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THE NOVY RAT VIRUS
ITS RECOVERY AND CHARACTERIZATION

By

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Several years ago in this laboratory, Dr. F.G. Novy isolated a virus from rats which was maintained by serial passage in normal rats. During the course of passage the virus was presumably lost. The purpose of this thesis is to report the results of studies designed to characterize an infectious agent, recovered by passage of glycerinated blood which had been obtained from rats infected with the above virus, and had been sealed in glass tubes and stored in a laboratory cold room. The existence of these tubes had been unsuspected for a number of years; they were found during changes associated with a reorganization of the laboratory. According to the labels found on the tubes the glycerinated blood mixture had been preserved by Dr. Novy in the period between 1914 and 1918.

The recovered filtrable infectious agent has been named the Novy Rat Virus. Although it was shown to be predominantly neurotropic, causing in susceptible animals the greatest amount of damage in the central nervous system, other organs of the body were involved to a certain degree. The virus caused fatal infection in young rats and mice when inoculated by any route; susceptibility to extraneural inoculation of virus was shown to decrease with increasing age of the animals. Guinea pigs, rabbits, chickens, and the developing chick embryo were susceptible to the lethal effects of the virus.

Methods used to characterize the infectious agent included ultracentrifugation studies which demonstrated that the virus could be sedimented at 42,000 times gravity. Electron microscopic examination of the sediment from allantoic fluid infected with the virus revealed spherical particles measuring 35 millimicrons in diameter. The virus was filtrable through Berkefeld and Seitz filters; its diameter as estimated by filtration through gradocol membranes, was determined to be approximately 18 to 26 millimicrons.

In studying the effect of hydrogen ion concentration on the infectious agent it was found to be most stable when suspended in buffer solutions ranging in pH between 7.3 to 9.7. Experiments designed to determine resistance of the virus to chemical disinfection under different conditions of temperature and exposure showed the virus to be resistant to 0.25 percent formalin for two hours at room temperature; it remained active in chloroform and phenol at 3-5°C for five and seven weeks respectively.

Brain suspensions of the virus resisted heating at 56°C for 30 minutes, but were inactivated at 70°C in ten minutes. The virus retained its infectivity after 11 months storage in the frozen state at -55°C and for the same length of time when mixed with glycerine and stored at 4°C.

Serological studies, which included virus neutralization and cross protection tests, indicated the Novy virus to be antigenically distinct from Mengo, Semliki Forest, Venezuelan, or western equine encephalomyelitis viruses.

Results of intracerebral cross protection tests showed that some immunological relationship exists between the Novy virus and eastern equine encephalomyelitis virus, since mice vaccinated against the Novy virus resisted 30,000 LD₅₀ of the eastern virus given intracerebrally. However, homologous challenge revealed a resistance index of 3,200,000 indicating a significant quantitative difference between the two viruses.

On the basis of its pathogenicity for experimental animals, its immunological reactions so far studied, and the lesions which it induces, the Novy virus is believed to be a new virus, significantly different from other known viral agents, but with some serologic relationship with the eastern equine encephalomyelitis virus.

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THE NOVY RAT VIRUS ITS RECOVERY AND CHARACTERIZATION

INTRODUCTION

Approximately forty years ago Dr. F. G. Novy isolated a virus from spirochete-infected rats which he maintained in the laboratory by serial passage in normal rats. During the course of passage, the virus was presumably lost and for many years attempts to reisolate the new infectious agent from laboratory rats failed.

Recently some glass tubes which, according to the labels, contained glycerinated blood from rats infected with the virus were found by Dr. W. J. Nungester. Apparently these had been prepared and stored by Dr. Novy in the period between 1914 and 1918. The purpose of this thesis is to report the results of studies designed to characterize an infectious agent recovered by passage of the stored blood in rats, and to establish physical, chemical, and immunological relationships, if any, of the recovered agent to known viruses.

HISTORY

The study of viruses as we know them today may be said to have had its beginning in 1892, when Iwanowski (1) reported that the juice of tobacco plants infected with mosaic disease retained its infectivity even after passage through a Berkefeld filter. Since Iwanowski's observation, the term "filtrable viruses" has been used to designate certain transmissible agents which produce pathological conditions in plants, insects, fish, birds, and mammals.

The first animal disease shown to be induced by a filtrable ultramicroscopic agent was reported in 1897 by Loeffler and Frosch (2). In studying the problem of immunity to foot-and-mouth disease, they attempted to free vesicular fluid or "lymph" from corpuscular elements by passing it through Berkefeld filters. The filtrates were found to be as typically infectious in cattle as was unfiltered material. They first considered the possibility of the filtrate containing a toxin, but later they devised a unique system of titration and showed that the disease was due to a multiplying agent. It is interesting to note that the first animal virus shown to be filtrable is also considered to be the smallest of the presently known mammalian viruses. There is little doubt that the reported findings of Loeffler and Frosch alerted contemporary investigators, and gave impetus to the search for new filter-passing agents.

At the turn of the century the dreaded disease yellow fever had gained great importance. In 1900 a United States Commission was appointed to study the transmission of this infection in Cuba. The notable achievements of the American workers Reed, Carroll, Agramonte, and Lazear (3,4) in establishing the mode of transmission by means of mosquitoes is

a landmark in medical science. Among other important findings, the commission showed that the blood of patients was infective during the first three days of fever, and that the infecting agent was able to pass through a Berkefeld filter. Filtrable disease-producing agents soon gained the attention of many notable scientific investigators throughout the world.

In South Africa an infectious disease of horses and mules characterized by edema of the subcutaneous tissues and hemorrhage of the internal organs had been known for a hundred years. However, it was not until 1900-01 that McFaydean (5,6), in attempting to determine the etiology of the disease, diluted infected blood with saline and filtered it through Chamberland and Berkefeld filters. The resulting bacteria-free filtrates produced typical infections when injected into susceptible horses. A valuable contribution, made as a result of studying this disease, was a prophylactic method of immunization (7,8). The procedure consisted of the injection of large quantities of immune serum intravenously, together with small quantities of virulent blood subcutaneously, followed by a second serum injection after the appearance of the febrile reaction. An interesting review of African horse sickness covering various aspects of the disease was made in 1921 (9).

In other parts of the world at about the same time, interest was centered on a bovine disease known as Rinderpest or "cattle plague". This disease has for many years caused great losses in Asia and Europe. At one time the malady was thought to be of spontaneous origin. Its etiology remained a mystery until 1902, when Nicolle and Adil-Bey (10) demonstrated that the causative agent of this disease could pass through porcelain filters and retain its infectivity. Since 1921 cattle plague

has been reported in various parts of Africa and Asia, and at one time was the most important cattle disease in the Phillipine Islands. The disease does not exist in the United States.

In 1902 two separate diseases of fowls, fowl pox and fowl plague, were being investigated. The former is a contagious epizootic disease in which epithelial nodules appear upon the skin, especially on the comb and wattles. Marx and Sticker (11), investigating fowl pox, demonstrated that the etiologic agent of this disease readily passed through a porcelain filter. Fowl plague, on the other hand, is an acute infectious disease characterized by petechial hemorrhages disseminated throughout the body, and is frequently fatal. Centanni (12) showed that experimental infections may be produced in chickens by the inoculation of filtered blood, or small amounts of nasal or cloacal secretions.

In 1903 Borrel (13), studying an acute infectious disease known as sheep pox, noted the occurrence of distinctive cellular bodies in the papulo-vesicular eruptions involving the mucous membranes of the eyes and respiratory passages. Investigating further, he found that infection could readily be transmitted to normal sheep by inoculating Chamberland filtrates of the serous fluid contained in the loculated vesicles. The following year Schweinitz and Dorset (14) reported an acute septicemic disease of swine known as hog cholera. The authors observed that the inciting agent could be transmitted from diseased to healthy animals by filtered blood. However, it was further pointed out that natural infection in hog cholera appears to occur by the ingestion of infective excreta, especially material contaminated with the urine of sick animals (15).

Perhaps one of the most world-wide and dreaded virus diseases is rabies. In 1844 Pasteur (16) and his colleagues found that the virus

has a special affinity for the central nervous system. The development of a rabies vaccine by Pasteur following his first observations on the pathogenesis of the disease, will remain forever a classical achievement in the science of experimental medicine. However, it was not until 1903 that Remlinger (17) demonstrated the filtrability of the rabies virus. The same year, Negri (18) discovered the cytoplasmic inclusion bodies which now bear his name and are considered by many to be pathognomonic for the disease. Negri's interest in so-called "inclusion bodies" led him to the study of vaccinia virus, which also produces a characteristic intracytoplasmic inclusion. Further, he showed that infected calf lymph could pass through coarse Berkefeld filters, the filtrates of which produced a true vaccinal keratitis on the cornea of rabbits, and characteristic lesions on udders of cows (19).

A virus disease of equines characterized by septicemia and a gradual destruction of red blood cells is known as infectious anemia of horses, or "swamp fever". The etiology of this disease was disputed for many years before 1904, when Vallée and Carré (20) demonstrated the true nature of the inciting agent. Saline-diluted blood passed through Chamberland candles and injected into susceptible horses produced characteristic infections. Approximately a year later, Carré (21) became interested in distemper, which is an acute infectious disease of young carnivorous animals. Carré demonstrated the virus etiology of this disease when he was able to initiate infection in young puppies with bacteria-free filtrates of catarrhal exudates from infected animals.

Numerous workers have long suspected that certain tumors might be caused by infectious agents (22). Molluscum contagiosum is a contagious epithelial neoplasm of man which is characterized by small tumors, which

are usually the color of normal skin, but which may become red and "warty" in appearance. In 1905, Juliusberg (23) succeeded in producing experimentally in human volunteers typical tumors with infected tissue suspensions passed through Chamberland filters. Two years later, Cuiffo (24) proved the virus of human warts, or verrucae, to be filtrable through a Berkefeld candle.

Two febrile diseases which occur in tropical or subtropical countries, and appear very similar clinically, are dengue fever and Pappataci or "sandfly fever". In 1907 Ashburn and Craig (25,26) showed that dengue fever was transmitted by mosquitos, and that the intravenous inoculation of small amounts of filtered blood from infected patients produced the disease in susceptible humans. The following year, Doerr (27) proved the causal agent of sandfly fever could pass through the pores of a Reichel earthenware filter. Later Birt (28,29) filtered infective blood through Chamberland candles, and reproduced the disease in a volunteer by inoculating the filtrate.

History of the Novy Virus

In 1907 Dr. F. G. Novy obtained a strain of spirochete isolated by Robert Koch from relapsing fever patients in Africa. Dr. Novy maintained it by serial passage in rats. At first there was no difficulty in doing this, but approximately two years later it was found that the rats were dying in 2 to 4 days after inoculation, and showed very few spirochetes in their blood. It became increasingly difficult to maintain the strain, and it appeared that it might be lost. In an attempt to save the spirochaetal strain, the rat blood was injected into an albino mouse. Two days following the injection the animal was bled,

and on microscopic examination the blood was shown to contain 2 spirochetes per field. Blood from the infected mouse was then injected into 2 albino rats, which subsequently developed a typical infection with many organisms in the blood. The "purified" strain was thus re-established, and is still being maintained by rat passage. The question then arose as to the nature of the "contaminant" which was lethal for rats. Direct examination of infected rats blood, including stained preparations, failed to reveal any organism other than the spirochete. All attempts by Dr. Novy at cultivation, under aerobic and anaerobic conditions, on a variety of liquid and solid media proved unsuccessful. These findings suggested the presence of a filtrable agent. By further study Dr. Novy and his colleagues demonstrated that injection of Berkefeld filtrates of blood from the animals infected with spirochetes of relapsing fever caused a fatal infection in normal rats inoculated intraperitoneally. In the decade which followed, an extensive study was made of certain of the properties of the filtrable agent. Brief reports were published in 1911 (30,31,32) in which filtration and centrifugation experiments were mentioned, emphasizing the small size of the pathogen. In 1929 reference to the virus was again made by McKinley (33), who described some of the more outstanding properties of the agent.

Experimental results obtained by Dr. Novy and his associates revealed the infectious agent to be extremely small and capable of passing through collodion sacs. Although the actual porosity of these sacs is not known, the concentration of collodion used in their preparation would indicate a very small average pore diameter. Further indications of the minuteness of the virus was obtained when Dr. Novy devised a unique and ingenious method of filtration through agar. This was accomplished

by passing through a Berkefeld filter hot melted agar and allowing it to cool. A filtering agar surface was thus obtained. Despite several such coatings of agar, the virus was often found in the filtrates.

Signs of infection in moribund rats following inoculation of the virus included tremors, hyperexcitability, clonicotonic contractions, convulsions and prostration. Particularly notable was a "bloody urine" and often the eyes and nose of dying rats were covered with a serous exudate; the virus could be demonstrated in both urine and exudate. Administration of the pathogen by feeding or instillation into the conjunctival sac led to death of several rats tested.

