Gave birth to 2 live young 21 days later</td>
</tr>
<tr>
<td>6</td>
<td>4S</td>
<td>Gave birth to 4 live young 19 days later</td>
</tr>
<tr>
<td>7</td>
<td>4S</td>
<td>Gave birth to 3 live young 10 days later</td>
</tr>
</tbody>
</table>

<sup>a</sup>1.0 ml. inoculated intracardially.

Table 20. Test of the identity of the agent reisolated from the guinea pig which aborted

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Virus titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Neutralization index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Virus plus 1L antiserum</td>
</tr>
<tr>
<td>1L</td>
<td>7.2</td>
<td>1.5</td>
</tr>
<tr>
<td>Guinea pig isolate</td>
<td>5.8</td>
<td>1.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as Log<sub>10</sub> TCID<sub>50</sub> per ml.
Table 21. Filtration studies of the 1L and 4S prototype virus strains

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Virus titer$^a$</th>
<th>220 μm APD$^b$</th>
<th>100 μm APD</th>
<th>50 μm APD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1L</td>
<td></td>
<td>8.2</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>4S</td>
<td></td>
<td>7.3</td>
<td>4.8</td>
<td>3.5</td>
</tr>
</tbody>
</table>

$^a$Expressed as Log$_{10}$ TCID$_{50}$ per ml.

$^b$Average pore diameter of the Millipore filter membrane.
Figure 20. Electron micrograph of the IL virus. X250,000. Negative staining with phosphotungstic acid.

Figure 21. Electron micrograph of the IL virus. X250,000. Negative staining with phosphotungstic acid.

Figure 22. Electron micrograph of the IL virus showing two "empty" virus particles. X250,000. Negative staining with phosphotungstic acid.
Figure 23. Electron micrograph of the 4S virus with some particles showing a polygonal outline. X200,000. Negative staining with phosphotungstic acid.

Figure 24. Electron micrograph of a clump of 4S virus particles. X250,000. Negative staining with phosphotungstic acid.
the two viruses gives a figure of about 25 μ for the diameter of the particles of both viruses. Some particles which appear to be polygonal in outline can be seen in Figure 23. Studies of noninfected tissue culture fluids failed to reveal any structures resembling the virus particles.

Results of clinical observations and laboratory studies of calves experimentally infected with the prototype viruses are shown in Figures 25 through 31. As shown in the above figures only two of the four inoculated calves developed diarrhea following inoculation. The virus was recovered from all calves, however, indicating that all were actually infected. Marked alterations in body temperature and total leucocyte counts did not occur following inoculation but in the two calves which developed diarrhea temperature readings were somewhat higher during the first three or four days after inoculation and total leucocyte counts decreased somewhat initially and then increased during the time when diarrhea occurred. These changes were certainly not marked, however, and may have been coincidental. No distinguishable patterns in temperature readings or leucocyte counts were observed in the two calves which failed to develop diarrhea after the initial inoculation or in any of the three calves following subsequent inoculation with the other prototype virus. Hematocrit studies of the blood from these animals ruled out dehydration and subsequent hemoconcentration as having any effect on
Figure 25. Clinical and laboratory data obtained on calf number 1 exposed to the IL prototype virus.

- --- Temperature
- --- Leucocytes per cmm.
Fig. 25—Clinical and laboratory data obtained on calf number 1 exposed to IL prototype virus.
Figure 26. Clinical and laboratory data obtained on calf number 2 exposed to the IL prototype virus.

--- Temperature

--- Leucocytes per cmm.
Fig. 26—Clinical and laboratory data obtained on calf number 2 exposed to IL prototype virus
Figure 27. Clinical and laboratory data obtained on calf number 2 exposed to the 4S prototype virus.

--- Temperature

--- Leucocytes per cmm.
Inoculation

Fig. 27—Clinical and laboratory data obtained on calf number 2 exposed to 4S prototype virus.

Inoculation
Figure 28. Clinical and laboratory data obtained on calf number 3 exposed to the 4S prototype virus.

