

STUDIES ON THE IN VITRO CULTIVATION OF
AVIAN ENCEPHALOMYELITIS VIRUS

by

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TABLE OF CONTENTS

INTRODUCTION	1
REVIEW OF LITERATURE	2
Definition and Nomenclature	2
History and Distribution	2
Etiology	4
Symptoms	5
Pathology	7
Differential Diagnosis	9
Epidemiology	10
Immunity	13
Cultivation of the Virus of Avian Encephalomyelitis	15
MATERIALS AND METHODS	17
Source of Experimental Birds	17
Virus Strains	20
Histopathological Examination	24
Preparation of Antiserum	25
Tissue Culture: Materials	26
Tissue Culture: Methods	29
EXPERIMENTAL WORK	35
Embryonic Chick Kidney Cell Cultures	35
Pig Kidney Cell Cultures	46
HeLa Cell Cultures	48
Suspended Cell Cultures: Maitland Technique	56
DISCUSSION	60

SUMMARY	66
ACKNOWLEDGMENT	69
REFERENCES	70
APPENDIX	79
MICROPHOTOGRAPHS	84

INTRODUCTION

The marked revival of interest within recent years in the specific disease entity of chickens known as avian encephalomyelitis (epidemic tremor) is a reflection not only of the widespread occurrence and economic importance of this disease, but stems also from a greater appreciation of the complex nature of the condition.

Fundamental knowledge regarding the virus, its mode of transmission and duration within the bird is essentially lacking. While considerable study has been made of the effects of the virus in young chicks, very little is known of the pathogenesis in adult birds or of the subsequent duration and level of immunity. Likewise, the degree or duration of passive immunity in chicks hatched from recovered or immune birds has not been clearly elucidated. Certain other associated phenomena such as the effect of the virus on egg production and subsequent egg quality, the occurrence of iridocyclitis and cataracts in birds following outbreaks of disease and the role of other avian species and even of fomites in the dissemination of the virus have not, as yet, been thoroughly investigated.

As a fundamental approach to the problem it was considered that a knowledge of the biology of infection at the cellular level was essential to an understanding of the disease, since it is the individual tissue cell rather than the intact animal which is the true host of the virus. In the bird, investigation at this level is extremely complicated, but tissue culture enables the isolation of the essential host from the physiological, nutritional and immunological mechanisms of the intact animal and opens the way for a study of the virus-host relationship at a level which would not otherwise be possible.

The specific purpose of this study, therefore, was to investigate the behavior and properties of avian encephalomyelitis virus when introduced into tissue culture systems that have been successfully employed for the propagation and study of certain other avian and mammalian viruses.

REVIEW OF THE LITERATURE

Definition and Nomenclature

Avian encephalomyelitis (AE) may be defined as a nervous disorder of viral etiology primarily affecting young chicks and manifested by depression, ataxia and paralysis, frequently accompanied by pronounced rapid tremor affecting different parts of the body.

Jones, in 1932 (47) first described the condition as an encephalomyelitis but later (1934) (48) chose the term epidemic tremor as being a more descriptive lay term. Although the latter term is still widely used and accepted, Van Roekel, et al. (99, 100) proposed the name, infectious avian encephalomyelitis because tremor was more frequently absent than present and nearly always appeared subsequently to the ataxia.

Finally in 1939 the binomial, avian encephalomyelitis, was recommended by the Committee on Poultry Disease Nomenclature of the American Veterinary Medical Association (5) and this has become the most commonly accepted term.

History and Distribution

A hitherto unrecognized disease of young chickens appeared in a number of New England flocks between 1930 and 1932. The condition was first seen in

Massachusetts and described in a preliminary report by Jones (47) in 1932. Extensive studies were undertaken over a four-year period and reported in detail in 1934 (48). By this time frequent reports indicated a widespread occurrence of AE throughout the New England states (48, 7).

During the following decade the disease apparently became well established throughout the North American continent (57, 98, 102) and was reported from Australia in 1940 (39). Subsequent surveys have been undertaken by Feibel (33) in 1951 and more recently by Taylor and Schelling (95) in 1960 in the United States and Canada. The latter authors used an immunity test based on the resistance of embryonating eggs from recovered (immune) birds to challenge with a calculated dose of AE virus. Tests conducted on eggs obtained from 46 States and Provinces indicated the presence of resistant birds in the majority of flocks tested.

Numerous reports in the literature testify to the almost world-wide occurrence of epidemic tremor at the present time. In Europe the disease has been reported from Great Britain (68, 69), Germany (36), France (9, 10, 37), Scandinavia (62), Switzerland (35), and the Netherlands (6), while outbreaks have also been reported from Israel (107) and various parts of Africa (24, 91). In many cases initial outbreaks have been ascribed to the importation of hatching eggs from the U. S.

Descriptions of the disease from widely disseminated sources indicate a marked similarity in its behavior. While not considered to be a major threat to the industry at large, its sudden appearance, often with high mortality and unpredictable duration, constitutes an economic problem to the broiler industry and a source of great concern to the individual breeder and hatchery man (88, 98, 109).

Etiology

The viral etiology of the disease was clearly established by Jones (48) and later confirmed by the work of Olitsky and Bauer (76). From an academic point of view, the virus of avian encephalomyelitis is of interest by virtue of its relationship to the poliomyelitis group of viruses (58). Burnet (13) tentatively classified it with this group on the basis of its neurotropic character and ether resistance.

Filtration studies have shown the virus to be capable of passing through Berkeveid V & N candles (48), Eiford collodion filters of average pore size 1.0μ (39), and also through Seitz filter pads (48, 99) but with a marked reduction in titer in the latter case (99).

Olitsky and Bauer in 1939 (76), using gradocol membrane filtration, estimated the virus to have a diameter of 20 to 30 $m\mu$ which is within the range of other virus encephalitides such as St. Louis encephalitis and the Eastern and Western strains of equine encephalomyelitis.

While centrifugation for one hour at 12,000 r.p.m. yielded virus satisfactory for filtration through gradocol membranes (73), Jungherr and Minard (57) were successful in obtaining a noninfective supernate following centrifugation at 20,000 r.p.m. for one hour, the sediment remaining infectious.

Jones (48) reported the survival of the infective agent after storage of brain material in 50% glycerol for at least 69 days and Olitsky (73) for at least 89 days. The virus was also extremely stable in the lyophilized state (73). Frozen brain material was found to be still infective after 438 days when inoculated as a 1:10 dilution intracerebrally into susceptible chicks (85), while a brain in saline suspension of the virus remained potent after

storage at 4° C for as long as 836 days (77). The reported (87) survival of the virus for 14 days at 37° C further testifies to its tenacity.

The virus is not susceptible to the action of antibiotics (penicillin, streptomycin or chlortetracycline) even in high concentrations (32). The differentiation of AE virus from the Eastern and Western strains of encephalomyelitis has been demonstrated by means of serological tests and lack of pathogenicity for various laboratory animals (73).

Although the virus of epidemic tremor has in the past been chiefly characterized by its obvious neurotropic and neuropathic properties, more recent work (18, 19) indicates that field isolates, less removed from their original hosts than artificially maintained strains, may also exhibit definite enterotropic characteristics. Up to the present time there has been no indication of the occurrence of antigenically different strains.

Symptoms

Natural infection with epidemic tremor has been reported in only two avian species, the domestic fowl (47) and the Mongolian pheasant (70). However, ring-necked pheasant and guinea fowl chicks are susceptible to artificial inoculation (70) as are turkey poults and ducklings (100). Laboratory animals such as rabbits, guinea pigs, white mice, and monkeys are refractory to artificial infection (73) and attempts to adapt the virus to rats and mice by serial intraocular passage have not met with success (103).

Avian encephalomyelitis is chiefly manifested by a nervous disorder in very young chicks, most commonly between 1-3 weeks of age, but infection is by no means confined to this age group. Spontaneous cases have been reported (47, 96, 99, 100) in chicks 24-48 hours after hatching but heaviest losses are

encountered in chicks between the ages of 2-6 weeks (47, 48, 57, 68, 98, 99). The incubation period following artificial inoculation varies according to the dose and route of administration, the strain of virus, and the susceptibility of the chick (39, 47, 48, 73, 84, 99, 100). Van Roekel et al. (99) reported an increase in virulence following repeated passage of the virus through chicks with a reduction in the average incubation time from 25 to 13 days, the shortest period observed being 5 days. Although several workers have reported (47, 48, 84, 99) the appearance of symptoms in some cases as long as 40 days after inoculation of a group of chicks, the possibility of contact transmission should be borne in mind (18, 82, 85).

The characteristic symptoms in young chicks have been described in detail by Jones (47, 48), Van Roekel et al. (98, 99, 100), Jungherr et al. (56, 57), and Ostendorf (78). Affected birds show no significant difference in body temperature or blood count (7). Morbidity is generally estimated at 5-10%, but losses are difficult to calculate under field conditions as many are destroyed by the owner. However, mortality rates of over 50% have been reported (48, 99, 107). Survivors frequently show persistent nervous symptoms but may attain normal egg production nevertheless (100, 109).

An interesting feature of the disease is the reported occurrence of iridocyclitis, and cataracts (usually bilateral) in birds 3 to 6 months of age and associated with previous outbreaks of AE (8, 34, 32, 81, 108). A similar condition has been produced experimentally by intraocular inoculation of chicks with AE virus (32, 108).

Natural infection in susceptible adult birds does not result in observable symptoms but, a temporary drop in egg production and simultaneous lowering of fertility during this period has been observed both under field (96, 109) and experimental (18) conditions.

Pathology

The need for accurate diagnostic criteria due to the similarity of AE to several other clinical entities in young chicks in which gross pathological lesions are absent, has emphasized the importance of specific histopathological changes. These changes, which have been described in considerable detail by Jones (47, 48), Olitsky (73), Jungherr (52), Jungherr and Minard (57), and Ostendorf (78) are essentially of a comparatively mild nature in spontaneous cases.

Olitsky (73) considered neuronal degeneration, mainly seen in the pons medulla and anterior horn cells of the spinal cord in the region of the lumbosacral enlargement, to be the most striking lesion seen in the CNS. Progressive changes consist of an initial swelling of both the cell body and nucleus, followed by nuclear eccentricity, tigrolysis, clearance of the Nissl substance from the cytoplasm and, in advanced cases, complete or almost complete disappearance of the entire neuron.

Degeneration of the Purkinje cells of the cerebellum, particularly in advanced cases, was noted by Jones (48), Olitsky (73), and Hart (39), while neuronophagia and satellitosis were also generally seen only in the chronic condition (57).

The distribution of focal lesions in the CNS exhibits a distinctly vascular orientation. Jungherr and Minard (57) observed that in naturally infected cases, 31% exhibited lesions in the cerebrum and 23% in the midbrain, cerebellum and medulla. Moreover, while these focal lesions were mainly observed in the grey matter of the brain, the spinal cord did not show this restriction. The smallest recognizable lesions, which are nevertheless considered to be of diagnostic significance, consist of hyperplasia of endothelial and

adventitial cells, together with varying degrees of perivascular infiltration, chiefly by lymphocytes. Astrocyte and microglial reactions are mainly confined to the periphery of the focal reaction. The intensity of the focal CNS lesions varies widely and quite independently of the severity of symptoms.

In experimental cases, following intracerebral inoculation, these lesions tend to become massively developed, particularly in the cerebrum while visceral lesions are either absent or much less pronounced (48, 57, 73, 78).

Occasional small collections of lymphoid cells normally occur quite irregularly distributed throughout avian tissues (57, 97). However, since the muscularis of the gizzard and proventriculus is ordinarily devoid of lymphoid follicles, their occurrence in these tissues is considered to be of diagnostic value. Likewise, since the normal myocardium may exhibit loose foci of extramedullary myelopoietic tissue, only definite lymphoid follicles are considered significant. These lymphoid aggregations exhibit either a loose irregular arrangement or occur as definite oval or round follicles circumscribed by a fine reticular membrane. These round, clearly delineated aggregations are considered rather characteristic of AE by Fritzsche (36).

A striking feature observed (48, 57) in natural infections is the occurrence of numerous circumscribed aggregates of lymphocytes in the pancreas especially in the vicinity of blood vessels.

In attempting a diagnosis on histopathological grounds, Jungherr (52) points out that visceral lesions can only be considered significant if accompanied by specific CNS lesions and also (57) that a certain percentage of cases may reveal symptoms but no specific focal lesions and vice versa.

Differential Diagnosis

In considering the diagnosis of epidemic tremor, a number of conditions manifested by nervous disorders, particularly in young chicks, may have to be taken into account.

Certain strains of Newcastle disease virus exhibiting neurotropic tendencies may give rise to symptoms and lesions not easily differentiated from AE (98). Ollitsky (73) demonstrated that AE was a completely distinct entity which could be differentiated from equine encephalomyelitis both serologically and by inoculation of mice and guinea pigs.

On the whole the microscopic lesions seen in chicks inoculated with the equine virus are relatively severe, with lymphoid aggregations, necrosis, polymorphonuclear (heterophile) infiltration and severe vascular changes (97).

Nutritional deficiencies (3) in young chicks, particularly avitaminosis A, avitaminosis E, riboflavin deficiency, ricketts and a cerebellar disorder of nutritional origin described by Pappenheimer and Goetsch (79) may give rise to similar clinical symptoms but with distinctive histopathology. Encephalomalacia caused by Vitamin E deficiency is characterized by edema, degeneration and necrosis of the Purkinje cells and small hemorrhages scattered through the central white matter of the cerebellum (80). Jungherr (50) noted cerebellar fibrosis as well as the occurrence of large areas of increased vascularity accentuated by various degrees of adventitial cell proliferation and intervascular gliosis.

On the other hand Jungherr (50, 51) considers that it may be difficult if not impossible to differentiate neural lymphomatosis involving the CNS from AE and Raggi et al. (82) stated that the CNS lesions resulting from experimental or contact infection with AE virus and a pathogenic strain of Mycoplasma could not easily be differentiated.