The virus was shown to be readily inactivated by alcohol. Glycerinated blood in sealed glass tubes remained active in the cold room for 227 days, whereas infectivity was not destroyed for 16 days at room temperature or 5 days at 37°C. The virus resisted inactivation at 55°C for 60 minutes or 60°C for 45 minutes. Dilutions of the virus prepared in saline solution proved less stable than dilutions made in glass-distilled water.

An increased resistance with age to the lethal effects of the virus was observed in rats, and young mice were shown to be more susceptible to intraperitoneal inoculation than were older animals. The M.L.D. of the rat virus was often 10^{-9} and sometimes reached a titer of 10^{-11} . Recently a comprehensive review covering the original isolation of the Novy rat virus and its characteristic properties has been prepared and is in press (34). In these early studies, the virus was maintained by serial passage in young rats, or infected blood mixed with an equal part of glycerine was hermetically sealed in glass tubes and stored in the cold room until needed. In the course of passing the virus in rats the

agent was accidentally "lost" and glycerine-preserved blood prepared by one of Dr. Novy's colleagues failed to produce infection in rats. Approximately 40 years after the supposed loss of the virus, some hermetically sealed glass tubes were found in the laboratory. These apparently contained glycerinated blood prepared and stored by Dr. Novy from virus-infected rats in the period between 1914 and 1918. When found, the tubes were at room temperature, although it is believed that at an earlier period they had been kept in the refrigerator. Although the first impulse was to discard the material, at Dr. W. J. Nungester's suggestion it was decided to investigate the contents of the tubes, and to determine whether the Novy virus which had been preserved for so many years was still active.

EXPERIMENTAL

A. Materials and Methods

Stored Virus. The "stored" virus consisted of 25 hermetically sealed glass tubes labeled as containing glycerinated (1:1) blood from rats infected with the Novy virus. The tubes were divided into groups of 5 each, washed with soap and water, and immersed in 70% ethanol for 1 hour. The contents of the 5 tubes of each group were then pooled and ground in a mortar, using as abrasive portions of the glass tubes on which particles of blood had dried during the sealing process. A portion of the suspension was cultured on blood agar and in thioglycollate broth with negative results except for 1 pool, which was discarded.

Animals Used. Rats used for the first of the current studies were 3-4-week-old males weighing from 45 to 60 grams from an albino strain raised in the Department of Pathology at the University Medical School. For subsequent experiments rats were obtained from departmental breeding colonies started from this strain.

Mice used in the experiments, unless otherwise stated in the protocols, were 3-4-week-old animals and were of a Webster strain raised in the Department of Bacteriology.

Virus Suspensions. Virus suspensions were prepared from brains of virus-infected mice or rats. The infected tissue was ground in a mortar using alundum as an abrasive and sufficient inactivated, undiluted rabbit serum to make a 10 or 20% suspension. The rabbit serum was obtained by cardiac puncture; the blood was allowed to clot and then stored at 4° to 6°C overnight. The next morning the serum was removed, heated at 56°C for 30 minutes, and filtered through a Seitz EK filter pad. The filtered

serum was then stored in a "deep freeze" until needed. Suspensions were centrifuged at approximately 2000 revolutions per minute in an International Refrigerated Centrifuge for 5 minutes to sediment the gross particles. Virus suspensions which were to be stored after centrifugation were distributed in 2.0 ml amounts in screw-capped vials, quickly frozen in a mixture of dry ice and alcohol and placed in the deep freeze. As virus was needed, one or more vials were removed from storage and thawed in a water bath at 37°C just before use. Portions of all suspensions were cultured on blood agar and thioglycollate broth for 48 hours to determine the absence of bacterial contamination.

Inoculation of Animals. Animals inoculated intravenously received 0.5 ml of a given dilution in the tail vein. To avoid leakage, pressure was applied to the tail afterwards until there was no bleeding; it was then wiped with diethyl ether and collodion applied to the point of inoculation.

Intracerebral inoculations were made under light ether anaesthesia, 0.05 ml being the inoculum for rats and 0.03 ml for mice. Intranasal inoculation of rats was performed by dropping 0.05 ml of the suspension onto the external nares of the etherized animal. Intraperitoneal inoculation of 1.0 ml of the above suspension was given without anaesthesia.

Collection and Preparation of Material from Infected Animals. For the collection of feces, rats were placed in cages with wire floors which permitted the material to fall through onto a paper-covered pan. The excreta was emulsified in broth (pH 8.0), centrifuged, and passed through a Seitz EK pad previously saturated with broth.

Urine was obtained by exposing the bladder and aspirating with a sterile needle and syringe. Sterility was determined by culturing in thioglycollate broth.

Since the intracerebral injection of undiluted urine is often toxic for rats, it was first diluted with 2 parts of physiological saline immediately before inoculation.

Blood was obtained from etherized rats by cardiac puncture using heparin (1:500) as the anticoagulant. After exsanguination, the thoracic cavity was opened and the descending aorta cannulated with a blunt needle. Approximately 35.0 ml of sterile saline was then forcefully injected to free the organs of most of the blood within their vessels. The heart, lungs, liver, spleen, kidneys, brain, and spinal cord were removed. Pools of each organ (except parts of the CNS) from 2 animals were washed in cold sterile distilled water and these were then tested for the presence of virus by intracerebral inoculation in mice. The brains of animals inoculated intranasally were sectioned through the cerebral hemispheres to approximate an isolation of the "anterior rhinencephalon" from the remainder of the brain. The anterior rhinencephalon consisted of the olfactory bulbs and approximately one-third of the brain substance immediately behind the bulbs; the remaining portion has been designated "remainder of brain". This technique for sectioning brain has been similarly applied to distribution studies of other neurotropic viruses (35,36).

Virus Titrations. Virus suspensions were titrated intracerebrally in mice using 10% rabbit serum-saline or sterile skimmed milk as the diluent. Tenfold serial dilutions were prepared in the above diluents using separate pipettes for each dilution. During the preparation of dilutions and inoculations of animals all materials were immersed in an ice-water bath. In titrating the virus, 4 to 6 mice were inoculated intracerebrally with 0.03 ml of each dilution. The LD₅₀ titers indicated in the

protocols were calculated by the method of Reed and Muench (37), the original blood or urine being regarded as 0 dilution and the 10% suspension of tissues as the 10^{-1} dilution. The total amount of virus in a given organ was calculated in the following manner: If the LD_{50} titer of a mouse brain suspension, for example, was $10^{-3.3}$, 0.03 ml of the 10% suspension (i.e., 10^{-1}) contained 2,000 (antilog of 3.3) LD_{50} of the virus. Then if the entire brain weighed 0.4 gram (suspended in 4.0 ml for the 10^{-1}), the amount of virus in the entire brain was

$$\frac{2,000 \times 4.0}{0.03} = 267,000 LD_{50}$$

Inoculation of Developing Chick Embryos. Fertile eggs obtained from a local source were incubated at $38^{\circ}C$ for 10 days prior to inoculation. The presence and location of the embryos was determined by transillumination. Eggshells were disinfected with a 7.0% tincture of iodine which was washed off with ether. A small hole was made in the shell by means of a sterile drill and 0.03 ml inoculated into the desired area of the embryo. Following inoculation, the hole in the shell was sealed with melted paraffin and the eggs were further incubated at $38^{\circ}C$. In studies of the distribution of virus throughout the eggs, the amniotic and allantoic fluids were aspirated by using a needle attached to a syringe. Titrations were done by inoculating decimal dilutions of the virus onto membranes of 10-day-old embryos and using 3 embryos for each dilution unless otherwise stated. Test eggs were kept under observation for 72 hours, although results were usually evident in 24 to 48 hours. Cultures were taken periodically to check for possible contamination and routine inoculations were made in mice to verify the presence of virus.

Neutralization Test in Mice. The technique used in the neutralization tests is that recommended by the Neurotropic Virus Commission in 1942 (38). The method of using undiluted serum with serial virus dilutions was chosen, since this is the most commonly employed for comparative studies, has fewer technical difficulties, and is relatively easy to standardize. Hyperimmune monkey sera used in these experiments were obtained through the courtesy of Dr. John Bugher and Dr. Kenneth C. Smithburn, and included Western equine encephalomyelitis (WEE), eastern equine encephalomyelitis (EEE), Semliki Forest (SFV), and Mengo antisera. Venezuelan equine encephalomyelitis virus (VEE) antisera (guinea pig) was kindly supplied by Dr. Roy Fothergill.

Tenfold serial dilutions of the virus were prepared in an ice bath using 10% inactivated rabbit serum in saline (pH 8.2) as diluent. From each of the dilutions to be tested, 0.2 ml was placed in each of 2 tubes. To one series of tubes (control) was added 0.2 ml of 10% normal inactivated rabbit serum. To the test series 0.2 ml of the undiluted hyperimmune serum to be tested was added. The tubes were incubated in a 37°C water bath for 2 hours and placed in an ice bath prior to injection. Five mice were used for each dilution tested. The control was tested with virus dilutions from 10^{-6} to 10^{-9} and test sera with dilutions from 10^{-4} to 10^{-7} .

Cross Protection Tests. The immunological relationships between viruses are often detected by the use of "cross resistance" or "cross protection" tests. In this procedure vaccinated animals are tested for resistance by the intracerebral or intraperitoneal routes.

Vaccine. Vaccine consisted of a 10% suspension from a pool of ten mouse brains. The virus was inactivated by the addition of 0.5% formalin (Merck USP) and the mixture kept in the refrigerator in glass-stoppered bottles. The material was tested for infectivity before use by intracerebral inoculation into mice. Mice 60 to 75 days old were used for the intracerebral protection tests. They were immunized against each of the viruses studied (Novy, EEE, and WEE) by means of 2 injections of formalinized vaccine followed by 3 injections of active virus (39). The schedule of vaccination is summarized below.

VACCINATION SCHEDULE FOR PROTECTION TESTS

Day	Material 10%	Amount, ml.	Route	Dilution
1	Vaccine	0.25	IP*	Undiluted
3	Vaccine	0.25	IP	Undiluted
10	Active	0.25	IP	10 ⁻⁴
15	Active	0.5	IP	10 ⁻⁴
20	Active	0.5	IP	10 ⁻²

* Intraperitoneally

Two weeks after the last inoculation the mice were challenged intracerebrally with 0.03 ml of the test viruses and observed for 21 days. Unvaccinated mice of the same age as the test animals served as controls.

Interpretation of "Resistance" Tests. The degree of protection given by a serum in the neutralization test is determined by the ratio between the 50% mortality titer of the test serum and the 50% mortality titer of the controls. This ratio is designated as the neutralization index.* Similarly, the degree of resistance conferred by a vaccine is represented by the ratio between the 50% mortality titer of the vaccinated mice and 50% mortality titer of the controls; this ratio is known as the resistance index (39).

* Both neutralization and resistance indices express the maximum number of MLD of protection given by a serum or vaccine as compared to the controls, for which the value of the index is always 1.

B. Results

Recovery of the Novy Rat Virus. Since the original isolation of the Novy virus was made during the serial passage of Spirochaeta novyi (40), it was considered advisable to inoculate the test animals with a spirochete prior to the inoculation of the stored blood. Further, it was believed that the spirochete might in some way alter the resistance of the rats, and thereby render them more susceptible to the stored virus.

For these tests 25 rats were divided into 5 groups. Each rat was injected intraperitoneally with 1.0 ml of heparinized Spirocheata novyi infected rat blood containing approximately 25 spirochetes per oil immersion dark field. This was followed in 24 hours by 1.0 ml intraperitoneally and 0.05 ml intracerebrally of undiluted stored blood from 1 of the 5 pools suspected of containing the Novy rat virus. Despite the fact that some of the animals showed convulsions following intracerebral inoculation of the ground-glass blood mixture, all survived the injection. Control animals of the same age and weight received the same dose of Spirochaeta novyi infected blood intraperitoneally, and 0.05 ml of a mixture prepared from normal rat blood and ground glass intracerebrally.

Serial Passage of Blood from Rats Showing Signs of Infection Following Injection of the Blood Mixture. Results of the first passage appear in Table 1.

From Table 1 it may be seen that in group 1, one animal showed signs of infection as early as the third day, and two rats had died by the sixth day following inoculation, whereas all control animals survived. In group 2, two rats were showing signs of infection on the fifth day, and in group 5, one rat was sick on the fourth day. It should be pointed

TABLE 1
FATE OF RATS FOLLOWING INJECTION
OF STORED BLOOD

Rat	Group					Controls
	1	2	3*	4	5	
1	6d	5+		5+	5+	S
2	3+	5+		15d	S	S
3	11d	8d		21d	4+	S
4	6d	8d		S	5+	S
5	S	S		S	S	S

* Group discarded due to bacterial contamination.

d = day of death

+ = signs of infection

S = survived

out that in group 4 the incubation period of one rat was prolonged for 21 days, and that at least one rat survived in each of the 4 groups used in the experiment.

