THE PATHOGENESIS OF POWASSAN VIRUS IN MICE AFTER AIRBORNE INFECTION

by

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B.Sc. University of Alberta 1961

M.Sc. University of Alberta 1966

A THESIS SUBMITTED IN PARTIAL FULFILMENT

OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in the Department

of

Microbiology

We accept this thesis as conforming to the

required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April, 1972

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Date JULY 13 1972

ABSTRACT

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Although arboviruses usually infect humans by bites of infected ticks or mosquitoes accidental infection with several agents has been encountered following inhalation of virus-laden aerosols generated accidentally during spillage of material in the laboratory. This project provides a model of accidental airborne infection of man by Powassan virus. The specific aims were:

1. to determine if mice could be infected with Powassan virus, a member of the Russian spring-summer complex of tick-borne group B arboviruses by a means which did not introduce the virus directly into the bloodstream;
2. to provide information on the dose-response relationships using various routes of inoculation;
3. to determine the pathogenesis of Powassan virus in mice after airborne infection.

Intranasal instillation of Powassan virus suspensions into anaesthetized mice induced a fatal infection by a route which did not introduce virus directly into the bloodstream.

Although mice developed fatal encephalitis following inoculation of Powassan virus by several routes, the smallest quantity of virus which induced an overt infection was injected either intracerebrally or intravenously. Fatal infections followed the subcutaneous injection of 20 times the minimum amount of virus. Mice could not be infected by the instillation of more than one million intracerebral doses of virus directly into the gastrointestinal tract. Infection by inhalation of aerosols required about 600 times the minimum virus dose, and infection was initiated by the intranasal instillation of 10,000 or more minimum intracerebral doses of virus.

The infectivity of Powassan virus suspensions and aerosols decayed rapidly under ultraviolet irradiation.

Aerosolized virus was slightly more stable under conditions of low relative humidity in contrast to high and intermediate relative humidities, when aerosols were aged up to five hours in the dark at 21°C.

After aerosol infection of mice with Powassan virus the following sequence of events occurred. Powassan virus invaded and multiplied within the epithelial cells and/or macrophages of the mouse lung. Subsequently, virus appeared in the blood. When the virus titre within the blood attained approximately 3 $\log_{10}$ mouse intracerebral LD_{50} per ml. at two days post exposure it appeared in other tissues including the brain where it multiplied to titres as high as 9 $\log_{10}$ mouse intracerebral LD_{50} per gram by the seventh day. Mice died with encephalitis seven to eight days after aerosol inhalation.

Using fluorescent antibody techniques, it was determined that virus present in the blood of the mouse invaded the cuboidal epithelium of the choroid plexus and later spread either from this tissue to the cerebrospinal fluid or directly by way of the vascular endothelium to the tissues of the brain.

Electron micrographs of nasal epithelium infected with Powassan virus presented very preliminary information on the possible morphogenesis of the virus within this tissue. Cytoplasmic vacuoles were observed which could serve as the site for synthesis of virus particles.

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ACKNOWLEDGEMENT

The author wishes to express his sincere thanks and appreciation to:

Dr. D. M. McLean, Professor and Head of the Division of Medical Microbiology in the Department of Microbiology, University of British Columbia, supervisor of the thesis project, for his guidance and encouragement;

Dr. J. J. R. Campbell, Professor and Head of the Department of Microbiology and Dr. I. McT. Cowan, Dean of the Faculty of Graduate Studies, University of British Columbia, for allowing the major portion of this research to be completed at the Defence Research Establishment Suffield, Ralston, Alberta;

The Defence Research Board for scholarship funds which helped to finance this research;

Mr. J. F. Currie, Head of the Microbiology Section at the Defence Research Establishment Suffield for his advice and encouragement;

Dr. D. S. Willoughby, formerly of the Defence Research Establishment Suffield and Dr. W. C. Stewart, Director of Research for the Defence Research Establishment Suffield, extramural advisors;

My wife, Marie, for typing this thesis;

The Instrumentation, Photography and Computer Section and the Information Services Group, Defence Research Establishment Suffield for the preparation of the final copies of the thesis.

HISTORICAL REVIEW

Introduction

Statement of Purpose

Human virus infections acquired in the laboratory, especially those due to arboviruses, have been attributed to the inhalation of infectious aerosols generated during accidents. Arboviruses are maintained in nature principally through biological transmission between susceptible vertebrate hosts by blood-sucking arthropods in whose tissues they multiply. The usual means by which humans contract arbovirus infections is through bites by infected arthropods, such as ticks and mosquitoes. Thus virus is introduced directly into the blood stream. However, laboratory infections with Venezuelan equine encephalitis virus (Korpowski and Cox, 1947), St. Louis encephalitis virus (Magnus, 1950), Russian spring-summer encephalitis virus (Haymaker et al, 1955), West Nile virus (Nir, 1959), Kyasanur forest disease virus (Morse et al, 1962), and Lassa virus (Frame et al, 1970), (Leifer et al, 1970), and other arboviruses have resulted from inhalation of virus-laden aerosols following laboratory accidents. The lung parenchyma has thereby become the portal of entry of virus.

Although abundant information is available regarding the pathogenesis of influenza virus which remains localized to the respiratory tract, relatively few investigations have been made on the fate of inhaled agents such as arboviruses where infection of vertebrates is regularly accompanied by viremia.

This project reports investigations of the pathogenesis in mice of Powassan virus, the only North American member of the Russian spring-summer complex of tick-borne group B arboviruses, following intranasal instillation of infectious droplets containing a suspension of Powassan virus and after inhalation of infectious Powassan virus aerosols. This provides a model of the multiplication and movement of a virus in man following accidental airborne infection with an agent which is naturally arthropod-borne. A necessary prerequisite to infection of mice with suspensions and aerosols of Powassan virus was the determination of the decay rate of virus infectivity in suspension and aerosols under various environmental conditions.

Arbovirus Group

Classification and Properties

Arboviruses are agents which are maintained in nature principally by cycles of infection involving vertebrates and hematophagous arthropods. They induce viremia in vertebrate hosts, at titres which infect arthropods which ingest blood. They multiply in the tissues of arthropods, and after an extrinsic incubation period, they are transmitted to other vertebrates by bites of arthropods (Casals and Reeves, 1965).

Provisional classification as an arbovirus is based on a combination of laboratory and epidemiologic determinants (Casals, 1961), including: circumstances of isolation, antigenic relationship to an established member, effects on laboratory animals and tissue cultures, and reduction in infectivity titre on exposure to ethyl ether (Andrewes and Horstmann, 1949), or sodium deoxycholate (Theiler, 1957).

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These ecologic and biologic criteria are different from the physical and chemical taxonomic characteristics used for establishing other viral groups (Andrewes, 1962), (Lwoff et al, 1962). There is no assurance that arboviruses will constitute a homogeneous class when their physical and chemical properties are more completely studied.

The number of currently catalogued arbovirus serotypes has increased dramatically from 34 in December 1949 to 228 in January 1969 -- American Committee on Arthropod-Borne Viruses (ACAV) (1969); 241 June 1970, ACAV (1970); 269 November 1971, ACAV (1971). Two hundred four arbovirus strains as described in the Catalogue of Arthropod-Borne Viruses of the World were dispersed among 24 serological groups (ACAV, 1969) (see Table 1). Subsequently new serological groups have been added (ACAV, 1970) (see Table 2). As of November 1971 two further serological groups have been added, namely Matariya and Palyam (ACAV, 1971).

Each antigenic group includes viruses that cross-react in one or more serological tests and thus presumably share common antigens. It was earlier proposed that the Tacaribe serological group be included in a newly defined virus group, the Arenoviruses (Rowe et al, 1970). This would effectively remove the Tacaribe complex from the arbovirus group of viruses. However, ACAV authorized a recommendation to broaden the name of the Catalogue to permit retention of viruses provisionally registered as arboviruses (such as the Tacaribe group, Lassa virus, etc.). Future editions of the Catalogue will contain (1) arthropod-borne viruses, (2) possible arboviruses, (3) non-arthropod-borne animal viruses.

The antigenic classification and knowledge of interrelationships have value beyond taxonomic utility since they may reflect the evolutionary

origin of related viruses and the immune response to these viruses. An infected host may develop an immune response both to the infecting and related viruses, but the first responses tend to be the most specific. Antigenic inter-relationships also facilitate the rapid identification through grouping of newly isolated virus strains; however they complicate the interpretation of results of serological tests on sera from suspected clinical cases or animals in areas where closely related viruses co-exist (Casals and Reeves, 1965).

Only four families of blood sucking arthropods, Culicidae, Ceratopogonidae, Psychodidae and Ixodidae are clearly implicated as natural vectors of arboviruses (Taylor, 1967). Detailed field investigation and laboratory studies of vector-virus associations have been limited largely to viruses that pose significant public health or veterinary problems.

Those arboviruses studied by electron microscopy exhibit mostly cubic symmetry and are surrounded by envelopes. However some viruses with helical or complex symmetry are included in the group by arbovirologists (McLean, 1968).

Arboviruses contain ribonucleic acid (RNA) and the majority range in size between 20 and 60 nanometers (nm.).

As previously mentioned, arboviruses are transmitted in nature from one vertebrate host to another by the bites of blood-feeding arthropods especially mosquitos and ticks. Before transmission can occur, however, sufficient time is required for the virus to multiply within the tissues of the arthropod and obtain a sufficiently high titre in the salivary glands to permit transfer of enough virus to infect the new host when saliva is introduced during the act of taking a second blood meal. In the arthropod the period between obtaining an infectious blood meal and development of the ability to transmit the virus by biting is termed the 'extrinsic incubation period'. Most arboviruses are transmitted by culicine mosquitos, however

members of the Russian spring-summer complex of group B tick-borne arboviruses of which Powassan is a member, are transmitted by bites of Ixodid ticks.

Table 3 is a modified list of the prototype strains of the Russian spring-summer complex of tick-borne group B arboviruses, year and location of initial isolation, geographical distribution, tick vector, mammalian reservoir and syndromes in man (McLean, 1968).

Because little work has been done along these lines, the following is a necessarily brief account of the various physical and chemical properties of the group B arboviruses. The infectivity of the group B viruses is relatively sensitive to heating, inactivation occurring at 56^{o} to 60^{o} C for 10 to 30 minutes, and is unstable at room temperature, especially at high dilutions. Inactivation can be retarded by the use of a protein diluent instead of saline, or the addition of protein in the form of bovine albumin (0.75 per cent) or animal serum (10 per cent). The infectivity and hemagglutinating activity are best preserved if crude extracts of the virus are lyophilized or stored frozen at -70^{o}C. Crude group B arbovirus suspensions are most stable at pH's ranging between 7.4 to 8.5 with infectivity being rapidly destroyed at extremely high or low pH.

Infectivity of group B arboviruses is destroyed or markedly lowered following treatment with ethyl ether and acetone, formalin, and deoxycholate 1:1000 for 30 minutes at 22^{o}C. As previously mentioned the latter inactivates all arboviruses thus far investigated. Members of the tick-borne encephalitis complex are inactivated by ethanol, hydrogen peroxide, trichloracetic acid, while treatment with fluorocarbons lowers infectivity only slightly.

The hemagglutinins of group B arboviruses are most stable in buffered saline at pH 9.0 and temperatures near 0^{o}C. They react with erythrocytes of geese, roosters and newly hatched chicks only in a narrow pH zone, the optimal

pH for most members being in the range 6.0 to 7.0. They are inactivated by treatment with ultraviolet light while treatment with acetone has little effect on activity. The hemagglutinin can be blocked by various inhibitors found in sera and tissue extracts (McLean, 1968), (Sokol, 1962), (Hammon and Sather, 1969), (Casals and Brown, 1954).

Data on size and morphology of the various members of the group vary depending on the methods of investigation indicating that more work is needed in this area with possible standardization of methods for the various investigation procedures.

Encephalitis caused by infection with tick-borne group B arboviruses occurs in either sporadic or epidemic form throughout Europe and Asia in areas located between the latitudes of 45^{o}N and 62^{o}N, extending from Scotland in the west to Kamchatka in the east.

Infection with the Russian spring-summer complex of viruses may be manifested either as a paralytic form of polioencephalomyelitis or as biphasic meningoencephalitis. The paralytic form results in flaccid paralysis. Bulbar centers may also be affected, causing paralysis of respiration, the palate and the pharynx. In fatal cases death usually occurs four to seven days after onset of illness. In patients who recover paralysis is frequently permanent (McLean, 1968).

The meningoencephalitis form shows a biphasic course in most cases. Fifty to 70 lymphocytes per cu. mm. appear in the cerebrospinal fluid. The pyramidal, thalamic, cerebellar, sympathetic and parasympathetic systems may all show disorders, but flaccid paralysis similar to poliomyelitis does not develop, Complete recovery is the rule but convalescence may be prolonged for two months or more (McLean, 1968).

Powassan Virus

Powassan virus is antigenically related to, but distinct from Russian spring-summer encephalitis virus (Casals, 1960) and is so far the only serotype among the group B tick-borne arboviruses which has been isolated in North America. The prototype strain LB was isolated from the brain of a child who lived in a forested area at Powassan, Ontario, Canada (latitude 46^{o}N, longitude 79^{o} 30'W) and who had contracted fatal encephalitis (McLean and Donohue, 1959). Subsequent isolations have been recorded in Colorado, (Thomas et al, 1960), South Dakota (McLean et al, 1964) and New York State (Whitney and Jamnback, 1965), with serological evidence of its presence in British Columbia (McLean et al, 1968), (McLean et al, 1969), (McLean et al, 1971). Field studies have revealed a natural cycle involving rodents such as ground hogs (Marmota monax) and red squirrels (Tamiascuris hudsonicus) as reservoirs with hard ticks such as Ixodes cookei (Packard), Ixodes marxi (Banks), (McLean et al, 1962), (McLean and Larke, 1963), (McLean et al, 1964a), (Whitney and Jamnback, 1965), (McLean et al, 1966), (McLean et al, 1967), and Dermacentor andersoni (Stiles) (Thomas et al, 1960), (McLean et al, 1964a), (McLean et al, 1968), (Chernesky, 1969), as vectors. Man and other vertebrates appear to be tangential hosts in the natural tick-rodent cycle.

Abdelwahab et al, (1964) disclosed during her investigations with Powassan virus infection of LLC-MK2 cells that the virus produced cytopathic effects comprising cell rounding and cytoplasmic vacuolation five days after inoculation, and that mixtures of Powassan virus and specific antiserum inhibited the appearance of cytopathic effects. Hemagglutinins for rooster erthrocytes first appeared in the supernatant fluid four days after inoculation, the optimum pH for the hemagglutination reaction was 6.4 at 22^{o}C.

Electron microscope studies of thin sections of infected tissue culture cells revealed particles 36 to 38 nm. These particles were usually arranged along the outer edges of cell membranes in a 'palisade' formation or within cytoplasmic vacuoles. Negatively stained preparations showed intact virus particles of 40 to 45 nm. in diameter. Each particle consisted of an inner electron dense core of 39 nm. diameter surrounded by a fine outer membrane of 2 nm. in width.

Newborn mice inoculated with suspensions of Powassan virus developed signs of acute encephalitis five days after inoculation. Weaned mice developed encephalitis on the seventh day after inoculation with the same material.

Pathogenesis and Pathology of Virus Diseases

Pathogenesis comprises the sequence of events in multiplication and movement of virus through the body which may sometimes culminate in production of disease. Pathology is concerned with the abnormalities in tissues which result from the reaction to injury.

Viruses multiply intracellularly and in the course of growth affect the physiologic state of the infected cell. The change is often registered as cell degeneration or sometimes proliferation. Secondary inflammation usually accompanies the primary degenerative or proliferative reaction and these combined changes often constitue a pathogenic picture characteristic of a specific group of viruses.

Pathogenesis may be described in four distinct although closely related phases. Phase 1, the entry of the virus from the outside into the host, can occur by several routes. The virus can gain entrance to the host by way of the mucus membranes of the nose and throat, lower respiratory tract, gastrointestinal tract, conjunctival sac or the genitourinary tract. The virus may breach the skin or subcutaneous and muscular tissue by the bites of arthropods or vertebrates. Vertical transmission may occur in cases where a viral infec-

tion is transmitted from mother to offspring across the placenta.

The 2nd phase of pathogenesis involves the spread of virus from the sites of initiation to specific target tissue. Spread can be mediated through passage of the virus from cell to cell by the formation of intercellular bridges. This type of spread is relatively unimportant in the overall scheme of viral pathogenesis.

One of the key roles in the spread of viruses from the site of initial infection must be ascribed to the macrophages of the reticuloendothelial system. Macrophages are situated in all major compartments of the body. Those lining the liver, spleen and bone marrow sinusoids monitor the blood and remove for instance, circulating effete red blood cells. Those lining the sinuses of the lymph nodes monitor the lymph, removing inert particles or microorganisms brought in by the afferent lymph stream. Those lining the pleural and peritoneal cavities monitor these cavities and those lining the respiratory tract monitor the respiratory fluid film ingesting inhaled dust particles and microorganisms. Finally, large numbers of macrophage live in, move through, and monitor the connective tissue spaces throughout the body. The outcome of virus penetration into the host is to a large extent determined by the fate of the virus once it has entered the macrophage. The virus, whether it is adsorbed to lymphocytes or taken up by wandering tissue macrophage is transported by the lymph to the lymph node. The infection may be overcome in the nodes through the interaction of antibody and macrophages lining the sinuses of the node, but often the virus is not inactivated at the lymph node and is carried by the lymph to the blood stream.

One of the most characteristic features of viral infections is the presence of viremia. This means that virus can be isolated from the blood of the infected animal. In the early stages of spread within the body,

viruses may enter the blood stream after initial multiplication at the site of entry; this is spoken of as primary viremia. A secondary viremia may occur later following another stepwise increase of virus in the viscera.

Neurotropic viruses can spread along nerve fibres. This has been investigated by titration of segments of nerve trunks at various intervals after peripheral injection, by transection experiments and by immunofluorescence techniques.

Phase 3 of pathogenesis involves the localization of virus in target organs. Viruses exhibit a wide range of variation in regard to the organs of the body on which they exert their main action. Each has a selective affinity for one or more such target organs and this property is termed tropism. Viruses therefore can be classified in terms of their tissue tropism, dermotropic, pneumotropic, neurotropic, etc. Some viruses may attack a variety of tissue and thus may be termed pantropic.

The final phase of pathogenesis involves the release of virus from the infected host. Viruses may be released from a variety of sites; the site of local multiplication, the upper or lower respiratory tract, the gastrointestinal or genitourinary tract. Virus may also be released in the blood -- an event which is of particular interest when one considers the transfusion of whole blood or when one considers the arthropod-borne group of viruses (Bang and Luttrell, 1961), (Buddingh, 1965), (Rhodes and Ditchfield, 1968), (Mims, 1964).

Viral Invasion of the Central Nervous System

The clinical manifestations of many virus infections are dependent on whether or not the virus gains access to susceptible cells within the central nervous system. If infection is limited to extraneural tissue, signs may be mild or inapparent, but infection of neural tissues may lead to meningitis, encephalitis, paralysis, and death. Therefore, the mechanisms by

which viruses penetrate the central nervous system are of prime importance in the understanding of the pathogenesis of virus diseases which have nervous system complications.

Infection of the central nervous system with viruses can no longer be explained on the basis of rare agents with a special predilection for neural tissues. Enteroviruses, mumps virus, herpes virus, measles virus, and arthropod-borne viruses are the agents most often responsible for infections involving the central nervous system. Serological evidence supports the facts that large segments of the population have been infected with viral agents but have not displayed any clinical signs of disease. Polioviruses and the arthropod-borne encephalitis viruses cause clinically significant diseases on relatively rare occasions. These diseases are frequently manifest by neural involvement. Herpes, mumps and measles infections usually present as mild illnesses, but may sometimes be complicated by central nervous system involvement (Meyer et al, 1960). Early development of antibody, or administration of antiserum at the time of extensive viral replication in the central nervous system may promote extensive neuronal damage and death of mice infected with Langat virus (Webb, et al, 1968), (Webb et al, 1968a).

In human infections it is not possible to trace the pathogenesis of encephalitic diseases caused by viruses, and in the case of fatalities caused by such infections, the disease is usually too widespread to reconstruct its evolution. Attempts have been made to study the tissue changes due to virus infections in experimental animals. This involves the examination of tissue collected at various times during the incubation period. Conventional histological examination of tissue is generally inadequate because morphological changes due to viral infections occur late in the course of infection, if they occur at all.

Fenner (1949) in his studies of ectromelia virus infections in mice (mousepox) used a more precise method to study the pathogenesis of the disease. Animals were dissected at regular intervals during the incubation period and organs were titrated to quantitate virus content. This method demonstrates the rate of viral multiplication and the sequence of infection of the various organs. However, it does not identify the cells within the tissue which are affected and in cases where there is a pronounced viremia, does not distinguish between the blood-borne virus and the virus produced in situ.

The development of the fluorescent-antibody technique (Coons et al, 1942), (Coons et al, 1950), provided a practical means for tracing viral infection at the cellular level. The technique essentially involves the production of a hyperimmune serum against the virus causing the infection; conjugation of the isolated hyperimmune gamma globulin fraction with a fluorescent label, and application of the labelled globulin to sections of tissue. The labelled globulin complexes with viruses in infected tissue revealing foci of specific fluorescence when examined using a microscope with an ultraviolet light source. A combination of the fluorescent-antibody technique together with the quantitation of virus in various tissues provide precise methods for the accurate portrayal of the pathogenesis of a virus disease (Mims and Subrahmanyan, 1966).

Neural Spread of Virus to the Central Nervous System

Observations have been made that the central nervous system represents a complicated meshwork of tightly packed cells and processes with virtually no free space with the exception of minute clefts of 100 - 200 Angstrom units between adjacent elements (Shcale and Ford, 1965). Presumably these tortuous clefts between neural elements would preclude the dispersion of viruses by way of the intercellular spaces of the central nervous system. How-

ever, Bodian (1948) studying the development of morphological lesions in experimental poliomyelitis and Luttrell and Bang (1958) with similar studies using Newcastle disease virus obtained evidence which indicated that the pathway of spread was neuronal, although it was not decided whether the spread was by way of the axon or intercellular space. Boyse et al (1956a), studying the spread of herpes simplex in the spinal cord of rabbits and Liu et al (1958) studying the poliovirus infections in monkeys concluded that the spread was from cell to cell through the intercellular space. It was further suggested by Boyse et al (1956) that an important factor supporting the dispersal of viruses or bacterial toxins through the cerebrospinal axis may be the repeated fluctuation of pressure conveyed onto the central nervous system by the impact of the arterial pulse.

In strong support of the theory of viral dispersion through the extracellular space are the observations that high doses of hyperimmune serum can limit or arrest the progress of an infective encephalitic process (Liu et al, 1959), (Schindler, 1961).

It was long believed that the normal hematoencephalic barrier was impermeable to most if not all viruses (Hurst, 1936). Early experimental studies with rabies and herpes simplex viruses suggested that viruses spread to the central nervous system along peripheral nerves (Marinesco and Dragenesco 1923), (Goodpasture and Teague, 1923), (Goodpasture, 1925).

The neural lymphatics have also been implicated as potential pathways for the spread of virus to the central nervous system, but anatomical studies conducted by Brierley (1950) have indicated that they lie outside the perineurium and do not communicate directly with the subarachnoid space. The subarachnoid space around nerve roots terminates in a cul-de-sac near the proximal pole of the ganglion. Field and Brierley (1948) (1948a) examined the diffusion of particles from India ink within the subarachnoid space and

found that they diffuse through the cuff of arachnoid surrounding the peripheral nerves and are taken up by the prevertebral lymphatics. The diffusion is normally centrifugal, but when abdominal pressure was applied, reversing the pressure gradient, particles were forced from the lymphatics into the dorsal root ganglia.

In a review of nerve trunks as pathways of infection Wright (1953) concluded that three conduits were available: axons, lymphatics and intercellular space of nerve fasicles. Axons were eliminated as a pathway because of the viscosity of axoplasm, the lymphatics because of the centrifugal flow of lymph (except under irregular conditions) thus leaving tissue spaces between individual nerve fibres as the logical pathway for the dispersion of viruses.

Radiopaque substances or dyes injected directly into a nerve fasicle spread quickly toward the subdural space (Dorang and Matzke, 1960) without leaking visibly into the epineurium. The perineurium therefore seems to be a rather tight semipermeable membrane. The dispersion occurs in tissue spaces of the endoneurium. The driving force of fluid within the fasicles is believed to arise from the pressure developed during normal physiological activity of the muscle (Wright, 1959).

Rabies has been quoted as the classical example of a virus disease which spreads to the central nervous system along peripheral nerves (Habel, 1964). It has been observed that there was a rapid dispersion of the virus to the central nervous system in spite of no (Johnson, 1965a), or limited (Schindler, 1961) replication at the site of inoculation. After multiplication within the central nervous system the virus extends centrifugally along peripheral nerves (Dean et al, 1963).

In Johnson's (1965) study using fixed-rabies virus in mice, no evidence of endoneural-cell infection was found. Spread of virus to the central nervous system appeared to be by way of the nerve since infection could be

prevented by transecting the appropriate peripheral nerve. When the nerve was maintained intact, the appropriate dorsal route ganglion cells were first to show evidence of infection upon fluorescent-antibody staining.

Johnson (1964) using routine titration procedures and fluorescent-antibody staining for the identification of infected cells showed that after intercerebral inoculation of herpes simplex virus, the virus rapidly dispersed into the cerebrospinal fluid, multiplied in the meninges and ependyma and then invaded the underlying parenchyma of the brain infecting both neurons and glia. Following extraneural inoculation he found that virus gained access to the central nervous system by hematogenous and neural pathways. After intraperitoneal and intranasal inoculation, virus was found to multiply in viscera and produce a viremia; foci of central nervous system infection then developed around cerebral vessels. Spread of virus was demonstrated along peripheral nerves after subcutaneous and intranasal inoculation. Johnson states that this spread resulted from centripetal infection of endoneural cells (Schwann cells and fibroblasts) and also that viral antigen was not found in axons even after infection of the corresponding ganglion cell perikaryon. It would appear therefore that infection of endoneural cells would provide a pathway for the spread of virus along nerves together with the alternative of spread within the interspaces of nerve fasicles (Field, 1952), (Dean et al, 1963), (Wildy, 1967).

There are several other viruses which have been shown to be capable of infecting and progressing along peripheral nerves: human poliovirus (Nathanson and Bodian, 1961), West Nile virus (Kundin et al, 1962), tick-borne encephalitis virus (Albrecht, 1962). Studies by Johnson with herpes simplex infection in mice showed that after intranasal inoculation virus could spread to the central nervous system by neural and hematogenous route simultaneously (Johnson, 1964).

Hematogenous Spread of Virus to the Central Nervous System

As previously mentioned the 'blood-brain barrier' was considered to be impermeable to viruses (Hurst, 1936). Extensive studies on the pathogenesis of poliomyelitis in the 1940's and 1950's (Bodian, 1955), (Sabin, 1956), (Bodian and Horstmann, 1965) provided the major impetus for the reconsideration of the hematogenous spread of viruses to the central nervous system.