Under experimental conditions, the difficulty of accurate diagnosis is further accentuated by the fact that apparently pure strains of AE virus may become contaminated during passage either in chick embryos or in embryo tissue culture with CEL0 virus or CEL0-like agents (46, 64, 66). These agents have been reported capable of producing symptoms and histologic lesions in chicks which might be confused with those produced by AE virus (22, 46, 105).

Other conditions which have been described as giving rise to similar symptoms and requiring differentiation from epidemic tremor are: congenital loco (61); congenital tremor (41); a sex-linked, semi-lethal nervous disorder described by Scott et al. (90) as "the shaker fowl"; certain forms of poisoning such as arsenic and lead (98), DDT (29), and nitrofurazone (49), as well as botulism, bacterial or mycotic osteomyelitis (103) and an ataxia of chicks associated with nephritis (28).

Epidemiology

Early studies (7) on the epidemiology of avian encephalomyelitis indicated that the disease was not hereditary, was not caused by variations of incubation or hatching temperature, and occurred under widely differing systems of brooding and feeding. Experiments (25) designed to determine the influence of thiamin deficiency on the susceptibility of chicks to artificial infection were inconclusive.

However, in 1938 Van Roekel et al. (99) in a preliminary report stated that the inoculation of eggs with infectious material at the beginning of the incubation period gave rise to chicks exhibiting symptoms soon after hatching. This suggested therefore, that the infectious agent may be egg-borne and that breeding stock served as reservoirs of infection.

While these results were again confirmed by Van Roekel (101) in 1941 using eggs obtained from a flock with no previous history of AE, and by Minard and Jungherr (71) using various routes of inoculation, Klügler and Ollitsky (60) questioned an egg-borne cycle since they were unable to demonstrate the presence of infective virus in eggs for longer than 72 hours following inoculation of 5-7 day-old embryos via the allantoic sac. Furthermore, while Jungherr and Minard (57) found that the unabsorbed egg yolk of spontaneously affected chicks contained the virus, Feibel (32) was unable to demonstrate the virus in the yolk sac of artificially inoculated eggs for longer than 5 to 6 days post-inoculation. The latter author was unable to reproduce the disease by intramniotic injection of embryos or to adapt the virus to multiplication in eggs by duplicate zig-zag embryo-chicken passages.

This apparent anomaly was finally resolved by Sumner et al. (94) in 1957 following earlier attempts (58) to adapt the virus to embryos via the intra-ocular route. It was found (93) that the end points of virus titrations in embryos differed markedly with eggs from different sources. They attributed lack of successful egg propagation by other workers to the fact that, from their survey, most flocks produced embryos somewhat resistant to AE infection and suggested that the variability of AE virus titrations in embryos from different sources might be due to parental immunity (94). Subsequent investigations (18, 65, 95, 88) proved this to be the case.

Van Roekel et al. (100) observed that when day-old chicks derived from actively infected flocks, were taken from fumigated incubators and exported immediately to a district where AE was not known to exist, many of them developed the disease. It is apparent from observations of field outbreaks of AE in chicks, that initial infection is usually the result of egg transmission from infected breeder stock (69, 71, 88, 96, 101, 108).

One of the major difficulties in making adequate observations of an outbreak in breeders has been the fact that most flocks show no signs of disease other than a temporary drop in egg production and hatchability (18, 94, 96, 109). Minard and Jungherr (71) were able to demonstrate the presence of a virus in the tissues of adult birds obtained from known AE infected flocks and in feces from a small percentage of presumably normal flocks. This agent produced brain lesions in inoculated chicks indistinguishable from those seen in spontaneous cases of epidemic tremor. An enteric virus, serologically indistinguishable from AE virus was later isolated by Burke et al. (11, 12) in chicken kidney tissue culture from the feces of an apparently normal bird.

Further evidence of an enteric route of infection in adult birds was reported by Calnek et al. (18) who demonstrated that the administration of AE virus to susceptible adult breeders in the drinking water produced a syndrome indistinguishable from that observed in field outbreaks. Not only was egg transmission observed but virus was eliminated in the feces of infected birds over a period of several days and at a level sufficient to infect young susceptible chicks by the oral route. The additional possibility of infection via the respiratory tract was, however, not ruled out.

Jones (48) observed that epidemic tremor may or may not occur in the same flock in successive years and may or may not affect consecutive hatches in any one year, a finding substantiated by several other observers (7, 39, 100, 107). A seasonal prevalence was indicated by Jungherr and Minard (57). Although epizootic periods tend to coincide with the principal hatchery seasons, the vast increase in broiler production in many states would tend to stabilize the incidence level throughout the year. This was, however, not found to be the case in a survey undertaken in Connecticut over a ten-year period (32) where a minor period of prevalence in the Fall was followed by a major one in the

Spring months. The natural tendency for AE to express itself in a biennial cycle within a closed flock has been described by Schaaf (87). Exposure to the disease as chicks causes the survivors to become immune and no outbreaks will occur among chicks hatched from them the following season. These second generation chicks, if reared to maturity may, however, produce AE infected chicks if exposed to the virus during the time when their eggs are being saved for hatching and the biennial cycle will continue.

The disease as observed in England (69), France (37, 63), Israel (107), and South Africa (24) has tended to occur in approximately 2-year cycles.

Immunity

Jones (48) and Bottorff (7) thought it unlikely that the survivors from an outbreak of epidemic tremor would be capable of transmitting the virus to their progeny. However, evidence for the development of serum antibodies following natural or artificial infection with AE was first presented by Olitsky (73) in 1939, by Jungherr and Minard (57) in 1942, and again by Feibel (32) in 1951. A practical method of determining immunity to AE by the use of serum neutralization tests in susceptible embryos was developed by Sumner and co-workers (65, 94), who demonstrated the presence of specific AE virus neutralizing antibodies in the sera and in the yolk of fresh eggs obtained from survivors of the disease.

The practical significance of this immunological response was first recognized by Schaaf and Lamoreaux (88) who suggested that since the survivors of AE in breeder flocks appeared to develop an immunity, they should not be discarded but retained as the most certain source of disease-free chicks. These and other findings led Jungherr (54) in 1958 to state: "The combined observations

changed the entire former concept of the disease. We now know that, (a) we are dealing with a widespread infection of laying flocks which goes unnoticed in many instances; (b) epidemic tremor in chicks occurs in the progeny of the exceptional, susceptible flock; and (c) we are dealing with a disease impossible to eradicate and only attackable through proper vaccination."

Schaaf and Lamoreaux (88) obtained promising but inconclusive results following wing web inoculation of young prospective breeding stock with infected brain material. Vaccination by the intramuscular route provided a greater degree of protection but also induced the highest number of clinical cases following inoculation (84). An embryo-adapted strain of virus was found to be too pathogenic for practical use when administered by the wing-web or intramuscular routes (16) but when given in the drinking water (18), clinical signs were not noticed subsequent to vaccination with the exception of a drop in egg production in two of five breeder flocks inoculated. Immune responses were comparable to those observed following natural outbreaks of AE.

The duration of immunity following vaccination with beta propiolactone (BPL) inactivated virus has not been established but both chick brain (86) and embryo (17) propagated strains were well tolerated. Using the serum-neutralization test (15) to determine the levels of immunity obtained, Calnek and Taylor (17) in 1960 concluded that consistent and high responses to a BPL inactivated virus can be obtained provided dosage is adequate.

Attempts to demonstrate hemagglutinating properties in infected brain material or HI antibodies in the serum of recovered birds, have not been successful (32).

Experimental studies to determine the presence of complement fixing antibodies in the sera of recovered or immunized birds have been undertaken (57, 103) but the results reported to date have been inconclusive. The highly

anti-complementary properties of brain tissue together with the difficulty in absorbing or otherwise destroying the basic brain tissue antibody have proved to be major obstacles in the development of a complement fixation test.

Cultivation of the Virus of Avian Encephalomyelitis

Early studies (48, 73, 100) on the propagation of AE virus indicated that this virus could only be grown by intracerebral inoculation of chicks. More recently it has been demonstrated, however, that the virus can be grown in chick embryos inoculated intra-ocularly (93) and even by the simpler technique of yolk sac inoculation (15, 58, 65, 104).

Many previous attempts to demonstrate multiplication of AE virus by the egg technique had failed and it is now known that the success or failure of embryo propagation depends neither on the route of inoculation nor the strain of virus used, but on the susceptibility of the embryo itself, which in turn is related to the susceptibility of the dam (15, 55, 93, 95).

Calnek and Jelnich (15) reported that peak virus titers were obtained 6-8 days following inoculation of 6-day-old embryos via the yolk sac. While embryo-lethal strain of AE virus has been developed (72) the majority of strains do not cause death of the embryo before the 19th-20th day of incubation (15, 20, 58, 104).

The pathological response of the developing chicken embryo to inoculation with AE virus has been described by Jungherr et al. (58) and Casorso and Jungherr (20). Characteristic macroscopic lesions were first observed on about the 18th day of incubation and consisted, essentially, of partial or complete immobilization, reduced size (dwarfing), stiffness or ankylosis of the limbs and abnormally placed toes; either gross atrophy or reduction of skeletal

musculature were seen, and severe internal hydrocephalus occurred in the terminal stages. Specific histological changes associated with the progressive pathogenesis of the infection have been described by Casorso and Jungherr (20) who suggested that the virus appeared to attack the CNS first, causing marked degenerative changes, followed by dorsal ganglionic atrophy and secondary muscular dystrophy. The neural lesions were characterized by severe local edema, gliosis, vascular proliferation and pyknosis. Terminally, little or no functional nervous tissue remained in the spinal cord or brain. Muscular changes consisted of progressive myopathy with little or no inflammatory response.

Inoculation of AE virus onto the chorio-allantoic membrane of embryonating eggs has been reported to produce lesions which may be differentiated from those characteristic of laryngotracheitis and CELV viruses by the absence of intranuclear inclusion bodies (66).

An apparent multiplication of the virus in Maitland type cultures of minced whole embryo tissue in vitro in the presence of chicken serum has been reported by Kligler and Olitsky (60). Thus, cultures, having a viral titer of 10^{-3} at the outset were shown by chick inoculation tests to have a titer of 10^{-2} in the 5th subplant, although the original virus itself was calculated as being diluted to 10^{-8} in this passage. Virus multiplication did not occur in cultures of minced chick embryo brain in the presence of 10% chicken serum or in whole embryo cultures in the absence of serum. These workers concluded that the virus had distinctive requirements for multiplication in contrast to the neurotropic viruses of poliomyelitis and rabies.

In 1959, Hwang, Luginbuhl and Jungherr (43) reported the successful propagation of AE virus in monolayer chick kidney cell cultures prepared by a modification (42) of the techniques described by Younger (106) for monkey kidney monolayers. The demonstration of plaque formation in a chicken embryo kidney

cell culture system was reported by the same authors (44) in 1959, and in a subsequent paper (45) they compared the results of in vitro virus neutralization tests on serum samples from breeding flocks with those of the embryo susceptibility test undertaken in eggs from the same flocks, and found them to be in agreement. For these reasons it was considered desirable to further investigate the dynamics of virus multiplication in vitro.

The lack of a uniform and readily discernable specific reaction in laboratory animals or embryos as well as the difficulty frequently encountered in rendering an etiological diagnosis on the basis of histopathological lesions has greatly hampered epidemiological studies. Much basic knowledge regarding the virus itself is lacking, while nothing is known of the possible relationship between this and other avian viruses in the pathogenesis of disease.

Accordingly it was decided to attempt the propagation of the virus in embryonic chick kidney monolayers using only minor modifications of techniques reported to be successful for multiplication of certain strains of Newcastle disease, vaccinia, vesicular stomatitis, swine influenza, and mouse encephalomyelitis (14) as well as laryngotracheitis (21) and infectious bronchitis (23, 31).

MATERIALS AND METHODS

Source of Experimental Birds

The chickens and adult birds used in various phases of this study were obtained from different sources as follows:

Group A:

Initial propagation of the Van Roekel (VR) strain of AE virus, which had been stored at approximately -12° C for two and a half years, was undertaken in 10-day-old sexed cockerels (cross-bred) obtained as day-old chicks from a commercial hatchery near Manhattan, Kansas. Three subsequent passages and titrations were undertaken in birds from the same source.

Group B:

A troupe of 25 three-week-old White Leghorn chickens, obtained as day-old chicks from the Kansas State University Poultry Husbandry flock, were used for preparation of a virus stock. These birds had been vaccinated at 1 day of age with infectious bronchitis (Massachusetts strain) and Newcastle disease (B-1 strain) given by the intranasal route.

Group C:

Adult cocks originally obtained as day-old from the Kansas State University Poultry Husbandry flock and raised in an isolation unit were maintained for the production of antiserum.

During this phase of the study the birds were housed on wire mesh floors in tiered brooders fitted with electrical heating units and located in an isolated area within the Pathology Department building. The birds were tended only by the investigator and observed twice daily for symptoms and mortality. Apart from those in Group B, none of the birds described above were subjected to any form of vaccination.

Group D:

Birds used for subsequent titrations and virus passages were obtained from a University of California Poultry Husbandry flock. Although maintained as a closed unit and known to be free from the common respiratory infections, epidemic tremor had been diagnosed in this flock on several occasions prior to the conduction of these experiments (82).

Group E:

A limited number of virus titrations were carried out for comparative purposes using eggs and chicks collected from a small flock of single comb White Leghorn hens maintained in a maximum security isolation building at the University of California, Davis. These birds were known to be fully susceptible to epidemic tremor. The limited number of fertile eggs available from this flock precluded their use as a general source of AE susceptible chicks. However, sufficient eggs were available for propagation of the virus in embryos for five generations, and for hatching a small group of susceptible chicks.