- - - - Temperature

- - - - Leucocytes per cmm.
Fig. 28 - Clinical and laboratory data obtained on calf number 3 exposed to 4S prototype virus
Clinical and laboratory data obtained on calf number 3 exposed to the IL prototype virus.

Temperature

Leucocytes per cmm.
Inoculation

Fig. 29—Clinical and laboratory data obtained on calf number 3 exposed to IL prototype virus
Figure 30. Clinical and laboratory data obtained on calf number 4 exposed to the 4S prototype virus.

--- Temperature

--- Leucocytes per cmm.
Fig. 30—Clinical and laboratory data obtained on calf number 4 exposed to 4S prototype virus
Figure 31. Clinical and laboratory data obtained on calf number 4 exposed to the IL prototype virus.

--- Temperature

--- Leucocytes per cmm.
Fig. 31 - Clinical and laboratory data obtained on calf number 4 exposed to IL prototype virus.
leucocyte counts.

Calf 1, a colostrum deprived calf, was inoculated with the IL virus when 6 days old and developed a mild diarrhea on the second and third day after inoculation but the consistency of the feces returned to normal on the fourth day. On the fifth day this calf developed a severe watery diarrhea but the condition persisted for only 12 to 18 hours and the feces then returned to a normal consistency for the rest of the observation period. The virus was reisolated from this animal on the fourth day after inoculation. The animal appeared rather weak and listless during the entire observation period but this was felt to be due to the nutritional problems resulting from the lack of colostrum and an initial low food intake rather than as a result of virus infection. The calf was killed eight days after it was inoculated and a necropsy performed. No gross lesions were observed which were thought to be due to the virus infection. The animal was found to be extremely emaciated and a mild hydrothorax and ascites were noted as well as some subcutaneous edema. These changes were felt to be a result of inadequate nutrition.

A second calf, colostrum fed, was inoculated with the IL agent when it was 27 days old. This animal remained normal during the following nine day observation period but the IL virus was recovered from rectal swabs collected on the 1st, 4th, and 7th days after inoculation. On the 34th day after
the initial inoculation this calf was again inoculated, this time with 4S virus. No clinical signs of illness developed during the following nine days. The 4S virus was reisolated from rectal swabs collected on the first through the seventh day following inoculation.

The third experimental calf was inoculated with the 4S virus when it was 27 days old. On the third day after inoculation this calf developed a profuse watery diarrhea with excess mucus present in the feces. At this time the animal appeared somewhat depressed and its appetite was diminished. Diarrhea persisted for six days and the calf became dehydrated and weak but after the first day of illness the appetite returned to normal. During the last two days of illness the animal was more alert and rapidly returned to normal. The virus was reisolated from this animal by means of rectal swabs on the fourth through the sixth days after inoculation. Thirty-five days after the initial inoculation this calf was inoculated with the IL agent. No clinical changes occurred during the following eleven days but the virus was reisolated from rectal swabs collected on the first through the sixth and the ninth and tenth days after inoculation.

A fourth experimental calf, colostrum fed, was inoculated with the 4S agent at an age of 27 days. This calf developed no clinical signs of illness during the following ten days but the virus was recovered from the feces on each of these
days. A subsequent inoculation of the 1L virus 23 days after the 4S inoculation also failed to produce any clinical signs of disease. The virus was reisolated from the animal on the first through the eighth days postinoculation.

The viruses reisolated from the experimental calves were subsequently tested against known positive antiserum in an attempt to prove their identity with the prototypes inoculated. Results of these tests are presented in Tables 22 and 23. From these results it can be seen that the reisolated viruses were, in general, neutralized to a high degree by the antiserum prepared against the prototype viruses which served as inocula. An exception to this is the 1L virus reisolated from calf 2. The titer of this virus was decreased 2 logs by the test serum while all other isolates tested were neutralized to a greater extent.

Studies of neutralizing antibodies in the serum of experimental calves were also conducted using paired preinoculation and postinoculation serum samples. As shown in Tables 24 and 25 neutralizing antibodies did not develop to any great extent in the test animals. In two cases, however, a significant rise in antibody titer did occur. The titers of virus plus diluent controls showed in many cases that preinoculation serum samples had some neutralizing activity against the virus strains.

