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Biological markers for the differentiation of Aujeszky's disease virus strains

by

Kenneth Bradley Platt

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of DOCTOR OF PHILOSOPHY

Department: Veterinary Microbiology and Preventive Medicine Major: Veterinary Microbiology

Approved:

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For the Graduate College

Iowa State University
Ames, Iowa

1977
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INTRODUCTION

During the period from 1969 to 1976 the incidence of Aujeszky's disease (AD) or pseudorabies in the Iowa swine population has increased dramatically. Prior to 1973, an average of five AD outbreaks were confirmed annually by the Veterinary Diagnostic Laboratory at Iowa State University. Twenty-eight outbreaks were confirmed in 1974, 48 in 1975, 165 in 1976 and 145 between January 1 and March 15, 1977. A similar increase in the incidence of this disease has been observed concurrently in other swine producing states (38).

This rise in the incidence of AD is particularly alarming because of the emergence of more virulent Aujeszky's disease virus (ADV) strains. Aujeszky's disease was not considered a severe disease of older pigs in the U.S.A. prior to its current rise in incidence (44). However, the mortality rates apparently have increased in ADV-infected pigs of all ages (38), and reproductive failures in ADV-infected sows are now commonly observed (38, 53). Consequently, swine producers are threatened with severe monetary losses if AD is not controlled. In one study involving 16 AD outbreaks among neighboring farms, the economic loss was conservatively estimated
The successful control and eventual eradication of AD will depend largely on epidemiological investigations. These investigations will be facilitated greatly if ADV strains can be reliably differentiated. The purpose of the following study has been to develop a basis for differentiating ADV strains by their biological markers. An additional goal of this study has been to identify specific markers which may be highly correlated with virulence. Such markers may then be used to screen populations of wild ADV strains that may be suitable vaccine viruses.
LITERATURE REVIEW

The current increase of Aujeszky's disease virus (ADV) infection in the swine population of the United States has created the need for effective control programs. Implicit in such programs are epidemiological investigations which are dependent upon virus strain identification. The following review of the literature directs itself to the task of describing and comparing known and potential biological markers of herpesviruses that may be of use in strain identification.

Social Behavior and Plaque Size

Several investigators have shown that the social behavior of specific cell types infected with some herpesviruses may be characteristic of the strain or type of the infecting virus. To date most studies comparing the social behavior and plaque-forming ability of strains have been directed toward hominid herpesvirus 1 (HH 1), hominid herpesvirus 2 and ADV. The hominid herpesviruses have been extensively reviewed by Nahmias and Dowdle (59). These authors concluded that HH 1 and HH 2 may be consistently identified on the basis of cytopathic effect (CFE) and plaque size. Non-genital
HH 1 strains produce small plaques and CPE characterized by tight adhesion of rounded cells. Hominid herpesvirus 2 strains induce large plaques surrounded by loose aggregates of rounded cells and syncytia. These differences were observed in HeLa, FL amnion, mouse embryo, chick embryo fibroblast (CEF) and rabbit kidney (RK) cells. The magnitude of the differences, however, depended on the cell line used (56).

Although syncytium formation is routinely associated with HH 2 and not with HH 1 strains, exceptions have been noted. No syncytia have been observed in HH 2-infected rabbit cornea fibroblast line cells (SIRC) (72) nor in HEp-2 cells infected with recently isolated HH 2 strains (21). Plummer et al. (62) reported that HH 2 isolates failed to induce syncytia in RK and human lung fibroblast cells. Munk and Donner (56) observed the formation of small syncytia in HeLa cells infected with 35 different HH 1 strains. Another HH 1 strain isolated from a clinical case of herpetic eczema induced large plaques and large syncytia identical to those seen in HH 2-infected cells.

The inconsistent results described above could have been due to differences in virus and cell strains. The level of passage through cells may also influence
the syncytium-inducing ability of specific virus strains. Kohlhage and Schieferstein (45) observed that 13 HH 1 strains induced syncytium formation in RK cells but only after four to nine passages. However, Plummer et al. (62) did not observe syncytium formation or any change in CPE after passaging HH 1 strains up to 20 times in RK cells.

The plaque size and social behavior markers of HH 1 and HH 2 appear to be stable but mutations can occur. No changes in HH 1-induced CPE were observed by Smith et al. (72) following 20 passages in SIRC cells. However, CPE induced by HH 2 was characterized by a slightly greater dispersion of rounded aggregated cells after the same number of passages. As previously mentioned, Plummer et al. (62) saw no changes in CPE in HH 1- and HH 2-infected RK cells after multiple passages. However, in later studies Plummer et al. (63) observed that one of five HH 1 strains induced large plaque and large syncytium formation in RK cells following multiple intracerebral passages in mice. No changes in CPE were observed in RK cells singly infected with the remaining four HH 2 strains. In all cases of mutation, no loss of serological identity was detected (46, 63, 72).
Distinct differences in CPE have been reported for variants of herpes B virus. Falke (22) simultaneously isolated two variants of herpes B virus from uninoculated monkey kidney cells. The FR variant induced the formation of giant cells containing as many as 100 nuclei. Variant rR was characterized by amitotic nuclear divisions that resulted in "small" giant cell formation in KB, HK, MK and FL amnion cells. HeLa cells infected with the rR variant formed giant syncytia. However, when the rR variant was re-isolated from HeLa cells and used to infect KB, HK, MK and FL amnion cells, "small" giant cell formation occurred which indicated that cell type influences phenotypic expression of the CPE marker.

Some success has also been achieved in differentiating strains of infectious bovine rhinotracheitis (IBR) virus by plaque size. Bagust (3) observed that a brain isolate of IBR produced smaller plaques on primary bovine testicle (BT) cells than three IBR strains isolated from outside the central nervous system (CNS). None of these strains differed antigenically. Buening and Gratzek (13) also used BT cells in plaque comparison experiments, but did not find distinct differences between two field isolates and two vaccine
strains of IBR virus.

Several investigators have reported distinct differences in plaque-forming ability and CPE caused by virulent and avirulent ADV strains. These differences were found to be dependent on the host cell type. Zuffa et al. (81) reported that calf kidney (CK) line cells infected with one or more TCID\textsubscript{50} per cell of either virulent or attenuated virus formed syncytia. Syncytia induced by virulent strains contained as many as 12 nuclei, whereas syncytia caused by attenuated strains were characterized by two to three nuclei.

Syncytium formation, however, is not a universal characteristic of cells infected with ADV. Tokumaru (75) compared a virulent and an avirulent variant of the same strain on MK cells. The former induced syncytium formation but the latter did not. Skoda and Jamrichová (70) compared both virulent (CV7D, SUCH-2) and avirulent (SUCH-1, BUK) strains on chick embryo fibroblast (CEF) cells. The CPE produced by all strains was indistinguishable. Infected cells were rounded, refractive and did not form syncytia.

More recently Golais and Sabó (32) compared the CPE induced by five virulent and four vaccine (BUK, SUCH-1, TK 900 and CK-PRV-X) ADV strains in nine
different cell lines. All strains caused syncytium formation in rabbit lung cells (ZP). However, these same strains produced only cell rounding in human amnion (Am), Detroit-6 (D6), BHK-21, L, and HeLa cells. Syncytia were present during the first two to three virus passages in Am, D6 and HeLa cells. Green monkey kidney (GMK) cells infected with virulent strains formed syncytia but responded by rounding when inoculated with the vaccine strains.

Golais and Sabó (32) also demonstrated that the strain of cell line influences CPE. Virulent and vaccine ADV strains caused only cell rounding in the PK-K line of pig kidney cells. In a second pig kidney cell line (PS), vaccine strains also induced cell rounding, but infection with different virulent strains resulted in either cell rounding or syncytium formation.

Two hypotheses have been presented that may account for the differences in social behavior of specific cell lines infected with different herpesvirus strains (8). First, the degree of cell fusion may be directly correlated to the activity of lysosomal enzymes on cytoplasmic membranes. This relationship, however, has not been consistently observed (25). Secondly, differences in social behavior may be due to different
complements of virus-specified glycoprotein that are present on cytoplasmic membranes.

The relationship between plaque size and virulence has been evaluated in different cell types. Tokumaru (75) cloned a small plaque and large plaque variant from MK cells and observed that the former was less virulent for rats than the latter. Bartha's naturally avirulent K strain was cloned as a small plaque variant from PK cells (4). Lomniczi (48) showed that the avirulent KP strain produced small plaques on PK cells and that one virulent strain produced large plaques. However, after evaluating five field isolates before and after laboratory attenuation, Žuffa and Grigelová (80) concluded that plaque size in PK cells was not correlated with virulence. Similarly, Skoda and Jamrichová (70) observed that there was no relationship between plaque size in CEF cells and virulence because small, intermediate and large plaques were produced by virulent and attenuated strains.

Temperature Markers

Effect of temperature on virus

Temperature, through its effect on the viral replicative cycle and/or physical inactivation of
virions has been used to differentiate virus strains within several families of viruses. Fenner (23) distinguished strains of vaccinia by exposing freshly prepared virus to 55°C for 40 minutes and calculating the log decrease in infectivity. Bedson and Dumbell (7) differentiated strains of variola minor in a similar manner. Lomniczi (49) compared Newcastle disease virus by measuring the time required for a two log decrease in virus titer at 50°C. He was able to characterize strains within virulence groups and concluded that thermal resistance was not correlated with virulence. Wild and vaccine strains of poliovirus have been identified by Boué (12) on the basis of yield differences of virus propagated at 36 and 40°C.

Employing an adaptation of Boué's technique, Ratcliff (65) and Longson (51) differentiated HH 1 from HH 2. These workers used an index system that compared virus yields at 39 and 40°C to yields at 34.5°C. Longson categorized strains as cold, warm and hot. Cold strains corresponded to HH 2. Warm isolates and hot strains corresponded to HH 1 isolates and laboratory modified hominid herpesviruses. The differences between yields of hot, warm and cold strains at 39 and 40°C may have been due to heat lability of the strains and/or temperature inhibition
of the replicative process. Figueroa and Rawls (24) speculated that yields of HH 1 strains were higher than yields of HH 2 strains in other growth tests because of the greater thermal stability of HH 1.

Hominid herpesvirus strains 1 and 2 have also been differentiated by the classical temperature sensitivity test. Plummer et al. (62) compared the thermal sensitivity of HH 1 and HH 2 by diluting cell propagated virus 1:10 in medium 199 and then heating the suspensions to 37°C. Ninety percent inactivation of HH 1 and HH 2 occurred within 20 and 10 hours respectively. Other investigators (24, 62, 63) have used similar methods and found that HH 1 strains were more heat resistant than HH 2 strains. However, Smith et al. (72) found that the HH 2 Arch strain was more heat resistant than the HH 1 Del strain.

Differences in heat lability may exist among strains of IBR virus. Buening and Gratzek (13) compared the thermosensitivity of two virulent field isolates and two vaccine strains by exposing virus to 37°C for 24 hours and measuring residual virus activity. No significant differences were observed among one
field isolate and the vaccine strains. The remaining field isolate was found to be slightly more heat labile and antigenically different from the other viruses tested. Bagust (3) heat tested four IBR strains that were slightly different antigenically, but was unable to demonstrate any differences in thermal stability.

Distinct differences in thermosensitivity have been recorded for European ADV strains. Bodon et al. (11) observed that Bartha's attenuated K strain was more heat resistant than a virulent laboratory strain and two field isolates. Bartha et al. (5) suggested that the heat marker was correlated with virulence. However, Tokumaru (75) failed to detect differences in heat sensitivity between an avirulent small plaque variant and a virulent large plaque variant derived from the same ADV strain. Golais and Sabó (31) reported that 9 of 10 virulent, and three of four vaccine strains were heat sensitive. It appears that the heat marker, although not correlated with virulence, may be of value in differentiating some ADV strains.

**Thermal inactivation kinetics**

Early researchers observed that log functions of survival ratios of physically or chemically inactivated
viruses or microorganisms formed straight line survival curves when plotted against time. This relationship was described as the exponential law (37). Thermal inactivation reactions generally followed this law at low temperatures (37 to 40°C). At higher temperatures (44°C and greater) survival curves did not always follow the exponential law. Instead, thermal inactivation curves were characterized by an initial fast, first order kinetic reaction followed by a slower first order reaction. This pattern of thermal inactivation has been observed for vaccinia (41), foot and mouth disease virus (2), poliovirus (19), rhinovirus (19), Newcastle disease virus (17), hominid herpesvirus (62) and cytomegalovirus (47).

Explanation for deviation from the exponential law has centered around two theories, the vitalistic and the mechanistic (37). The former contends that the slow component of virus thermal inactivation curves is due to the presence of a constant proportion of heterogeneous virus more heat resistant than the majority of the population. The mechanistic theory contends that rate differences are due to factors operating within the thermal inactivation process.

The vitalistic theory has not been widely accepted
even though heat resistant poliovirus strains have been cloned from heat labile virus populations following heat treatment. Voese (78) contends that if the slow phase of thermal inactivation were due to population heterogeneity, then the proportion of surviving virus should be independent of temperature which, as far as he determined, was not the case. Instead, Woese postulated that the rate differences were due to inactivation of nucleic acids existing in two different forms, one more stable than the other. Dimmock (19) concluded, from his experiments involving poliovirus and rhinovirus, that the fast phase of the thermal inactivation curve was due to protein denaturization and the slow component to RNA inactivation.

Factors affecting thermal sensitivity

The cell type in which virus is propagated affects the thermal sensitivity of hominid herpesvirus. Plummer et al. (63) demonstrated that the difference between survival rates of identically treated HH 1 and HH 2 was markedly smaller when these strains were propagated in human fibroblast cells than when the strains were propagated in RK or MK cells.

The chemical composition of the heating medium
also affects thermal stability. Smith et al. (72) were unable to demonstrate any difference between thermal stability of HH 1 and HH 2 when these viruses were heat treated in their growth media. However, distinct differences in heat sensitivity were observed when the viruses were diluted in phosphate-buffered saline prior to heat treatment.

Wallis and Melnick (76) reported that hominid herpesvirus is more heat stable in 1M sodium sulfate or 1M dibasic sodium phosphate than in distilled water. It was also shown that tris buffer, Earle's salts and spent growth medium increased the thermal sensitivity of the virus. Proteins and amino acids partially reduced the thermosensitizing effect of Earle's salt and were most effective between pH 6.5 and 6.9. The thermosensitizing effect of spent growth medium was eliminated by diluting stock virus 100 fold in distilled water. No thermal stabilizing nor sensitizing effects were observed when hominid herpesvirus was heat-treated in 1M magnesium chloride, 1M magnesium sulfate, 1M monobasic potassium phosphate or 2M potassium chloride.
Trypsin Sensitivity

Trypsin sensitivity has been used as a marker for differentiating virus groups and strains. Cheng (14) reported that the genus Alphavirus of the Togaviridae was trypsin resistant, thus differentiating this group from the trypsin sensitive Flavivirus genus. Gresser and Enders (35) demonstrated that influenza A virus was trypsin resistant while influenza B virus was trypsin sensitive. The same authors tested three strains of hominid herpesvirus in the same manner and found all three strains to be trypsin sensitive. This observation differed from that of Amos (1) who described a trypsin resistant HH strain. Gresser and Enders concluded that in view of Amos' finding, trypsin sensitivity may be a strain characteristic of hominid herpesvirus.