The hematogenous route for the dissemination of viruses is of paramount importance in the pathogenesis of diseases caused by members of the arthropod-borne group of viruses. However, the wide range of hosts together with the variations in pathogenicity of the multitude of viruses which compose the arbovirus group makes tenuous generalizations on pathogenesis with respect to the various diseases; therefore this outline will be confined to some of the arboviruses which are known to cause central nervous system infections in man and animals such as tick-borne encephalitis viruses, of which Powassan virus is a member.

Most arboviruses have a broad range of susceptible animals. However some species are resistant to virus multiplication (Casals and Clarke, 1965), (Clarke and Casals, 1965), (McLean, 1968). Some species can become infected with a virus and show evidence of widespread systemic involvement with negligible neural involvement. This type of virus-host relationship is found primarily in the natural vertebrate host animal and probably represents natural selection which has taken place over long periods of close association between the host and virus (Fenner, 1965). It was learned that the Princeton Rockefeller Institute (PRI) strain of mice was highly resistant to all members of Casals' group B arboviruses, but not to the group A arboviruses. The factor controlling this resistance behaved in cross-breeding experiments as a single dominant autosomal gene, which depressed

the multiplication of the virus in the brains of resistant mice. It was concluded that the resistance was due to the inability of the viruses from the group B arboviruses to infect the macrophages of the resistant mice (Sabin, 1952), (Goodman and Koprowski, 1962). Thus it would appear that in the case of the above experiments resistance to virus disease may be under genetic control which operates by the ability of viruses to infect and destroy certain key cells.

Mims (1964) in his excellent review on the pathogenesis of virus diseases provides information on the clearance of viruses from the blood by the macrophages of the reticuloendothelial system. He states that particle size affects the clearance rate. Large viruses such as vaccinia or vesicular stomatitis are cleared much more rapidly than the smaller viruses. He mentioned that another characteristic feature noted with respect to virus clearance curves is that clearance does not continue at a constant rate, hence the curve flattens out leaving an 'uncleared tail.' He suggests that this uncleared tail may be due to the fact that some viruses become associated with the cellular elements of the blood stream or that the uptake of particles by the reticuloendothelial system macrophages is partly reversible with an equilibrium being reached between circulating and phagocytosed particles.

In most virus infections, organs and tissues are involved which are distant from the site of initial entry of the virus into the body and spread to these organs takes place via the blood stream. In the case of arbovirus diseases adequate viremia is essential for the transfer of virus to new hosts by blood feeding arthropods. Viruses, as mentioned earlier, may become associated with the formed elements of the blood stream cells such as thrombocytes, lymphocytes, monocytes and leukocytes. One important aspect of white cell associated viremias is that leukocytes are able to carry viruses with them in their migration through the body. In some viremias virus becomes

associated with the erythrocyte. Rift Valley fever virus is an example of this type of association (Mims, 1956). Viruses may also be released free into the fluid or plasma portion of the blood. This type of plasma viremia is subject to clearance by the reticuloendothelial system. If the rate of entry of virus into the blood stream equals the rate of clearance then the level of viremia remains constant. If the clearance rate is rapid in relation to the rate of entry there may be no detectable viremia. Obviously a rise or fall in the rate of entry of virus into the blood would be accompanied by a rise or fall in the degree of viremia. Many arbovirus infections display the above features. Some viruses are known for their ability to infect and damage vascular endothelium. A search for a close association between vascular endothelium and arboviruses has been pursued in order to explain the high levels of viremia caused in arbovirus infections; also if arboviruses had the ability to grow through vascular endothelium the pathway of invasion of the central nervous system by various members of the arboviruses could more easily be understood.

Fluorescent-antibody studies by various researchers reveal evidence of viral antigen in vascular endothelium in the cases of some arbovirus infections, e.g., Sindbis virus (Johnson, 1965); together with lack of antigen in vascular endothelium in the cases of others, e.g., tick-borne encephalitis virus in mice Albrecht (1962) indicated that viremia invariably follows subcutaneous inoculation of virus. He states that the origin of the viremia is obscure. Some authors believe that multiplication of the virus occurs in the reticuloendothelial system, others were only able to demonstrate that virus proceeded quickly to the central nervous system with subsequent pouring of virus into the blood circulation. Still other authors were able to demonstrate that oral administration of virus caused infection of the alimentary canal with subsequent spread to the central nervous system and no involvement

of the reticuloendothelial system. The multiplicity of results obtained were explained on the basis of differences in the concentration of virus in different tissue; these variations being due to inactivation of virus by tissue enzymes during processing and titration or the presence of various volumes of viremic blood present in the different organs at the time of processing and titration. However, examination of tissue using the fluorescent-antibody technique revealed specific fluorescence in practically all neuronal cells in the brain, medulla and spinal cord after subcutaneous or oral infection of suckling mice with tick-borne encephalitis virus. Furthermore, specific fluorescence was observed in the 'ground substance' pointing to continuous release of virus from nerve cells with involvement of glial cells since bright rings of fluorescence were observed around many glial nuclei. The propagation and dissemination of virus along nerve tracts could be well demonstrated in the optic nerve. The most intensive fluorescence was observed in the peripheral nervous system comprising all the sympathetic and parasympathetic ganglia. The affinity of the virus for non-nervous tissue was also noted especially in the secretory glands where nests of fluorescent cells were observed in the parotids, naso-lacrymal glands, pancreas, in small groups of cells in the gastric and pyloric mucosa and colon, Kidneys and various endocrine glands also showed specific fluorescence. In the spleen, lymph nodes and bone marrow single or clusters of hemopoietic cells displayed ring-shaped fluorescence. Smooth and striated muscle was also shown to be involved.

It was found that in men autopsied after fatal infections due to Russian spring-summer encephalitis virus involvement of the peripheral sympathetic and parasympathetic nervous system was usual.

Further studies on the pathogenesis of tick-borne encephalitis virus in mice by Malkova (1962) and Malkova and Smetana (1966) revealed that

when tick-borne encephalitis virus was administered subcutaneously the virus was transported into the regional lymph vessels and then via these, penetrated the blood stream. A direct translocation of the virus into the blood stream was never observed. It was found that tick-borne encephalitis virus spread first to the lymphatics subsequently circulated in the lymph and blood and then invaded other organs including the central nervous system. When peak titres were observed in the brain most other organs and tissues were observed to contain virus.

In rabbits, however, which are resistant even to intracerebral inoculation with tick-borne encephalitis virus, infection was limited to the lymphatic and blood circulatory systems.

In Czechoslavakia a high incidence of human infection with tick-borne encephalitis virus was observed where some 600 people became ill due to the contamination of cows' milk with several litres of tick-borne encephalitis virus infected goats' milk. Pogodina (1962) revealed that tick-borne encephalitis virus administered orally to mice resulted in a paralytic infection. The pathogenetic pathway outlined was penetration of virus into the tissues of all parts of the gastrointestinal tract, followed by multiplication mainly in the intestine with subsequent viremia and invasion of the central nervous system.

A variety of histological and fluorescent-antibody studies indicate that infection of the central nervous system occurs by the translocation of virus across the blood-brain barrier at a variety of locations concurrent with viremia and that virus enters the brain early in the course of the infection during the rise of viremia followed by diffuse encephalitis in two to three days depending on the arbovirus concerned (Kundin et al, 1962), (Albrecht, 1962), (Johnson, 1965).

It appears that a certain level of viremia is required before virus can cross the blood-brain barrier. In the case of the Peking strain of Japanese B encephalitis virus infection in mice, viremias in the region of log $10^{1.8}$ LD_{50}/0.03 ml. were required to cause encephalitis (Huang and Wong, 1963). A comparison of the proliferation capacity of West Nile virus, tick-borne encephalitis virus (Hypr), Venezuelan equine encephalitis virus and Japanese B encephalitis virus (high and low neuroinvasive strain) in peripheral and neural tissue seems to indicate that neuroinvasiveness is invariably linked to the ability of the neuroinvasive virus to multiply in the peripheral tissue which results in high levels of viremia (Albrecht, 1968).

Olfactory Spread of Virus to the Central Nervous System

The possible mechanisms of spread of viruses across the nasal mucosa, submucosal tissue and cribriform plate are similar to those discussed for neural spread, however several significant anatomical differences exist. As with the spinal root, a cuff of arachnoid surrounding the olfactory-nerve fibre perforates the cribriform plate and dura and forms a cul-de-sac in the submucosal connective tissue. Carbon particles or labelled proteins can diffuse through this cuff and enter the lymphatic channels in the olfactory submucosa (Brierley, 1950), (Field and Brierley, 1948). The normal diffusion of cerebrospinal fluid across the olfactory area has been shown to be ten times greater than the diffusion of cerebrospinal fluid from spinal nerve roots in prevertebral lymphatics (Courtice and Simmonds, 1951). Electron microscopy of infant rabbits has shown that olfactory receptors extend into and beyond the olfactory epithelium, therefore a very direct communication of nerve fibres with external environment exists in the olfactory mucosa. Dyes dropped into the nasal cavities of rabbits have been found to reach the olfactory bulbs in one hour presumably by way of interstitial spaces of the

perineural sheath of these olfactory nerves (de Lorenzo, 1960).

Fluorescent-antibody studies in mice have confirmed the olfactory spread of viruses to the central nervous system. In Johnson's (1964) study of herpes simplex infection in mice by intranasal instillation the pattern of fluorescent cells showed two types of spread into the olfactory bulbs. Infection extended directly from nasal mucosa and submucosal tissue through the meninges into the subarchnoid space; this gave rise to widespread infection of meninges, similar to that seen after intracerebral inoculation. In other mice however, infection of the olfactory bulb was observed with no involvement of the meninges; fine lines of fluorescence were found traversing the cribriform plate and meninges in association with olfactory nerve fibres.

Aerosol infection of mice with West Nile virus, a group B arbovirus, showed no infection of the nasal mucosa by immunofluorescent techniques yet initial infection of the olfactory bulbs occurred. This suggests that virus may spread along olfactory fibres without infection of submucosal perineural and endoneural cells (Nir et al, 1965).

Airborne Virus Infections

Extensive surveys have been published on laboratory infections listing etiological agents, frequency rates for different scientific institutions, the usual laboratory techniques involved in causing infections and methods of control. (Sulkin and Pike, 1951), (Sulkin and Pike, 1951a), (Chatigny, 1961), (Phillips, 1965). A large proportion of these infections are due to the inhalation of microorganisms due to accidental creation of infectious aerosols.

A mucociliary blanket covers much of the surface of the respiratory tract. Airborne particles are deposited on the mucoid surface and ciliated epithelium carry the entrapped particles upward to the trachea and oropharynx.

Distal to the respiratory bronchioles the mucociliary blanket is absent and macrophages are implicated as playing an important role in the uptake of inhaled foreign particles in this area, carrying antigen to the pulmonary lymph nodes (Mims, 1964), (Danes et al, 1969), (Benda and Danes, 1969). Certain viruses (enteroviruses, tick-borne encephalitis virus) carried upward by the mucociliary blanket may subsequently infect the gastrointestinal tract after swallowing.

Penetration into and retention of airborne viruses in the lung is dependent on the size of the inhaled particle. Experiments conducted along this line report that penetration into the pulmonary air spaces is nearly zero for particles of 10 microns in diameter but reaches a maximum at and below 1 micron and also that the percentage deposition of particles which have penetrated to the alveolar air spaces is maximum for particles between 1 and 2 microns. It was noted, however that the probability for deposition of particles 1 micron and less decreases until particle sizes of 0.5 to 0.25 microns are reached when the probability for deposition again rises. It was pointed out that those particles in the 1 to 2 micron range which do reach the alveoli settle out by gravity whereas particles 0.5 microns and less are precipitated by forces of diffusion, the diffusion forces increasing as the particle size further diminishes (Hatch 1961), (Hatch and Gross, 1964).

The majority of the laboratory infections acquired by workers engaged in research with arboviruses appears to be due to the accidental creation of infectious aerosols. The types of accidents leading to the production of infectious aerosols are: careless use of or handling of pipettes, homogenizers, centrifuges, syringes and excretions from infected animals. Laboratory infections caused by the following arboviruses have been reported: Venezuelan equine encephalitis virus (Koprowski and Cox, 1947), St. Louis encephalitis virus (Magnus, 1950), Russian spring-summer encephalitis virus

(Haymaker et al, 1955), West Nile virus (Nir, 1959), and Kyasanur forest disease virus (Morse et al, 1962), Lassa virus (Frame et al, 1970),(Leifer et al, 1970).

The study of the pathogenesis of respiratory infections caused by viruses by the experimental inoculation of laboratory animals with aerosols of infectious viruses has provided information with regard to dose-response relationships, modes of spread of viruses, viremia, target organs involved and subsequent antibody production.

Danes et al (1962) found that the approximate inhalation lethal dose of a virus aerosol of tick-borne encephalitis virus in mice was equivalent to 10 to 40 intracranial LD_{50} and that the incubation period was the same as that following intranasal instillation of the virus; whereas the incubation period for the intraperitoneal and intravenous method of inoculation was less. In the animals exposed to infectious aerosols, virus multiplied first in the lungs where it reached a titre between 10^3 and 10^4 LD_{50}/0.03 ml. On the third day it appeared in the blood and one day later in the brain where its concentration increased until the death of the animal. High doses of virus compared to low doses tended to cause the virus to appear earlier in the various organs.

Johnson (1964) in his fluorescent-antibody studies of the pathogenesis of herpes virus encephalitis by various routes of inoculation found the following: a droplet containing 50,000 plaque forming units of virus was necessary to cause 100 per cent mortality (as compared to 3 plaque forming units for the intracerebral and 14 plaque forming units for the intraperitoneal routes of inoculation), mice remained well for four or five days then encephalitis developed with median time of death at five days; organ titrations were similar to those obtained after intraperitoneal inoculation but viremia was lower ranging from less than $10^{1.3}$ to $10^{2.9}$ plaque forming units per ml. as

compared to $10^{2.5}$ to $10^{3.7}$ plaque forming units per ml. following the intraperitoneal route of inoculation. After intracerebral inoculation however, virus was irregularly recovered from the blood, liver or spleen; virus titres rose rapidly in the brain to $10^{6.7}$ plaque forming units per gm. by the third day resulting in death of the mice. Initial rapid multiplication in the lungs was followed by hematogenous spread to the liver and spleen on the second day. Again virus was not detectable in the central nervous system until the third day after inoculation. Fluorescent foci were found in lungs, but no antigen was found in the mucosa of the stomach or intestines. Fluorescent cells were not found within the central nervous system before the fourth day. The patterns of fluorescence in the central nervous system indicated that infection of the nasal mucosa and submucosa tissues through the meninges into the subarchnoid space resulted in widespread infection of the meninges. Also, infection of olfactory nerve fibres with subsequent spread through the cribriform plate to the olfactory bulb was observed.

Nir et al (1965) in their study of the pathogenesis of West Nile virus after exposure of mice to aerosols of the virus stated that immediately after exposure, demonstrable quantities of virus were detectable in the lungs only. It was only after 48 hours that virus appeared in other organs. Fluorescent foci were not seen in most slides of liver, spleen and kidney tissue taken from 0 to 144 hours after exposure to the aerosol. On the other hand positive staining for antigen was noted in macrophages in all sections of lung tissue 24 hours after exposure. Antigen in the brain was observed at 48 hours and reached a maximum at 120 hours post-exposure. They postulate that invasion of the central nervous system does not appear to stem from early multiplication in the lung. No virus was found in cervical lymph nodes or the blood before it first appeared in the brain, which seems to rule out the hematogenous spread of virus from the lungs to the central nervous system.

Virus titrations of various parts of the brain seem to point to the invasion of the central nervous system along the olfactory pathway. Conventional and fluorescent-antibody staining of olfactory bulbs tend to substantiate the above postulate.

Henderson et al (1967) studying the pathogenesis of Semliki forest virus in hamsters indicated that virus was first detected in the olfactory lobe at eight hours post-exposure, at 14 hours in the main portion of the brain, at 26 hours in the spinal cord after aerosol exposure to 1,000 viral units (retained lung dose) of Semliki forest virus. Pathological changes were also observed in the olfactory mucosa and the liver where an acute hepatitis was produced late in the course of the disease. No damage appeared in lung epithelium. They postulate the olfactory pathway for the spread of virus to the central nervous system after aerosol inoculation of Semliki forest virus. Contact infection between infected and non-infected hamsters was seen to occur. Because fluorescent-antibody was not used in this study to reveal the presence of antigen, viral multiplication in various tissues could have been overlooked while using conventional histological techniques.

Miller (1966) studying the host-parasite relationship between Venezuelan equine encephalitis virus and white Carneau pigeons found that inhalation of virus in small particles by normal birds resulted in infections when the inhaled dose in one minute was more than 135 $MICLD_{50}$ units (mouse intracranial lethal doses 50 per cent end point). The infection was characterized by viremia of two or three days duration followed by the development of serum neutralizing antibodies. The presence of the antibodies offered protection against subsequent aerosol challenge. Virus was also found to be present in the oral cavity, however air exhausted from the cage of infected pigeons failed to cause infection in normal birds upon introduction of the potentially infectious air. Non-specific respiratory resistance factors were

invoked as the reason for failure to infect normal birds with the exhaust air of infected birds because it was noted that subcutaneously inoculated birds responded with an immediate occurrence of viremia whereas aerosolized birds showed only a partial response with a delayed onset. It was concluded that the critical factor in the induction of viremia and stimulation of neutralizing antibody formation was dose-dependent; the dose required necessarily being greater than the non-specific inactivation of the virus.

Hruskova et al (1969), found that the inhalation LD_{50} for guinea pigs to Venezuelan equine encephalitis was equivalent to 3.5 guinea pig intracerebral LD_{50} and for mice; equivalent to 135 mouse intracerebral LD_{50} or 100 guinea pig LD_{50}.

Later in a fluorescent-antibody study Hruskova et al (1969a), stated that the course of the infection after aerosol challenge was acute and the nasal mucosa could be considered as: the site of primary virus multiplication, the reservoir for viremia, and the region from which the central nervous system was infected.

Waldman et al (1970), following the antibody response in humans when exposed to aerosols of influenza virus vaccine showed that (1) the level of neutralizing antibody after aerosol immunization was about six times pre-immunization levels a year afterwards; (2) the response in patients with chronic lung disease was as good or better than normal individuals and they suffered no ill effects from aerosol procedures; (3) the antibody response in children three to ten years of age was of a lesser magnitude than those in the seven to ten age group or adults; (4) the immunoglobulin class of neutralizing antibodies was IgG in the case of serum and IgA in the case of secretions; (5) the particle size of the aerosol had a dramatic effect on the antibody response. The smaller particle sizes (1.5 micron) produced a better serum antibody (IgG) response; the larger particle sizes (40 - 100

microns) produced a better secretion antibody (IgA) response; the entire spectrum of sizes (1.5 micron) produced a better serum antibody (IgG) response; the larger particle sizes (40 - 100 microns) produced a better secretion antibody (IgA) response; the entire spectrum of sizes (1.5 to 100 microns) stimulated sputum antibody responses. The specific aims were: (1) to determine if mice could be infected with Powassan virus, a member of the Russian spring-summer complex of tick-borne group B arboviruses by a means which did not introduce the virus directly into the bloodstream; (2) to provide information on the dose-response relationships using various routes of inoculation; and (3) to determine the pathogenesis of Powassan virus in mice after airborne infection.

MATERIALS AND METHODS

Virus

Powassan virus, prototype strain LB third mouse brain passage, obtained from Dr. D. M. McLean, Department of Microbiology, University of British Columbia, Vancouver 8, Canada exhibited a titre of $10^{7.8}$ mouse intracerebral lethal doses 50 per cent end point ($MICLD_{50}$) per milliliter (ml.) when titrated using three- to four-week-old Swiss Webster mice.

Stocks of virus were prepared by inoculating groups of 50 suckling mice intracerebrally with 125 $MICLD_{50}$ in 0.02 ml. of the above virus, harvesting infected brain tissue, preparing 10 per cent homogenates and removing disrupted cellular debris by centrifugation. Stocks of the LB prototype strain of Powassan virus in its fourth mouse brain passage were stored in 0.1 ml. aliquots at $-70^{o}C$ and used for all experiments.

In order to determine the optimum time at which to harvest stocks of Powassan virus from infected suckling mice, a growth curve was constructed in the following manner: 65 suckling mice were inoculated intracerebrally with 125 $MICLD_{50}$ of the Powassan virus prototype strain LB third mouse brain

passage. At 12 hour intervals for 144 hours, five mice were sacrificed, their brain tissue removed, pooled and placed in a pre-weighed 10 to 12 ml. screw-capped sterile vial and stored at -70°C in a Revco ultra low temperature freezer (Model ULT 656 Revco Incorporated, Deerfield, Michigan). Subsequently the contents of each vial were placed in a pre-cooled mortar at 4°C and ground with a pestle. Two hundred rotary movements were executed for each sample treated. Microscopic examination of samples of the various homogenates indicated that the majority of tissue cells were disrupted after such treatment. A 10 per cent suspension in Earle's balanced salt solution (EBSS) containing 20 per cent heat inactivated (56°C for one hour) calf serum, 200 international units per ml. penicillin and 200 micrograms per ml. streptomycin, pH 7.5, was prepared for each 12 hour time period.

Cellular debris was sedimented using the Beckman preparative ultracentrifuge Model L2HV using the type 30 fixed angle rotor (Beckman Instrument Incorporated, Palo Alt, California) at 10,000 x g. for 10 min. at 4°C. The supernatant was decimally diluted in EBSS. Each dilution was examined for infectivity by the intracerebral inoculation of three- to four-week-old weaned mice using 0.03 ml. per inoculation and six mice per dilution. The $MICLD_{50}$ was calculated by the method of Reed and Muench (1938).

Preparation of Powassan virus antigens, hemagglutination, and hemagglutination-inhibition tests were performed according to the procedures described in Diagnostic Procedures for Viral and Rickettsial Infections, Hammon and Sather (1969).

Fluorescent Antibody

Hyperimmune serum to Powassan virus was prepared in the following manner: $10^{7.7}$ $MICLD_{50}$ in 5 ml. were inoculated intravenously into the marginal ear vein of a 2.7 kilogram female rabbit. The primary inoculation was followed by four similar doses given intraperitonealy spaced one week apart.

One week subsequent to the final inoculation 50 ml. of blood was removed from the marginal ear vein by piercing the vein with a sterile lancet and collecting extravasated blood in a graduated cylinder. The blood was allowed to clot, rimmed with a sterile 1.0 ml. plastic pipette, placed in a Milner refrigerator Model RSS45 (Edward Milner Co., Ltd., Toronto, Canada) at $4^{o}C$ overnight to allow for clot retraction. Serum was removed with a Pasteur pipette, placed in a cellulose nitrate centrifuge tube and centrifuged in the type 30 rotor using the Beckman L2HV preparative ultracentrifuge at 10,000 x g. for 10 minutes at $4^{o}C$.

To 20.0 ml. of antiserum was added an equal quantity of 0.15 M sodium chloride solution. A flask containing the diluted serum was held at $4^{o}C$ in the Milner refrigerator. Forty ml. of saturated ammonium sulfate was added to the diluted serum at a rate of 60 drops per minute. During, and 30 minutes after the addition of ammonium sulfate to the dilute serum the mixture was stirred using a Cenco (Central Scientific Co., Chicago) magnetic stirring apparatus. The suspension was then poured into pre-cooled cellulose nitrate centrifuge tubes and held at $4^{o}C$ for two hours. The precipitated gamma globulin suspension was then centrifuged at 10,000 x g. for 10 minutes at $4^{o}C$. The supernatant was discarded and the precipitate was washed with an additional 10 ml. of cold half-saturated ammonium sulfate and recentrifuged. The supernatant from the second centrifugation was also discarded and the precipitate was redissolved in 6.0 ml. of sterile deionized water.

The solution of immune gamma globulin was placed in a length of dialysis tubing of 0.625 inch diameter (inflated) (Fisher Scientific Co.) and dialyzed against phosphate buffered saline pH 7.2 (P.B.S.) containing 0.145 M sodium chloride 0.01 M phosphate and consisted of 8.5 g NaCl, 1.07 g Na_2HPO_4, and 0.39 g $NaH_2PO_4 \cdot 2H_2O$ per liter of deionized water. Four (100 volume) changes of the external solution over a 64 hour period was

sufficient to remove contaminating ammonium sulfate when a 10.0 ml. sample of the external solution failed to show the presence of ammonium ion after being mixed with three drops of Nessler's reagent.

The immune globulin was placed in a sterile 10 to 12 ml. screw-capped vial and stored at -20^{o}C in a Milner freezer Model RSS 45 SF until conjugated with fluorescein isothiocyanate (FITC) (Nutritional Biochemicals Corp. Cleveland).

Prior to FITC conjugation the nitrogen content of the globulin was determined using the Perkin Elmer No. 240 elemental analyzer (Perkin-Elmer, Norwalk, Connecticut), Prezioso (1965). A commercial preparation of rabbit gamma globulin COHN fraction II 95 per cent purity (Nutritional Biochemicals Corp., Cleveland) was used as a standard. The Powassan virus hyperimmune rabbit gamma globulin was conjugated with FITC according to the method outlined by Nairn (1969) and Fothergill (1969).

The removal of unreacted fluorescent material was accomplished by gel filtration with a Sephadex G-25 column in phosphate buffered saline. The dimensions of the column were 15 millimeters by 150 millimeters. The Sephadex was washed three times with phosphate buffered saline pH 7.2 to remove the 'fines.' After the third wash the gel beads were allowed to swell overnight in the refrigerator at 4^{o}C. A slurry of the G-25 Sephadex in phosphate buffered saline was added to a K15/30 chromatographic column (Pharmacia Canada Ltd., Montreal, P.Q.) until the desired bed height was reached. Excess buffer was allowed to percolate through the column. The conjugate was added to the column when the buffer became level with the bed surface; it was allowed to enter the bed completely before additional buffer was added. Collection of the eluate began when the first visible band of dye began to leave the column and was discontinued when the eluate became colorless. The eluate was approximately twice the volume of the conjugate which was added originally

to the Sephadex G-25 column. The volume of the eluate was therefore reduced to the original (6 ml.) with the aid of ultrafiltration using a Diaflo membrane XM 50, a Model 12 and Model 8MC ultrafiltration cells (Amicon Corporation, Lexington, Mass.) operated at 60 psi. One hundred milligrams (mg.) /ml. of lyophilized mouse brain powder was added to the conjugate and stirred at 23°C for two hours. The mixture was subsequently centrifuged at 10,000 x g. for 10 minutes at 4°C. The pellet was discarded and the supernatant conjugate was tested for Powassan specific antibody using the neutralization test (Nt) and hemagglutination-inhibition test (HI) as previously noted (Hammon and Sather, 1969).

Lyophilized liver and/or brain powder was prepared in the following manner: liver tissue was removed from ten healthy four-week-old mice. The tissue was washed clear of blood with phosphate buffered physiological saline pH 7.2 both before and after dicing. An equal volume of fresh cold physiological saline was added to the diced tissue and the suspension was homogenized in a high speed Waring blender for five minutes at 4°C. The homogenate was treated to three alternate cycles of washing with cold physiological saline and centrifugation at 5000 x g. for 10 minutes at 4°C. The final centrifugation was at 10,000 x g. for 10 minutes at 4°C. The finely separated tissue was resuspended for the last time in fresh volume of saline, dispensed into 2 ml. freeze-dry ampules and lyophilized using the Virtis Model 10-147-MR-BA Freeze-drier (Virtis Co., Gardiner, N.Y.)