Group F:

For the final titration of tissue culture passaged brain material, a batch of 200 sexed White Leghorn cockerels was obtained as two-day-old birds from a large commercial hatchery in Northern California. These chicks were the progeny of dams that had been vaccinated as young growing birds with AE virus by the method described by Schaaf (84, 88). The chicks, themselves, were not subjected to any form of vaccination and were raised in strict isolation and under careful supervision until the time of the experiment.

The experimental birds in Groups D, E, and F were housed in isolation units, either in wire-floored cages or on a litter composed of clean wood shavings. Access to these units was by way of a small anteroom provided with foot and hand baths containing disinfectant and entry was strictly limited to the investigator alone. Sterile protective clothing was worn at all times. Upon conclusion of an experiment, all equipment, litter, etc. were removed and the unit thoroughly disinfected and hosed down.

None of the birds in Groups D and E, nor their dams, were subjected to any form of vaccination.

Virus Strains

Two strains of avian encephalomyelitis virus (AEV) were used for the in vitro propagation studies on tissue culture.

Massachusetts (Van Roekel) Strain. This strain of avian encephalomyelitis virus had originally been isolated by Dr. H. Van Roekel from a field outbreak in Massachusetts and was designated the VR strain. A sample of this virus had been received by Dr. J. L. West (Pathology Department, Kansas State University) at the 150th passage level of brain to brain transmission through chicks. It was subsequently passaged in turkey poults by intracerebral inoculation of brain material for five generations (103) and had been stored at -12° C for two and a half years as a 50% suspension of infected turkey brain in physiological saline prior to use in the present experiment. This strain of virus was then designated, AE(VR), 155th brain passage.

California (Schaaf) Strain. This strain of virus was originally isolated from a field outbreak of epidemic tremor in 1949 and subsequently maintained by serial brain to brain passage in chicks. It was obtained from Dr. Kermit Schaaf, Kimber Farms Incorporated, Niles, California.

The virus was received as a 20% suspension of brain material in 50% buffered glycerin diluent and represented the 9th chick passage. It was designated AE (S).

Preparation and Titration of Virus Strains. Serial transfer of the VR strain of AE virus by intracerebral injection of infective brain material was carried out approximately every 2 months using batches of chicks ranging in age from 3 days to 22 days. The inoculum consisted of 0.05 ml. or 0.1 ml. of a 1 in 10 suspension of brain material in phosphate buffered saline or nutrient broth containing 5 mg. of dihydrostreptomycin and 50,000 units of penicillin

per ml. Chicks more than 2 weeks old received the larger dose. A proportionate number (5 to 10%) of uninoculated birds served as controls in each batch. Infected chicks were generally destroyed 2 to 3 days after the initial appearance of symptoms or when the birds had become paralyzed and incapable of reaching feed and water.

The heads were removed and dipped in isopropyl alcohol, allowed to dry and the brains removed aseptically. Phosphate buffered saline (27) or nutrient broth was added to the pooled brain material at the rate of 4 ml. of diluent per gram of tissue and the mixture blended in a homogenizer. The suspension was then rapidly frozen and thawed and reground in the homogenizer for a further period, the procedure being repeated 3 or 4 times in order to disrupt the cell membranes. A small quantity of the homogenate was removed for bacteriological examination and the remainder stored at -60° C in 4 ml. amounts in screw-capped glass vials or rubber-stoppered tubes. Aliquots from certain batches were also stored at -60° C.

Sterility tests were undertaken on the brain tissue from each batch in the following manner:

1. Tubes of Thioglycollate medium (Difco) and tryptose broth were inoculated and examined for evidence of growth after two days and again at 7 days.
2. Blood agar plates were streaked, examined after 24 hours incubation at 37° C and again at 3 days and 7 days for presence of bacterial or mycotic colonies.
3. Specific examination for the presence of mycoplasma species was undertaken by inoculating tubes of PPL0 broth (Difco) containing 1% yeast autolysate (Albiml), 0.5% glucose and 10% horse serum (1, 2). Following incubation for 3 days at 37° C, 2 or 3 loopfuls of broth were streaked onto 10% horse serum agar plates which were then incubated at 37° C for a further 3-4 days. The

plates were examined by oblique light under a dissecting microscope for mycoplasma colonies.

4. Inoculation of embryonating eggs via the chorio-allantoic cavity was not a routine procedure but was undertaken with some hatches as an additional precaution. In the event of mortality occurring before the 7th day post-inoculation allantoic fluid was harvested and examined for hemagglutinating properties with washed chicken erythrocytes. Subsequent egg passages were undertaken when considered necessary.

Titration of each batch of prepared virus material was undertaken by intracerebral inoculation of young chicks with consecutive tenfold dilutions of brain material in a suitable diluent using a minimum of 5 chicks per dilution. Observations were recorded for a total period of 28-35 days following inoculation except that deaths occurring before 24 hours were considered nonspecific, i.e., caused by trauma or shock. Although several cases were encountered in which histopathological lesions were observed in the absence of clinical symptoms, a standardized procedure was adopted for titration purposes whereby only those chicks exhibiting clinical manifestations of AE were considered infected. Histopathological examination was used only to confirm the diagnosis in clinically affected chicks. By this means an approximation of the infective dose for 50% of the chicks (ID₅₀) was calculated using the statistical method described by Reed and Muench (83).

The results of titration experiments undertaken on various batches of stock AE virus are summarized in Table I.

Propagation of the Virus in Chicken Embryos. Before attempting propagation of the VR strain of virus in embryos, fertile hatching eggs obtained from the small experimental flock described above (Group E) were examined for susceptibility to infection with the chick brain propagated virus, by the method

Table 1. Results of titrations of various passage levels of stock AE virus used for tissue culture propagation studies.

Virus pool	Age of chicks	Dose	Dilutions								Estimated CID ₅₀ titer/ml. **
			10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	Control	titer/ml.	
156th chick brain passage (VR strain)	9 days	0.05 ml.	8/8* (10.0)**	8/8 (10.0)	8/8 (11.5)	7/8 (12.4)	3/8 (12.4)	1/8 (14.0)	0/8	10 ^{6.0}	
157th chick brain passage (VR strain)	5 days	0.05 ml. ---	4/4	5/5	3/5	2/5	0/5	0/5	0/5	10 ^{5.7}	
158th chick brain passage (VR strain)	15 days	0.1 ml.	5/5 (8.0)	5/5 (9.8)	5/5 (10.4)	5/5 (12.4)	0/4	>10 ^{5.0}			
160th chick brain passage (VR strain)	2 days	0.05 ml. ---	8/8 (12.8)	7/8 (14.7)	6/9 (17.3)	1/9 (18.0)	0/5	10 ^{5.5}			
10th chick brain passage (Schaaf strain)	7 days	0.05 ml.	4/4 (13.2)	5/5 (14.8)	5/5 (17.3)	4/4 (19.0)	2/5 (23.8)	0/5 (20.0)	10 ^{6.5}		

*The numerator indicates the number of chicks affected; denominator represents number of chicks inoculated.

**Numbers in parenthesis represent average incubation period in days.

***Chicken infective dose 50% titer estimated by the Reed-Muench method (83).

described by Sumner and co-workers (93, 94). Tenfold dilutions of infective brain material (160th passage) having an ID₅₀ for chicks of $10^{5.5}$ per ml. were inoculated in 0.2 ml. amounts into the yolk sac of 7-day-old embryonating eggs. The embryos were examined alive on the 20th day of incubation for signs of infection as evidenced by immobility, dwarfing, and muscular atrophy, curling of the neck, and misplaced toes. On the basis of these findings and using 5 eggs per dilution, the 50% embryo infective dose (EID₅₀) was found to be greater than $10^{5.0}$ per ml. On the other hand when a similar titration was undertaken in embryonating eggs obtained from a commercial hatchery in Northern California, the infectivity pattern was found to be so erratic that an embryo infective dose for 50% could not be calculated.

Having established the susceptibility of the Group E experimental flock, the virus was subsequently maintained for 4 passages by inoculation of 6- or 7-day-old fertile eggs using embryonic brain material harvested on the 7th day post-infection from the previous passage. Unfortunately further passages or titrations could not be undertaken in eggs from this source due to a decline in fertility.

Histopathological Examination

All in vivo experiments in chickens, including routine titration of different passage levels of virus were supported by histopathological examination of tissues from randomly selected chicks of both clinically affected and non-affected groups. Tissues routinely selected for detailed study included the brain, spinal cord, proventriculus, gizzard, pancreas, and myocardium. The brain was either sectioned transversely so as to obtain cross-sections of the cerebellum, midbrain and medulla or, in some cases, paramedian sections were

cut on the longitudinal axis. The spinal cord was removed from the vertebral column after initial fixation for 2 to 3 days in 10% formalin and transverse sections cut, chiefly in the lumbar region.

Following fixation in 10% buffered formalin, the tissues were dehydrated, cleared and embedded in paraffin blocks. Sections were cut at 3 microns and routinely stained with hematoxylin and eosin.

Preparation of Antiserum

Separate lots of antiserum were prepared at various times during the experiment, using different passage levels of AE virus (VR strain).

Lot A. Hyperimmune Serum. Six young adult cocks (Group C) were given an initial immunizing dose of formalin inactivated virus by the intraperitoneal route. This was followed 15 days later by a similar dose of viable virus (156th passage). Thereafter, the birds received increasing doses of virus material every 3 to 4 days for a total of 6 injections. After a further period of 2 weeks they were given a final booster injection. The birds were bled twice at weekly intervals and finally exsanguinated four weeks after the last injection.

Lot B. Hyperimmune Serum. A group of 11-week-old cockerels (Group A) constituting survivors from a titration experiment undertaken 8 weeks previously were injected with 1 ml. of virus material given in 0.5 ml. amounts into the pectoral muscles. The inoculum consisted of equal parts of complete Bacto adjuvant (Difco) and a 10% suspension of virulent brain material (158th passage).

The birds were exsanguinated one month following inoculation.

Lot C. Immune and Hyperimmune Serum. A group of eight 4-month-old birds (Group D) were inoculated intraperitoneally with 0.25 ml. of the supernatant

fluid obtained following centrifugation of a 10% suspension of virulent brain material (160th passage). By the end of one month, 3 birds had died after exhibiting symptoms of incoordination and progressive paralysis. All of the remaining birds exhibited varying degrees of incoordination or ataxia at this time and a mental disturbance characterized by alternating periods of dullness and hyperexcitability. The birds were bled on the 30th and 40th days. They were then challenged with a further dose of virus given intraperitoneally and finally exsanguinated 40 days later.

In addition, antisera were prepared in rabbits and guinea pigs following a series of three intramuscular injections with supernatant fluid from centrifuged brain suspensions (160th passage level).

Normal serum was obtained from the small flock of known susceptible birds in Group E.

All the above mentioned serums were tested for ability to inhibit agglutination of chicken red blood cells by the GB (Texas) strain of Newcastle disease virus, and tested for presence of agglutinins to the S6 strain of Mycoplasma gallisepticum.

Tissue Culture: Materials

Preparation of Glassware. Although slight modifications were introduced from time to time, the basic procedures used in the washing and preparation of glassware remained the same.

1. All glassware that had been used for propagation of cell cultures was either rinsed in tap water immediately after use or allowed to soak overnight. All virus infected glassware was subjected to autoclaving prior to further washing.

2. Glassware coming in direct contact with tissue cells was subjected to a detergent (7X:Limbro Chemical Co., New Haven, Conn.) in a concentration of 1% by autoclaving or boiling. At intervals (4 to 6 weeks) all glassware was treated with a 1:100 dilution of sodium carbonate in order to remove the slight film which tended to form following continued use of detergent.

3. Each piece was rinsed in running tap water followed by a rinse in distilled water. A final three rinses were carried out in Deeminized distilled water having a chloride content of less than 0.25 p.p.m.

4. The glassware was then dried in an oven maintained at low heat, capped with aluminum foil and finally sterilized at a temperature of 160° C for one hour in the dry air oven.

Circular coverslips to be used in the preparation of permanent mounts were washed in 95% methyl alcohol, dried with gauze and placed in position in the vials prior to sterilization.

Regular black rubber stoppers were used throughout and were subjected to the same washing procedures. They were then drained and dried at room temperature, packaged in convenient numbers in aluminum foil and autoclaved at 20 lbs. pressure.

Tissue Culture Media: Lamb Serum. Whole blood from a group of freshly killed lambs was obtained from a local slaughterhouse and the serum harvested. After centrifugation to remove any cells, the serum was warmed to 45° C and sterilized by filtration through a 2-liter Hercules filter using a 140 mm. ST-3 pad under positive air pressure. The serum was then tested for sterility in thioglycollate broth and tryptose broth and stored, without addition of antibiotics, at ordinary refrigerator temperature or frozen at -6° C.

Ox Serum. Whole blood obtained from young animals at a local slaughterhouse was treated as described above and the serum stored at -6° C.

Chicken Serum. Blood was obtained from a small group of birds maintained in isolation (Group E) and apparently fully susceptible to AE. The serum was sterilized by Seitz filtration and inactivated by heating to 56° C for 30 minutes in a waterbath prior to use in certain Maitland type cultures.

Swine Serum Ultra-filtrate. This was obtained from a stock previously prepared (4) for use in Simms-Sanders medium (92).

Tissue Culture Media: Salt and Buffer Solutions. Hanks Basic Salt Solution (38) was prepared as a stock 10X concentrate (appendix formula II) with phenol red added and stored in the refrigerator. Sufficient working solution was prepared for about two weeks work by adding one part of the concentrated salt to 9 parts of triple distilled water, and sterilized in the autoclave at 10 lbs. steam pressure. Antibiotics were added shortly before use and the solution buffered by addition of sodium bicarbonate solution to a final concentration of 0.035%.

Earle's solution (30) (modified). A 10X concentrated solution was prepared according to formula III (appendix) and sterilized by positive pressure Seitz filtration. The stock solution was dispensed in 200 ml. quantities and stored in the refrigerator.