Bodon et al. (11) differentiated ADV strains on the basis of their sensitivity to trypsin at 37°C. The avirulent Bartha K strain was highly resistant. A fully virulent field isolate was completely inactivated by trypsin under identical conditions. Two other field isolates of lower virulence were less trypsin sensitive than the fully virulent strain, but more sensitive than
the K strain.

Bartha et al. (5) confirmed the observation of Bodon et al., and evaluated the trypsin sensitivity of IBR virus as well. Although the IBR vaccine strain was more resistant to trypsin than the virulent strain, the difference was not as marked as with the attenuated and virulent ADV strains. These workers hypothesized that the trypsin resistance marker could be used to select innocuous ADV strains from wild virus isolates.

Additional trypsin-based markers for ADV may exist. Itoh et al. (40) demonstrated that the addition of trypsin to the medium of virus-infected Vero cells increased the yield of certain strains of influenza and parainfluenza viruses. Other strains of both viruses were not affected. Tobita et al. (74) reported that 11 strains of influenza A virus produced plaques on Madin Darby canine kidney (MDCK) cell monolayers under agar overlay containing 20 μg/ml trypsin. Only five of these strains produced plaques in the absence of trypsin.

Animal Susceptibility

The extensive literature reviews of Galloway (28) and Baskerville et al. (6) illustrate that the host
range of ADV is broad. Virtually all North American farm animals have been shown to be susceptible to the virus; these include swine, cattle, sheep, goats, dogs, cats and horses. In addition feral rats, mink, monkeys, opossum, porcupine and jackal also have been infected with ADV. Geese, ducks, buzzards, sparrow hawks, pigeons and chickens also have been infected with this virus. Pigeons and chickens are the most susceptible birds (28). Laboratory animals including rabbits, hamsters, gerbils, guinea pigs, rats, mice and one-day-old chicks are also suitable hosts for ADV.

Among laboratory animals, the rabbit is considered to be the most susceptible to ADV. McFerran and Dow (55) demonstrated that the rabbit was as susceptible to the virus as either primary rabbit or pig kidney (PK) cells. However, susceptibility of rabbits appears to be dependent on age, breed and "origin" (28). The incubation period in the rabbit varies with dose and route (55). Rabbits subcutaneously inoculated with $10^3$ TCID$_{50}$'s ADV developed pruritus and convulsions at 90 hours post inoculation (pi). Rabbits that received a 1000-fold greater dose developed similar clinical signs at 66 hours pi. Intracerebral inoculation reduced incubation periods by approximately 12 hours.
Mortality and the presence or absence of pruritus in ADV-infected rabbits appear to be directly correlated with a strain's virulence in pigs. Bodon et al. (11) compared Bartha's avirulent K strain to three ADV strains of varying degrees of virulence as determined by pig inoculation. Mortality rates were lower and incubation periods were longer in rabbits inoculated with the K strain and the less virulent field isolates. Skoda and Jamrichová (70) compared laboratory attenuated BUK and the naturally avirulent SUCH-1 vaccine strains of ADV to two virulent field isolates. All four strains caused death in rabbits but only the virulent isolates caused pruritus. Prior to its attenuation, the BUK strain also caused pruritus.

Both Galloway (28) and Glover (30) considered mice and rats to be less susceptible to ADV than rabbits. Fraser and Ramachandran (26) concurred with Galloway and Glover with respect to the sensitivity of adult mice and rats, but expressed the opinion that unweaned mice may be as sensitive as rabbits. These authors based their opinions on the observation that unweaned mice, one to seven days old, were approximately 1000 times more sensitive to ADV than adult mice 8 to 13 weeks old. They also found that unweaned mice were as
sensitive as PK 15 cells to ADV.

Fraser and Ramachandran (26) also observed that the response of mice to ADV varied with the dose and route of inoculation. The mean incubation period of the virus in adult mice inoculated via the intracerebral, intramuscular, intravenous or intraperitoneal routes, ranged from 28 to 48 hours. The mortality rate in these groups was 100 percent. The mean incubation period in adult mice infected with ADV via the nasal and ocular routes increased to 61 and 83 hours respectively while the mortality rates decreased to 88 and 63 percent respectively.

The genetic strain of mice probably influences susceptibility to ADV. Gowen and Shott (34) observed that the survival rates of different mouse strains inoculated with ADV ranged from 52 to 8 percent. These workers further illustrated the effect of genetic homogeneity by randomly dividing non-genetically defined mouse populations into groups. The groups were then infected with ADV. The resulting group survival rates extended over the range bracketed by the survival rates of the most resistant and susceptible mouse strains.

Of the remaining laboratory animal species, the guinea pig has been reported to be 100 to 1000 times
less susceptible to ADV than the rabbit (28). However, Glover (30) found the guinea pig to be only five times less susceptible. Bodon et al. (11) presented limited data that indicated hamsters may be as susceptible to ADV as rats and mice.

Ramachandran and Fraser (64) reported that one-day-old chickens inoculated intracerebrally were as sensitive to ADV as adult mice and rats. However the susceptibility of the chicken to ADV decreases rapidly with age.

The differences in sensitivity to ADV among and between animal species varies with the virus strain. These differences have permitted investigators to characterize and distinguish ADV strains on the basis of virulence. Bodon et al. (11) characterized four strains of ADV on the basis of the mortality patterns observed in rabbits, rats, golden hamsters, mice and pigs. Skoda and Jamrichova (70) differentiated the BUK strain from the SUCH-1 strain on the basis of the former strain's virulence for cats. Remlinger and Bailey (67) commented on a Brazilian isolate that was more virulent for cats than for other species. Lomniczi (48) characterized virulent and avirulent ADV strains on the basis of an intracerebral pathogenicity index derived from the intracerebral inoculation of one-day-old chicks.
Strains have also been characterized according to tissue tropism. Baskerville et al. (6) reported that four ADV strains are recognized in Northern Ireland based on this criterion. Strain NIA-1 is primarily neurotropic and strain NIA-2 principally pneumotropic. Strain NIA-3 is not considered exclusively neurotropic or pneumotropic but causes a higher mortality rate than either of the first two strains. Finally, strain NIA-4 is naturally avirulent and has not been recognized as possessing specific tissue tropism.

Other Markers Associated with Aujeszky's Disease Virus (ADV)

Golais and Sabó (31) studied the relationship between urea sensitivity and virulence among ten virulent and four attenuated ADV strains. Marked differences in urea sensitivity were observed among the different strains, but no correlation with virulence was evident. Although the authors did not comment on the feasibility of utilizing the urea sensitivity marker as a strain differentiator, their results indicate that the marker could be used for this purpose.

Lomniczi (48) demonstrated that avirulent ADV strains were more sensitive to interferon than virulent strains. Avirulent strains also induced higher
interferon titers than virulent strains in 24-hour-old CEF cells. However, some virulent ADV strains induced interferon production as efficiently as avirulent strains in 48-hour-old CEF cell cultures.

Munk and Fischer (57) used the fluorescent antibody technique (FAT) to categorize HH strains into groups based on the intracellular distribution of viral antigen. Zuffa et al. (81) compared the distribution of viral antigen in CK cells singly infected with virulent and avirulent ADV strains. At 20 hours pi, nuclear fluorescence was observed in cells infected with all virulent strains and one avirulent strain. Extensive cytoplasmic fluorescence was observed only in cells infected with avirulent strains.

Dilovski (18) described four different strains of ADV which differed in sensitivity to ultraviolet irradiation. Similar differences have been observed between HH 1 and HH 2 strains (60, 72). Intratypic hominid herpesvirus strain differences in ultraviolet sensitivity have not been reported.
Potential ADV Markers

Plummer et al. (62) compared the rate of CPE formation caused by HH 1 and HH 2 in the presence of heparin. These workers concluded from their results that HH 1 is more sensitive to heparin than HH 2. The apparent mode of action of heparin and other acid mucopolysaccharides is against attachment of the virion to the host cell (59). Consequently, differences in heparin sensitivity are most likely associated with the architecture of the virion surface. Such differences have been shown to exist between HH 1 and HH 2 (68).

Differences between virion surfaces have also been demonstrated using column chromatography. Attenuated LSc and 2 ab strains of poliovirus have been differentiated from the virulent Mahoney strain by column chromatography using diethylamino DEAE-cellulose (39) and aluminum hydroxide gel (79). Hominid herpesviruses 1 and 2 have been distinguished on the basis of their elution patterns from calcium-phosphate-gel columns (68).

Numerous other chemical agents have been used with varying degrees of success to differentiate strains of
herpesviruses. Attempts to differentiate HH 2 from HH 1 strains via ethyl ether and chloroform inactivation kinetics were not successful (68). However, HH 2 was found to be more resistant than HH 1 to inactivation by antimetabolite iododeoxyuridine (52, 59) and by the polycyclic aromatic hydrocarbon, 7, 12-dimethylbenzan-thracene (20). Vaccine strains of equine rhinopneu-monitis virus have been differentiated from virulent field isolates via differences in strain sensitivity to the reducing agent dithiothreitol (42, 43). Gainer et al. (27) demonstrated that ADV also was sensitive to dithiothreitol inactivation which may provide the basis of another effective biological marker for Aujeszky's disease virus.
MATERIALS AND METHODS

Medium Preparation

Growth medium

Growth medium (GM) was prepared by dissolving powdered Eagle's minimum essential medium with Earle's salts, L-glutamine and non-essential amino acids\(^1\) in deionized, double distilled water. Sodium bicarbonate was added at the rate of 220 mg percent and the mixture was filter sterilized by passing it through an 02 Selas filter at five psi pressure. The medium was dispensed in 500 ml aliquots and stored at 4\(^\circ\)C until used. Ten ml fetal calf serum\(^2\) (FCS), two ml antibiotic solution\(^3\) (ABS) and 0.33 mg amphotericin B\(^4\) were added to each 100 ml of medium prior to use.

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\(^1\)Catalog number F-15, Grand Island Biological Co., Grand Island, New York.

\(^2\)Virus tested, catalog number 629, Grand Island Biological Co., Grand Island, New York.

\(^3\)10,000 units penicillin B/ml, 10,000 ug streptomycin sulfate, Grand Island Biological Co., Grand Island, New York.

\(^4\)Fungizone, E. R. Squibb and Sons, New York.
Maintenance media

Maintenance medium-1 (MM-1) was prepared and stored in the same manner as GM except that the FCS content was reduced to two percent.

Maintenance medium-2 (MM-2) was prepared by adding two ml ABS, 0.33 mg amphotericin B and two ml FCS to each 100 ml of commercially prepared Eagle's minimum essential medium containing Earle's salts, non-essential amino acids and 25 mM hepes.

Plaque assay medium

Plaque assay medium (PAM) was prepared by mixing equal amounts of 2X GM and 1.6 percent gum tragacanth solution. Two ml of FCS, two ml of ABS and .33 mg amphotericin B were added to each 100 ml of PAM.

Plaque cloning medium

Plaque cloning medium (PCM) was prepared by mixing equal amounts of 45C 2X GM and two percent liquified special agar Noble maintained at 45C. Two ml of FCS, two ml of ABS and .33 mg amphotericin B were added to each 100 ml of PCM.

1MEM (Eagle's) catalog number 236, Grand Island Biological Co., Grand Island, New York.

2Fischer Scientific Co., Fair Lawn, New Jersey
Cell Culture

Cell lines

Madin-Darby bovine kidney (MDBK), Mengeling-Vaugh pig thyroid (MVPT), PK-15, ST and the MARU strain of Vero line cells were used in these investigations.

Cell propagation

Confluent cell monolayers were removed from 75 mm plastic tissue culture flasks\(^1\) with trypsin-versene solution (Table 1), washed and resuspended in 60 ml of GM. Twenty ml of this suspension was used to inoculate new flasks. Individual wells of Linbro tissue culture plates\(^2\) were inoculated with two ml of cell suspension. Leighton tubes containing 10 x 35 mm glass coverslips were inoculated with one ml of cell suspension. Cells were incubated at 37°C in a humidified atmosphere containing five percent carbon dioxide.

Viruses

Strains

The origins and history of all viruses used in the study are summarized in Table 2. The passage levels of

\(^1\)Lux Scientific Corp., Thousand Oaks, California.

\(^2\)Model FB-6-TC, Linbro Chemical Co., Inc., New Haven, Connecticut.
Table 1. Preparation of trypsin-verse (ATV) solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>750 ml</td>
<td>Triple distilled</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.09 g</td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>0.49 g</td>
<td></td>
</tr>
<tr>
<td>Dextrose</td>
<td>1.09 g</td>
<td></td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.69 g</td>
<td>Added as aqueous paste</td>
</tr>
<tr>
<td>Trypsin</td>
<td>0.50 g</td>
<td>Entire solution incubated one hour at 37°C</td>
</tr>
<tr>
<td>Versene</td>
<td>0.29 g</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>q.s. 1000 ml</td>
<td>ATV filter sterilized and stored at -20°C</td>
</tr>
</tbody>
</table>

^Disodium ethylene diamine tetra-acetate. Nutritional Biochemicals Corp., Cleveland, Ohio.

all viruses used in the following experiments are as shown unless otherwise indicated. Strains K and BUK are attenuated European vaccine strains. Strains Be, Dr, Pa and S62/26 have been shown by pig inoculation to be highly virulent viruses. All other low-passage viruses were isolated from pigs that had died during
Table 2. Summary of origins and history of virus strains used in present study

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Host species originally isolated from and year</th>
<th>Geographic area of original host</th>
<th>Times plaque cloned (cell type used)</th>
<th>Total number passages in MDBK cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>Pig 1961</td>
<td>Europe</td>
<td>1 (MDBK)</td>
<td>7a</td>
</tr>
<tr>
<td>BUK</td>
<td>NA\textsuperscript{b} 1961</td>
<td>Europe</td>
<td>3 (CEF) 1 (MDBK)</td>
<td>6c</td>
</tr>
<tr>
<td>1664</td>
<td>Pig 1973</td>
<td>Iowa</td>
<td>3 (MDBK)</td>
<td>12\textsuperscript{d}</td>
</tr>
<tr>
<td>429</td>
<td>Pig 1973</td>
<td>Iowa</td>
<td>3 (MDBK)</td>
<td>11\textsuperscript{d}</td>
</tr>
<tr>
<td>S62/26</td>
<td>Pig 1974</td>
<td>Iowa</td>
<td>2 (MDBK)</td>
<td>7\textsuperscript{d}</td>
</tr>
<tr>
<td>Be</td>
<td>Pig 1970</td>
<td>Iowa</td>
<td>3 (MDBK)</td>
<td>12\textsuperscript{d}</td>
</tr>
<tr>
<td>Dr</td>
<td>Cow 1969</td>
<td>Iowa</td>
<td>3 (MDBK)</td>
<td>12\textsuperscript{d}</td>
</tr>
<tr>
<td>Pa</td>
<td>Dog 1966</td>
<td>Iowa</td>
<td>3 (MDBK)</td>
<td>18\textsuperscript{d}</td>
</tr>
<tr>
<td>Code</td>
<td>Source Date</td>
<td>Source Location</td>
<td>Age</td>
<td>Proliferation</td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
<td>-----------------</td>
<td>-----</td>
<td>---------------</td>
</tr>
<tr>
<td>1608</td>
<td>Pig 1968</td>
<td>Iowa 3 (MDBK)</td>
<td>10d</td>
<td></td>
</tr>
<tr>
<td>S67</td>
<td>Pig 1974</td>
<td>Iowa 3 (MDBK)</td>
<td>10d</td>
<td></td>
</tr>
<tr>
<td>8003</td>
<td>Pig 1974</td>
<td>Iowa 3 (MDBK)</td>
<td>10e</td>
<td></td>
</tr>
<tr>
<td>11105</td>
<td>Pig 1974</td>
<td>Iowa 3 (MDBK)</td>
<td>12e</td>
<td></td>
</tr>
</tbody>
</table>

*Received from Dr. J. B. McFerran, Veterinary Research Laboratories, Stormont, Belfast, Northern Ireland.*

*NA - Not available.*

*Received from Dr. D. Burger, Washington State University, 1975. The virus was originally obtained by Dr. Burger from the Institute of Virology, Bratislava, Czechoslovakia. Subsequently, it was plaque purified three times in CEF and serially passaged ten times in CEF and two times in Vero cells.*

*Received from Dr. C. J. Mare, Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, Iowa.*

*Received from Dr. H. Hill, Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa.*
Aujeszky's disease outbreaks and are also considered to be "virulent" rather than attenuated viruses.