Routes of Infection

(i) Intracerebral Inoculation

Powassan virus was assayed using the intracerebral lethal dose 50 per cent end point in three- to four-week-old mice. Decimal dilutions of virus suspensions were inoculated intracerebrally into mice using 0.03 ml. of inoculum per mouse and at least five mice per dilution. Time to death was

recorded over an observation time of ten days. The lethal dose 50 per cent (LD_{50}) was determined statistically by the method of Reed and Muench (1938). The strength of the virus suspensions therefore was recorded as mouse intracerebral lethal dose 50 per cent end point per milliliter ($MICLD_{50}$/ml.). This was defined as: the minimum amount of virus suspension required to cause death in 50 per cent of the mice inoculated by the intracerebral route using 0.03 ml. of virus suspension during an observation period of ten days.

(ii) Intranasal Instillation

Preparatory to studying the pathogenesis of Powassan virus in mice infected using an infectious aerosol, it was necessary to determine if mice could be infected by a route which did not introduce virus particles directly into the blood stream. Therefore, several mice were subjected to the intranasal instillation of various concentrations of Powassan virus under light ether anaesthesia. After it became apparent that mice could indeed be infected by this route the LD_{50} was determined. Also, a limited study of virus levels and time of appearance in various target organs was undertaken.

The procedures used were as follows: Powassan virus LB strain in its fourth mouse brain passage containing $10^{9.3}$ $MICLD_{50}$ per ml. was diluted decimally in EBSS from 10^{-1} to 10^{-8}. Using nine mice per dilution, one drop of virus suspension (0.037 ml.) was instilled into the external nares of each mouse with the aid of a calibrated Pasteur pipette.

The course of infection, over a ten day period was followed recording time to death. The mouse intranasal lethal dose 50 per cent ($MINLD_{50}$) was defined as the minimum amount of Powassan virus per ml. of suspension which caused death in 50 per cent of mice.

The time of appearance and levels of Powassan virus in various target tissues was studied in the following manner: 45 mice were inoculated with an intranasal instillation of 10 $MINLD_{50}$ of Powassan virus. Each 24

hours for seven days, five mice were sacrificed and the following tissues were pooled and examined for the presence of virus: nasal epithelium (Turbinates), lung tissue, blood, spleen and brain tissue. The lung, nasal, spleen and brain tissues were washed free of blood both before and after dicing. Ten per cent homogenates of the tissues were prepared in EBSS. Decimal dilutions were prepared of the homogenate supernatants, and these were inoculated intracerebrally in 0.03 ml. amounts into groups of six mice per dilution. Whole blood was divided into two aliquots. One aliquot was allowed to clot and the serum was retained and stored at -20^{o}C for later serological examination for the presence of Powassan virus specific antibodies. The other aliquot was lysed by freeze-thawing. Decimal dilutions of this aliquot were examined for infectivity as above.

(iii) Subcutaneous Inoculation

(iv) Intravenous Inoculation

(v) Intraperitoneal Inoculation

(vi) Per Os Inoculation

The LD_{50}'s by the above routes of inoculation were determined in a similar manner as follows: mice under light anaesthesia were inoculated with decimal dilutions of Powassan virus LB strain in its fourth mouse brain passage. This virus stock contained $10^{7.76}$ $MICLD_{50}$ per ml.

For the subcutaneous route of inoculation 0.03 ml. of decimal dilutions of Powassan virus was inoculated subcutaneously into the dorsal region of three- to four-week-old mice using nine mice per dilution.

For the intravenous route of inoculation 0.03 ml. of decimal dilutions of Powassan virus was inoculated into the lateral tail vein of three- to four-week-old mice using nine mice per dilution.

For the intraperitoneal route of inoculation 0.03 ml. of decimal dilutions of Powassan virus was inoculated through the ventral body wall and

into the peritoneal cavity of three- to four-week-old mice using nine mice per dilution.

For the per os route of inoculation 0.05 ml. of decimal dilutions of Powassan virus was instilled directly into the gastrointestinal tract using a specially prepared 24 gauge needle 1.25 inches long which had been blunted by adding a drop of brass to the end and grinding the blunted needle smooth. The needle was introduced into the mouth, down the esophagus and into the stomach. Nine mice three- to four-weeks-old were used for each dilution. The virus diluent for this experiment was sterile skimmed milk. A duplicate series of the above dilutions were inoculated intracerebrally into three- to four-week-old mice using 0.03 ml. of inoculum and six mice per dilution in order to determine if skimmed milk had any detrimental effects on the viability of the virus.

The LD_{50}'s by various routes were designated as follows: mouse subcutaneous lethal dose 50 per cent end point per ml. ($MSCLD_{50}$/ml.), mouse intravenous lethal dose 50 per cent end point per ml. ($MIVLD_{50}$/ml.), and mouse intraperitoneal lethal dose 50 per cent end point per ml. ($MIPLD_{50}$/ml.). All LD_{50}'s were determined using the method of Reed and Muench (1938) and are related to the intracerebral LD_{50} ($MICLD_{50}$/ml.) in three- to four-week-old mice.

(vii) Aerosol Inoculation

The LD_{50} by this route of infection was based on the theoretical amount of virus inhaled by exposed mice (calculated on the basis of the minute respiratory volume of three- to four-week-old mice, Guyton (1947), the concentration of virus in the aerosol, and the length of time of exposure of mice to the infectious aerosol). The actual retained dose of virus in the lungs of individual mice after aerosol exposure of groups of mice to a known concentration of virus for various periods of time was also examined.

During the first respiratory inoculation three- to four-week-old mice were exposed to aerosols of Powassan virus for two, five and ten minutes using five mice per exposure. All mice succumbed after seven or eight days even when exposed for the minimal time of two minutes.

During the second respiratory exposure the following time periods -- 15, 30, 45, 60, 75, 90, 105 and 120 seconds -- using ten mice for each exposure period were examined. Eight mice were held for ten days recording time to death. Two mice from each exposure period were sacrificed, lung tissues were removed, homogenized and pooled. Cellular debris was sedimented, decimal dilutions of the supernatants were prepared and assayed for infectivity by intracerebral inoculation of three- to four-week-old mice.

Subsequently, because recovery of virus from lung homogenates during the above exposures was too low to quantitate by the relatively insensitive LD_{50} titration method, mice were exposed to infectious aerosols of Powassan virus for more extended periods of time. Hence, mice were exposed for 5, 10 and 15 minutes using five mice per exposure. Lung tissues were removed, weighed and 10 per cent homogenates were prepared. Cellular debris was sedimented by centrifugation and the supernatants were quantitated for virus content.

Aerosol Techniques

A special facility (Figure 1) was designed in order to expose experimental animals to dynamic aerosols of Powassan virus plus study the aerosol characteristics of microorganisms while providing a maximum of protection to personnel engaged in various experimental aerosol techniques. The facility consisted of a suite of laboratories provided with a conditioned air supply which remained negative in pressure with respect to the rest of the laboratory building (i.e., all air flow was controlled so that when a breach of the aerosol suite was made by opening a door or autoclave, the air flow was directed into the aerosol suite rather than the reverse). Furthermore

all air leaving the suite passed through banks of ultraviolet lamps which emitted 1600 micro watts per square centimeter (1600 microwatts/cm^2) at the germicidal wave length of 2537 Angstrom units. Subsequently air was then directed through an air incinerator which was operated at a temperature at the bottom of a 66 foot high stack of 650^oF one foot above the flame, to 450^oF at the top. The length of time required for a particle to traverse the stack was approximately 80 seconds. The efficacy of the incinerator to take care of residual infectious aerosols was tested using Bacillus subtilis var niger spores (Davids, 1971 unpublished data).

Within the facility was located an insulated temperature and relative humidity controlled chamber. It was this chamber that housed the aerosol decay test apparatus (Figure 1, Plate 1) and also the aerosol animal exposure unit (Plate 2). All laboratories within the aerosol suite were provided with regular, emergency and ultraviolet germicidal lamps mounted in the ceiling. Each laboratory had controls for ultraviolet lamps but the regular and emergency (in case of power failure) lights were controlled by one main switchbox just outside the entry door to the aerosol suite.

Communication to all the microbiology laboratories outside the suite was provided by a vocal and warning light system so that in case of accident proper recovery procedures could be instituted immediately.

Two autoclaves were provided, one small unit, 18" x 18" x 36" which was situated between the aerosol suite and the hallway of the general microbiology laboratories. All materials requiring wash-up were autoclaved and presented through this autoclave. The other autoclave, a large model, 24" x 36" x 60" was used to autoclave all disposable material (plastic animal cages, dead infectious laboratory animals, etc.).

All doors separating the suite from the general laboratories were equipped with specially designed seals. Access and egress were accomplished through door 'A'; experimental animals and other supplies were admitted

through door 'B' and sterile disposable material was exited through this door as well.

Each laboratory within the suite was supplied with filter sterilized and humidity conditioned breathing air which was delivered to overhead bayonet-type hose connectors. Investigators, therefore, using specially designed positive pressure plastic hoods (Plate 3) were able to attach themselves by long flexible hoses to a separate sterile breathing air supply. Decontamination showers were provided and used before personnel returned to the general laboratory area. Showers were also equipped with ultraviolet germicidal lamps for shower stall decontamination.

A dressing procedure was observed when the aerosol suite was to be used. Street clothes were removed in the outside dressing room, laboratory clothes (coveralls, gown, footwear, etc.) were put on in the inside dressing room. The reverse procedure interrupted by showering was observed when leaving the suite.

Modified Horsfall-type units (Plate 4) were installed in the animal holding room. Animals which had been exposed to aerosols were placed in plastic disposable cages within the Horsfall-type units. Air entering and leaving the Horsfall-type units was filter sterilized, passed through banks of ultraviolet germicidal lamps and the air incinerator before release to outside.

All dissections and other manipulations with regard to infected animals were confined to the aerosol suite.

(i) Powassan Virus Decay Studies

Various decontamination procedures were examined for purposes of safety. The effectiveness of the ultraviolet germicidal lamps for the decontamination of accidental spills was examined in the following manner: Powassan virus containing $10^{8.0}$ $MICLD_{50}$/ml. in EBSS was maintained both in

the dark and under ultraviolet irradiation at 20 microwatts/cm^2 which was the minimum amount of radiation encountered on the floor in the aerosol suite when all the ultraviolet lamps were activated. Thin films (2 mm. deep) of Powassan virus suspensions were placed in 100 mm. petri dishes. Samples of the suspensions were taken at 30 minute intervals, under the dark condition and at 15 minute intervals under the ultraviolet radiation light condition for a total elapsed time of 180 minutes and assayed for virus infectivity using three- to four-week-old mice. A decay curve of Powassan virus under the two conditions was plotted.

The biological decay of Powassan virus at low, intermediate and high relative humidity (RH) and a constant temperature of 21°C (69.8°F) was examined using the 500 litre rotating aerosol drum (Goldberg et al, 1958).

Powassan virus suspensions containing $10^{8.0}$ $MICLD_{50}$ per ml. under controlled RH and temperature conditions were disseminated for ten minutes into the 500 litre rotating (3 - 4 rpm) stainless steel drum using a Collison atomizer operated at 26 pounds per square inch. This atomizer generates aerosol particles in the 1 to 5 micron range at the above operating pressures (Green and Lane, 1964).

Samples were taken every hour for a total elapsed time of five hours using 12/30 all glass impingers (AGI 12/30) (Brachman et al, 1964) containing 10 ml. of EBSS complete with ten per cent heat inactivated calf serum. The impingers were operated at 12.5 litres per minute and the sample time was one minute in duration. All impinger samples were transferred to 10 ml. sterile screw-capped ampules and stored at -70°C until the completion of the investigation.

Dissemination fluids (both before and after atomizer refluxing) and impinger fluids were examined for infectivity using mice.

Three experiments were completed for each RH range examined (i.e.,

20 per cent RH, 50 per cent RH, 80 per cent RH at 21°C).

The biological decay of Powassan virus aerosols in the presence of germicidal ultraviolet radiation was determined using the following techniques and equipment: Powassan virus containing 10^8 $MICLD_{50}$/ml. was dispersed into an aerosol drum using a Collison atomizer operated at 26 psi. The virus aerosols were aged within the drum for three hours. Samples of the aerosol were taken at prescribed intervals using an AGI 12/30 operated at 12.5 litres per minute for one minute. Impingers contained 10 mls. of EBSS with 10 per cent heat inactivated calf serum added. Three experiments under an ultraviolet radiation intensity of 400 microwatts/cm^2 average plus three under the dark condition were completed. Aliquots of the various dissemination and impinger fluids from related time periods for the two experimental conditions were assayed for virus infectivity in the usual manner.

(ii) Cross-Infection

Thirty mice, three- to four-weeks old, were exposed to Powassan virus aerosols and held for one day in Horsfall-type units to allow grooming practices. An equal number of marked uninfected mice were placed with the exposed group and the colony was maintained for 30 days allowing intimate contact to take place between infected and non-infected mice.

(iii) Aerosol Exposure of Mice

A restraining cage holding five mice was designed which oriented the mice in the direction of an incoming infectious aerosol (Plate 2). The infectious aerosol was generated using a Collison atomizer operated at 26 psi and was directed into a mixing tube (equipped with a water manometer so that the atmospheric over or under pressure could be controlled using a separate conditioned air supply) just prior to being admitted to the mouse exposure chamber. Residual aerosol (aerosol distal to the exposure chamber) was removed under vacuum, filtered, subjected to ultraviolet irradiation and

air incineration before being released to the outside. Investigators wore protective clothing plus the positive pressure hood.

Mice were exposed to an infectious aerosol of Powassan virus in the following manner: under light ether anaesthesia five mice at a time were exposed to an aerosol containing $10^{5.4}$ $MICLD_{50}$/litre for five minutes until 50 mice had been exposed. The following tissues were removed from four of the five mice sacrificed for each 24 hour time period post exposure for a total elapsed time of eight days for the purpose of tissue virus content determinations:

(1) nasal epithelium (Turbinates)
(2) lung tissue
(3) thymus tissue
(4) whole blood
(5) sera
(6) spleen tissue
(7) liver tissue
(8) kidney tissue
(9) gastrointestinal tract tissue
(10) striated muscle tissue
(11) cardiac muscle tissue
(12) smooth muscle tissue
(13) brain tissue

Tissues, with the exception of whole blood and serum, were removed, pooled, washed free of blood with sterile buffered physiological saline pH 7.0, weighed, diced, washed three more times with saline and homogenized using sterile, Pyrex brand glass tissue grinders (Fisher Scientific Co.) at $2^{o}C$. Ten per cent suspensions were cleared of cellular debris by centrifugation as noted earlier. Decimal dilutions of the tissue homogenate supernatants

were examined for virus by the intracerebral inoculation of mice. Blocks of the above tissues were taken from the remaining mouse and saved for microscopic examination.

Histopathology

Mouse tissues were subjected to a variety of histological techniques in an endeavour to picture overt pathology caused by Powassan virus and also to confirm the types of tissue cells in which the virus was replicating.

(i) Histological Techniques

Duplicate blocks of the various types of infected tissues were processed in order that they could be examined using fluorescent antibody (Powassan virus specific FITC conjugated rabbit gamma globulin) and/or electron microscope techniques.

(ii) Sectioning Techniques

Blocks of tissues were removed from infected mice and snap-frozen in liquid nitrogen. Tissue blocks were then placed in vials and stored at -70^{o}C until sectioned. Tissues were applied to the chuck of a SLEE (South London Electrical Equipment Co. Ltd.) Model HRM microtome cryostat using a drop of saline. With the temperature of the cryostat set at -22^{o}C, ribbons of sections were cut at six microns thickness employing the antiroll bar. Sections were picked up and placed on clean, cooled (-22^{o}C) 22 x 40 mm. coverslips using a camel hair brush. Tissue was fixed to the coverslip by applying a finger to the underside of the cold coverslip directly beneath the tissue section. Sections were air dried at 37^{o}C for one hour then stained with fluorescent-antibody. Tissue sections scheduled for hematoxylin and eosin (H and E) staining were placed directly into the stain for two minutes.

A modification of the H and E stain as described by Humason (1967) was used to stain snap-frozen sections of mouse tissue. Sections

of various tissue were treated in the following manner:

(1) Harris' hematoxylin - two minutes;

(2) wash in tap water;

(3) dip in acid-alcohol;

(4) wash in tap water;

(5) dip in Scott's solution until blue;

(6) check microscopically for proper differentiation;

(7) alcohol-eosin three to five minutes;

(8) wash in tap water;

(9) ten dips in 95 per cent ethyl alcohol;

(10) ten dips in 99 per cent ethyl alcohol (molecular sieves);

(11) ten dips in 99 per cent ethyl alcohol (molecular sieves);

(12) xylene three minutes;

(13) mount with permount.

(iii) Fluorescence Microscope Techniques

For fluorescence microscopy, sections were treated according to the following protocol:

a) Powassan virus specific FITC conjugated rabbit gamma globulin, showing specific staining (direct staining method).

b) Unconjugated Powassan virus specific rabbit gamma globulin, washed, conjugated Powassan virus specific rabbit gamma globulin showing no staining (blocking test).

c) Unconjugated non-specific gamma globulin (control globulin), washed, conjugated Powassan virus specific rabbit gamma globulin, showing specific staining (control blocking test).

d) Unconjugated Powassan virus specific rabbit antiserum, washed, conjugated goat anti-rabbit globulin, showing specific staining (indirect staining method).

e) Unconjugated non-specific rabbit antiserum, washed, conjugated goat anti-rabbit globulin showing no staining (antiserum control).

Tissues processed for fluorescence examination were viewed using a Leitz Wetzlar Ortholux fluorescence microscope equipped with an Osram HBO 200 high pressure mercury lamp after which a heat filter, UG1 ultraviolet transmission filter, a BG 38 red suppression filter plus two K430 ultraviolet suppression filters attached to an eight power periplan binocular. A D.O. 80 Leitz dark field condenser was used for fluorescence microscopy.

Sections were photographed with a Zeiss Contarex S 35 mm. camera equipped with interchangeable backs for black/white and colour photography. Kodak Plus X, ASA 125, was used for black/white photographs. Kodak high speed Ektachrome, ASA 160 was used for colour photographs. Exposure times were determined using a Zeiss Ikophot M exposure meter equipped with a microscope adapted slide-in sensor which indicated light intensities directly from the section.

H and E stained sections were observed using the same microscope equipped with a five volt tungsten light source and a Leitz No. 601 swing-out brightfield condenser.

(iv) Electron Microscope Techniques

Various types of tissue from various elapsed time periods were placed in a glass petri dish under 2 per cent glutaraldehyde in phosphate buffer pH 7.4. The tissue was sliced into cubes 1 mm. on the edge. Tissues were fixed for two hours in 2 per cent glutaraldehyde then two hours in a 1 per cent osmium tetroxide preparation. The following protocol was followed for the staining and embedding of the various tissues:

(1) tissue washed five times with 0.1 M phosphate and 0.2 M sucrose buffer pH 7.4;

(2) fixed with 1 per cent osmium tetroxide in 0.1 M phosphate 0.2 M sucrose buffer for two hours;

(3) wash five times 0.1 M phosphate, 0.2 M sucrose buffer;

(4) dehydrate in 30 per cent ethanol 10 minutes;

(5) dehydrate in 70 per cent ethanol 10 minutes;

(6) dehydrate in 80 per cent ethanol 10 minutes;

(7) dehydrate in 95 per cent ethanol 10 minutes;

(8) dehydrate in absolute ethanol (with molecular sieves) 10 minutes;

(9) dehydrate in Propylene oxide (with molecular sieves) 15 minutes;

(10) dehydrate in Propylene oxide (with molecular sieves) 15 minutes;

(11) dehydrate in Propylene oxide and Epon 812 ratio 4:1 one hour;

(12) dehydrate in Propylene oxide and Epon 812 ratio 1:1 one hour;

(13) embed in Epon 812.

The formulation of the epoxy resin was as follows:

Mixture A:	Epon 812 (Fisher Scientific)	62 ml.
	Dodecenyl succinic anhydride	100 ml.
Mixture B:	Epon 812	100 ml.
	Nadic methyl anhydride	89 ml.

The above mixtures were compounded in a ratio of 4A : 6B, 1.5 per cent DMP (2-, 4-, 6-tri dimethylaminomethyl phenol) accelerator was added to the epoxy resin.

Cubes of tissue were dried thoroughly and placed in the extreme end of beam capsules (Fisher Scientific Co.) epoxy resin was added using a 10 ml. syringe equipped with a 14 gauge needle. Capsules were placed in a 60^{o}C oven for 48 hours. Sections were cut using an LKB Ultrotome type 4801A (Stockholm, Sweden). Ribbons of sections were applied to copper electron microscope grids and post-stained with uranyl acetate (5 gms. of uranyl acetate in 100 ml.

distilled water, mix 1 ml. of the latter with 1 ml. of absolute alcohol) and lead citrate (0.04 gms. NaOH in 50 ml. distilled water, mix 25 ml. of the latter with 0.125 gms. of lead citrate). Grids were viewed using an AEI 801 high resolution electron microscope (AEI Scientific Apparatus Ltd., Harlow, Essex, England).

Photomicrographs were taken using Ilford 3¼" x 3¼" plates and LR Ilfoprint LR 4 IP photographic paper.

RESULTS

Virus

Figure 3 represents the increase in Powassan virus content in the brain tissue of three-day-old suckling mice, at 12 hour intervals after the intracerebral inoculation of 125 $MICLD_{50}$ of Powassan virus. Optimum concentrations of virus in the mouse brain occurred at between five and six days, where the titre was always approximately 10^9 $MICLD_{50}$. If tissue was titrated after the death of the animal, recovery of virus was usually less than the optimum.

Sucrose-acetone extracts of Powassan virus-infected suckling mouse brain, routinely provided Powassan virus hemagglutinin with an average reciprocal hemagglutinin titre of from 256 to 512 at optimum pH, usually 6.4

Fluorescent Antibody

Powassan virus specific rabbit gamma globulin after conjugation with FITC, purification and concentration using G25 Sephadex and Diaflo ultrafiltration respectively neutralized 10^4 $MICLD_{50}$/0.03 ml. and a reciprocal hemagglutination inhibition titre of 1024 for eight hemagglutinating doses of virus.

Routes of Infection

Table 4 reveals the minimum dose of Powassan virus per ml. required

to cause death in 50 per cent of infected animals after infection by various routes. It should be noted that the same dose of Powassan virus is required to cause infection and death in mice regardless of whether the virus is administered by the intracerebral or the intravenous route of inoculation.

Mice could not be infected by the introduction of high concentrations ($10^{6.5}$ $MICLD_{50}$) of Powassan virus directly into the gastrointestinal tract (see Plate 5), using a specially prepared needle to limit the advent of accidental tissue inoculation.

Intranasal Instillation

After it was determined that mice could be infected with Powassan virus by the intranasal instillation of suspensions of virus, various key mouse tissues were examined for levels of Powassan virus at various time intervals after inoculation.

This portion of the study was started and completed at the University. Intranasal instillation of the virus was used instead of aerosol inoculation because the University of British Columbia did not have an aerosol safe facility.

Figure 4 reveals the levels of Powassan virus in the turbinates (nasal epithelium), lung tissue, blood, spleen tissue, and brain tissue after the intranasal instillation of 10 $MINLD_{50}$ of Powassan virus.

It should be noted that 3 log of virus was recovered from the lung after inoculation whereas undetectable levels were observed in other tissues immediately after inoculation. Hence, a sizeable inoculum was introduced directly into the mouse lung. As mentioned above, high concentrations of Powassan virus introduced directly into the gastrointestinal tract of mice could not initiate an infection. This would then indicate that the mice subjected to intranasal instillation of Powassan virus suspensions became infected by way of the lung. Virus levels in the nasal epithelium began to rise

immediately; 24 hours after inoculation 2.6 log of Powassan virus appeared in this tissue.

There was a lag of between one to two days before appreciable levels of virus were detectable in the blood. On the second day 3.2 log of Powassan virus appeared in the blood. Between days two and three after inoculation, levels of Powassan virus in the spleen rose appreciably.

As soon as appreciable quantities of virus were detected in the blood, comparable levels were detected in the brain. Multiplication within the brain tissue was observed from the second day after inoculation until a maximum titre of 9.2 log was attained at seven days.

Aerosol Inoculation

The minimum dose of Powassan virus (administered by the aerosol route of inoculation) required to initiate infection and death in 50 per cent of mice exposed was found to be 640 $MICLD_{50}$. Figure 5 reveals the apparent retained dose received by mice when they were exposed to Powassan virus aerosols for periods up to 15 minutes.

Recovery of virus from lung homogenates was not possible for exposure times of less than four to five minutes. Furthermore retained dosage of Powassan virus appeared to be far lower than the calculated theoretical minimum aerosol lethal dose 50 per cent.

A 20 minute exposure was attempted, but atomizer fluid became exhausted before the 20 minute exposure time was completed.

It is interesting to note that the retained dose approximately doubles for each five minute extension of the exposure time.

Powassan Virus Decay Studies

In the interest of personal safety and also in an endeavour to learn a little about the aerosol characteristics of Powassan virus a representative of the group B arboviruses as compared with the aerosol charac-

teristics of Semliki Forest virus, a representative of the group A arboviruses which was studied earlier (Gaunt, 1968, 1969), various aspects of the biological decay of Powassan virus under a variety of conditions were examined. The results of these investigations are presented below.

Figure 6 presents data on the effect exerted on shallow (2 mm.) suspensions of Powassan virus when they are subjected to ultraviolet irradiation.

'Normal' decay of Powassan virus suspensions maintained in the dark at 21^{o}C in a balanced salt solution containing serum was approximately 1 log per ml. of suspension after three hours. Whereas Powassan virus suspensions subjected to ultraviolet irradiation at an intensity of 20 microwatts/cm.^{2} decayed rapidly so that after 90 minutes of exposure virus could not be detected in the suspensions.

The curves are derived from data obtained from three separate experiments for each condition. These data are presented numerically in Table 8.

The biological decay of Powassan virus aerosols under various conditions of relative humidity and a constant temperature is illustrated in Figure 7. The numerical presentation of these data appear in Table 9.

The physical decay of the aerosol as revealed by the Bacillus subtilus spore was essentially zero. It can be stated that Powassan virus aerosols are slightly more stable at low relative humidity than at high or intermediate relative humidities. Very little loss of viability occurs after one hour of cloud age. By far the greatest loss of viability occurs within the first hour, in fact, within the first few minutes after aerosol dissemination.

Samples of spray fluid were examined both before and after dissemination in order to determine if the refluxing action of the Collison atomizer

had a detrimental effect on the viability of the virus. On the average, spray fluids after dissemination showed slightly higher levels of virus content than spray fluids assayed before dissemination of the aerosol.

Points on the curves are in terms of mean $MICLD_{50}$ per ml. of impinger or collecting fluid. These values can be converted to $MICLD_{50}$ per litre of air by multiplying by 0.8.

Figure 8 presents the decay data in terms of relative humidities hour by hour; Figure 9 and Table 10 present data on the biological decay of Powassan virus aerosols in the dark and under ultraviolet irradiation.

