CHARACTERIZATION OF A BOVINE VIRAL DIARRHEA AGENT

by

Donald Eugene Gutekunst

A Thesis Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
MASTER OF SCIENCE

Major Subject: Veterinary Bacteriology

Iowa State University
Of Science and Technology
Ames, Iowa

1964

TABLE OF CONTENTS

INTRODUCTION

In recent years, considerable attention has been directed to a newly recognized group of disease syndromes occurring in cattle which have somewhat similar clinical and pathological manifestations. The first syndrome observed was given the name "virus diarrhea." Later, a more severe clinical manifestation of this syndrome was designated "mucosal disease." Today, the syndrome is usually referred to as the bovine viral diarrhea-mucosal disease complex. Even though the etiological agents involved in this complex are serologically related, it appears that the terms viral diarrhea and mucosal disease can best be used in describing the clinical manifestation of the disease complex as it occurs in the field.

The disease syndrome probably has been present for a number of years and has not been recognized or undoubtedly mis-diagnosed in the field. The significance of this disease complex is not fully known, but undoubtedly it plays a major role in economic loss to the cattle industry. At the present time, the disease is known to have world-wide distribution.

At the National Animal Disease Laboratory in 1962, a clinical case of mucosal disease occurred in the experimental herd. A cytopathogenic virus was isolated from various tissues collected at necropsy, which was designated the NADL-MD strain of bovine viral diarrhea-mucosal disease virus.

The objectives of this study are to characterize the NADL-MD virus and to partially elucidate the disease syndrome in the involved herd by virological and serological procedures.

REVIEW OF LITERATURE

In 1946, Olafson et al. (45) described the occurrence of a highly contagious disease of cattle in New York characterized by high morbidity, fever, nasal discharge, erosions of the alimentary canal and diarrhea. They could experimentally transmit the disease to calves, and noted that certain clinical signs of illness resembled those caused by rinderpest. They termed this disease syndrome "bovine virus diarrhea" (BVD) of cattle (46).

Later, Walker and Olafson (63) in 1947 proved conclusively by both cross-immunity and neutralization tests that there was no antigenic relationship between rinderpest virus and the causative agent of the new disease syndrome.

Ramsey and Chivers (50) in 1953 and Ramsey (49) in 1954 reported the occurrence in Iowa and surrounding states of a newly recognized syndrome in cattle. They named the syndrome :mucosal disease" (MD) of cattle because it mainly affected the mucosal surfaces. Their description of the disease differs from that reported for BVD by more pronounced lesions in the alimentary tract, greater differences in morbidity and mortality rates, and the epizootiology of the syndrome. However, they were not able to reproduce the typical disease.

In 1954, Baker et al. (3) isolated two strains of virus from field cases resembling those described by Olafson et al. (45). They designated these isolates New York 1 (NY 1)

and Maine 2 (M 2). When calves were inoculated intravenously or intranasally with infective blood or splenic emulsion, a diphasic temperature response accompanied by leukopenia and general malaise occurred. Diarrhea occured in about half of the inoculated calves, but oral lesions were observed only in an occasional animal. By cross-protection tests in calves, they found both viruses to be antigenically related and concluded that these isolates were similar to Olafson's isolate even though the original material was not available for comparison. They were unable to detect neutralizing antibodies by injecting calves with mixtures of splenic emulsion and serum from inoculated calves. Likewise, they found no fixation of complement when using the infective splenic preparations as antigen in the complement-fixation test. The infective agents would not grow in embryonating eggs or guinea pigs, but could be transferred in rabbits. After 75 serial passages in rabbits, the NY 1 virus became modified and only produced a slight decrease in leukocytes and mild temperature elevation when inoculated into calves. The modified virus immunized the calves against fully virulent virus challenge.

In 1956, Pritchard et al. (48) described the widespread occurrence in Indiana of a syndrome which closely resembled BVD as reported by Olafson (45). They isolated a virus from a field case and were able to reproduce the syndrome experimentally in calves. They named the agent Indiana 143 and

found immunological differences when compared to the reference NY 1 virus by quantitative cross-protection tests in calves.

Lee and Gillespie (41) in 1957 were able to grow and maintain the NY 1 strain isolated by Baker et al. (3) for 20 consecutive passages in bovine embryonic skin-muscle tissues prepared in roller tubes by the plasma-clot method. The virus was maintained for an additional 15 transfers in bovine embryonic kidney cell cultures. The concentration of virus in fluids from these tissue-cultured cells were virulent for calves at dilutions of 10^{-5} to 10^{-7}. However, the NY 1 strain did not produce any evidence of cytopathic effect (CPE) in the tissue culture systems.

Underdahl et al. (62) in 1959 were the first to isolate and propagate cytopathogenic viruses from field cases of MD. They isolated and cultivated in bovine embryonic kidney cells two cytopathogenic viruses from separate herds which they designated M-833 and ISC-1. Cytopathic changes in the tissue cultures were observed 7 to 10 days following inoculation, with small vacuoles forming within the cells and increasing in size until the cell layer was completely destroyed by 10 to 14 days. The viruses were inactivated by temperatures above 50 C for 15 minutes and would pass an 03 Selas filter. Both viruses stimulated formation of neutralizing antibodies in sheep, but they failed to determine if their viruses would produce the experimental disease in calves. Subsequent studies by

Gillespie et al. (24) in 1961 have shown that Underdahl's isolates are immunologically related to the reference NY 1 virus.

Noice and Schipper (44) in 1959 also isolated a cytopathogenic virus in bovine kidney cell cultures from field cases diagnosed as MD. Intravenous inoculation of two calves produced a mild syndrome characterized by a single temperature elevation 6 to 10 days postinoculation (PI). The calves developed neutralizing antibodies to the virus in approximately 15 to 25 days. This virus has been referred to as the North Dakota agent. As far as can be determined, the North Dakota virus is similar or identical to infectious bovine rhinotracheitis as reported by Tyler (60).

In 1959 Gillespie and Baker (22) demonstrated by reciprocal immunity tests in calves that NY 1 virus and the Indiana 46 virus were immunologically related and produced similar syndromes in experimental calves. They propagated both strains in tissue cultures but observed no evidences of CPE.

The isolation of a cytopathogenic virus by Gillespie et al. (23) in 1960 was one of the first major advances in the study of this disease syndrome. The virus was isolated from splenic tissue from a calf which had succumbed to a syndrome similar to BVD. The agent was designated Oregon C24V. After several passages in tissue cultures, CPE appeared in 2

or 3 days but was much more pronounced in 6 to 8 days. Affected cells became rounded and gradually detached from the glass surface, with complete detachment by the 12th to 15th day. Stained cells showed homogeneity of the cytoplasmic vacuoles of varying size were commonly seen in infected cultures. Reciprocal cross-immunity tests in calves showed that the agent was immunologically related to NY 1 strain. Serum neutralization tests using tissue-cultured Oregon C24V virus indicated not only a relationship with NY 1 but also with the Indiana 46 strain of virus.

Since this time numerous cytopathogenic and non-cytopathogenic BVD-MD viruses have been isolated and there is serological evidence of the disease throughout the world.

Robson et al. (53) in 1960 studied the correlation between a neutralization test and immunity to BVD in 59 experimentally infected calves. All calves were serologically negative at the time of the first inoculation, and all developed "typical signs of BVD." The calves were serologically positive 2 to 4 weeks following the first inoculation. Following challenge with virulent virus, none of the calves showed signs of illness. They concluded that the neutralization test for immunity was at least 95% accurate in the determination of the immune status of the animal. Even though they did not indicate, it is assumed the neutralization tests utilized the Oregon C24V virus.

In 1964 Coggins (9) studied the neutralization test for BVD using Oregon C24V tissue-cultured virus. He found that from 30 to 300 $TCID_{50}$ units of virus would give consistent results and yet permit maximum sensitivity. In the neutralization test, an increase or decrease of 1 log unit of virus would result in an inverse change of 0.44 log unit in the serum titer. He concluded that the neutralization test was accurate within a 3-fold dilution if procedures were followed which controlled variables.

Kniazeff and Pritchard (39) in 1960, used the plaque assay procedure to conduct neutralization tests on various BVD-MD antiserums against Oregon C24V virus. Antiserums used in this study were obtained from experimental animals exposed to known viral isolates. Fourteen antiserums against NY 1 strain of BVD were obtained from Florida, Indiana and New York, and an additional antiserum from an unidentified isolate was obtained from Nebraska. A total of 45 calf antiserums to MD agents were obtained from Indiana, Iowa, North Dakota and England. All of the preinoculation serums were found to have no detectable neutralizing antibodies when tested against Oregon C24V virus. They found an antigenic relationship between Oregon C24V virus and all of the anti-BVD and anti-MD serums tested. They concluded that the BVD-MD agent are members of an antigenically related group. The Oregon C24V

virus was also utilized in the neutralization test against serums obtained from cases of bluetongue, hog cholera, infectious bovine rhinotracheitis, bovine infectious ulcerative stomatitis, winter dysentery, malignant catarrhal fever, sporadic bovine encephalitis and bovine mycotic stomatitis. Even though these diseases have some similarity in their pathogenesis to the BVD-MD syndrome, no antigenic relationship was found by neutralization.

Kniazeff et al. (38), in 1961, reported that Oregon C24V virus was also neutralized by antiserums against BVD-MD viruses from Scotland and West Germany. They therefore concluded that BVD-MD agents occurring in various parts of the world are antigenically related.

In 1961, Gillespie et al. (24) compared different strains of BVD-MD viruses by neutralization tests using antiserum against NY 1 virus. The viruses tested were Oregon C24V, a Nebraska BVD strain, a Nebraska MD strain, and three strains of BVD isolated in New York and designated as C55F, C60F and C80K. All of the viruses showed an antigenic relationship when compared by the neutralization test. They suggested that serological tests should be used before new and different disease entities are postulated on the basis of clinical and pathological findings.

Darbyshire (14), in 1962, investigated the agar double diffusion technique as a method of diagnosis for MD. A

total of 1323 tissues of various types were tested against immune MD serum from 223 clinically affected cattle. Of these, tissues from 85 of the animals reacted with the immune serum and 14 additional animals gave a doubtful reaction. The tissues from 20 healthy cattle did not show any lines of precipitation. He concluded that the agar double diffusion technique would be useful in the diagnosis of MD.

Gutekunst and Malmquist (30), in 1963, separated a soluble antigen from infective virus particles by ultra-centrifugation of infective tissue culture fluids. They showed that by concentrating the soluble antigen, a single line of precipitation would occur when tested in the agar double diffusion plates against immune serums. The infective virus "pellet," however, failed to show a precipitin line.

In 1964, Gutekunst and Malmquist (29) reported a complement-fixation test for BVD-MD using soluble antigen prepared from infective tissue culture fluids. They were able to detect complement-fixing antibodies in experimentally infected animals, and demonstrated that the soluble antigen alone was antigenic in calves.

Gratzek (28), in 1962, reported on a virus interference test which he used to detect noncytopathogenic BVD viruses in tissue cultures. In this system, tissue culture cells

infected with either NY 1 or Indiana 46 viruses were temporarily resistant to superinfection with vesicular stomatitis virus.

In 1962, Gillespie et al. (25) reported a plaque inhibition assay method for detecting noncytopathogenic BVD viruses in tissue cultures. They found that cell cultures became resistant to 50 plaque-forming units of cytopathogenic C60F virus if noncytopathogenic strains of BVD viruses had been inoculated at least 3 days earlier. This cellular resistance was neutralized by BVD antibodies. They suggested that this cellular resistance was explained by the principle of interference.

Gutekunst and Malmquist (29) using a modification of the interference test described by Gillespie et al. (25) found that tissue cultures infected with noncytopathogenic strains of BVD viruses would show almost 100% interference when the cultures were challenged with 10^3 $TCID_{50}$ of cytopathogenic NADL-MD virus. They were able to do neutralization tests with the noncytopathogenic viruses by the use of this interference principle.

In 1963 Inaba et al. (36) described the use of the END method to detect noncytopathogenic strains of BVD. They were able to titrate noncytopathogenic viruses in bovine testicular cells by the "exaltation" of CPE by newcastle disease virus as

compared to its effect in non-infected cultures. They also described a neutralization test using this technique.

Fernelius (21) in 1964 was able to detect and titrate noncytopathogenic BVD viruses by the use of immunofluorescence. He found that fluorescein-conjugated serum globulins against either a cytopathogenic or noncytopathogenic BVD-MD virus would stain infected cells of various noncytopathogenic BVD-MD viruses. Titration of noncytopathogenic viruses by immunofluorescence correlated with the results obtained by the interference technique described by Gutekunst and Malmquist (29).

In 1962 Malmquist* observed that animals which succumb to MD failed to develop either neutralizing or complement-fixing antibodies. He demonstrated a viremia in clinical cases for a period of 4 months prior to the animals' death. In each case, no antibodies against the virus were found, whereas other "healthy" animals in the herd developed significant levels of antibodies. He suggested that the inability of the clinical cases of MD to develop antibodies resembles the "immune tolerance or paralysis" phenomenon.

Dinter et al. (18) in 1962 and Borgen (5) in 1963 reporting on virological and serological studies of herds

---

*Malmquist, W. A., National Animal Disease Laboratory, Ames, Iowa. Observations on immune tolerance associated with the bovine viral diarrhea-mucosal disease syndrome. Private communication. 1962.

with evidence of MD, found no antibodies present in animals from which they isolated MD viruses. Other animals in the herd had no clinical signs of the disease but had high levels of neutralizing antibodies. Thomson and Savan (59) in 1963 also observed in several cases that animals which became diseased and died were serologically negative. They suggested that the animals may have been incapable of an antibody response. They observed clinical signs in one case for over 2 months and the animal was still serologically negative immediately prior to death, at which time it still yielded virulent virus.

The first evidence of a possible relationship between the causative agents of BVD-MD and hog cholera (HC) was reported by Darbyshire (15, 16) in 1960 and 1962. Using tissues from infected animals as antigens, he observed a line of identity in agar double diffusion plates when the antigens were tested against either HC or BVD-MD immune serums. In absorption experiments, he found that the HC antigen removed the antibodies from anti-hog cholera serum as well as the BVD antibodies in the bovine serum. Likewise, the MD antigen removed the precipitating antibodies from both types of antiserums. Normal tissues did not remove the precipitating activity of either antiserum and furthermore, the antigens were not affected by normal serums. Neither antiserums nor tissue antigens from infected animals

with canine distemper, canine hepatitis, myxomatosis, rabbit fibroma, swine pox, vaccinia, rinderpest, teschen and foot-and-mouth diseases showed any serological relationship with BVD-MD or HC in the agar double diffusion plates.

Bechenhauer et al. (4) in 1961 reported that pigs inoculated with Oregon C24V BVD virus were resistant to challenge with virulent HC virus 14, 24 and 37 days later. They found that two other isolates, Nebraska BVD and Nebraska MD, did not protect against subsequent challenge with virulent HC virus. Serial passage of Oregon C24V virus every 7 days in pigs did not cause it to increase in virulence, but the pigs were susceptible to HC after 2 to 3 passages. They "hyperimmunized" 2 pigs with Oregon C24V virus and found that large doses of the antiserum would protect pigs against challenge with virulent HC virus. They concluded that the immunity produced in pigs by the BVD virus was related to antibody formation rather than some type of blocking mechanism.

In 1962 Sheffy et al. (56) found that SPF pigs inoculated with NY 1 or Oregon C24V BVD viruses would withstand challenge with virulent HC strain A virus. No signs of illness were noted following inoculation of BVD viruses and the pigs developed a low level of neutralizing antibodies against BVD virus, but no neutralizing antibodies against HC virus. Seven days after challenge with HC virus, high levels of

neutralizing antibodies were present against both BVD and HC viruses. After challenge with HC virus, the control pigs developed signs of illness and died in 8 to 15 days, whereas the pigs initially given BVD viruses showed a lesser elevation of temperature for 1 to 2 days and returned to normal. Likewise, a similar pattern was observed when calves were inoculated with HC virus and subsequently challenged with BVD virus. Serums which contained BVD antibodies did not neutralize HC virus and, in reciprocal tests, serums that contained HC antibodies would not neutralize BVD viruses. They concluded that since BVD and HC viruses fail to cross-neutralize, the mechanism of protection appears to be related to an accelerated secondary response induced by prior exposure to a heterotypic virus.

Kumagai et al. (40) in 1962 investigated the antigenic relationship between BVD and HC viruses by cross-neutralization tests. They found the two viruses were antigenically different when convalescent serums were used in the neutralization tests. However, they detected a slight cross-neutralization when hyperimmune serums were used in the test. They concluded that the two viruses share common "minor" antigens and the secondary response reported by Sheffy et al. (56) might be partly, if not entirely, due to the booster effect of these minor antigens common to both viruses.

In 1963 Gutekunst and Malmquist (30) separated a soluble antigen and infective virus particles from tissue culture fluids of NADL-MD and C24V viruses. They found that the soluble antigen of the BVD viruses would form a single line of identity in the agar double diffusion plates when reacted with anti-hog cholera and anti-BVD serums. However, infective virus particles failed to form precipitin lines with either type of antiserum. No neutralizing antibodies were found in the anti-HC serums when tested against the BVD viruses except in a commercially prepared serum. They suggested the relationship between BVD and HC viruses was due to the sharing of a common soluble antigen.

Gutekunst and Malmquist (29) in 1964 demonstrated that the soluble antigen of BVD viruses could be used to assay complement-fixing antibody levels in anti-HC serums. Normal swine serums failed to fix complement when tested with the BVD soluble antigen.