Simms-Sanders salt solution was prepared as the 10X concentrated solutions described in the appendix (formula IV). Working solution was prepared by adding 25 cc. of each salt to 450 cc. of distilled water and the pH adjusted to approximately 7.2 with CO₂ gas from a cylinder.

Tyrodes solution was prepared as described in the appendix (formula V) and sterilized in the autoclave at 10 lbs. pressure.

The phosphate buffer solution (PBS) was prepared according to the formula of Dulbecco & Vogt (27) as described in the appendix (Formula I).

Tissue Culture Media: Trypsin Solution. Trypsin solution for primary cell dispersion was prepared from bactotrypsin 1:250 (Difco) in a final concentration of 0.25% by weight using PBS as diluent. Following sterilization by filtration, the solution was stored at -6° C in 100 ml. quantities, and thawed in a waterbath shortly before use.

Tissue Culture Media: Antibiotics. Both penicillin and streptomycin were used with most tissue culture procedures at a concentration of 1-200 units or micrograms respectively. Initially, nystatin (Mycostarin, Abbott Laboratories) was also added to monolayer cultures at the rate of 50-100 μ g per ml. but was later replaced by erythromycin lactobionate (Erythrocin, Abbott Laboratories) at 100 μ g per ml. for certain phases of the study when micrococci were found to be more troublesome than moulds as contaminants in the cell culture.

Tissue Culture: Methods

Chick Embryo Kidney Cell Cultures. The method of obtaining embryonic kidney cell cultures was mainly adopted from Younger's method (106) for trypsinizing monkey-kidney tissue and growing cells in a monolayer.

Viable 18- to 20-day-old embryonating eggs were aseptically opened and the embryos removed, leaving the yolk sac and membranes in situ. The embryos were exsanguinated by decapitation, the limbs being removed simultaneously so as to immobilize the body. Following evisceration, the kidneys were aseptically removed to a wash medium consisting of Hank's solution (Formula III) with bicarbonate and antibiotics added. Care was taken to avoid the inclusion of blood and mesenteric or capsular tissues. A minimum of 6 to 8 embryos was used on each occasion.

The kidney tissue was broken down into small fragments by first shaking vigorously and then expelling the tissues several times from a 10 ml. pipette. The kidney fragments in suspension were then allowed to sediment to the bottom of the tube and supernatant fluid removed. A further quantity of wash medium (30-35 ml.) was added and the process repeated a second time.

Trypsinization was carried out by either of the following methods:

1. After the final wash the tissue was suspended in 0.25% trypsin solution at the rate of 5 ml. per pair of kidneys in a tightly stoppered 250 ml. Erlenmeyer flask. Trypsinization was carried out in a refrigerated room for 15 to 16 hours, the tissue being gently agitated by a teflon covered magnetic bar activated by a Magnestir. At the end of this period, a drop of the suspension was examined under the microscope to determine the degree of cell dispersion present.

2. The second, or rapid method consisted of adding trypsin solution, prewarmed to 37° C to the washed tissue at the rate of 7 ml. per kidney pair. The suspension was shaken vigorously at intervals over a period of 20-30 minutes, the flask being held at approximately 37° C. The desired degree of trypsinization was determined by examining a drop of the suspension under the microscope.

Following trypsinization by either method, the cell suspension was filtered through two layers of 32/40 sterile gauze to remove any coarse tissue fragments or fibrous membranes. The trypsin solution was then decanted following centrifugation at 800 r.p.m. for 10 minutes, and replaced by wash medium containing 5% lamb serum in Hank's solution (with added bicarbonate and antibiotics).

After thorough dispersion of the trypsinized cells in this medium, the suspension was centrifuged lightly and the supernatant fluid withdrawn. A second round of washing was undertaken and the cells then resuspended in approximately

10 ml. of growth medium without serum in a 15 ml. graduated conical centrifuge tube. The growth medium consisted of Hank's basic salt solution enriched with 0.5% lactalbumin hydrolysate,* 0.01% yeast extract** and buffered with 0.5% of 7.5% sodium bicarbonate. Lamb serum and antibiotics were added according to the dictates of the experiment.

The volume of sedimented cells obtained following centrifugation at 800 r.p.m. for exactly 10 minutes constituted the packed cell volume. These cells were then resuspended in growth medium (without serum) to make a 1% suspension by volume. After thorough mixing, 0.5 ml. of this suspension was transferred to a Wasserman tube to which was added 1 ml. of Eagles cell counting solution (Appendix Formula VI).

The mixture was shaken vigorously at intervals over a period of 15 minutes and a drop of the stained cell suspension transferred to a bright-line Neubauer counting chamber. The number of viable cells was calculated by multiplying the average number of cells counted per square millimeter (taken from at least 2 counts) by 30,000.

Although cell counts were regularly undertaken during the earlier stages of the work, it was found that an average count of approximately 7×10^6 cells per ml. was consistently obtained in a 1 percent suspension. In most cases, therefore, subsequent dilutions were based on this figure. The cell suspension was diluted with growth medium containing 10 percent lamb serum to the correct concentration of cells required within a given volume for seeding into test tubes, vials, or bottles, depending on the experiment.

Prescription bottles (8 oz.) were seeded with 15 ml., and square-sided milk dilution bottles with 10 ml. of cell suspension adjusted to contain

*National Biochemical Company

**Difco Laboratories

approximately 1.5 million cells per ml. and incubated in a horizontal position. Cell sheets generally formed within 48 to 72 hours at which time the medium was replaced with maintenance medium, the latter having the same formula as growth medium except that the bicarbonate content was doubled and the serum reduced to either 5 or 2 percent.

Rubber stoppered tubes (16x125 mm.) were charged with 2 ml. of cell suspension and incubated in special racks designed to hold the tubes at a slight angle from the horizontal. An initial seed containing 1 million cells per ml. usually resulted in the formation of a confluent monolayer within 48 hours whereas concentrations of 6- to 800,000 cells/ml. gave rise to less confluent but somewhat more durable cell sheets. The stage at which growth medium was replaced was therefore largely determined by microscopic examination of the cell sheet and the degree of acidity as shown by the phenol red indicator.

Permanent stained mounts were prepared from cell cultures grown on circular coverslips having a diameter of 18 mm. These were placed inside flat-bottomed glass vials with an internal diameter just sufficient to admit the coverslip. Vials with coverslips in situ were capped with aluminum foil and sterilized by dry heat prior to seeding after which the foil was replaced by tightly fitting #3 stoppers. The usual inoculum consisted of 2 ml. of growth medium containing approximately 750,000 cells per ml., the spent medium being replaced after 48 or 72 hours by maintenance medium. At daily intervals during the course of an experiment, coverslips were removed from a suitable number of vials, washed in normal saline and fixed in absolute methyl alcohol for one hour.

After a number of initial trials with various staining procedures it was found that staining for approximately 18 hours in a weak dilution of Giemsa (7 drops in 10 ml. distilled water) gave excellent detail on cellular morphology

with the additional advantage of simplicity. A transverse slit made in the end of a cork served to hold the coverslips in place so that they could be vertically suspended in the staining solution. They were then washed, air-dried, and mounted on slides in 50 percent piccolyte.

HeLa (Gay) Cell Culture. HeLa cell cultures were derived from a continuous line of HeLa cells maintained in the Department of Avian Medicine, University of California, Davis and serially passed every 7 to 10 days. Following trypsinization and harvesting of the donor culture, the cells were washed, counted and suspended in growth medium to make a final concentration of 250,000 cells per ml. In this case the growth medium consisted of lactalbumin hydrolysate, 0.5 percent, and yeast extract 0.01 percent in Earle's basic salt solution with antibiotics added and was buffered with 1 percent of 7.5 percent sodium bicarbonate.

Stationary cultures were prepared by seeding 16 x 120 mm. test tubes with 500,000 cells contained in 2 ml. of growth medium. The tightly stoppered tubes were incubated (37° C) at a slight angle from the horizontal for 48 to 72 hours.

At this time the growth medium was decanted and the cell sheets examined under the 10X objective prior to inoculation of virus or virus passage fluids. Any tubes showing less than a 95 percent complete cell monolayer were discarded. Maintenance medium contained 5 percent lamb serum instead of 10 percent ox serum as used in the growth medium. Cell cultures were examined daily under the 10X objective for evidence of specific degenerative changes.

Permanent stained mounts were prepared on coverslips as described for chick embryo kidney cultures, except that the vials were seeded with 500,000 cells suspended in 2 ml. of growth medium.

Pig Kidney Cell Cultures. Primary monolayer cultures were first prepared by trypsinization of kidney cortical fragments obtained from young healthy

swine. After washing with Hanks' solution, the cells were suspended in growth medium containing 10 percent ox serum at the rate of 1 ml. packed cells to 150 ml. of growth medium. Roux bottles were inoculated with 70 ml. of this suspension and incubated for 4 to 5 days at 37° C.

Although primary monolayers prepared by this method may be used for propagation of viruses, the preparation of a first subculture had been found (4) to produce 'cleaner' and more homogeneous monolayers, more suitable for microscopic investigation of specific cytologic changes. Accordingly, cells were removed from the Roux bottle after 5-7 days growth by the addition of warm trypsin-versin medium (Formula VII), the bottles being held at 37° C for 30 to 40 minutes. Trypsinized primary cells were washed twice with Hanks' solution containing 10 percent ox serum and resuspended in growth medium (as used for HeLa cell cultures).

After counting in a hemocytometer chamber, the cell suspension was diluted to approximately 500,000 cells per ml. of growth medium containing 10 percent bovine serum. Vials containing coverslips, and test tubes, were charged with 2 ml. of the above suspension, tightly stoppered and incubated at 37° C.

A confluent, homogeneous cell sheet was obtained in 48 hours, at which time the growth medium was decanted and 0.2 ml. of virus suspension inoculated. Maintenance medium, containing 5 percent lamb serum instead of ox serum was added at the rate of 1.8 ml. per vial. Coverslips from a suitable number of vials were prepared and stained each day in the manner described above.

Suspended Cell Cultures - Maitland Technique. Minced embryonic tissue was prepared from decapitated 8-to 9-day old chicken embryos and approximately 0.1 gram of tissue suspended in 10 or 12 ml. of the nutrient medium in a 22 x 150 mm. tube. Two series were run in parallel using 2 different media:

Series A: Simms-Sanders (modified) medium was composed of 80 parts of Simms-Sanders buffered salt solution, 1 part of a saturated solution of p-amino-benzoic acid, 1 part of a 5 mg. percent solution of cysteine hydrochloride and 18 parts of swine serum ultrafiltrate (92). Penicillin and streptomycin were added to a concentration of 100 units and 100 μ g. per ml. of medium. The pH was adjusted with carbon dioxide gas to 7.2.

Series B: Tyrodes medium contained 10 percent chicken serum in Tyrodes salt solution at pH 7.4. Penicillin was added to a concentration in the medium of 100 units per ml. The chicken serum had been obtained from a small flock of experimental birds (Group E) of known susceptibility to AE and was inactivated by heating at 56° C for 30 minutes prior to sterilization and use.

The cultures were subjected to bacteriological examination following each virus passage and the pH of the medium in each tube checked in a Beckman model G pH meter.

EXPERIMENTAL WORK

Embryonic Chick Kidney Cell Cultures

Experiment 1. Bottle Cultures. Chick embryo kidney cultures were prepared in 8 oz. prescription bottles by the method previously described (page 29). Each of 6 bottles were inoculated with 15 ml. of growth medium containing approximately one million cells per ml. and incubated in a horizontal position at 37° C.

A dense sheet of almost confluent epithelial-like cells was formed by the end of 48 to 52 hours incubation at which time the bottles were gently rocked to remove any loose-lying cells and the fluid medium carefully decanted.

The virus inoculum consisted of a 1:10 dilution of infective brain material from the 156th chick passage of the VR strain on AEV in phosphate buffered saline, the coarser tissue particles having been removed by centrifugation at 1000 r.p.m. for 10 minutes. An aliquot of this virus, when subsequently titrated in 9-day-old sexed cockerels by intracerebral inoculation was found to have a chicken infective dose 50 (CID₅₀) titer of $10^{6.1}$ per ml.

Three bottle cultures received 1.5 ml. each of the virus suspension, while the remaining 3 bottles received 1.5 ml. of sterile phosphate buffer solution. The bottles were then gently rocked in the horizontal position to obtain an even dispersion of virus throughout the monolayer and allowed to remain at room temperature for approximately 35 to 40 minutes prior to addition of maintenance medium at the rate of 13.5 ml. per bottle.

Both control and infected cultures were examined daily under the 10X objective for evidence of cytologic changes. This experiment was repeated on five consecutive occasions but with the following modifications:

Trial 1. As outlined above.

Trial 2. The virus inoculum consisted of a 1:100 dilution of infective brain material.

Trial 3. Cell cultures were prepared with a suspension of 800,000 cells per ml. instead of 1,000,000 cells per ml. as above; virus inoculum as in Trial 1.

Trial 4. Repeat of Trial 3.

Trial 5. Cell cultures were prepared with a suspension of 500,000 cells per ml. and inoculated at 72 hours instead of 48 hours.

Results of Experiment 1. Early degenerative changes in the cell monolayer were first observed approximately 48 hours following the change to maintenance medium in both infected and control cultures. These changes consisted of a

granular appearance of the cytoplasm and a tendency for the cells to "bank up" on top of each other, particularly in the more densely covered areas. Increasing granularity of the cytoplasm was followed by pyknosis of the nucleus and sloughing of the cell so that by the 3rd or 4th day following the change of media, approximately 70-80 percent of the cell sheet had become detached from the glass leaving scattered fibroblast-like cells still attached. A few small islands of relatively normal cells usually remained attached to the glass for up to 6 days but showed little evidence of active growth apart from fibroblast-like extrusions of the cytoplasm.

While certain individual cultures had a slightly better survival rate than others, both infected and non-infected monolayers on the average showed similar progressive degenerative changes; no specific cytopathogenic effects were observed in the virus infected cultures.