In an attempt to determine the origin of the neutralizing
Table 22. Test of the identity of agents reisolated from experimental calves following inoculation with the IL prototype virus

<table>
<thead>
<tr>
<th>Virus source</th>
<th>Virus titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Neutralization index</th>
<th>Virus identity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Virus plus 1L antiserum</td>
<td></td>
</tr>
<tr>
<td>Calf 1</td>
<td>6.8</td>
<td>1.5</td>
<td>5.7</td>
</tr>
<tr>
<td>Calf 2</td>
<td>6.8</td>
<td>4.8</td>
<td>2.0</td>
</tr>
<tr>
<td>Calf 3</td>
<td>7.2</td>
<td>1.8</td>
<td>5.4</td>
</tr>
<tr>
<td>Calf 4</td>
<td>8.5</td>
<td>2.8</td>
<td>5.7</td>
</tr>
<tr>
<td>1L prototype</td>
<td>7.2</td>
<td>1.5</td>
<td>5.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as Log<sub>10</sub> TCID<sub>50</sub> per ml.
Table 23. Test of the identity of agents reisolated from experimental calves following inoculation with the 4S prototype virus

<table>
<thead>
<tr>
<th>Virus source</th>
<th>Virus titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Neutralization index</th>
<th>Virus identity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Virus plus 4S antiserum</td>
<td></td>
</tr>
<tr>
<td>Calf 2</td>
<td>7.3</td>
<td>3.8</td>
<td>3.5</td>
</tr>
<tr>
<td>Calf 3</td>
<td>6.2</td>
<td>1.5</td>
<td>4.7</td>
</tr>
<tr>
<td>Calf 4</td>
<td>7.0</td>
<td>3.2</td>
<td>3.8</td>
</tr>
<tr>
<td>4S prototype</td>
<td>7.2</td>
<td>2.5</td>
<td>4.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as Log<sub>10</sub> TCID<sub>50</sub> per ml.
Table 24. Neutralization of the IL virus by preinoculation and postinoculation serum samples from IL infected calves

<table>
<thead>
<tr>
<th>Serum source</th>
<th>IL&lt;sup&gt;a&lt;/sup&gt; virus titer&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Neutralization index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Virus plus preinoculation serum</td>
<td>Virus plus postinoculation serum</td>
</tr>
<tr>
<td>Calf 1</td>
<td>5.8</td>
<td>5.8</td>
</tr>
<tr>
<td>Calf 2</td>
<td>6.2</td>
<td>7.2</td>
</tr>
<tr>
<td>Calf 3</td>
<td>5.2</td>
<td>5.5</td>
</tr>
<tr>
<td>Calf 4</td>
<td>5.5</td>
<td>5.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Control titer of IL virus 7.5.
<sup>b</sup>Expressed as Log<sub>10</sub> TCID<sub>50</sub> per ml.

Table 25. Neutralization of the 4S virus by preinoculation and postinoculation serum samples from 4S infected calves

<table>
<thead>
<tr>
<th>Serum source</th>
<th>4S&lt;sup&gt;a&lt;/sup&gt; virus titer&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Neutralization index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Virus plus preinoculation serum</td>
<td>Virus plus postinoculation serum</td>
</tr>
<tr>
<td>Calf 2</td>
<td>6.5</td>
<td>3.8</td>
</tr>
<tr>
<td>Calf 3</td>
<td>7.0</td>
<td>5.2</td>
</tr>
<tr>
<td>Calf 4</td>
<td>4.5</td>
<td>5.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Control titer of 4S virus 6.8.
<sup>b</sup>Expressed as Log<sub>10</sub> TCID<sub>50</sub> per ml.
Table 26. Virus neutralizing activity of the colostrum fed to experimental calves