Mengeling et al. (43) in 1963 demonstrated the antigenic relationship between BVD-MD and HC viruses by immunofluorescence. By using fluorescein-conjugated serum globulins against either BVD or HC viruses, they were able to stain infected tissue cultures of either BVD or HC viruses. They found the staining to be specific and no fluorescence was observed when normal bovine or swine serum was used.

In 1963 Dinter (17) reported HC and BVD-MD viruses to share similar physical and chemical properties. They were both approximately 50 mμ in size, ribonucleic acid (RNA) type, sensitive to ether and chloroform, moderately sensitive to trypsin and were not stabilized in cationic solutions. In reciprocal neutralization tests he found some cross-neutralization if the antiserums were "very high" in titer, however, some would show no neutralization toward the heterologous virus.

Coggins and Seo (10) in 1963 using rabbit antiserums against HC and BVD viruses, found no cross-neutralization. Inoculation of either virus followed by challenge with the other produced a secondary response to both. They suggested this to be additional proof of "heterotypic" relationship.

In 1962 Gratzek (28) reported the adsorption rate of Oregon C24V virus in calf testicular cells to proceed rapidly and is essentially complete in 10 minutes. He noted a tendency towards accelerated adsorption at 37 C over 26 C but suggested these differences were within the confidence limits of the plaque assay method. A multicyclic growth curve of this virus in embryonic bovine kidney cells revealed maximal titers to occur from 3 to 7 days PI. Using calf testicular cells, he could detect cell-associated virus as early as 8 to 10 hours PI and found infective virus in the extracellular fluid approximately 4 hours later. He

suggested that the testicular cells were more susceptible to this virus than were the kidney cells.

Gillespie et al. (26) in 1963 obtained maximum viral infectivity of C24V or C60F virus in extracellular fluids 72 to 96 hours PI, with a marked decline in the titer following subsequent incubation. They found the virus in the fluid phase to exceed that of the cell-associated phase by at least one log during the growth of these viruses. In contrast, Coggins (9) reported the growth and release of C24V virus in tissue culture to be very gradual and reaching a maximum titer only after 6 days' incubation.

In 1949 Andrewes and Horstmann (2) were the first to observe that different viruses varied in their sensitivity to ethyl ether. Since this time, sensitivity to ether has been one of the major criteria used in the classification of viruses. Several workers have studied the susceptibility of BVD-MD viruses to ether. Hermodsson and Dinter (32) in 1962 and Gillespie et al. (26) in 1963 reported C24V virus to be sensitive to 20% ether when incubated at 4 C for 18 hours. However, Taylor et al. (58) in 1963 reported C24V virus to be stable in the presence of ether.

In 1961 Feldman and Wang (20) found that viruses which were sensitive to ether were also sensitive to the action of 5% chloroform when incubated at room temperature for 10 minutes. They suggest that chloroform is more efficient

than ether because of its greater polarity as a lipid solvent, and offers the advantage of being readily separable by sedimentation. Hermodsson and Dinter (32) have reported that C24V BVD virus is inactivated by chloroform.

The type of nucleic acid of viruses has been investigated by the use of inhibitors of deoxyribonucleic acid (DNA) synthesis. In 1960 Salzman (54) found the growth of vaccinia in tissue culture to be inhibited by the addition of 5-fluorodeoxyuridine (FUDR), whereas poliovirus was resistant. Herrmann (33) in 1961 reported that the addition of 5-iododeoxyuridine (IUDR) or 5-bromo-deoxyuridine (BUDR) would inhibit the plaque formation of vaccinia and herpes simplex viruses, but had no effect on the RNA-containing viruses, West Nile and newcastle disease. Hermodsson and Dinter (32) and Ditchfield and Doane (19) found C24V BVD virus to multiply in tissue culture in the presence of IUDR or BUDR. They concluded that the BVD-MD viruses were RNA in type.

Since the development of the collodion membrane filters by Elford in 1929, many workers have used various types of filters to estimate the size of virus particles. Hermodsson and Dinter (32) using millipore filters found C24V virus to readily pass the 100 mμ filter and reduced amounts of infective virus passed the 50 mμ filter. Taylor et al. (58) reported C24V virus would pass millipore filters or graded asbestos filter pads having a porosity of 100 mμ. However,

they were unable to detect any infective virus in the filtrate when the 50 mμ filters were used.

Hermodsson and Dinter (32) using the electron microscope observed spherical particles with a diameter of approximately 40 mμ from ultra-thin sections of concentrated C24V virus pellet. However, they thought the concentration of infective virus in the pellet was too low to permit an identification of the spherical particles as BVD viruses. Ditchfield and Doane (19) observed particles in electron micrographs of ultra-thin sections of tissue culture cells infected with C24V or MAC-A BVD viruses. The particles were approximately 150 to 250 mμ in size with periodic projections of 10 mμ. The internal component was in a form of a helix and had a diameter of 18 mμ. On this basis, they suggested that the BVD viruses belong to the myxovirus group.

In 1962 Wallis and Melnick (64) described a new property for enteroviruses. They found that high concentrations of cations would stabilize enteroviruses whereas they would enhance the thermal inactivation of certain viruses in the other major groups. They suggested that this characteristic is a key criterion in the differentiation of enteroviruses from others with similar properties. Dinter (17) in 1963 found that the thermal inactivation of C24V BVD was enhanced in molar solutions of Ca^{++} or Mg^{++} as compared to the control. Ditchfield and Doane (19)

reported C24V to be completely inactivated in both the molar Ca++ solution and the control when incubated for one hour at 50 C.

A number of investigators have reported the results of thermal inactivation tests of BVD viruses under a variety of temperatures, times of exposure and other conditions not stated. Gratzek (28) found the titer of infective C24V BVD virus would decrease approximately one log in 24 hours when held at either 37 C or 27 C. Thermal inactivation of the virus at 58 C resulted in a two-component curve which indicated the possibility of heat resistant variants. He found a small amount of infective virus remains following 1 hour's incubation. Taylor et al. (58) reported C24V virus to be completely inactivated by 96 hours when incubated at 37 C. They found the virus to survive at 56 C for only 20 to 35 minutes. Coggins (9) reported no appreciable loss in titer when C24V virus was held at 25 C for 24 hours.

In 1958 Cheng (8) found that trypsin, chymotrypsin or papain would inactivate both the hemagglutinin and infectivity of the group B arboviruses, whereas they had no appreciable effect on the group A arboviruses. He suggested this characteristic would aid in the differentiation of the group A and B arboviruses. Dinter (17) found C24V BVD virus to be moderately sensitive to the enzymatic action

of trypsin, and thought this virus to resemble the group B arboviruses in this characteristic.

In 1956 Peterson and Sober (47) and Sober et al. (57) first described the preparation of DEAE- and ECTEOLA-cellulose ion exchangers and their use in column chromatography of proteins. Hoyer et al. (35) were the first to use DEAE- and ECTEOLA- cellulose anion exchangers for column chromatography and purification of animal viruses. Since this time, numerous workers have used chromatographic separation techniques to purify viral preparations and in the separation of viral components.

In 1951 Brakke (7) described a new separation technique, density gradient centrifugation. Using different densities of sucrose layered in a centrifuge tube, he was able to purify and separate potato yellow-dwarf virus from other debris on the basis of their bouyancy in sucrose solutions during high speed centrifugation. Since that time, various modifications of this technique have been used to separate and purify viral components.

PART I. VIRUS CHARACTERIZATION

MATERIALS AND METHODS

Since a variety of tissue culture media and buffer solutions were utilized in this study, a description of each is included in the appendix.

Tissue Culture System

Primary bovine embryonic kidney (BEK) cell cultures were used throughout this study. Cell cultures were prepared by the general method of Youngner (65). Kidneys were collected aseptically from selected 6-9 months bovine fetuses and placed in a sterile container of Hanks' balanced salt solution (HBSS). The capsule was removed from the kidney, and the cortical region separated from the medullary region. The cortical tissue was minced until approximately 1 mm^3 pieces were obtained. The minced cortical tissue was washed 3 times with cold GKN solution, with the washing fluids being discarded. The tissues were placed in a sterile trypsinization flask* containing a sterile teflon-covered magnetic stirring bar. Sterile trypsin solution (0.25%) chilled to 4 C was added to the trypsinization flask and incubated at 4 C for 4 to 6 hours. After incubation the supernatant fluid which contains toxic debris was discarded and fresh cold trypsin was added. Trypsinization was

---

*Bellco Glass Company, Vineland, N. J.

continued for an additional 18 hours at 4 C. Following trypsinization, fibrous tissue was separated from the trypsinized cells by filtration through sterile cheesecloth. The cells were sedimented by centrifugation at 1,000 x g in a refrigerated centrifuge and the supernatant fluid was discarded. The cells were then washed 3 times with cold GKN and diluted in HBSS containing 10% specific pathogen-free (SPF) calf serum to give a final cell concentration of approximately 1 x 10^6 cells/ml. The BEK cells were propagated in sterile tissue culture tubes, 4-oz prescription bottles or Blake tissue culture bottles depending on the requirements for the experiments. The cells were planted in medium containing 89.5% HBSS, 0.5% lactalbumin hydrolysate, 10.0% SPF calf serum, and penicillin and streptomycin at a concentration of 100 units and 100 µg/ml, respectively. After 3 days' incubation at 37 C, the medium was changed to 89.5% Earles' balanced salt solution (EBSS), 0.5% lactalbumin hydrolysate, 10% SPF calf serum, and also containing antibiotics.

After complete monolayers had formed in approximately 5 days, the medium was removed and the cultures were washed 3 times with warm phosphate buffered saline solution (PBS). Depending on the experiment, the cultures were washed with warm PBS following inoculation and adsorption period and Eagles' basal medium (EBM) containing 2.0% SPF calf serum

was added for maintenance. The cultures were incubated in stationary racks at 37 C and observed twice daily for the duration of the experiment.

Viruses Used

The bovine viral diarrhea (BVD) virus used for characterization in this study was isolated from a clinical case occurring in a group of yearling Holstein heifers at the National Animal Disease Laboratory, and was designated NADL-MD strain of BVD virus. The virus was isolated from the following tissues: oesophageal mucosa, abomassal mucosa, Peyer's patch, mesenteric lymph node, spleen, bone marrow, rectal and nasal swabs. Primary isolations were made in BEK cell cultures. Virus neutralization tests and typical cytopathic effect (CPE) demonstrated that all isolates were identical.

The virus was purified by 3 terminal endpoint dilutions, and virus passaged 8 or 9 times in primary BEK cell cultures was used throughout the experiment unless otherwise stated.

Since most research on BVD viruses has been with Oregon C24V strain, isolated by Gillespie et al. (23), this virus was used in certain experiments as a control and to compare it with the test NADL-MD virus.

Assay Procedures

Infective virus

Cytopathogenic viruses were titrated in BEK cell tube cultures. The infective fluids were separated from cellular debris by centrifugation for 30 minutes at 2,000 x g in a refrigerated centrifuge. Serial 10-fold dilutions of the clarified tissue culture fluids were prepared in cold HBSS. Two-tenths ml of each dilution was inoculated into 5 BEK tube cultures. The cultures were incubated in a stationary rack at 37 C and observed twice daily for evidence of cytopathic effect (CPE).

Noncytopathogenic viruses were titrated using the principle of viral interference described by Gillespie et al. (25) and modified by Gutekunst and Malmquist (29). Serial 10-fold dilutions of noncytopathogenic viruses were prepared in cold HBSS, and 5 BEK cell tube cultures were inoculated with 0.2 ml of each dilution. The cultures were incubated in stationary racks at 37 C for at least 72 hours to allow time for the higher dilutions to completely infect the culture. Following 72 hours' incubation, the medium was aspirated and the cultures were washed twice with warm PBS. One ml of EBM containing approximately 10^3 $TCID_{50}$ of cytopathogenic NADL-MD virus was then added to each culture. The cultures were incubated at 37 C and observed twice daily for evidence of CPE. If the noncytopathogenic virus

had propagated in the cultures there was almost 100% interference with the challenge cytopathogenic virus. Likewise, typical CPE of the challenge virus could be observed if the noncytopathogenic virus was not present in the culture. Fifty percent infective virus endpoints were calculated by the method of Reed and Muench (51).

Serum neutralization

The beta method of constant virus-varying serum dilutions was the procedure of choice for neutralization tests in this study. Test serums were heat inactivated in a 56 C water bath for 30 minutes. Serial 2-fold dilutions of each serum were prepared in HBSS, then an equivalent amount of HBSS containing approximately 100 $TCID_{50}$ per 0.1 ml of cytopathogenic virus was added to the serum dilutions. The mixtures were incubated at 37 C for 30 minutes, and 0.2 ml of each dilution was inoculated into each of 5 BEK cell tube cultures. The cultures were incubated at 37 C and observed twice daily for evidence of CPE.

For neutralization tests involving noncytopathogenic viruses, the principle of interference as described previously was employed. Serial 2-fold dilutions of test serums were mixed with equal amounts of HBSS containing approximately 100 $TCID_{50}$ per 0.1 ml of noncytopathogenic virus. The mixtures were incubated at 37 C for 30 minutes, and 0.2 ml of each dilution was inoculated into each of 5 BEK

cell tube cultures. After 96 hours' incubation at 37 C, the fluids were removed and the cultures washed twice with warm PBS. The medium in each tube was replaced with 1 ml EBM containing approximately 10^3 $TCID_{50}$ cytopathogenic NADL-MD virus. The presence of unneutralized noncytopathogenic virus could then be detected by its interference with the cytopathogenic challenge virus. Fifty percent neutralization endpoints were calculated by the method of Reed and Muench (51).

Complement fixation

The complement source for the complement fixation (CF) test was prepared from pooled guinea pig serum by the procedure of Kabat and Mayer (37). Blood was collected in individual sterile containers from approximately 50 guinea pigs and allowed to clot for 1 to 2 hours at room temperature. Any natural hemolytic antibodies were removed by absorbing twice with sheep erythrocytes. One-hundred ml of ice-cold guinea pig serum was mixed with 3 ml of packed sheep erythrocytes which had been washed 4-5 times in isotonic saline. The mixture was kept at 0 C for ten minutes, then centrifuged at 2,000 x g for 10 minutes at 0 C and the serum removed. The absorbed guinea pig serum was distributed into chilled ampules and promptly frozen at -60 C.

Hemolysin was prepared by inoculation of rabbits with boiled sheep erythrocyte stromata as described by Kabat and

Mayer (37). One liter of sheep blood was collected in 250 ml of 3.8% sodium citrate with continuous mixing. The blood was filtered through sterile cheesecloth to remove clumps, and the erythrocytes were sedimented by centrifugation at 4 C. The erythrocytes were then washed in two volumes of isotonic saline. Erythrocytes were "laked" by slowly adding the erythrocytes, with constant vigorous mixing, to 10 liters of ice-cold acidified distilled water (4 ml glacial acetic acid to 10 liters distilled water). The mixture was stirred for approximately 10 minutes and allowed to stand at 4 C overnight to permit the stromata to settle. The supernatant fluid was discarded and the stromata were washed 4-6 times with cold 0.001 M acetate buffer, pH 5. After each washing, the stromata were sedimented by centrifugation at 2,000 x g at 4 C. Following the final sedimentation the stromata were suspended in an equal volume of cold 0.15 M NaCl. After washing three times in 0.15 M NaCl, to remove the acetate, the stromata were suspended in 0.15 M NaCl to a total volume of 300-400 ml. The stromata were heated for 1 hour in a 100 C water bath. The mixture was allowed to cool, then stirred by a magnetic stirring apparatus until a smoothly dispersed suspension was obtained. Rabbits were immunized with this suspension by intravenous inoculations according to the following sequence: One injection of 0.1 ml, five injections of 1 ml, and five injections of 2 ml, each. The series of 11 injections covered

a period of about 2 weeks, with not more than 2 consecutive days between any two injections. The rabbits were bled 4 to 5 days following the last inoculation. The serum collected was inactivated at 56 C for 30 minutes and stored at -20 C.

The sheep erythrocytes used in the CF test were obtained by collecting the blood in an equal volume of sterile, modified Alsever's solution. The sheep blood was stored at 4 C and allowed to age for at least 1 week prior to use. After this stabilization period, a volume of blood sufficient for a day's work was withdrawn aseptically and centrifuged. The plasma and buffy coat were carefully aspirated and the sedimented cells were washed 3 times with 10 volumes of veronal-buffered saline containing Ca++ and Mg++. Following the final washing, the erythrocytes were suspended in veronal-buffered saline to a final concentration of 2%.

The CF technique was essentially the same as described by Schmidt and Lennette (55) and modified by Gutekunst and Malmquist (29). The specific soluble antigen used in this study was prepared as previously described by Gutekunst and Malmquist (30). The soluble antigen was prepared from tissue culture fluids of NADL-MD virus and clarified of cellular debris by centrifugation for 30 minutes at 2,000 x g in a refrigerated centrifuge. Clarified tissue culture fluids were centrifuged for 2 1/2 hours at 98,500 x g in a Spinco Model L preparatory ultracentrifuge. Following centrifugation

the supernatant fluid was carefully decanted and concentrated approximately 100-fold by pressure dialysis at 4 C using cellulose casing under nitrogen pressure. Chloroform was added to the resulting concentrated soluble antigen to a final concentration of 5% to inactivate any remaining infective virus particles. This emulsion was gently mixed for 30 minutes at room temperature and the excess chloroform was removed by centrifugation at 2,000 x g for 20 minutes in a refrigerated centrifuge. The supernatant fluid was dialized against frequent changes of veronal-buffered saline at 4 C for 4 to 5 days then stored at -40 C.