Cross-Infection

Table 5 reveals the results observed when 30 non-infected mice were placed with 30 mice which had been exposed to an infectious Powassan virus aerosol. All of the mice that were subjected directly to the infectious aerosol were dead by the 11th day post-exposure. Of the 30 non-infected mice 13 succumbed to Powassan virus infection contracted because of close contact with the infected group. The non-specific deaths were due to two mice which suffocated because they became caught in the cage air exhaust port. These non-specific deaths occurred too early to determine if they had received a sufficient dose of virus to become infected.

Aerosol Exposure of Mice

Figure 10 and Table 8 illustrate the levels of virus content in various mouse tissues after aerosol inoculation. Each value shown is the mean of five separate experiments for each tissue examined. Values were within two sigma units. It should be noted that there was a two-day lag before an increase in the level of Powassan virus in the nasal epithelium was observed. This is in contrast to an immediate increase after intranasal instillation of the virus.

Increase of virus within the lungs began immediately after aerosol inoculation. It rose from undetectable levels initially to more than 5 log by the fifth day after aerosol inoculation.

A 24 hour lag period took place before appreciable viremia was

observed. At this point approximately 2- to 3-log of virus were present in the lung. After levels in the blood had risen significantly virus began to appear in other tissues. Levels of virus in the spleen lagged behind, but closely paralleled levels of virus within the blood. If they appear slightly higher it is because of characteristic pooling of blood with the tissues of the spleen.

During the course of this investigation, various aliquots of blood were examined for blood cell-associated viremia as compared to plasma-associated viremia. It was found that Powassan viremia was essentially plasma-borne and not red or white cell-associated (Mims, 1964).

Blood cells were separated from plasma, ground in diluent and inoculated into mice to provide information whether the virus was red or white cell-associated or was contained solely in the plasma portion of the blood.

Levels of virus within liver tissue were low; the time of increase approximated the rise of virus levels in the blood.

Levels of Powassan virus in thymus, kidney, skeletal, cardiac and smooth muscle tissue strongly reflected the trends of viremia.

Multiplication of virus within the brain was first detected three days after aerosol inhalation. This was one day after viremia became manifest. Levels of virus within the brain far exceeded levels in all other tissues, and attained about 9 log five days or more after aerosol inoculation.

Histopathology

Thin sections of various tissues were stained with H and E for use with light microscope techniques, FITC, conjugated Powassan virus specific rabbit gamma globulin for use with fluorescence microscope techniques and osmium tetroxide followed by post-staining with uranyl acetate and lead citrate for use with electron microscope techniques.

Plate 6 shows an H and E stained section of an uninfected mouse lung. Plates 7 and 8 show H and E stained sections of mouse lungs five days after aerosol inoculation with Powassan virus. Plates 6 and 7 are magnified 160 x; Plate 8, 320 x.

In infected lung, alveolar walls were swollen with accumulation of macrophages and capillaries were dilated (Plates 7 and 8). The walls were thickened in contrast to uninfected lung (Plate 6). Thus infected lung showed patches of bronchopneumonic consolidation.

Within the cerebellum, virus infected tissues showed infiltration with macrophages, especially surrounding degenerating neurones (Plates 10, 11) in contradistinction to the lack of infiltration in uninfected tissues (Plate 9).

Within the cerebrum (Plates 12, 13, 14) widespread neuronal degeneration accompanied by macrophage accumulation was observed in subcortical areas and adjacent to cleavage planes.

Plates 15 and 16 are cross-sections of Powassan virus infected mouse lung stained with fluorescent-antibody three and five days after aerosol inoculation. This was an important finding and is probably the manner in which Powassan virus invades the blood stream.

Plates 17 to 20 are fluorescent-antibody stained sections of Powassan virus infected mouse brain three days after aerosol inoculation showing examples of the cuboidal epithelium of the choroid plexus. These cells show Powassan virus specific fluorescence and probably represent the means by which Powassan virus crosses the blood-brain barrier to enter the cerebrospinal fluid and thus promote its dissemination through the central nervous system.

Plate 21 shows Powassan virus specific fluorescence in cerebral cortex cells adjacent to blood vessels in the arachnoid layer of the meninges just beneath the skull three days after aerosol inoculation. However, no immunofluorescence was demonstrated in the olfactory bulbs or tracts before it was observed elsewhere in the brain.

Plate 22 is a sagittal section of infected mouse brain showing widespread Powassan virus specific fluorescence in cells of the cerebral cortex six days after aerosol inoculation.

Plate 23 is an electron micrograph of what is believed to be a cell of the reticuloendothelial system in the mouse lung three days after aerosol inoculation with Powassan virus. Attention is called to structures which resemble phagolysosomes structures known to be active in phagocytic cells in the presence of foreign material.

Plates 24, 25, and 26 are electron micrographs of nasal epithelium three days after intranasal instillation of Powassan virus. Virus specific structures were observed in infected epithelium but never in the uninfected epithelium that was examined.

DISCUSSION

The pathogenecity of Powassan virus for the suckling mouse was always more rapid and intense than in the adult. Virus titres reached a peak in six days in the suckling mouse, compared to eight days in the adult. The hind limbs of infected mice with rare exception became paralyzed. The paralysis ascended to the thoracic region of the mouse which induced a 'hunched back' appearance before death. Midway in the infection mice were hyperesthetic to light, sound and touch. This slowly diminished until the infected mouse became insensitive to several stimuli. The above sequence was followed by coma and death.

Because a reliable tissue culture plaque assay method (Cooper, 1967) for Powassan virus has not as yet been developed, the assessment of the virus content of Powassan virus suspensions was based on the sigmoid dose-response curve. Since an absolute dosage (one that will just affect all entities tested) cannot be measured, the 50 percentile, or the dosage

causing an effect (in the case of Powassan virus lethality in mice) in 50 per cent of entities tested was used.

These data were analyzed by the statistical method of Reed and Muench (1938). Brown (1964) in a paper on variance estimation using the Reed and Muench 50 per cent end point determination stated that if the number of susceptible animals used for the determination was constant, and greater than five for each dilution examined for virus content, the two sigma limits computed are comparable with those obtained using other procedures in which estimates of sample variation accompany the procedure (Bliss, 1938), (Wilson and Worcester, 1943), (Miller and Tainter, 1944), (Finney, 1947), (Litchfield and Wilcoxan, 1949), (Finney, 1952).

Nairn (1969) recommended negative pressure dialysis for the concentration and purification of FITC conjugated gamma globulin after G-25 Sephadex filtration. It was found during this study that Diaflo ultrafiltration techniques using XM50 membranes (retains all molecules with a molecular weight greater than 50,000) were superior for the purification and concentration of conjugated globulins. No chance rupture of dialysis tubing and the consequent loss of sample can occur using the Diaflo technique.

Lyophilized uninfected suckling mouse brain was used for adsorbing non-virus antibody from FITC conjugated gamma globulin. Liver preparations were found generally to contain unacceptably high concentrations of hemoglobin. The hemoglobin tended to mask fluorescence on fluorescent-antibody treated tissue sections.

Determination of the minimum lethal dose by various routes of inoculation revealed that the intracerebral and intravenous routes require the same dose. Mims (1960), who carried out experiments on intracerebral injections in mice, stated that the pressures exerted during routine intracerebral injections ranged from 200 to 300 cm. of water or 20 to 30 times

normal cerebrospinal fluid pressure. It would not be surprising if anatomical barriers were broken under such high injection pressures. Mims believed that the first barriers to break down as the pressure rose were the arachnoid villi.

The large endocranial venous sinuses are entirely enclosed by thick walls of dura matter except in definite places, chiefly in the sagittal sinus of the falx (the process of dura which intervenes between the cerebral hemispheres) where the dura is perforated by numerous protrusions of the arachnoid membrane, through each of which a finger-like evagination of the arachnoid mesothelium is thrust into the lumen of the sinus. This is an archnoid villus. Its cavity, which contains a small amount of loose arachnoid tissue is in free communication with the subarachnoid spaces, so that here the fluid of these spaces is separated from the blood of the sinus only by a thin mesothelial membrane.

The arachnoid villi provide the main pathway for the outflow of cerebrospinal fluid directly into the venous circulation; once they have been 'blown open' there is a free flow of injected material into the blood stream and from this point on the injection becomes an intravenous one.

The differences in lethal dose that were observed between the subcutaneous and the intraperitoneal routes of inoculation can probably be ascribed to the differences in structure, phagocytic index, motility, enzyme content of lysosomes, oxygen requirements and other metabolic and biochemical characteristics of macrophages in the subcutaneous tissues as compared to the peritoneal cavity of the mouse (Johnson, 1964), (Fenner, 1968), Nelson, 1969).

As mentioned mice could not be infected by the instillation of high concentrations of Powassan virus into the stomach. This event is in strong contrast to the fact that a related virus tick-borne encephalitis virus can

cause a fatal infection in mice when introduced into the gastrointestinal tract (Pogodina, 1962), (Moritsch and Kovack, 1962). Tick-borne encephalitis virus penetrates and replicates in the intestine followed by viremia with invasion and multiplication in the central nervous system.

In the case of Powassan virus the gut cells either do not possess receptor sites for the virus or it becomes inactivated by stomach acids or the active ingredients of bile (sodium deoxycholate) (Theiler, 1957) within the intestine.

When mice were subjected to Powassan virus infection by the intranasal instillation of virus they contracted a fulminating infection and died within eight days.

Because later studies indicated that mice could not be infected by the instillation of large concentrations of Powassan virus directly into the lumen of the gastrointestinal tract it must be assumed that the presence of virus in the fluid which bathed the nasopharynx and entered the lung caused the infection. The fact that virus containing fluid entered the lung is proven by the presence at zero time of approximately 3 log of virus. Subsequently, the Powassan virus titre in the lung tissue exceeded 6 log.

A drop of virus-containing fluid was placed on the external nares of lightly anaesthetized mice. Upon breathing the fluid was aspirated directly into the lungs; also the nasopharynx was bathed in the infectious virus suspension. Nasal epithelium became infected and the important observation was that the virus began to multiply immediately with no intervening lag period.

Viremia became measurable after a lag period of from one to two days after inoculation. The infected tissues contributing to this viremia were with little doubt the epithelial tissues of the nasopharynx and lungs. After a similar lag period virus appeared in the central nervous system and

multiplied to a high level.

Virus levels in the spleen reflect the time sequence of viremia and are slightly higher because of the characteristic pooling of blood within splenic tissue.

The apparently large virus dose required to initiate infection and cause death in mice inoculated by the intranasal instillation of Powassan virus was probably due to the ability of the ciliated epithelium of the tracheobronchial tree to remove a considerable quantity of the inoculum to the region of the oropharynx where it could be expectorated or swallowed. In the stomach of course it was shown that the virus in the inoculum was inactivated. The absence of immunofluorescence in the olfactory bulb neurones before it was demonstrated elsewhere in the brain suggests that infection of the central nervous system did not result from entry of virus through the olfactory plate.

The mechanism of upper respiratory tract clearance (exclusive of nasal passages) is dependent upon proximal movement of a blanket of mucinous fluid which is maintained by the ciliary activity of columnar epithelium lining the tracheobronchial mucus membrane. This ciliated epithelium extends from the inferior aspect of the epiglottis down to the terminal bronchiole. Not all these cells, of course are ciliated. In the rat the ratio of non-ciliated (goblet cells) to ciliated cells is 3:5. The beat frequency of the cilia is about 1300 per minute. This activity results in the propulsion of the overyling mucinous fluid at an average rate of approximately 13.5 mm. per minute (Hatch and Gross, 1964).

The calculated lethal dose 50 per cent by the aerosol route of inoculation was 640 $MICLD_{50}$. This is probably an over-estimate of the actual retained dose of Powassan virus when mice were subjected to infectious aerosols. The 640 $MICLD_{50}$ assumes that all the aerosol that was inhaled by the mouse in a prescribed time was retained. It should be noted that an experimental animal both inhales and swallows microbes as a result of respiratory exporuse. Goldberg and Leif (1950) exposed mice to a test aerosol

of P32-labelled Pasteurella pestis and found that whereas the total retention was over 80 per cent of the calculated dose per mouse only 30 per cent of the retained material (i.e., 24 per cent of the total) was in the respiratory tree and 70 per cent was in the gastrointestinal tract.

Between species, lung volume is proportional to body weight, whereas alveolar surface area correlates linearly and directly with metabolic activity (Tenney and Remmers, 1963). Two animals of equal body size will have lungs of the same volume, but if one has a higher rate of metabolism its alveoli will be smaller. Particle size is therefore an extremely critical factor for respiratory penetration and retention in laboratory animals. With a homogenous aerosol of 1 to 2 micron sized particles, average respiratory retention is 15 per cent in the mouse (Dimmick and Ackers, 1969), (Danes et al, 1962). If this was applied to the theoretical LD_{50} by an aerosol it would amount to about 96 $MICLD_{50}$ or approximately equivalent to the LD_{50} by way of intraperitoneal inoculation.

Because the calculated LD_{50} by aerosol inoculation was determined to have been received after 22 seconds of exposure to the aerosol, the Powassan virus retention curve appears to far underestimate the actual dose retained after five minutes of exposure to the aerosol.

It would of course be impossible to recover the total number of retained $MICLD_{50}$ from exposed lungs. During the process of tissue grinding released enzymes would probably inactivate some of the virus, thermal and light inactivation would also take its toll; also possibly a large proportion of virus would be adsorbed to disrupted lung tissue and would be sedimented with unwanted cellular debris during centrifugation (Albrecht, 1962), (Appleyard, 1967).

In some of the longer duration exposures much of the virus could have entered cells to exist in the eclipse phase of replication.

The curve does show, however, that the longer the mouse is held in the aerosol the greater the degree of retention. The dose-retention curve may more closely approximate the actual happenings.

With the reciprocating pattern of air flow into and out of the respiratory system, only a fraction of each tidal volume reaches the pulmonary air spaces; the rest fills the nasopharyngeal chamber and airways of the upper respiratory tract. Clearly no more aerosol can be carried to the pulmonary air spaces than is contained in the fraction of new air. It has also been shown that only limited mixing takes place from breath to breath and this fact has interesting implications with respect to depth of penetration into and site of deposition of aerosol particles in the pulmonary air spaces. Since the bulk of new air does not mix volumetrically with lung air it follows that non-diffusible particles (greater than 0.5 micron) will penetrate only as far as the new air goes. Sub-micron (i.e., less than 0.5 micron) having significant diffusion velocities will to some degree move independently into the static lung air just as do gas molecules.

There can be significant differences with aerosol particle size in the site of deposition within the ultimate pulmonary unit. The coarser particles will be deposited in greater degree high up in the unit (along respiratory bronchioles and alveolar ducts); whereas the sub-micron particles will have a relatively greater probability of being deposited in the alveolar sacs and alveoli (Hatch and Gross, 1964).

Powassan virus either in suspensions or in aerosols proved to be highly sensitive to inactivation by ultraviolet light. Appleyard (1967) found that Semliki Forest virus and to a lesser extent Sindbis virus, two group A viruses, Murray Valley encephalitis virus, a group B virus, influenza type A and rabbit pox were sensitive to natural or artificial daylight. The active wave lengths were in the region of from 3300 to 4700 Angstrom units.

Kleczkowski (1957), Gard and Maaloe (1959), Kleczkowski (1968) described action spectra as curves obtained by plotting wave lengths of radiations against their relative efficiencies in causing inactivation. Coincidence between a maximum of the action spectrum and a maximum of the absorption spectrum of some component of the virus may be considered as an indication that the component might be concerned in the mechanism of inactivation.

Action spectra of vaccinia virus, of a few bacteriophages and of influenza A all resemble the absorption spectrum of nucleic acid. It could be concluded therefore that the nucleic acid components of the viruses are involved in the mechanism of inactivation of these viruses by ultraviolet irradiation.

It was found that two peaks in the action spectrum of a Megatherium bacteriophage a broad and prominent one at around 2600 Angstrom units corresponding with the maximum adsorption for nucleic acids, and a smaller one at 2800 Angstrom units corresponding with tyrosine and tryptophan residues of protein. There was also a sharp rise of the action spectrum curve as the wave length fell below 2300 Angstrom units where adsorption by aliphatic amino acids and the peptide bond became pronounced.

It was also revealed that a wave length of 2652 Angstrom units was the most efficient in inactivating the ability of influenza virus to infect and multiply, whereas that of 2803 Angstrom units was the most efficient in inactivating its hemagglutinating ability. The nucleic acid component of the virus therefore may be primarily concerned with loss of ability to infect and multiply, whereas the protein component may be concerned with loss of the ability to hemagglutinate.

Powassan virus aerosols were slightly more stable at low relative humidity than at intermediate and/or high relative humidities. Similar observations were made when Semliki Forest virus aerosols were examined for bio-

logical decay with time (Gaunt, 1968, 1969). Semliki Forest virus, however, was slightly more sensitive to biological decay at 80 per cent relative humidity than at 50 per cent relative humidity which was demonstrated with Powassan virus.

Harper (1961) examining the airborne survival of vaccinia, influenza, Venezuelan equine encephalomyelitis and poliomyelitis viruses, found better survival at low relative humidities for vaccinia, influenza and Venezuelan equine encephalomyelitis; on the other hand, poliovirus survived better at high relative humidity.

Webb et al, (1963) studied the effect of relative humidity on aerosols of pigeon pox virus and Rous Sarcoma virus in the presence and absence of inositol. Pigeon pox virus was found to be stable in aerosols and was little affected by changes in relative humidity. Rous Sarcoma virus however, was extremely sensitive to relative humidity. Webb has suggested that the death of airborne bacteria is a direct result of loss of water molecules bound to cell nucleoproteins and that the action of protective compounds, such as inositol, results from their ability to take over the role of bound water in maintaining the vital structure of these cell components. When Rous Sarcoma virus was aerosolized from a six per cent solution of i-inositol the recovery of virus at 30 per cent relative humidity increased from 1 per cent to 90 per cent. This protective ability of inositol was apparent at all relative humidity values below 70 per cent (values above which Rous Sarcoma virus was most stable).

Dejong and Winkler (1968) working with aerosols of poliovirus stated that an organism sprayed in air is exposed to (1) the stress of spraying, (2) quick evaporation until the droplets are in equilibrium with with ambient air, and (3) to the decay in the stable aerosol during storage. They also said that the mechanisms of inactivation during these three

phases might differ.

Their data revealed that the spray loss was large at low relative humidity and decreased with rising humidity, being minimum at 55 per cent relative humidity. The storage loss rate was low below 35 per cent relative humidity and above 70 per cent relative humidity, but high between 40 and 55 per cent relative humidity. The range of maximal storage loss rate coincides with the range of decrease of the spray loss.

In order to determine the rate of oxidation, poliovirus was aerosolized in the presence of nitrogen and in the presence of oxygen. As well as following the biological decay of the intact poliovirus particle, infectious ribonucleic acid was also extracted from samples of virus taken at different time intervals during aerosol storage.

The observations of Dejong and Winkler were that: (1) the ribonucleic acid in the virus decays simultaneously with the whole virus particle, (2) oxidation does not play a significant role, (3) L-cystine which stabilizes aqueous solutions of poliovirus against thermal inactivation at $50^{o}C$ by reacting with SH-groups and stabilizing structure, does not protect poliovirus aerosols from decay.

On the strength of these observations it was concluded that in aerosols denaturation of viral ribonucleic acid is the cause of the inactivation of poliovirus.

Like Powassan virus, Benbough (1971) showed that Langat virus, a member of the group B tick-borne arboviruses, did not survive as well in aerosols held at low, intermediate and high relative humidites when the spray fluid contained salts. He later found survival was appreciably better for Langat virus when the virus was nebulized from desalted solutions.

Benbough believes that the susceptibility of viruses in aerosols to the environmental conditions must be related to the partition of bound and unbound water between virus, other constituents of the aerosol particle containing the virus, and the surrounding atmosphere. He maintained that following aerosolization there is a period of equilibration during which the concentration of solutes in the aerosol particle increases to levels which may be toxic to the virus.

Benbough noted little effect on the biological decay of poliovirus regardless of whether the virus was aerosolized from salted or desalted solutions. He postulated that the inactivation of arboviruses nebulized from fluids containing salts could be due to the dissolution of the lipoproteins which are present in the virus. This breakdown of lipoproteins of viruses in aerosols may be caused by chloride ions displacing bound water in membrane systems and that arboviruses having variable amounts of lipoprotein content could be expected to have different susceptibilities to salts in aerosols.

My interest was in determining the decay of Powassan virus in the types of fluid suspensions in which the virus is ordinarily handled and in the life of the virus when aerosols either accidentally or intentionally were created from such suspensions.

In the cross-infection study aerosol exposed mice were placed in a separate cage for 24 hours so that they could complete characteristic grooming practices which were observed to occur after full body exposure of mice to infectious aerosols of Powassan virus. The grooming, both individually and collectively involved the licking of fur plus the licking of front feet in combination with a washing action. These grooming practices sometimes continued for several hours after aerosol exposure. It is an educated guess that any virus adhering to fur would be removed by these

practices or would become inactivated naturally within 24 hours.

After 24 hours uninfected mice and infected mice were placed together in a new, clean cage which was fed with a filtered, humidified air supply. Mice were maintained for 30 days.

This limited study indicated that uninfected mice can become cross-infected by mice who have been exposed and become infected by aerosols of Powassan virus. The possibility is fairly high that mice became infected by nose-to-nose contact; that is the accidental inhalation by an uninfected mouse of particles produced by an aerosol infected mouse upon snuffling or sneezing. The other possibilities are that mice become infected by the inhalation of aerosols of urine or feces. This is not as likely because mice were removed regularly to clean cages with care being exercised so that accidental aerosols with regard to feces or urine were not created. Also any urine was soon adsorbed by clean new shavings and it was noted that feces from mice seldom became powdery (because of controlled humidity), a situation which would be conducive to the creation of infectious dusts.

Aerosol infected mice succumbed eight to ten days after exposure. Cross-infected mice succumbed 10 to 14 days after that; indicating possibly that uninfected mice received relatively small doses of virus from their aerosol infected counterparts which eventually multiplied to significant levels in the lungs and subsequently caused their deaths 10 to 14 days later.

These experiments provide a model of a situation whereby virus may be transferred to a close human contact by a person who has become infected accidentally by inhalation of an aerosol.

Druett et al (1956) found that there was a positive correlation between the number of deaths in control animals and the number of dead or dying experimentally infected animals with which the controls were in contact. The size of particles infecting experimental animals also influenced the cross-infection rate. Deaths in control animals were approximately four times greater after contact with animals exposed to particles containing

only one cell than after contact with animals previously exposed to multicellular particles of 12 microns in diameter.

The above of course can be correlated with deposition of large aerosol particles in the upper respiratory tract and clearance by mucociliary mechanism.

It was noticed after exposure of mice to aerosols of Powassan virus that growth within the turbinates lagged, for from two to three days before a noticeable increase in virus was observed. At this point in time levels in the blood were between 3 and 4 log. This suggests that the infection of the nasal epithelium was a result of viremia rather than direct contact between nasal epithelium and the infectious aerosol. Similarly, the central nervous system was not infected by transfer of virus through the olfactory plates.

The Collison atomizer produces particles in the 1 to 5 micron size range. Particles of this size penetrate deeply into the lung. Particles in the 10 micron range and above are usually trapped in the nasopharynx (Hatch and Gross, 1964). When virus was instilled into the external nares growth in the nasal epithelium began immediately. This indicates that under the conditions of nasal instillation of virus, the epithelium which was bathed in the inoculum became infected directly rather than by way of viremic blood.

Multiplication of virus within the lung after aerosol inoculation began immediately and levels rose from undetectable amounts to greater than 5 log in five days. This was in contrast to mice which were infected by the intranasal instillation of virus where 3 log was recovered directly from the lung tissue immediately after inoculation.

The rise of virus in the thymus tissue may be as a result of multiplication of virus within this tissue or within many of the thoracic lymph nodes which are closely associated with the thymus gland and which were probably removed with the gland when it was separated from tissues of the lungs,

heart and large vessels. Alternatively the virus titre may simply reflect the concentration of virus in the blood which was incompletely removed during processing of tissue.

Bloom and Fawcett (1968) report that in the thymic reticulum, macrophages are located especially in the vicinity of blood vessels. These macrophages could engulf virus particles and initiate an infection in thymus tissue. The blood supply to the thymus (in man) is from arteries which arise from the internal mammary and the inferior thyroid veins. The lymphatics run mainly in interlocular connective tissue and empty into anterior mediastinal and trachiobronchial lymph nodes (nodes closely associated with thymus tissue). Blood supply and drainage in the mouse would be comparable. Infectious blood would soon reach thymus tissue from the site of initial mutliplication -- the lung. Because the thymus gland is intimately involved in the development of immunological competence in young mice (Blood and Fawcett, 1968) contact with bacteria and viruses would seem to be a necessity; therefore it is not unreasonable that the tissues of the thymus gland and lymph nodes became infected with Powassan virus and was instrumental in keeping levels of virus within the blood high. Levels of Powassan virus in the liver were low. It is known (Thieler, 1957), (Albrecht, 1962) that arboviruses are highly susceptible to bile salts (sodium deoxycholate) or possibly destructive enzymes released during tissue grinding when extracting Powassan virus from tissue.

The gastrointestinal tract could not be infected by instillation of Powassan virus directly. After aerosol infection of mice the level of Powassan virus in gut tissue was followed; minimal levels were detected solely on the fifth day.

Rising levels of virus in the kidney tissue could mean that multiplication took place in this organ. The increase closely follows the time sequence for rising levels in nasal epithelium and thymus gland tissue.

Levels of virus in striated muscle and cardiac muscle reflect viremia. Total removal of viremic blood from these tissues would be impossible by the methods that were used (fine dicing with successive washings). A more complete removal of blood from various tissues can be accomplished by perfusion of the whole animal prior to removal and processing of infected tissues. Determinations of hemoglobin in tissues after whole body perfusion experiments have been as low as 10 per cent of the original hemoglobin and as high as 50 per cent (Albrecht, 1962).

Of interest is the increase of levels of virus in smooth muscle. The representative tissue used for smooth muscle was the urinary bladder of the mouse. Levels of virus in this tissue could stem from multiplication in the smooth muscle tissue of the urinary bladder or the epithelial layer of cells forming the mucosal surface of the bladder. The submucosa, the layer of connective tissue interposed between the lamina propria of the mucosa and the outer band of smooth muscle making up the bladder is highly interspersed with blood vessels (Bloom and Fawcett, 1968). Viremic blood could initiate an infection in this tissue. Alternatively, infection of the epithelial cells of the kidney could have taken place by the passage of viremic blood through the glomeruli of the kidney.

The capsule of Bowman develops around a tuft of glomerular capillaries as a double walled cup composed of squamous epithelium. The wall closely applied to the glomerulus is the visceral layer (glomerular epithelium) the outer wall, the parietal layer (capsular epithelium) and the slit-like cavity between them is the capsular space (Bowman's space).

At the vascular pole of the renal corpuscle, the visceral layer is reflected off the glomerular vessels to become continuous with the squamous epithelium of the parietal layer. At the urinary pole, the capsular epithelium is continuous with the cuboidal epithelium in the neck of the proximal

convoluted tubule.