**Virus cloning**

Confluent MDBK or Vero cell monolayers were inoculated with virus diluted in saline G (Table 3) containing one percent FCS. Inoculums were removed after virus adsorption and replaced with PCM. Plaques were allowed to develop at 37°C and were cloned with Pasteur pipettes approximately 48 hours after inoculation.

**Virus propagation**

Confluent 24- to 48-hour-old monolayers of MDBK, ST or Vero cells were inoculated with virus at a multiplicity of infection of at least 0.5. Virus was adsorbed to cells for a minimum of 90 minutes. Inoculums were removed and replaced with MM-2. Some lots of virus were propagated in MM-1.

Virus-infected cells were incubated 24 to 48 hours at 37°C in a five percent carbon dioxide atmosphere and then harvested. Virus was freed from intact cells by rapidly shell freezing in liquid nitrogen and thawing in 37°C water, three times. The virus suspension was clarified by low speed centrifugation, adjusted to
Table 3. Preparation of saline G

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>750 ml</td>
<td>Triple distilled</td>
</tr>
<tr>
<td>Dextrose</td>
<td>1.20 g</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>8.00 g</td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>0.40 g</td>
<td></td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>0.15 g</td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.15 g</td>
<td></td>
</tr>
<tr>
<td>MgSO₄·6H₂O</td>
<td>0.15 g</td>
<td></td>
</tr>
<tr>
<td>Phenol red</td>
<td>1.20 mg</td>
<td></td>
</tr>
<tr>
<td>Lactalbumin hydrolysate a</td>
<td>1.00 g</td>
<td>Previously dissolved by heating in water</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>16.00 mg</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>q.s. to 1000 ml</td>
<td>Autoclaved at 15 pounds pressure, 30 minutes</td>
</tr>
</tbody>
</table>

aSolubilized, Nutritional Biochemical Corp., Cleveland, Ohio.

pH 7.4 with normal sodium hydroxide or hydrochloric acid, and filtered through a 450 nm pore size filter.¹ One ml aliquots of the virus preparation were dispensed into

¹Millipore Corporation, Bedford, Massachusetts.
sterile screw cap one dram glass vials and stored at -70°C. All virus strains that were compared in any group of experiments were propagated and stored concurrently.

Virus assay

Standard procedure Virus activity was assayed by the plaque method. Serial dilutions of virus were made in saline G containing two percent calf serum. Three quarters of a ml of each virus dilution was inoculated in triplicate onto 24- to 48-hour-old MDBK cell monolayers in six-well tissue culture Linbro plates. Inoculums were removed after a 90 minute adsorption period and replaced with two ml of PAM.

Virus-infected cell monolayers were incubated for 48 hours at 37°C in a humid atmosphere containing four percent carbon dioxide. Cell sheets were fixed by adding two ml of 20 percent formalin to each well. The contents of each well were removed after 20 minutes and the cell sheets stained with five percent crystal violet dye prepared in 20 percent ethanol. The plaques were counted and the virus titer was expressed in PFU/ml.
Evaluation of 90-minute adsorption period

Three wells of each of eight different Linbro plates were inoculated with 0.75 ml suspensions of ADV strains K and Be. The plates were placed in a 37°C incubator. At 15 minute intervals, beginning at 30 minutes post inoculation (pi) and ending at 135 minutes pi, individual Linbro plates were removed from the incubator and the virus inoculums replaced with PAM. The mean plaque counts of both viruses were plotted against time. The experiment was repeated twice.

Proportionality of plaque count to virus concentration

Strain S62/26 was diluted in saline G to yield approximately 400 PFU/ml. Subsequently, two separate sets of six serial two-fold dilutions were made. Each dilution was assayed for virus activity. The mean number of plaques and the coefficient of variation (CV) for each dilution was determined. Linear regression analysis was performed to determine if virus concentration was proportional to plaque count. The experiment was repeated two times on the same day.

Experimental Animals

Weanling, 18- to 22-day old White Swiss mice were obtained from three sources at Iowa State University:
the Department of Veterinary Microbiology and Preventive Medicine (VMPM), the Veterinary Diagnostic Laboratory (VDL) and the Veterinary Medical Research Institute (VMRI).

Immature female New Zealand White rabbits weighing two to three pounds were supplied by a commercial rabbitry located in central Iowa.

Thermal Inactivation Kinetics

Selection of test medium

The following solutions were evaluated as virus diluents for the thermal inactivation kinetic (TIK) test: saline G; potassium chloride, 2M; sodium sulfate, 1M; magnesium sulfate, 1M; sodium chloride, 0.85 percent; glucose, 0.11 percent; Hank's balanced salts; Earle's balanced salts; Earle's balanced salts without calcium and magnesium; and sodium phosphate buffer, 0.15M.

Aujeszky's disease virus strains K and Be were separately diluted 1000 fold with each diluent to yield approximately 5,000 PFU/ml. The pH of each virus suspension was adjusted to 6.8 with normal hydrochloric acid and/or sodium hydroxide and the pre-treatment virus activity assayed. Duplicate two ml samples of
each virus suspension were dispensed into five dram sterile glass screw-top vials. The vials were capped and immersed in a 50°C water bath to a depth of 30 mm. After one hour all vials were simultaneously transferred to an ice bath and mean residual virus activity was determined. The diluent in which the maximum difference in percent survival occurred between strains was selected as the standard medium for the TIK test.

**Selection of optimum temperature**

Stock virus K and Be were separately diluted 1000 fold in saline G, pH 6.8 to yield approximately 5000 FFU/ml. The diluted viruses were maintained at 2°C to 5°C and initial virus activity was assayed. Two ml aliquots of each virus were dispensed into five dram sterile glass vials. The vials were closed and duplicate samples of each virus suspension were immersed to a depth of 30 mm in water baths pre-set at 45.5 ± 0.2; 47 ± 0.1; 48 ± 0.1; 49 ± 0.1; and 50 ± 0.1°C. Heat treatment was stopped after 45 minutes by transferring the vials to an ice bath. Residual virus activity in each vial was assayed and the mean of duplicate samples was calculated. The temperature at which the maximum difference in percent virus survival occurred between
strains, was selected as the standard for the TIK test. The experiment was repeated on three different days.

**Selection of optimum pH**

Strains K and Dr were diluted 1000 fold in saline G to yield approximately 5,000 PFU/ml. Each virus suspension was divided into five, 20 ml aliquots and maintained at 2C to 5C. The pH of the aliquots of each virus was adjusted to 6.2, 6.5, 6.8, 7.0 and 7.3 respectively. The initial virus titer activity of all pH-adjusted viruses was determined.

Duplicate, two ml samples of each treated virus were dispensed into five dram glass vials. The vials were closed and heat treated for 45 minutes as previously described. The mean percent virus survival of duplicate samples was calculated. The pH at which the maximum difference in percent virus survival occurred, was selected as the standard for the TIK test. The experiment was repeated on three different days.

**Procedure for TIK test**

Individual stock viruses (Table 2) were diluted initially with maintenance medium so that a subsequent
1000-fold dilution in saline G, pH 6.9 would yield a virus concentration of approximately 5000 PFU/ml. The pH of each virus suspension was readjusted to pH 6.9 as needed with 1 N hydrochloric acid or sodium hydroxide solution. All virus suspensions were maintained in an ice bath prior to and after heat treatment.

Duplicate seven ml aliquots of virus suspension were dispensed into 16 x 125 mm disposable glass tissue culture tubes. Tubes were closed with rubber plugs and their contents mixed seven to ten seconds with a vortex mixer at a speed just below that which would cause foaming of the medium. A one ml aliquot was removed from each tube for pre-heat-treatment virus assay.

The TIK test was initiated by placing the virus-containing tubes to a depth of 100 mm in a 48 ± 0.1°C water bath. As many as six different viruses could be tested in this manner at one time by sequentially placing tubes in the water bath at 45-second intervals. One ml aliquots were removed from each tube at 10- or 15-minute intervals beginning at 15 minutes and ending at 55 or 60 minutes post immersion. The mean residual virus activity of duplicate samples at each sampling time was assayed.

16.5 ml of 0.15M sodium phosphate buffer, pH 7.0 was added to each 200 ml of saline G prior to adjusting the pH to 6.9.
and plotted against time forming TIK curves.

Comparison of thermal inactivation kinetic curves of Aujeszky's disease virus strains

Viruses The TIK curve was determined a minimum of three times on separate days for ADV strains K, BUK, 1664, 429, S62/26 and Be. The TIK curve of strains 8003, Dr, Pa, 1608, S67 and 11105 was determined on two separate days.

Method of comparison Thermal inactivation kinetic curves of these strains were compared by visual inspection and by the combined comparison of each strain's thermal inactivation rate (K) and predicted survival value ($S_{30}$) after 30 minutes of heat treatment. These parameters were determined by the following method.

The percent survival data, represented by the TIK curves of each replicate of all strains, were transformed to natural logarithmic values. These values were regressed on time using the equation, $y = bx + a$, where $y$ equals $\log_e$ of percent survival +1, $x$ equals time, $b$ equals regression coefficient and $a$ equals the $y$ axis intercept. The regression coefficients that were obtained from the resulting regression equations of each replicate of all strains were used as estimators of $K$. Predicted virus survival at 30 minutes ($S_{30}$) was calculated from
the individual regression equations.

Thermal inactivation rates \( K \) and \( S_{30} \) were transformed to standardized values by the following equations:

\[
K' = \frac{K - \bar{K}}{s_K},
\]

where \( K' \) equals the standardized thermal inactivation rate \( K \) of an individual replicate of strain, \( \bar{K} \) equals the mean \( K \) of all replicates of the strains tested and \( s_K \) equals the standard deviation of \( K \) and

\[
S' = \frac{S_{30} - \bar{S}}{s_{S}},
\]

where \( S' \) equals the standardized \( S_{30} \) of an individual replicate of a strain, \( \bar{S} \) equals the mean \( S_{30} \) of all replicates of all strains tested and \( s_{S} \) equals the standard deviation of \( S \).

The standardized \( K \) and \( S_{30} \) of each replicate of each strain were plotted on the ordinate and abscissa respectively. The resulting points

\[ \bar{K} \text{ and } \overline{S} \text{ were calculated from the log transformed TIK curves obtained from all experiments except those evaluating the effect of virus concentration and the effect of hepes buffer on TIK of ADV.} \]
representing each replicate of all strains were then visually compared.

Effect of initial virus concentration on TIK test

Three experiments were conducted to determine the effect of initial virus concentration on thermal inactivation kinetics of ADV. In experiment one, three aliquots of stock strain K were diluted in MM-2 so that a subsequent 1000-fold dilution in saline G of each aliquot yielded virus concentrations of 5,378; 3,000 and 1,678 PFU/ml.

In experiment two strain K was similarly diluted and yielded virus concentrations of 12,906; 6,764 and 3,214 PFU/ml. In experiment three strain 8003 was diluted in the same manner and yielded virus concentrations of 8,922; 5,111 and 2,522. Thermal inactivation kinetic tests were conducted simultaneously on all concentrations of each virus. The TIK curves of each experiment were compared as previously described.

Effect of hepes buffer on TIK

Three lots of ADV strain K were prepared. Maintenance medium-1 was used in the preparation of two of these lots and MM-2, containing 25 mM hepes buffer, was used in the preparation of the third lot. All
three lots of strain K were simultaneously tested on each of three separate days. The TIK curves of each replicate were compared as previously described.

Trypsin Inactivation Kinetics

Test components

Saline G, without lactalbumin hydrolysate or phenol red, was adjusted to pH 6.9 with normal hydrochloric acid and/or sodium hydroxide and used as diluent for trypsin and virus in the trypsin inactivation kinetic (TRIK) test. Stock trypsin solution was prepared at a concentration of five mg/ml in diluent, readjusted to pH 6.9, dispensed in five ml aliquots and stored at -70°C until used.

Selection of trypsin concentration and temperature for TIK

Aujeszky's disease virus strains K and Be were diluted to approximately 5000 PFU/ml. Duplicate samples of both strains were exposed to trypsin concentrations of 0.04, 0.02 and 0.01 mg/ml for 30 minutes at both 30°C and 37°C. The inactivation reaction was stopped by removing one ml aliquots from the test solutions and depositing them into cold (2°C to 5°C) nine ml saline G dilution blanks containing 20 percent sheep serum.
Virus-trypsin controls were maintained at 4°C and sampled in an identical manner at time zero and 30 minutes. Virus-diluent controls were maintained at 4°C and at the test temperatures and were sampled at time zero and 30 minutes. The trypsin concentration and reaction temperature at which the greatest difference in percent survival was observed between strains, were selected as standard parameters of the TSIK test.

**Procedure for trypsin inactivation kinetic test**

Stock virus was initially diluted in MM-2 so that a subsequent 1000-fold dilution in diluent would yield a virus concentration of approximately 4,000 to 5,000 PFU/ml. Diluted virus stock was maintained at 2°C to 5°C and adjusted to pH 6.9. Trypsin stock was diluted to 0.1 mg/ml in cold diluent and adjusted to pH 6.9. One ml amounts of diluted trypsin were placed in duplicate 10 x 125 mm disposable glass tubes which were kept in an ice bath. Nine ml of the diluted virus were added to each tube which was plugged with a rubber stopper. The contents of all tubes were mixed for seven to ten seconds with a vortex mixer at a speed just below that which would cause foaming. One ml aliquots were removed from individual tubes and placed in cold
nine ml saline G dilution blanks, containing 20 percent sheep serum, for pre-treatment virus assay.

The TRIK reaction was initiated by placing tubes containing virus-trypsin mixture to a depth of 100 mm in a 30C water bath. As many as six different viruses could be prepared and tested in this manner at one time by sequentially placing samples in the water bath at 45-second intervals. One ml aliquots were removed from individual tubes and deposited into dilution blanks at 10 minute intervals for virus assay. Sampling was initiated at 15 minutes and terminated at 55 minutes post immersion. The mean percent virus survival was calculated from duplicate samples and plotted against time forming TRIK curves.