The dose of soluble antigen to be used in the CF test was determined by "block" titration, with several dilutions of antigen concurrently titrated against successive dilutions of bovine anti-NADL-MD serum. One unit of antigen was taken as the highest dilution which would give 50% lysis with the highest dilution of antiserum. Two units of antigen contained in 0.2 ml were utilized in the test. The antigen was also tested for non-specific hemolytic or anticomplementary activity. Control antigen consisted of tissue culture fluids from normal BEK cell culture and prepared in the same manner as described for the specific soluble antigen.

The guinea pig complement was titrated in the presence of soluble antigen and 2 exact units in a volume of 0.2 ml

were used in the test. The incubation period for the titration was 1 hour at 37 C.

The ability of normal bovine serum to enhance the fixation of complement to certain virus-antibody complexes has been noted by numerous workers. In this study, unheated SPF bovine serum was added to optimally diluted complement at a concentration of 5% as described by Boulanger et al. (6). At this concentration, the normal bovine serum had neither anticomplementary nor non-specific activity with the soluble antigen.

Hemolysin was titrated using 2 units of complement in 0.2 ml containing 5% SPF bovine serum and 0.25 ml of a 2% sheep erythrocyte suspension. Two units of hemolysin in 0.25 ml were used in the CF test.

Sensitized erythrocytes were prepared by mixing equal volumes of a 2.0% suspension of washed sheep erythrocytes and a dilution of hemolysin containing 2 hemolytic units in a volume of 0.25 ml. The mixture was allowed to stand at room temperature for 10 minutes to become optimally sensitized.

All test serums were heat inactivated at 56 C for 30 minutes prior to testing. Serial 2-fold dilutions of each serum were prepared in cold veronal-buffered salin. Two-tenths ml of each dilution was dispensed into test tubes placed in an ice bath. Two units of complement in 0.2 ml

containing 5% normal bovine serum was then added, followed by 2 units of antigen in a volume of 0.2 ml. Antigen, control antigen, serum, complement and hemolytic controls were added to the system to determine the presence of any non-specific activity of the reagents or test serums. Fixation was allowed to proceed at 4 C for approximately 18 hours. Following the fixation period, the tubes were warmed in a 37 C water bath for 10 minutes, then 0.5 ml of sensitized erythrocytes was added to each tube, and secondary incubation at 37 C was continued for 20-30 minutes depending on the complement controls. The CF titer of the serums tested was expressed as the highest initial dilution of serum showing 50% lysis.

Growth Cycle

The growth cycle of NADL-MD virus was determined in primary BEK cell cultures. Thirty 4-oz prescription bottles containing monolayers of BEK cells were washed 3 times with warm PBS. Each culture was inoculated with 1 ml of NADL-MD virus (3×10^6 $TCID_{50}$/ml). After 1 hour of adsorption at 37 C, excess fluids were removed and the cultures washed 3 times with warm PBS. These fluids were pooled and titrated for unadsorbed virus. Fifteen ml of EBM containing 2% SPF calf serum was added to each culture for maintenance, then further incubated.

Two cultures were removed from incubation at 3-hour intervals for the first 12 hours and at 6-hour intervals for the next 96 hours. The supernatant fluids were harvested and clarified of cellular debris by centrifugation at 2,000 x g in a refrigerated centrifuge. The fluids were stored at -80 C until assayed for extracellular virus.

The cells which remained attached to the glass were washed twice with warm PBS and disrupted in 15 ml of HBSS by several cycles of freezing and thawing in an alcohol-dry ice bath. The cellular debris was sedimented by centrifugation at 2,500 x g for 1 hour in a refrigerated centrifuge and the resulting supernatant fluids titrated for cell associated virus.

Ether and Chloroform Sensitivity

The procedure for determining the ether sensitivity of the virus was essentially the same as described by Andrewes and Horstmann (2). Absolute diethyl ether* was added to infective tissue culture fluids to a final concentration of 20% and allowed to react for 18 hours at 4 C. Following the refrigeration period, the mixture was centrifuged at 2,000 x g for 20 minutes and the other layer removed. Residual ether was removed by vacuum. Oregon C24V virus

---

*Mallinckrodt Chemical Works, St. Louis, Missouri

was also tested for ether sensitivity in the manner described above. Controls consisted of both viruses to which 20% PBS replaced the ether in the procedure. Both the ether treated and control preparation were assayed for infective virus.

Sensitivity of NADL-MD and C24V viruses to chloroform was determined by the method described by Feldman and Wang (20). Sufficient analytical reagent grade chloroform* was added to infective tissue culture fluids to make a final concentration of 5%. Following 10 minutes of continuous shaking at room temperature, the mixture was centrifuged at 500 x g for 5 minutes. The sedimented chloroform appeared at the bottom of the tube and the clear supernatant fluid was removed and titrated for infective virus.

Nucleic Acid Type

The type of nucleic acid possessed by NADL-MD virus was investigated by the procedure of Hermodsson and Dinter (32) which employed two inhibitors of deoxyribonucleic acid (DNA) synthesis, 5-iodo-deoxyuridine (IUDR)** and

---

*Mallinckrodt Chemical Works, St. Louis, Missouri.

**Nutritional Biochemicals Corporation, Cleveland, Ohio.

5-bromo-deoxyuridine (BUDR)*. A known DNA virus, vaccinia**, as well as a known ribonucleic acid (RNA) virus, para-influenza 3***, served as controls. Each DNA inhibitor was aseptically added to EBM containing 2% SPF calf serum at a concentration of 50 µg per ml of medium. The medium was removed from 3 racks (64 tubes per rack) of BEK cultures and replaced with EBM containing IUDR in first rack, BUDR in the second rack, and no inhibitors in the remaining rack. Following 2 hours' incubation, 0.2 ml of each virus (approximately 10^3 $TCID_{50}$/ml) was inoculated into 15 BEK cultures of each of the 3 sets of media. The supernatant fluids of 5 replicate tubes from each of the 3 viruses in the 3 different media were pooled immediately after inoculation and assayed for virus content. The remaining cultures were incubated at 37 C and observed twice daily for evidence of CPE. Sampling procedures were repeated at 3 and 6 days PI in order to determine the effect of the inhibitory chemicals.

Ultrafiltration

Tissue culture virus suspensions of NADL-MD and C24V viruses were passed through Millipore filters/ of different

---

*Nutritional Biochemicals Corporation, Cleveland, Ohio.

**Smallpox vaccine, Lederle Laboratories.

***Obtained from Shipping Fever Project, NADL, Ames, Iowa.

/ Millipore Filter Corporation, Bedford, Massachusetts.

pore size to estimate the size of the infective virus particles. The following filters sizes were used in this experiment: 220 ± 0.2 mμ, 100 mμ ± 8 mμ, 50 mμ ± 3 mμ and 10 mμ ± 2 mμ. The filters were used directly from the sterile packages to avoid possible damage by autoclaving. Nitrogen pressure was used to facilitate filtration when the smaller pore size filters were used. The original virus sample and the filtrates were assayed for infective virus.

Cationic Stability

Since thermal stabilization by the addition of high concentrations of cations is a property of the enteroviruses, an experiment was conducted to test the cationic stability of NADL-MD virus by the procedure of Wallis and Melnick (64). Two molar solutions of $MgCl_2$ and of $CaCl_2$ were prepared in distilled water and sterilized by autoclaving. Two ml of tissue culture virus was added to equal volumes of each cation solution making a final 1 molar solution. Equal quantities of sterile water and tissue culture virus served as controls.

Immediately after mixing a portion was removed for assay and the remaining was placed in a 50 C water bath and titrated for infective virus following 30 and 60 minutes' incubation.

PH Stability at 56 C

The sensitivity of NADL-MD virus to thermal inactivation at 56 C was determined in isotonic veronal-acetate buffer solutions at various pH values. This buffer system was chosen because of its' wide range of pH values and elimination of possible specific ion effects when using different buffer systems. Since precipitates formed in buffers stored at 4 C for periods of 24 hours or longer, it was necessary to prepare buffers fresh each day. Buffer solutions ranging from pH 3 to 9 were sterilized by filtration through a 220 mμ Millipore filter. The pH of freshly prepared buffers was stable throughout the thermal inactivation period of 56 C.

NADL-MD virus for this experiment was prepared in BEK cultures containing EBM alone since serum proteins have buffering activity. To facilitate the adjustment of the pH of the virus preparation, the infective tissue culture fluids were dialized against frequent changes of 0.005 M phosphate buffered saline (pH 7.0) at 4 C for 3 days. The dialyzed virus was dispensed in 1 1/2 ml volumes into vials and stored at -80 C.

Two volumes of isotonic veronal-acetate buffer (3 ml) were added to 10 vials of virus material for each pH value. A preincubation sample was immediately adjusted to pH 7.0 by the addition of 3 ml of appropriate isotonic veronal-acetate buffer and titrated for virus content. The

remaining vials were placed in a 56 C water bath. Samples were removed from the water bath at 15 minute intervals and immediately placed in an ice bath. The pH of the same was adjusted to 7 by the addition of 3 ml of appropriate buffer and then the samples were titrated for infective virus.

The thermal stability of NADL-MD virus at 56 C was determined in buffers at pH's of 3, 4, 5, 6, 7, 8 and 9. The pH of the buffers were checked during thermal inactivation and found to vary not more than 0.2 pH units from the original pH value.

Trypsin Sensitivity

The sensitivity of NADL-MD and Oregon C24V viruses to the enzymatic action of trypsin was determined by the procedure of Dinter (17). Three times crystallized trypsin* was dissolved in 0.02 M phosphate buffered saline, pH 7.8, at a concentration of 1.0 mg per ml. The trypsin solution was sterilized by passage through a 220 mμ Millipore filter. Infective tissue culture fluids were dialized against frequent changes of 0.02 M phosphate buffered saline, pH 7.8, at 4 C for 3 days. Equal volumes of trypsin solution and virus preparation were mixed and additional samples of virus were mixed with equal volumes of the same buffer to serve as controls. Portions were removed for assay prior to

---

*Nutritional Biochemicals Corporation, Cleveland, Ohio.

incubation and the remaining placed in a 37 C water bath for 1 hour. Prior to assay, the trypsin activity in each sample was inhibited by the addition of 0.2% 5X crystallized soybean trypsin inhibitor* in 0.02 M phosphate buffered saline, pH 7.0. The samples were placed in an ice bath and titrated for infective virus in BEK cultures.

Hemagglutination Procedure

To determine if NADL-MD virus material possessed properties of hemagglutination, a procedure was used according to the general method described by Cunningham (13). Three different preparations of viral antigens were used in the hemagglutination (HA) test: 1) infective tissue culture virus, 2) infective tissue culture fluids concentrated approximately 100 times by pressure dialysis followed by dialysis against frequent changes of PBS at 4 C, and 3) concentrated soluble antigen as prepared for the CF technique.

Erythrocytes from various species were used in the HA test. Blood was collected aseptically into an equal volume of Alsever's solution from the following species: bovine, chicken, day-old chicken, guinea pig, rabbit, mouse, sheep, swine, hamster and human type O.

---

*Nutritional Biochemical Corporation, Cleveland, Ohio.

The erythrocytes were washed 3 to 4 times in 10 volumes of PBS. Following the final washing, a 0.5% suspension of each species of erythrocytes was prepared in sterile PBS.

Duplicate sets of serial 2-fold dilutions of each antigen preparation were made in PBS. An equal volume of each dilution was added to 0.25 ml of PBS followed by 0.25 ml of erythrocyte suspension. One set was incubated at room temperature for 2 hours and the other was refrigerated at 4 C for approximately 18 hours. Patterns of sedimented erythrocytes were observed for evidence of hemagglutination.

Chromatographic Separation

Chromatographic procedures have been useful in separating various components from crude virus preparations. In this experiment, an anion exchange cellulose column was utilized to separate NADL-MD viral antigens by increasing the molarity of NaCl in a buffer solution as described by Hoyer et al. (35). Ten grams of diethylaminoethyl (DEAE)-cellulose (0.91 meq/g)* were washed in 250 ml of sterile water. The cellulose was allowed to settle at 4 C and the fine particles were aspirated with the supernatant fluids. The washing process was repeated 3 times. Following the last washing, the DEAE-cellulose was equilibrated with

---

*Bio-Rad Laboratories, Richmond, California.

several changes of 0.01 M tris(hydroxymethyl)aminomethene-HCL*, pH 7.4 at 4 C. A slurry of DEAE-cellulose in tris buffer was added to a 1 cm chromatographic column and carefully packed to a depth of 7.5 cm by the aid of nitrogen pressure. The column was further equilibrated by allowing approximately 250 ml of tris buffer to pass through the column.

NADL-MD virus material for chromatographic separation was prepared in BEK cultures in EBM medium without serum or phenol red indicator. The infective tissue culture fluids were concentrated at least 100 times by pressure dialysis at 4 C, then dialyzed against frequent changes of tris buffer for 3 days.

The chromatographic procedure was conducted at 4 C. The previously equilibrated DEAE-cellulose column was loaded with 1 ml of concentrated virus material. The column was then eluted stepwise with 4 ml aliquots of tris buffer containing increasing molarities of NaCl. The flow rate through the column was adjusted to approximately 20-30 drops per minute by the use of nitrogen pressure. Eluates were collected dropwise in 2 ml volumes. The infectivity of each sample was determined by titration in BEK cultures.

---

*Sigma Chemical Company, St. Louis, Missouri.

The complement-fixing activity of each sample was determined by the CF technique.

Sucrose Density Gradient Centrifugation

Sedimentation in a density gradient has been a very useful technique for separation and purification of viral components. The sucrose density gradient technique was used in this experiment for separation of NADL-MD virus components by a procedure described by Brakke (7). Analytical reagent grade sucrose* solutions were prepared in sterile distilled water at concentrations of 15, 30, 45 and 50% respectively. Gradient columns were prepared by carefully layering 1 ml of each aqueous sucrose solution in a sterile centrifuge tube. The gradient tubes were allowed to equilibrate at 4 C for at least 24 hours. The viral material was prepared in the same manner as for the chromatographic separation procedure except the material was dialyzed against frequent changes of sterile saline at 4 C for 2 days. After the gradient tubes had equilibrated, 0.5 ml of concentrated virus material was carefully overlaid on the sucrose gradient. An additional gradient column was overlaid with 0.5 ml of crystallized bovine albumin (10 mg protein nitrogen/ml)** to serve as a control. The gradients

---

*Mallinckrodt Chemical Works, St. Louis, Missouri.

**Armour and Company, Chicago, Illinois.

were centrifuged in a SW-39L swinging bucket rotor of the Spinco Model L ultracentrifuge for 18 hours at 35,000 rpm. Rapid acceleration with non-braking deceleration was used to minimize convection and stirback.

Various techniques were investigated for collecting samples from the gradient tubes. The technique which gave clear-out separation of the samples with the least amount of mixing was used in this study. It consisted of individual 0.5 ml pipettes held in a fixed vertical position and connected to a 2 ml syringe with non-collapsible tubing. Gradient tubes were held in a glass receptacle affixed to an adjustable stage for vertical positioning of the sample in reference to the fixed pipettes. Successive 0.5 ml aliquots were carefully collected from just below the meniscus by slowly elevating the gradient column. Each aliquot was assayed for infective virus in BEK cultures and for soluble antigen in the CF test. Protein nitrogen determination of each sample containing bovine albumin was made by the biuret method (27).

Relationship of NADL-MD Agent to other Bovine Viral Diarrhea Viruses

The relationship of NADL-MD virus to other viruses of the BVD-MD complex was determined by neutralization tests.

The cytopathogenic viruses, NADL-MD and Oregon C24V (23)* were compared by the use of anti-NADL-MD serum prepared in an SPF calf. The following noncytopathogenic viruses were titrated and compared in the neutralization test by the principle of interference: NY 1 (3)*, Indiana 46 (48)*, Merrell (61)*, Sanders (52)* and CG-1220 (29).

The soluble antigens of NADL-MD, Oregon C24V and CG-1220 viruses were compared in the CF test. Two CF units of each were used to titrate the complement-fixing antibody content in anti-NADL-MD and anti-CG-1220 serums prepared in SPF calves.

Relationship of NADL-MD Agent to other Virus Groups

Reciprocal cross-neutralization tests with other viruses and their antiserums were performed to determine possible relationship with NADL-MD virus. The following viruses and antiserums were tested; infectious bovine rhinotracheitis (IBR) virus and antiserum**, parainfluenza-3 and antiserum**, vesicular stomatitis***, vaccinia, anti-hog cholera

---

*Obtained from Doctor D. E. Tyler, Iowa State University, Ames, Iowa.

**Obtained from Shipping Fever Project, NADL, Ames, Iowa.

***Obtained from Doctor L. O. Mott, NADL, Ames, Iowa.

serum*, anti-transmissible gastrointerititis serum (TGE)*, anti-group B arborvirus serum**, and anti-Lymphocytic choriomeningitis (LCM) serum***.

The antiserums listed above were also tested against the soluble antigen of NADL-MD in the CF test. Procomplementary activity of the anti-hog cholera and anti-TGE serums were removed by the procedure described by Cowan (12). One ml of each test serum was heat inactivated at 56 C for 30 minutes. The serum was allowed to cool at room temperature, then 0.2 ml of a 1:80 dilution of formalin was added to each test serum. The mixture was incubated for 45 minutes at 37 C. Immediately after incubation, the serum was serial 2-fold diluted in cold veronal-buffered saline and used in the CF test.

Animal Experimentation

An SPF calf was inoculated with NADL-MD virus to study and characterize the experimental disease.

Experimental animal

A Holstein calf was procured by the animal quarantine section at the National Animal Disease Laboratory under

---

*Obtained from Doctor A. L. McClurkin, NADL, Ames, Iowa.