The visceral layer becomes extensively modified; it bears filtration slits about 250 Angstrom units wide, while glomerular endothelium is perforated by pores 500 to 1000 Angstrom units in diameter (Bloom and Fawcett, 1968).

It is understandable that kidney epithelium would be relatively easy to infect under these conditions from an anatomical basis alone. It is known also that Powassan virus will adsorb to and multiply within a variety of renal epithelial tissue (Abdelwahab et al, 1964), (Taylor, 1967).

It is also reasonable that after virus has multiplied within the renal epithelium upon release from infected cells virus could be carried by the ureters to the urinary bladder, the mucosal surface of which would be open to infection.

It was observed that when levels of virus within the kidney increased substantially on the fourth to fifth day, a sudden rise in virus levels within bladder tissue was observed between four and seven days.

During the course of examination of infected tissues using a variety of histological techniques, it was difficult to recognize any overt pathological changes in sections of the various tissues examined with the exception of the brain using the H and E method of staining.

After the infection of mice with aerosols containing Powassan virus, titration of Powassan virus levels in various tissues plus the visualization of Powassan virus specific foci first in cross-sections of mouse lung would seem to implicate the mouse lung as the primary site of viral invasion and multiplication.

In concert with the rise of Powassan virus in the blood stream, fluorescent-antibody stained sagittal sections of mouse brain three days after aerosol exposure showed Powassan virus specific fluorescence in cuboidal epi-

thelium of the choroid plexus.

There are four places where the wall of the brain retains its embryonic character as a thin non-nervous epithelium. This part of the brain wall is the lamina epithelialis. The pia mater which covers it is extremely vascular and otherwise modified to form a choroid plexus. The lamina epithelialis is closely joined to the choroid plexus and the whole is called the tela choroidea, or less exactly, choroid plexus.

These choroid plexuses are found in the roof of the third and fourth ventricles and in a part of the wall of the two lateral ventricles. In each case the tela choroidea is much folded and invaginated into the ventricle so that the free surface exposed to the ventricular fluid is large with tortuous vessels and a rich capillary net.

In animals repeatedly injected intravenously with vital dyes, such as trypan blue, the epithelium of the choroid plexus stores large amounts of the dye. Also in the perivascular connective tissue core of the plexus are many fixed macrophages, which store large amounts of the dye (Bloom and Fawcett, 1968).

On the boundary between adjacent epithelial cells in electron micrographs, there is a juxtaluminal functional complex that appears to seal the intercellular space. The capillaries beneath the epithelium are unlike those elsewhere in the brain, in that they are thin walled and have fenestrations or pores closed by thin diaphragms. The junctions between endothelial cells also appear to be more permeable.

A protein extracted from horse radish (peroxidase) when injected into the femoral vein of mice was demonstrable within the choroid plexus. Peroxidase was pinocytosed by the fenestrated endothelium but apparently did not penetrate the fenestral diaphragms. It is as yet uncertain whether endothelial cell junctions (Albrecht, 1968) completely excluded this protein, but

enough crossed the endothelium, presumably by vesicular transport to penetrate the perivascular basement membrane. Peroxidase then moved between epithelial cells until stopped by conical structures of the interspace near the ventricular surface (Brightman, 1967).

Smaller particles, such as ferritin, can be traced shortly after intravenous injections, as they make their way through the system of endothelial vesicles and through the basement membrane. After injecting large doses of carbon into rats and mice, it was found in the fenestrated endothelium in many organs. However, the concentration of carbon was never high, presumably because of the competition of the reticuloendothelial system in clearing carbon particles. In later experiments perfusion of an organ with colloid solutions in order to minimize the effects caused by the reticuloendothelial system, uptake of particulate matter by the endothelium could be much more easily demonstrated. Impounding of large droplets even measuring several microns by the endothelial cells of large vessels and capillaries of the brain was regularly observed. This might explain how large molecules the size of viruses and rickettsia are taken up by endothelial cells. Phagocytosis in a broad sense is a common phenomenon in endothelial cells. However, the phenomenon is different from that performed for example by Kupffer cells, because it leads primarily to transport across the cell rather than to intracellular storage and digestion (Albrecht, 1968).

Fluorescence was not detected in the vascular endothelium during these studies. However, if the virus was merely passed through these cells it is not likely that they would show specific fluorescence. Other arboviruses which cause central nervous system disease where infection of the vascular endothelium could not be demonstrated are Japanese B encephalitis virus, tick-borne encephalitis virus, Semliki Forest virus and dengue virus (Albrecht, 1968).

Nir et al (1965) exposed mice to aerosols of West Nile virus (also a member of the group B arboviruses). Their results in one experiment show that West Nile virus infectivity appeared in the lung at day zero and was at its height at 72 hours at 4.5 log. It appeared first in the blood at 48 hours at 0.2 log, in the brain at 48 hours at 0.1 log and in the nasal epithelium at 72 hours at 0.1 log.

These findings are similar to the Powassan virus studies contained in this thesis. That is, primary site of multiplication in the lung resulting in a viremia in 24 hours followed by the presence of the virus in other tissues at 48 hours.

In a second experiment, Nir et al described a situation where 2.6 log of West Nile virus appeared in the olfactory bulb of the brain at 36 hours after inoculation and attained a level of 4.5 log 48 hours after inoculation and in the cerebellum at less than 1 log 48 hours after inoculation. Unfortunately no record of viremia or the appearance of virus in other tissues was included. Judging from past experience, the levels of virus and the time of appearance as presented in the second experiment would indicate that the mice had received a substantially higher dose of virus than they did in the first experiment. This would have the effect of advancing the sequence of events and amplifying the levels of virus titrated in each tissue (Danes et al, 1962).

It is also interesting that in the first experiment West Nile virus was present in the brain 24 hours before it was observed in the nasal epithelium. Also that under the conditions of the second experiment, 4- to 6 log was present in the brain at 48 hours, whereas in the first experiment only 0.1 log was present in the brain at 48 hours. Nir et al concluded that West Nile virus invades the central nervous system of the mouse through infection of the nerves of the olfactory mucosa.

The pathogenesis of Powassan virus in mice after airborne infection occurred in the following sequence: infectious virus particles were aspirated into the lung, invaded and multiplied within the tissues of this organ, establishing a viremia 24 hours after inoculation. When the level of Powassan virus within the blood attained a threshold titre of approximately 3 logs (Japanese B encephalitis 1.8 log per 0.03 ml. in mice, Huang and Wong, 1963), infectivity appeared in other tissues including the brain.

Fluorescent antibody studies indicate that the epithelial cells of the choroid plexus may be implicated in the spread of the virus to the central nervous system. Although fluorescence in vascular endothelium was not observed, (". . . the level of multiplication of virus within vascular endothelium may be so low as to escape detection by the not-very-sensitive fluorescent-antibody technique . . ." Albrecht, 1968), passage of virus by way of the vascular endothelium may also be of importance in the infection of the central nervous system.

Studies would indicate that the virus is capable of multiplying within a variety of tissues, notably tissues which have a high epithelial cell content. Powassan virus multiplies within the brain of the mouse to extremely high levels eventually causing death of the mouse. Fluorescent-antibody studies indicate wide involvement of tissues of the lung and brain, with less specific fluorescence being seen in other organs of the mouse.

Electron micrographs reveal the presence of virus specific structures in macrophages of the lung and in the nasal epithelial cells of mice infected by intranasal instillation of virus. (Nelson, 1969a)

These structures are reminiscent of similar structures observed by Grimley et al (1968), Tan (1970), who studied the morphogenesis of Semliki Forest virus and Venezuelan equine encephalomyelitis virus (Garcia-Tamayo, 1971), both representatives of the group A arboviruses.

TABLE 1

ANTIGENIC GROUPS OF ARBOVIRUSES**

GROUP	ABBREVIATION
A	A
B	B
C*	C
African horse sickness	AHS
Anopheles A	ANA
Bakau	BAK
Bluetongue	BLU
Bunyamwera*	BUN
Bwamba*	BWA
California*	CAL
Capim*	CAP
Changuinola	CGL
Epizootic hemorrhagic disease of deer	EHD
Guama*	GMA
Koongol*	KOO
Patois*	PAT
Phlebotomus (sandfly) fever	PHL
Piry	PIRY
Quaranfil	QRF
Simbu*	SIM
Tacaribe	TCR
Timbo	TIM
Turlock	TUR
Vesicular stomatitis	VSV

* The Bunyamwera supergroup includes members of the following nine groups, among which some interrelations have been shown:
Bunyamwera, Bwamba, Group C, California, Capim, Guama, Koongol, Patois and Simbu.

** ACAV, 1969

TABLE 2

ANTIGENIC CLASSIFICATION OF ARBOVIRUSES**

GROUP

A
African horsesickness
Anopheles A
Anopheles B
B
Bakau
Bluetongue
Bunyamwera Supergroup
    Bunyamwera
    Bwamba
    C
    California
    Capim
    Guama
    Koongol
    Patois
    Simbu
    Tete
    Unassigned
Changuinola
Congo
Epizootic hemorrhagic disease
Ganjam
Hughes
Kaisodi
Kemerovo
Mapputta
Mossuril
Nyando
Phlebotomus fever
Qalyub
Quaranfil
Timbo
Turlock
Uukuniemi
Vesicular stomatitis

GROUP

Ungrouped

Tacaribe*
Matariya***
Palyam***

* Evidence for an arthropod cycle in nature is scant for all the viruses in this group.

** ACAV, 1970

*** ACAV, 1971

TABLE 3

PROTOTYPE STRAINS OF THE RUSSIAN SPRING-SUMMER COMPLEX OF TICK-BORNE GROUP B ARBOVIRUSES

Prototype Virus	Initial Isolation			Geographical		Mammal	
Name	Strain	Year	Location	Distribution	Tick Vector	Reservoir	Syndrome in Man
Louping ill (LI)	Original	1929	Scotland	Scotland, Ulster	I. ricinus	voles, mice sheep	Aseptic meningitis
Russian spring-summer encephalitis (RSSE)	Sofjin	1937	Far eastern U.S.S.R.	U.S.S.R. (European Siberia) Far East	I. persulcatus	forest rodents	Encephalitis
Omsk hemorrhagic fever (OMSK)	Kubrin	1947	Omsk Oblast	U.S.S.R. (Siberia)	D. pictus	forest rodents	Fever, hemorrhages
Negishi (NEG)	Original	1948	Japan	Japan			Encephalitis
Tick-borne encephalitis (TBE)	Absettarov	1951	Leningrad Oblast	U.S.S.R. (European)	I. ricinus & I. persulcatus	forest rodents goats (milk borne)	Aseptic meningitis
	Hypr Hansalova Kumlinge	1953 1948 1959	Czechoslovakia Finland	Austria, Czechoslovakia, Finland Hungary, Poland, Sweden	I. ricinus	forest rodents	Meningoencephalitis
Langat (LGT)	TP21	1956	Malaysia	Malaysia	I. granulatus	forest rodents	
Kyasanur Forest Disease (KFD)	W371	1957	Mysore, India	India	I. granulatus H. spinigera and other Haemaphysalis sp.	monkeys forest rodents	Hemorrhagic fever
Powassan (POW)	LB	1958	Ontario, Canada	Canada and U.S.A.	i. cookei	forest rodents	Encephalitis

TABLE 4

MINIMUM LETHAL DOSES FOR VARIOUS ROUTES OF INFECTION

ROUTE OF INFECTION	DOSAGE $MICLD_{50}$*
Intracerebral inoculation	1
Intravenous inoculation	1
Subcutaneous inoculation	18
Intraperitoneal inoculation	98
Aerosol inoculation	640
Intranasal instillation	10,000
Per Os instillation	No infection after 3×10^6

* $MICLD_{50}$ = mouse intracerebral LD_{50} = the minimum amount of Powassan virus per ml. required to cause death in 50 per cent of three- to four-week-old mice.

TABLE 5

CROSS-INFECTION RECORD

Days Post-Exposure	Deaths Due To Infectious Aerosol	Contact
8	9	
9	9	
10	11	
11	1	
16		1 NS
17		
18		2
19		4
20		4
21		
22		1
23		2
24		1 NS

Aerosol Deaths 30/30

Contact Deaths 13/30

NS - non-specific -- may or may not have been infected; 43 per cent of control mice infected because of intimate contact with aerosol-infected mice.

TABLE 6

LEVELS OF POWASSAN VIRUS IN VARIOUS MOUSE TISSUES

AFTER INTRANASAL INSTILLATION OF 10 MINLD$_{50}$ OF VIRUS

Mouse Tissue	Days After Infection						
	0	1	2	3	4	5	6	7
Turbinates	0	2.76	2.76	3.76	5.28	5.28	5.28	5.0
Lung	3.0	4.0	5.28	6.28	6.28	5.28	5.28	4.69
Blood	0	0	3.72	4.98	4.77	3.48	2.97	2.97
Spleen	0	0	0	5.69	4.69	4.28	3.52	3.28
Brain	0	0	3.3	4.52	6.0	8.0	8.77	9.28

Figures presented represent the average of three separate experiments for each tissue examined.

TABLE 7

LEVELS OF POWASSAN VIRUS

IN 10 PER CENT TISSUE HOMOGENATES

LOG_{10}/ML.

	Day After Aerosol Inoculation							
Tissue	0	1	2	3	4	5	6	7	8
Turbinates	0	+	0	2.0	+	4.5	5.8	5.0	+
Lung	0	1.8	2.8	4.5	4.8	5.3	4.8	5.2	3.2
Thymus	0	0	+	2.8	2.8	3.3	3.8	4.2	4.8
Blood	0	0	3.2	4.1	4.0	+	4.0	4.1	2.9
Spleen	0	0	1.8	2.8	4.3	4.5	4.2	2.8	4.2
Liver	0	0	0	+	1.8	2.0	2.3	2.0	0
Kidney	0	0	+	2.8	2.3	5.0	4.0	4.0	3.0
Gut	0	0	0	0	0	+	0	0	0
Skeletal Muscle	0	0	0	2.0	+	3.3	3.0	4.0	2.8
Cardiac Muscle	0	0	0	+	3.0	3.0	2.8	4.2	3.0
Smooth Muscle	0	0	0	0	0	3.0	4.3	6.0	3.0
Brain	0	0	+	4.0	4.0	5.0	7.8	8.5	7.8

Figures presented represent the average of five separate experiments for each tissue examined.

+ = virus present, but too low to quantitate

TABLE 8

BIOLOGICAL DECAY OF POWASSAN VIRUS SUSPENSIONS

IN THE DARK AND UNDER ULTRAVIOLET IRRADIATION

Suspension Condition		Time In Minutes 0	30	60	90	120	150	180
Dark	L	7.77	7.28	7.52	7.28	6.77	6.55	6.52
21°C	H	8.0	7.77	7.77	7.52	7.0	6.77	7.0
		7.8 ± 0.24*	7.5 ± 0.41	7.6 ± 0.24	7.4 ± 0.24	6.9 ±0.24	6.7 ± 0.24	6.8 ± 0.34
		0	15	30	45	60	75	90
UV 20 micro-		7.77	6.0	5.52	2.52	2.77	+	0
watts/cm^2		8.0	6.52	6.0	3.77	3.27	+	0
21°C		7.9 ± 0.24*	6.3 ± 0.24	5.77 ± 0.24	3.69 ± 0.24	3.0 ± 0.29	+	0

* = Mean $\log_{10}$ LD_{50}/ml. based on three measurements with ± 2 sigma limits.

+ = Virus present but too low to quantitate.

L = Lowest value.

H = Highest value.

TABLE 9

BIOLOGICAL DECAY OF POWASSAN VIRUS AEROSOLS AT LOW, INTERMEDIATE AND HIGH RELATIVE HUMIDITIES AND CONSTANT TEMPERATURE

LOG_{10} LD_{50}/ML. OF IMPINGER FLUID

Aerosol Condition		Time In Hours 0	1	2	3	4	5
20% RH	L	5.27	4.0	3.77	4.0	3.77	4.0
21°C	H	5.76	4.27	4.27	4.27	4.0	4.0
		5.02 ± 0.41*	4.1 ± 0.24	4.1 ± 0.24	4.1 ± 0.24	3.94 ± 0.24	4.0 ± 0.0
50% RH		4.77	3.0	3.0	3.0	3.0	2.77
21°C		5.0	3.52	3.27	3.27	3.27	3.27
		4.85 ± 0.24	3.35 ± 0.47	3.1 ± 0.24	3.2 ± 0.24	3.2 ± 0.24	3.02 ± 0.41
80% RH		4.52	3.52	3.52	3.52	3.52	3.27
21°C		5.0	4.0	3.77	3.77	3.77	3.52
		4.85 ± 0.24	3.85 ± 0.47	3.6 ± 0.24	3.68 ± 0.24	3.6 ± 0.24	3.44 ± 0.24
Tracer		4.98	4.93	4.9	4.95	4.89	4.89
21°C		5.18	5.0	5.0	5.08	4.95	4.98
		5.06 ± 0.7	4.97 ± 0.69	4.94 ± 0.23	5.0 ± 0.41	4.92 ± 0.30	4.92 ± 0.18

* = Mean $\log_{10}$ LD_{50}/ ml. based on three measurements and ± 2 Sigma limits.

L = Lowest value.

H = Highest value

TABLE 10

BIOLOGICAL DECAY OF POWASSAN VIRUS AEROSOLS

IN THE DARK AND UNDER ULTRAVIOLET IRRADIATION

Aerosol Condition		Time In Minutes 0	60	120	180
Dark					
20% RH 21°C	L	4.0	3.27	3.0	3.27
	H	5.0	4.0	4.0	4.0
		4.69 ± 0.95*	3.78 ± 0.84	3.76 ± 0.88	3.79 ± 0.93

	Time In Minutes 0	5	10	15	30
U.V.					
400 microwatts/cm^2	4.77	+	0	0	0
20% RH 21°C	5.0	+	0	0	0
	4.9 ± 0.63*	1.79	0	0	0

* = Mean $\log_{10}$ LD_{50}/ml. based on three measurements and ± 2 Sigma limits.

+ = Virus present but too low to quantitate.

L = Lowest value.

H = Highest value

FIGURE 1

Diagram of aerosol suite.

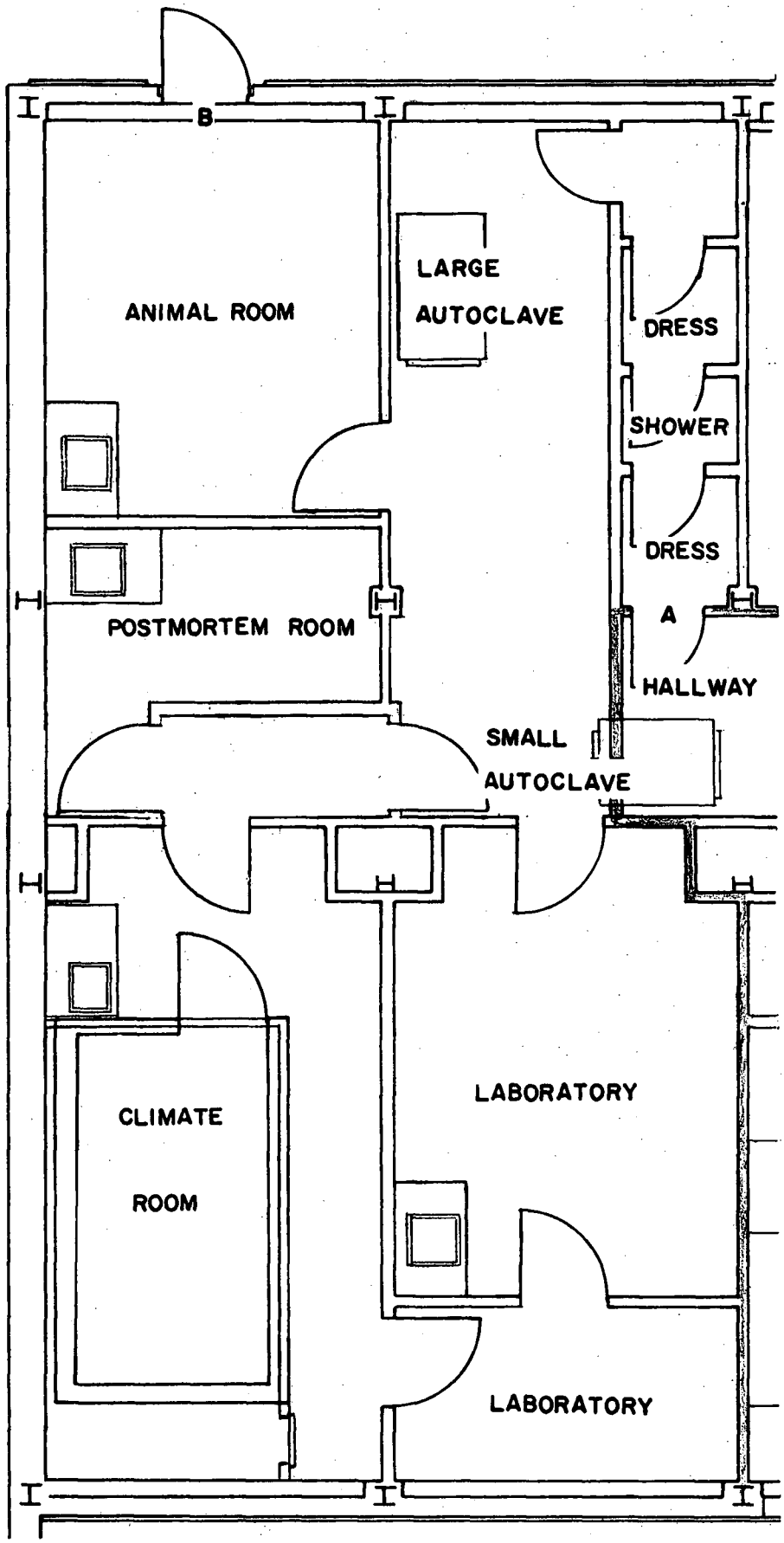

B
ANIMAL ROOM
LARGE
AUTOCLAVE
DRESS
SHOWER
DRESS
A
HALLWAY
POSTMORTEM ROOM
SMALL
AUTOCLAVE
CLIMATE
ROOM
LABORATORY
LABORATORY

FIGURE 2

Diagram of the aerosol animal exposure unit and the aerosol aging drum.

Legend

A 500 liter aerosol drum

B aerosol mixing tube

C water manometer

D air flow meter

E air flow regulator

F particulate filter

G heat exchanger

H hot/cold water mixer regulator

I aerosol humidity control tank

J Collison atomizer

L all glass impinger

M animal exposure container

N dry bulb thermometer

P wet bulb thermometer

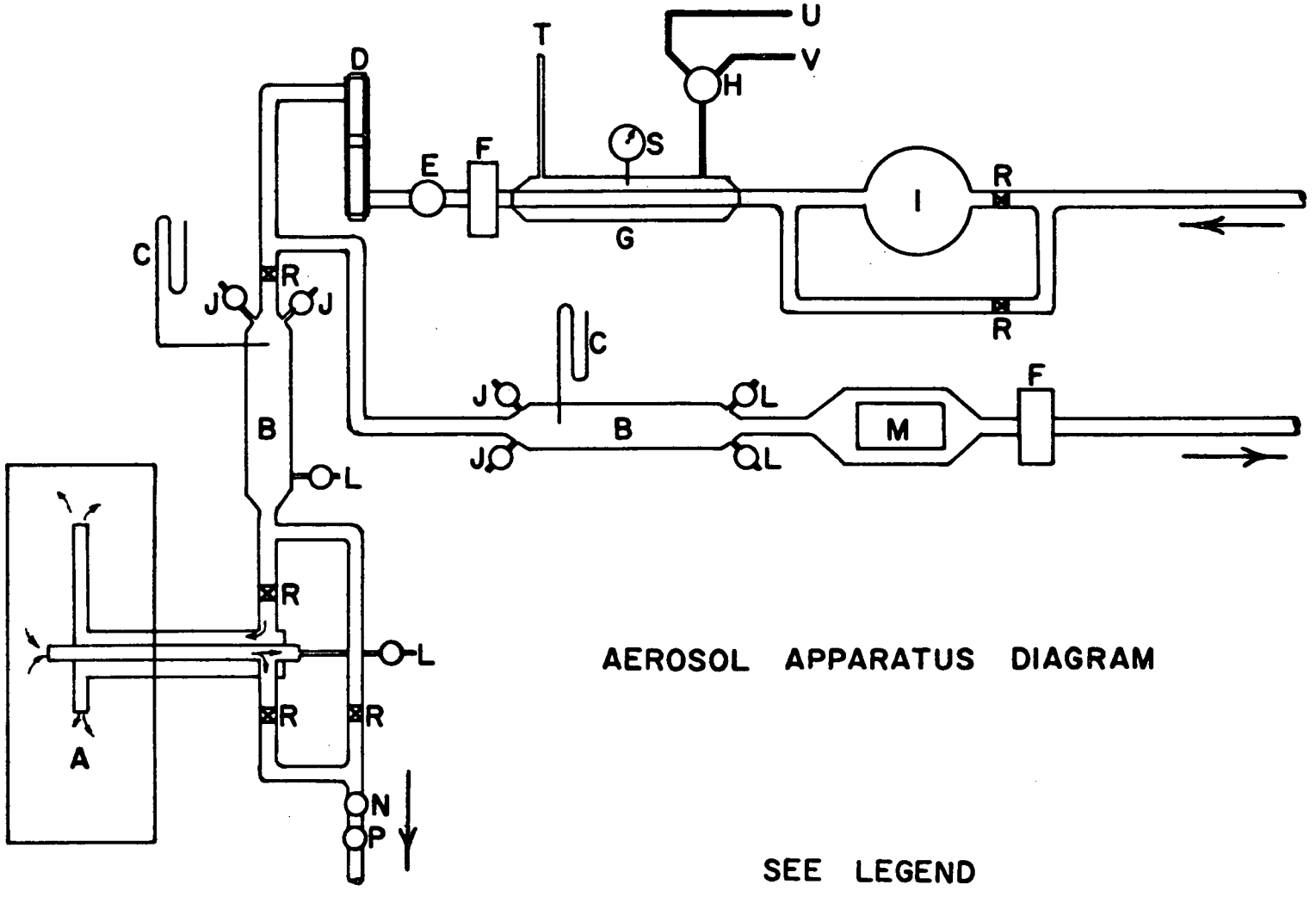
R valves

S heat exchanger thermometer

T heat exchanger drain

U hot water line

V cold water line



AEROSOL APPARATUS DIAGRAM

SEE LEGEND

FIGURE 3

Growth curve of Powassan virus in suckling mouse brain.