Signs of infection consisted of ruffled fur, listlessness, and apparent lack of interest in food and water. As the disease progressed, rats developed tremors, clonicotonic contractions, and convulsions. Some rats showed a marked hyperexcitability. Reddish-brown encrustations was common around the nose and both front paws. A characteristic hematuria in rats dead of infection with the Novy virus has been described by Dr. Novy (34). Hematuria in certain of the rats following intraperitoneal injection of the recently isolated virus has been inconstantly observed. One or two days after the first signs of infection, animals became prostrate and death rapidly ensued.

Both rats in group 2 showing signs of infection were lightly etherized, and 2.0 ml of blood was removed from each animal by cardiac puncture. The heparinized blood from rats 1 and 2 was then pooled, shaken gently, and 0.1 ml placed on a blood agar plate and incubated at 37°C for 48 hours. No growth was obtained. The rats were sacrificed and the brains were aseptically removed, pooled, and a 10% suspension prepared.

Each of five rats, 3 to 4 weeks old and weighing approximately 50 grams, was injected intraperitoneally with 1.0 ml of the heparinized blood from rats 1 and 2. Darkfield examination of this blood, prior to injection, showed the presence of several spirochaetal forms per field. Each of the rats was then injected intracerebrally with 0.05 ml of the 10% brain suspension from rats 1 and 2.

Serial passage was made 5 times, pooling portions of blood and brains of each of two rats showing signs of infection. Prior to each

passage, the blood collected by cardiac puncture was examined for the presence of spirochetes by darkfield examination and cultured for possible bacterial contamination. Following each passage of blood and brain suspension, three or four of the rats from each group became prostrate and died of infection, or were sacrificed for further passage material.

Filtration Experiments. After it had been demonstrated that injection of blood and brain suspensions from spirochete infected rats was uniformly fatal for normal rats of the same age and strain, an experiment was designed to determine whether death of the animals was due to the presence of the spirochetes or to a virus which might have been present in the original stored glycerinated blood.

On the fifth serial passage, two rats which 5 days previously had received 1.0 ml of undiluted rat blood containing spirochetes and 0.05 ml of pooled brain suspension and which had developed signs of infection were sacrificed by bleeding from the heart. Approximately 2.0 ml of heart's blood was collected from each animal and pooled. Darkfield examination of the heparinized blood showed the presence of several spirochetes per field. The blood was then centrifuged at 1500 r.p.m. for 5 minutes. The plasma was removed and passed through a Berkefeld (W) filter with aid of suction. The filtrate showed no spirochetes on darkfield examination, and inoculation of 0.1 ml produced no growth on a blood agar plate. However, approximately 1.0 ml of the spirochete-free filtrate injected intraperitoneally into each of three 40-gram rats produced typical signs of infection in two of the rats as early as the fourth day, and one animal died on the fifth day. Darkfield examination of heart's blood from these animals failed to demonstrate the presence of spirochetes. The infectious agent also passed a Seitz EK filter pad, and produced death of 3- to 4-week-old rats injected intraperitoneally with the filtrate.

Control Experiment. The passage of blood or tissue suspensions in series from one animal to another is a procedure which must be undertaken with a full realization of the many possible pitfalls. The presence of latent infectious agents which are made active by repeated animal passage, as well as the possibility of an active infection in one or more animals, must be considered. In order to investigate the possibility of a latent infectious agent in the stock of laboratory rats used in these experiments, the following procedure was carried out. For these tests, 25 rats of the same age, sex, and weight as those used in the previous experiments were divided into 5 groups of 5 animals each. They were injected intraperitoneally with 1.0 ml of heparinized Spirochaeta novyi infected rat blood containing approximately 30 spirochetes per darkfield. The spirochetes used were from the same source and strain as those employed in the experiment from which the Novy virus was re-isolated. In order to produce an irritation comparable to that produced by the administration of old blood and ground glass, the following technique was employed. A 20-ml portion of citrated human blood, which had been collected approximately 7 months before and stored in the refrigerator, was placed in a mortar containing glass particles, and the mixture ground. Each of the 25 rats, which had received the spirochete infected rat blood 24 hours previously, was inoculated with the citrated-human-blood-ground-glass mixture, 0.05 ml intracerebrally and 1.0 ml intraperitoneally. The rats were observed for 60 days.

At 4 days following intraperitoneal and intracerebral inoculation of the blood-glass mixture none of the rats were showing any evidence of infection. At this time, 2 rats from group 1 were selected at random and exsanguinated by cardiac puncture. Approximately 2.0 ml of heparinized

blood was obtained, pooled, and examined by darkfield illumination for the presence of spirochetes. Several spirochetes were noted per field. The brains of the 2 sacrificed rats were aseptically removed, and a 10% suspension was prepared in cold sterile saline. Each of 3 normal rats was then inoculated intracerebrally and intraperitoneally as described in the original isolation experiment. The collection of blood, the preparation of brain suspension, and the inoculation of normal rats was conducted in exactly the same manner with the four remaining groups. Even though the above procedure was repeated through 5 serial passages, none of the inoculated rats showed any apparent evidence of virus infection.

Gradocol Membrane Filtration. After it had been shown that the Novy virus would pass through Berkefeld and Seitz filters, the next step was to determine the size of this virus more accurately by filtration through graded collodion membranes. Collodion membranes used in these studies were obtained from two different sources,* and had average pore diameters (A.P.D.) ranging from 85 to 50 millimicrons. Membranes were sterilized by placing them in cold glass-distilled water and steaming for 1 hour at 100°C. The A.P.D. values were calculated from data obtained by measuring the time required for a known amount of distilled water to flow through a membrane of known area and thickness. Knowing the rate of flow (R.F.W.) and the pressure producing the flow, it is possible to calculate the A.P.D. (41). All filtrations were conducted under a positive nitrogen pressure of 100 cm of mercury. The effective filtration area of the membranes was about 4 cm², and the amount of filtrate collected through such an area varied from 5 to 8 ml.

* One series of membranes was purchased from St. Mary's Hospital, London, England. The others were kindly supplied by Dr. Evelyn Tilden, Northwestern University.

Brains of mice showing signs of infection were used as a source of virus. In preparing material for filtration, 10 brains were ground and suspended in a diluent of 20% inactivated rabbit serum in saline (pH 8.2). The suspension was then centrifuged and passed through a Seitz EK filter pad. The LD₅₀ titer of the filtrate was 10^{-7.8}. Portions of this stock filtrate were passed through a series of collodion membranes of varying porosities. In order to reduce the extent of adsorption of the virus by the membranes, 2.0 ml of the 20% rabbit serum in saline were passed through each membrane prior to filtration of the virus. The presence of virus in the filtrates was determined by intracerebral injection of mice, 4 animals of varying ages being used for each filtrate.

Results of the filtration experiments are summarized in Table 2. As seen from this table, the virus passed through all membranes with an A.P.D. greater than 55 millimicrons, while membranes with a smaller A.P.D. gave uniformly negative results. However, the amount of virus passing through membranes between 60 and 55 millimicrons appeared to be greatly diminished. In four subsequent experiments conducted with membrane No. 6, which had a calculated A.P.D. of 55 millimicrons, one series gave entirely negative results; whereas in another experiment only 1 of 4 mice injected died. In the remaining two experiments 3 of 4 mice died following injection of the membrane filtrate. The filtration end point in these experiments, therefore, seems to be approximately 55 millimicrons.

It should be pointed out that collodion membranes have a strong adsorptive affinity for proteins, and in filtration experiments usually little protein passes through a membrane before this affinity is satisfied. This adsorption naturally reduces the diameter of the pores, the extent being dependent on the amount of cohesion of protein molecules.

TABLE 2
GRADOCOL FILTRATION EXPERIMENTS USING
NOVY VIRUS INFECTED MOUSE BRAINS

Membrane No.	Average pore diameter, millimicrons *	Amt. of Filtrate Collected, ml.	Results in mice inoculated with dilutions of filtrate**				
			10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵
Seitz			4/4	4/4	4/4	4/4	2/4
1	85	5	4/4	4/4	3/4	1/4	0/4
2	75	7	4/4	4/4	3/4	0/4	0/4
3	70	6	4/4	4/4	3/4	0/4	0/4
4	65	5	4/4	3/4	1/4	0/4	0/4
5	60	8	3/4	2/4	0/4	0/4	0/4
6	55	5	3/4	1/4	0/4	0/4	0/4
7	50	7	0/4	0/4	0/4	0/4	0/4

* Calculated to the nearest whole number.

**In this and subsequent tables, the numerator represents the number of mice that succumbed to infection; the denominator, the number of mice used in the test.

taking place in the capillary channels of the membrane. In view of this phenomenon, Elford (41) has estimated that in filtration experiments with membranes of greater porosity than 10 millimicrons, a particle must have a diameter only from one-half to one-third of that of the pore in order to traverse it. Since the Novy virus was found to pass collodion membranes with an A.P.D. of 55 millimicrons, but was completely held back by those of 50 millimicrons, the application of Elford's principle to the above experiments indicates the probable size of the Novy virus to be between 18 and 26 millimicrons in diameter.

Multiplication and Spread of the Novy Virus in the Rat Following Various Routes of Inoculation. Experimental encephalitis due to a virus etiology involves many factors, some of which are well recognized but nevertheless inadequately understood. In this study experiments were designed to learn something about the fate and distribution of the Novy virus from the time it is injected until the animal dies of infection. Such information is essential for a better understanding of any virus disease, and aids in the interpretation of postmortem pathological findings.

Results are summarized in the following four tables, which show the distribution of virus throughout the rat at various intervals after inoculation by different routes.

Distribution of the Virus Following Intranasal Instillation. Following instillation of virus onto the external nares of etherized animals, virus is aspirated through the nose and into the mouth; some of it is swallowed and some reaches the lungs. Reference to Table 3 shows that 4 and 8 hours following nasal instillation of the virus, it was recovered from the lungs. At 24 hours there is evidence of presence of

TALBE 3

DISTRIBUTION OF THE NOBY VIRUS IN SELECTED TISSUES
OF RATS AT VARIOUS INTERVALS FOLLOWING INTRANASAL INSTILLATION

Material Tested	Tested for presence of virus by intracerebral injection in mice. Results in hours following .03 ml of a 10% brain suspension.				
	4	8	24	48	60(prostrate)
<u>Nervous</u>					
Anti.Rhinen.*	0/3	0/3	2/4	3/4	4/4
Rem. Brain**	0/3	0/3	0/4	3/4	4/4
Sp. Cord	0/3	0/3	0/4	1/4	3/4
<u>Blood and Viscera</u>					
Blood	0/3	0/3	0/4	2/4	3/4
Lungs	3/3	2/3	1/4	2/4	1/4
Heart	0/3	0/3	0/4	2/4	2/4
Liver	0/3	0/3	0/4	0/4	1/4
Spleen	0/3	0/3	0/4	1/4	2/4
<u>Urinary</u>					
Urine	0/3	0/3	0/3	0/3	0/3
Kidneys	0/3	0/3	0/3	0/3	0/3
<u>Intestinal</u>					
Feces	0/3	0/3	0/3	0/3	0/3

* Ant. Rhinen = "anterior rhinencephalon"

** Rem. Brain = "remainder of brain"

virus in the anterior rhinencephalon but not in the remainder of the brain. There appears to be a decrease in the amount of virus present in the lungs. At 48 hours the amount of virus in the brain was increased and spread to the remainder of the brain and spinal cord. Virus was demonstrated in the heart, blood, liver and spleen for the first time. The presence of virus in these organs at this time is probably due to a hematogenous spread of the pathogen. At 60 hours, virus was present in all materials tested except urine, kidneys, and feces.

Distribution of the Virus Following Intravenous Inoculation.

The course of events following injection of the virus into the general circulation are summarized in Table 4. At 4 hours after intravenous inoculation of virus into the general circulation, none was recovered from the brain or spinal cord. As might be expected, the blood and heart showed the greatest amounts, with trace quantities being recovered from the lungs, liver, and spleen. At 8 hours the brain and spinal cord remained free of detectable virus and the blood and viscera which contained the virus at 4 hours were still positive. Infection of urine, which had until this time shown no virus, caused death of 1 to 3 mice tested. At 24 hours the brain and spinal cord showed an increase in the multiplication of virus, whereas virus had disappeared from the liver but was again detected in the lungs. At 42 hours, brain and cord suspensions prepared from prostrate rats killed all the mice tested. The blood and viscera which contained the virus at 24 hours were still positive, and the kidneys, hitherto negative, became positive.

Distribution of the Virus Following Intracerebral Injection.