An initial seed of 500,000 cells per ml. (trial 5) resulted in the formation of an only partially complete monolayer by the end of 72 hours, although the cells generally remained attached to the glass for a longer period than with more heavily seeded cultures.

Experiment 2. Viability Study. In order to determine the presence of virus in the culture system following incubation for 3 days at 37° C, the cells and fluid media from three bottle cultures inoculated in Experiment 1 (trial 4) were harvested and pooled. After thorough mixing with a 10 ml. pipette, 5 ml. of the suspension was removed to a screw cap glass vial and subjected to 3 consecutive treatments of rapid freezing in a dry ice-alcohol mixture followed by thawing in a 37° C water bath. The suspension was then centrifuged at 1000 r.p.m. for 10 minutes in an International refrigerated centrifuge at 6° C. Tenfold dilutions of the supernatant fluid were prepared in chilled phosphate

buffer solution and inoculated into 3-day-old sexed cockerels (Group B) via the intracerebral route using 5 birds per dilution.

A sample of undiluted material was subjected to routine bacteriological examination and also to specific cultural tests for PPL0.

Results. Bacterial and PPL0 sterility tests were negative.

The CID_{50} of the tissue culture material in 3-day-old chicks was calculated as $10^{2.8}$ per ml. after an observation period of 35 days.

Experiment 3. Test Tube Cultures. Monolayer cultures of embryonic kidney cells were prepared in 16 x 125 mm. pyrex tubes by seeding with approximately 2 million cells contained in 2 ml. of growth medium (plus 10 percent lamb serum). When a confluent cell sheet had formed, generally after 48 hours, the growth medium was removed and half of the tubes inoculated with 0.2 ml. of centrifuged or filtered suspension of infected brain material from different passage levels of the VR strain.

Both control and inoculated tubes were then left for 30 to 40 minutes at room temperature prior to addition of maintenance medium to give a final volume of 2 ml. per tube.

In this series three different virus passage levels were studied.

Trial 1. An ampule of infective brain material from the 156th chick passage was thawed, centrifuged at 800 r.p.m. for 10 minutes, and 0.5 ml. of the supernatant fluid added to 4.5 ml. of Hanks' solution to make a final dilution of 1:100. The titer of this material had been previously estimated by i/c inoculation of 9-day-old cockerels as $10^{6.1}$ CID_{50} per ml. Ten cultures received 0.2 ml. of the diluted virus material with an equal number remaining as uninoculated controls.

Trial 2. In this test, an inoculum constituting a 1:10 dilution of virus material from the 158th chick passage was used. The original harvested

material had become contaminated and was therefore sterilized by filtration through a Millipore filter disc of 0.45μ aperture size. The filtrate when titrated in 15-day-old commercial chicks was found to have a titer greater than $10^{5.0}$ CID₅₀ per ml. (endpoint not determined). As in Trial 1, ten tubes were inoculated with virus; control tubes received 0.2 ml. of sterile PBS.

Trial 3. A sample from the 160th chick passage, representing a 1:5 suspension of brain material in nutrient broth was further diluted by addition of an equal quantity of Hanks' solution, and 0.2 ml. of the lightly centrifuged supernatant fluid inoculated into each of 20 tubes. Ten cultures receiving the same amount of sterile nutrient broth acted as controls. The titer of this virus was subsequently estimated in 2-day-old chicks (Group D) as $10^{5.5}$ CID₅₀ per ml.

Results of Trials 1, 2 and 3. The tubes were examined twice daily for evidence of cellular degeneration. By the third day following inoculation (p/i), commencing non-specific degeneration of the kidney cell monolayer was indicated by cytoplasmic granulation and sloughing of cells in both control and infected tubes, this progressing rapidly so that by the end of the 5th day p/i only very few viable cells remained attached to the glass. Since approximately the same degree of cellular degeneration was shown by both control and infected tubes at any one time, none of the observed effects could be ascribed to the action of the virus.

Experiment 4. Serial Passage in Test Tube Cultures. An attempt was made to adapt the virus to tissue culture by serial passage of infective brain material through successive chicken embryonic kidney cell cultures.

Cultures were prepared by seeding 16 x 125 mm. test tubes with 2×10^6 cells contained in 2 ml. of growth medium. After 48 hours, when a full cell sheet had formed, the growth medium was decanted and cultures inoculated with

0.2 ml. of fluid from the previous passage. The inoculum was dispersed over the cell sheet by rocking the tubes at intervals over a period of 30-40 minutes. Maintenance medium was then added to make up the final volume to 2 ml.

The tubes were incubated for a further 72 hours at 37° C after which they were frozen and thawed once to help release the cells from the glass surface. Any cells remaining attached to the walls of the tube were removed by vigorous pipetting with a 1 ml. pipette and the entire contents harvested, pooled and placed in 5 ml. quantities in small screw-cap vials which were immediately stored at -6° C. In addition, samples from the 1st, 3rd, 6th, and 10th subcultures were stored in a dry-ice cabinet.

Ten cell culture tubes were prepared for each passage, six inoculated with material from the previous passage and four acting as controls. Observations were made daily to determine the presence of cellular changes.

A 1:10 suspension of filtered brain material from the 158th chick brain passage, having a CI_{50} titer of greater than 10^5 per ml. constituted the virus material used for the initial inoculum.

Results. Altogether 10 consecutive subcultures of the original infective brain material were undertaken. No specific cytopathogenic effects were seen at any stage, although varying degrees of early cellular degeneration were observed in both control and inoculated cultures between the 2nd and 3rd days following change to maintenance medium.

Experiment 5. In Vivo Activity of Tissue Culture Passaged Material. In order to determine the presence of infective virus in the tissue culture system at different passage levels, intracerebral inoculations were carried out in known AE susceptible chicks hatched from the small group of birds maintained in a maximum security isolation unit (Group E). The small number of hens available combined with a poor fertility rate limited the number of available chicks

Table 2. Results of experiment 5, intracerebral inoculation of 9- to 10-day-old susceptible chicks with tissue culture (C.E.K.C.) passaged virus.

Inoculum*	Chick : wing band : 484	Incubation : period (days) : 8	Time to :		Symptoms :	Histopathology (brain) :
			death (D) or : S - 10	slaughter (S) : S - 13		
Primary brain filtrate 1:10 dilution in P.B.S.	489	11	S - 10	S - 13	Ataxia, weakness	Slight "cuffing" and gliosis Slight endothelial proliferation
1st passage tissue culture fluid and cells (undiluted)	491 487	10 10	S - 13 D - 13	S - 13 D - 13	Ataxia, tremor, weakness Ataxia, progressive weakness	"Cuffing", gliosis, some neuron degeneration Advanced autolysis
2nd passage tissue culture fluid and cells	Not done Not done					
3rd passage tissue culture fluid and cells	492 485	13 14	D - 15 S - 18	D - 15 S - 18	Ataxia, weakness Ataxia, tremor, paresis	No specific lesions observed "Cuffing", slight gliosis
4th passage tissue culture fluid and cells	486 488	--- ---	--- ---	--- ---	(Accidental death 2nd day) No symptoms over 30 day period	Not examined No lesions observed
5th passage tissue culture fluid and cells	490 493	--- ---	--- ---	--- ---	No symptoms over 30 day period No symptoms over 30 day period	No lesions observed No lesions observed
Control, sterile buffer solution	494	---	---	---	No symptoms over 30 day period	No lesions observed

*Dose: 0.05 ml. given into the right cerebral hemisphere.

to 11, which were divided into 5 groups of two each with one acting as uninoculated control.

Group 1 received .05 ml. of the original filtered brain material (158th passage) used to initiate the tissue culture passage series in Experiment 4. Groups 2, 3, 4, and 5 were inoculated with the undiluted fluids (cells and maintenance medium) from the 1st, 3rd, 4th, and 5th tissue culture passages, respectively. This material had been held in a dry-ice cabinet since the time it was harvested.

The chicks were housed as individual groups in wire cages in an isolated unit and observed daily for 30 days following inoculation at 10-days of age. In order to obtain fresh specimens for histological examination, certain birds showing typical symptoms of AE were killed in extremis.

Results. The results are shown in Table II. While 3rd passage material contained sufficient infective virus to cause typical disease after a slightly prolonged incubation period, no evidence of infection was shown in the 2 chicks inoculated with 5th passage material or the single remaining chick given 4th passage fluid.

Experiment 6. Titration of Tissue Culture Passaged Virus in Chicks. An aliquot of the tissue culture fluids harvested from 16 tubes at the 10th passage of the virus in chick embryo kidney cultures (exp. 4) was tested at a later date for infectivity in chicks.

This material had been stored in a dry-ice cabinet for 98 days before suitable chicks became available. The latter were 29-day-old sexed cockerels (Group F) obtained as 2-day-old chicks from a large commercial hatchery in Northern California and were the progeny of stock vaccinated as young growing birds with the Schaaf strain of AEV (86).

Tenfold dilutions were prepared in chilled growth medium (without serum) and 0.1 ml. inoculated by the intracerebral route, using 5 birds per dilution. In addition, 5 chicks received the same dose of undiluted tissue culture material while 5 controls received sterile growth medium.

They were housed as a group in an isolation unit and observed daily for any abnormal symptoms.

Results. Five birds, including one control, died within 48 hours of inoculation from trauma or shock; the remainder showed no symptoms over a period of 31 days. Three birds selected from the group receiving undiluted tissue culture fluids and 2 controls were killed for histopathological study. Examination of brain and spinal cord sections revealed no evidence of neuronal degeneration or lymphocytic perivascular cuffing.

Parallel titrations of the VR strain (160th chick passage) and Schaaf strain (10th chick passage) of AEV in chicks from the same hatch resulted in titers exceeding 1×10^6 CID_{50} in both cases.

Experiment 7. Studies with Chick Embryo Propagated Virus. Tubes seeded with slightly less than 2×10^6 cells and forming complete monolayers in 48 hours were inoculated with a suspension of fetal brain material representing the 4th embryo passage of the VR strain of virus. The brain material was diluted 1:10 by volume with growth medium and each tube inoculated with 0.2 ml. so as to give a final dilution of 1:100 when maintenance medium was added to the tubes to make up the volume to 2 ml.

While the titer of the 4th embryo passage material was not determined, the chick brain propagated virus used to initiate embryo passages had an embryo infective dose 50 (EID_{50}) of $10^{5.3}$ per 0.2 ml. when titrated in eggs from the same source i.e. susceptible (Group E) birds.

Serial passage was undertaken every 5th day for 5 passages using cells plus supernatant fluid from the previous cultures.

Results. Degenerative changes, occurring in the 5 control and 10 infected tubes, were observed from about the beginning of the 3rd day p/i in most cases. By the 4th day it appeared that these changes, consisting of vacuolation and a granular appearance of the cytoplasm with rounding or contraction of the cells, were somewhat more marked in the control tubes. However, by the 5th day, extensive areas of sloughing were present in both infected and control tubes. No specific CPE were seen following 5 serial passages.

Experiment 8. Microscopic Examination of Permanent Stained Mounts. In order to determine the effect of AE virus on chick embryo kidney cultures at the cellular level, cultures were prepared in flat-bottomed vials containing circular coverslips. These were seeded with cell concentrations ranging from 1.5×10^6 to 2×10^6 contained in 2 ml. of growth medium resulting in formation of an almost confluent monolayer on the coverslips in approximately 48 to 72 hours. At this time, the growth medium was removed, an appropriate number of vials inoculated with infective brain material or tissue culture passaged brain material, and after a 30 minute incubation period (room temperature), replenished with maintenance medium.

Coverslips were then removed and stained at 24-hour intervals from a representative number of vials.

In a prior series of experiments to determine optimum conditions for maintenance of the cell sheet in this system, it was found that while an initial seed of 2×10^6 cells gave rise to a confluent cell monolayer within 48 hours, the cell sheet underwent very rapid degeneration, probably due to overcrowding. An initial seed of 1×10^6 cells on the other hand resulted in a sheet which remained 'patchy', never forming a complete monolayer before cellular degeneration

became evident at about the 4th to 5th day. When approximately 1.5×10^6 cells were used, although the monolayer never became completely confluent, areas of the cell sheet remained in good condition for 3 to 4 days.

For maintenance of the culture following virus inoculation, the more strongly buffered Earle's salt appeared to give slightly better results than Hanks' salt solution.

Separate studies were conducted on the cytopathological effects of infective brain material from the 158th and 160th chick passages of the VR strain as well as tissue culture fluids from the 1st, 3rd, 6th, and 10th passage levels of the virus in embryonic kidney cell cultures (expt. 4).

Results. In most of the trials undertaken, nonspecific degenerative changes in the monolayer could be detected within 48 hours following replacement of growth medium with maintenance medium. Indeed with heavily seeded cultures (2×10^6 or $>$ cells) sloughing of the entire cell sheet occurred between 72 and 96 hours. The longest period that recognizable areas of epithelial-like cells could be maintained without replenishing the media was approximately 5 days although the cells were by no means contiguous at this time. The period during which specific cytopathogenic effects (CPE) resulting from viral infection could have been detected was therefore limited.

Nonspecific degenerative changes consisted of an apparent contraction of the monolayer causing the cells to bank up into numerous multilayered clumps or tufts containing many cells with dark-staining, shrunken nuclei. The cells in the intervening spaces tended to become elongated and arranged in parallel or radiating strands. Progressively increasing vacuolation of the cytoplasm and condensation of the nuclear chromatin was accompanied, by sloughing of numerous cells, leaving an irregular patchwork of gradually coalescing areas on the coverslip from which the majority of cells had become detached. On

the whole, the central portion of the coverslip became denuded before the periphery.

However, while the rate of degeneration and sloughing of the cell sheet varied considerably between experiments depending inter alia on the initial seed concentration, no significant differences could be detected during any single experiment in the rate or degree of cellular degeneration between inoculated and control cultures.