**Obtained from Doctor T. H. Works, Chief, Virology Section, Communicable Disease Center, Atlanta, Georgia.

***Diagnostic Reagents Section, Communicable Disease Center, Atlanta, Georgia.

conditions which approximated SPF production methods as described by Edward et al.* The calf was deprived of colostrum and held approximately 5 months in a unit completely separated from other animals. Subsequent neutralization tests with preinoculation serum against the agent under study proved the complete absence of antibody to the NADL-MD virus. Daily observation and establishment of basal temperature and leukocyte counts were performed over a 4-day period preceding artificial exposure.

Inoculum and exposure

NADL-MD virus was passed twice in BEK cultures then 5 ml tissue culture fluid containing approximately 10^6 $TCID_{50}$ were inoculated intravenously into the calf. Twelve weeks following the first inoculation, a second intravenous injection of 5 ml NADL-MD virus was given.

Collection of data

Beginning 24 hours PI and continuing daily for approximately 3 weeks, blood samples were obtained for total and differential leukocyte counts. Serums recovered from weekly bleedings were used for neutralization and CF tests. Citrated blood was collected for buffy coat cultures each day for 1 week and at weekly intervals thereafter, with the

---

*Edward, A. G., Miles, G. D. and Calhoun, J. R., National Animal Disease Laboratory, Ames, Iowa. Production of colostrum deprived specific pathogen-free calves. Private communication. 1962.

exception of the 4th day PI when a pre-scapular lymph node biopsy was performed. Swabs were used to collect material from intestinal contents and nasal secretions at the same time as the blood for the buffy coat cultures. Rectal temperatures and clinical signs were recorded 3 times daily.

Virus isolation technique

Buffy coat cultures were prepared by a procedure similar to that described by Malmquist and Hay (42) for the propagation of swine leukocytes. Approximately 25 ml of blood was collected aseptically from the experimental calf into 5 ml of a 10% sodium citrate solution. Citrated blood samples were centrifuged at 2,000 x g for approximately 30 minutes and the plasma aspirated. Recovered buffy coat cells were washed 2 to 4 times in a Ca free salt solution (GKN), care being taken to exclude as many erythrocytes as possible from each recovery. Four washings were used when the presence of antibodies was anticipated. Buffy coat cells were planted with a growth medium containing 80% EBM and 20% isologous serum. In most instances paired sets of cultures media were prepared, one incorporating preinoculation serum and the other with serum collected at the same time as the buffy coat cells. After 5 to 7 days' incubation at 37 C the buffy coat cultures were reinoculated into BEK cultures. Rectal and nasal swabs were washed out in EBM which was clarified by centrifugation and inoculated into

BEK cultures. The pre-scapular lymph node biopsy material was minced with sterile scissors after which it was distributed into tubes of growth medium and incubated as explant cultures. After the lymph node cells had formed monolayers, they were then subcultured into BEK cell cultures. The production of a CPE in the cultures similar to that produced by the original virus inoculum served as criterion of successful recovery of the virus. The isolates were further identified by virus neutralization tests.

Serum neutralization and complement fixation

Serums collected at weekly intervals from the experimental calf were inactivated at 56 C for 30 minutes and serial 2-fold dilutions of each were prepared. The serums were tested against infective NADL-MD virus in the neutralization test and against the soluble antigen in the CF test.

Necropsy

The calf was necropsied 16 weeks following the first inoculation. Various tissues were aseptically collected for virus isolation and buffy coat and bone marrow cultures were also prepared. A thorough search for gross lesions was made and those observed were described.

RESULTS

Growth Cycle

Ninety-two percent of the virus in the inoculum was adsorbed to the BEK cells during a 1 hour incubation at 37 C. The first appreciable cell associated virus was detectable at 9 hours PI and reached a peak at 18 hours PI when the first evidence of CPE was observed. Over 99% of the total virus yield was released into the supernatant fluids by the time the cultures evidenced 50% CPE. The release of virus into the extracellular fluids appears to be by gradual release, rather than by "burst," as occurs with bacteriophages. The complete cell layer was destroyed within 48 hours PI (Figure 1).

Ether and Chloroform Sensitivity

NADL-MD and Oregon C24V viruses were completely inactivated by treatment with 20% ether for 18 hours at 4 C, and no detectable infective virus remained in the fluids following the removal of ether (Table 1).

Both viruses were sensitive to chloroform at a concentration of 5% when incubated at room temperature for 10 minutes (Table 1).

Figure 1. Growth cycle of NADL-MD virus in bovine embryonic kidney cell cultures

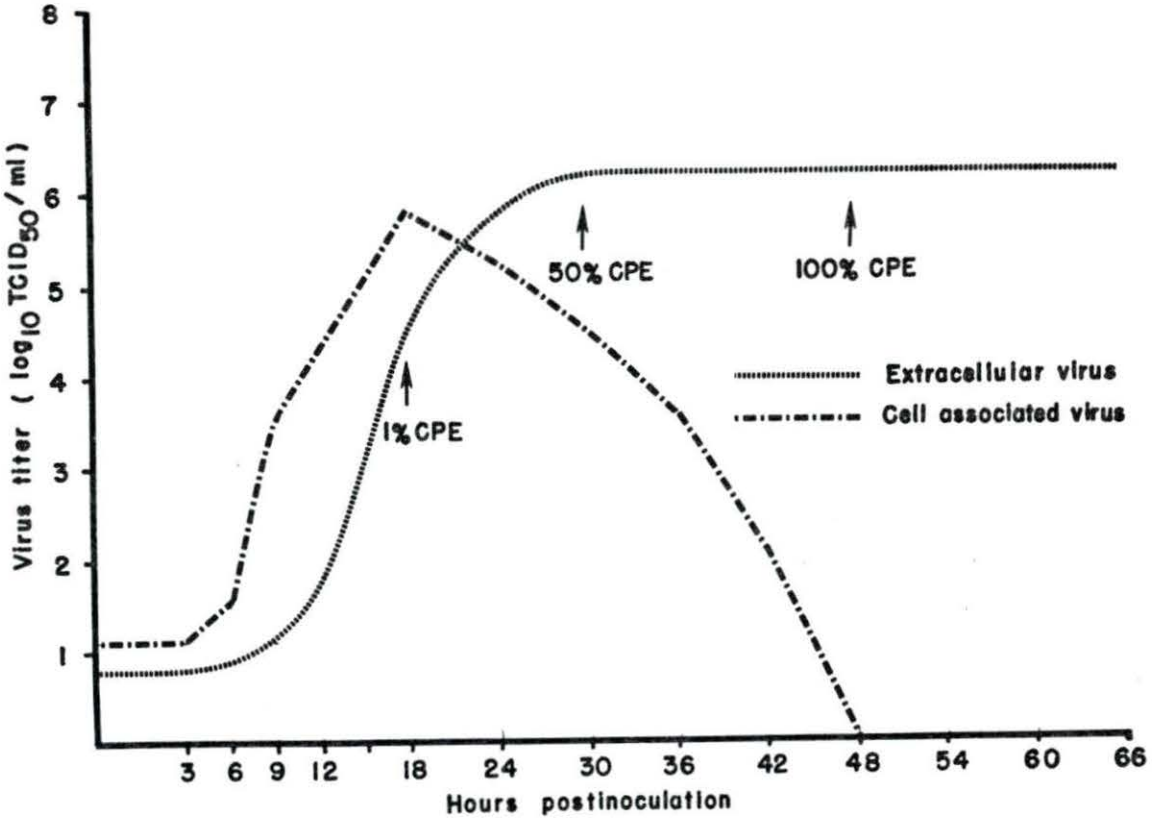

Virus titer (log10 TCID50 / ml)
8
7
6
5
4
3
2
1
1% CPE
50% CPE
100% CPE
Extracellular virus
Cell associated virus
3 6 9 12 18 24 30 36 42 48 54 60 66
Hours postinoculation

Table 1. Ether and chloroform sensitivity of NADL-MD and Oregon C24V

Virus	Ether[a] control	Ether[a] treated	Chloroform[b] control	Chloroform[b] treated
NADL-MD	5.8[c]	<1.0	6.2	<1.0
Oregon C24V	5.5	<1.0	5.8	<1.0

[a] 20% ether for 18 hours at 4 C.

[b] 5% chloroform for 10 minutes at room temperature.

[c] Titers expressed as $\log_{10} TCID_{50}$/ml.

Nucleic Acid Type

Inhibitors of DNA synthesis, IUDR and BUDR, did not inhibit the replication of NADL-MD virus in BEK cultures at a concentration of 50 µg per ml of medium. However vaccinia, a DNA virus, was inhibited by both IUDR and BUDR. The RNA virus, parainfluenza-3, showed a normal replication in the presence of the 2 DNA inhibitors (Figure 2).

Ultrafiltration

Both NADL-MD and Oregon C24V viruses readily passed through the 220 mµ and 100 mµ millipore filters. A small fraction of both viruses passed the 50 mµ filter, but no infective virus was present in the filtrate when the 10 mu filter was used (Table 2).

Figure 2. Nucleic acid type determination of NADL-MD using DNA inhibitors, IUDR and BUDR, in BEK cell cultures

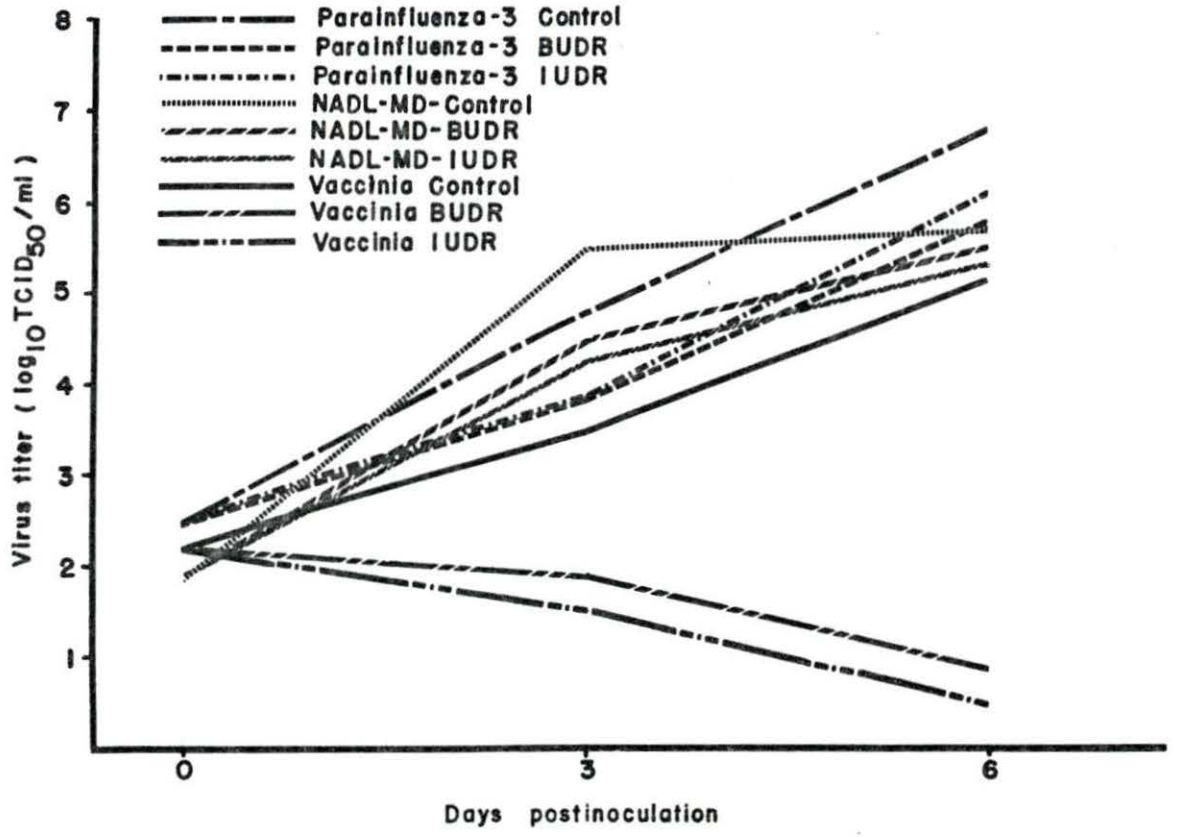

Parainfluenza-3 Control
Parainfluenza-3 BUDR
Parainfluenza-3 IUDR
NADL-MD-Control
NADL-MD-BUDR
NADL-MD-IUDR
Vaccinia Control
Vaccinia BUDR
Vaccinia IUDR
Virus titer (log10 TCID50/ml)
8
7
6
5
4
3
2
1
0
3
6
Days postinoculation

Table 2. Ultrafiltration studies of NADL-MD and Oregon C24V viruses

Filter size	NADL-MD control	NADL-MD filtrate	Oregon C24V control	Oregon C24V filtrate
220 mμ	6.2[a]	5.8	5.5	4.8
100 mμ	6.2	4.5	5.5	3.8
50 mμ	6.2	1.2	5.5	1.2
10 mμ	6.2	<1.0	5.5	<1.0

[a]Titer expressed as $\log_{10}$ $TCID_{50}$/ml.

Cationic Stability

The thermal inactivation of NADL-MD virus was enhanced when incubated at 50 C in the presence of 1 M solutions of either Ca++ or Mg++ as compared to inactivation of the virus in equal quantities of water (Table 3). Ca++ appeared to have a greater effect on the therma inactivation of the virus than the Mg++.

Table 3. Cationic stability of NADL-MD virus to thermal inactivation at 50 C in presence of 1 M Ca++ or Mg++

Incubation time (minutes)	$MgCl_2$[a]	$CaCl_2$[b]	H_2O
0	6.2[c]	6.0	6.2
30	4.5	4.2	5.8
60	3.2	2.8	4.5

[a]One molar solution at 50 C.

[b]One molar solution at 50 C.

[c]Titer expressed as $\log_{10}$ $TCID_{50}$/ml.

PH Stability at 56 C

NADL-MD virus was relatively stable to thermal inactivation at 56 C in buffer solutions of pH 6 to 8, with a small amount of infective virus remaining after 2 hours' incubation. A two component inactivation curve at these pHs indicate possible heat resistant variants. The inactivation of the virus at 56 C was greatly enhanced at pH 5 or below (Figure 3).

Trypsin Sensitivity

Both NADL-MD and Oregon C24V viruses were moderately sensitive to the enzymatic action of trypsin. Ninety-nine percent or more of each virus was inactivated when incubated for 1 hour at 37 C in the presence of trypsin (Table 4).

Figure 3. Thermal inactivation of NADL-MD virus at 56 C in buffer solutions at various pH values

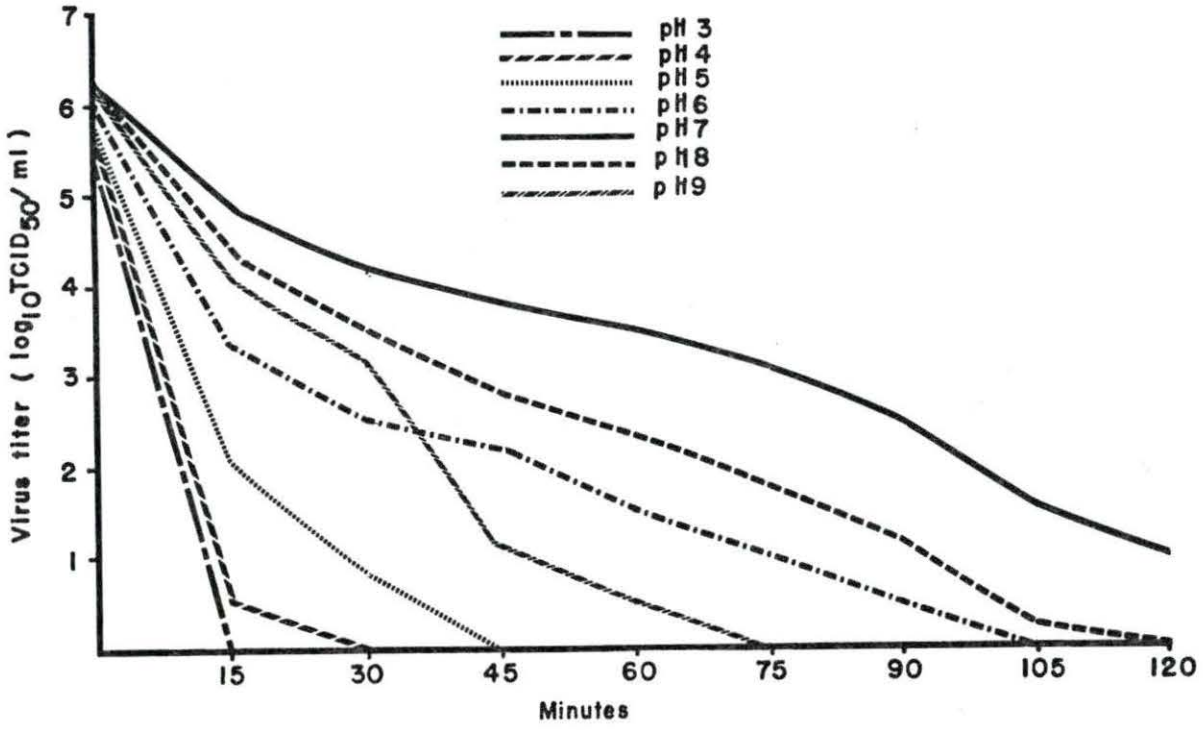

pH 3
pH 4
pH 5
pH 6
pH 7
pH 8
pH 9
Virus titer ($log_{10}TCID_{50}$/ ml)
7
6
5
4
3
2
1
15
30
45
60
75
90
105
120
Minutes

Table 4. Sensitivity of NADL-MD and Oregon C24V viruses to the enzymatic action of trypsin for 1 hour at 37 C

Virus	Control	Trypsin Treated	Difference
NADL-MD	5.2[a]	2.5	2.7
Oregon C24V	4.8	2.8	2.0

[a]Titer expressed as $\log_{10}$ $TCID_{50}$/ml.