It may be seen in Table 5 that 4 hours after injecting virus directly into the central nervous system, none could be detected in any of the materials

TABLE 4

DISTRIBUTION OF THE NOVY VIRUS IN SELECTED TISSUES OF RATS
AT VARIOUS INTERVALS FOLLOWING INTRAVENOUS INOCULATION

Material Tested	Tested for presence of virus by intracerebral injection in mice. Results in hours following .03 ml of a 10% brain suspension.			
	4	8	24	42(Prostrate)
<u>Nervous</u>				
Brain	0/3	0/3	4/4	4/4
Sp. Cord	0/3	0/3	2/4	4/4
<u>Blood and Viscera</u>				
Blood	2/3	1/3	1/4	3/4
Lungs	1/3	1/3	0/4	2/4
Heart	2/3	1/3	0/4	2/4
Liver	1/3	1/3	0/4	0/4
Spleen	1/3	1/3	1/4	2/4
<u>Urinary</u>				
Urine	0/3	1/3	1/3	0/3
Kidneys	0/3	0/3	0/3	1/3
<u>Intestinal</u>				
Feces	0/2	0/2	0/2	0/2

TABLE 5

DISTRIBUTION OF THE NOVY VIRUS IN SELECTED TISSUES OF RATS
AT VARIOUS INTERVALS FOLLOWING INTRACEREBRAL INJECTION

Material Tested	Tested for presence of virus by intracerebral injection in mice. Results in hours following .03 ml of a 10% brain suspension.			
	4	8	24	42(Prostrate)
<u>Nervous</u>				
Brain	1/3	2/3	4/4	4/4
Sp. Cord	0/3	1/3	3/4	4/4
<u>Blood and Viscera</u>				
Blood	0/3	0/3	3/4	2/4
Lungs	0/3	0/3	0/4	1/4
Heart	0/3	0/3	1/4	2/4
Liver	0/3	0/3	0/4	1/4
Spleen	0/3	1/3	2/4	2/4
<u>Urinary</u>				
Urine	0/3	0/3	0/3	1/3
Kidneys	0/3	1/3	1/3	1/3
<u>Intestinal</u>				
Feces	0/2	0/2	0/2	0/2

tested except for a small amount in the brain, which was probably no more than what might be expected as residual of the 0.05-ml inoculum. At 8 hours, 2 of 3 mice injected with the virus died of infection, and the agent had multiplied sufficiently to reach the spinal cord. The presence of a small amount of virus in the spleen and kidneys indicated that some of the virus had found its way into the general circulation. At 24 hours, the amount of virus in the spinal cord and brain appeared to have increased further, and the blood, heart, and kidneys became positive. At 40 hours, brain and cord suspensions of prostrate rats rapidly killed all mice. The blood and viscera were positive and the only place where virus could not be found was in the stools.

Distribution of the Virus Following Intraperitoneal Inoculation.

Results of these tests are summarized in Table 6.

At 4 hours following intraperitoneal inoculation of the virus, none could be detected in the brain or spinal cord, despite the fact that virus was recovered from the blood, heart, and liver. At 8 hours, blood and viscera which contained virus at 4 hours were still positive, with the exception of the liver; on the other hand, the kidneys became negative, and for the first time in the entire experiment the stools were positive. It should be pointed out, however, that the presence of virus in the stool might well have been a result of the route of inoculation rather than of dissemination of the pathogen by other means; moreover, virus was not again demonstrable in the feces either at 24 or 56 hours, when the rat became prostrate. At 24 hours, virus has reached the brain and spinal cord. Little change has occurred in the blood and viscera, although the kidneys and urine have become positive. At 56 hours the C.N.S. was strongly positive. It is interesting to note that the virus

TABLE 6

DISTRIBUTION OF THE NOVY VIRUS IN SELECTED TISSUES OF RATS
AT VARIOUS INTERVALS FOLLOWING INTRAPERITONEAL INOCULATION

Material Tested	Tested for presence of virus by intracerebral injection in mice. Results in hours following .03 ml of a 10% brain suspension.			
	4	8	24	56(prostrate)
<u>Nervous</u>				
Brain	0/3	0/3	2/4	4/4
Sp. Cord	0/3	0/3	1/4	4/4
<u>Blood and Viscera</u>				
Blood	2/3	3/3	2/4	1/4
Lungs	0/3	0/3	0/4	1/4
Heart	1/3	1/3	2/4	1/4
Liver	1/3	0/3	1/4	0/4
Spleen	0/3	2/3	1/4	0/4
<u>Urinary</u>				
Urine	0/3	0/3	1/3	0/3
Kidneys	1/3	0/3	1/3	1/3
<u>Intestinal</u>				
Feces	0/2	1/2	0/2	0/2

content of the blood appears to have diminished, and that the spleen which was positive at 24 hours was now negative.

To summarize the course of events following inoculation of the Novy virus by different routes, it would appear that virus may invade the central nervous system by various pathways. Following nasal instillation, virus was recovered from the anterior rhinencephalon, which is located nearest the nasal and olfactory mucosae, before it was detected in the blood. This would suggest that nasally instilled virus gains entrance into the C.N.S. by first penetrating the neuronal endings of the nasal mucosa, multiplying, and then invading the brain by way of the olfactory tract. If the virus had first invaded the blood stream from the nasal mucosa and then localized in the C.N.S., it could have in all probability been recovered from other parts of the brain at the same time, or perhaps before it was recovered from the anterior rhinencephalon.

The detection of virus in almost all the tissues tested at 48 hours indicates that the agent has multiplied sufficiently in the C.N.S. to spill into the blood stream, and is thus disseminated by way of the general circulation.

After intravenous inoculation of the virus it appears that the blood stream may be "cleared" of virus particles by the reticulo-endothelial system, and that the primary site of multiplication is in the C.N.S. These data do not indicate that multiplication occurs in the viscera or blood vessels, nor is it certain that persistence of the virus in these sites is not due to a low-grade multiplication. The pathological lesions in the C.N.S. of rats dying of encephalitis following intravenous inoculation were widespread and diffuse. It is possible that introduction of virus directly into the general circulation results in the establishment of several foci in the C.N.S. where virus may localize and multiply.

The injection of large amounts of virus directly into the brain results in sufficient multiplication after 8 hours to be detected in the spinal cord. The presence of virus in the spleen at this time suggests that the pathogen has reached the general circulation and is being disseminated by the blood. On the other hand, virus was detected in the blood 4 hours after intraperitoneal injection, but was not demonstrable in the brain until 20 hours later. Following intraperitoneal inoculation it appears that the virus reaches the C.N.S. by way of the blood stream; it is entirely possible, however, that the virus invades the local nerves at the site of inoculation, and proceeds to the central nervous system by means of neuronal pathways.

Pathological Lesions Induced by the Novy Virus.* Pathological changes induced by the Novy virus have been studied in the rat following various routes of inoculation. Although the central nervous system appears to suffer the greatest damage, other organs of the body are involved to a certain degree. In animals studied early following the recovery of the Novy virus, and before its repeated passage, cerebral lesions were characterized by an acute passive congestion of the meninges and brain substance with hemorrhage into the meninges accompanied by a mild leukocytic infiltration, the findings characteristic of an early acute meningitis. With the exception of the liver, the visceral changes in these early studies were not marked. The lungs showed evidence of an acute passive congestion, with areas of patchy emphysema and hemorrhage by diapedesis into the alveoli. A moderate degree of passive congestion was the only change noted in the spleen. Frozen sections of the liver,

* The author wishes to express his gratitude to Dr. Carl V. Weller of the Department of Pathology for help in the study and interpretation of the above sections.

on the other hand, showed a marked acute passive congestion accompanied by cloudy swelling. Fat stain of a frozen section showed degenerative fatty infiltration of the reticulo-endothelial cells of the sinusoids in the intermediate zones of the lobules.

As the virus was rapidly passed in series, the tissue reaction appeared to increase in severity. In the cerebrum karyorrhexis of nerve cells and neuronophagia was apparent, accompanied by advanced chromatolysis of pyramidal cells. Many basophilic granules were seen in pyramidal cell layers, and there were some areas in which active focal gliosis was detected. No inclusion bodies were identified in inspection of routinely stained material. Sections of the spinal cord showed some edema, but changes were much less intense than in the cerebrum. No involvement of the anterior horn cells could be detected.

Sections of kidney were characterized by acute passive congestion, cloudy swelling, and simple necrosis of the epithelium of the convoluted tubules. The spleen was mildly congested and showed marked hyperplasia of reticulo-endothelial cells around the periphery of each follicle with very active hematopoiesis in the splenic pulp.

These findings indicate that following injection of the Novy virus rats developed an acute meningo-encephalitis, accompanied by severe degenerative parenchymatous lesions of the brain, particularly in the pyramidal cell layers. There was an acute passive congestion of all organs, and in the renal tubules there was degeneration of the epithelium.

Experimental Infection and Host Range. Since the albino rat had been the only animal used for passage of the Novy virus, it was considered of importance to determine the adaptability of the virus to other

species commonly used in the laboratory. Included in this group were mice, guinea pigs, rabbits, chickens, and the developing chick embryo.

Mice: Each of five 7- - 10-gram mice was injected intraperitoneally with 1.0 ml and intracerebrally with 0.05 ml of infected rat brain suspension. Only three of the animals showed any evidence of infection. Serial passage of blood and brain suspensions obtained from mice showing signs of infection was continued for five passages. By the sixth passage clinical signs began to increase in severity, and by the seventh passage, infected brain suspensions injected intracerebrally in mice produced a uniformly fatal disease in 48 to 72 hours.

Generally speaking, it may be said that young animals are more susceptible to virus infection than are old animals (42). For example, young rabbits readily succumb to infection with the Borna disease virus, while older animals are quite resistant (43). Similarly, young monkeys are more susceptible to intracerebral injection of poliomyelitis virus, whereas older animals may become insusceptible (44). In order to determine the effect of age on the susceptibility of mice injected with the Novy rat virus, mice of varying ages were employed. The virus used in this and subsequent host-range studies had undergone 12 intracerebral mouse passages, and the LD₅₀ of the virus suspension before storage in the deep freeze was $10^{-9.3}$. The inoculum and route of inoculation in mice are indicated in Table 7.

It may be seen that no appreciable difference in susceptibility exists between young and old mice when the virus is injected intracerebrally or instilled into the nose. The virus appears to be as effective in mice 3 weeks of age as it is in mice 5 months old. It was noted, however, that the incubation period in older mice following intranasal

TABLE 7
SUSCEPTIBILITY OF MICE TO VARIOUS ROUTES OF INOCULATION
WITH THE NOVY VIRUS

Age of Mice in Weeks	Routes of Inoculation				
	IC .03ml*	IP 0.5ml	IN .05ml	IV 0.5ml	IM 0.5ml
3	3/3	3/3	3/3	2/3	3/3
5	3/3	2/3	3/3	3/3	2/3**
7	3/3	1/3	3/3	2/3	1/3
10	3/3	0	2/3	1/3	1/3
15	3/3	0	1/3	1/3	0/3
22	3/3	0	2/3	0/3	0/3

* Inoculum from a 20% suspension of virus infected mouse brain.

** Developed flaccid paralysis

IC = intracerebral

IP = intraperitoneal

IN = intranasal

IV = intravenous

IM = intramuscular

instillation of the virus was sometimes 48 to 56 hours longer than that seen in younger animals.

Resistance of mice to the lethal effects of the virus given intravenously was first observed at about 10 weeks after birth, and 3 mice injected when 22 weeks old were completely refractory to infection. Resistance of mice to intraperitoneal inoculation of the virus was first seen in animals between 5 and 7 weeks old. Insusceptibility to the virus by this route appeared to increase as the animals grow older. Increased resistance of mice to the virus administered by the intramuscular route appeared to begin when the animal was between 7 and 10 weeks old, and according to the data present here, older mice seem to be entirely refractory to the lethal effects of the virus injected by this route.