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Colostrum fed to</th>
<th>Virus titera</th>
<th>Neutralization index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Virus plus colostrum</td>
</tr>
<tr>
<td>1L</td>
<td>Calf 2</td>
<td>7.2</td>
<td>5.5</td>
</tr>
<tr>
<td>1L</td>
<td>Calf 3</td>
<td>7.2</td>
<td>2.8</td>
</tr>
<tr>
<td>1L</td>
<td>Calf 4</td>
<td>7.2</td>
<td>3.5</td>
</tr>
<tr>
<td>4S</td>
<td>Calf 2</td>
<td>6.5</td>
<td>6.8</td>
</tr>
<tr>
<td>4S</td>
<td>Calf 3</td>
<td>6.5</td>
<td>5.2</td>
</tr>
<tr>
<td>4S</td>
<td>Calf 4</td>
<td>6.5</td>
<td>3.2</td>
</tr>
</tbody>
</table>

aExpressed as Log_{10} TCID_{50} per ml.
substances in the preinoculation serum samples, colostrum fed to the last three experimental calves was tested to determine its ability to neutralize the prototype viruses. The results of these tests are presented in Table 26. It can be seen that the colostrum contained substances capable of neutralizing the prototype viruses. It also is obvious that this neutralizing activity varied not only among colostrum samples but also within a single sample when tested against the two prototype strains.
DISCUSSION

A general consideration of the properties of the viruses isolated leaves little doubt that they are enteroviruses. Their isolation from the intestinal tract, typical cytopathic effect in bovine kidney cell cultures, small particle size and resistance to treatment with ethyl ether or chloroform all support this classification. The possibility that these agents might be reoviruses has been considered but their particle size, lack of hemagglutinating activity and failure to produce intracytoplasmic inclusion bodies clearly exclude them from this group.

Electron micrographs of these agents indicate that they have a particle size of approximately 25 μ, there being no differences observed between the LL and 4S strains in this respect. These findings are in agreement with the results of the filtration studies. There are also indications that the virus particles are not spherical but may be polygonal in shape suggesting the presence of some type of cubic symmetry. La Placa and Portolani (30) have made similar observations regarding the shape of bovine enteroviruses and also report particle sizes in the range of 23 μ to 25 μ which closely approximate results reported above. Caspar and Klug (15) have studied the structure of an ECHO virus in more detail and report that it possessed icosahedral symmetry.

Observations of infected cells stained with acridine
orange revealed a gradual accumulation of RNA in the cytoplasm. It was further observed that this material was subsequently rather rapidly released. This suggests that the virus multiplies and accumulates within the cytoplasm and is later released, perhaps at the time of cell death. The accumulation of RNA with no evident change in quantities of DNA within the cell would also indicate that these are RNA-containing viruses. Studies of this type using human enteroviruses have yielded similar results.

Magnesium chloride stabilization to heat inactivation has been proposed by Wallis and Melnick (55) as an additional characteristic of the enterovirus group. In these studies the 4S virus was definitely stabilized in the presence of 1 molar magnesium chloride but the 1L prototype showed marked stability to heat even in the absence of magnesium chloride (Figures 18 and 19). The significance of this finding is difficult to assess because so few bovine viruses have been tested that no general statement regarding their uniformity in this respect can be made. It may also be that subsequent studies will reveal that heat stable strains do not conform in this test. No attempt was made to test the virus at higher temperatures because it has been shown that as the temperature is raised above 50 C the stabilizing effect of magnesium chloride decreases. The studies of Pollard (49) and Woese (57) indicating differential thermal inactivation
of viruses would also make one hesitant to compare results of tests conducted at higher temperatures with those obtained in the standard test.

The serological classification of bovine enteroviruses is still very incomplete because there have been no extensive studies carried out. In addition to this the lack of standardization of serological tests has made it difficult to compare available results. The frequent occurrence of neutralizing substances in normal sera also poses problems in the serological comparison of agents of this type. These situations point out the need, not only for wide-spread serological studies of these agents, but also for a standardization of techniques and exchange of prototype strains so that a classification can be developed.

Attempts to produce immune sera against the prototype viruses used in this study served to illustrate some of the problems caused by the presence of neutralizing substances in preinoculation serum samples. As shown in Table 5, these substances were found to be present in the sera of rabbits and guinea pigs to a sufficiently high titer that if adequate controls were not used they could be mistaken for specific antibodies. A comparison of the results presented in Tables 4 and 5 also gives some indication that the presence of these neutralizing substances may prevent the development of specific antibodies during hyperimmunization. Neutralizing
substances were not found in preinoculation chicken serum and as this species responded well to hyperimmunization it appears to be a logical choice for use in the production of typing sera. These observations are in agreement with those made previously by Evans (20) after studying the occurrence of neutralizing substances in the sera of a number of species. It might also be noted at this time that the difference in serological response of rabbits and guinea pigs to hyperimmunization with the 1L and 4S viruses indicates a further difference between these prototype strains.