Since it was felt that failure to demonstrate viral multiplication or specific cytopathogenic effects in the CEKC system may have been associated with inability to maintain a viable cell sheet for more than 3 to 5 days following inoculation, an attempt was made to propagate the virus in two other cell culture systems, namely pig kidney and HeLa cell monolayers.

Pig Kidney Cell Cultures

Experiment 1. Test Tube Cultures. Primary subcultures of pig kidney cells were prepared in 16 x 120 mm. tubes as described in the section on materials and methods (page 33). A suitable number of tubes (5 to 10) containing well developed cell monolayers were inoculated with 0.2 ml. of a 1:10 dilution of infective brain material from the 156th passage of the VR strain of AEV, an equivalent number of uninfected tubes acting as controls.

Tubes were examined daily for 5 days under the 10x objective for cytologic changes. Three similar but separate trials were undertaken.

Results. Progressive degeneration of the cell sheet starting from about the third day after inoculation was observed equally in both virus-infected and control cultures. By the 5th day in most cases, large areas of the cell

sheet had become detached from the glass surface. No specific changes were observed in the virus inoculated cultures.

Experiment 2. Cultures Grown on Coverslips. Cultures were prepared in vials containing circular coverslips by the method described on page 33. On the 3rd day (approximately 64 hours) following seeding, when the cells appeared to have formed a contiguous monolayer, the medium was decanted and 10 vials inoculated with 0.2 ml. of infective brain suspension, as described in Experiment 1. An equal number of uninfected cultures were used as controls. Following approximately 30 minutes incubation at room temperature, maintenance medium was added and the vials incubated at 37° C. Coverslips were removed from one inoculated and one control culture on successive days. These were then fixed in absolute methanol, stained overnight with Giemsa and mounted on glass slides for detailed microscopic examination.

Results. Nonspecific degenerative changes could be detected in both control and inoculated cell cultures starting from about the 4th day following the change to maintenance medium. Microscopically, these changes consisted of progressive granularity and vacuolization of the cytoplasm accompanied by contraction and darkening of the nucleus.

While individual variations occurred between coverslips selected on the same day, there was no overall difference in the rate or degree of cellular degeneration observed in either infected or control cultures.

By the 8th day p/i almost complete sloughing of the monolayer had occurred in the remaining cultures from both groups and the experiment was terminated.

HeLa Cell Cultures

Experiment 1. Serial Virus Passage in HeLa Cell Cultures Grown in Test

Tubes. Subcultures of the stock HeLa cell line were prepared in 16 x 120 mm. test tubes twice weekly by the method described (page 33). Cell concentrations for seeding were calculated to give rise to confluent monolayers that could be utilized for virus inoculation at 48 and 72 hours respectively. By alternating the time of inoculation to 8 a.m. and 5 p.m., respectively, the period of incubation of any one passage level of virus was adjusted to approximately 82+/- hours. Following this period of incubation (37° C), the cell sheet was removed from the glass by scraping with a platinum loop followed by vigorous shaking of the tube. From each batch of 5 tubes inoculated, the cells and fluid medium from 3 tubes were pooled in preparation for serial transfer to fresh cultures, while the remaining 2 tubes together with 5 uninoculated control tubes were reincubated for a further 3 days and examined daily for cytologic changes.

Each sub-inoculum consisted of 0.2 ml. (per culture tube) of the pooled suspensions harvested from the previous passage, the actual transfer being accomplished within 10 minutes.

Parallel serial transfers (passages) were made with 4 different original inocula described below. Each series was passaged at least 10 times in monolayer HeLa cell cultures at approximately 80-84 hour intervals.

Routine sterility tests were conducted at each passage level by inoculating harvested material onto 10 percent blood agar plates and into thioglycollate medium. These were checked for the presence of bacterial growth following 48 hours incubation and then discarded.

The following materials were studied:

1. Tissue culture fluid (i.e., cells plus maintenance medium) harvested at the 10th passage of AE virus (VR strain) in chick embryo kidney cell cultures (page 39). This material had been stored at -60° C for 21 days, the inoculum consisting of an aliquot of the pooled suspension obtained from 16 tube cultures.

2. Infective brain material from the 160th chick passage of the Van Roekel strain of AEV diluted 1:10 in Dulbecco's buffered saline (PBS). This virus stock when previously titrated in 2-day-old chicks (Group D) had been found to have a Log ID₅₀ titer of $10^{-4.2}$ per 0.05 ml. equivalent to $10^{4.8}$ doses per 0.2 ml. The suspension was not centrifuged prior to inoculation at the rate of 0.2 ml. per tube.

3. Infective brain material from the 10th chick passage of the Schaaf (California) strain of AEV diluted 1:10 in tryptose broth. This virus had a titer of $>10^5$ CID₅₀ per 0.1 ml. in Group D chicks. Cultures were inoculated with 0.2 ml. of the whole tissue suspension.

4. Normal chick brain suspension. The brains were aseptically collected from a small group of 3-week-old chicks that had been hatched in the laboratory from eggs obtained from the small susceptible flock (Group F) previously described. The initial inoculum consisted of a 1:10 suspension of brain material in tryptose broth ground in a Ten Broeck tissue grinder.

Sterility tests were conducted on all of the above materials, using 10 percent blood agar plates incubated in a candle jar, thioglycollate medium (Difco) and serum-enriched PPL0 broth (Difco). After 3 days incubation, sub-inoculations were made from the PPL0 broth onto serum agar plates which were then examined at subsequent intervals under the dissecting microscope.

Results. The results of all bacteriological tests conducted on the original brain inocula and different passage levels in tissue culture were negative.

In view of subsequent findings (Expt. 2), however, it should be pointed out that while the original inocula were specifically examined for mycoplasma infection, similar tests were not performed on the HeLa cell passaged material.

Although occasional individual cell cultures showed more rapid degeneration or sloughing than others, this same phenomenon was also noted with control cultures. At no time was it possible to designate any of the changes seen as specific when comparison was made with either uninoculated control cultures or cell monolayers inoculated with the serially passed normal brain tissue. It was apparent during the first passage that normal brain tissue in a dilution of 1:100 (final dilution in the maintenance medium) caused increased granularity of the cytoplasm of the cells but this effect was not subsequently seen in further passages, presumably due to the tenfold dilution factor.

Experiment 2. Microscopic Examination of Stained Coverslip Preparations.

HeLa cell subcultures were grown on coverslips placed in flat-bottomed vials using an initial seed of approximately 180,000 cells contained in 2 ml. of growth medium. Antibiotic supplementation was limited to 150 µg. of streptomycin per ml.

After 48 hours incubation the growth medium was removed and the vials inoculated with 0.2 ml. each of the tissue culture fluids harvested from the 10th HeLa cell culture passage of each of the four inoculum groups described in Experiment 1 above. Six vials were used for each group, viz.:

Group A: Chick embryo tissue culture passaged AE virus (VR strain).

Group B: AE virus (VR strain).

Group C: AE virus (Schaaf strain).

Group D: Normal chicken brain.

Following a 30-minute period of incubation at room temperature, maintenance medium was added and the vials tightly stoppered and incubated at 37° C. Maintenance medium in this case contained 2.5 percent added lamb serum instead of the usual 5 percent, and 200 units of penicillin, 200 µg. of streptomycin, and 100 µg. of erythromycin per ml.

One or two vials from each group were selected on the 1st, 3rd, 4th, 5th, and 6th days following inoculation and the coverslips removed for preparation of stained mounts.

Results. Microscopic examination of Giemsa stained coverslips prepared from 2 uninoculated cultures 48 hours after initial seeding revealed the presence of occasional small clusters of minute granular or cocco-bacillary bodies occurring chiefly within the cytoplasm of the cells. These bodies could not be detected in coverslips prepared from 24-hour cultures.

However, when stained coverslips prepared from vials 24 hours following inoculation were examined under oil immersion, similar minute coccoid bodies were present in markedly increased numbers. In general they appeared to be mainly confined to the cytoplasm, several hundred occurring within any one cell and varying in size from approximately 0.5 to 1 µ. While the coccoid forms predominated, comma, ring and short-rod forms were occasionally also seen.

Cultures from all four inoculum groups were equally affected. By the second day following inoculation only occasional small groups of coccoid bodies could be detected, but evidence of cellular degeneration was present in the form of cytoplasmic vacuolation, pyknosis of a small percentage of nuclei and an overall reduction in the cell sheet.

This was followed by an apparent recovery of the cell monolayers. Cultures from each group examined on the 4th, 5th, and 6th days p/i showed no

further evidence of cellular degeneration, the cells forming a confluent monolayer in each case. No coccoïd bodies could be detected by examination of stained coverslips under the oil immersion lens on the 4th or 5th days. However, a few minute, darkly staining bodies were again noted on 2 coverslip preparations on the 6th day following inoculation.

In order to determine the nature and possible origin of these small bodies, a sample of the stock HeLa line cell culture from which these subcultures had been derived was inoculated onto PPL0 agar (Difco) enriched with 10 percent horse serum and 1 percent yeast autolysate.

When the plates were examined under a dissecting microscope after 4 days incubation at 37° C, numerous small, homogeneously granular and slightly raised colonies could be detected. The size of individual colonies varied from as little as 0.05 to approximately 0.2 mm. in diameter but did not appear to increase in size after the 6th to 7th day of incubation.

The fact that they could not be washed off the plate with saline indicated that the colonies were growing into the agar. Colonial morphology was further studied by removing a small block of agar, placing it face down on a clean slide and flooding with Bouins fixative. After removing the agar block, the slide was stained with a weak dilution of Giemsa. Pleomorphic coccoïd, bacillary or ring forms were noted, all of minute size.

These were tentatively diagnosed as mycoplasma on the grounds of general appearance and colonial morphology.

Cultural examination of several subsequent passages of the stock HeLa cell line again revealed the presence of an insidious mycoplasma infection. No attempt was made at this time to classify the species of mycoplasma isolated. The organism did not produce regular or lasting cytopathogenic effects in HeLa cell monolayers. Infected cell cultures inoculated intracerebrally into

3-week-old chicks failed to induce clinical symptoms during a 30-day observation period and no microscopic lesions could be detected in brain sections prepared at the end of this period.

Experiment 3. In Vivo Activity of HeLa Passaged AE Virus. Tests were undertaken to determine the presence of infective virus in HeLa cell cultures following serial passage of infective brain material.

A batch of 150, sexed, day-old cockerels was obtained from Kimber Poultry Farms, Inc., Niles, California and raised in strict isolation until 20 days of age. The chicks were the progeny of dams that had previously been vaccinated with the Schaaf strain of virus as young growing birds (86). This was considered preferable to using chicks from an unvaccinated flock in order to obtain a more uniform level of susceptibility to the virus.

The chicks were wingbanded and divided into 5 groups of 30, each group being housed in individual floor units on wood shaving litter. Each unit contained an anteroom in which boots and hands could be disinfected in detergent solution on entering or leaving.

The inoculation schedule was as follows:

Group I.

The inoculum consisted of the Van Roekel strain of AE virus in a suspension of brain tissue from the 160th chick brain passage. This was an aliquot of the same virus material used for original infection of HeLa cell and chick kidney tissue cultures and had been stored at -60° C. Tenfold dilutions, ranging from 10^{-1} through 10^{-5} were prepared in tryptose broth, each dilution being injected intracerebrally into 5 chicks at a dosage rate of 0.1 ml. Five control chicks were given intracerebral injections of sterile tryptose broth.

Group II.

AE virus (Schaaf strain) consisting of infective brain material from the 10th chick brain passage was inoculated into the second group using the same dilutions, dosage and procedure as for Group I.

Group III.

The inoculum was composed of cells and supernatant fluid from the 10th HeLa cell passage of the chick embryo kidney tissue culture passaged virus (page 49). Five chicks were inoculated with 0.1 ml. each of the undiluted material. Tenfold serial dilutions of this material were also prepared in Earle's basic salt solution from 10^{-1} through 10^{-4} and injected into 4 groups of 5 chicks. Five control chicks were given identical intracerebral injections of sterile diluent.

Group IV.

The test material consisted of a suspension of cells plus supernatant fluid (maintenance medium) from the 12th HeLa cell culture passage of the Van Roekel strain of virus. Dilutions, dosage and inoculation procedure were the same as used in Group III.

Group V.

Chicks were inoculated with a suspension of the cells and maintenance medium from the 10th HeLa cell passage of the Schaaf strain of virus, using the same procedure as described above.

All inoculations were performed on the same day, care being taken to use a different set of sterile equipment for each group. The various inocula were held at ordinary refrigerator temperature until shortly before use, all dilutions having been prepared that same day. The injections were given into the right cerebral hemisphere using 5/8", 25 gauge, disposable needles attached to a 1 ml. tuberculin syringe.

Observations were made daily for 30 days for any abnormal symptoms, particularly those related to CNS disturbance (dullness, ataxia, tremors, etc.). In order to obtain fresh specimens for histological examination, a few birds were killed in the final stages of disease when symptoms had been shown for at least 3 days. At the termination of the observation period, 10 percent of the remaining birds from each group were selected for histopathological examination of the brain and spinal cord.

Finally, 12 birds from the Group IV experiment that had received HeLa passaged virus material were challenged on the 40th day by intracerebral inoculation with 0.1 ml. of a 1:100 dilution of infective brain material (VR strain).

Results. The results were somewhat marred by the fact that 29 chicks (nearly 20 percent) died within 3 days following inoculation. While a certain proportion of these losses were undoubtedly caused by trauma or shock resulting from intracerebral inoculation, an error in management was at least a contributing factor. Initially the water fountains had been placed on 1 foot high stands to prevent soiling with litter and it was only on the 2nd day that it was realized that many of the chicks had not yet located this source. Cultural examination of brain material from several of these chicks failed to reveal any bacteria or mycoplasma. In addition, since losses were fairly evenly distributed amongst all dilutions and all groups including control chicks, these early deaths were attributed to nonspecific factors.