Guinea pigs: To establish the Novy virus in guinea pigs, 0.05 ml of a 20% rat brain suspension was injected intracerebrally and 1.0 ml intraperitoneally into each of two animals, which died 5 and 7 days respectively following inoculation. Injection into mice of brain suspensions prepared from the dead guinea pigs confirmed the presence of virus. To determine more accurately the susceptibility of guinea pigs to the virus, 25 animals were inoculated as indicated in Table 8. Although these experiments were of necessity done with a very small number of animals, the results suggest that young and old guinea pigs are equally susceptible to the lethal effects of the virus when it is given intracerebrally. It may be seen that a 10^{-7} dilution of the virus killed both animals tested by this route despite their differences in age. The incubation period in the older animal, however, appears to have been prolonged. Intranasal instillation of the virus, on the other hand, failed to kill young guinea pigs at a dilution higher than 10^{-5} . Furthermore it should be pointed

TABLE 8

SUSCEPTIBILITY OF THE GUINEA PIG TO INOCULATION OF THE NOVY VIRUS
BY VARIOUS ROUTES OF INOCULATION

Route of Inoculation using 20% rat brain suspension	Dilution of Virus	150 to 175 Grams	400 to 600 Grams
IC .05 ml			
1	10 ⁻¹	n.t.*	4
2	4	3**	
3	5	3	5
4	6	5	
5	7	6	9
6	8	0	
IN 0.5 ml			
1	10 ⁻¹	n.t.	6
2	4	4	
3	5	6	0
4	6	0	
5	7	0	0
6	8	0	
IP 1.0 ml			
1	10 ⁻¹	5	0
2	2	1	
3	4	0	
4	5	0	0
5	6	0	
6	7	0	0

* n.t. = not tested

** Number indicates day of death of individual guinea pig, 0 signifies survival.

out that only 1 of the 3 older animals inoculated intranasally died. In animals which survived inoculation of the virus, a febrile reaction occurred in 48 to 72 hours. Blood taken at this time and injected intracerebrally into mice produced a fatal encephalitis in 3 of 5 animals tested.

Following intraperitoneal inoculation of the virus, it appears that young guinea pigs may be susceptible only if large amounts of the pathogen are given. On the other hand, the data suggest that older animals are resistant to any amount of the virus by this route.

Rabbits: To determine the susceptibility of rabbits to infection with the Novy virus, 4 animals were used, ranging in weight from 2 to 4 kilograms. Of these, 2 rabbits were inoculated intracerebrally with 0.5 ml of a 20% suspension of virus; the other 2 received 2.0 ml intraperitoneally. Both animals receiving the virus directly into the brain died of encephalitis, 1 on the seventh and 1 on the eighth day after inoculation. The rabbit which succumbed to infection on the eighth day developed a flaccid paralysis of the lower extremities 6 days following injection of the virus. Both rabbits receiving the virus intraperitoneally survived the inoculation, and attempts to recover virus from the blood were unsuccessful. However, when both rabbits which survived the intraperitoneal inoculation of virus were challenged by the intracerebral route 2 weeks later, one of the rabbits died in 9 days, whereas the second animal became ill and showed a febrile reaction but survived the injection.

Fowl: The young fowl employed in this study were "discards" which had been used for immunization against certain tumors, and weighed between 750 and 900 grams. Each chicken received 0.25 ml intracerebrally

and 1.0 ml intraperitoneally of a 20% brain suspension. One of the chickens survived; of the two that died, one bird became progressively weaker following inoculation and died after 5 days. The second animal was found dead on the morning of the eighth day without having previously shown signs of illness. Presence of virus was confirmed by intracerebral passage in mice.

The data presented, covering experimental infection and host range of the Novy virus, suggest that a wide variety of susceptible animals exists. The broad host range of the virus further emphasizes its great pathogenicity and virulence. The studies reported here indicate that no apparent difference in susceptibility exists between young and old mice inoculated intracerebrally, while only a slight variation is indicated when the virus is nasally instilled. As mice grow older they acquire a resistance to peripheral inoculation of the virus which begins about 5 weeks after birth. In general it may be said from the data presented here that young and old guinea pigs are equally susceptible to the lethal effects of the virus given directly into the brain in dilutions ranging from 10^{-1} to 10^{-7} . Following nasal instillation of the virus young guinea pigs succumbed, but greater concentrations of the virus were required to kill older animals. Rabbits and young chickens died of encephalitis following injection of the virus by the intracerebral route. Because of the small number of animals used in these experiments, no definite conclusions can be drawn as to the effect of age on the susceptibility of rabbits or chickens following inoculation of the Novy virus.

Propagation of the Novy Virus in the Developing Chick Embryo.

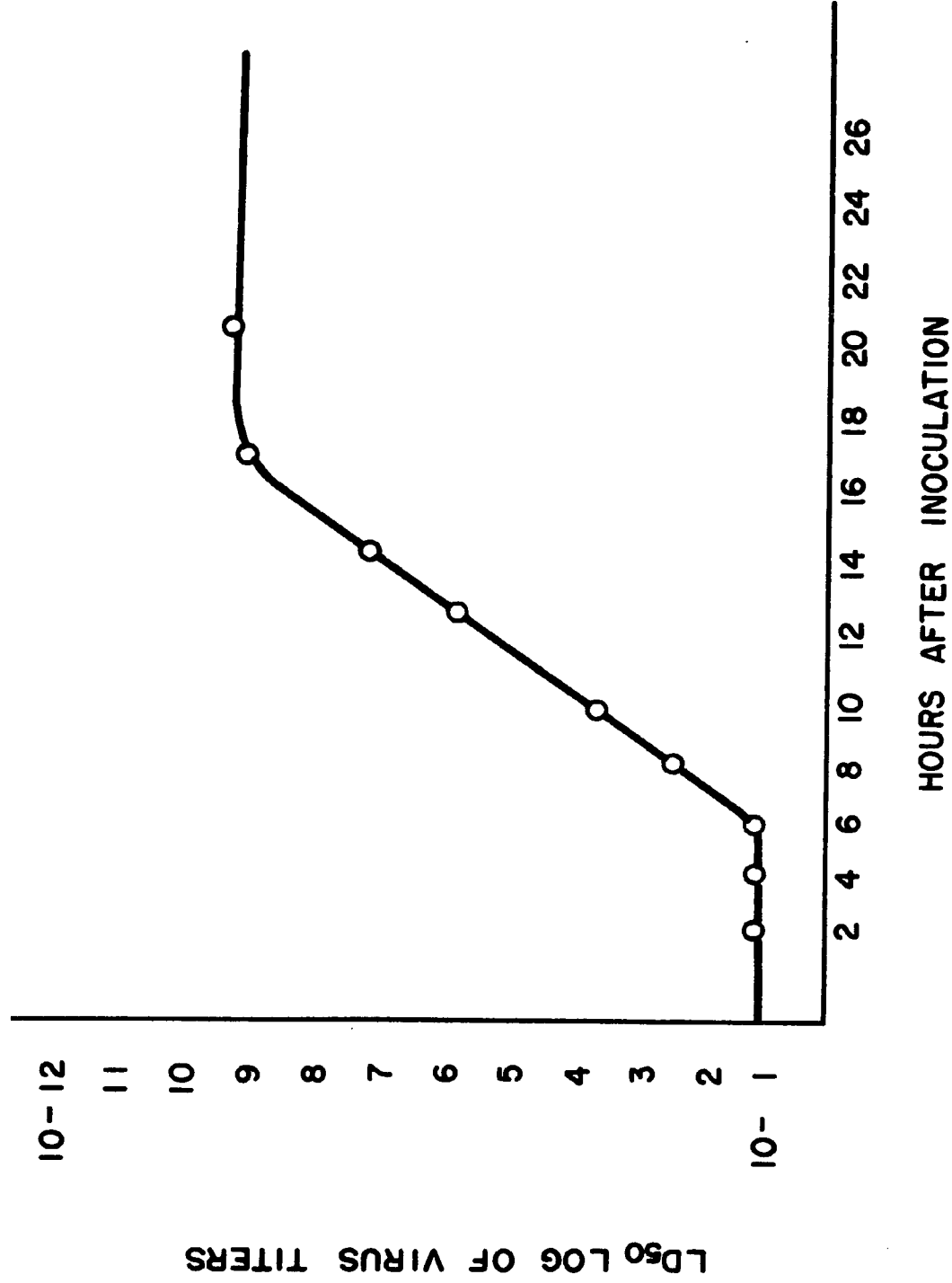
In 1931 Woodruff and Goodpasture (45) successfully cultivated the virus

of fowl pox on the chorioallantoic membrane (CA) of the developing chick embryo. Since then a number of viruses have been propagated either on the membranes or in the embryo per se. Certain of these agents show a preference for the membranes, while others grow better in the embryo. The cultivation of any virus in chick embryos is a convenient method of obtaining large quantities of virus in considerable concentration. It is also a means by which viruses may be passed in series without the use of more expensive laboratory animals. Furthermore, it is a simple method of obtaining virus relatively free of specific animal tissues for use in physical or immunological studies.

Multiplication of the Virus. A typical multiplication curve obtained by inoculation 10-day-old embryos on the chorioallantois and then titrating a 20% suspension of pooled embryos is shown in Figure 1.

The lag seen in the multiplication curve probably represents the time necessary for the virus to establish itself on the membrane and then gain entrance into the embryo. It is interesting to note that no virus was detected in embryos tested during the first 4 hours after inoculation. Virus was first demonstrable at 6 hours after inoculation, when 1 of 3 eggs inoculated with a 10^{-1} suspension of embryo developed typical infection and died. Between 6 and 16 hours, the titer of the virus increased rapidly. The peak of multiplication was reached at about 20 hours after inoculation, after which the growth curve flattened out. The flattening of the curve is probably associated with the beginning of generalized destruction of the embryo. It has been observed that with the death of the embryo, virus multiplication slows down until the embryo begins to disintegrate, after which the titer decreases.

FIG. 1
 MULTIPLICATION OF THE NOBY VIRUS IN THE DEVELOPING CHICK EMBRYO
 FOLLOWING INOCULATION ONTO THE CHORIOALLANTOIC MEMBRANE



20% SUSPENSION POOLED EMBRYOS
 3 EGGS PER DILUTION

Effect of Age. It has been observed (46) that rabies virus can be propagated on the choriallantoic membrane of embryonated eggs if the embryos are 8-days-old or less at the time of inoculation. The findings were confirmed (47) and extended to show that with vesicular stomatitis virus 7-day-old embryos were much more susceptible to infection than 10-day old embryos. On the other hand, it has been observed (48) that the propagation of influenza virus in 10- or 11-day-old embryos yielded better results than did younger or older embryos. It seemed of interest, therefore, to carry out experiments which would show the relationship between the age of the embryo or hatched chick and the activity of the Novy virus. Decimal dilutions of a 20% suspension of 10-day-old embryos were inoculated onto the chorioallantoic membranes of embryos and intracerebrally into hatched chicks of varying ages. Figure 2 and Table 9 summarize the results of this experiment.

It appears from Figure 2 that the CA membrane gradually becomes more resistant to infection with the Novy virus, since the titers obtained at 18 days were somewhat lower than those obtained at 10 or 11 days. However, it should be pointed out that this increased resistance is merely relative. In certain instances, when virus was inoculated 48 to 72 hours before hatching, this was followed by hatching and subsequent death of the chick due to the virus.

From the data presented in Table 9 it appears that hatched chicks up to 18 days after hatching are equally susceptible to the lethal effects of the virus injected intracerebrally.

Distribution of the virus in embryos following various routes of inoculation: Data showing the degree of spread of the virus throughout the egg are summarized in Table 10. Inoculum consisted of 0.03 ml of a 10^{-4} dilution of virus.