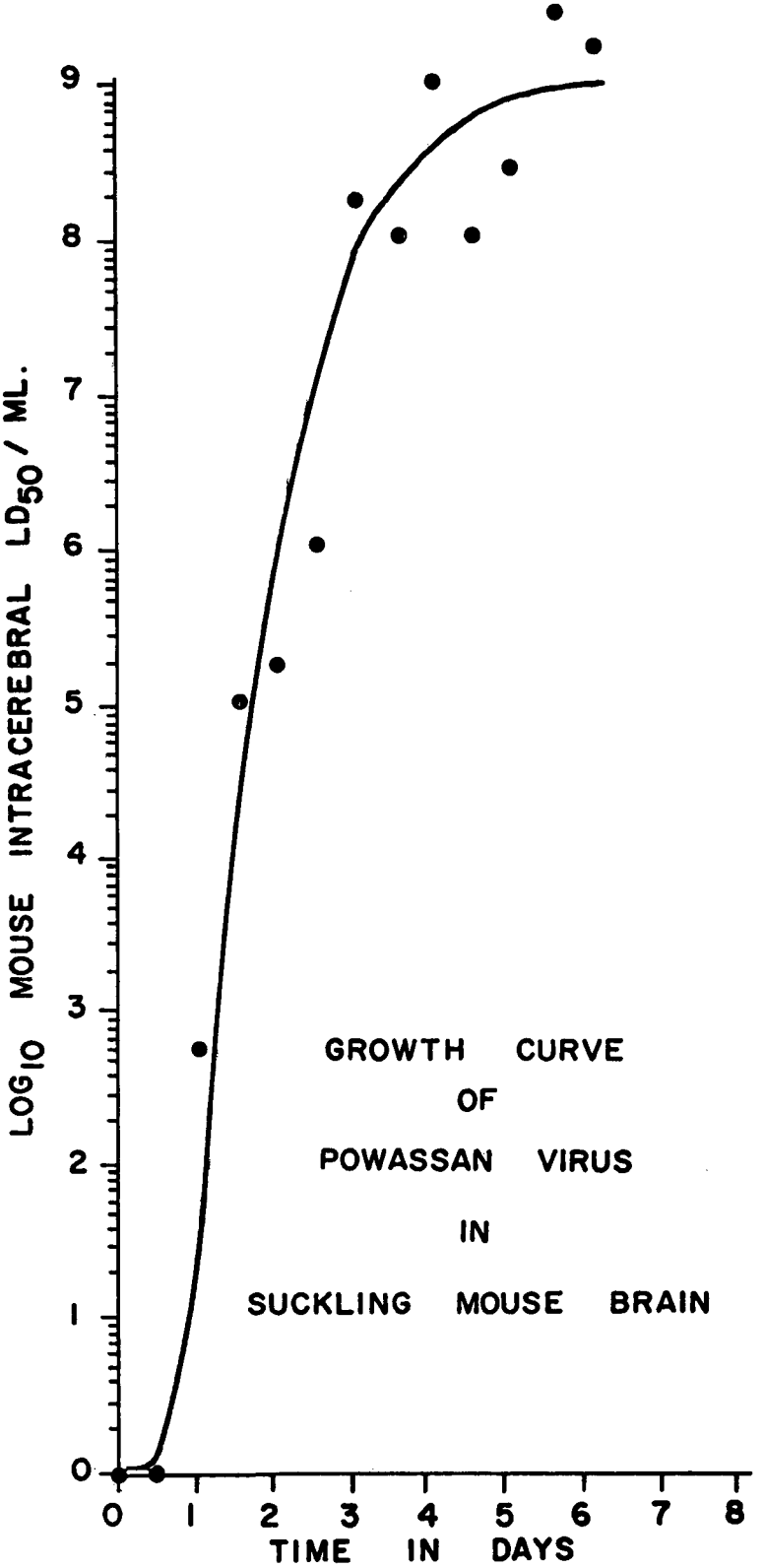
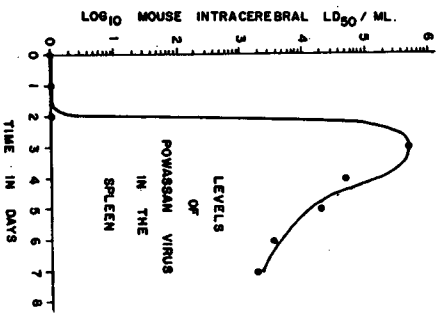
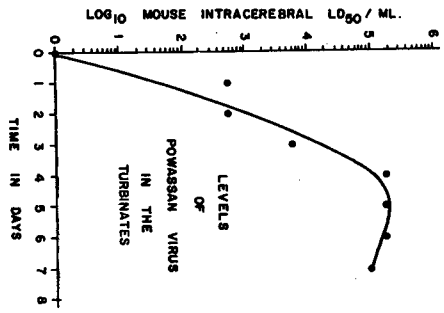
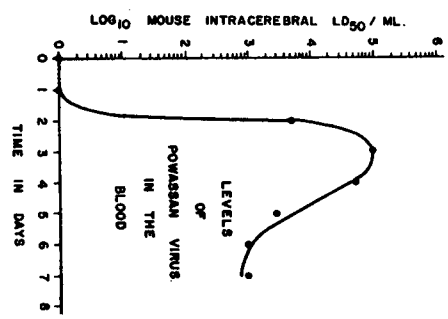

LOG10 MOUSE INTRACEREBRAL LD50 / ML.
0
1
2
3
4
5
6
7
8
9
TIME IN DAYS
0
1
2
3
4
5
6
7
8
GROWTH CURVE
OF
POWASSAN VIRUS
IN
SUCKLING MOUSE BRAIN

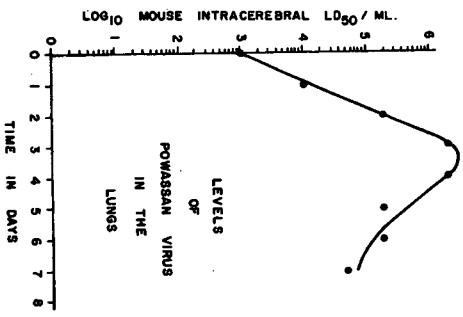
FIGURE 4

Levels of Powassan virus in various mouse tissues after intranasal instillation of 10 $MINLD_{50}$ of Powassan virus.


LOG10 MOUSE INTRACEREBRAL LD50 / ML.
TIME IN DAYS
LEVELS OF POWASSAN VIRUS IN THE SPLEEN


LOG10 MOUSE INTRACEREBRAL LD50 / ML.
TIME IN DAYS
LEVELS OF POWASSAN VIRUS IN THE TURBINATES


LOG10 MOUSE INTRACEREBRAL LD50 / ML.
TIME IN DAYS
LEVELS OF POWASSAN VIRUS IN THE BLOOD


LOG10 MOUSE INTRACEREBRAL LD50 / ML.
TIME IN DAYS
LEVELS OF POWASSAN VIRUS IN THE LUNGS

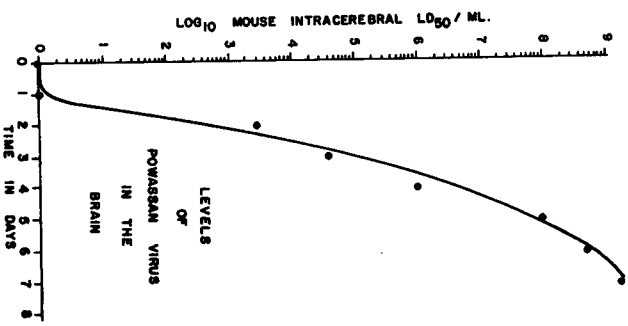

LOG10 MOUSE INTRACEREBRAL LD50 / ML.
TIME IN DAYS
LEVELS OF POWASSAN VIRUS IN THE BRAIN

FIGURE 5

Curve of the retention of Powassan virus in the lungs of mice after aerosol inoculation.

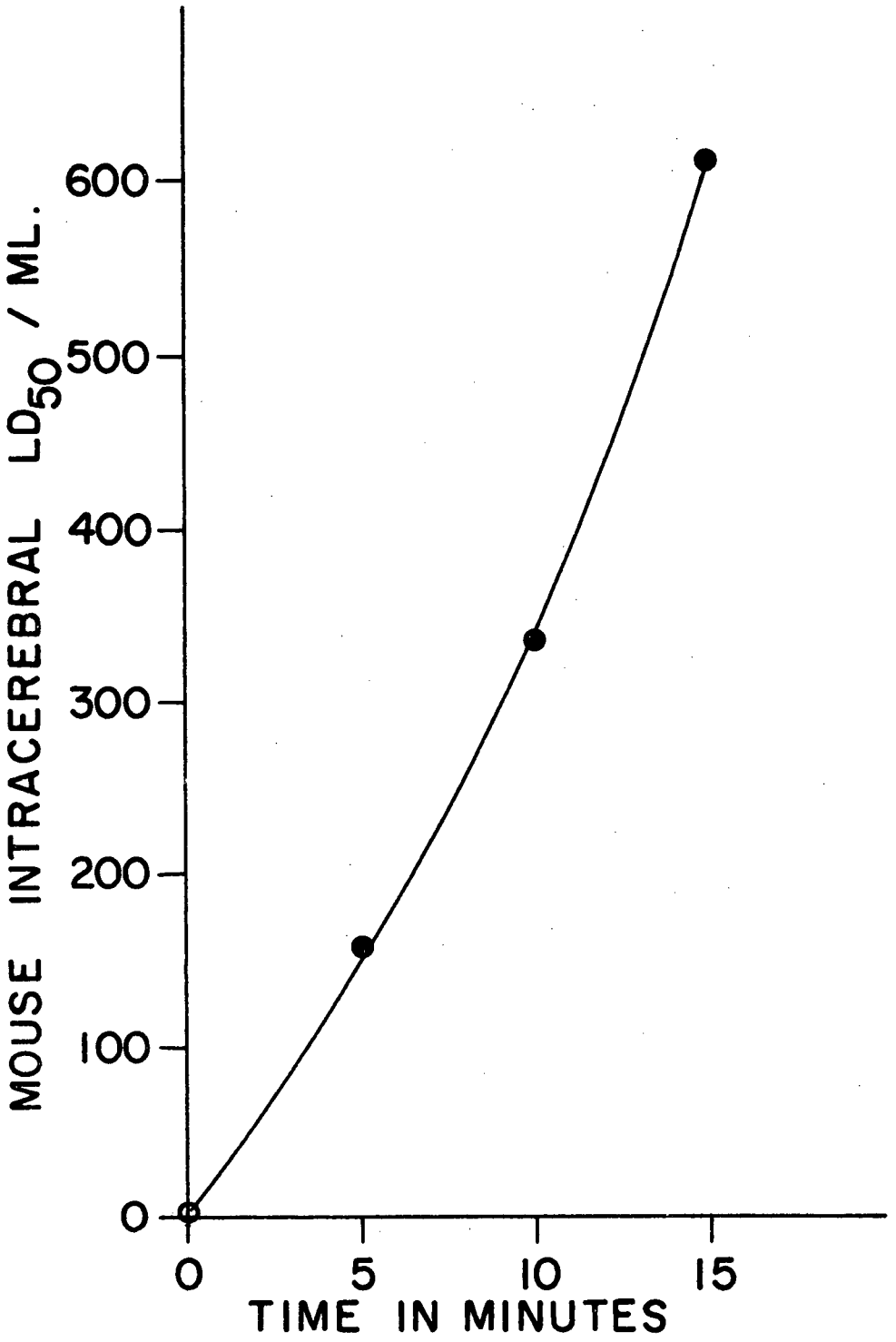

MOUSE INTRACEREBRAL LD_{50} / ML.
600
500
400
300
200
100
0
0
5
10
15
TIME IN MINUTES

FIGURE 6

Biological decay of Powassan virus suspensions in the dark and under ultraviolet irradiation.

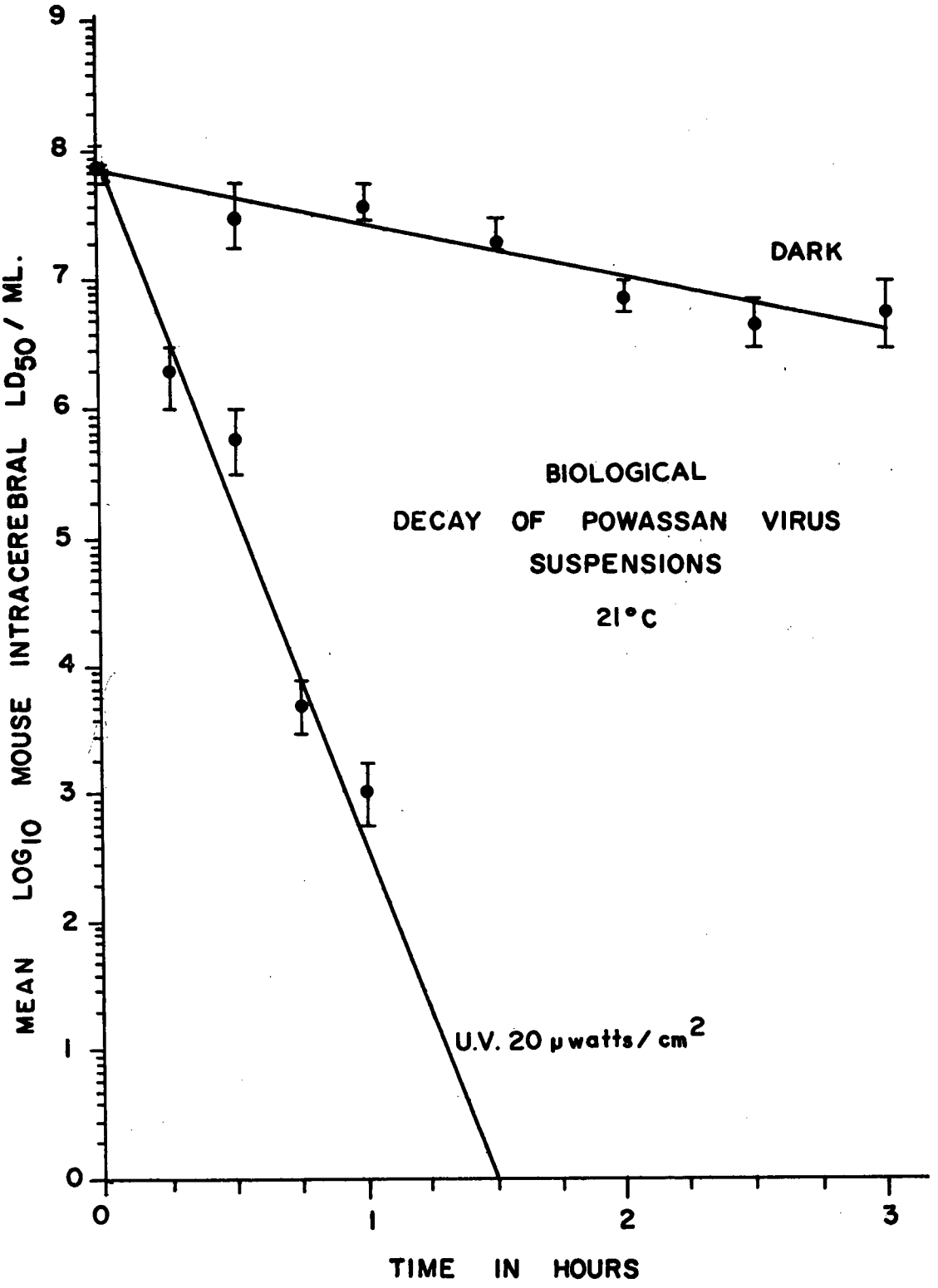

MEAN LOG10 MOUSE INTRACEREBRAL LD50 / ML.
TIME IN HOURS
DARK
U.V. 20 μ watts / cm2
BIOLOGICAL
DECAY OF POWASSAN VIRUS
SUSPENSIONS
21°C
0
1
2
3
4
5
6
7
8
9

FIGURE 7

Biological decay of Powassan virus aerosols at low, intermediate and high relative humidites and at a constant temperature of 21°C.

Tracer = Bacillus subtillus (var. niger) spores which are not susceptible to biological decay.

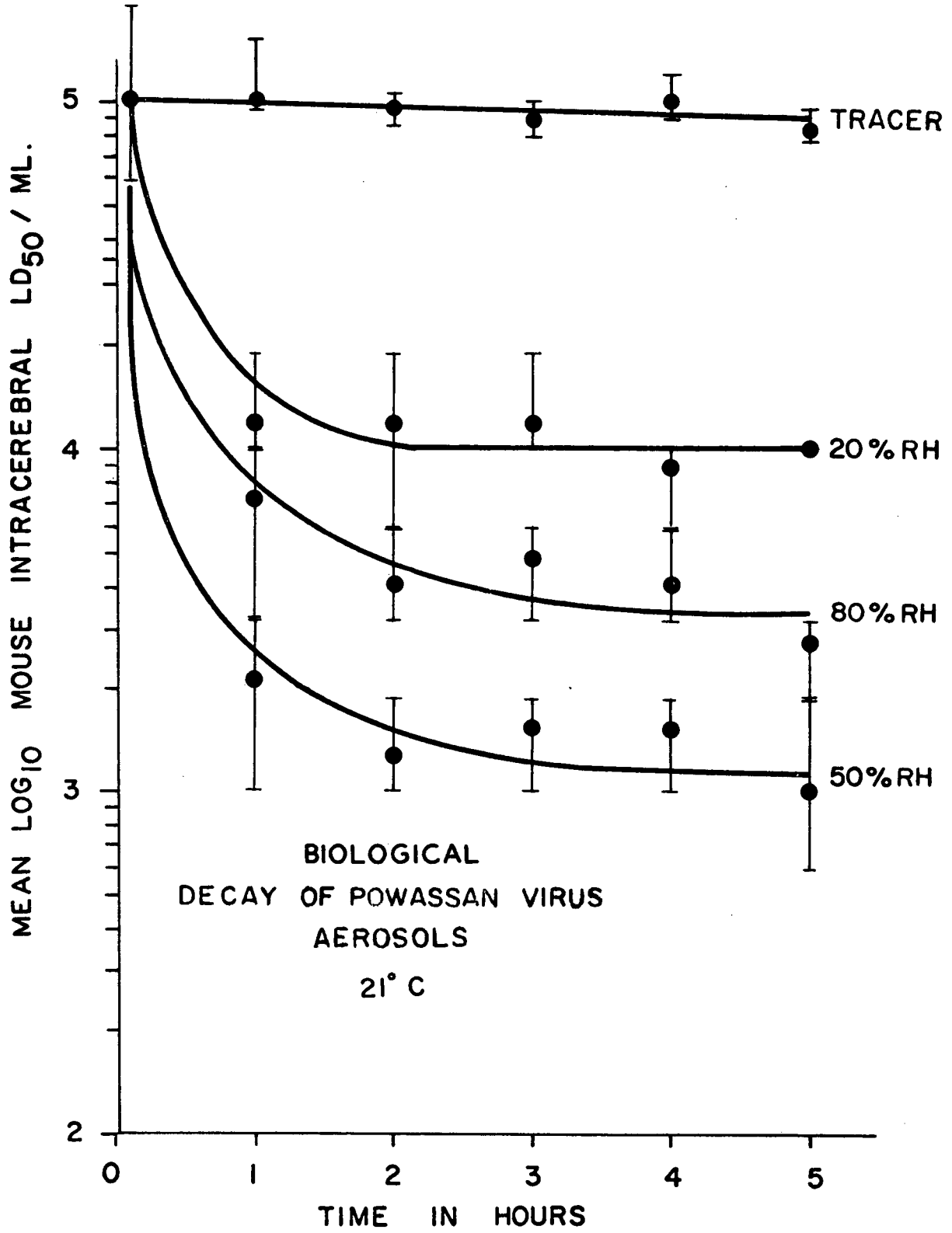

MEAN LOG10 MOUSE INTRACEREBRAL LD50 / ML.
5
4
3
2
0
1
2
3
4
5
TIME IN HOURS
TRACER
20% RH
80% RH
50% RH
BIOLOGICAL
DECAY OF POWASSAN VIRUS
AEROSOLS
21° C

FIGURE 8

Biological decay of Powassan virus aerosols at various relative humidities.

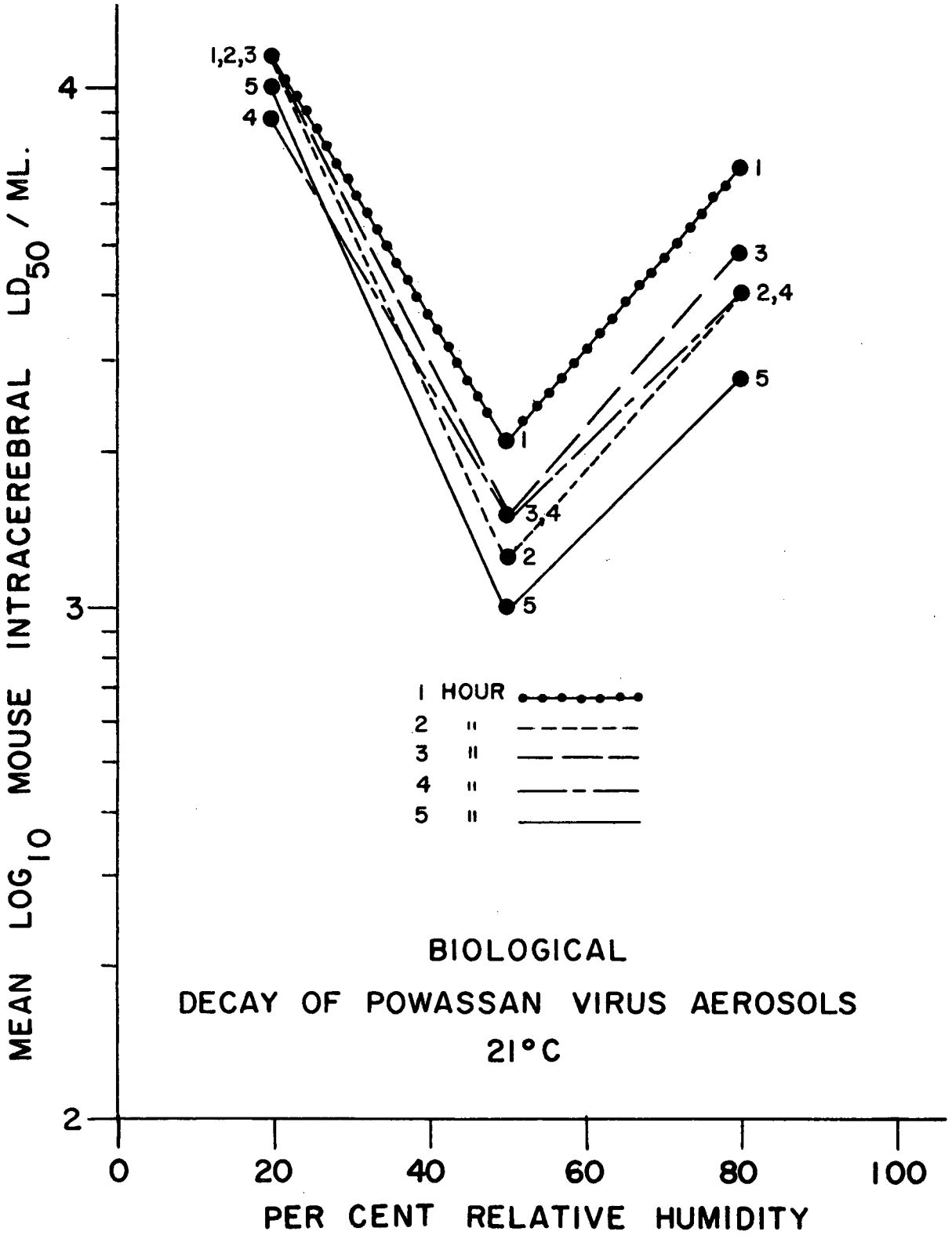

MEAN LOG10 MOUSE INTRACEREBRAL LD50 / ML.
4
3
2
1,2,3
5
4
1
3
2,4
5
1
3,4
2
5
1 HOUR
2 "
3 "
4 "
5 "
BIOLOGICAL
DECAY OF POWASSAN VIRUS AEROSOLS
21°C
0
20
40
60
80
100
PER CENT RELATIVE HUMIDITY

FIGURE 9

Biological decay of Powassan virus aerosols in the dark and under ultraviolet irradiation.

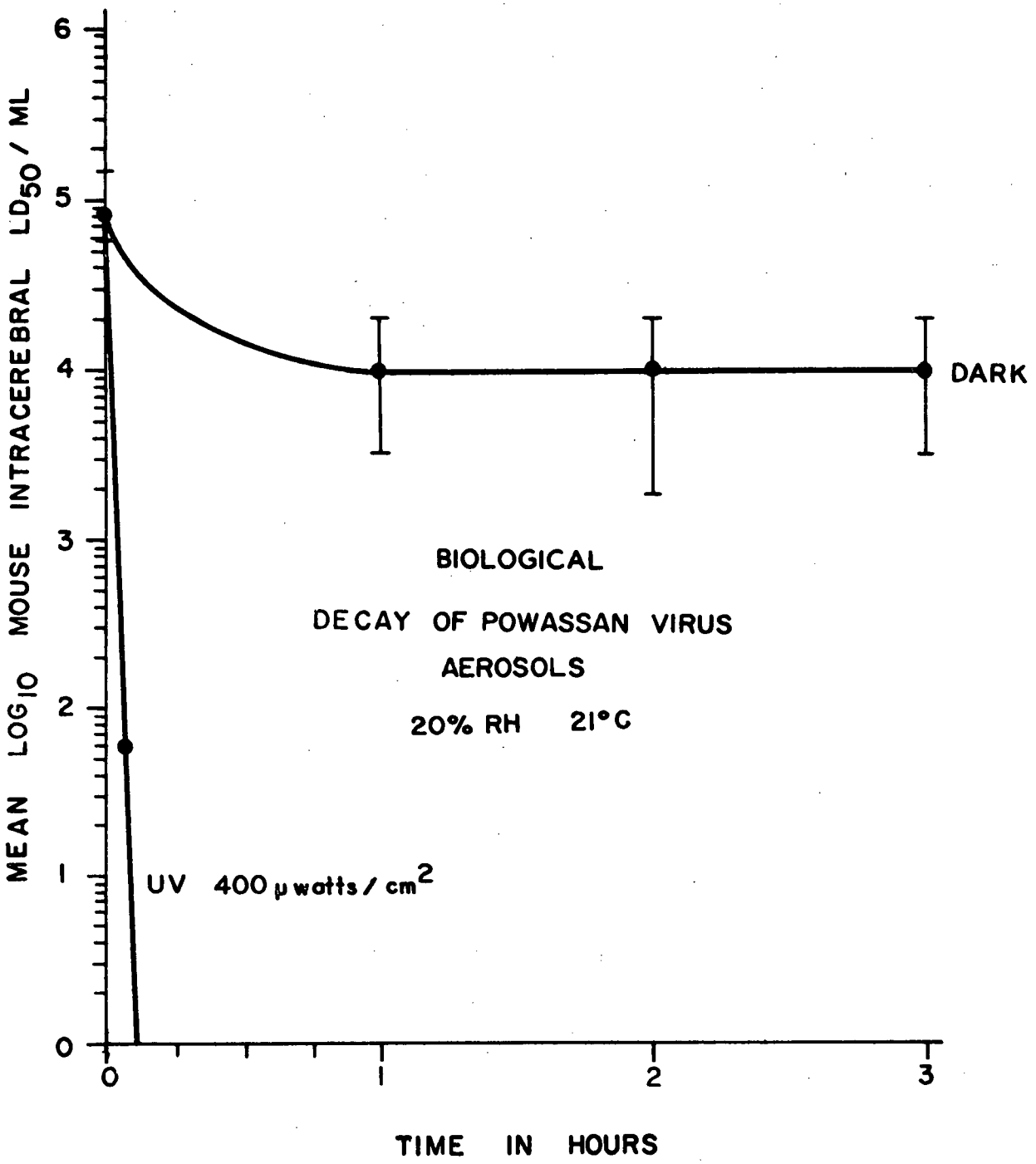

MEAN LOG10 MOUSE INTRACEREBRAL LD50 / ML
6
5
4
3
2
1
0
0
1
2
3
TIME IN HOURS
DARK
BIOLOGICAL
DECAY OF POWASSAN VIRUS
AEROSOLS
20% RH 21°C
UV 400 μ watts / cm2

FIGURE 10

Levels of Powassan virus in various tissues after aerosol inoculation of virus.