Comparison of trypsin inactivation kinetic curves of Aujeszky's disease virus strains

The TRIK curve was determined three times on separate days for ADV strains K, BUK, 1664, 429, S62/26 and Be. These curves were compared by the same methods used for TIK curves.

Effect of virus concentration on trypsin inactivation kinetics

Three aliquots of strain K were initially diluted with MM-2 so that subsequent 1000-fold dilutions of
each aliquot with TRIK test diluent yielded virus concentrations of 6,542; 4,115; and 2,540 PFU/ml. The same procedure was repeated with ADV strain Dr and virus concentrations of 3,280; 1,617; and 1,178 PFU/ml were obtained.

The TRIK test was conducted on each virus preparation. Results were expressed as percent survival of PFU/ml and plotted against time. The TRIK curves representing the three concentrations of each virus were compared as previously described.

Plaque Growth Rate

Selection of cell type and plaquing medium

Vero, MVPT, MDBK, ST and PK 15 line cells were propagated in Linbro plates. Confluent 48-hour cell monolayers were inoculated in duplicate with ADV strains K, Be and Pa. Virus was diluted to contain approximately 50 PFU per 0.75 ml inoculum. Following a 90-minute adsorption period, the inoculums were replaced with specific plaquing mediums described in Table 4. Plaques were allowed to develop for 48 hours and the cell sheets were fixed and stained as previously described. The cell plaquing medium combination that demonstrated the largest difference in plaque size
Table 4. Plaquing media and cell types evaluated for plaque growth rate studies

<table>
<thead>
<tr>
<th>Plaquing medium</th>
<th>Percent</th>
<th>Cell types overlaid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar (Noble)</td>
<td>1.00</td>
<td>MDBK, MVPT, Vero, ST, PK</td>
</tr>
<tr>
<td>Gum Tragacanth</td>
<td>0.80</td>
<td>MDBK, MVPT, Vero, ST, PK</td>
</tr>
<tr>
<td>CMC</td>
<td>0.37</td>
<td>MDBK, Vero</td>
</tr>
</tbody>
</table>

\(^{a}\) In addition to the component listed above, each plaquing medium contains MEM, two percent FCS, 200 units penicillin G/ml, 200 ugm streptomycin sulfate/ml and 3.3 ugm amphotericin B/ml.

\(^{b}\) Sodium carboxymethylcellulose, Grand Island Biological Co., Grand Island, New York.

between strains as determined by visual inspection was selected for use in the plaque growth rate (PGR) test.

Plaque growth rate test

Aujeszky's disease virus strains K, BUK, 1664, 429, Be and S62/26 were diluted in virus diluent to yield approximately 50 plaques per 0.75 ml inoculum after a 90-minute adsorption period. Linbro plates containing 48-hour old Vero cell monolayers were divided into four groups. Three wells in each group were inoculated with the same virus suspension. Following adsorption, inoculums were replaced with plaquing medium containing CMC (Table 4).
All Linbro plates were placed in a 37°C humidified incubator containing five percent carbon dioxide. At 24-hour intervals pi, one group of Linbro plates was removed from the incubator and the cell monolayers fixed and stained as previously described.

Plaques were measured by projecting the image of the stained cell sheets on a white paper grid. All plaques were measured in mm. The mean plaque diameter for all viruses at each day pi was calculated and plotted against time. The rates of plaque growth of all strains were compared. The experiment was repeated twice.

Comparison of Cytopathic Effect Induced by Field and Vaccine Strains of ADV in MDBK and Vero Cells

Madin Darby bovine kidney and Vero cells were separately propagated on glass coverslips in Leighton tubes. Forty-eight-hour confluent cell monolayers were infected with ADV strains K, BUK, 429, Be and S62/26 at a multiplicity of infection equal to or greater than one, and maintained in MM-2. Virus-infected cells were fixed with a cold, 1:1 mixture of acetone and absolute methanol at eight to 54 hours pi, depending on the rate of cell deterioration.
Cell monolayers were stained with hematoxylin-eosin, mounted on glass slides, examined microscopically and photographed.

Virulence of ADV Strains in Laboratory Animals

Comparison of ADV strain virulence in mice

Aujeszky's disease virus strains K, BUK, 1664, 429, Be and S62/26 were assessed for virulence by determining the LD\textsubscript{50} of each strain for weanling Swiss albino mice. Virulence was also assessed by calculating the mean-time-to-death (MTD) for mice inoculated with virus. Each virus strain was initially diluted with MM-2 to yield approximately 10\textsuperscript{5} PFU/ml. Virus was then serially diluted in 10-fold increments with MM-2. Groups of weanling mice were inoculated subcutaneously in the nape of the neck with 0.1 ml of each virus dilution. Mice were observed daily for 12 days. All deaths and clinical signs were recorded. The LD\textsubscript{50} for each ADV strain was calculated by the method of Reed and Muench (66). Mean-time-to-death was calculated for mice that received $10^{3.2}$ to $10^{3.8}$ PFU of virus.

The above experiment was repeated two times. In the first experiment mice were provided by the Department of Veterinary Microbiology and Preventive Medicine.
Mouse-inoculation groups in this experiment contained five principals each. Mice for the second experiment were supplied by the Veterinary Medical Research Institute. Mouse-inoculation groups in this experiment contained six principals each.

Comparison of ADV strain virulence in rabbits

Rabbits were divided into groups of three. All rabbits in each group were inoculated subcutaneously in the right rear flank with approximately $10^{4.0}$ PFU of ADV strain BUK, S62/26, 1664, 429 or Be. Only one rabbit was inoculated with strain K. Rabbits were observed daily for clinical signs and death. Dead rabbits were necropsied and gross lesions recorded. Lung, liver and brain tissue was recovered from each dead rabbit for virus isolation and identification.

Effect of Passaging ADV in Different Cell Lines on Viral Markers

Aujeszky's disease virus strain Dr was serially passaged 114 times through MDBK, Vero and ST cell lines at 37°C and is hereafter referred to as Dr-M, Dr-V and Dr-ST respectively. The final passages of Dr-M, Dr-ST and Dr-V were coordinated so that all were propagated, harvested, processed and stored concurrently.
Individual lots of strains Dr and K were also produced concurrently with the modified Dr strains. Thermal sensitivity, PGR and CPE markers were defined two times on separate days for the above viruses. Strains Dr and K served as reference controls. A separate suspension of strain Dr served as heat control for the TIRI test.

The virulence marker was assessed as previously described in mice and rabbits for Dr-M, Dr-ST, Dr-V and Dr. Mice and rabbits were divided into equal groups composed of five and four principals respectively. The rabbit experiment was conducted once and mouse experiments two times. Mice were supplied by the Veterinary Diagnostic Laboratory.

Statistical Analysis

Data from experiments that consisted of three or more treatment groups were evaluated by the analysis of variance procedure (73). Treatment means were separated by Duncan's new multiple-range test or orthogonal comparisons as indicated (73). Student's t-test was used to compare data of experiments that consisted of two treatment groups (73). Inactivation rates of the same virus strain under different
experimental conditions were analyzed by the analysis of variance in regression procedure described by Ostle (61).
RESULTS

Viruses

**Virus propagation**

Virus titers of $10^6$ to $10^7$ PFU/ml were routinely obtained from virus-infected ST, Vero and MDBK cell cultures.

**Virus assay**

All strains of ADV with the exception of strain BUK, consistently produced clearly visible plaques approximately 1.5 to 2.0 mm in diameter by 48 hours pi. Plaques produced by strain BUK during the same time period were not as distinct as the plaques produced by all other strains and measured approximately 0.5 to 1.0 mm in diameter.

The relationship between virus concentration and plaque counts is illustrated in Table 5. Assay error, as measured by the coefficient of variation, was lowest for plaque counts around 400. Error appeared to be constant between plaque counts that ranged from 60 to 240. The highest error occurred when plaque counts were less than 60.

Virus adsorption curves for ADV strains K and Be
<table>
<thead>
<tr>
<th>Consecutive Two-fold Virus Dilution</th>
<th>Replicate I Mean Count</th>
<th>Replicate I Coefficient Variation</th>
<th>Replicate II Mean Count</th>
<th>Replicate II Coefficient Variation</th>
<th>Mean of Replicates I and II Plaque Count</th>
<th>Mean of Replicates I and II Coefficient Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>430</td>
<td>3.6</td>
<td>406</td>
<td>3.9</td>
<td>418</td>
<td>3.8</td>
</tr>
<tr>
<td>2</td>
<td>234</td>
<td>11.3</td>
<td>208</td>
<td>8.8</td>
<td>221</td>
<td>10.1</td>
</tr>
<tr>
<td>3</td>
<td>122</td>
<td>7.5</td>
<td>108</td>
<td>6.6</td>
<td>115</td>
<td>7.1</td>
</tr>
<tr>
<td>4</td>
<td>65.8</td>
<td>8.2</td>
<td>60.2</td>
<td>10.1</td>
<td>61.3</td>
<td>9.2</td>
</tr>
<tr>
<td>5</td>
<td>36.0</td>
<td>13.7</td>
<td>31.8</td>
<td>11.0</td>
<td>24.9</td>
<td>12.4</td>
</tr>
<tr>
<td>6</td>
<td>19.8</td>
<td>16.1</td>
<td>15.7</td>
<td>18.8</td>
<td>17.8</td>
<td>17.4</td>
</tr>
</tbody>
</table>

Correlation Coefficients .9196 .9110 .9142
are presented in Figure 1. Virus was adsorbed rapidly to MDBK cells during the first 45 to 60 minutes post inoculation (pi). Thereafter, the adsorption rate decreased until 105 minutes pi when the adsorption rate again increased. Ninety minutes was chosen as the standard adsorption time.

Thermal Inactivation Kinetics

Selection of optimum test medium

The percent survival of strains K and Be following thermal inactivation in different mediums is summarized in Table 6. The largest difference in percent survival between the two strains occurred when virus was heat treated in saline G. Saline G was then selected as the standard diluent for the thermal inactivation kinetic (TIK) test.

Selection of optimum temperature

Strain K was more resistant to thermal inactivation than strain Be at all temperatures evaluated (Figure 2). The maximum difference between the percent survival of both strains occurred at 48°C. Forty-eight degrees C was then chosen as the standard temperature for the TIK test.
Figure 1. Adsorption kinetics of ADV strains K and Be on MDBK cells at 37°C. The initial fast phase of adsorption was followed by a relatively stationary phase between 75 and 105 minutes. After 105 minutes there appeared to be an increase in adsorption rate.
Figure 2. The effect of temperature on thermal inactivation of ADV strains K and Be at pH 6.9. The largest difference between survival rates occurred at 48°C.
Table 6. Percent survival of vaccine strain K and virulent field strain Be in different media after one hour at 50°C, pH 6.8

<table>
<thead>
<tr>
<th>Media</th>
<th>% Survival (PFU/ml) strain K</th>
<th>% Survival (PFU/ml) strain Be</th>
<th>Difference in % survival of strains K &amp; Be</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline G</td>
<td>35.0</td>
<td>0.1</td>
<td>34.9</td>
</tr>
<tr>
<td>Na₂SO₄ 1M</td>
<td>9.0</td>
<td>0.1</td>
<td>8.9</td>
</tr>
<tr>
<td>MgSO₄ 1M</td>
<td>3.0</td>
<td>0.1</td>
<td>2.9</td>
</tr>
<tr>
<td>Glucose .11g%</td>
<td>2.5</td>
<td>0</td>
<td>2.5</td>
</tr>
<tr>
<td>Phosphate buffer .15M</td>
<td>0.4</td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>Hanks balanced salts</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Earle's balanced salts</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Earle's balanced salts without Ca++ and Mg++</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Selection of optimum pH

Thermal resistance of strains K and Be increased as the pH of the medium increased (Figure 3). Maximum thermal resistance of strains K and Be occurred at pH 6.8 and 7.0 respectively. Thermal resistance of both strains decreased in higher pHs. The maximum difference in percent survival between strain K and Be occurred at pH 6.9. This pH was then used as the standard for the TIK test.
Figure 3. The effect of pH on thermal inactivation of ADV strains K and Be at 48°C. Note that the largest difference between survival rates occurred at pH 7.0.
PERCENT VIRUS SURVIVAL (PFU/ML) AFTER 48H AT 48°C

PH OF MEDIUM

△ REPLICATE 1
○ REPLICATE 2
× REPLICATE 3

--- THERMAL RESISTANT ADV STRAIN K
--- THERMAL SENSITIVE ADV STRAIN Dr
Comparison of thermal inactivation kinetic curves of Aujeszky's disease virus strains

The mean TIK curves of European vaccine strains K and BUK and 10 different ADV strains are presented in Figure 4. The range of thermal sensitivity varied from the highly thermal resistant (Tr) strain K to the extremely thermal sensitive (Ts) strains BUK, Be and Dr. Strains 429 and 1664 are clearly intermediate (Ti) with respect to thermal sensitivity.

Visual inspection of the log-transformed curves of all virus strains tested revealed that a lower rate of virus thermal inactivation occurred during the first 15 minutes of heat treatment than that which occurred thereafter. Correlation coefficients that were obtained from the regression analyses of the log-transformed TIK curves of each replicate of each strain between 15 minutes and the termination of heat treatment, are presented in Table 7.