Hemagglutination Test

No hemagglutination was detectable when either of the 3 antigens were tested in the HA test with the erythrocytes of various species and incubated at room temperature or at 4 C for 18 hours (Table 5).

Table 5. Hemagglutination tests of NADL-MD viral antigens with various species' erythrocytes

Erythrocytes	Tissue culture virus		Concentrated virus		Soluble antigen	
	25 C	4 C	25 C	4 C	25 C	4 C
Bovine	-	-	-	-	-	-
Chicken	-	-	-	-	-	-
Day-old chicken	-	-	-	-	-	-
Guinea pig	-	-	-	-	-	-
Rabbit	-	-	-	-	-	-
Mouse	-	-	-	-	-	-

Table 5 (Continued)

Erythrocytes	Tissue culture virus 25 C	Tissue culture virus 4 C	Concentrated virus 25 C	Concentrated virus 4 C	Soluble antigen 25 C	Soluble antigen 4 C
Sheep	-	-	-	-	-	-
Swine	--	-	-	-	--	-
Hamster	-	-	-	-	-	-
Human type O	-	-	-	-	-	-

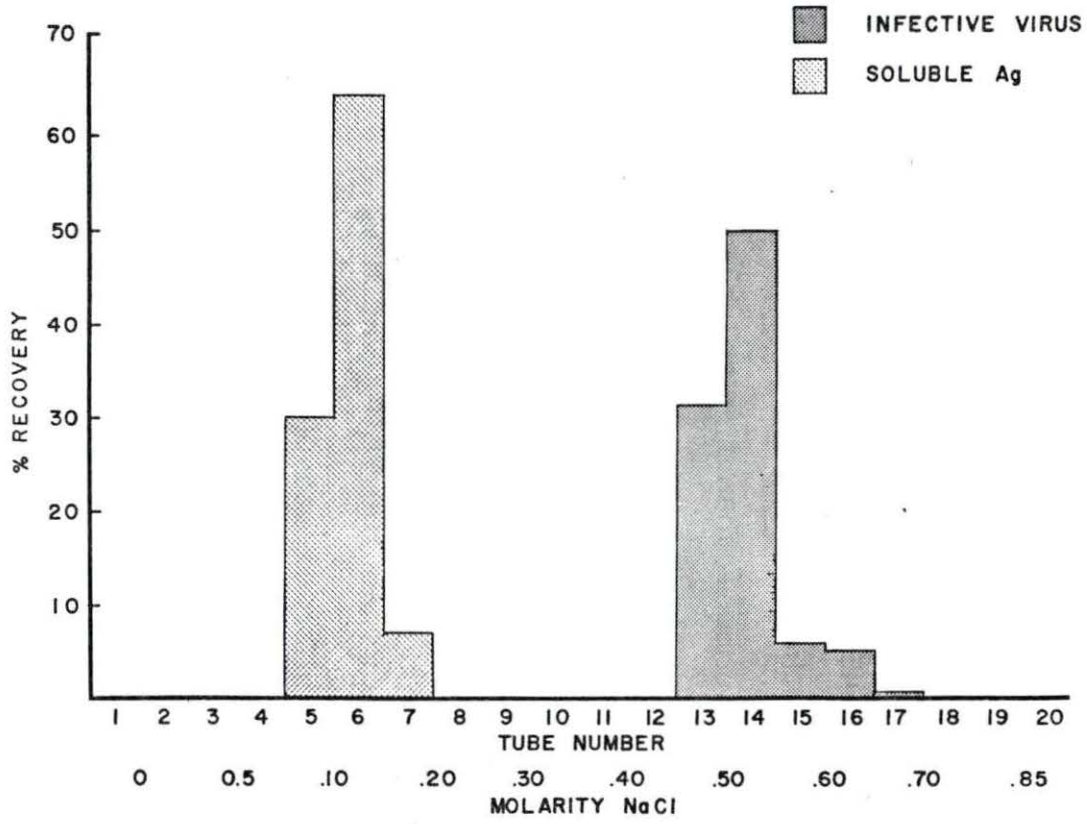
Chromatographic Separation

Separation of the soluble antigen from the infective virus particle was accomplished by elution from a DEAE-cellulose column with increasing molarities of NaCl. Most of the soluble antigen was eluted from the column in the range of 0.1 and 0.2 M NaCl in 0.01 M tris buffer, pH 7.4. Elution of infective virus required 0.5 M NaCl in tris buffer and a "virus trail" followed with the addition of higher molarities of NaCl (Figure 4). The soluble antigen was assayable in the CF test, however, the infective virus failed to fix complement.

Sucrose Density Gradient Centrifugation

Sedimentation in a sucrose density gradient yielded partly purified soluble antigen and infective virus particles. Infective virus, as determined by titration in BEK cultures,

Figure 4. Chromatographic separation of NADL-MD viral components in DEAE-cellulose by increasing molarity of NaCl in 0.01 M tris buffer pH 7.4


INFECTIVE VIRUS
SOLUBLE Ag
% RECOVERY
70
60
50
40
30
20
10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
TUBE NUMBER
0 0.5 .10 .20 .30 .40 .50 .60 .70 .85
MOLARITY NaCl

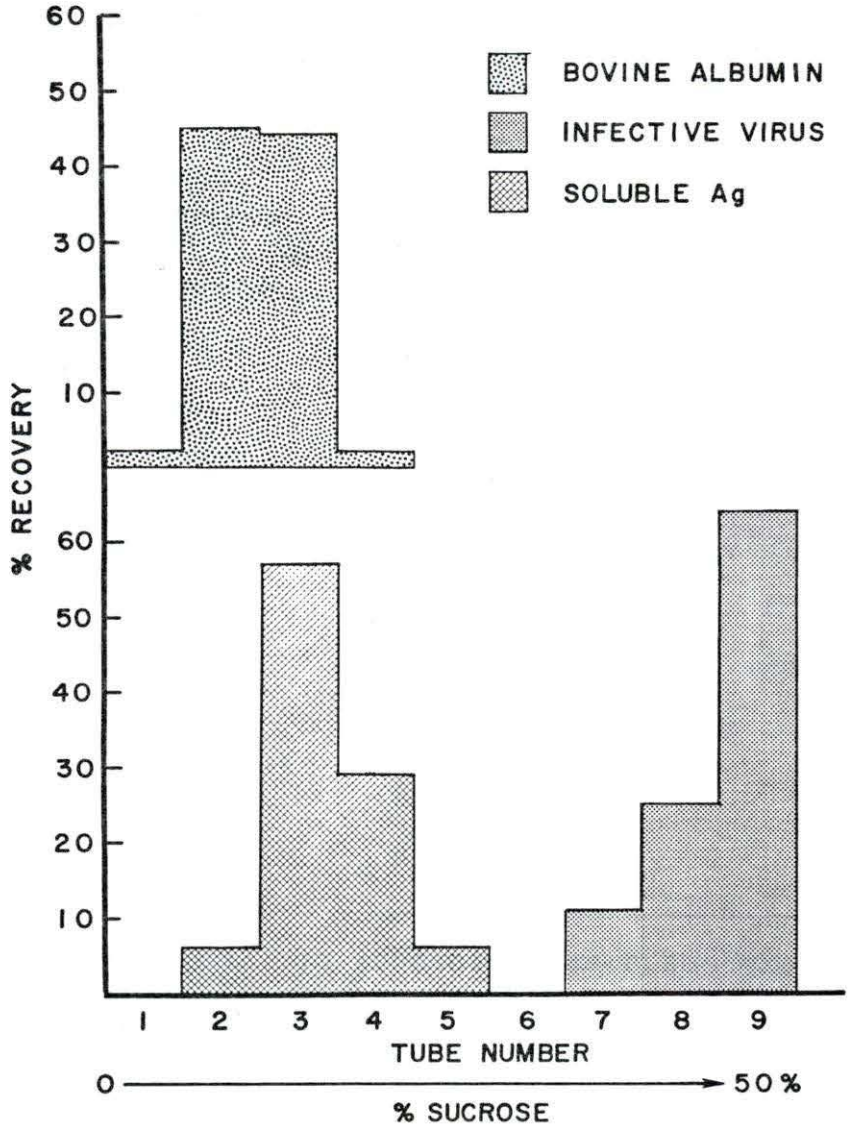
was located near the bottom of the gradient column in the higher densities of sucrose. The complement-fixing soluble antigen was located near the top of the gradient in the lower densities of sucrose (Figure 5). Sample number 9, taken from the bottom of the gradient tube, showed anti-complementary activity in the CF test. However, after the sucrose was removed by dialysis, no anticomplementary activity remained. As in the chromatographic procedure, the samples containing the infective virus particles failed to fix complement. The bovine albumin was found in slightly lower densities of sucrose than the soluble antigen (Figure 5).

Relationship of NADL-MD Agent to other Bovine Viral Diarrhea Viruses

Neutralization tests using serial 2-fold dilutions of anti-NADL-MD serum against 100 $TCID_{50}$ of Oregon C24V, NY 1, Indiana 46, Sanders, Merrel and CG-1220 viruses showed an antigenic relationship, but to a lesser degree than the homologous NADL-MD virus. The cytopathogenic Oregon C24V virus showed a lower neutralization titer than any of the noncytopathogenic viruses when tested against anti-NADL-MD serum (Table 6).

Soluble antigens prepared from NADL-MD, Oregon C24V and CG-1220 viruses and each standardized in the CF test

Figure 5. Sucrose density gradient centrifugation of NADL-MD virus components


60
50
40
30
20
10
% RECOVERY
BOVINE ALBUMIN
INFECTIVE VIRUS
SOLUBLE Ag
60
50
40
30
20
10
1 2 3 4 5 6 7 8 9
TUBE NUMBER
0
50 %
% SUCROSE

yielded results within one dilution titers of each when tested against anti-NADL-MD and anti-CG-1220 serums (Table 7).

Table 6. Neutralization tests using serial 2-fold dilutions of anti-NADL-MD serum against 100 $TCID_{50}$ of different bovine viral diarrhea viruses

Viruses	Serum	
	Preinoculation	Postinoculation
Cytopathogenic		
NADL-MD	<4[a]	5,400
Oregon C24V	<4	128
Noncytopathogenic[b]		
New York 1	<4	256
Indiana 46	<4	180
Sander	<4	320
Merrell	<4	512
CG-1220	<4	1,530

[a]Titers expressed as reciprocal of final serum dilution.

[b]Interference test utilized to titrated and detect.

Table 7. Comparison of the soluble antigen of NADL-MD, Oregon C24V and CG-1220 in the complement-fixation test with anti-NADL-MD and CG-1220 serums

Soluble antigen	Anti-NADL-MD serum			Anti-CG-1220 serum		
	Preinoculation	4 week	6 week	Preinoculation	4 week	6 week
NADL-MD	<5	160[a]	1280	<5	320	640
Oregon C24V	<5	80	1280	<5	320	640
CG-1220	<5	160	1280	<5	160	640

[a]Titers expresses as reciprocal of the initial dilution exhibiting 50% lysis.

Relationship of NADL-MD agent to other Virus Groups

No demonstrable neutralizing antibodies were detected when NADL-MD virus was tested against anti-IBR, anti-parainfluenza-3, anti-hog cholera, anti-TGE, anti-group B arbovirus and anti-LCM serums. Likewise no neutralizing antibodies were detectable when IBR, parainfluenza-3, vesicular stomatitis and vaccinia viruses were tested against anti-NADL-MD serum.

Anti-hog cholera serum had a complement-fixing titer of 320 when tested against the soluble antigen of NADL-MD virus, whereas no detectable complement fixation was found in the other antiserums listed above.

Animal Experimentation

The SPF calf inoculated with NADL-MD virus showed a clinical response similar to that reported for other BVD-MD agents.

Clinical observations

An initial temperature rise of 2.5 degrees occurred in the experimental calf 24 hours following inoculation with NADL-MD virus. Following a brief remission, a definite rise occurred on the 6th and 7th days PI. The temperature slowly returned to normal in the subsequent 4 days with an additional temperature rise occurring 2 weeks PI. Thereafter the temperature remained relatively normal even after challenge with virulent NADL-MD virus 12 weeks following the initial exposure (Figure 6).

With the initial temperature rise the calf was depressed and showed lack of appetite. A mild lacrimation and mucous nasal discharge were present. Four days PI the pre-scapular lymph nodes were enlarged approximately 3 times and a biopsy was performed at this time. On the 4th to 7th day PI, the calf exhibited lamititis and would stand with an arched back, moving only with difficulty. The calf became very weak and depressed with a complete loss of appetite. Excessive salivation and lacrimation with increased respiration were observed. The calf had a slight diarrhea on the 6th and 7th days with excessive amounts of mucous present in the discharge.

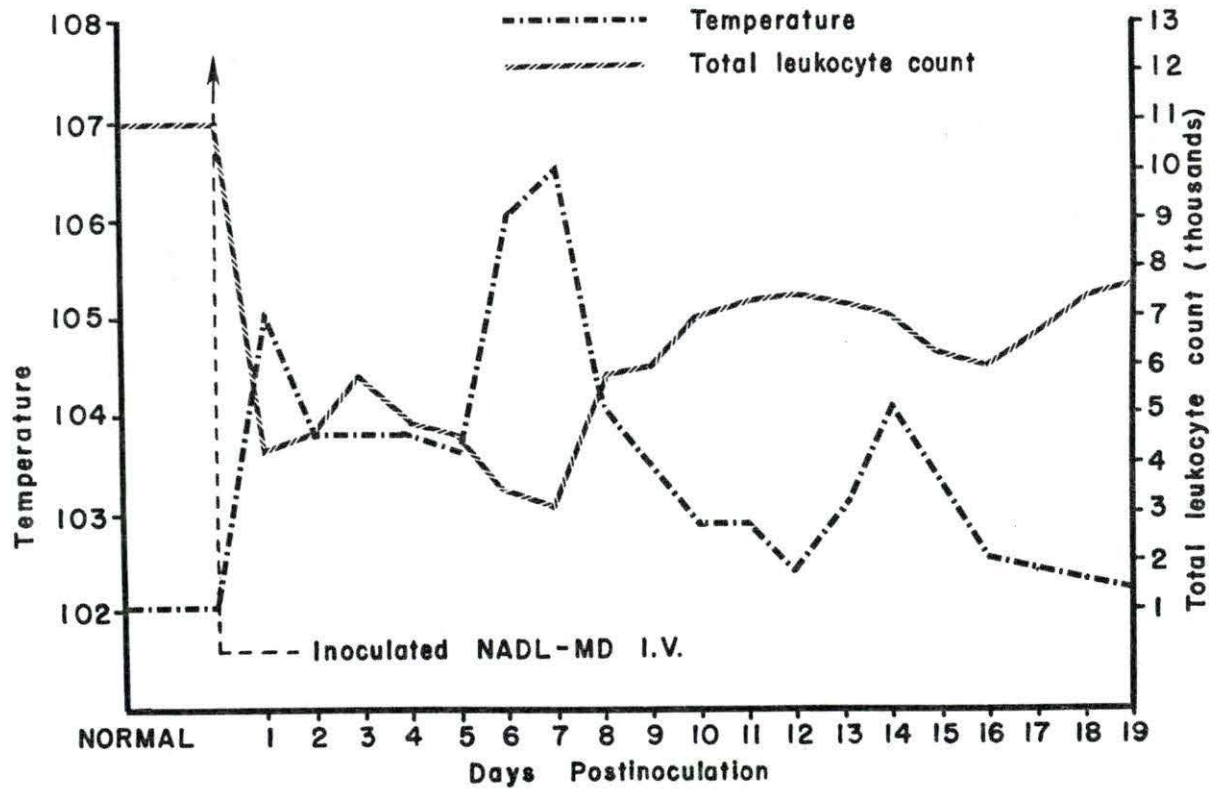
On the 8th to 10th days, erythemia of the mucous membranes of the buccal cavity was noted. The calf's muzzle had a "boiled appearance." The calf was very dehydrated and had lost approximately 100 to 150 lbs. of weight. After 2 weeks PI, the calf began eating and drinking and rapidly returned to normal. No clinical signs were observed following challenge with virulent virus 12 weeks after the initial exposure.

Hematologic observation

The total leukocyte count fell to approximately 40% of the preinoculation level 24 hours PI, then rose to about 60% of the normal in 3 days. A marked leukopenia occurred on the 6th and 7th days when total leukocyte counts were approximately 70% below the normal. The total leukocyte count slowly returned to normal in approximately 4 weeks, with the exception of a slight depression occurring on the 15th and 16th days PI. No significant change in total leukocyte counts were observed when the calf was challenged with virulent NADL-MD virus. The depressions in total leukocyte counts closely correlated with the rises in temperature (Figure 6).

Differential leukocyte counts indicated a drop in total lymphocytes and neutrophils as compared to the normal. At the time of greatest total leukocyte depression, total lymphocytes were approximately 41% below the normal and a

Figure 6. Leukocyte and temperature response in an SPF calf inoculated with NADL-MD virus


Temperature
Total leukocyte count
108
107
106
105
104
103
102
Temperature
13
12
11
10
9
8
7
6
5
4
3
2
1
Total leukocyte count (thousands)
Inoculated NADL-MD I.V.
NORMAL
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Days Postinoculation

decrease of 49% was observed in the total neutrophil count. Other leukocyte numbers did not vary appreciably from pre-inoculation counts.