LOG10 MOUSE INTRACEREBRAL LD50 / ML.
TIME IN DAYS
LEVELS OF POWASSAN VIRUS IN THE TURBINATES
LEVELS OF POWASSAN VIRUS IN THE LUNGS
LEVELS OF POWASSAN VIRUS IN THE THYMUS
LEVELS OF POWASSAN VIRUS IN THE BLOOD
LEVELS OF POWASSAN VIRUS IN THE SPLEEN
LEVELS OF POWASSAN VIRUS IN THE BRAIN
LEVELS OF POWASSAN VIRUS IN THE LIVER
LEVELS OF POWASSAN VIRUS IN THE KIDNEY
LEVELS OF POWASSAN VIRUS IN THE SKELETAL MUSCLE
LEVELS OF POWASSAN VIRUS IN THE CARDIAC MUSCLE
LEVELS OF POWASSAN VIRUS IN THE SMOOTH MUSCLE

PLATE 1

Photograph of the aerosol aging apparatus within the climate control room of the aerosol suite.

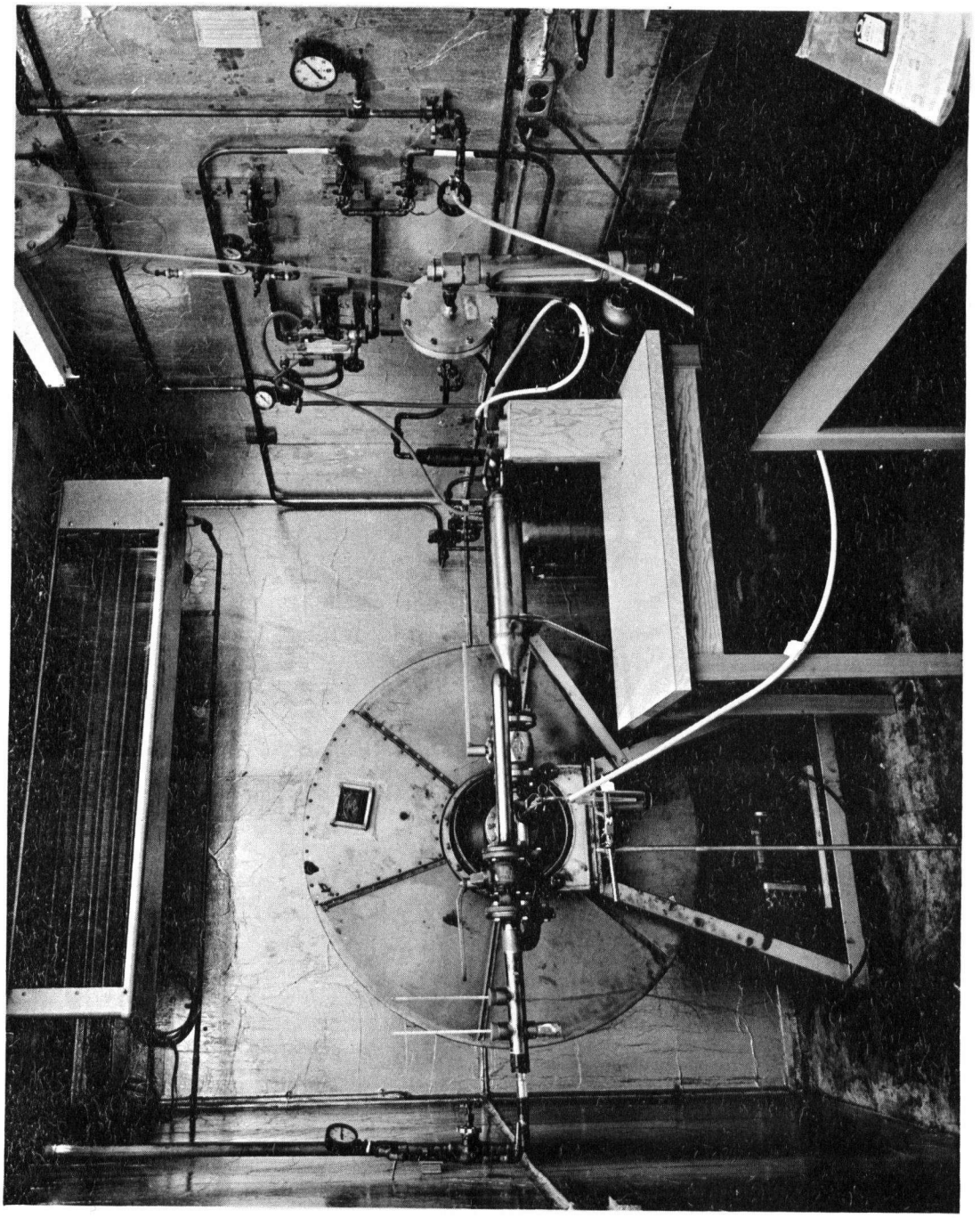


PLATE 2

Photograph of animal exposure apparatus within the climate room of the aerosol suite.

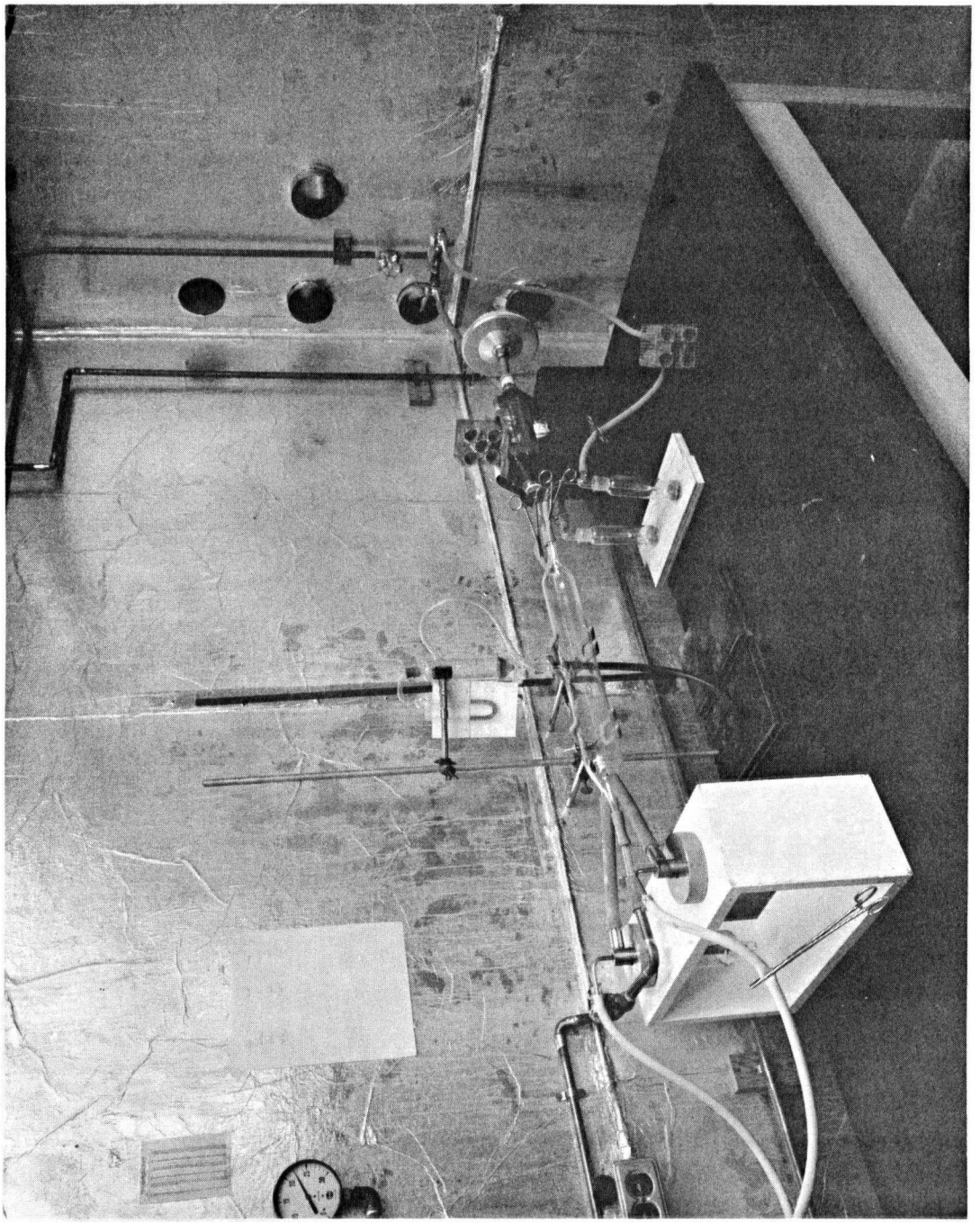


PLATE 3

Photograph of researcher wearing protective clothing plus the positive pressure safety hood which is attached to the separate, filtered, conditioned air supply within the climate room of the aerosol suite.

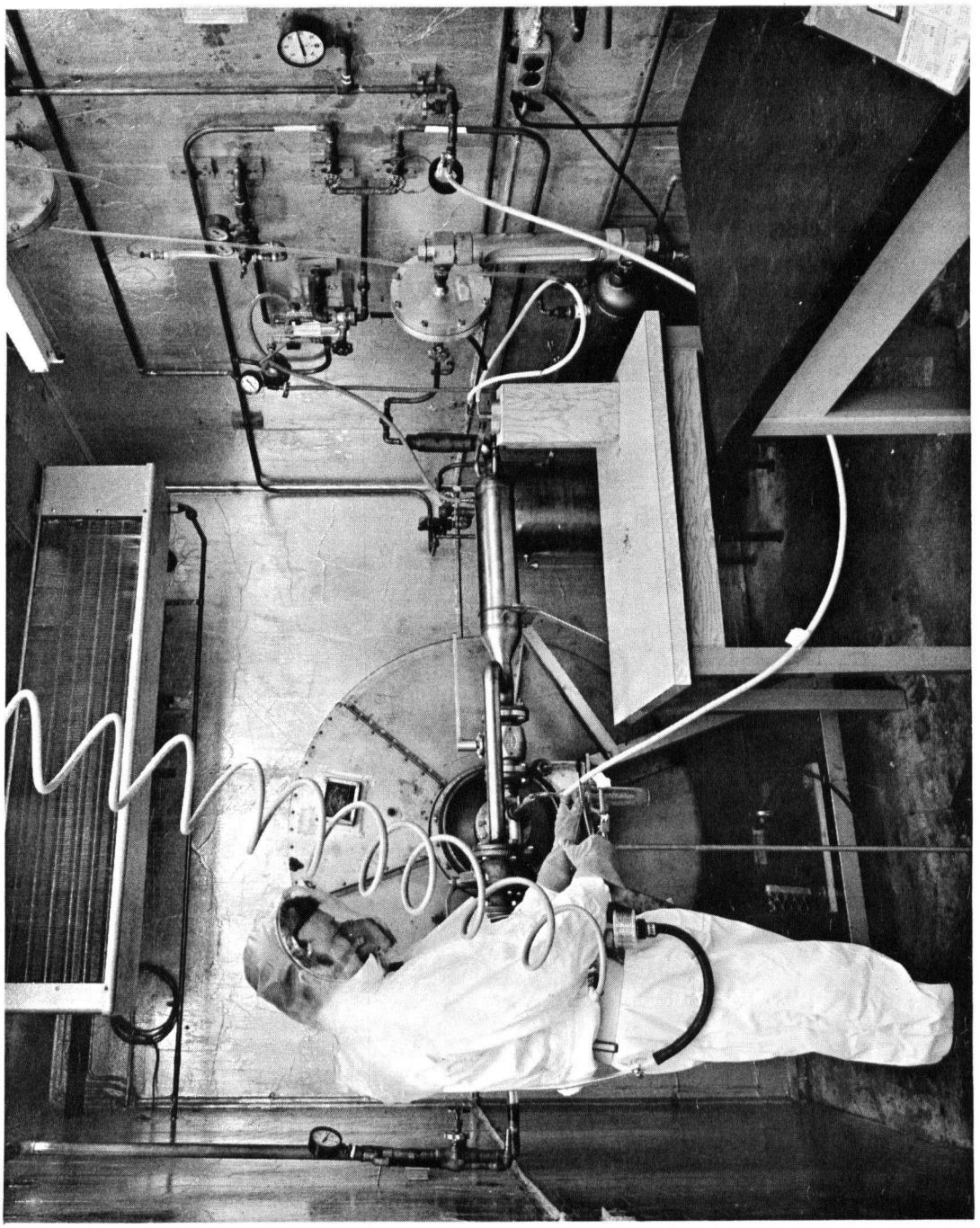


PLATE 4

Photograph of the modified Horsfall cages used for the containment of mice exposed to infectious aerosols of Powassan virus. The air entering and leaving the unit is filter-sterilized. The air leaving the units is also subjected to ultraviolet irradiation of 1600 microwatts/cm^2 plus air incineration at 450^oF.

PLATE 5

Photograph of the special needle used for the instillation of Powassan virus into the gastrointestinal tract of mice.

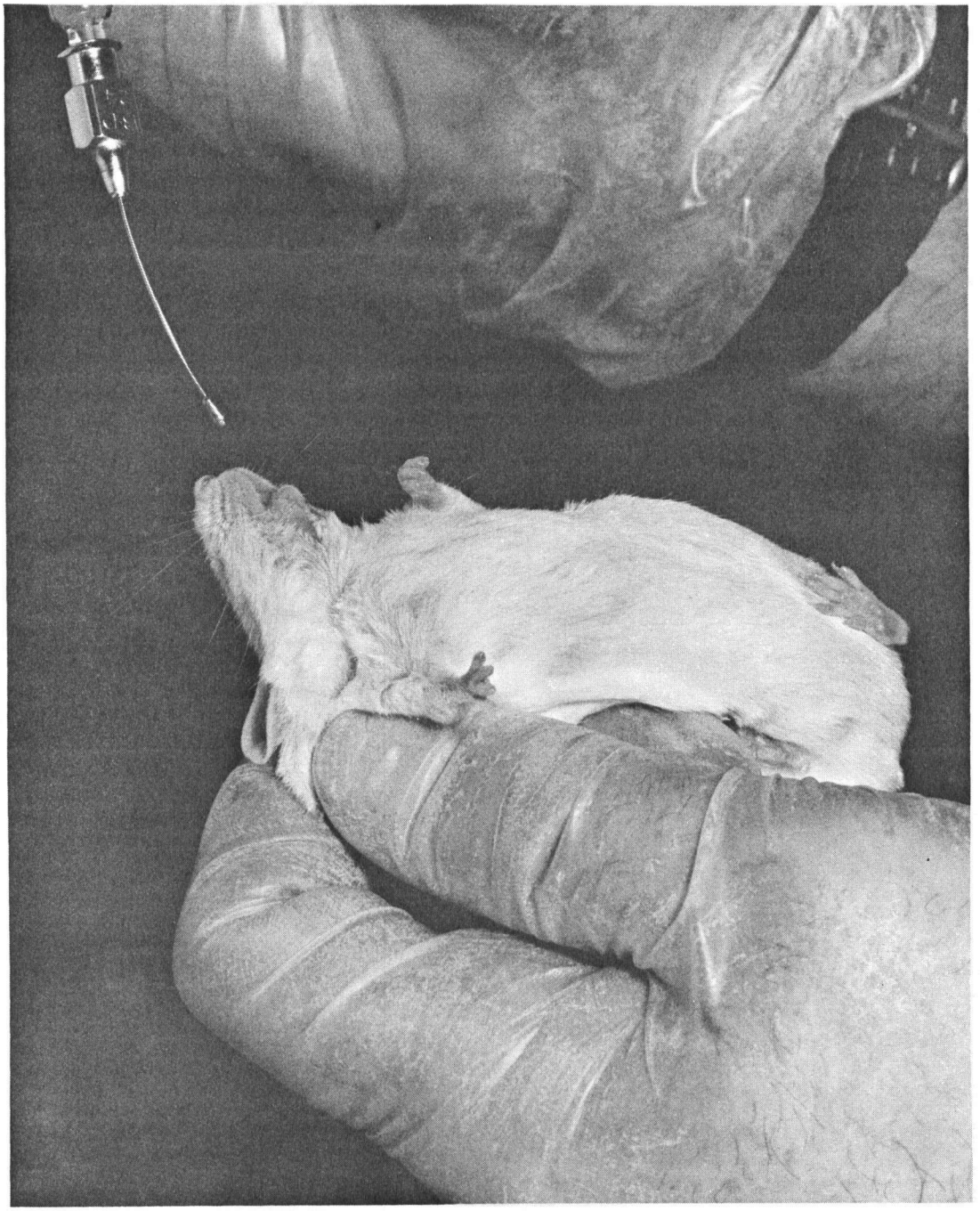
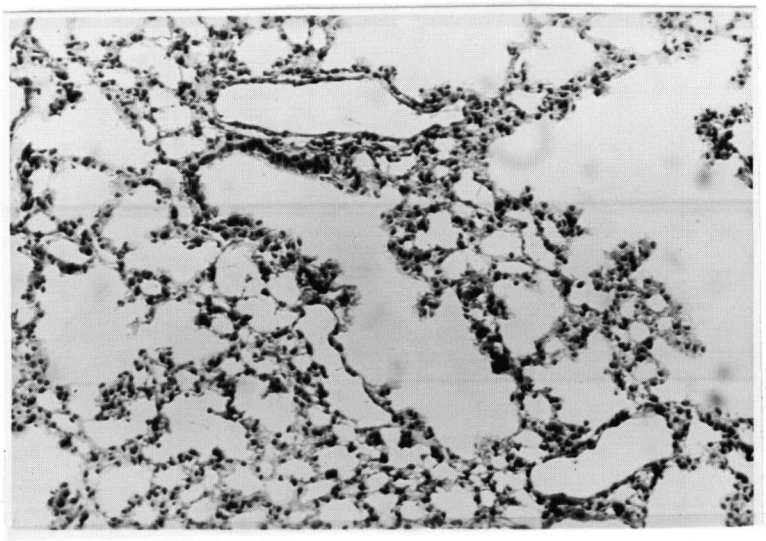


PLATE 6

Cross-section of uninfected mouse lung, stained with H and E, magnification 320 x.

PLATE 7

Cross-section of Powassan virus infected mouse lung five days after aerosol inoculation, stained with H and E, magnification 320 x.



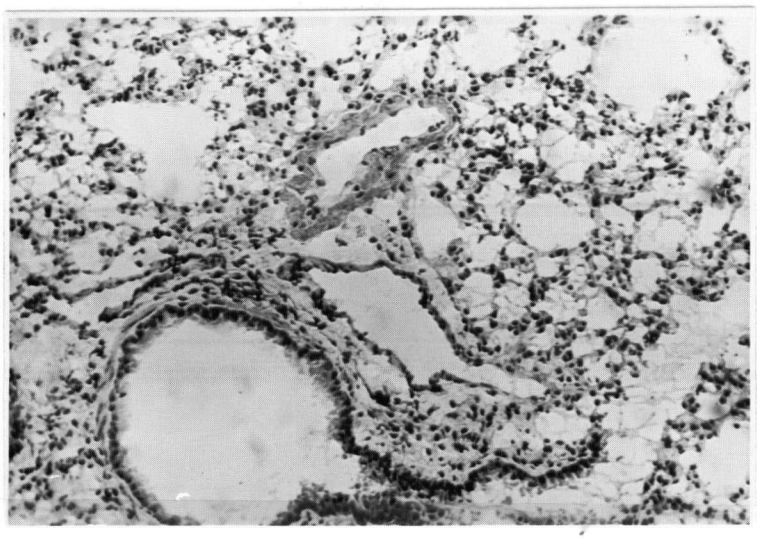
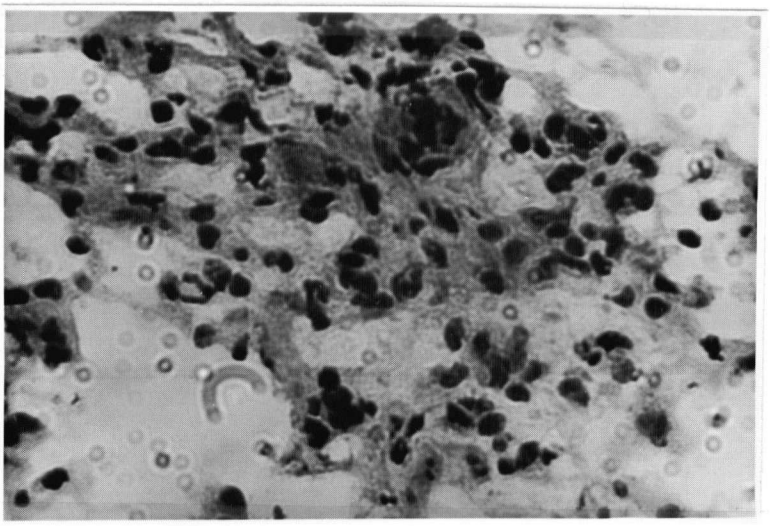


PLATE 8

Cross-section of Powassan virus infected mouse lung five days after aerosol inoculation, showing thickening of alveoli epithelium and accumulation of macrophages, stained with H and E, magnification 640 x.

PLATE 9

Sagittal section of uninfected mouse cerebellum, stained with H and E, magnification 640 x.



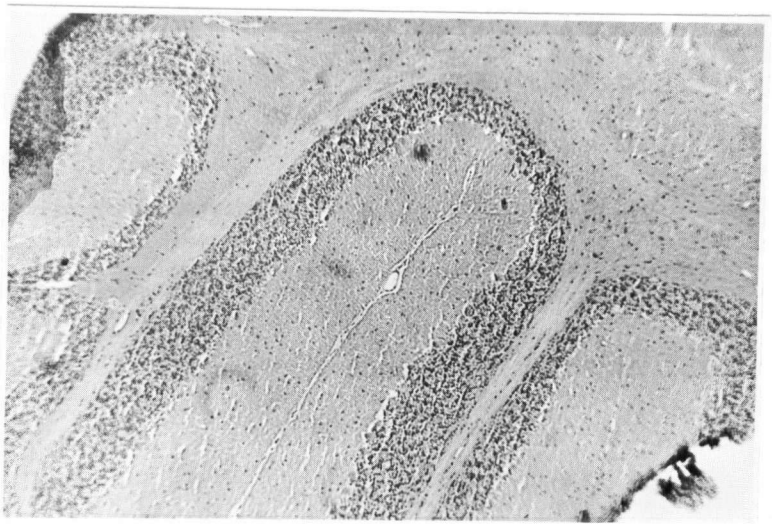


PLATE 10

Sagittal section of Powassan virus infected mouse cerebellum three days after aerosol inoculation, showing the beginning of cellular degeneration; stained with H and E, magnification 320 x.

PLATE 11

Sagittal section of Powassan virus infected mouse cerebellum eight days after aerosol inoculation. Note widespread cellular degeneration; stained with H and E, magnification 320 x.

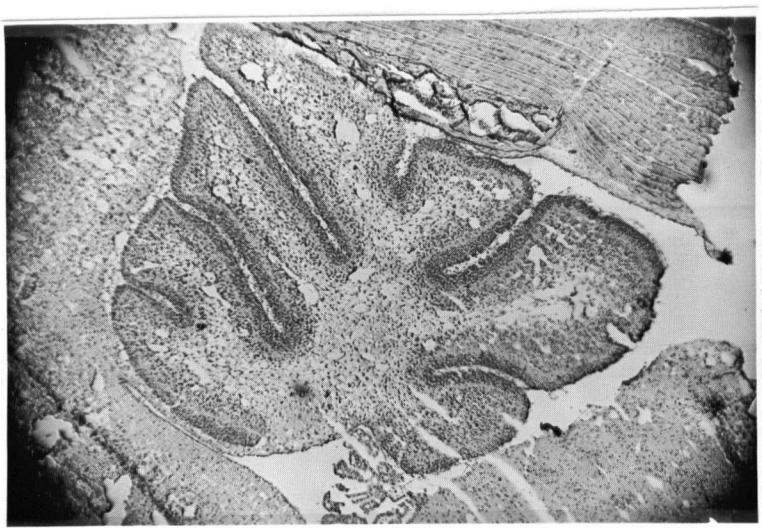


PLATE 12

Sagittal section of Powassan virus infected mouse cerebrum six days after aerosol inoculation showing cellular degeneration and infiltration of inflammatory cells; stained with H and E, magnification 320 x.

PLATE 13

Sagittal section of Powassan virus infected mouse brain at the site of cleavage planes between adjacent portions of the brain. Note cellular degeneration plus inflammatory cell infiltration; stained with H and E, magnification 320 x.

PLATE 14

Sagittal section of Powassan virus infected mouse brain at the site of cleavage planes between adjacent portions of the brain. Note cellular degeneration plus inflammatory cell infiltration; stained with H and E, magnification 320 x.

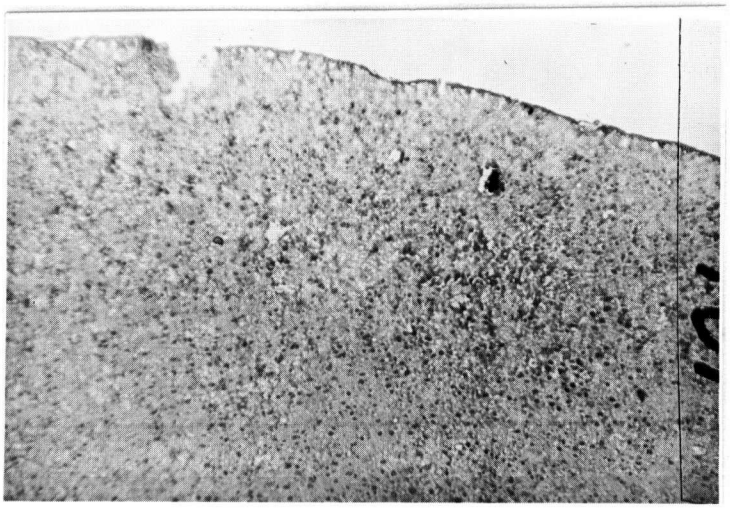
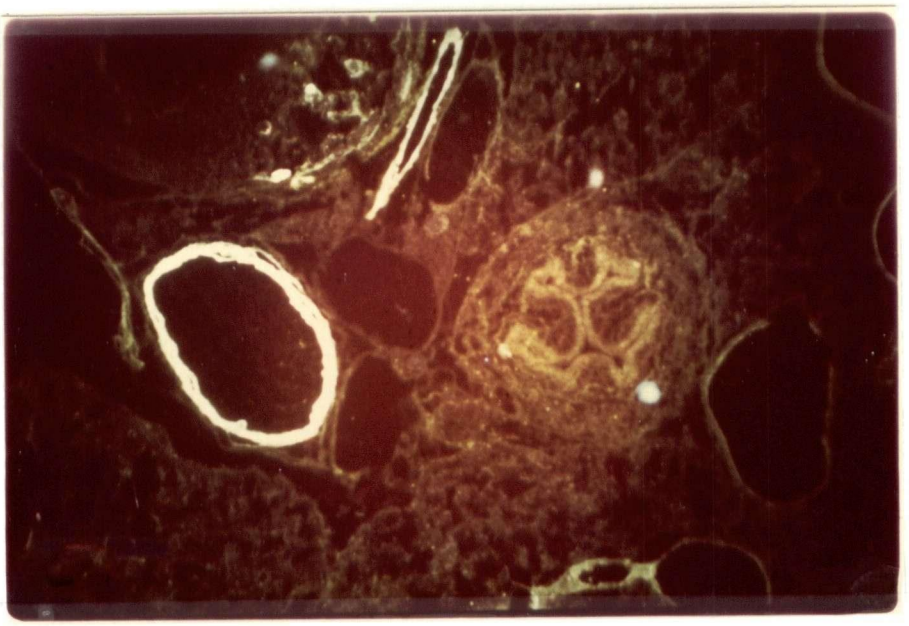


PLATE 15

Cross-section of Powassan virus infected mouse lung two to three days after aerosol inoculation, showing Powassan virus specific fluorescence in lung epithelial cells. Stained with FITC conjugated Powassan virus specific rabbit gamma globulin, magnification 640 x.