Final results are given in summarized form in table 3, the data pertaining only to observations made from the fourth day post-inoculation to the 30th day, inclusive. The incubation period in an individual chick was determined as the time interval from inoculation to the first appearance of any abnormal symptoms no matter how slight, but provided that subsequent observations showed

either a progression of the first symptom manifested or the appearance of some other characteristic sign of epidemic tremor. Except for chicks slaughtered in extremis for histopathological examination, all birds manifesting clinical symptoms eventually succumbed although several remained alive for as long as 6 to 7 days.

In Group I (VR strain of virus) only one chick, apart from control birds, survived to the end of the observation period, while in the group inoculated with the Schaaf strain, only 2 failed to show evidence of infection. From these results it was concluded that the 20-day-old cockerels were fully susceptible to both strains of virus used in tissue culture studies. In addition it appeared that the incubation period was more or less directly related to the amount of virus present in the inoculum, a phenomenon previously noted in other titration experiments.

None of the birds inoculated with tissue culture passage virus showed any clinical manifestations of epidemic tremor during the observation period or for a further period of 10 days. Histological examination of brain sections prepared from approximately 10 percent of these birds, selected at random, failed to reveal any significant lesions. A number of birds from Group IV challenged at this time with virulent brain material all succumbed within 3 weeks.

Suspended Cell Cultures: Maitland Technique

Two series of suspended cell cultures of the Maitland type were run in parallel.

Series A consisted of minced whole chick embryo plus a modified Simms-Sanders medium containing swine serum ultrafiltrate.

Table 3. Titration of chick brain and tissue culture passage AE virus in three-week-old cockerels.

Inoculum	Dilutions						
	10 ⁻⁰	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	Control
160th chick brain passage (VR strain)	---	2/2* (6.0)**	5/5 (8.6)	3/3 (9.7)	4/4 (14.2)	2/3 (13.5)	0/4
10th chick brain passage (Schaaf strain)	---	3/3 (12.3)	3/3 (14.7)	5/5 (18.4)	3/4 (17.3)	3/4 (24.3)	0/5
10th HeLa cell culture passage (CEKC passaged virus)	0/5	0/5	0/3	0/5	0/5	---	0/4
12th HeLa cell culture passage (VR strain)	0/4	0/3	0/4	0/4	0/3	---	0/4
10th HeLa cell culture passage (Schaaf strain)	0/5	0/4	0/5	0/5	0/4	---	0/4

*The numerator indicates the number of chicks affected; denominator represents number of chicks inoculated and alive at 4th day post-inoculation.

**Numbers in parenthesis represent average incubation period in days.

Series B consisted of minced whole chick embryo suspended in Tyrodes salt solution with 10 percent chicken serum added.

Following a short period (4-6 hours) to allow for tissue respiration and pH adjustment, two cultures in each series were inoculated with 0.2 ml. of a 10^{-1} suspension of infective brain material from the 161st chick passage of the VR strain of AE virus. The viral suspension as well as a sample of the inoculated culture from each series was titrated for determination of viral titer in 8 day old chicks at the outset.

The cultures were then incubated at 37° C for a period of five days at which time they were again titrated for virus activity and subinoculations made into fresh media. The transfer material consisted of 0.25 ml. of a mixture of equal parts of supernatant fluid from two cultures in each series, the latter being maintained separately. Each series was carried through 6 passages so as to exclude any possible dilution factor of the original viral inoculum.

At the end of the third and fifth passages, respectively, titrations were again undertaken in chicks to determine the ID_{50} of the supernatant material in each series.

Titrations were performed by intracerebral inoculation of chicks obtained from the University of California poultry flock (Berkeley), using 5 chicks per log dilution. Chicks less than 2 weeks old received 0.05 ml., older chicks 0.1 ml., while control birds received an identical dose of the equivalent medium or diluent.

Results. The results are summarized in Table 4 and reveal that while the virus showed a high degree of survival in both types of culture when incubated for 5 days at 37° C, no activity was detectable through the third or fifth subpassages, the second passage representing the point beyond which the virus would have been diluted out of its original activity.

Table 4. Viral activity in minced chick embryo cultures at progressive intervals.

Titer of stock virus used for seeding cultures	Media	Titer of virus plus media			
		Immediately prior to incubation	After 5 days incubation at 37°	At end of 3rd passage	At end of 5th passage
5.8*	Series A Mod. Simms-Sanders +swine serum ultra ¹ filtrate	3.8	>3.0**	0	0
	Series B Tyrodes solution +chicken serum	3.1	>3.0	0	0

*Titers are expressed as the Log₁₀ ID₅₀ per ml. of inoculum.

**Exact end point not determined.

Supernatant fluid from the 6th passage in each series, when passaged in embryonating eggs, failed to induce mortality or dwarfing of the embryos following two subpassages at 6 day intervals using pooled allantoic fluid.

Table 5 shows the pH readings obtained in both control and inoculated cultures with the Beckman model G pH meter at the end of each incubation period. There was no significant difference in reaction between infected and uninfected cultures although it appeared that the final pH of the medium following incubation may have been related to the amount of chopped embryonic tissue used, since this varied slightly in different cultures.

Table 5. Reaction (pH) of inoculated cultures at consecutive passage levels compared with uninfected control cultures.

pH	: Simms-Sanders + serum ultra :		Tyrodes solution +	
	: filtrate (Series A) :		: chicken serum (Series B)	
	: Virus	: Control	: Virus	: Control
Initial pH of media	---	7.2	---	7.4
pH at end of				
1st passage	6.9	6.8	6.5	6.9
2nd passage	6.8	6.9	6.9	6.9
3rd passage	7.1	7.0	6.9	6.8
4th passage	6.9	6.8	6.9	6.9
5th passage	6.7	6.8	6.8	7.0
6th passage	6.8	6.9	6.8	6.8

DISCUSSION

Subsequent to the original communication by Hwang et al. (43) in which successful propagation of AE virus in monolayer chick kidney cell cultures was reported, considerable doubt was thrown on these studies by the finding that the material used to prepare hyperimmune serum contained both AE virus and a CELO-like agent (46). It appears that the tissue cultures had, in fact, been contaminated with the CELO or a prototype virus (26, 64) which probably caused the artifact of growth and CPE by AEV. The CELO agent was apparently capable of producing ataxia and histologic lesions in chicks which could be confused with those produced by AE virus.

At least two strains of CELO virus have also been shown to produce cytopathogenic effects when inoculated into chick embryo kidney cell cultures (4, 22), while the virus has been found, on occasion, to be present in apparently normal embryos (105).

The original purpose of this study had been to investigate the dynamics of viral reproduction in vitro with a particular view to adapting the AE virus to an in vitro serum neutralization test. Also, since Hwang et al. had reported a reduction in virulence for chickens of their tissue culture propagated agent (43), it was felt that the possible utilization of a tissue culture attenuated strain for immunization against AE should be investigated. At that time it was not known that the latter authors had been working with a contaminant virus and that there was no conclusive evidence that AEV produces CPE (64).

Initial experiments with the VR strain in chick embryo kidney cultures, failed to indicate cytopathogenic effects following infection with different passage levels of the virus. The virus apparently survived in the culture but did not cause degeneration of the cell monolayer over a period of 3 days.

Although considerable effort was then made to extend the survival period of the embryonic kidney cell monolayer, it was found that, without repeated changes of medium, the cells almost invariably underwent degeneration and became detached from the glass surface after a period of between 4 to 6 days. After seeding tubes with the appropriately diluted cell suspension, an initial lag phase of 10-18 hours was followed by a period of vigorous cell multiplication to form an even, confluent monolayer within 36-48 hours. Replacement of the growth medium at this stage with a maintenance medium containing reduced amounts of serum and increased bicarbonate, assisted in maintaining a more or less even monolayer provided that the medium was replaced at frequent intervals. However, when only a single change of media was used, that is, following inoculation with virus, the cells gradually began to form multilayered clumps with only a sparse covering of elongated cells in the intervening spaces. Within three to four days degenerative changes manifested by a

granular appearance of the cytoplasm and large vacuolated areas became apparent in both virus infected and uninoculated tubes. Since these changes were of a nonspecific nature, further observation for specific CPE was limited to gross changes only.

Viral synthesis is known to take place only in a healthy cell population maintained under optimum conditions (67). An attempt was therefore made to adapt the virus to growth in tissue culture by rapid serial passage in healthy kidney cell monolayers at three day intervals. In order to maintain maximum viral activity, both cells and supernatant from the previous culture were used as transfer material. When the viral activity of passaged material was tested by inoculation of fully susceptible chicks via the intracerebral route, it became clear that while the virus had survived for 9 days at 37° C (3 passages), there was no measurable activity beyond the point of dilution of the original inoculum.

Microscopic study of stained, infected cell monolayers grown on coverslips showed no changes in the cell structure that could not be attributed to normal developmental or degenerative changes.

Thus, while chick embryonic kidney cells cultured in vitro on a glass surface have been found suitable for the propagation and study of several avian viruses (4, 14, 21, 31, 67) and, indeed, were being used at the time of this study in the same laboratory for successful cultivation of Newcastle disease and laryngotracheitis viruses, it was concluded that this particular procedure was unsuitable for synthesis of the strain of AEV in question.

The advantages of continuously propagating cell lines over the use of raw or primary cultures have become increasingly recognized in recent years, particularly with regard to ease of manipulation and reproducibility of results. The HeLa cell line (Gey) of malignant human epithelial cells, in

particular, has found wide use in the study of poliomyelitis and several other viral encephalitides (89). Unfortunately an attempt to propagate two strains of AE virus by serial subcultivation in HeLa cells was somewhat negated by the subsequent discovery of a contaminant organism, apparently a species of mycoplasma (PPL0) in the HeLa cell line. It was not possible to determine how long the stock cell line had been contaminated since the infection was inapparent, causing little, if any, cell damage and only revealed by direct microscopic examination under high power magnification. The microorganism was not pathogenic for chickens when inoculated via the intracerebral route and did not kill embryos inoculated via the yolk sac.

Hearn et al. (40) quote several recent reports of PPL0 contamination of many cell lines in different laboratories throughout the world. In most cases the contamination is insidious and difficult to detect because the organism either fails to infect all cultures or produces only an occasional irregular cytopathogenic effect. This particular strain of PPL0 exhibited a remarkable degree of resistance to streptomycin.

Since no suitable experimental chicks were available during the early part of this experiment to determine the rate of survival of the two virus strains in HeLa cell cultures, and since the stage at which the stock cell line became contaminated is not known, the possibility of competitive or indirect interference with viral synthesis must be considered. Nevertheless, when chicks were available, no viral activity could be detected when material from the 10th subculture was inoculated into chicks by the intracerebral route. Although the chicks used in this study were hatched from immunized dams and might therefore be expected to have some residual passive immunity (85) they were found to be fully susceptible to the stock viruses used, when challenged by the intracerebral route.

It should be pointed out, however, that failure to observe cytopathogenic effects in a monolayer cell culture does not necessarily indicate the absence of virus synthesis. Kaplan and Melnick (59) have indicated that failure to observe cytologic changes in the presence of virus multiplication may in some instances be due to the relatively small number of cells in the culture capable of supporting growth of the virus. Thus, the degeneration of only a few cells would be difficult to detect, even microscopically. On the other hand the extremely narrow host range of AE virus, the long incubation period and lack of a uniform and readily differentiated specific reaction in inoculated animals, places severe limitations on the procedures used for determination of viral activity in an unknown sample. An adequate supply of embryonating eggs from known susceptible birds was not available for work in this study so that intracerebral inoculation of chicks obtained from various sources had to be relied upon for virus assay work. Wherever possible these chicks were tested for susceptibility to AE virus by challenge with graded doses of a stock virus suspension.

Kligler and Olitsky (60) obtained somewhat inconclusive evidence of virus multiplication in minced embryonic tissues using Tyrodes solution plus 10 percent chicken serum as medium. They reported a titer of 10^{-2} in the fifth subplant although the original virus was calculated to have been diluted out of activity at this passage level. However their method of identification of this passaged agent is not recorded, nor was specific neutralization with homologous antiserum undertaken.

An attempt to reproduce the above findings by using almost identical procedures was not successful. The virus was found to survive without appreciable loss of titer for 5 days in the culture held at 37° C but no synthesis could be detected following serial transfer. Since it might be argued that the

chicken serum used in the Tyrodes medium could contain either specific or non-specific neutralizing power, a parallel series of cultures was run using the modified Simms-Sanders buffered salt containing 18 percent swine serum ultrafiltrate. The latter medium has been successfully used in the cultivation of a number of viral agents (4, 92). Here again identical results were obtained, no viral activity being detected beyond the second subplant.

It is clear that considerable further work will be needed in order to completely elucidate the apparently conflicting results reported by Hwang et al. (43, 44, 45, 46) and Klügler and Olitsky (60) and those reported in this study. Virus strains of more recent origin or having a broader spectrum of activity for different tissue cells than the highly neurotropic VR strain may be more suited to growth in tissue culture. A method for in vitro propagation of AE virus would be of inestimable value from both a research and diagnostic point of view while subsequent adaptation of the virus to cells of nonavian origin would eliminate or at least minimize the hazard of contamination with endogenous viruses of chicken origin.

A point of some interest brought out during the course of these studies was that although numerous titration experiments were conducted in chicks using fully virulent virus, uninoculated contact control chicks never became infected during the period of observation (30-40 days). In several instances these control chicks were challenged by intracerebral inoculation at the end of the experiment or some weeks later and invariably found to be susceptible. Although Van Roekel (101) found that chicks inoculated with AEV were able to transmit the disease to uninoculated chicks in cohabitation, it was later stated (98) that clinical manifestations appeared only to a limited degree in noninjected contacts. Schaaf (85) noted that uninoculated controls, held in contact with vaccinated chicks received sufficient exposure to develop some degree of

immunity without developing signs of AE. One of the main factors determining the rate of spread among susceptible chicks under experimental conditions was found by Calnek et al. (18) to be the character of the strain or isolate used. Previous work by these authors (16) using the VR strain (150th chick passage subsequently adapted to embryos), indicated that a very high oral dosage was required to stimulate an immune response. Furthermore, contact controls held in the same room with inoculated chicks remained susceptible for several weeks.