When standardized K and S30 of each replicate of each strain were plotted, a linear distribution that represented a thermal sensitivity spectrum (TSS) occurred (Figure 5). No distinct breaks were observed in this continuum. However, when boundaries were arbitrarily placed to separate the two most thermal sensitive strains, BUK and Be, and the most thermal resistant strain, K, from all other strains, a convenient method of
Figure 4. Thermal inactivation kinetics of two European vaccine strains and ten virulent field strains of ADV at 48°C and pH 6.9.
PERCENT SURVIVAL OF VIRUS (PFU/ML)

TIME IN MINUTES

STRAIN K *

STRAIN 1664

STRAIN Be

STRAIN 429

STRAIN S62/26

STRAIN BUK

\[ \text{\#2 STANDARD ERRORS} \quad \text{N \ NUMBER OF REPLICATES} \]

* EUROPEAN VACCINE STRAINS
Figure 4. Continued.
Figure 5. The thermal sensitivity spectrum of ADV strains characterized by the thermal inactivation kinetic test. Note that the European vaccine strains K and BUK are at the opposite ends of the spectrum.
TEST CONDITIONS:
48°C, pH 6.9

STANDARDIZED PERCENT SURVIVAL

LOW VIRUS SURVIVAL

1 VACCINE STRAIN K
2 VACCINE STRAIN BUK
3 STRAIN 1664
4 STRAIN 429
5 STRAIN Be

HIGH VIRUS SURVIVAL

6 STRAIN S62/26
7 STRAIN Pa
8 STRAIN 11105
9 STRAIN 1608
10 STRAIN S67
11 STRAIN 8003
12 STRAIN 2626
13 STRAIN Dr
14 STRAIN Dr -ST
15 STRAIN Dr -M
16 STRAIN Dr -V
Table 7. Regression and correlation coefficients of log-transformed thermal inactivation kinetic (TIK) curves

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Range of regression coefficient</th>
<th>Mean regression coefficient</th>
<th>Range of correlation coefficient</th>
<th>Mean correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>.0069 to .0345</td>
<td>.0176</td>
<td>.900 to .996</td>
<td>.960</td>
</tr>
<tr>
<td>BUK</td>
<td>.0560 to .0869</td>
<td>.0738</td>
<td>.907 to .997</td>
<td>.961</td>
</tr>
<tr>
<td>429</td>
<td>.0401 to .0558</td>
<td>.0492</td>
<td>.984 to .996</td>
<td>.993</td>
</tr>
<tr>
<td>1664</td>
<td>.0364 to .0617</td>
<td>.0498</td>
<td>.977 to .997</td>
<td>.989</td>
</tr>
<tr>
<td>S62/26</td>
<td>.0720 to .0841</td>
<td>.0753</td>
<td>.994 to .998</td>
<td>.996</td>
</tr>
<tr>
<td>Be</td>
<td>.0762 to .0887</td>
<td>.0839</td>
<td>.985 to .997</td>
<td>.992</td>
</tr>
<tr>
<td>Dr</td>
<td>.0852 to .0911</td>
<td>.0882</td>
<td>.986 to .968</td>
<td>.977</td>
</tr>
<tr>
<td>Pa</td>
<td>.0477 to .0574</td>
<td>.0526</td>
<td>.993 to .999</td>
<td>.996</td>
</tr>
<tr>
<td>2686</td>
<td>.0450 to .0470</td>
<td>.0460</td>
<td>.996 to .997</td>
<td>.996</td>
</tr>
<tr>
<td>8003</td>
<td>.0293 to .0338</td>
<td>.0319</td>
<td>.989 to .990</td>
<td>.990</td>
</tr>
<tr>
<td>1608</td>
<td>.0505 to .0696</td>
<td>.0601</td>
<td>.993 to .994</td>
<td>.994</td>
</tr>
<tr>
<td>S67</td>
<td>.0445 to .0522</td>
<td>.0484</td>
<td>.993 to .999</td>
<td>.996</td>
</tr>
</tbody>
</table>
categorizing strains as Ts, Ti or Tr was established. (Figure 5).

**Effect of initial virus concentration on TIK test**

The mean value for the initial virus concentration of all TIK tests was 4,977 PFU/ml. The range extended from 2,142 to 12,738 PFU/ml. Eighty-one percent of all initial virus concentration values ranged from 3,166 to 6,551 PFU/ml.

No marked differences were observed among the thermal inactivation rates or TSS position of strains K and 8003 when virus concentration was varied over the range that occurred during TIK tests (Figures 6 and 7).

**Effect of hepes buffer on TIK**

No significant differences were observed between the thermal inactivation rates or TSS position of strain K that was prepared in the presence of 25 mM hepes buffer and strain K that was concurrently prepared in the absence of hepes buffer (Figures 8 and 9).

**Trypsin Inactivation Kinetics**

**Selection of trypsin concentration and reaction temperature**

The effect of trypsin concentration and the reaction temperature on virus inactivation are summarized in Figure 10. The maximum difference between the percent survival of strains K and Be occurred at a trypsin
Figure 6. The effect of virus concentration on the thermal inactivation kinetics of two strains of ADV at 48°C, pH 6.9. No statistically significant differences were detected between the thermal inactivation rates of ADV in experiment 1, $F(2,6) = .90$; experiment 2, $F(2,9) = .49$; and experiment 3, $F(2,6) = .67$. 
Figure 7. The effect of virus concentration on thermal inactivation kinetics of two strains of ADV at 48°C, pH 6.9, as demonstrated by the thermal sensitivity spectrum.
TEST CONDITIONS
48°C, pH 6.9

STANDARDIZED PERCENT SURVIVAL

LOW VIRUS SURVIVAL —> HIGH VIRUS SURVIVAL

STRAIN K REP 1 @ 5,378 PFU/ML
STRAIN K REP 1 @ 3,000 PFU/ML
STRAIN 8003 @ 8,922 PFU/ML
STRAIN 8003 @ 5,111 PFU/ML
STRAIN 8003 @ 2,522 PFU/ML
STRAIN K REP 2 @ 12,906 PFU/ML
STRAIN K REP 2 @ 6,764 PFU/ML
STRAIN K REP 2 @ 3,214 PFU/ML
STRAIN 8003 @ 5,378 PFU/ML
Figure 8. The effect of hepes buffer on the thermal inactivation kinetics of ADV strain K at 48°C, pH 6.9. No statistically significant differences were detected between the thermal inactivation kinetic curves in replicate 1, F (2,9) = .06; replicate 2, F (2,9) = .042; and replicate 3, F (2,9) = 1.22.
percent survival of virus (pfu/ml)

rep 1

rep 2

rep 3

0 15 30 45 60

0 15 30 45 60

0 15 30 45 60

time in minutes

100

75

50

25

o---o strain k lot 1 stored in mem with bicarbonate buffer

o---o strain k lot 2 stored in mem with bicarbonate buffer

o---o strain k lot 2 stored in mem with hepes buffer
Figure 9. The effect of hepes buffer on the thermal inactivation kinetics of ADV strain K at 48°C, pH 6.9, as demonstrated by the thermal sensitivity spectrum.
TEST CONDITIONS
48°C, pH 6.9

REPLICATE 1
REPLICATE 2
REPLICATE 3

LOT 1 STORED IN MEM WITH BICARBONATE BUFFER
LOT 2 STORED IN MEM WITH BICARBONATE BUFFER
LOT 2 STORED IN MEM WITH HEPES BUFFER
Figure 10. The effect of trypsin concentration at pH 6.9 on the inactivation of ADV strains K and Be at 30C and 37C. Note that the maximum difference between the survival rates of strains K and Be occurred when these strains were subjected to .01 mg/ml trypsin at 30C.
TRYPSIN CONCENTRATION IN MG PER ML

PERCENT VIRUS SURVIVING (PFU/ML) 40 MINUTES TRYPSIN INACTIVATION

K—K TRYPSIN RESISTANT STRAIN K TREATED AT 37°C

K—K TRYPSIN RESISTANT STRAIN K TREATED AT 30°C

B—B TRYPSIN SENSITIVE STRAIN Be TREATED AT 37°C

B—B TRYPSIN SENSITIVE STRAIN Be TREATED AT 30°C
concentration of 0.01 mg/ml and a reaction temperature of 30°C. No reduction in virus titer was detected in controls at 30°C. A trypsin concentration of 0.01 mg/ml and the reaction temperature of 30°C were then selected as standard parameters for the trypsin inactivation kinetic (TRIK) test.

Comparison of trypsin inactivation kinetic curves of ADV strains

The mean TIK curves of the two European strains (K, BUK) and four virulent field strains are presented in Figure 11. Strains K and 1664 are clearly the most trypsin resistant (TRr), whereas strain BUK is the most trypsin sensitive (TRs). All other strains are intermediate (TRi) with respect to trypsin sensitivity.

When the standardized K and $S_{30}$ of each replicate of each TIK tested strain were plotted, a trypsin sensitivity spectrum (TRSS) analogous to the TSS, was observed (Figure 12). Arbitrary division of the TRSS by locating boundaries midway between the closest replicates that represented an arbitrarily defined TRi strain and a TRs or TRr strain provided a means of categorizing ADV strains according to their trypsin sensitivity.

Correlation coefficients that were obtained from
Figure 11. Trypsin inactivation kinetics of two European vaccine strains and four virulent field strains of ADV.
TEST CONDITIONS: 30°C, pH 6.9, 0.01 mg trypsin per ml

- REP 1
- REP 2
- REP 3

* EUROPEAN VACCINE STRAINS

△ MEAN CONTROL
Figure 12. The trypsin sensitivity spectrum of two European vaccine strains and four virulent field strains of ADV. Note that the European vaccine strains K and BUK are at the opposite ends of the spectrum.
TRYPSIN CONC. 0.01 MG/ML
REACTION TEMP. 30 C
pH 6.9

STANDARDIZED PERCENT SURVIVAL
1 VACCINE STRAIN K 4 STRAIN 429
2 VACCINE STRAIN BUK 5 STRAIN Be
3 STRAIN 1664 6 STRAIN S62/26

LOW VIRUS SURVIVAL → HIGH VIRUS SURVIVAL
the regression analyses of each log-transformed replicate of each strain, between 15 minutes and termination of trypsin inactivation, are presented in Table 8.

### Table 8. Correlation and regression coefficients of log-transformed trypsin inactivation kinetic (TRIK) curves

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Range of regression coefficient</th>
<th>Mean regression coefficient</th>
<th>Range of correlation coefficient</th>
<th>Mean correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>.003 to .009</td>
<td>.006</td>
<td>.743 to .908</td>
<td>.800</td>
</tr>
<tr>
<td>BUK</td>
<td>.051 to .070</td>
<td>.062</td>
<td>.957 to .996</td>
<td>.978</td>
</tr>
<tr>
<td>429</td>
<td>.047 to .059</td>
<td>.053</td>
<td>.939 to .966</td>
<td>.951</td>
</tr>
<tr>
<td>1664</td>
<td>.024 to .032</td>
<td>.028</td>
<td>.990 to .998</td>
<td>.995</td>
</tr>
<tr>
<td>S62/26</td>
<td>.039 to .090</td>
<td>.066</td>
<td>.960 to .986</td>
<td>.976</td>
</tr>
<tr>
<td>Be</td>
<td>.039 to .062</td>
<td>.053</td>
<td>.880 to .996</td>
<td>.956</td>
</tr>
</tbody>
</table>

**Effect of initial virus concentration on TRIK test**

The mean initial virus concentration of all TRIK tests was 4,033 PFU/ml. The range extended from 1,164 to 6,471 PFU/ml. Seventy-five percent of all initial virus concentration values ranged from 3,182 to 6,471 PFU/ml.

No marked differences were observed among the mean trypsin inactivation curves (Figure 13) or TRSS position (Figure 14) of strains K and Dr when virus concentration was varied over the range that occurred during TRIK tests.
Figure 13. The effect of virus concentration on trypsin inactivation kinetics of two strains of ADV at 30°C, pH 6.9. No obvious differences exist among the inactivation curves of strain K or strain Dr.
VIRUS CONCENTRATION

**STRAIN Dr**
- 3,280 PFU/ML
- 1,618 PFU/ML
- 1,178 PFU/ML

**STRAIN K**
- 8,053 PFU/ML
- 4,115 PFU/ML
- 2,540 PFU/ML

**TEST CONDITIONS:** 30°C, pH 6.9, 0.01 MG TRYPSIN PER ML
Figure 14. The effect of virus concentration on trypsin inactivation kinetics of two ADV strains at 30°C, pH 6.9, as demonstrated by the trypsin sensitivity spectrum.
TRYPSIN CONC. 0.01 MG/ML
REACTION TEMP. 30°C
pH 6.9

STANDARDIZED PERCENT SURVIVAL
LOW VIRUS SURVIVAL → HIGH VIRUS SURVIVAL

Dr-1 3280 PFU/ML  K-1 8053 PFU/ML
Dr-2 1617 PFU/ML  K-2 4115 PFU/ML
Dr-3 1178 PFU/ML  K-3 2540 PFU/ML
Plaque Growth Rate

Selection of cell type and plaquing medium for plaque growth rate (PGR) comparison

No marked size difference was observed between plaques produced by strain K and the virulent field isolate Be in PK-15 and ST line cells under agar or GT overlays. Conversely, marked differences in size were observed between plaques produced by strains K and Be in MVPT, Vero and to a lesser extent in MDBK cell lines (Figure 15). Similar size differences were observed between plaques produced by these same virus strains in Vero cells overlaid with 0.375 percent carboxymethylcellulose (CMC) plaquing medium.

Virus plaques that developed under CMC were larger than plaques that developed under GT but smaller than plaques that developed under agar. The shape of plaques under CMC was markedly more uniform than the shape of plaques which formed under agar (Figure 16).

Plaque growth kinetics of two vaccine and five virulent strains

Distinct differences in plaque growth rates (PGR) were observed between strains K, BUK, 429, 1664 S62/26 and Be (Figure 17). The PGR of strains K and BUK were statistically different (P < .05) from each
Figure 15. The influence of cell type on the size of plaques produced by the ADV vaccine strain K and the virulent strain Be. Plaques produced by strain K in MVPT, MDBK and PK-15 cells under agar overlay are illustrated by pt-K, bk-K and pk-K respectively. Plaques produced by strain Be in MVPT, MDBK and PK-15 cells under agar overlay are illustrated by pt-B, bk-B and pk-B respectively. Note the marked difference between the size of plaques produced by strain Be and strain K in MVPT cells. The size difference is less pronounced in MDBK cells and practically inapparent in PK-15 cells. Plaques were allowed to develop for 48 hours. (X 1.8)
Figure 16. The influence of overlay medium on the development of plaques produced in Vero cells by ADV strains K and Be. Plaques produced by strain K under gum tragacanth (GT), carboxymethylcellulose (CMC) and agar overlays are illustrated by g-K, m-K and a-K respectively. Plaques produced by strain Be under GT, CMC and agar overlays are illustrated by g-B, m-B and a-B respectively. Note the distinct difference between the size of plaques produced by strains K and Be under CMC overlay. Marked size differences are also seen when plaques form under agar overlay but the shape of these plaques is not uniform. Plaque size differences are not as marked when plaques develop under GT overlay. Plaques were allowed to develop for 48 hours. (X 1.9)
Figure 17. Plaque growth kinetics of two European vaccine strains and four virulent field strains in Vero cells at 37°C. Significant differences exist among the plaque growth rate (PGR) of the individual strains, $F(5, 6) = 13.5$, $P < .005$. Orthogonal comparisons show that the PGR of vaccine strains are different from each other, $F(1, 6) = 6.3$, $P < .05$ and from the PGR of virulent field strains, $F(1, 6) = 56.8$, $P < .005$. No differences occur among the PGR of the virulent field strains, $F(3, 6) = 1.53$. 
The graph shows the mean plaque diameter (in mm) over time (in hours) for different strains of Andromyxa parathermophila. The strains are differentiated by different markers and line styles. The graphs are labeled as REP 1, REP 2, and MEAN.

- **STRAIN Be** (open circles, solid line)
- **STRAIN S62/26** (black circles, dashed line)
- **STRAIN 1664** (white squares, dotted line)
- **STRAIN 429** (black squares, solid line)
- **VACCINE STRAIN K** (white triangles, dashed line)
- **VACCINE STRAIN BUK** (black triangles, solid line)

The time points for measurement are 0, 24, 48, 72, and 96 hours.
other and from the virulent strains. No statistical
differences were found among the virulent field strains.
However, the PGR of strain 429 appeared to be slightly
lower than the PGR's of other field strains.

Comparison of Cytopathic Effect Induced
in MDBK and Vero Cells by Virulent
Field and Vaccine Strains of ADV

Madin-Darby bovine kidney cell monolayers that
were infected with virulent ADV strains 429, 1664,
S62/26 and Be, were characterized by the formation
of cell free voids as early as eight hours pi. These
voids were frequently transversed with slender cyto-
plasmic strands. Distinct eosinophilic intranuclear
inclusion bodies were observed at 18 to 20 hours pi.
Syncytia containing more than 25 nuclei were also
observed at this time (Figure 18). All virus-infected
cell monolayers had detached from glass coverslips by
24 hours pi.