PLATE 16

Cross-section of Powassan virus infected mouse lung five days after aerosol inoculation. Note widespread Powassan virus specific fluorescence. Stained with FITC conjugated Powassan virus specific rabbit gamma globulin, magnification 640 x.



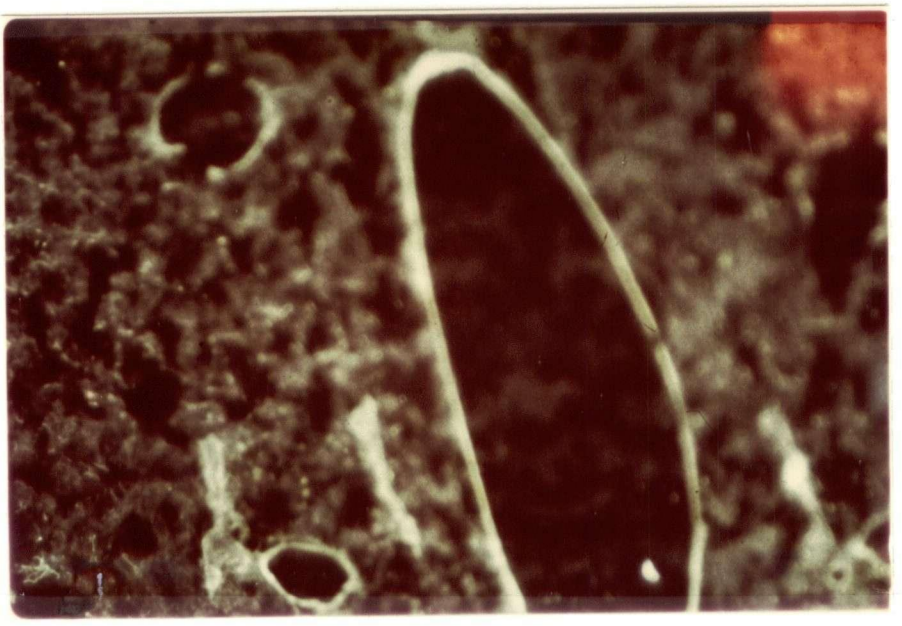


PLATE 17

Sagittal section of Powassan virus infected mouse brain showing cells of the choroid plexus of the mouse as they protrude into the fourth ventricle of the brain close to the cerebellum. Stained with FITC conjugated Powassan virus specific rabbit gamma globulin, magnification 160 x.

PLATE 18

Sagittal section of Powassan virus infected cells of the choroid plexus of the mouse three days after aerosol inoculation. Note Powassan virus specific fluorescence of cuboidal cells of the choroid plexus. Stained with FITC conjugated Powassan virus specific rabbit gamma globulin, magnification 640 x.

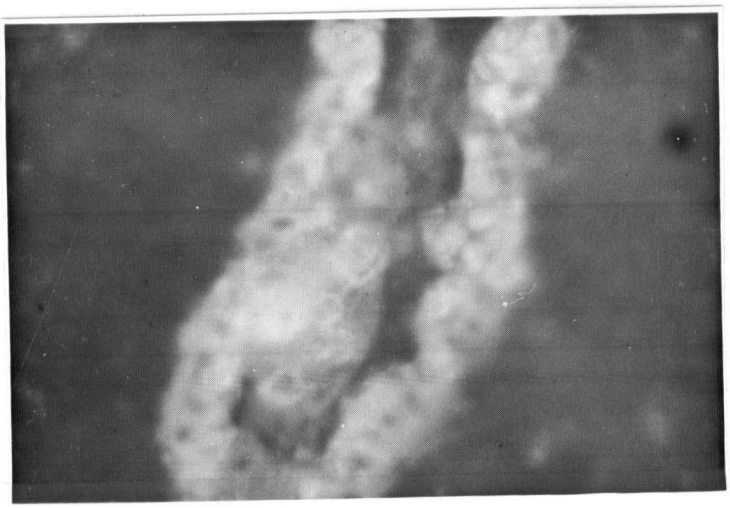
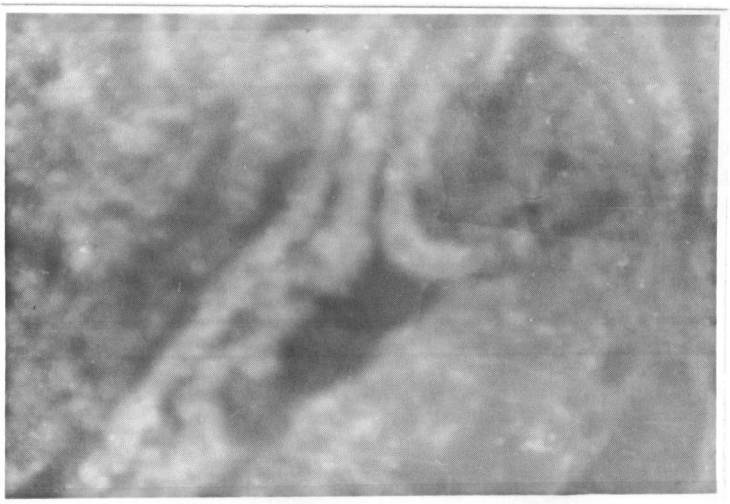


PLATE 19

Sagittal section of Powassan virus infected cells of the choroid plexus of the mouse three days after aerosol inoculation, showing Powassan virus specific fluorescence. Stained with FITC conjugated Powassan virus specific rabbit gamma globulin, magnification 640 x.

PLATE 20

Sagittal section of Powassan virus infected cells of the choroid plexus of the mouse three days after aerosol inoculation, showing Powassan virus specific fluorescence. Stained with FITC conjugated Powassan virus specific rabbit gamma globulin, magnification 640 x.



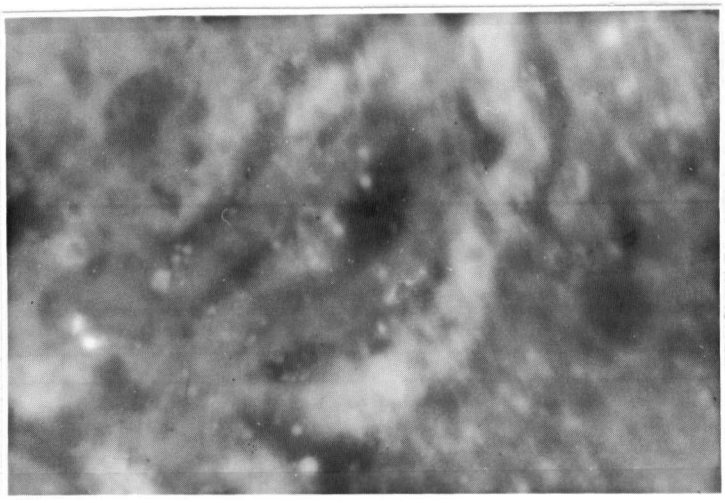
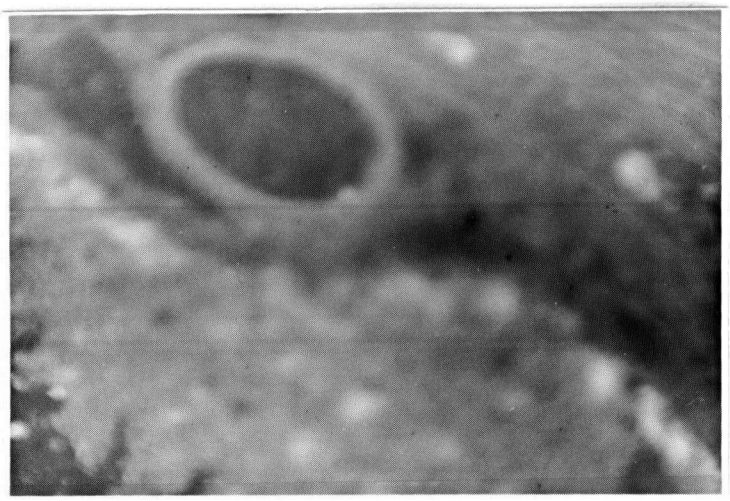


PLATE 21

Saggital section of Powassan virus infected mouse brain at the interfaces between the archnoid and pia mater; the pia mater and cerebral cortex three days after aerosol inoculation. Note points of fluorescence in cells of the cerebral cortex. Stained with FITC conjugated Powassan virus specific rabbit gamma globulin, magnification 640 x.

PLATE 22

Sagittal section of Powassan virus infected mouse brain six days after aerosol inoculation showing widespread Powassan virus specific fluorescence in tissues of the cerebrum. Stained with FITC conjugated Powassan specific rabbit gamma globulin, magnification 320 x.



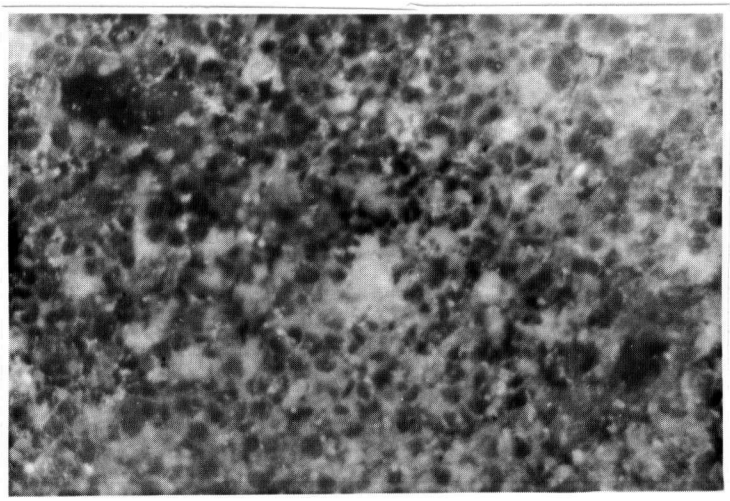


PLATE 23

Electron micrograph of what is believed to be a macrophage of the lung three days after intranasal instillation of Powassan virus, showing what appear to be phagolysosomes structures (arrows) which ordinarily destroy foreign material aspirated into the lung. Stained with osmium tetroxide, magnification approximately 50,000 x.

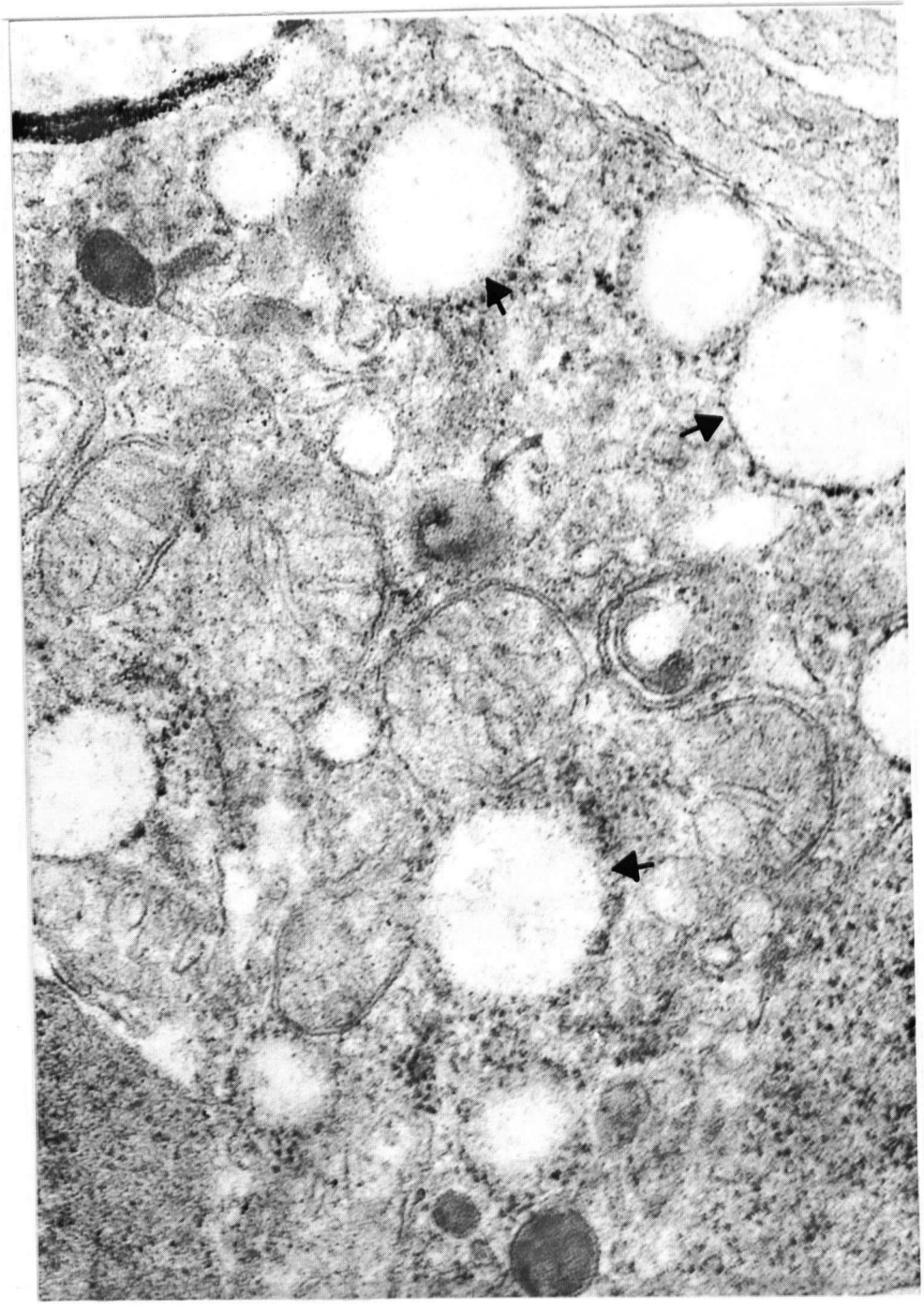


PLATE 24

Electron micrograph of nasal epithelium six days after intranasal instillation of Powassan virus. Note structures which appear to be virus particles (arrows) arranged around vacuoles within the cytoplasm of epithelial cells. Stained with osmium tetroxide, magnification approximately 50,000 x.

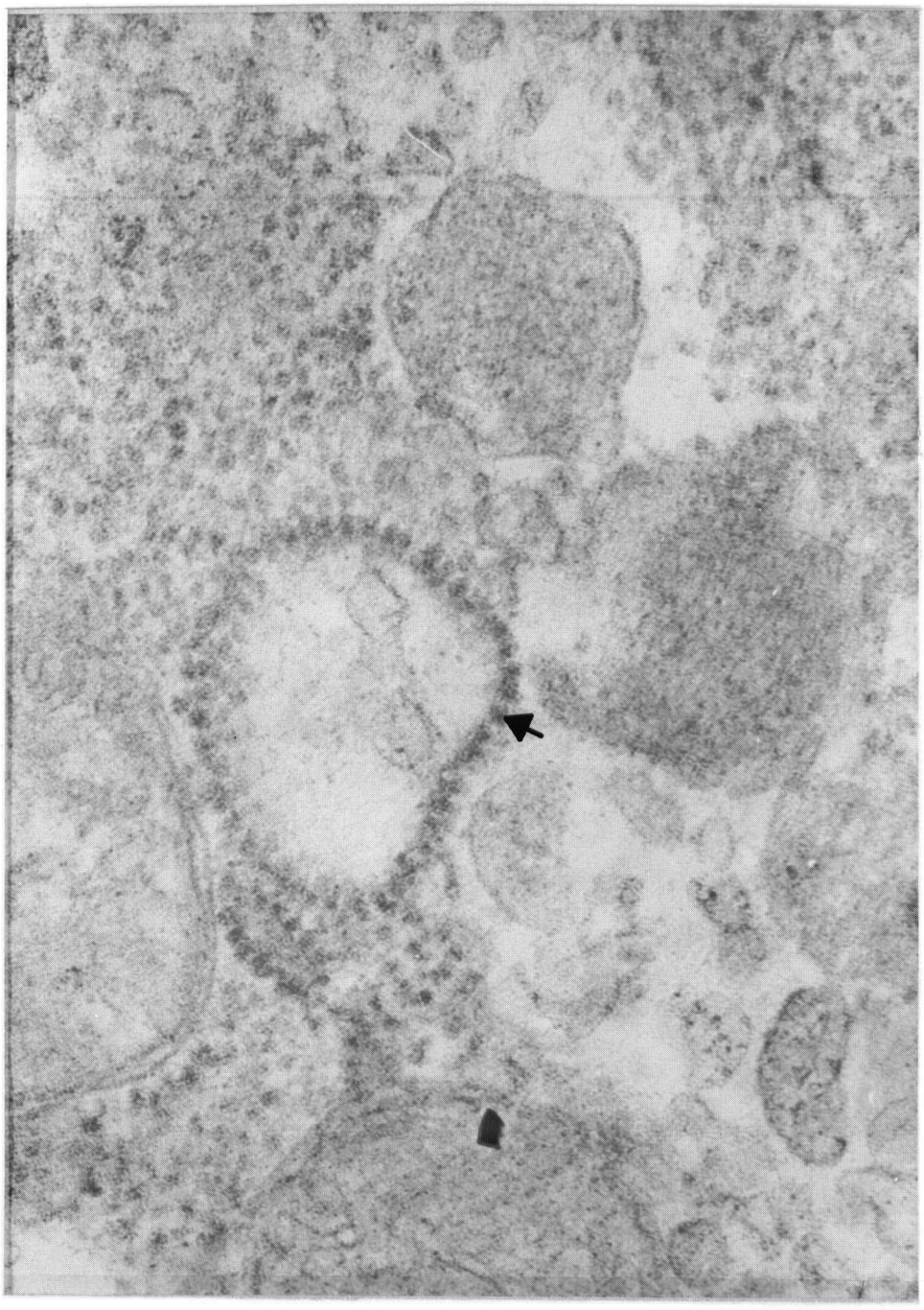


PLATE 25

Electron micrograph of nasal epithelium six days after intranasal instillation of Powassan virus. Note structures which appear to be virus particles arranged around vacuoles within the cytoplasm of epithelial cells. Stained with osmium tetroxide, magnification approximately 50,000 x.

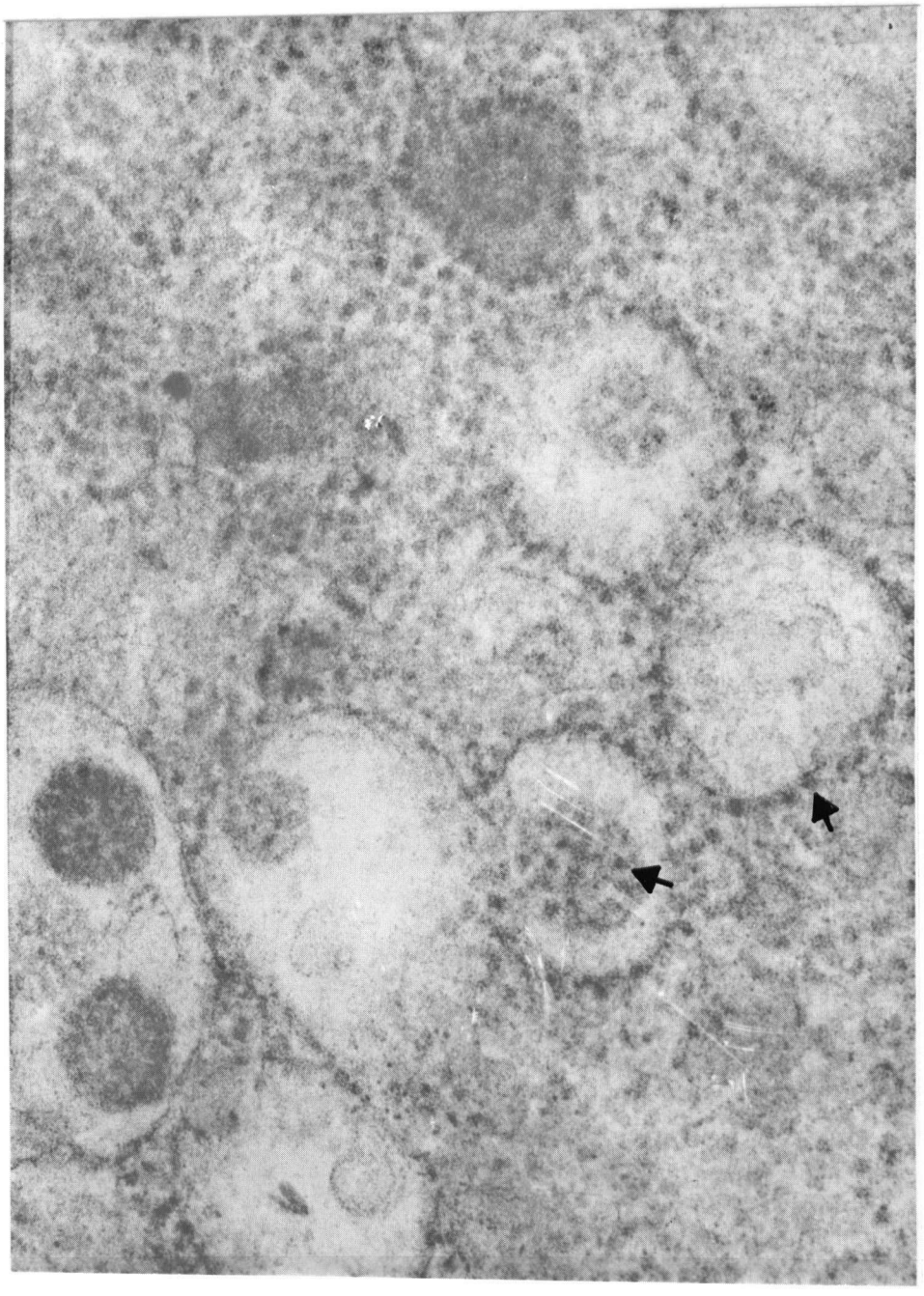
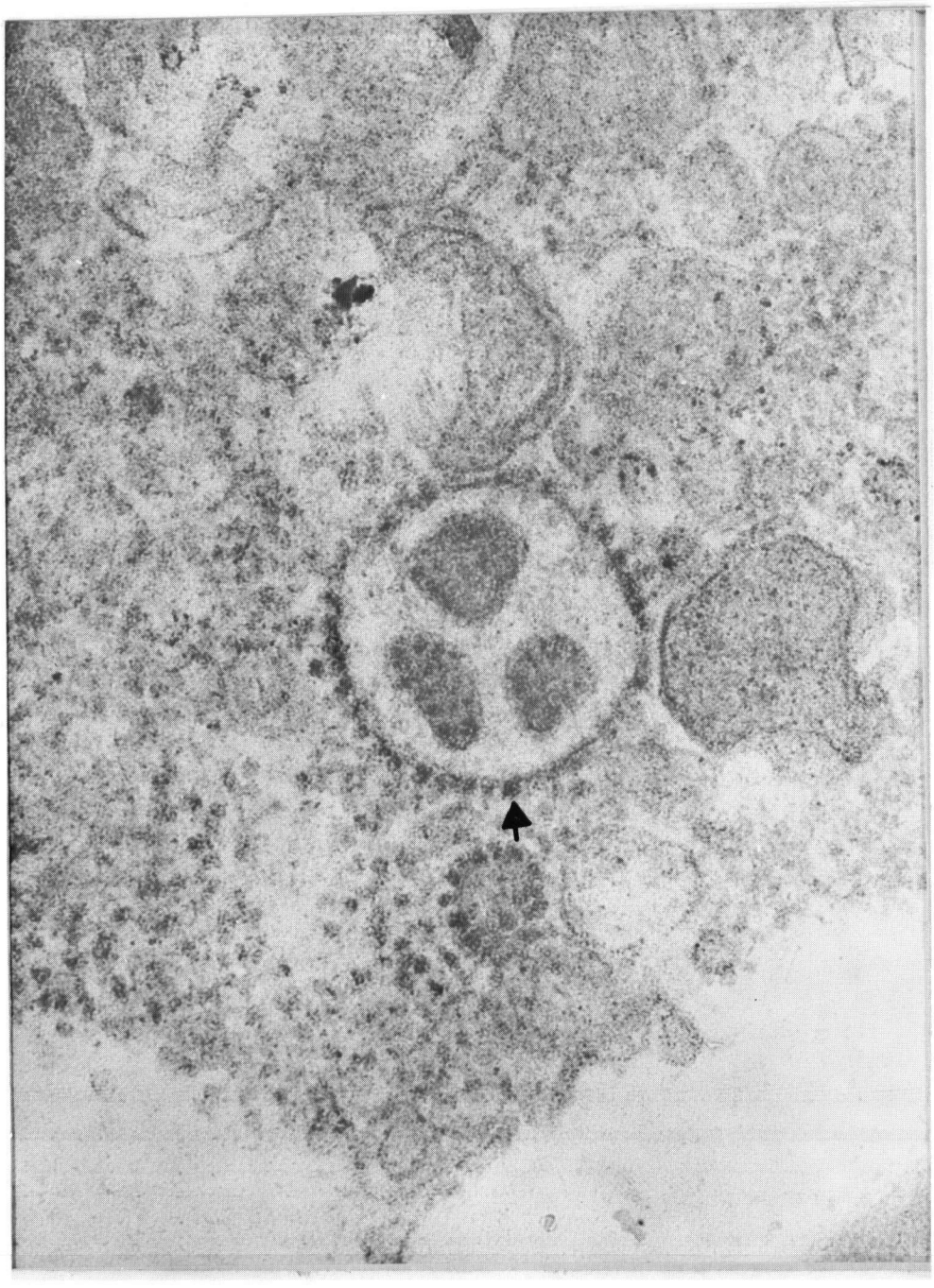


PLATE 26

Electron micrograph of nasal epithelium six days after intranasal instillation of Powassan virus. Note structures which appear to be virus particles arranged around vacuoles within the cytoplasm of epithelial cells. Stained with osmium tetroxide, magnification approximately 50,000 x.



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