Subsequent serological studies of the eleven isolates allowed a separation into two groups consisting of five agents, each represented by a prototype virus, while the remaining isolate was placed into a third group. It should be pointed out, however, that there was not a complete antigenic separation of the two prototype strains. The 1L serum showed a high neutralizing activity when tested against the homologous virus but also showed some activity when tested against the 4S prototype strain. The 4S antiserum, on the other hand, was active against the 4S virus but showed no activity against the 1L virus. This would indicate a one way antigenic relationship between the two strains. It is rather difficult at the present time to decide whether the two prototype strains should be considered as separate antigenic strains or not. The final decision in this matter will have to await a more
complete study of bovine enteroviruses and the development of a standard quantitative test with agreement upon the significant limits for determining new antigenic types. The latest and most complete scheme of classification, proposed by Moscovici and La Placa (45), provides little help in solving this problem because antiserum dilutions used would not have revealed the one way antigenic relationship observed in this study. It might be pointed out, however, that a similar situation exists among the human enteroviruses in that one way antigenic relationships exist between ECHO virus type 12 and ECHO virus type 1 and also between ECHO virus type 12 and ECHO virus type 8. Due to the high titer of ECHO 12 antiserum against the homologous virus and low titers against the ECHO virus types 1 and 8 the three viruses were nonetheless designated as separate types. In this study it can be seen that there is about a 4.0 log higher activity of the IL antiserum against the homologous virus than against the 4S virus. This difference plus some obvious dissimilarities in other biological properties indicates that the two prototype strains should at least be tentatively assigned to different antigenic types.

If this classification is used it becomes apparent that no antigenic differences could be found between the large and small plaque types separated from the original isolates and consequently that no animals were found to be excreting more
than one antigenic type of virus at the time the rectal swabs were collected. It is also obvious that the isolations from animals in the Westrum and Dairy Nutrition herd included both antigenic types of the virus. The single isolate obtained from the Veterinary Obstetrics herd was the 6L agent which could not be classified with either of the above groups.

The antigenic relationships of the 1L and 4S viruses to the LCR4 strain were also investigated. Using the antiserum prepared against the prototype viruses a close relationship between the LCR4 and 1L virus could be determined but no indication of a relationship between the LCR4 and 4S virus was found. When LCR4 antiserum was used all three of the virus strains were neutralized to about the same extent but neutralization indexes were quite low. Unfortunately no preinoculation serum samples were available so the presence of nonspecific neutralizing antibodies could not be eliminated.

The inoculation of experimental calves and the subsequent reisolation of the same virus following each inoculation leaves little doubt concerning their ability to invade and multiply within the intestinal tract. Rectal swabs collected prior to inoculation ruled out the possibility of pre-existing viral infections and serological studies of the reisolated viruses, with one exception, confirmed their

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1 Supplied by Dr. C. M. Kunin, University of Virginia Medical School, Charlottesville, Virginia.
identity with the inoculated strain. In the one exception the reisolated virus was neutralized to some extent by anti-serum to the prototype virus but the neutralization index was much lower than those found for the other reisolated viruses. The possibility of the presence of unrelated viruses in the calf must be considered but seems unlikely. Although each calf was inoculated with both prototype strains during the course of these studies the virus isolation in question occurred after the initial inoculation, thus eliminating the possibility of the activation of a latent infection resulting from a previous inoculation. The introduction of a virus from outside sources is also unlikely because in all cases the isolation procedures were sufficient to prevent this. McFerran (35) has reported antigenic variations among his isolates and it may be that this has also occurred in this case. During the course of the calf inoculation studies it was also found that the presence of neutralizing substances in the blood did not prevent infection nor did the inoculation of one virus interfere with subsequent infection with the other prototype strain.