Madin-Darby bovine kidney cells that were infected
with strain BUK at a multiplicity of infection of one
or higher, rounded and detached from glass coverslips
between 8 and 12 hours pi. Occasionally syncytia
Figure 18. Cytopathic effect of virulent ADV in MDBK cells. A. Normal MDBK cells prior to infection with ADV. B. Madin-Darby bovine kidney cells 20 hours after infection with a virulent ADV strain at a multiplicity of infection greater than one. Note that the syncytia contain more than 25 nuclei. Nuclear inclusions (I) can be seen in most cells. Stained with hematoxylin-eosine (X 560).
containing up to eight nuclei were seen prior to cell detachment. Nuclear inclusion bodies were present but not clearly delineated.

When the multiplicity of infection of strain BUK-infected MDBK cells was reduced to less than 0.1, cell monolayers remained intact for up to 36 hours pi. At 18 to 20 hours pi infected cells were rounded and clumped together. Occasionally small syncytia containing less than 10 nuclei were observed (Figure 19). Most cells contained distinct eosinophilic nuclear inclusion bodies.

Cell free voids were not observed in cell monolayers infected with strain K until 18 to 20 hours pi. By 36 hours pi cells had rounded and clumped together forming islands. Eosinophilic nuclear inclusion bodies were prevalent among these cells and clearly delineated. Syncytia containing up to five nuclei were occasionally observed. See Figure 20.

Vero cell monolayers inoculated with ADV strains K, BUK, 429, 1664, Be and S62/26 were characterized by cell free voids at eight hours pi. These voids were transversed frequently by thin cytoplasmic strands. Distinct eosinophilic nuclear inclusion bodies were observed in all cells of the virus-infected monolayers (Figure 21).
Figure 19. Cytopathic effect of European ADV vaccine strain BUK in MDBK cells. A. Madin Darby bovine kidney cells 20 hours after infection with ADV strain BUK at a multiplicity of infection less than 0.1. Note the syncytium (S) that contains less than 10 nuclei. B. The same cell monolayer. Note that virus-infected cells are piled one upon another. Nuclear inclusions (I) can be seen in most cells. Stained with hematoxylin-eosine (X 560).
Figure 20. Cytopathic effect of European ADV vaccine strain K in MDBK cells. A. Madin-Darby bovine kidney cells 20 hours after being infected with ADV strain K at a multiplicity of infection greater than one. Note the syncytium (S) containing five nuclei. Nuclear inclusions (I) are visible in most cells. B. Madin-Darby bovine kidney cells 36 hours after infection with strain K. Note how the virus-infected cells are piled one upon another. Stained with hematoxylin-eosine (X 560).
Figure 21. Cytopathic effect of virulent ADV in Vero cells. A. Normal Vero cell monolayer prior to infection with ADV. B. Vero cells eight hours after infection with ADV at a multiplicity of infection greater than one. Note the large nuclear inclusion bodies (I). C. Vero cells 20 hours after infection with a virulent ADV strain at the same multiplicity of infection. Note that the large syncytium (S) contains between 10 and 20 nuclei. Nuclear inclusions (I) can be clearly seen in most cells. Stained with hematoxylin-eosine (X 560).
No syncytia were found.

By 20 hours pi syncytia were observed in Vero cells infected with strains 1664, 429, Be and S62/26 (Figure 21). These syncytia usually contained 10 to 20 nuclei and occasionally more than 50 nuclei. No inhibition or enhancement of syncytium formation was observed at 20 or 36 hours pi in Vero cells that were inoculated with strain S62/26 when the multiplicity of infection was reduced to less than 0.1.

Vero cell monolayers infected with strain BUK were rounded and clumped together by 20 hours pi. Small syncytia containing three to five nuclei were occasionally observed at this time (Figure 22). No enhancement or inhibition of syncytium formation was observed at 20 or 36 hours pi when the multiplicity of infection was reduced to less than 0.1.

Vero cell monolayers that were infected with strain K were rounded and clumped together by 20 hours pi. No syncytia were observed at this time (Figure 23). At 36 and 48 hours pi the rounded cells were more densely aggregated and small syncytia containing three to five nuclei were occasionally observed. No enhancement or inhibition of syncytium formation was observed at 20, 36 or 48 hours pi when the multiplicity of infection
Figure 22. Cytopathic effect of European ADV vaccine strain BUK in Vero cells. A. Vero cells 20 hours after infection with ADV strain BUK at a multiplicity of infection greater than one. Nuclear inclusion bodies (I) are visible in most cells. Note the small syncytium (S) that contains four nuclei. B. The same cell monolayer. Note that the virus-infected cells are rounded and piled one upon another. Stained with hematoxylin-eosine (X 560).
Figure 23. Cytopathic effect of European ADV vaccine strain K in Vero cells. A. Normal Vero cell monolayer prior to infection with ADV. B. Vero cells 20 hours after infection with strain K at a multiplicity of infection greater than one. Note that the cells are rounded and piled one upon another and nuclear inclusions are present in most. Stained with hematoxylin-eosine (X 560).
was reduced to less than 0.1.

Virulence of ADV
Strains in Laboratory Animals

Comparison of ADV strain virulence in mice

The mean mouse LD$_{50}$'s of specific ADV strains are summarized in Table 9. The mean LD$_{50}$'s of strains K, BUK and S62/26 appear to be different from the mean LD$_{50}$'s of strains 429, 1664 and Be. The overall mean LD$_{50}$ in experiment one was significantly different (P<.02) from the overall mean LD$_{50}$ of experiment two.

The mean time-to-death (MTD) of mice inoculated with different ADV strains is summarized in Table 10. The MTD of mice inoculated with strain K was significantly longer (P<.05) than the MTD of mice inoculated with all other ADV strains in both experiments. The MTD in mice inoculated with strain BUK was not significantly different from the MTD of mice inoculated with the four virulent strains in experiment one but was different (P<.05) in experiment two. No significant differences were observed among the MTD of mice infected with virulent field strains in either experiment.

Clinical signs of ADV infection preceded death by 24 hours or less in mice inoculated with strains BUK,
Table 9. Virulence of two European vaccine and four virulent field strains of ADV as measured by their LD$_{50}$ for subcutaneously inoculated weanling Swiss albino mice

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Experiment 1 mouse source 1$^a$</th>
<th>Experiment 2 mouse source 2$^b$</th>
<th>Mean experiment 1 &amp; 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>34</td>
<td>503</td>
<td>268.5</td>
</tr>
<tr>
<td>BUK</td>
<td>59</td>
<td>1014</td>
<td>536.5</td>
</tr>
<tr>
<td>S62/26</td>
<td>36</td>
<td>489</td>
<td>262.5</td>
</tr>
<tr>
<td>429</td>
<td>19</td>
<td>88</td>
<td>53.5</td>
</tr>
<tr>
<td>1664</td>
<td>17</td>
<td>52</td>
<td>34.5</td>
</tr>
<tr>
<td>Be</td>
<td>5</td>
<td>93</td>
<td>49.0</td>
</tr>
<tr>
<td>Experiment mean$^c$</td>
<td>28.3</td>
<td>373.2</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Department of Veterinary Microbiology and Preventive Medicine, Iowa State University.

$^b$Veterinary Medical Research Institute, Iowa State University.

$^c$Experiment means are significantly different at P < .05, t = 2.25, d.f. = 10.

S62/26, 429, 1664 and Be in the above experiments. These strains caused severe pruritus characterized by extensive self-mutilation at the injection site. Affected mice would intermittently race about their
Table 10. Virulence of six ADV strains as measured by the mean time-to-death (MTD) in subcutaneously inoculated weanling Swiss albino mice

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Experiment 1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Experiment 2&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount virus inoculated (log&lt;sub&gt;10&lt;/sub&gt; PFU)</td>
<td>MTD&lt;sup&gt;c&lt;/sup&gt; (days)</td>
</tr>
<tr>
<td>K</td>
<td>3.76</td>
<td>7.2&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>BUK</td>
<td>3.77</td>
<td>3.0&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>S62/26</td>
<td>3.18</td>
<td>3.2&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>429</td>
<td>3.78</td>
<td>3.0&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>Be</td>
<td>3.32</td>
<td>2.8&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>1664</td>
<td>3.91</td>
<td>2.6&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Experiment means<sup>d</sup> 3.63 3.93

<sup>a</sup>Mice supplied by the Department of Veterinary Microbiology and Preventive Medicine, Iowa State University.

<sup>b</sup>Mice supplied by the Veterinary Medical Research Institute, Iowa State University.

<sup>c</sup>Experiment 1, standard error = .2199, error d.f. = 24. Experiment 2, standard error = .2708, error d.f. = 30. Treatment means that do not have a superscript (A, B and C) in common are different at P<.05 (Duncan's multiple range test).

<sup>d</sup>Experiment means were not significantly different, t = 1.41, d.f. = 10.

cages in an explosive uncoordinated manner, abruptly stop, scratch, tumble over backwards and lie on their sides in an apparent state of exhaustion.
In contrast, clinical signs developed 36 to 48 hours prior to death in mice infected with strain K. Severe pruritus and erratic behavior patterns were not observed. Affected mice were characterized by a "hunched up" somnolent attitude that was occasionally disturbed by feeble attempts to scratch the injection site.

Strain virulence in rabbits

The virulence of six ADV strains was evaluated by observing the clinical signs and measuring the MTD in rabbits inoculated with each strain. Results of the experiment are summarized in Table 11.

The MTD of rabbits inoculated with the European vaccine strain BUK was significantly greater (P<.05) than the MTD in rabbits inoculated with the virulent field strains 429, 1664, S62/26 and Be. No difference was apparent among the MTD of rabbits inoculated with these virulent strains. European vaccine strain K did not cause clinical signs or death. Severe pruritus as evidenced by extensive self mutilation at the injection site occurred within 24 hours of death in all rabbits inoculated with the virulent field strains. Generalized trembling and
Table 11. Virulence of ADV strains as measured by clinical response and the mean time-to-death (MTD) in subcutaneously inoculated white female New Zealand rabbits

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Amount virus inoculated (log\textsubscript{10} PFU)</th>
<th>Rabbit number</th>
<th>Time of death(^a)</th>
<th>Range of MTD(^a)</th>
<th>Mean(^b) MTD</th>
<th>Pruritus</th>
</tr>
</thead>
<tbody>
<tr>
<td>S62/26</td>
<td>4.0569</td>
<td>1</td>
<td>55-58</td>
<td>56-60</td>
<td>58.0(^B)</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>55-58</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>58-64</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Be</td>
<td>3.5682</td>
<td>4</td>
<td>55-58</td>
<td>52-56</td>
<td>54.0(^B)</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>50-55</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>50-55</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>429</td>
<td>3.9542</td>
<td>7</td>
<td>58-64</td>
<td>57-61</td>
<td>59.0(^B)</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>50-55</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>9</td>
<td>64-65</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1664</td>
<td>4.1271</td>
<td>10</td>
<td>50-55</td>
<td>53-58</td>
<td>55.5(^B)</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>50-55</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>58-64</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUK</td>
<td>4.1399</td>
<td>13</td>
<td>99-102</td>
<td>97-99</td>
<td>98.0(^A)</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>99-102</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>92-93</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>4.1959</td>
<td>16</td>
<td>survived</td>
<td></td>
<td></td>
<td>no</td>
</tr>
</tbody>
</table>

\(^a\)Hours post-inoculation.

\(^b\)Treatment means with different superscripts are different at \(P < .05\), (Duncan's multiple range test), \(s_\overline{x} = 3.3\), error d.f. = 12.
occasionally slight jerking movements of the legs were observed in some rabbits following the development of pruritus.

Strain BUK did not cause pruritus. Death occurred suddenly and was not preceded by clinical symptoms.

Extensive ecchymotic hemorrhages were found in the lungs of all rabbits infected with each ADV strain except strain K.

Effect of Passaging ADV in Different Cell Lines on Viral Markers

Effect of high cell passage on thermal sensitivity marker of strain Dr

Thermal sensitivity of strain Dr appeared to decrease after 114 passages through MDBK cells (Figures 24 and 25). No apparent decrease in sensitivity occurred following multiple passage through ST or Vero cells.

Effect of high cell passage on plaque growth rates (PGR)

The mean PGR of strain Dr before high cell passage was 0.349 mm dia/day. Following multiple passage through MDBK, ST and Vero cells, the mean PGR increased to 0.352, 0.399 and 0.426 mm dia/day, respectively (Figure 26). These differences were not statistically significant. The difference between the PGR of strain K (0.224 mm dia/day) and the PGR of strain Dr and its
Figure 24. Thermal inactivation kinetics of ADV strain Dr at 48°C, pH 6.9 before and after 114 passages through ST, Vero and MDBK cells. Note that the thermal sensitivity of strain Dr following passage through MDBK cells apparently decreased.
REP 1

STRAIN K REF CONTROL

Dr PASSED, 114X IN MDBK CELLS

REP 2

STRAIN K

X REF CONTROL Dr

TI 0 15 30 45 60 0 15 30 45 60

TIME IN MINUTES

STRAIN Dr PASSED, 114X IN VERO CELLS

VACCINE STRAIN K

STRAIN Dr PASSED, 114X IN MDBK CELLS

STRAIN Dr PASSED, 114X IN ST CELLS

PERCENT SURVIVAL OF VIRUS (PFU/ML)
Figure 25. The distribution of ADV strain Dr in the thermal sensitivity spectrum before and after 114 passages through ST, Vero and MDBK cells. Note that passage through Vero and ST cells apparently did not affect the thermal sensitivity of strain Dr.
TEST CONDITIONS: 48C, pH 6.9

STANDARDIZED PERCENT SURVIVAL

LOW VIRUS SURVIVAL

HIGH VIRUS SURVIVAL

K STRAIN K REFERENCE CONTROL
Dr STRAIN Dr REFERENCE CONTROL
Dr-ST STRAIN Dr PASSED 114X IN ST CELLS
Dr-V STRAIN Dr PASSED 114X IN VERO CELLS
Dr-M STRAIN Dr PASSED 114X IN MDBK CELLS

1 REPLICATE 1
2 REPLICATE 2
MEAN OF REPLICATE 1 AND 2
Figure 26. Plaque growth kinetics of ADV strain Dr in Vero cells at 37°C before and after 114 passages through ST, Vero and MDBK cells. No statistically significant differences were detected among the plaque growth rates (PGR) of strain Dr before and after high cell passage as determined by orthogonal comparisons, $F(3,5) = 1.22$. The PGR of strain K is significantly different from the PGR of strain Dr and its modifications, $F(1,5) = 16.5$, $P < .01$. 
REP 1

STRAIN Dr PASSED 124X IN MDBK CELLS

REP 2

STRAIN Dr PASSED 114X IN ST CELLS

STRAIN Dr PASSED 114X IN VERO CELLS

MEAN

VACCINE STRAIN K

MEAN PLAQUE DIAMETER (MM)

TIME IN HOURS

0 24 48 72 96 0 24 48 72 96
modifications was significant ($P < .01$).