SUMMARY

In order to investigate the behavior and properties of avian encephalomyelitis in various tissue culture systems that have been successfully employed for propagation and study of other avian and mammalian viruses, a series of experiments were conducted with AE virus using the following systems:

1. Chick embryo kidney cell (CEKC) monolayer cultures. No specific CPE could be detected either macroscopically or microscopically following inoculation of tissue cultures with different passage levels of chick brain propagated AE virus or with embryo passaged virus. Infected tissue culture fluids harvested after 72 hours incubation at 37° C had a reduced infectivity titer for chicks compared with the seed virus. An attempt to adapt the virus to tissue culture by rapid serial subcultivation in CEKC failed to induce gross or microscopic CPE after 10 passages. Supernatant fluid and cells harvested at the 1st and 3rd passages remained infective for chicks while 4th and 5th passage material failed to indicate either clinical or histopathological evidence of infection.

The 10th passage material, stored for 98 days at -60° C and titrated in a different batch of chicks again failed to reveal the presence of virus.

2. HeLa cell cultures. Serial subcultivation of 3 different strains of AE virus was undertaken in parallel with CEKC-passaged material and normal chick brain tissue. Specific CPE attributable to viral activity could not be demonstrated, while chicks inoculated with supernatant fluid and cells from the 10th (Schaaf and embryo passaged strains) and 12th (VR strain) tissue culture passages, respectively, failed to become infected. On the other hand, identical chicks from the same batch were found to be fully susceptible when inoculated with the stock (brain propagated) viruses.

When stained coverslip preparations were examined for microscopic CPE, an inapparent infection of the stock HeLa cell line with mycoplasma (PPL0) was brought to light. The latter organism appeared to induce only a mild and transient effect on the HeLa cells, and was not pathogenic for chicks following intracerebral inoculation.

3. Pig kidney cell cultures. Primary subcultures of pig kidney cells inoculated with AE virus failed to reveal gross or microscopic cytopathogenic changes of a specific nature during a period of 5 days incubation.

4. Suspended cell cultures. Serial transfers of AE virus were undertaken in Maitland type cultures consisting of minced chicken embryonic tissues suspended in either Tyrodes solution containing chicken serum or a modified Simms-Sanders solution containing swine serum ultrafiltrate. The virus showed a high degree of survival in both types of culture when incubated at 37° C for 5 days but no activity beyond the second subpassage as indicated by failure to infect chicks.

From these studies it was concluded that the strains of AE virus used could not be readily propagated by various tissue culture techniques currently in use for growth of a number of avian and mammalian viruses.

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APPENDIX

FORMULAS OF VARIOUS TISSUE CULTURE SOLUTIONS USED.

Formula I

PHOSPHATE BUFFER SOLUTION (Duibecco's).

Salt #1

Sodium Chloride, NaCl	8.0 gm.
Potassium Chloride, KCl	0.2 gm.
Sodium phosphate di-basic, Na_2HPO_4	1.15 gm.
Potassium phosphate monobasic, KH_2PO_4	0.2 gm.
Distilled water*	800.0 ml.

Salt #2

Calcium Chloride anhydrous, CaCl_2	0.1 gm.
Distilled water	100.0 ml.

Salt #3

Magnesium Chloride, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	0.1 gm.
Distilled water	100.0 ml.

Solutions 1, 2, and 3 were autoclaved, combined when cool, and the fluid loss made up.

Formula II

HANKS' BASIC SALT SOLUTION (10X concentration).

Solution A.

Sodium Chloride, NaCl	80.0 gm.
Potassium Chloride, KCl	4.0 gm.
Magnesium sulphate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	1.0 gm.

* In all cases "distilled water" refers to demineralized, triple-distilled water.

Solution A. continued

Magnesium Chloride, $MgCl_2 \cdot 6H_2O$		1.0 gm.
Distilled water	approx.	200 ml.

Solution B.

Calcium Chloride, $CaCl_2$		1.4 gm.
Distilled water	approx.	200 ml.

Solution C.

Sodium phosphate dibasic, $Na_2HPO_4 \cdot 2H_2O$		0.6 gm.
Potassium phosphate monobasic, KH_2PO_4		0.6 gm.
Glucose (dextrose)		10.0 gm.
Phenol red solution 1 percent		20.0 ml.
Distilled water	approx.	400 ml.

Solutions A, B, and C were dissolved separately, then combined and brought to a total volume of 1 liter with distilled water.

Formula IIIEARLE'S SOLUTION (10X concentration).

Sodium Chloride, $NaCl$		70.0 gm.
Potassium Chloride, KCl		4.0 gm.
Calcium Chloride, $CaCl_2$		2.0 gm.
Magnesium Sulphate, $MgSO_4 \cdot 7H_2O$		2.0 gm.
Sodium Phosphate, monobasic, $NaH_2PO_4 \cdot H_2O$		1.4 gm.
Glucose (dextrose)		25.0 gm.
Phenol red solution 1 percent		5.0 ml.
Distilled water	q.s.	1 liter

Ingredients were dissolved in the order given and the solution sterilized by filtration.

Formula IVSIMMS-SANDER'S SALT SOLUTION (10X concentration).

		<u>Salt #1</u>
Sodium Chloride, NaCl		160 gm.
Potassium Chloride, KCl		4 gm.
Calcium Chloride, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$		2.94 gm.
Magnesium Chloride, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$		4.06 gm.
Distilled water	q.s.	1 liter

Salt #2

Sodium bicarbonate, NaHCO_3		20.2 gm.
Sodium Phosphate dibasic, Na_2HPO_4		4.26 gm.
Dextrose		20.00 gm.
Phenol red		0.10 gm.
Distilled water	q.s.	1 liter

The stock solutions were prepared and stored separately.

Formula VTYRODE'S SOLUTION

Sodium Chloride, NaCl		8.00 gm.
Potassium Chloride, KCl		0.2 gm.
Calcium Chloride, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$		0.2 gm.
Magnesium Chloride, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$		0.1 gm.
Sodium Phosphate monobasic, NaH_2PO_4		0.05 gm.
Sodium bicarbonate, NaHCO_3		1.00 gm.
Glucose		1.00 gm.
Distilled water	q.s.	1 liter

Formula VIEAGLE'S CELL COUNTING SOLUTION

Dissolve 7.68 gm. desiccated citric acid in 200 ml. of deeminized water.

Add 4 cc. of a 0.1 percent crystal violet solution and mix. The solution was stored in brown glass medicine bottles.

Formula VIITRYPSIN-VER SIN MEDIUM

Sodium Chloride, NaCl	8.0 gm.
Potassium Chloride, KCl	0.4 gm.
Dextrose	1.0 gm.
*Versin (1:5000)	0.2 gm.
**Trypsin (1:400)	2.5 gm.
Sodium bicarbonate	0.35 gm.
Phenol red 1 percent solution	0.5 ml.
Distilled water	1000 ml.

Ingredients were dissolved in the order shown and sterilized by Seitz filtration.

* Disodium dhydrogen ethylene diaminetetra acetate dihydrate
manufactured by Frederick Smith Chemical Co., Columbus, Ohio.

** Bactotrypsin (Difco).

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MICROPHOTOGRAPHS

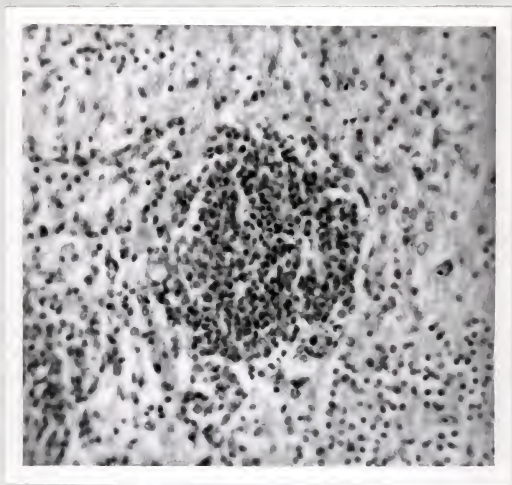


Fig. 1. Perivascular lesion in cerebrum. Chick #491 (expt. 5), ataxic for 3 days. X 450.

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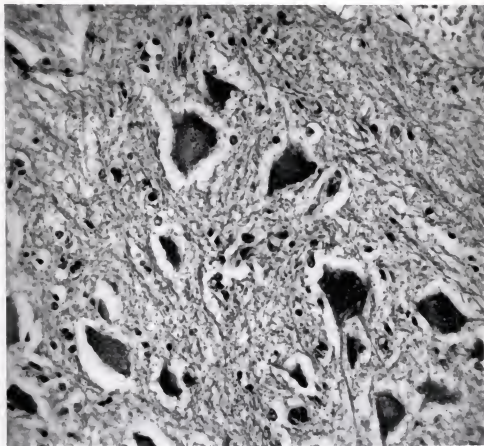


Fig. 2. Anterior horn cells of the spinal cord in the lumbosacral region showing early degenerative lesions. Chick inoculated with VR strain of AEV from 160th chick passage; incubation period 12 days; ataxic for 6 days. X 450.

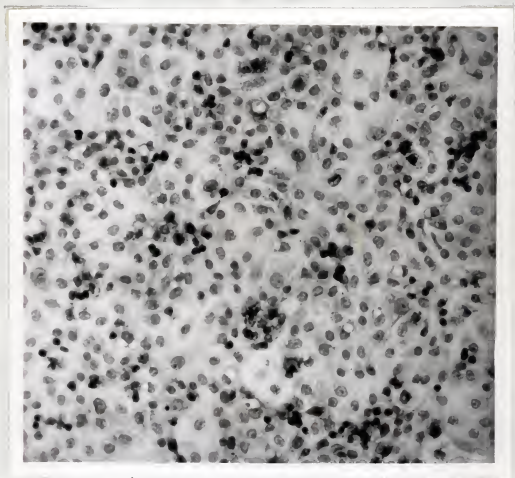


Fig. 3. Primary subculture of pig kidney cells on sixth day following inoculation of chick brain propagated AE virus. Note absence of C.P.E. Giemsa stain. X 500.

142

142

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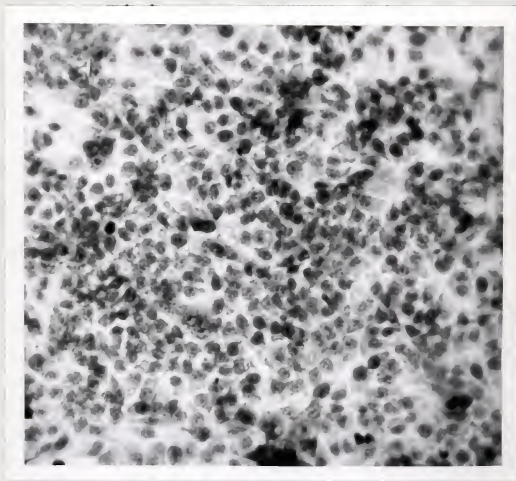


Fig. 4. HeLa cell monolayer stained at 96 hours following inoculation of AE virus from the third HeLa cell passage. No specific C.P.E. could be detected. Giemsa stain. X 500.

STUDIES ON THE IN VITRO CULTIVATION OF
AVIAN ENCEPHALOMYELITIS VIRUS

by

JOHN RUSSELL PHILIP

B.V.Sc., University of Pretoria, South Africa, 1954

AN ABSTRACT OF
A MASTER'S THESIS

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requirements for the degree

MASTER OF SCIENCE

Department of Pathology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1962

Avian encephalomyelitis (epidemic tremor) is a virus disease of chickens of widespread distribution and a cause of considerable economic loss to the poultry industry. A description of the disease and a review of some recent findings are given in the first part of the paper. It appears that research has been seriously handicapped by a basic lack of knowledge regarding the virus and an inadequacy of reliable diagnostic tools.

The specific purpose of this study was to investigate the behavior and properties of avian encephalomyelitis virus in various tissue culture systems that have been successfully employed for propagation and study of other avian and mammalian viruses.

The experimental procedure adopted was as follows: Virus strains were obtained from two different sources and maintained by serial intracerebral passage in chicks, one strain being also propagated in embryonated eggs. Stock suspensions of homogenized brain tissue were titrated for infectivity in young chicks following intracerebral inoculation.

In the first series of experiments, monolayer cell cultures of embryonic chicken kidney cells were grown on the surface of bottles, tubes, and coverslips and subsequently infected with virus harvested from different chick passages. Attempts were made to adapt the virus to tissue culture by rapid serial passage using both brain and embryo propagated strains. Microscopic studies were made of infected cell monolayers grown on coverslips for detection of specific cytopathic effects.

A similar series of experiments was undertaken using monolayer cultures prepared from a line of malignant human epithelial cells (HeLa) which were inoculated with two different strains of avian encephalomyelitis virus. In addition, the effect of the virus on primary subcultures of porcine kidney

cells grown on monolayers on glass was studied.

Finally attempts were made to propagate the virus in a system previously reported to have been successful, namely in Maitland-type cultures of minced embryonic tissues suspended in Tyrode's and modified Simms-Sander's solutions. Determination of viral activity was undertaken by intracerebral inoculation of chicks and the findings confirmed by histopathological examination.

The results of this work indicated that virus multiplication as determined by the ability to infect chicks did not occur, although the strains of virus used were capable of survival for several days in different tissue culture systems.

The inability of the virus to cause cytopathic effects in different cells cultivated in vitro, and the inability of the virus to multiply within these cells, is in direct contradiction to previously published work on this subject. The above conclusions and some minor observations made during the course of the study are discussed in relation to other published works on the subject.