The development of significant antibody titers following inoculation can be more closely correlated with the presence of preinoculation neutralizing substances than with the production of clinical disease. Only those animals whose pre-inoculation serum was virtually devoid of neutralizing activity
showed an increase in antibody titer after inoculation. When considering the failure of Calf 1, which did not receive colostral antibody, to develop an antibody titer following inoculation it should be kept in mind that this calf was killed eight days after inoculation so antibody titers may not have had a chance to develop.

A possible source of the neutralizing substances in the preinoculation serum would be the colostrum which the calves were fed. This is supported by some correlation between the antibody levels of colostrum and those subsequently found in the calf serum. Serum from the colostrum-deprived calf showed, however, that nonspecific neutralizing substances were also present. Oliver (16) has studied colostral antibodies against bovine enteroviruses and has shown that the ingestion of these antibodies is followed by the appearance of antibodies in the serum. He has also demonstrated the presence of neutralizing substances in serum collected from newborn calves prior to the ingestion of colostrum.

The occurrence of diarrhea in two of the four experimental calves following virus inoculation indicates the pathogenic potentialities of these agents. There is little difficulty in implicating the virus as the cause of this reaction when one considers the time at which the diarrhea occurred and the concurrent virus isolations. These results indicate that bovine enteroviruses can cause clinical disease but
whether they actually do under normal rearing conditions remains unknown. The short clinical course of the disease and the failure to cause death of the test animals would indicate that if these agents are involved in outbreaks of calf diarrhea under field conditions it is probably as an initial invader which allows secondary infections to develop. The reasons for failure of the experimental animals to develop these secondary infections are obvious when one considers the excellent sanitation and the strict conditions of isolation in which the animals were reared.

The results following inoculation of pregnant guinea pigs are somewhat unique, not that abortion occurred, but that the agent was reisolated at the time of abortion. Unsuccessful attempts by other workers to reisolate the virus may have been due to their failure to examine the uterus of the female. This reisolation of the virus helps to confirm the connection between virus inoculation and subsequent abortion and also indicates that the multiplication of the virus and subsequent damage resulting in abortion may occur within the uterus instead of the fetus.

These findings, along with the report by Moll and Finlayson (43) associating bovine enteric viruses with abortions in cattle make it obvious that the role of these viruses in abortions should be investigated more thoroughly. Recent reports by Bögel, Straub and Dinter (8) and Straub and
Böhm (54) that viruses isolated from the urogenital tract of cattle are very similar to bovine enteroviruses support this statement. These indications that enteroviruses may play a role in reproductive as well as enteric disease emphasizes their potential importance as pathogens.
SUMMARY

Seven viral isolates were obtained from 45 rectal swabs collected from cattle in three herds. These isolates were obtained only from the younger animals sampled. Studies of the physical and biological properties of these agents clearly established their identity as enteroviruses.

There were no differences in cytopathic changes caused by the seven isolates but they could be divided into eleven substrains on the basis of plaque size. These eleven substrains were subsequently placed into three groups on the basis of their serological relationships. Groups 1 and 2 each contained five agents while the third group consisted of a single virus which could not be satisfactorily included within either of the other groups. On the basis of this serological classification it became apparent that strains isolated from one individual were of the same serological type in spite of their differences in plaque size and thus no animals were found to be excreting virus of more than one antigenic type. Members of both groups 1 and 2 were isolated from animals in two herds while the single strain placed into group 3 was the only isolate from a third herd. The group 1 prototype strain was found to be closely related to the LCR4 virus, a strain which has been found to be antigenically related to many other bovine enteroviruses from various parts of the world. The group 2 prototype was apparently unrelated to
this agent.

The inoculation of the two prototype viruses into experimental calves resulted in the establishment of infection in each case. The presence of antibodies in the calves serum prior to inoculation did not protect against infection nor did the inoculation of one prototype virus prevent infection from a subsequent exposure to the other strain. Two calves developed diarrhea after inoculation indicating some pathogenicity on the part of these agents.

The occurrence of abortion following the inoculation of pregnant guinea pigs indicates a possible involvement of these agents in reproductive diseases.


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