**Effect of high cell passage on CPE**

The CPE caused by strains Dr, Dr-M, Dr-V and Dr-ST in MDBK and Vero cells was indistinguishable from the CPE caused by strains 429, 1664, S62/26 and Be with one exception. Large syncytia were not as frequently observed in Vero cells infected with strain Dr-ST.

**Effect of high cell passage on animal virulence markers**

Differences between the mouse LD$_{50}$'s of strain Dr before and after multiple cell passage were not statistically significant (Table 12). However, the LD$_{50}$ of strain Dr was markedly greater than the LD$_{50}$ of Dr-M and Dr-V in both experiments.

The MTD of rabbits inoculated with strain Dr increased from 68.3 hours to 87.4 hours following multiple passage of this strain through Vero cells (Table 13). This increase was significant at $P < .05$. Passage through ST and MDBK cells did not affect the strain's virulence.

**Characterization of ADV strains by marker profiles**

The markers of ADV strains K, BUK, 429, 1664, S62/26 and Be are summarized in Table 14. Six distinct marker profiles are demonstrated.
Table 12. Virulence of ADV strain Dr before and after 114 passages in MDBK, ST and Vero cell lines as demonstrated by the LD$_{50}$ of each strain in subcutaneously inoculated weanling Swiss albino mice

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Experiment 1 $^b$</th>
<th>Experiment 2 $^b$</th>
<th>Mean$^a$ experiment 1 and 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr</td>
<td>1960</td>
<td>351</td>
<td>1155</td>
</tr>
<tr>
<td>Dr-ST</td>
<td>93</td>
<td>308</td>
<td>201</td>
</tr>
<tr>
<td>Dr-M</td>
<td>23</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Dr-V</td>
<td>29</td>
<td>14</td>
<td>23</td>
</tr>
<tr>
<td>Experiment mean$^c$</td>
<td>526</td>
<td>174</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Treatment means were not significantly different (Duncan's multiple range test), $s_x = 485$, error d.f. = 4.

$^b$Mice supplied by Veterinary Medical Research Institute, Iowa State University.

$^c$Experiment means were not significantly different, $t = .723$, d.f. = 6.
Table 13. Virulence of ADV strain Dr after 114 passages in MDBK, ST and Vero cells as measured by the clinical response and the mean time-to-death (MTD) in subcutaneously inoculated white New Zealand rabbits

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Amount virus inoculated (log_{10} PFU)</th>
<th>Rabbit number</th>
<th>Time of death</th>
<th>Range of MTD</th>
<th>Mean MTD</th>
<th>Pruritus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr</td>
<td>4.1780</td>
<td>1</td>
<td>60-68</td>
<td>66.0-70.0</td>
<td>68.3^B</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>72</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>73-74</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>60-68</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dr-M</td>
<td>4.3263</td>
<td>5</td>
<td>71</td>
<td>72.5-76.3</td>
<td>73.5^B</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>76-75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>60-68</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>84-85</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dr-ST</td>
<td>4.1894</td>
<td>9</td>
<td>60-68</td>
<td>62.8-69.0</td>
<td>65.9^B</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>60-68</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>11</td>
<td>71-72</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>60-68</td>
<td></td>
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</tr>
<tr>
<td>Dr-V</td>
<td>4.2236</td>
<td>13</td>
<td>95-96</td>
<td>85.8-89.2</td>
<td>87.4^A</td>
<td>yes</td>
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<td></td>
<td></td>
<td>14</td>
<td>82-84</td>
<td></td>
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<td>15</td>
<td>82-84</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>16</td>
<td>84-92</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^aHours post-inoculation.

^bTreatment means with different superscripts are different at P < .05, (Duncan's multiple range test), s_x = 3.3, error d.f. = 12.
Table 14. Marker profiles of two European vaccine strains and four virulent field isolates of ADV

<table>
<thead>
<tr>
<th>ADV virus markers</th>
<th>European vaccine strains</th>
<th>Virulent field isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K  BUK</td>
<td>1664  429  Be  S62/26</td>
</tr>
<tr>
<td>Heat</td>
<td>R  S</td>
<td>I  I  S  I</td>
</tr>
<tr>
<td>Trypsin</td>
<td>R  S</td>
<td>R  I  I  I</td>
</tr>
<tr>
<td>PGR</td>
<td>M  L</td>
<td>H  H  H  H</td>
</tr>
<tr>
<td>CPE in MDBK cells</td>
<td>SS  SS-D</td>
<td>LS  LS  LS  LS</td>
</tr>
<tr>
<td>CPE in Vero cells</td>
<td>SS  SS</td>
<td>LS  LS  LS  LS</td>
</tr>
<tr>
<td>Virulence in mouse</td>
<td>MV  MV</td>
<td>V  V  V  MV</td>
</tr>
<tr>
<td>Virulence in rabbit</td>
<td>NV  MV</td>
<td>V  V  V  V</td>
</tr>
</tbody>
</table>
R  Highly resistant to inactivation by heat or trypsin.
S  Highly susceptible to inactivation by heat or trypsin.
I  Moderately susceptible to inactivation by heat or trypsin.
H  High plaque growth rate.
M  Intermediate plaque growth rate.
L  Low plaque growth rate.
LS Induces rapid formation of syncytia that contain 20 or more nuclei.
SS Induces slow formation of syncytia that contain 10 or less nuclei.
SS-D Induces rapid formation of syncytia that contain 10 or less nuclei. Cell monolayers are consistently destroyed within 12 hours post infection.
V  Requires relatively small LD$_{50}$ to infect mice. Causes pruritus and death in rabbits.
MV Requires relatively high LD$_{50}$ to infect mice. Kills rabbits but does not produce pruritus.
NV Not virulent.
DISCUSSION

Thermal and Trypsin Inactivation

Comparison of ADV strains

Differences between thermal sensitivity of ADV strains have been previously demonstrated by other investigators (5, 11, 31). However, these investigators have not attempted to develop a classification system based on heat sensitivity and other markers that could ultimately be used for the identification and differentiation of ADV strains.

The three distinct categories of thermal sensitivity represented in the thermal sensitivity spectrum (TSS) indicated that a classification system based on marker profiles is feasible. Consequently, strains K, 429, 1664, S62/26, Be and BUK were selected for further characterization by other markers because they represented the three zones of the TSS.

When the above strains were grouped according to their thermal and trypsin sensitivity markers, five marker profiles were observed (Table 14). The differentiation of strain 429 from 1664 via their trypsin sensitivities was considered to be highly significant because these strains were virtually indistinguishable on the basis of their thermal sensitivity markers.
(Figures 4 and 5).

Thermal inactivation of different viruses (17, 19, 41, 78) including the cytomegalovirus (47) has been attributed to protein denaturization. These observations provide circumstantial evidence that thermal inactivation of ADV is also due to protein denaturization.

The number and strength of covalent, ionic and hydrogen bonds that determine the stability of proteins are determined by the number and location of specific amino acid sequences within the protein molecule (77). Since heat adversely affects hydrogen bonds (77), the differences in thermal sensitivity that were observed between ADV strains may have been a reflection of protein stability moderated by quantitative and qualitative differences in their protein components. Such differences have been demonstrated between the envelope proteins of different hominid herpesvirus strains (29). Similar differences may exist between ADV strains.

The proteolytic activity of trypsin is directed specifically against the peptide bonds in which carboxyl groups of arginine and lysine participate (77). Consequently, it appears that the relative position of ADV strains in the trypsin sensitivity spectrum (TRSS) may reflect the number and/or position of arginine and lysine molecules within the affected protein.
Differentiation of strain 429 from strain 1664 by the TRIK test may indicate that qualitative differences exist between the protein components of both strains, and that these do not noticeably affect thermal stability. It is also conceivable that the difference between these strains that was demonstrated by the TRIK test reflected restricted access of trypsin to the affected protein. This theory would account for the similarity of the mean TRIK curve of strain 429 to the mean TRIK curve of strain 1664 following the initial refractory period (Figure 11). Access of trypsin to protein could be hindered by the location of the protein within the virion or by a physical barrier of some other component of the virion.

Virus assay

The large correlation coefficients that were obtained from the regression of virus plaque counts on corresponding two-fold dilutions of virus suspension indicated that virus concentration and plaque counts were proportional (Table 5). Consequently, the validity of the plaque assay procedure used in the preceding study was confirmed (15).

Ninety minutes was selected as the standard adsorption time for the virus assay procedure because it represented the approximate mid point of the flattest
segment of the virus adsorption curve. Consequently, any error that occurred due to small variation in adsorption time was minimized.

Log transformation of the first and second segment of the virus adsorption curves presented in Figure 1 demonstrated a linear relationship between virus adsorption and time. This relationship was indicative of a first order kinetic reaction that characterizes virus adsorption to cells (50).

The increased virus adsorption rate that was represented by the third segment of the virus adsorption curves (Figure 1) was unexpected. The reason for this late increase in virus adsorption was not clear. Changes in the virus concentration of inocula were probably not responsible because virus adsorption is independent of virus concentration (50). The increased adsorption rate may have occurred as a result of increased MDBK cell susceptibility to virus attachment. It is conceivable that an undefined physiological change occurred in MDBK cells as a result of prolonged exposure to saline G. Increased cell susceptibility to virus attachment in some cell-virus systems has been shown to be related to the age of the cells (50).

**Establishment of test conditions**

The sensitivity of the TIK test was maximized by
selecting the pH, diluent and temperature that revealed the greatest difference between the heat sensitivity of a known heat resistant vaccine strain and a highly heat sensitive field isolate. The effects of pH, medium constituents and temperature on thermal-inactivation kinetics which were observed in preceding experiments have been previously described for herpesviruses (76).

Although the thermo-stabilizing effect of water has been demonstrated for hominid herpesvirus, water was not used as a diluent in the present study because of the difficulty in adjusting and maintaining the pH in this medium.

The initial virus concentration of approximately 5,000 PFU/ml was arbitrarily selected for the TIK and TRIK tests because it minimized the time, cost and potential assay errors because of the smaller number of dilutions which were required for virus assay.

**Effect of hepes buffer on TIK**

Initially all lots of individual virus strains that were compared in the TIK test were propagated in cell cultures maintained with MM-1. However, during the harvest procedure, the pH would frequently rise as much as 0.5 pH unit. To avoid pH variation between virus strains, MM-1 was replaced with MM-2 for virus propagation.

The presence of 25 mM hepes in MM-2 apparently did
not affect the TIK of ADV according to the experimental results summarized in Figures 8 and 9. These observations were further supported when no significant differences were detected between the mean inactivation rates of replicates representing strains K, 1664, 429 Be and S62/26 which were prepared in MM-1 and the same ADV strains which were prepared in MM-2.

**Initial virus concentration**

Unavoidable variation of stock virus titer occurred between vials of the same lot. This variation accounted for the variability of initial virus concentration that was observed in the TIK and TRIK tests. The results of experiments summarized in Figures 6 and 7 indicate that variation of initial virus concentration did not affect the repeatability of the TIK test. Similarly, results of experiments summarized in Figures 13 and 14 indicate that this variation did not affect the repeatability of the TRIK test.

**Methods of comparing thermal and trypsin inactivation kinetics of ADV**

Thermal- and trypsin-inactivation kinetic curves were converted to linear functions by log transformation to facilitate comparison between strains. The large correlation coefficients that were obtained from regressing
log transformed survival data on time are an indication that log transformation of TRIk and TIK curves were successful (Table 7). Time zero was not included in any transformation procedure to avoid the effect of temperature equilibration that occurred during the initial stages of the inactivation tests.

Differences between individual inactivation curves of ADV strains were demonstrated more clearly by the thermal and trypsin sensitivity spectrums than by visual comparison of non-transformed curves. In addition, visual comparison of log-transformed inactivation curves within these spectrums provided a more sensitive method of differentiating ADV strains than by comparison of their inactivation rates. This was apparent when the thermal inactivation rates of strains BUK, Be and S62/26 were compared (Table 15). The mean K of each strain indicated that strain Be was more sensitive to heat than either strains BUK or S62/26. However, when the TSS position (Figure 5) and the mean TIK curves (Figure 4) of these strains were compared, it was clear that the heat sensitivity of strain BUK was slightly greater than the heat sensitivity of strain Be and markedly greater than the heat sensitivity of strain S62/26.
Table 15. Comparison of three highly heat sensitive ADV strains by their non-standardized thermal inactivation rates as estimated by regression coefficients of log-transformed thermal inactivation kinetic (TIK) curves

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Range</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUK</td>
<td>.0560 to .0890</td>
<td>.0738</td>
</tr>
<tr>
<td>S62/26</td>
<td>.0720 to .0841</td>
<td>.0753</td>
</tr>
<tr>
<td>Be</td>
<td>.0762 to .0887</td>
<td>.0839</td>
</tr>
</tbody>
</table>

Similar sensitivity was demonstrated by the TRSS. Differences in trypsin sensitivity between strains Be and BUK were more clearly demonstrated by the TRSS (Figure 12) than by visual comparison of non-transformed TIK curves (Figure 11).

Categorization of virus sensitivity within TSS and TRSS

Thermal sensitivity zones were arbitrarily established because the TSS (Figure 5) represented a gradient of heat sensitive strains. The TSS position of all replicated T_r and T_S strains were contained in their respective zones. Similarly, the TSS position of T_i strains was contained within the thermal intermediate zone.
with the exception of one replicate of strain S62/26. Consequently, it appeared that the zone boundaries were optimally placed to permit a consistent method of categorizing strains according to their heat sensitivity. However, because these zones were established post priori, the appropriateness of their boundaries must be confirmed with additional TIK data of the same and different ADV strains.

Zones in the TRSS (Figure 12) were also arbitrarily established because the ADV strains tested represented gradients of sensitivity to trypsin. The establishment of these zones was based principally on visual comparison of non-transformed curves. The boundary that separates the resistant from the sensitive zone (Figure 12) was drawn to include both strains 1664 and K because both strains were initially resistant to trypsin inactivation (Figure 11). Determining the location of the boundary that separated the intermediate from the sensitive zone was not as obvious.

The position of replicates of strain Be in the TRSS would justify a classification of trypsin sensitive or intermediate. This strain was arbitrarily defined as intermediate to avoid dividing replicates of the TR 1 strain S62/26 between the intermediate and sensitive zones of the TRSS. The inclusion of strain Be in the
sensitive zone would have been equally justified on the basis of its mean non-transformed TRIK curve (Figure 11). It is apparent that additional replications of strain Be, S62/26 and other ADV strains will be required before the arbitrary division between the sensitive and intermediate zones of the TBSS can be firmly established.

**Experimental error associated with thermal and trypsin virus inactivation**

The distribution of points within the TSS (Figure 5) and TBSS (Figure 12) that represent TIK and TRIK test replications of individual ADV strains, is an indication of the magnitude of experimental error. The relative closeness of these points indicates uniform test conditions. The experimental error that did occur was attributed to several sources which included the plaque-assay procedure, pH, reaction temperature, medium components, errors of execution and in the TIK test, trypsin concentration.