FIG. 2

EFFECT OF AGE OF EMBRYO ON PROPAGATION OF THE NOBY VIRUS
FOLLOWING INOCULATION ONTO THE CHORIOALLANTOIC MEMBRANE

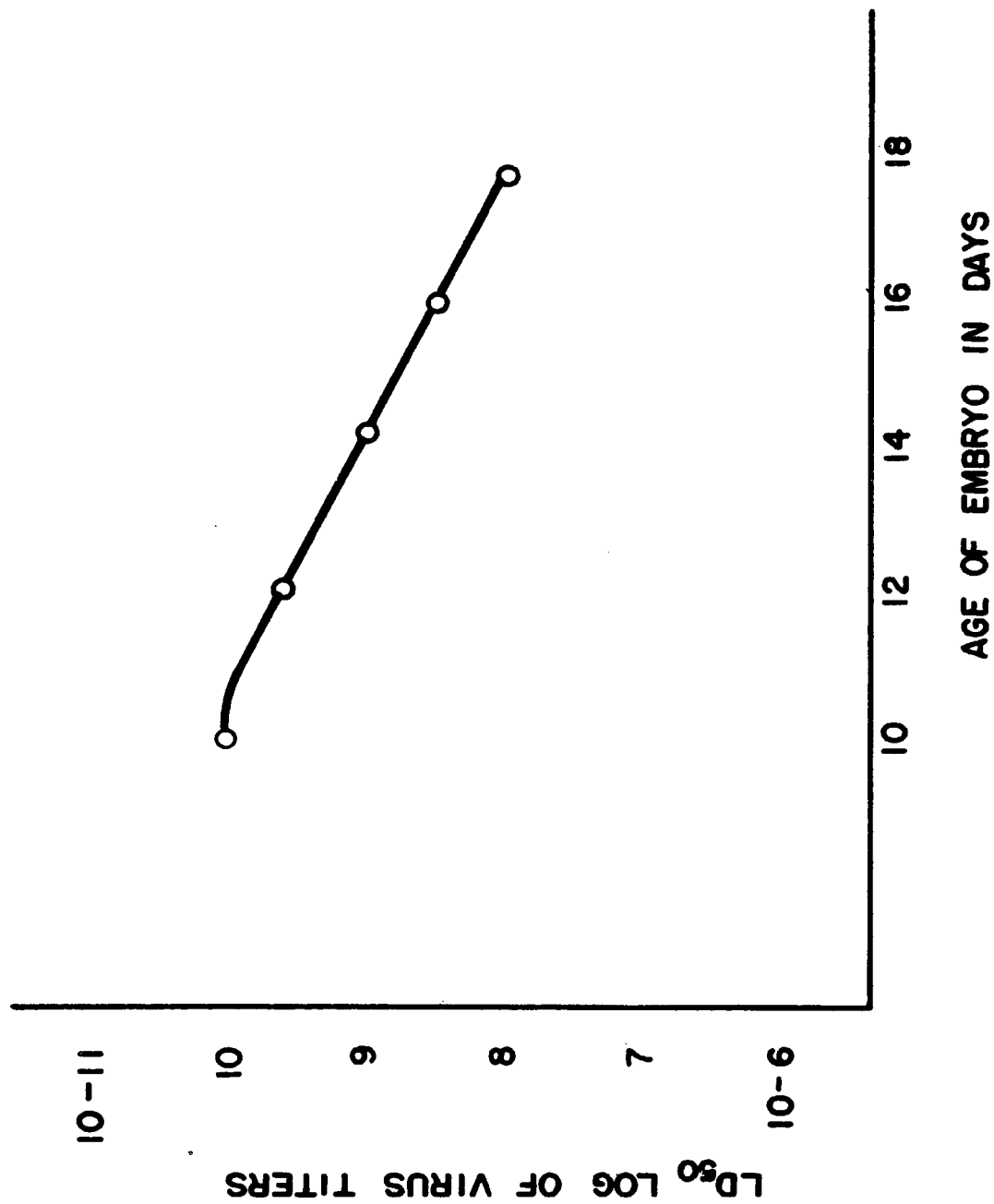


TABLE 9
SUSCEPTIBILITY OF THE HATCHED CHICK FOLLOWING INTRACEREBRAL
INJECTION WITH THE NOVY VIRUS

Dilution of Virus Tested	Age of Chick in Days								
	2	4	6	8	10	12	14	16	18
10 ⁻⁴ *	2/2	2/2	2/2	2/2	2/2	2/2	0/2	2/2	2/2
10 ⁻⁶	2/2	2/2	2/2	2/2	2/2	2/2	2/2	0/2	1/2
10 ⁻⁸	2/2	2/2	2/2	2/2	2/2	1/2	1/2	0/2	1/2
10 ⁻¹⁰	2/2	1/2	0/2	0/2	0/2	1/2	0/2	1/2	0/2

* Dilution of a 20% suspension of 10-day-old embryos.

From data presented in Table 10 it appears that after inoculation onto the CA membrane, the virus multiplied rapidly and was present in high concentration throughout the egg. The largest amounts of virus were present in the embryo and CA membrane and the smallest amounts in the yolk sac. Eggs inoculated by the yolk sac may survive several hours longer than those inoculated on the chorioallantois whereas virus introduced into the amniotic cavity (49) may be more rapidly lethal to the embryo than inoculation on the CA membrane. The LD_{50} of the virus can be stated for practical purposes to be within the range of 10^{-8} to 10^{-10} , but titers of 10^{-12} have been obtained in a few instances.

Pathological Lesions in Infected Embryos. Viruses are often characterized by the kind of lesions they produce, which in turn indicates varying susceptibility to different host cells. Similar information on the changes brought about by the Novy virus was considered desirable in order to compare its pathologic manifestations with those of known viruses. For this study 14 embryos, 10 days old, were inoculated on the CA membrane with a 10^{-4} suspension of the tenth-passage chick embryo virus. Another series of 6 was inoculated with serum-saline as controls. After incubation 2 eggs of each lot were examined for macroscopic changes at the intervals shown in Table 11. Macroscopically, virus-infected embryos inoculated with a 10^{-4} suspension of egg-passage material showed their earliest change in 12 to 16 hours. Lesions on the membranes consisted of small foci showing varying degrees of hyperemia and hemorrhage. This suggests an early destruction of vascular endothelium. Examination of the embryo showed numerous small hemorrhages over the head and legs, which increased in number and distribution as the infection progressed. Microscopically, thrombi were found in small vessels of the membranes and

TABLE 10
DISTRIBUTION OF THE NOVY VIRUS IN EGGS INOCULATED
BY VARIOUS ROUTES

Route of Inoculation*	Age of Embryo (days)	Incub. period (hrs)	LD ₅₀ Titers in Eggs				
			Susp. of Embryo	Ch. Al. Membr.	Allantoic Fluid	Amniotic Fluid	Yolk Sac
CA Membrane	10	20	12**	8.9	7.2	7.0	6.0
Amnoitic Sac	10	20	9.3	7.0	3.3	8.0	5.0
Yolk Sac	7	20	9.0	8.0	6.1	6.3	6.1

* Inoculum was .03 ml of a 10^{-4} dilution of chick embryo virus.

** Figures are logarithms of the 50% mortality end point.

TABLE 11

STATE OF VIABILITY AND GROSS APPEARANCE OF EMBRYOS
AT VARIOUS INTERVALS AFTER INOCULATION OF THE NOVY VIRUS
ONTO THE CHORIOALLANTOIC MEMBRANE

Interval After Inoculation* hours	Macroscopic Appearance of Embryos	Viability of Embryos
1	Normal	Both living
4	Normal	Both living
8	Normal	Both living
12	Very slight hyperemia	Both living
16	Moderate hyperemia. Several small petechiae	1 living, 1 dead
20	Marked hyperemia. Numerous small and large hemorrhages	Both dead
24	Several small foci showing vascular collapse, thrombosis hyperemia and hemorrhage	Both dead

* Inoculum was 0.03 ml of a 10^{-4} dilution of chick embryo virus.

throughout the tissue of the embryo, but no tissue showed any special susceptibility to the action of the virus and no inclusion bodies were seen in any of the sections studied.

Experimental data presented in the preceding section indicated that infection of the chick embryo with the Novy virus results in a rapidly disseminated fulminating disease. It appears that following inoculation of the virus by any route, the pathogen multiplies rapidly and reaches a high concentration in all portions of the egg, especially in the embryo itself. Age of the embryo or the hatched chick had little or no influence on the lethal effect of the virus, although some resistance to infection was observed with an increase in age. Histopathological studies of infected eggs suggested a hematogenous spread of the virus with rapid destruction of the endothelium, although no tissue showed any special susceptibility. The LD₅₀ titer of embryo and membrane suspensions remained within the range of $10^{-6.1}$ to 10^{-12} , with the maximum reached in chick embryos approximately 20 hours after inoculation of the virus.

The course of infection in the egg following exposure to the Novy virus appears to resemble that described for other encephalitogenic agents. However, there are certain characteristic differences produced in eggs infected with this agent which are not observed with others. The rate of multiplication of the virus, on the basis of studies reported here, resembles that of the equine (50,51) and the West Nile (52) viruses, but differs from that described for the St. Louis encephalitis virus (53). The Novy virus was present in appreciable amounts 16 hours after inoculation, and the LD₅₀ titers obtained appear to be higher than any thus far reported with other viruses which have been cultivated in the developing chick embryo. The virus multiplied rapidly until it reached its peak in

20 hours and from then on remained constant until disintegration of the embryo, which occurred 3 to 5 hours after death. The highest concentration of virus following CA inoculation was found in the embryo, although the allantoic fluid and CA membrane contained great quantities of the infectious agent. On the other hand, in the case of Japanese B virus a higher virus content was reported to have been found in the embryo following inoculation of the pathogen into the yolk sac rather than on the CA membrane (54). The distribution of the Novy virus in fertile eggs follows in general, the pattern established for other neurotropic viruses in which the largest amounts of the virus are encountered in the embryo pre se.

Immunologic Relationship to Other Viruses. In order to study the immunologic relationship, if any, between the Novy virus and other viruses with comparable biological properties, virus neutralization and cross protection tests were made. Information gained by tests of this nature is, in general, the most important evidence for establishing identity or close similarity among viruses. It is usually desirable to perform both types of test, since the neutralization test may fail to reveal minor relationships which may become apparent with cross protection tests (55,56).

Neutralization tests: Specific antisera against known viruses were tested for their capacity to neutralize the Novy virus. Table 12 summarizes the results of these tests.

The results indicate that there was little or no neutralization by any of the sera tested, with one exception. There was some evidence of a slight relationship between EEE and the Novy virus on the other hand, the tests gave little or no indication of any relationship between the Novy virus and the viruses of WEE, Mengo, VEE, or Semliki Forest.

TABLE 12

NEUTRALIZATION TESTS USING THE NOVY VIRUS AND VARIOUS HYPERIMMUNE SERA

Antisera tested	Dilution of Virus Tested					LD ₅₀	Neutralization Index
	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	
Venezuelan Equine*	5/5**	5/5	5/5	3/5	0/5	7.1	10.0
Western Equine	5/5	5/5	5/5	4/5	0/5	6.8	15.0
Eastern Equine	5/5	5/5	3/5	2/5	0/5	6.5	32.0
Semliki Forest	5/5	5/5	3/5	5/5		0	
Mengo	5/5	5/5	5/5	5/5		0	
Rabbit control serum			5/5	4/5	3/5	0/5	8.0

* The Venezuelan antiserum was produced in a guinea pig; other antisera used were produced in monkeys.

** The denominator indicates the number of animals inoculated and the numerator indicates the number of deaths.

Cross protection tests: Viruses selected for comparison in the cross protection tests were the EEE and WEE viruses. These agents were chosen because of their similarity to the Novy virus with respect to certain of their physical and biological properties; furthermore, the type of disease produced in experimental animals by the Novy virus more closely resembles that produced by the equine viruses than any others. It would have been desirable to include the virus of Venezuelan equine encephalomyelitis in these resistance tests; however, the high infectivity for man of the VEE virus made its use in the laboratory inadvisable.

The intracerebral method was used to test for resistance after vaccination because Swiss mice, regardless of age, are highly susceptible to intracerebral injection of the 3 viruses being investigated. It should be emphasized, however, that the intracerebral challenge of mice is so rigorous that small degrees of resistance which might be revealed by other routes of challenge may be missed.

Table 13 summarizes the results of the intracerebral cross protection tests. The degree of cross resistance shared by the Novy and EEE viruses is greater than that indicated by the neutralization test. This is understandable, since, as has already been pointed out, the rigorous nature of the intracerebral cross protection tests masks the "minor" differences which may exist in the antigenic pattern of viruses and emphasizes their similarities. The Novy vaccine in mice gave an extremely high degree of immunity, protecting the animals against 3,200,000 MLD of the homologous virus. On the other hand, EEE-vaccinated mice resisted 2,000 intracerebral MLD of the Novy virus, whereas in the reciprocal tests Novy-vaccinated mice resisted 50,000 intracerebral MLD of the eastern

TABLE 13

RESULTS OF CROSS PROTECTION TESTS IN MICE VACCINATED AGAINST NOVY, EEE AND WEE
ENCEPHALOMYELITIS VIRUSES AND CHALLENGED INTRACEREBRALLY

Mice Vaccinated against	Challenge virus	Mortality Ratio of Mice Inoculated with Virus Dilution										LD ₅₀	Resistance index
		10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	10 ⁻¹⁰		
Novy	Novy		6/6	1/6	1/6	0/6	0/6					3.0	3,200,000
	EEE		6/6	6/6	6/6	2/6	1/6	0/6				4.8	50,000
	WEE						6/6	6/6	3/6	0/6		8.0	32.0
EEE	Novy					6/6	4/6	0/6	0/6			6.2	2,000
	EEE	6/6	2/6	2/6	0/6	0/6						2.0	2,500,000
WEE	Novy				6/6	6/6	6/6	4/6	0/6	0/6		8.2	20.0
	WEE	3/6	2/6	0/6	0/6							1.3	2,000,000
Unvaccinated controls													
Novy							6/6	6/6	6/6	6/6	0/6	9.5	
EEE				6/6	6/6	6/6	6/6	6/6	5/6	0/6		8.4	
WEE					6/6	6/6	6/6	5/6	2/6	0/6		7.6	

virus. It should be emphasized, however, that in mice which received the Novy vaccine and were subsequently challenged with the homologous virus, the resistance index was much greater than that obtained with the eastern agent. Mice vaccinated with the western virus exhibited no significant degree of resistance to the fatal effect of the Novy virus.