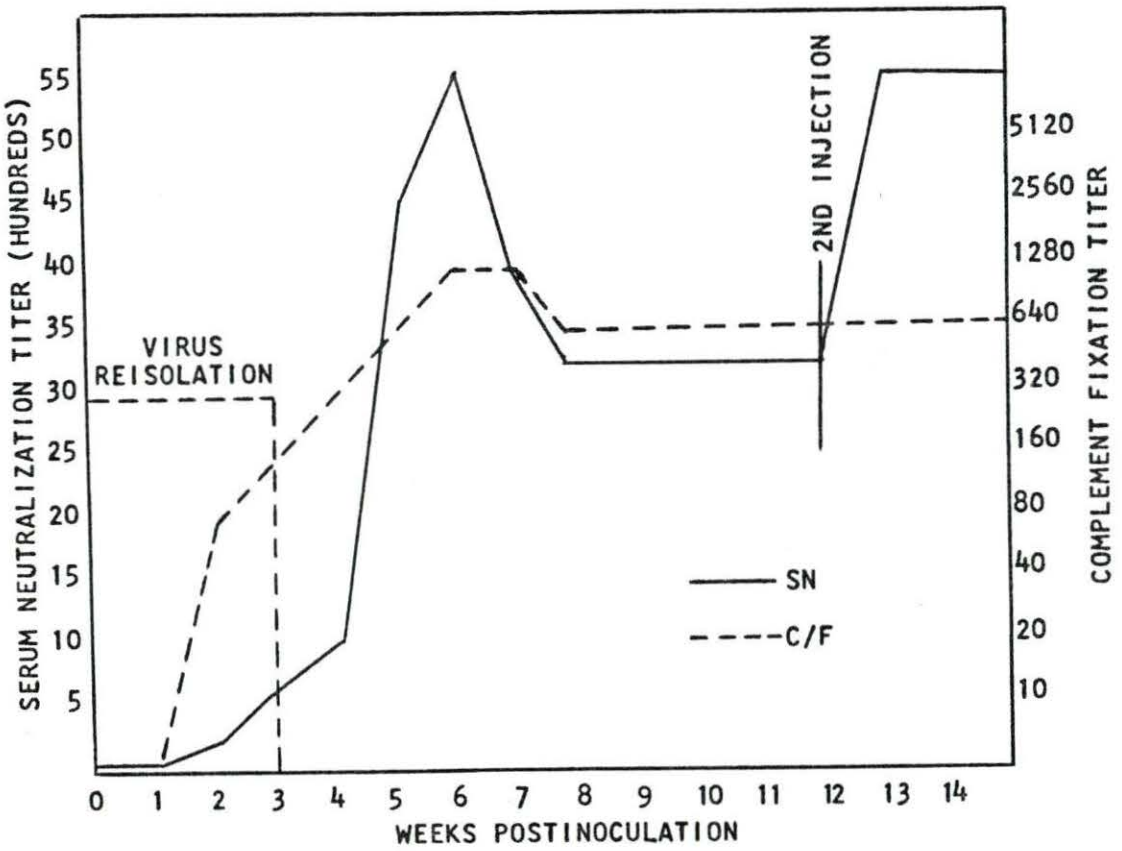
Antibody response

Neutralizing antibodies were detectable in the serum 2 weeks PI and rapidly increased to a peak titer at 6 weeks. Complement-fixing antibodies were detected prior to neutralizing antibodies and reached their highest levels at 6 weeks PI, thereafter they remained relatively constant for the duration of the experiment. There was a rapid rise in neutralizing antibody levels following the second injection of virulent NADL-MD virus, with no apparent effect on the complement-fixing antibody level (Figure 7).

Virus isolation

The virus was recoverable for only 1 week PI when serum collected at time of buffy coat preparation was utilized in the growth medium, however, the virus was isolated during 3 weeks PI when preinoculation serum was used in the medium (Table 8). It should be noted that the recovery of virus at 2 and 3 week PI occurred at a time when there was significant neutralizing and complement-fixing antibodies present (Figure 7). The virus was not recoverable at any time following the second injection of virulent virus.

Figure 7. Antibody response and virus recovery from an SPF calf inoculated with NADL-MD virus


SERUM NEUTRALIZATION TITER (HUNDREDS)
55
50
45
40
35
30
25
20
15
10
5
VIRUS
REISOLATION
2ND INJECTION
SN
C/F
COMPLEMENT FIXATION TITER
5120
2560
1280
640
320
160
80
40
20
10
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
WEEKS POSTINOCULATION

Culture of the biopsy material from the pre-scapular lymph nodes 4 days PI demonstrated the virus to be present at that time (Table 8).

No virus was recovered from daily rectal or nasal swabs (Table 8).

Recovered viruses were considered to be identical to the original agent when typical CPE was observed in tissue cultures and confirmed by virus neutralization.

Table 8. Virus recovery from an SPF calf exposed to NADL-MD virus

Days Postinoculation	Buffy coat	Lymph node	Rectal swab	Nasal swab
1	positive	N.T.[a]	negative	negative
2	"	"	"	"
3	"	"	"	"
4	N.T.	positive	"	"
5	positive	N.T.	"	"
6	"	"	"	"
7	"	"	"	"
14	"[b]	"	"	"

[a]Not tested.

[b]Positive when preinoculation serum was used in the medium but negative with simultaneously collected serum.

Table 8 (Continued)

Days Postinoculation	Buffy coat	Lymph node	Rectal swab	Nasal swab
21	"[b]	"	"	"
28	negative[c]	"	"	"
35	"[c]	"	"	"

[c]Negative when preinoculation or simultaneously collected serum was incorporated in the medium.

Necropsy

The calf was necropsied 16 weeks following the original exposure to NADL-MD virus. Six healed ulcers were present in the oral mucosa, occurring mainly on the dental pad and tongue. Numerous elongated areas of necrosis and erosion were present in the epithelial lining of the esophagus, being more prominent in the posterior region. Almost the entire epithelial surface of the abomasum was denuded and hyperemic. A marked catarrhal enteritis was observed in the small intestine, with the most severe lesions in the duodenum and jejunum. The mucosa was hyperemic and edematous with excessive catarrhal exudate in the lumen. The Peyer's patches were edematous and enlarged approximately 2 times. The enlarged Peyer's patches were distinctly outlined by marked hyperemia with an occasional erosion on the mucosal surface. The remaining portion of the intestine appeared

to be normal except for a small amount of catarrhal exudate. The mesenteric lymph nodes were enlarged and edematous with a few petechial hemorrhages on the surface. The spleen was approximately 1/2 normal size and appeared to be largely devoid of lymphoic material. No other gorss lesions were observed.

Bone marrow cultures prepared from the femur at time of necropsy yielded a virus which was identical to the original agent in CPE and by virus neutralization. No virus was recovered from the other tissues tested or by buffy coat cultural technique.

DISCUSSION

The general characteristics of this new isolate most closely resembles the BVD-MD group of viruses. However, the CPE produced by the NADL-MD agent in cell cultures is more rapid and complete as compared to the most studied BVD virus, C24V. Likewise, the replication and release of this agent is more rapid than that of C24V. In examining the growth cycle of NADL-MD virus in BEK cell cultures, over 99.9% of the total virus yield is released into the supernatant fluid by 24 hours PI when approximately 50% of the cells show evidences of CPE. In comparison, Gratzek (28) observed maximum titers of C24V in the supernatant fluids from 3 to 7 days PI and likewise Gillespie et al. (26) obtained maximum yield in 3 days but reported a marked decline in titer following subsequent incubation. However, Coggins (9) reported the multiplication of C24V virus as being very gradual and reaching maximum titer only after 6 days' incubation.

In this study, a substantial amount of the virus was found to be released into the extracellular fluid at the first evidences of CPE which occurred at 18 hours PI. The complete cell layer was destroyed at 48 hours PI. However one must remember that in comparing growth cycles of viruses, the initial multiplicity of infection of the culture will have a bearing on the rapidity of CPE and

the occurrence of maximum titer in the supernatant fluids.

Some virus was detectable in early extracellular and cell-associated samples when theoretically not enough time had elapsed for viral replication. Apparently the 3 cycles of washings following the initial adsorption period does not remove all of the "free" virus and some remains with the cell layer. This "free" virus could represent some that was adsorbed and subsequently released into the supernatant fluids of the samples collected at 0, 3, 6 and 9 hours' PI. The small amount of virus in the cell-associated phase at 0, 3 and 6 hours' PI could represent adsorbed virus which was not washed off or possibly virus that had not degraded prior to the eclipse phase. For these reasons, an accurate determination of the first appearance of cell associated and extracellular virus could not be made. However, an appreciable amount of cell associated virus was present at 9 hours' PI and reached a maximum titer at 18 hours' PI. An increase in detectable extracellular virus was noted at 12 hours PI with a rapid rise to near maximum virus yield at 24 hours' PI. Using bovine testicular cells, Gratzek (28) was able to detect cell associated C24V virus at 8 hours' PI and extracellular virus as early as 12 hours' PI. In experiments conducted by Gillespie et al. (26) with C24V virus, the cell associated virus curve followed a

similar pattern as the extracellular virus curve, but failed to reach as high titers.

It would appear that bovine viral diarrhea viruses contain lipids which are essential for infectivity. The sensitivity of these viruses to treatment with ether or chloroform in this study compares with those reported by Hermodsson and Dinter (32) and confirmed by Gillespie et al. (26). However, Taylor et al. (58) reported C24V virus as being stable in 20% ether at 4 C for 24 hours. Even though the infective virus particles of NADL-MD are sensitive to chloroform no decrease in complement-fixing activity of the soluble antigen is apparent following inactivation of the infective virus particles. Chloroform treatment appears to be a more suitable procedure for establishing the sensitivity of viruses to lipid solvents because it requires smaller quantities, shorter incubation period and is more readily separable from the virus containing fluids as compared to ether treatment.

It is apparent from this study that NADL-MD virus will multiply in tissue cultures containing inhibitors of DNA synthesis. However the control DNA virus, vaccinia, failed to replicate but parainfluenza 3, a RNA virus, showed nearly normal rate of growth. These results are similar to those found for C24V virus by Dinter (17) and Ditchfield and

Doane (19). It can be concluded that BVD-MD agents are RNA viruses.

At the present time, the size and structure of BVD-MD viruses have not been adequately determined. In this study, both NADL-MD and C24V viruses readily passed the 100 mμ millipore filter and reduced amount passed the 50 mμ filter. These results are in accord with those reported by Hermodsson and Dinter (32) for C24V virus. However, Taylor et al. (58) reported C24V virus to pass through the 100 mu filter but not the 50 mu filter. Using ultra-thin sections of C24V virus infected BEK cells, Hermodsson and Dinter (32) observed spherical particles approximately 40 mu in size by electron microscopy. However they could not be assured that these particles were the viruses. Recently, Ditchfield and Doane (19) observed particles with helical internal components approximately 150 to 250 mμ in size in ultra-thin sections of infected BEK cells. They suggest that the viruses of BVD are members of the myxovirus group and closely resemble the rinderpest-measles-distemper subgroup. Even though they had no serological evidences that the viruses they were working with were BVD agents, it would seem highly unlikely that the yield of infective particles which passed the 100 mμ and 50 mμ filters could be accounted for solely by infectious RNA. It appears

that further work will be necessary to elucidate the structure and size of these viruses.

The thermal inactivation of NADL-MD and C24V viruses is enhanced in the presence of molar solutions of Ca++ or Mg++ when incubated for 1 hour at 50 C. The results of this study compares closely with those reported by Dinter (17) for C24V, however Ditchfield and Doane (19) found C24V to be completely inactivated in the presence of the cationic solution or in the control after incubation for 1 hour at 50 C.

Since high concentrations of cations stabilizes enteroviruses (64) but enhance thermal inactivation of the NADL-MD virus, this is additional evidence that the viruses of BVD-MD complex are not members of the enterovirus group.

A considerable variation in the thermal inactivation rate of BVD viruses have been reported in the literature. Taylor et al. (58) found that C24V virus would not survive at 56 C for periods longer than 20 to 30 minutes. Ditchfield and Doane (19) reported it to be completely inactivated at 50 C for 1 hour. In contrast, Gratzek (28) observed a biomodial inactivation curve with a small amount of infective virus remaining after 1 hour's incubation at 58 C. Preliminary studies with NADL-MD virus in tissue culture medium indicated the thermal inactivation pattern was similar to that reported by Gratzek for C24V virus,

however it was noted that a considerable variation in the pH of the medium occurred during the incubation period. For this reason, the thermal inactivation of NADL-MD virus was determined in a buffer solution which did not vary in pH during the incubation period. In a buffer solution at pH 7.0, the virus was inactivated at a logarithmic rate during the first 45 minutes at 56 C and at a slower rate thereafter. A small amount of infective virus was detectable after 2 hours' incubation. This two-component curve indicates the possible presence of heat stable variants. The thermal inactivation is greatly enhanced in buffer solutions of pH 5.0 or below as compared to the virus in pH 7.0 buffer solution. The virus appears to be acid labile since it is unstable in buffer solution at pH 3.0. NADL-MD virus appears to be relatively stable to thermal inactivation when the pH of the medium is carefully controlled as compared to inactivation in tissue culture medium.

The susceptibility of arboviruses to enzymatic action of trypsin is a useful criterion in the differentiation of members of the A and B groups. Cheng (8) has shown that the B group of arboviruses, unlike the A group, are sensitive to the enzymatic action of trypsin. Dinter (17) has reported C24V virus to be moderately sensitive to the effects of trypsin. The results of this study substantiates those of Dinter in regard to C24V virus and further supports

the similarity of NADL-MD agent to the BVD-MD group. In this characteristic, the BVD agents resemble the B group of arboviruses more closely than the A group.

Hemagglutinin production was not detectable with NADL-MD virus by the technique employed in this study. However this does not exclude the possibilities of their presence since an exhaustive study was not done. Also Taylor et al. (58) found no hemadsorption of guinea pig erythrocytes to C24V virus infected tissue cultures.

Both sedimentation in a sucrose density gradient and chromatographic separation appear to be excellent procedures for the separation of soluble antigen from the infective virus particles. These techniques, alone or in combination with others, could be of use in future studies of BVD-MD viruses. With purified viral components, mono-specific antiserums could be prepared and utilized in studies to better characterize the viral components as well as to elucidate the role of each antigen in the production of antibody in animals.

The results of this study are suggestive that the strains of BVD-MD viruses share closely related if not identical soluble complement-fixing antigens but vary somewhat in neutralizing antigens. In neutralization studies, homologous virus and antibody systems usually yield the highest neutralization titer, whereas

complement-fixation titers are similar regardless of whether the soluble antigen is prepared from homologous or heterologous BVD viruses.

The relationship of BVD-MD viruses to hog cholera viruses appears to be limited to the sharing of soluble antigens. No antigenic relationship to other viruses was found in this study by the techniques employed.

The NADL-MD virus was recovered during 3 weeks PI by culturing the buffy coat cells from an experimentally infected calf. Because of the presence of significant levels of antibodies 2 weeks PI, this recovery of the agent would indicate an intracellular site for the virus in the circulatory system. The isolation of the NADL-MD virus by culturing the marrow of the calf's femur at necropsy is of considerable interest. Even though the virus was not isolated from any other tissues, this would indicate the persistence of the virus even in the presence of high levels of circulating antibodies. However, the bone marrow is known to have decreased circulation which could possibly allow the virus to reside and multiply in the bone marrow cells without being neutralized. The virus could have been residual virus following the second injection 4 weeks prior to necropsy, however no virus was isolated by buffy coat cultures nor did the animal exhibit a temperature rise or leukopenia suggestive of viremia.

The high levels of antibodies maintained after clinical recovery, especially the complement-fixing antibodies, is added evidences of a "carrier" state.

In recent years, the characterization and classification of animal viruses have been the concern of many prominent researchers in the field of virology. Cooper (11) and Andrewes et al. (1) have emphasized the importance of stable physical and chemical properties such as nucleci acid type, size, structure and ether sensitivity, as prime characteristics to be used for virus differentiations.

Although the knowledge of the physical, chemical and serological properties of the viruses in the BVD-MD group is far from complete, an attempt to classify these agents according to the scheme of Cooper (11) and modified by Hamparian et al. (31) could be useful in a better understanding of these viruses.

The NADL-MD strain of BVD-MD virus has RNA as its nucleic acid type which is the first criterion of the classification scheme. The agent is ether and chloroform sensitive and is acid labile, which are secondary and tertiary criteria. From the preceding characteristics, this virus should tentatively fall into the arbovirus or myxovirus groups. The arboviruses usually have a size of 30-50 mμ with a range of 20 to 100 mμ. Whereas, the myxoviruses are in the size range of 100-120 mμ but

varying from 50 to 500 mμ in some filementous forms. It would appear that the size of the NADL-MD agent would approximate that of the arbovirus group rather than the myxoviruses. Using trypsin sensitivity as a characteristic to differentiate group A from group B arboviruses, NADL-MD virus reacts similar to the B group.

Various workers have suggested different classifications for the BVD-MD group of viruses. Taylor et al. (58) suggested that they belong to the adenovirus group, Dinter (17) thought they were similar to the arbovirus group, and Ditchfield and Doane (19) suggested they resembled myxoviruses.

Since the BVD-MD viruses are sensitive to both ether and chloroform and are RNA viruses, these characteristics alone would exclude them from the adenovirus group.