Plaque assay errors were greatest when the mean plaque counts were less than 70 (Table 5). Consequently, dilution blanks were adjusted to yield plaque counts above 100 whenever possible. Adjusting dilution blanks to yield high plaque counts was particularly important for estimations of initial virus concentration. Because virus survival was
expressed in percent, the assay of initial virus concentration was proportionately represented at all points on virus inactivation curves. Therefore, any marked over or under estimation of this value was proportionately reflected in the elevation of the curves. The $S_{30}$ would be similarly affected because it is directly proportional to curve elevation. An example of this kind of error may be represented by the large horizontal variation between replicates of strains 2626 and S67 in the TSS (Figure 5).

Plaque counts of less than 50 that represented percent virus survival values of 10 percent and less, were invariably observed following 45 minutes of heat or trypsin inactivation of highly sensitive ADV strains. The error that resulted from these relatively low plaque counts was not considered significant because it represented a maximum of only two percentage points of the actual percent-virus-survival value.

The pH of test medium for the TIK and TRIK tests was checked and adjusted if necessary on the day of each test procedure. The inherent error of the pH meter was $\pm 0.05$ pH units under ideal conditions. Consequently, a pH difference of 0.1 or larger may have existed between test replications. However, no differences in pH were observed between virus samples within test replications.
The uniformity of pH was attributed to the 1000-fold dilution of stock virus with test medium that had been previously adjusted to the desired pH.

The thermosensitivity of hominid herpesvirus can be affected by undefined components of spent cell culture medium in which the virus is propagated (76). Consequently, virus for the TIK test was diluted a thousand-fold to minimize potential adverse effects of spent medium. Virus for TRIK was similarly diluted for the same purpose.

The temperature of the water bath was not visibly affected by the sequential addition of virus samples. Consequently, the reaction temperature between strains within replicates was uniform. However, temperature varied as much as 0.1°C between replications of the TIK and TRIK tests because of difficulty in adjusting the thermostat of the water bath.

Virus assay sampling procedures in the TIK and TRIK tests were usually completed in 15 to 20 seconds. Occasionally more time was required for sampling because of difficulty in attaching and/or removing pipette tips from the automatic syringe that was used in the sampling process. When variation in sampling time did occur, the resulting error was minimized because duplicate preparations of each strain were concurrently tested.
Trypsin concentration between strains and test replications was subject to some variation because two pipetting procedures were required to achieve the final concentration (see page 44). The marked vertical deviation from the linear distribution of replicated strains within the TRSS represented by strains S62/26 and Be (Figure 12) may have resulted from this kind of error. This was likely because all other test conditions were uniform within replications and no other strains exhibited similar variation.

Correlation of thermal and trypsin markers with virulence

It was concluded from the present study that virulence was not correlated with the thermal and trypsin sensitivity markers. This conclusion was based on the following observations. First, the European vaccine strains were located at the opposite extremes of the sensitivity spectrums. Secondly, multiple passage of strain Dr through Vero cell cultures resulted in an apparent increase in its virulence for mice (Table 12) and a significant reduction in its virulence for rabbits (Table 13). These changes in mouse and rabbit virulence were not accompanied by any visible change in the thermal or trypsin sensitivity markers.

These findings agree with the observations of
Colais and Sabó (31) with respect to the thermal sensitivity marker but do not support the hypothesis presented by Bodon et al. (11) and Bartha et al. (5) that virulence and trypsin resistance are correlated. The reason for this discrepancy probably was the small number of avirulent strains that were evaluated by Bodon, Bartha and their associates.

Cytopathic Effect and Plaque Growth Rate Markers

The use of CPE and PGR markers did not increase the number of distinct marker profiles (Table 14). However, the marked differences that were demonstrated between strain K, strain BUK and the field isolates, contributed to the uniqueness of individual profiles which will facilitate categorization of additional ADV isolates.

The relationship between CPE of ADV-infected MDBK and Vero cell lines and strain virulence that was observed in the present study, suggests that these markers may be correlated. Other investigators have reported similar relationships in ADV-infected PK (4, 48), CK (10, 11, 76) and MK (74) cells. However, this relationship may not be absolute. Virulent ADV strains have induced both cell rounding and syncytium formation in PS and PK-K pig kidney derived line cells (32). A definitive conclusion
regarding correlation between virulence and CPE in
in MDBK and Vero cells cannot be made in view of the
relatively small number of strains evaluated.

Some investigators have suggested that syncytium
formation by herpesviruses is dependent on the multi­
plicities of infection while other researchers have failed
to demonstrate this relationship (16). The results of
the current study (see page 109) tend to indicate that
syncytium formation by ADV-infected cells is not
related to the multiplicity of infection. The increased
occurrence of syncytia in MDBK cell monolayers inocu­
lated with a low multiplicity of strain BUK (see page
102) probably occurred because the cell monolayer
remained intact longer than it did when a high multi­
plicity of strain BUK was used. Consequently the
probability of observing syncytia was increased.

The relatively low PGR's of strains K and BUK and
the large PGR's of the virulent field isolates (Figure
17) suggest that a possible correlation may also exist
between plaque size in Vero cells and virulence of ADV
in rabbits. However, two observations made in the
present study contradict this suggestion.

First, the PGR of strain BUK was significantly
smaller than the PGR of strain K (Figure 17). If a
correlation existed between PGR and virulence, the PGR of strain K should have been less than the PGR of strain BUK because strain K is less virulent for rabbits than strain BUK (Table 11). This reasoning assumes that the expression of the PGR marker is proportional to virulence. However, it is conceivable that PGR is regulated by multiple factors that prevent a proportional response within a narrow range of virulence.

Secondly, the PGR of strain Dr increased slightly (Figure 26) while its virulence in rabbits decreased (Table 13) following multiple passage in Vero cells. However, it is possible that the slight increase in PGR represented an adaptation of strain Dr to replication in Vero cells, while the moderate reduction in rabbit virulence was not great enough to affect the PGR.

In any event, it appears that the PGR's of additional virulent and avirulent ADV strains must be compared before a firm conclusion can be made. At this time, however, the evidence tends to indicate that no correlation exists between PGR in Vero cells and virulence, which agrees with the findings of other investigators who studied the relationship of virulence to plaque size in PK cells (81) and chick embryo cells (71).
Animal Virulence Markers

Vaccine strains of ADV have been differentiated from virulent strains by their reduced virulence in different animal species (11, 48). However, no comparative studies of the mouse virulence markers of virulent ADV strains have been documented.

The comparison of ADV strains by their mouse LD$_{50}$'s in the present study may have established a sixth marker profile (Table 13), if the difference between the LD$_{50}$ of strain S62/26 and the other field isolates is real (Table 9). This difference could not be statistically evaluated because the susceptibility of the mice to ADV in the two experiments was different, as indicated by the significant difference ($P < .02$) between the mean LD$_{50}$ of both experiments. However, the data in both experiments suggested that the virulence of strain S62/26 in mice as determined by the LD$_{50}$ was equal to the virulence of the vaccine strains and less than the virulence of the field isolates.

The difference between the susceptibility of mice to ADV in the above experiments (Table 9) may have occurred if different ages were not equally represented in both experiments. The mice used in these experiments (Table 9) ranged in age from 18 to 23 days. This age
range may have represented a wide variation in susceptibility to ADV since this susceptibility can decrease a 1000-fold in mice from birth to maturity (26). This is also possible that the difference in mouse susceptibility was due to the separate origins of the mice because susceptibility to ADV is probably determined by genetic background (34).

The efficiency of the mouse virulence markers was enhanced by measuring the mouse MTD (Table 10). This parameter provided a means of separating strain K from strains BUK and S62/26 which was not possible by comparison of LD<sub>50</sub>'s.

The mean MTD for each ADV strain was calculated from the data of both experiments summarized in Table 10 because no significant differences were detected between the overall mean MTD's. This apparent homogeneity of mice between experiments may indicate that susceptibility, as determined by the LD<sub>50</sub>, and clinical response, as measured by MTD, are not correlated in some mouse strains.

In the present study, strain BUK killed rabbits without producing pruritus. Škoda and Jamrichová (70) have reported similar findings. The inability to kill or produce clinical signs in rabbits inoculated with strain K was not expected. Bodon et al. (11) reported death in
two of three rabbits 7 to 9 days following inoculation with strain K. The lack of virulence of strain K for rabbits in the present study may have been due to the virus dose, the susceptibility of the rabbits tested, or possibly to differences between the cell passage level of strain K used in Bodon's laboratory and the cell passage level of strain K evaluated in this laboratory. It is also possible that if more rabbits were included in the experiment summarized in Table 11, some of the principals may have died.

The failure of the rabbit virulence marker to detect differences among the four virulent field strains does not reduce this marker's potential as a strain differentiator. Gordon and Luke (33) observed that ADV isolates from three separate field outbreaks in England, required two to three serial passages in rabbits before pruritus was observed. All ADV isolates studied in this laboratory have produced pruritus in rabbits on the first inoculation. However, it is possible that field strains that do not readily induce pruritus in rabbits may also exist in this hemisphere.
Effect of Passaging ADV in Different Cell Lines on Viral Markers

The marked increase in thermal stability of strain Dr that occurred after multiple passages of the virus in MDBK cells (Figures 24 and 25) may have resulted from spontaneous mutation. It is also possible that the increase in thermal stability resulted from the gradual accumulation of thermal resistant properties acquired from the host cell.

Cumulative changes in antigenic properties of hominid herpesvirus have been demonstrated following a high number of passages in cells (36). Similar changes in the electrophoretic properties of IBR and IPV viruses have also been reported (54). Because protein is intimately associated with antigenic and electrophoretic properties of virus as well as thermal stability, the possibility that cumulative changes in thermal sensitivity of strain Dr occurred during passage of the virus in MDBK cells is real.

Cumulative changes in the thermal sensitivity of strain Dr did not occur following passage in Vero and ST cells (Figures 24 and 25). This observation may indicate that ADV should be propagated in either of these cell lines if it is to be characterized by the TIK test.
Vero, ST and MDBK cell lines appear to be equally suitable for propagating virus that is to be used in the evaluation of the CPE marker (see page 126). These cell lines also appear to be suitable for propagating virus strains for PGR comparison (Figure 26). Although passage of strain Dr through ST and Vero cells did result in a slight increase in the PGR after 114 passages, the low number of passages through either of these cell types to which ADV strains would be subjected prior to marker evaluation would not be expected to noticeably affect PGR.

Conclusions and Recommendations

This study has shown that cloned populations of ADV strains can be differentiated by their marker profiles (Table 14). It also has been shown that the rabbit virulence, mouse virulence, PGR and CPE markers can be used to screen wild virus populations for potential vaccine viruses.

Should the marker profiles summarized in Table 14 be found to remain stable under field conditions, they will serve as a valuable tool in epidemiological investigations. It is recommended that the stability of these marker profiles be studied in both vaccinated
and non-vaccinated swine populations to determine whether mutation occurs or whether the markers remain unaffected.

Additional markers should be defined to enhance the effectiveness of differentiating ADV strains by marker profiles. Urea (31), DDT (27) and heparin (62) sensitivity may prove to be useful markers. The mouse virulence marker should be further defined to increase its sensitivity. This refinement may permit finer distinction between virulent strains of ADV. Virulence markers for other laboratory animals should be defined. Such markers may prove to be better differentiators of virulent ADV strains than the rabbit and mouse virulence markers.
SUMMARY

The thermal sensitivity, trypsin sensitivity, CPE, PGR and animal virulence markers were characterized for the European ADV vaccine strains K and BUK. These markers were also characterized for virulent ADV field strains 1664, 429, S62/26 and Be. In addition, the thermal sensitivity marker of six other virulent field strains was described.

Thermal sensitivity differences between ADV strains were markedly influenced by pH, temperature and the components of the medium in which virus was heat treated. Marked differences between strains were demonstrated at 48°C, in saline G, pH 6.9. Each strain was classified as thermal resistant, thermal intermediate or thermal sensitive according to its position in the thermal sensitivity spectrum. This spectrum was demonstrated by transforming thermal inactivation kinetic curves to a natural log function of time and plotting standardized inactivation rates against standardized 30-minute survival values. The European vaccine strains K and BUK were located at the opposite extremities of the spectrum with strain K being the most heat resistant.
Trypsin sensitivity differences between ADV strains were demonstrated by subjecting virus to 0.01 mg/ml trypsin in modified saline G, pH 6.9 at 30C. Each strain was classified as trypsin resistant, trypsin intermediate or trypsin sensitive according to its position in the trypsin sensitivity spectrum. Strain K was the most resistant and strain BUK was the most sensitive to trypsin inactivation.

The magnitude of the difference between the mean size of plaques produced by different ADV strains was influenced by the cell type and the composition of the plaquing medium used. Significant differences were observed between PGR's of strain K and BUK in Vero cells overlaid with plaquing medium containing 0.375 percent CMC. These PGR's were also statistically different from the PGR's of the virulent field strains. Differences between the PGR's of some field strains appeared to exist although they were not statistically significant.

The CPE in MDBK and Vero cells infected at a multiplicity of greater than one with virulent field strains of ADV, was characterized by large syncytia. Vero cell syncytia commonly contained between 10 and 20 nuclei. Occasionally as many as 50 nuclei were seen.
Syncytia in MDBK cells usually contained at least 25 and frequently more than 50 nuclei.

The CPE in these cell lines infected at the same multiplicity with strains K and BUK was primarily characterized by rounding and piling up of cells. Occasionally syncytia containing fewer than five nuclei were observed in Vero cells and syncytia containing fewer than 10 nuclei were observed in MDBK cells.

Vaccine strains K and BUK were differentiated from the virulent field strains by their virulence in rabbits. Field strains induced pruritus and death. Strain BUK killed rabbits without causing pruritus and strain K neither killed rabbits nor caused clinical signs.

Apparent differences were observed between mouse LD$_{50}$'s of strains K, BUK and S62/26 and strains 429, 1664 and Be. Strain K was differentiated from strain BUK and the field strains by the mean time-to-death of mice separately inoculated with similar amounts of the different ADV strains.

Six distinct marker profiles were described when all of the above markers of each ADV strain were collectively compared. This observation may provide
the basis for a strain identification system that can be used in epidemiological investigations.

The thermal sensitivity, PGR, CPE and animal virulence markers were evaluated for ADV strain Dr following 114 passages through MDBK, Vero and ST line cells. No changes were observed in the PGR and CPE markers. An apparent decrease in thermal sensitivity occurred following passage through MDBK cells but no apparent changes resulted from passage through Vero or ST cells. The virulence of strain Dr for mice as measured by the LD$_{50}$ appeared to increase following passage in MDBK and Vero cells. Virulence in rabbits as measured by the mean time-to-death was significantly reduced by passaging strain Dr in Vero cells.
REFERENCES


ACKNOWLEDGMENTS

I extend my sincere appreciation to Dr. C. J. Maré for his guidance and support throughout the course of this study. I also appreciate the support given me by Dr. R. A. Packer and the other members of my committee, Drs. G. Beran, D. Durand, J. Kluge and W. Rowley. I also extend my thanks to Drs. P. Hinz and L. Dumenil for their valuable statistical advice.

This study would not have been possible without the support and understanding of my wife, Jean, and our children, Adriana and Bradley. I am also grateful to my mother, Eileen Huzzy, and my grandparents, Mr. and Mrs. Thomas L. Bradley, whose love and guidance during my childhood are reflected in the accomplishment of this dissertation.

The typist for this dissertation was my wife, Jean E. Platt.