Varying degrees of antigenic similarities have been reported for many viruses. Among the neurotropic agents, Russian spring-summer encephalitis of man and louping ill virus of sheep have been shown to be closely related antigenically. In cross protection tests in mice, Russian virus vaccine gave moderate protection against louping ill virus (55). Similar studies on St. Louis encephalitis, Japanese B, and West Nile viruses have shown these agents to be related, sharing common antigenic components (56).

The results obtained in these resistance tests may be summarized by saying that a relationship exists between the Novy and eastern viruses; however the Novy agent is unrelated to other viruses studied. The degree of cross protection in ~~EEE~~-vaccinated mice challenged with the Novy virus was less than that seen in the reciprocal test.

C. Physiochemical Properties of the Novy Virus

pH Stability of the Virus. Studies on several viruses have indicated that for each virus there is a range of hydrogen ion concentration over which the virus is stable, and that higher or lower pH values markedly affect virus activity. It therefore was of importance to determine the hydrogen ion concentration at which the Novy agent was most stable.

The LD₅₀ titer of the virus suspension used in this study was 10^{-9.8}. Buffers of desired hydrogen ion concentration were prepared by

adding either 10% sodium hydroxide or 3N hydrochloric acid to a solution 0.5M with respect to glycine and to disodium phosphate. The buffers were then autoclaved for 15 minutes at 15 pounds. After sterilization and shortly before they were to be used, the pH of the various solutions was checked in a Beckman pH meter. Immediately after preparation 1.0 ml of the 10% suspension of virus in serum saline was added to 9.0 ml of buffer of the desired pH (Table 14), and the mixture was then stored at 4°C. The various buffer mixtures were titrated for virus by injecting dilutions intracerebrally into mice after 24 hours at 4°C and again after 5 days. The results appear in Table 14.

It will be seen that the virus is very unstable below pH 2.5, since no virus was present after 24 hours. After 5 days no virus could be detected in the buffer solution which had a pH below 4.4 or above 11.5. From the data presented it would appear that the virus is most stable between a pH range of 7.3 to 9.7, and that at the more extreme pH values there is a rapid inactivation with a marked drop between 9.7 and 10.3. It is interesting to note that the inactivation of the virus was very abrupt in solutions at a pH higher than 9.7, whereas inactivation in the lower pH range was a gradual process. The striking feature about these studies is that the virus retained some infectivity for 24 hours in a pH range 2.5 to 11.5 and it remained active for 5 days in a range of 4.4 to 10.3. The above data is represented graphically in Figure 3.

Effect of Heat on the Virus. Experiments undertaken to determine the effect of various temperatures on the virus included the testing of a 20% rat brain suspension in buffered (pH 8.2) serum-saline solution, the supernatant fluid of the above after centrifugation, and

FIG. 3

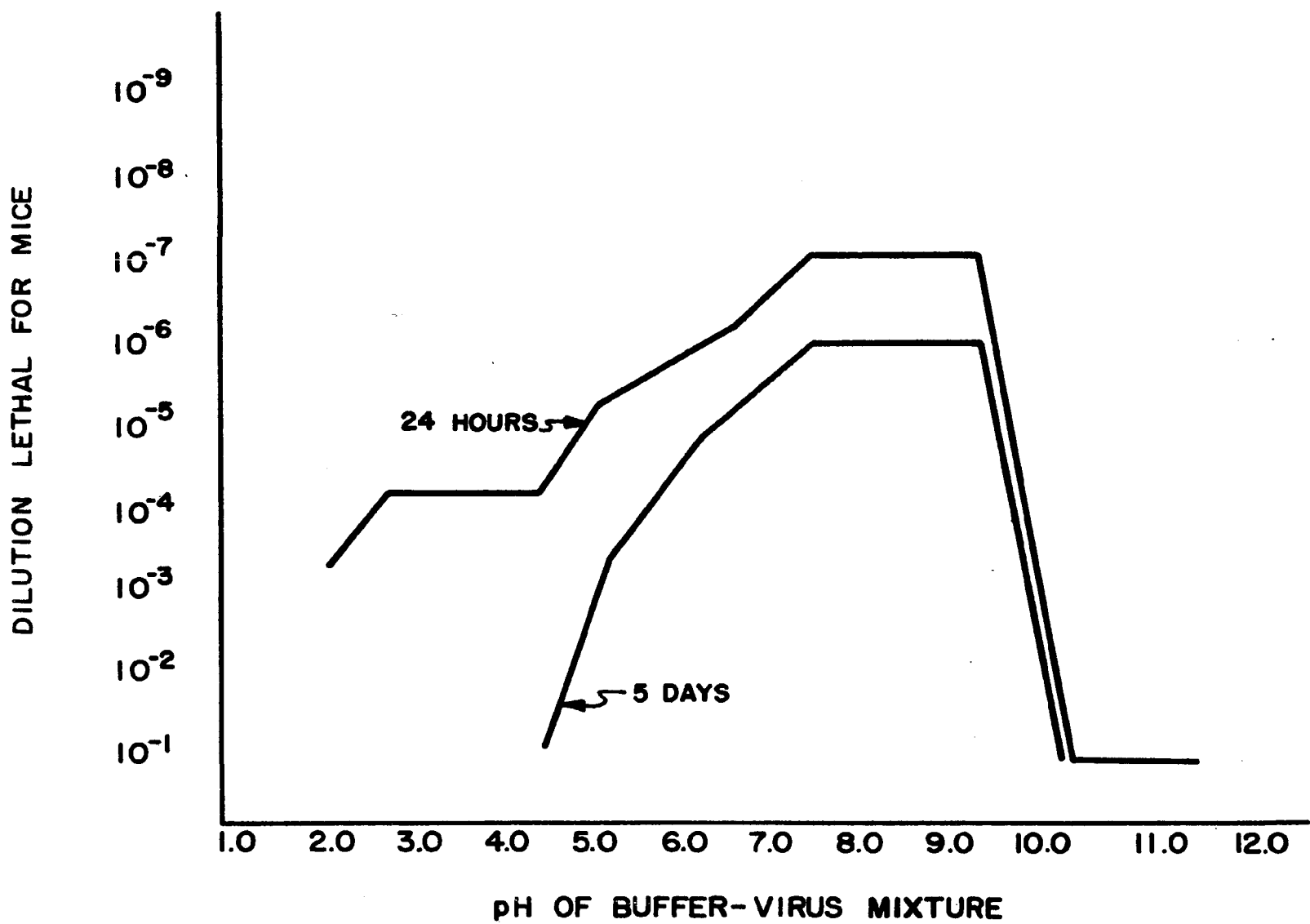


TABLE 14

EFFECT OF HYDROGEN ION CONCENTRATION ON THE NOVY RAT VIRUS

pH of Buffer	1 day	5 days	Mixture Titrated After	Lethal Dilution* for Mice
1.0	1.0	1.0	1 day 5 days	
2.5	2.5	2.5	24 5	10 ⁻³
3.5	3.3	3.3	24 5	10 ⁻⁴
4.5	4.5	4.4	24 5	10 ⁻⁴ 10 ⁻¹
5.5	5.0	5.0	24 5	10 ⁻⁵ 10 ⁻³
6.5	6.5	6.5	24 5	10 ⁻⁶ 10 ⁻⁵
7.4	7.3	7.2	24 5	10 ⁻⁷ 10 ⁻⁶
8.8	8.5	8.3	24 5	10 ⁻⁷ 10 ⁻⁶
9.7	9.7	9.7	24 5	10 ⁻⁷ 10 ⁻⁶
10.6	10.4	10.3	24 5	10 ⁻¹ 10 ⁻¹
11.5	11.5	11.5	24 5	10 ⁻¹

* Dilutions were made in saline containing 10% rabbit serum which possessed sufficient buffering capacity to bring all preparations to about pH 7.5.

the filtrate of this supernatant fluid. All materials were contained in glass test tubes and heated in a thermostatically controlled water bath. After heating these were tested for the presence of virus by intracerebral injection in mice. The data are summarized in Table 15.

The suspension was unaffected by heating at 56°C for 30 minutes. There was some destruction of virus heated at 60°C for 10 minutes, but the suspension was noninfectious after remaining 10 minutes at 70°C. The supernatant fluid was partially inactivated by heating at 60°C for 10 minutes, but all activity was destroyed at 65°C. The UF sintered-glass filtrate was no longer infectious after heating at 56°C for 30 minutes.

Resistance to Chemical Disinfection. The following experiments were undertaken to test the action of certain chemicals on the Novy virus. These included formalin (37% HCHO), chloroform, and phenol. The percent indicated in each experiment represents the final concentration of each of the total volume. The mixtures were tested directly by injecting 1.0 ml intraperitoneally into mice.

Resistance to chemicals at room temperature: Data summarized in Table 16 indicate that the virus suspension was unaffected by 0.25% formalin for 2 hours at room temperature. It was partially inactivated in a concentration of 0.5%, but was completely destroyed after 2 hours in 1.0% formalin. Identical results were obtained with chloroform. The virus was partly destroyed in 2% phenol and was no longer infectious in a 3% concentration after 2 hours.

Resistance to certain disinfectants at 3 - 5°C: In this study glass-stoppered bottles containing the suspension in contact with the disinfectant were kept in the cold room and examined periodically for the presence of infectious virus. On occasion, in the case of phenol, test

TABLE 15
EFFECT OF EXPOSURE OF THE NOVY VIRUS TO DIFFERENT TEMPERATURES

Material tested in mice	50°C for 10 min	56°C for 10 min	56°C for 20 min	56°C for 30 min	60°C for 10 min	65°C for 10 min	70°C for 10 min	Controls
20% Brain Susp.	4/4	4/4	4/4	4/4	3/4	2/4	0/4	2/2
Supernatant 1500 r.p.m.	4/4	4/4	4/4	4/4	1/4	0/4	0/4	2/2
Filtrate UF Sintered glass	3/4	3/4	2/4	0/4	0/4	0/4	0/4	2/2

TABLE 16

RESISTANCE OF THE NOVY VIRUS TO CHEMICALS
AT ROOM TEMPERATURE FOR 2 HOURS

Material tested in mice	Formalin, %				Chloroform, %				Phenol, %		
20% Brain Suspension	0.1	0.2	0.5	1.0	0.1	0.2	0.5	1.0	1.0	2.0	3.0
	3/3	3/3	1/3	0/3	3/3	3/3	1/3	0/3	3/3	2/3	0/3

material failed to kill any of the mice injected intraperitoneally up to 42 days. However, subsequent tests of material taken from the same bottle killed mice when injected at a later date. From the results presented in Table 17 it appears that under conditions of the experiments the Novy virus is not readily destroyed by the disinfectants tested. The agent remained active for 35 days in contact with a 0.1% solution of formalin at refrigerator temperature. Suspensions of the virus are not easily destroyed under these conditions by chloroform or phenol, since the virus remained active for 5 and 7 weeks respectively in the presence of these reagents.

Preservation of the Virus. Considering the unusual circumstances under which the Novy virus was recovered following years of storage, it was considered of interest to ascertain the length of time the virus would remain viable when stored under different conditions. Brains and blood from infected rats were collected and pooled separately. The brains were cut into small pieces (approximately 1.0 cm³) and after being placed in separate screw-capped bottles were quickly frozen and stored in the deep freeze; some were mixed (1:1) with buffered (pH 8.5) glycerine and placed in the cold room protected from light. Mouse brains from infected animals were removed and treated in the same manner. Blood was mixed (1:1) with glycerine. At monthly intervals the frozen and glycerinated virus material was removed from storage and tested for infectivity by intracerebral injection in rats. The inoculum was 0.05 ml of a 10% suspension of brain; 0.05 ml of glycerinated blood was injected undiluted. Table 18 summarizes the results on virus preservation studies conducted up to the present time.

TABLE 17
RESISTANCE OF THE NOBY VIRUS
TO CHEMICALS AT 3 - 5°C

Chemical*	Percent	Dilution	Active in days
Formalin	0.1	1:1	35
Chloroform	1.0	1:1	39
Phenol	1.0	1:1	42

* 20% brain suspension exposed to chemicals and then injected intra-peritoneally into mice.

TABLE 18
PRESERVATION OF THE NOVY VIRUS
UNDER DIFFERENT CONDITIONS

Material Preserved	Interval Months	Storage Temperature, °C	Results IC in Rats
Rat Brain + Buffered Glycerine	10	3 to 5	4,6,6*
Blood + Buffered Glycerine 1:1	10	3 to 5	5,6,7
Rat Brain	10	-60	3,3,4
Mouse Brain	8	-60	3,3,3**

* Numbers represent day of death

** Tested for infectivity in mice

It is apparent that the Novy virus stored under conditions of the experiment retained its infectivity in the cold room or deep freeze even after storage for 10 months. It may be seen in Table 18 that in rats which received the glycerinated blood and brain preparations stored at 3 - 5°C, the incubation period was prolonged to 6 and 7 days. On the other hand, there was a comparatively short incubation period in mice which were injected with suspensions of either infected mouse or rat brains which had been stored at -60°C.