Both arboviruses and myxoviruses are of RNA type, ether labile and acid labile; the same properties exhibited by the BVD-MD agents. The viruses appear to be in the general size range of the arboviruses, however a great variation in size exists in both the arbovirus and myxovirus groups.

The myxoviruses exhibit properties of hemagglutination, of hemadsorption and of virus elution. These properties have not been demonstrated for the NADL-MD virus. However hemagglutinins have not been detected from canine distemper and rinderpest, which are exceptions to the myxovirus group.

The characteristic cytopathology produced by myxoviruses consists mainly of cell lysis with the formation of giant cells or syncytia. In the author's experiences, no giant cells or syncytial formation have been observed with NADL-MD virus in tissue culture systems.

It appears that the general characteristics of the BVD-MD agents resemble more closely those of the group B arboviruses than any other group. However, it is not known if arthropods are associated with the BVD-MD syndrome which is a distinctive characteristic of the arboviruses. Even though no relationship was demonstrated between NADL-MD virus and broad spectrum group B arbovirus antiserum and complement-fixing antigen, this would not exclude them from the arbovirus group.

Our knowledge is insufficient at the present time to classify these viruses, however it appears they most closely resemble the group B arboviruses. Since BVD-MD and hog cholera viruses have similar physical and chemical properties and have a common soluble antigen, it would appear that they might eventually be placed in the same subgroup. However, the final classification of these viruses will require further intensive investigation.

SUMMARY AND CONCLUSIONS

The physical, chemical and antigenic characteristics of a previously undescribed virus (NADL-MD) isolated from a clinical case of mucosal disease are reported.

The growth cycle of NADL-MD virus was determined in BEK cell cultures. The first cell associated virus appeared 9 hours PI and reached a peak at 18 hours. The first extracellular virus was observed 12 hours' PI and over 99% of the total virus yield was released into the extracellular fluids at 24 hours at a time when the cultures evidenced approximately 50% CPE. The cell sheet was completely destroyed in 48 hours.

NADL-MD virus was sensitive to treatment with 20% ether for 18 hours at 4 C. Likewise, it was inactivated after exposure to 5% chloroform for 10 minutes.

The NADL-MD virus replicated in BEK cell cultures in the presence of IUDR and BUDR, inhibitors of DNA synthesis. Vaccinia, a DNA virus, failed to multiply in the presence of these inhibitors whereas parainfluenza 3, an RNA virus, showed normal replication.

Infective tissue culture fluids of NADL-MD virus readily passed the 220 mμ and 100 mμ millipore filters. A reduced amount of infective virus passed the 50 mμ filter, but no virus was detectable in the filtrate when a 10 mμ filter was used.

The thermal inactivation of the virus at 50 C was enhanced in the presence of molar Ca++ or Mg++ solutions, whereas high concentrations of cations stabilizes enteroviruses to thermal inactivation.

NADL-MD virus was relatively thermostable at 56 C in buffer solutions at pH 6.0 to 8.0, with a small amount of infective virus remaining after 2 hours' incubation in a buffer solution at pH 7.0. A two component inactivation curve indicated possible heat stable varients. The inactivation of the virus was greatly enhanced in solutions of pH 5.0 or below.

The virus was moderately sensitive to the enzymatic action of 1% trypsin when incubated for 1 hour at 37 C.

No hemagglutination was detectable when various antigen preparations were tested with a variety of different species' erythrocytes and incubated at room temperature for 2 hours or at 4 C for 18 hours.

The soluble antigen was readily separable from the infective virus particles by chromatographic procedure in a DEAE-cellulose column. Increasing molarities of NaCl in 0.01 M tris buffer solution were employed for elution. The soluble antigen was eluted with 0.1 and 0.2 M NaCl solution, while the infective virus particles were eluted with 0.5 M NaCl solution. A "virus trail"

occurred with the subsequent addition of solutions with increasing molarities of NaCl.

Sedimentation in a sucrose density gradient likewise separated the soluble antigen from the infective virus particles. The infective virus particles were located near the bottom of the gradient in the higher densities of sucrose, whereas the soluble antigen was near the top in the lower densities. In the control tubes, the bovine albumin fraction was located near the top of the gradient, which corresponded closely to the position of the soluble antigen fraction.

NADL-MD virus is antigenically related to other strains of BVD-MD viruses as determined by neutralization and complement-fixation tests. Homologous virus and antiserum systems have higher neutralizing titers as compared to heterologous systems. However, no differentiation could be made on this basis among the strains of cytopathogenic or noncytopathogenic viruses. NADL-MD, C24V and noncytopathogenic CG-1220 viruses possess common soluble antigens as determined by the complement-fixation test using anti-NADL-MD and anti-CG-1220 serums.

No relationship was detectable with other viruses by neutralization or complement fixation except with hog cholera. The anti-hog cholera serum had a high level of complement-fixing antibodies when tested against the

soluble antigen of NADL-MD virus, whereas no neutralizing antibodies were detectable when tested against infective NADL-MD virus.

A calf experimentally infected with NADL-MD virus responded with a typical diphasic temperature reaction which was accompanied by a marked depression in the number of circulating leukocytes. Complement-fixing antibodies appeared prior to the neutralizing antibodies, but both reached a peak 6 weeks PI. A rapid rise in neutralizing antibodies was observed following the second injection of NADL-MD virus with no apparent effect on the complement-fixing antibody level. The virus was recovered by culturing the buffy coat cells during 3 weeks PI, at times when significant levels of both neutralizing and complement-fixing antibodies were present. The virus was also recovered by culturing the calf's femor at necropsy, 16 weeks after inoculation.

The Oregon C24V bovine viral diarrhea virus, which was used as a control in some of the procedures, was found to have similar properties to the NADL-MD strain with the exception that the cytopathology produced in tissue cultures was not as pronounced or complete as that of NADL-MD virus.

The general characteristics of the NADL-MD virus definitely places it in the BVD-MD group of viruses, and resembles most closely those of the group B arboviruses.

PART II. HERD STUDIES

MATERIALS AND METHODS

After the occurrence of the clinical case of "mucosal disease" and subsequent isolation of the NADL-MD virus, a virological and serological study was conducted on the involved herd.

Herd History

The animal which exhibited clinical signs suggestive of MD and yielded the NADL-MD virus at necropsy in January 1962, was in a group of 20 yearling Holstein heifers in the experimental herd at the National Animal Disease Laboratory. The experimental herd originated at the research station in Beltsville, Maryland, and had no additions in the past 70 years. The herd's history gave no indication of the presence of bovine viral diarrhea-mucosal disease syndrome except varying degrees of diarrhea were observed in the calf crop for a 2 to 3 week period in early 1961. The herd was moved to the new facilities at Ames, Iowa, in May 1961.

Examination of the remaining 19 heifers following the occurrence of the clinical case revealed no clinical signs that were suggestive of the BVD-MD syndrome.

Virus Isolation

Individual buffy coat cultures were prepared from citrated blood collected from the remaining 19 heifers.

Buffy coat cells were collected at intervals for 4 months following the clinical case of MD: approximately 2 months (3/20/62), 3 months (4/25/62) and 4 months (5/24/62). The cultures were prepared as described in part I with the exception that SPF calf serum was incorporated into the growth medium. After 5 to 7 days' incubation, the supernatant fluids from each culture was serially passed at least 3 times in BEK cell cultures before they were recorded as negative. Any virus recovered was identified by CPE and virus neutralization.

Specimens of nasal secretions and intestinal contents were collected at the same time as the blood for buffy coat culture preparation. The specimens were handled in the same manner as described in part I.

Serum Neutralization

Individual serum samples which had been collected (5/23/61) at Beltsville prior to the herd's movement to Ames were tested for neutralizing antibodies against NADL-MD virus. Serum for neutralization tests was collected at 2, 8, 12, 16 and 28 weeks following the occurrence of the clinical case. The 19 heifers were then used for experimental purposes and no subsequent samples could be obtained. Each serum sample was heat inactivated at 56 C for 30 minutes and serial 2-fold dilutions were prepared.

Neutralization tests were conducted as previously described against 100 $TCID_{50}$ of NADL-MD virus.

RESULTS

Virus Isolation

Buffy coat cultures prepared from the 19 apparently normal heifers 2 months following the clinical case yielded virus from 4 individual animals (4978, 4991, 5008 and 5048). An additional isolation was made from heifer 4993 by buffy coat cultures at 3 months. No isolations were made 4 months following the clinical case (Table 9). All 5 isolates produced CPE characteristic of the original NADL-MD virus and appeared to be identical by virus neutralization.

Viruses were isolated from fecal material from 12 of the 19 heifers. Ten isolations were made 2 months following the clinical case, 9 isolations at 3 months and 2 isolations at 4 months. Usually the same individual animal continued to shed virus in the feces as indicated by consecutive isolations. In general, agents isolated from rectal swabs produce a very rapid and similar type CPE, usually in 24 hours. Two of the isolates, the only 2 tested, were not neutralized by anti-NADL-MD serum. No viruses were recoverable from the nasal secretions.

Serum Neutralization

Serum samples collected from the 20 animals prior to movement to the National Animal Disease Laboratory which was approximately 8 months before the occurrence of the

clinical case revealed the presence of significant amounts of neutralizing antibodies in all but 2 animals, 5030 and 5045. Animal 5030 developed clinical signs of mucosal disease and subsequently yielded the NADL-MD agent, and animal 5045 developed high levels of neutralizing antibody on the first serum collected following the occurrence of the clinical case in 5030. The individual neutralization titers of serum samples collected at various intervals are presented in Table 9. In general, the animals from which viruses were isolated from buffy coat cultures had relatively low levels of neutralizing antibodies present at the time of isolation, but subsequent samples indicated a substantial rise in the antibody levels. The mean neutralization titer of the 5 animals from which virus was isolated from buffy coat cultures is compared with the mean titer of the remaining 14 animals in figure 8 to better illustrate the differences in antibody level.

Table 9. Serum neutralization titers of 20 animal preceding and following the occurrence of a clinical case of mucosal disease

Animal Number	5/23/61	2/8/62	3/20/62[a]	4/25/62[a]	5/24/62[a]	8/22/62
4965	160[b]	104	76	128	256	645
4978	300	58	30[c]	400	2048	2650
4980	840	840	600	256	645	400
4985	75	300	256	160	210	1645
4987	840	1185	1590	840	>4096	>4096
4988	635	400	855	635	2650	1210
4991	75	30	58[c]	840	>4096	>4096
4993	645	160	75	30[c]	2650	3600
4994	2650	2650	3600	3600	>4096	2650
4998	855	1190	3600	2650	>4096	1200
5004	>4096	2650	3600	>4096	>4096	>4096
5008	30	58	30[c]	1024	>4096	>4096
5017	600	840	1600	3600	>4096	>4096
5023	210	840	1024	1600	2650	3600
5024	600	840	300	2650	>4096	4096

[a]Buffy coat cultures were prepared only at these times.

[b]Titers expressed as reciprocal of final serum dilution giving 50% neutralization.

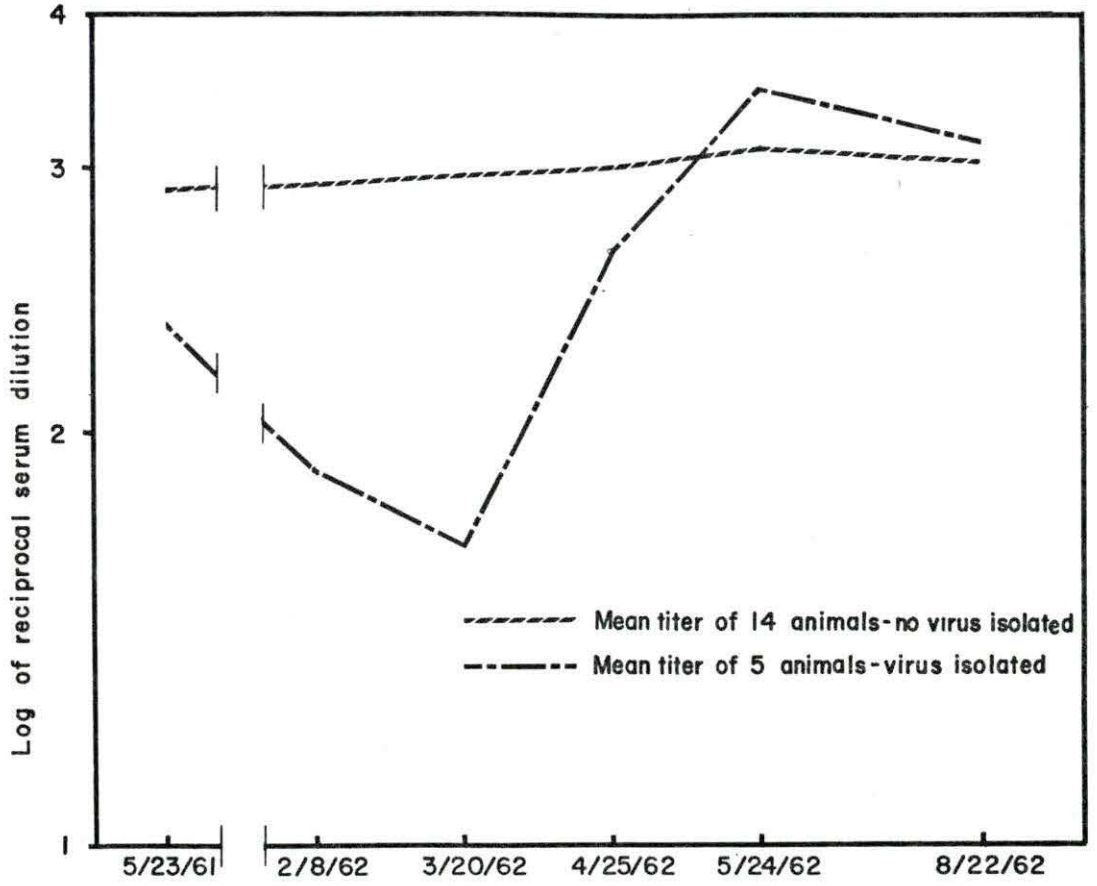
[c]Virus identical to NADL-MD were isolated from buffy coat cultures of these animals.

Table 9 (Continued)

Animal Number	5/23/61	2/8/62	3/20/62[a]	4/25/62[a]	5/24/62[a]	8/22/62
5030	< 4[d]	-	-	-	-	-
5033	256	600	210	840	2650	3600
5037	840	1024	600	1200	3600	>4096
5045	< 4	1024	640	1024	>4096	>4096
5048	160	25	16[b]	1024	>4096	>4096

[d]Animal which succumbed to mucosal disease and yielded NADL-MD virus.

Figure 8. Comparison of mean neutralization titers of animals from which NADL-MD virus was isolated with those of animals from which no virus was isolated


4
3
2
1
Log of reciprocal serum dilution
5/23/61
2/8/62
3/20/62
4/25/62
5/24/62
8/22/62
Mean titer of 14 animals-no virus isolated
Mean titer of 5 animals-virus isolated

DISCUSSION

It is apparent from this study that "normal" cattle may be "carriers" of the virus without showing clinical signs of the BVD-MD syndrome. Why one animal had such marked lesions and finally succumbed to the disease whereas other animals in the herd failed to show any clinical evidence of the syndrome is still unknown.

It has been observed by various researchers (4, 18, 59) that animals that show clinical signs of "mucosal disease" and subsequently succumb, fail to develop any neutralizing or complement-fixing antibodies and may have a viremia for a considerable period of time. Whereas others animals in the herd or in other herds may show only a mild clinical form of the disease and quite often appear normal. These animals usually develop high levels of neutralizing and complement-fixing antibodies.

In this study, the virus that was isolated from the clinical case was also recovered from 5 "normal" cattle. However, the 5 "normal" cattle had low levels of neutralizing antibodies present at the time of virus isolation, which increased in subsequent serum samples.

The various immunological and clinical manifestations present in the BVD-MD complex appears to have similarity to the variety of responses which occur in LCM virus

infections of mice. Mice can be infected in utero with LCM virus and carry the agent for many months. Inoculation of newborn mice may show only a slight affect whereas the same dose of virus given to adults approaches 100% mortality. The mice may become immune tolerant and have a persistent virus infection. The LCM virus can cause an extremely wide spectrum of clinical and pathological effects in the adults, ranging from inapparent, long-lasting infections through mild to severe illness with almost 100% mortality (34). The mechanism of immune tolerance in LCM virus infected mice might be similar to that which occurs in the BVD-MD syndrome.

The viruses isolated from the fecal material in this study are probably enteric cytopathogenic bovine orphan viruses and no serological association with the disease agent could be determined.

Further research is required to elucidate the mechanism of "immunological tolerance or paralysis" in animals which fail to develop specific antibodies and finally succumb to the BVD-MD syndrome, and why other animals may show only a mild syndrome and respond with antibody formation.

SUMMARY

A study was conducted on the herd from which the NADL-MD virus was recovered from a clinical case of MD.

Viruses identical to the NADL-MD agent were isolated from 4 animals by culturing their buffy coat cells 2 months following the occurrence of the clinical case and from a 5th animal at 3 months. No virus was recoverable from the 19 animals by buffy coat cultures collected 4 months following the clinical case.

Serum samples collected approximately 8 months prior to the occurrence of the clinical case revealed that all but 2 of the 20 animals had significant levels of neutralizing antibodies present against NADL-MD agent. Of the 2 animals which had no detectable neutralizing antibodies, one developed clinical signs of mucosal disease and subsequently yielded the NADL-MD agent and the other had significant levels of antibodies when tested at the time of the occurrence of the clinical case.

In general, the 5 animals from which the NADL-MD agent was isolated had very low levels of neutralizing antibodies prior to and at the time of virus recovery. However a significant rise in antibody levels occurred in subsequent serum samples.

Viruses were recovered from fecal material from 12 of the 19 animals. However, these viruses produced rapid CPE

and did not appear to be serologically related to the NADL-MD virus. In general, the animals from which viruses were isolated usually continued to shed virus in the feces as indicated by consecutive isolations from the same individual.

No clinical signs suggestive of the BVD-MD syndrome were observed in the remaining 19 animals following the occurrence of the clinical case.

LITERATURE CITED

1. Andrewes, C. H., Burnet, F. M., Enders, J. F., Gard, S., Hirst, G. K., Kaplan, M. M. and Zhdanor. Taxonomy of viruses infecting vertebrates: present knowledge and ignorance. Virology 15: 52-55. 1961

2. Andrewes, C. H. and Horstmann, D. M. The susceptibility of viruses to ethyl ether. General Microbiology Journal 3: 290-297. 1949.

3. Baker, J. A., York, C. J., Gillespie, J. H. and Mitchell, G. Virus diarrhea in cattle. American Journal Veterinary Research 15: 531-535. 1954.

4. Bechenhauer, W. H., Brown, A. L., Lidolph, A. A. and Norden, C. J., Jr. Immunization of swine against hog cholera with a bovine enterovirus. Veterinary Medicine 56: 108-112. 1961.

5. Borgen, H. C. Mucosal disease in Dänemark. II. Virologische und serologische befunde in einem bestand. Nordisk Veterinarmedicin 15: 346-356. 1963.

6. Boulanger, P. and Bannister, G. L. A modified direct complement fixation test for the detection of antibodies in the serum of cattle previously infected with vesicular stomatitis virus. Journal of Immunology 85: 368-374. 1960.

7. Brakke, M. K. Density gradient centrifugation: a new separation technique. American Chemical Society Journal 73: 1847-1848. 1951.

8. Cheng, P.-Y. The inactivation of group B arthropod-borne animal viruses by proteases. Virology 6: 120-136. 1958.

9. Coggins, L. Standardization of virus-neutralization test for bovine virus diarrhea. American Journal Veterinary Research 25: 103-107. 1964.

10. Coggins, L. and Seo, S. Serological comparison with rabbit antisera of hog cholera virus and bovine virus diarrhea virus. Society Experimental Biology and Medicine Proceedings 114:778-780. 1963.

11. Cooper, P. D. A chemical basis for the classification of animal viruses. Nature 190: 302-305. 1961.

12. Cowan, K. M. Immunological studies on african swine fever virus. I. Elimination of the procomplementary activity of swine serum with formalin. Journal of Immunology 86: 467-470. 1961.

13. Cunningham, C. H. A laboratory guide in virology. 4th ed. Minneapolis, Minn., Burgess Publishing Company. 1960.

14. Darbyshire, J. H. Agar gel diffusion studies with a mucosal disease of cattle. I. Preliminary results with the technique. Research in Veterinary Science 3: 118-124. 1962.

15. Darbyshire, J. H. Agar gel diffusion studies with a mucosal disease of cattle. II. A serological relationship between a mucosal disease and swine fever. Research in Veterinary Science 3: 125-128. 1962.

16. Darbyshire, J. H. A serological relationship between swine fever and mucosal disease of cattle. Veterinary Record 72: 331. 1960.

17. Dinter, Z. Relationship between bovine virus diarrhoea virus and hog cholera virus. Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene 188: 475-486. 1963.

18. Dinter, Z., Hansen, H.-J. and Roneus, O. Untersuchungen uber mucosal disease. I. Isolierung und identifizierung des virus der virusdiarrhoe. Zentralblatt fur Veterinarmedizin 9: 739-747. 1962.

19. Ditchfield, J. and Doane, F. W. The properties and classification of bovine viral diarrhea virus. Canadian Journal Comparative Medicine and Veterinary Science 28: 148-152. 1964.

20. Feldman, H. A. and Wang, S. S. Sensitivity of various viruses to chloroform. Society Experimental Biology and Medicine Proceedings 106: 736-738. 1961.

21. Fernelius, A. L. Noncytopathogenic bovine viral diarrhea viruses detected and titrated by immunofluorescence. Canadian Journal Comparative Medicine and Veterinary Science 28: 121-126. 1964.

22. Gillespie, J. H. and Baker, J. A. Studies on virus diarrhea. Cornell Veterinarian 49: 439-443. 1959.

23. Gillespie, J. H., Baker, J. A. and McEntree, L. A cytopathogenic strain of virus diarrhea virus. Cornell Veterinarian 50: 73-79. 1960.

24. Gillespie, J. H., Coggins, L., Thompson, J. and Baker, J. A. Comparison by neutralization tests of strains of virus diarrhea and mucosal disease. Cornell Veterinarian 51: 155-159. 1961.

25. Gillespie, J. H., Madin, S. H. and Darby, N. B., Jr. Cellular resistance in tissue culture, induced by noncytopathogenic strains, to a cytopathogenic strain of virus diarrhea virus of cattle. Society Experimental Biology and Medicine Proceedings 110: 248-250. 1962.

26. Gillespie, J. H., Madin, S. H. and Darby, N. B., Jr. Studies on virus diarrhea virus of cattle, with special reference to its growth in tissue culture and its ether susceptibility. Cornell Veterinarian 53: 276-282. 1963.

27. Gornall, A. G., Bardawill, C. J. and David, M. M. Determination of serum proteins by means of the biuret reaction. Biological Chemistry Journal 177: 751-766. 1949.

28. Gratzek, J. B. Characteristics of two bovine viral diarrhea agents. Unpublished Ph.D. thesis, Madison, Wisconsin, Library, University of Wisconsin. 1962.

29. Gutekunst, D. E. and Malmquist, W. A. Complement-fixing and neutralizing antibody response to bovine viral diarrhea and hog cholera antigens. Canadian Journal Comparative Medicine and Veterinary Science 28: 19-23. 1964.

30. Gutekunst, D. E. and Malmquist, W. A. Separation of a soluble antigen and infectious particles of bovine viral diarrhea viruses and their relationship to hog cholera. Canadian Journal Comparative Medicine and Veterinary Science 27: 121-123. 1963.

31. Hamparian, V. V., Hilleman, M. R. and Ketler, A. Contributions to characterization and classification of animal viruses. Society Experimental Biology and Medicine Proceedings 112: 1040-1050. 1963.

32. Hermodsson, S. and Dinter, Z. Properties of bovine virus diarrhoea virus. Nature 194: 893-894. 1962.

33. Herrmann, E. C., Jr. Plaque inhibition test for detection of specific inhibitors of DNA containing viruses. Society Experimental Biology and Medicine Proceedings 107: 142-145. 1961.

34. Hotchin, J. The biology of lymphocytic choriomeningitis infection: virus-induced immune disease. Cold Spring Harbor Symposia on Quantitative Biology 27: 479-499. 1962.

35. Hoyer, B. H., Bolton, E. T., Ormsbee, R. A., LeBouvier, G., Ritter, D. B. and Larson, C. L. Mammalian viruses and richettsiae. Science 127: 859-863. 1958.

36. Inaba, Y., Omori, T. and Kumagai, T. Detection and measurement of non-cytopathogenic strains of virus diarrhea of cattle by the END method. Archiv für die Gesamte Virusforschung 13: 425-429. 1963.

37. Kabat, E. A. and Mayer, M. M. Experimental immunochemistry. 2nd ed. Springfield, Illinois, Charles C. Thomas. 1961.

38. Kniazeff, A. J., Huck, R. A., Jarrett, W. F. H., Pritchard, W. R., Ramsey, F. K., Schipper, I. A., Stober, V. M. and Liess, B. Antigenic relationships of some bovine viral diarrhea-mucosal disease viruses from the United States, Great Britain and West Germany. Veterinary Record 73: 768-769. 1961.

39. Kniazeff, A. J. and Pritchard, W. R. Antigenic relationships in the bovine viral diarrhea-mucosal disease complex. United States Livestock Sanitary Association Proceedings 1960: 344-350. 1960.

40. Kumagai, T., Morimoto, T., Shimizu, T., Sasahara, J. and Watanabe, M. Antigenic relationship between hog cholera virus and bovine viral diarrhea virus as revealed by cross-neutralization. National Institute Animal Health Quarterly 2: 201-206. 1962.

41. Lee, K. M. and Gillespie, J. H. Propagation of virus diarrhea virus of cattle in tissue culture. American Journal Veterinary Research 18: 952-956. 1957.

42. Malmquist, W. A. and Hay, D. Hemadsorption and cytopathic effect produced by african swine fever virus in swine bone marrow and buffy coat cultures. American Journal Veterinary Research 21: 104-108. 1960.

43. Mengeling, W. L., Gutekunst, D. E., Fernelius, A. L. and Pirtle, E. C. Demonstration of an antigenic relationship between hog cholera and bovine viral diarrhea viruses by immunofluorescence. Canadian Journal Comparative Medicine and Veterinary Science 27: 162-164. 1963.

44. Noice, F. and Schipper, I. A. Isolation of mucosal disease virus by tissue cultures in mixture 199, Morgan, Morton and Packer. Society Experimental Biology and Medicine Proceedings 100: 84-86. 1959.

45. Olafson, P., MacCallum, A. D. and Fox, F. H. An apparently new transmissible disease of cattle. Cornell Veterinarian 36: 205-213. 1946.

46. Olafson, P. and Rickard, C. G. Further observations on the virus diarrhea (new transmissible disease) of cattle. Cornell Veterinarian 37: 104-106. 1947.

47. Peterson, E. A. and Sober, H. A. Chromatography of proteins. I. Cellulose ion-exchange adsorbents. American Chemical Society Journal 78: 751-755. 1956.

48. Pritchard, W. R., Taylor, D. B., Moses, H. E. and Doyle, L. P. A transmissible disease affecting the mucosae of cattle. American Veterinary Medical Association Journal 128: 1-5. 1956.

49. Ramsey, F. K. The pathology of a mucosal disease. American Veterinary Medical Association Proceedings 1954: 162-166. 1954.

50. Ramsey, F. K. and Chivers, W. H. Mucosal disease of cattle. North American Veterinarian 34: 629-633. 1953.

51. Reed, L. J. and Muench, H. A simple method of estimating fifty percent endpoints. American Journal Hygiene 27: 493-497. 1938.

52. Richter, W. R. The pathology of experimental infection produced by a filterable agent, isolated from a field case of mucosal disease of cattle. Unpublished M.S. thesis. Ames, Iowa, Library, Iowa State University of Science and Technology. 1962.

53. Robson, D. S., Gillespie, J. H. and Baker, J. A. The neutralization test as an indicator of immunity to virus diarrhea. Cornell Veterinarian 50: 503-509. 1960.

54. Salzman, N. P. The rate of formation of vaccinia deoxyribonucleic acid and vaccinia virus. Virology 10: 150-152. 1960.

55. Schmidt, N. J. and Lennette, E. H. A complement fixation test for polionyelitis. Experimental Medicine Journal 102: 133-150. 1955.

56. Sheffy, B. E., Coggins, L. and Baker, J. A. Relationship between hog cholera virus and virus diarrhea virus of cattle. Society Experimental Biology and Medicine Proceedings 109:349-352. 1962.

57. Sober, H. A., Gutter, F. J., Wyckoff, M. M. and Peterson, E. A. Chromatography of proteins. II. Fractionation of serum proteins on anion-exchange cellulose. American Chemical Society Journal 78: 756-763. 1956.

58. Taylor, D. O. N., Gustafson, D. P. and Claflin, R. M. Properties of some viruses of the mucosal disease-virus diarrhea complex. American Journal Veterinary Research 24: 143-149. 1963.

59. Thomson, R. G. and Savan, M. Studies on virus diarrhea and mucosal disease of cattle. Canadian Journal Comparative Medicine and Veterinary Science 27: 207-214. 1963.

60. Tyler, D. E. Comparative pathologic, immunologic and clinical responses produced by selected agents of the bovine mucosal disease-viral diarrhea complex. Unpublished Ph.D. thesis. Ames, Iowa, Library, Iowa State University of Science and Technology. 1963.

61. Tyler, D. E. The influence of avitaminosis A on the susceptibility of calves to mucosal disease. Unpublished M.S. thesis. Lafayette, Indiana, Library, Purdue University. 1960.

62. Underdahl, N. R., Grace, O. D. and Hoerlein, A. B. Cultivation in tissue-culture of cytopathogenic agent from bovine mucosal disease. Society Experimental Biology and Medicine Proceedings 94: 795-797. 1957.

63. Walker, R. V. L. and Olafson, P. Failure of virus diarrhea of cattle to immunize against rinderpest. Cornell Veterinarian 37: 107-111. 1947.

64. Wallis, C. and Melnick, J. L. Cationic stabilization--a new property of enteroviruses. Virology 16: 504-506. 1962.

65. Youngner, J. S. Monolayer tissue cultures. I. Preparation and standardization of suspensions of trypsin-dispersed monkey kidney cells. Society Experimental Biology and Medicine Proceedings 85: 202-205. 1954.

ACKNOWLEDGMENTS

The author wishes to thank Doctor R. A. Packer for his excellent guidance and endless hours of counseling throughout the work and during the preparation of the manuscript.

Thanks also go to Doctor W. A. Malmquist for his inspiration and helpful suggestions during the research.

The author wishes to acknowledge the assistance of the central service branch at the National Animal Disease Laboratory in the preparation of tissue cultures and some of the reagents used in this study, and to Mr. Ralph Glaizer for the excellent photographs.

Lastly, thanks goes to my wife for her help in the preparation of the manuscript and her patience and understanding which made this work possible.

APPENDIX

Hanks balanced salt solution (HBSS)

NaCl	8.00 grams per liter
KCl	0.40 " " "
$MgSO_4.7H_2O$	0.20 " " "
$Na_2HPO_4.H_2O$	0.06 " " "
Glucose	1.00 " " "
KH_2PO_4	0.06 " " "
$CaCl_2$	0.14 " " "
$NaHCO_3$	0.35 " " "

Earle balanced salt solution (EBSS)

NaCl	6.80 grams per liter
KCl	.40 " " "
$MgSO_4$	.10 " " "
NaH_2PO_4	.125 " " "
$NaHCO_3$	2.20 " " "
Glucose	1.00 " " "
$CaCl_2$	10.0 " " "
Lactalbumin hydrolysate	5.0 " " "

Eagle Basal Medium (BSM)

NaCl	6.8 grams per liter
KCl	.4 " " "
$NaH_2PO_4.H_2O$	.14 " " "
$NaHCO_3$	2.2 " " "
$CaCl_2$	0.2 " " "

Eagle Basal Medium (EBM) (Continued)

$MgCl_2.6H_2O$	0.17 grams per liter
Glucose	1.0 " " "
Arginine	.021 " " "
Cystine	.012 " " "
Histidine	.008 " " "
Isoleucine	.026 " " "
Leucine	.026 " " "
Lysine	.026 " " "
Methionine	.008 " " "
Phenylalanine	.016 " " "
Threonine	.024 " " "
Tryptophan	.004 " " "
Tyrosine	.018 " " "
Valine	.024 " " "
Glutamine	.300 " " "
Biotin	1.0 milligrams per liter
Choline	1.0 " " "
Folic Acid	1.0 " " "
Nicotinamide	1.0 " " "
Pantothenic Acid	1.0 " " "
Pyridoxal	1.0 " " "
Thiamin	1.0 " " "
Riboflavin	0.1 " " "
Phenol Red	.04 " " "

GKN solution

NaCl	8.0 grams per liter
KCl	0.4 " " "
Glucose	1.0 " " "

Trypsin solution

Trypsin	2.0 grams per liter
NaCl	8.0 " " "
KCl	0.4 " " "
Glucose	1.0 " " "
$NaHCO_3$	0.84 " " "

Dulbecco phosphate-buffered saline (PBS)

NaCl	8.0 grams per liter
KCl	0.2 " " "
Na_2HPO_4	1.15 " " "
KH_2PO_4	0.2 " " "
$CaCl_2$	0.1 " " "
$MgCl_2.6H_2O$	0.1 " " "

Modified Alsever's solution

Glucose	20.5 grams per liter
Sodium citrate	8.0 " " "
NaCl	4.2 " " "

Adjust to pH 6.1 with citric acid.

Veronal-buffered saline

NaCl	8.5 grams per liter
Na-5,5-diethyl barbiturate	.0375 " " "

Veronal-buffered saline (Continued)

5,5-diethyl barbituric acid .0575 grams per liter

Add 0.5 ml of a stock solution containing 1.00 M $MgCl_2$ and 0.30 M $CaCl_2$.

Isotonic veronal-acetate buffer solution of Michaelis

Solution A

Sodium acetate	9.714	grams per 500 ml H_2O
Na-5,5-diethyl barbiturate	14.714	" " " " "

Solution B

NaCl	8.5	grams per liter H_2O

To obtain the pH solution desired, add 5 ml of solution A to 2 ml of solution B plus x ml (from table below) of 0.1 Normal hydrochloric acid and 18 minus x ml of H_2O.

x	pH	x	pH
0.0	9.64	6.5	6.12
0.25	9.16	7.0	5.32
0.5	8.90	8.0	4.93
0.75	8.68	9.0	4.66
1.0	8.55	10.0	4.33
2.0	8.18	11.0	4.13
3.0	7.90	12.0	3.88
4.00	7.66	13.0	3.62
5.0	7.42	14.0	3.20
5.5	7.25	15.00	2.62
6.0	6.99	16.0	

All solutions were sterilized by passage through a 220 mμ millipore filter.