In preparing virus for deep freeze storage it will be recalled that brains were cut into small pieces before freezing and stored in separate containers. This was to avoid the harmful effects on the Novy virus of freezing and thawing at a time when it was to be tested. It has been observed in certain instances that if the frozen virus material is allowed to thaw slowly at room temperature and is then refrozen and again thawed, the infectivity titer may drop precipitously. No critical studies have been undertaken to determine the harmful effects of freezing and thawing of this virus, but it is believed that the LD₅₀ titer may drop from 10^{-9.5} to approximately 10⁻³ after freezing and thawing two times.

Electron Microscopic Studies of the Novy Virus. "Purified virus" for study in the electron microscope was obtained by centrifugation and filtration of virus-infected allantoic fluid. For this experiment 20 chick embryos were inoculated with egg-adapted virus after which the allantoic fluid was harvested when the embryos were still alive, though moribund. Fluid was collected in glass tubes and immediately centrifuged at 2500 r.p.m. to sediment the blood cells and gross particles.

To the resulting clear supernatant fluid was added 2 grams of 512 celite filter aid for each 50-ml volume and the suspension filtered through a thin mat (1-2 mm) of 503 celite supported in a Büchner funnel. Throughout the procedures all materials were kept cold and centrifugation was carried out at refrigerator temperature. Controls consisted of allantoic fluid harvested and prepared in exactly the same manner as was the test material.

A 15-ml portion of the filtrate was placed in metal tubes, capped, and spun in a Sorvall centrifuge for 30 minutes at approximately 9000 x gravity. Practically no sediment was formed. The supernatant fluid was removed with a sterile capillary pipette to collodion tubes and spun in a "Rockefeller" model centrifuge at 20,000 x gravity for 30 minutes. The small quantity of sediment formed was resuspended in 0.25 ml of Ringer solution (pH 8.5) and the tubes placed in the refrigerator. The supernatant fluid was removed to collodion tubes, capped, and sealed in the chilled rotar of a Spinco (Model D) ultracentrifuge and spun at 42,000 x gravity for 1 hour. Following this run the supernatant fluid was removed and the small translucent gel-like pellets were overlayed with 0.25 ml of buffered Ringer solution. With fine-tipped pipettes equipped with rubber bulbs, the fluid was gently pumped over the pellets, which quickly went into suspension. For examination in the electron microscope, Ringer solution containing the virus from the above supernatant fluids, or resuspended sediments, was placed on a thin collodion film supported on a wire screen. After a short drying period, salt was removed from the dried preparations by lightly washing them with water from a fine pipette or by dipping the screen and collodion film in water several times (59). The small screen supporting the virus was then

To the resulting clear supernatant fluid was added 2 grams of 512 celite filter aid for each 50-ml volume and the suspension filtered through a thin mat (1-2 mm) of 503 celite supported in a Büchner funnel. Throughout the procedures all materials were kept cold and centrifugation was carried out at refrigerator temperature. Controls consisted of allantoic fluid harvested and prepared in exactly the same manner as was the test material.

A 15-ml portion of the filtrate was placed in metal tubes, capped, and spun in a Sorvall centrifuge for 30 minutes at approximately 9000 x gravity. Practically no sediment was formed. The supernatant fluid was removed with a sterile capillary pipette to collodion tubes and spun in a "Rockefeller" model centrifuge at 20,000 x gravity for 30 minutes. The small quantity of sediment formed was resuspended in 0.25 ml of Ringer solution (pH 8.5) and the tubes placed in the refrigerator. The supernatant fluid was removed to collodion tubes, capped, and sealed in the chilled rotar of a Spinco (Model D) ultracentrifuge and spun at 42,000 x gravity for 1 hour. Following this run the supernatant fluid was removed and the small translucent gel-like pellets were overlayed with 0.25 ml of buffered Ringer solution. With fine-tipped pipettes equipped with rubber bulbs, the fluid was gently pumped over the pellets, which quickly went into suspension. For examination in the electron microscope, Ringer solution containing the virus from the above supernatant fluids, or resuspended sediments, was placed on a thin collodion film supported on a wire screen. After a short drying period, salt was removed from the dried preparations by lightly washing them with water from a fine pipette or by dipping the screen and collodion film in water several times (59). The small screen supporting the virus was then

"shadow-cast" (57,58). In this technic a very thin film of uranium is evaporated at an oblique angle of 35° onto the specimen mounted on its supporting membrane. This coats the virus with a very thin film of metal. When such virus particles are examined in the electron microscope, they cast a shadow which greatly contrasts the particle from its surroundings, giving it a third-dimensional effect.

Figure 4 is an electron micrograph of the Novy virus photographed shortly after purification. The picture was taken in an RCA electron microscope at 10,000 diameters and enlarged photographically to 50,000 diameters. The images are remarkably uniform in size and shape. Individual particles appear to be round, uniform in density, and in considerable contrast with the background.

Studies were made of the particle size of the virus by measurements of image diameters in the electron micrographs. For this purpose the virus was mixed with a suspension of polystyrene latex indicator particles and the preparation than shadow-cast as described above. The polystyrene spheres used in this study measured approximately 26,000 angstroms ($\text{\AA}$). Measurements were made of virus image diameters in the photographs. Each of 15 images in 5 different areas was measured to obtain the mean diameter of the virus particles. The following formula was used to calculate the size of the Novy virus (60):

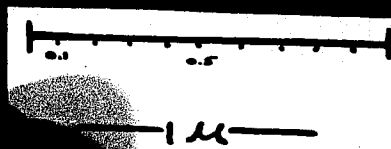
$$D_v = R \times D_{ps} ,$$

where R equals the ratio of the diameter of virus particles (D_v) to the diameter of the polystyrene particles (D_{ps}) which averages 26,000 $\text{\AA}$.

The ratio was found to be 1:75. Application of the above formula to data obtained from electron micrographs yields an estimated diameter of the Novy virus of 35 millimicrons.



Fig. 4. Electron Micrograph of the Novy Virus



It will be recalled that the diameter of the Novy virus by gradocol filtration was found to be 18 to 26 millimicrons. It should be pointed out that the differences in values obtained in measurements is probably due to the methods used. The finding of different values using these methods is consistent with observations reported by others (61,62). Viruses measured by collodion filtration remain in a relatively unaltered state, since infectivity of the particles is maintained even after passage through the membrane. On the other hand, viruses prepared for electron micrographs undergo rather drastic treatment before being photographed; moreover, since electrons cannot penetrate air and the entire electron path must be in a vacuum, such a procedure may well lead to an erroneous conclusion.

DISCUSSION

From the data presented, it appears that a filtrable infectious agent has been recovered from blood collected and stored from virus-infected rats by Dr. F. G. Novy approximately 40 years ago. The virus exerts its principal pathogenic effects on the central nervous system of all animals known to be susceptible to it. The agent is readily filtrable and studies of its size by ultrafiltration and the electron microscope indicate that it is among the smallest of the known viruses. It may be transmitted in series in rats, mice, guinea pigs, and fowl and has been cultivated in the developing chick embryo. Its natural host, the mode of transmission of the agent outside the laboratory, and its ability to cause disease in man are unknown. On the basis of its pathogenicity for experimental animals, the lesions which it induces, and immunological reactions thus far studied, the Novy virus is believed to be significantly different from all other known viral agents with the possible exception of the equine encephalomyelitis viruses.

The Novy virus elicits reactions in experimental animals which are similar to those produced by other neurotropic agents. Careful consideration of its properties nevertheless serves to differentiate this virus from other members of the neurotropic group. Since, however, the group of viruses affecting the central nervous system is so large, and individual members show considerable overlapping in many of their characteristics, only those agents possessing properties closely analogous to the Novy virus will be considered for discussion.

Although the course of infection in certain laboratory animals following inoculation of the Semliki Forest virus resembles that seen in animals infected with the Novy agent, rabbits and guinea pigs which readily succumb to infection with the Novy virus are unaffected by the Semliki virus (63). Mengo virus, though producing effects in many respects similar to those of the Novy agent following inoculation into mice, causes only inapparent infection following injection of massive doses into albino rats (64). Furthermore, neutralization tests have shown these viruses to be unrelated.

Although louping ill virus is not regularly infectious for rats, changes in the central nervous system of mice infected with it are similar to those seen in rats dead of the Novy virus; moreover such properties as resistance to heat and chemicals, common to both viruses, suggest a possible relationship between the two agents. Distinction between these two pathogens is easily made, however, since rabbits and guinea pigs are insusceptible to infection with louping ill virus (65).

It is interesting to note that rats from which the Novy virus was originally isolated were probably obtained from dealers located in the St. Louis, Missouri, area (40), where in 1932 and then again in 1937 there occurred an epidemic of encephalitis (66). The course of experimental infection in mice with St. Louis encephalitis virus is similar to that seen with the Novy virus, which suggests a possible relationship between these two agents. Although no comparison of possible antigenic similarities between these agents has been made, there are differences by which they may be distinguished from each other. Rats are insusceptible to the lethal effects of the St. Louis encephalitis virus (67), and in mice, which are susceptible, the incubation period is longer and the titers considerably lower than those obtained with the Novy agent.

Other viruses with properties similar to those of the Novy agent include the Japanese B encephalitis virus (68) and the West Nile virus (69). These two agents, however, do not include in their host range rabbits or guinea pigs, and rats may be infected only if massive doses are used in very young animals. The Russian spring-summer encephalitis virus differs in important respects from the Novy agent. Guinea pigs show only fever after inoculation with the Russian virus and the disease in them is not transmissible in series (70); moreover, attempts to infect rabbits and rats have been unsuccessful (71).

The behavior of the Novy agent in animals resembles that of the North American and Venezuelan equine encephalomyelitis viruses more closely than that of any other viruses. All four have similar host ranges; however, with the eastern and western agents most animals included in their host ranges are resistant to virus given by peripheral routes other than the nasal (72,73,74). Moreover, young fowls, following exposure to the eastern and western viruses, exhibit a viremia but show no overt signs of infection (75,76). Intracerebral titers of the equine viruses, other than Venezuelan, range between 10^{-8} and 10^{-9} , rarely as high as 10^{-10} . Although the North American equine viruses grow readily in eggs (77,50), titers obtained are in the same range as those stated above; furthermore, the agents have been reported to produce intranuclear inclusions in embryos following their inoculation (78). In contrast, distinctive features of the Novy virus include its intense virulence for susceptible animals when injected by either the intracerebral or all peripheral routes tested. It has been shown that young fowls are susceptible to its lethal effects; in chick embryos titers as high as 10^{-10} or 10^{-12} were

obtained and titers in mice and rats were determined at 10^{-11} . The Novy virus, as seen by the neutralization tests, is antigenically distinct from the Venezuelan and western viruses. The cross serologic reactivity between the Novy virus and the eastern virus will require a more complete comparison between these two agents especially since only one strain of eastern virus was used in these studies.

Perhaps the most outstanding property of the Novy virus is its unparalleled resistance to inactivation following approximately 40 years of storage. It is impossible to be certain under what exact temperature conditions the virus had been stored through all these years, although it is likely that most of the time the virus had been kept between 5 and 10°C (40). The activity of many viruses is rapidly destroyed when kept at 5 - 10°C or at room temperatures; some retain their infectivity under these conditions only for periods of a few months, while others become inactivated within a very few days.

The method used by Dr. Novy to preserve virus-infected blood by placing it in glass tubes mixed with equal volumes of glycerine and sealing in a blast lamp was undoubtedly the best method available at that time for preserving virus material. It is generally believed that viruses are more resistant to glycerine than to other preservatives; its extensive use for the preservation of viruses is probably largely dependent on the fact that viruses are usually susceptible to the release of inactivating substances from autolyzing tissues; glycerine acts as a dessicant and retards autolysis of the tissues containing the virus.

It is interesting to note that Landsteiner and Levaditi's first experiment on the transmission of poliomyelitis to monkeys in 1909 was

carried out with nervous tissue that had been preserved in glycerine (79). At about the same time Dr. Novy isolated the virus which now bears his name; in storing his newly isolated virus, he was among the first to use glycerine as a preservative. The virus of poliomyelitis has been shown by various workers to survive glycerination for months (80) and even years (81) if kept at refrigerator temperature. Results of a thorough search of the literature on preservation of animal viruses indicate that up to the present time the longest period of survival has been reported by Rhoads (82), who kept a mixture of two different strains of poliomyelitis virus for 8 years in 50% glycerine at 4°C. At the end of this period infectivity was demonstrated by intracerebral inoculation in monkeys. That glycerine is a good preservative, however, is not universally true. For example, Virus III and the salivary gland virus of guinea pigs (83) are inactivated after remaining in 50% glycerine for only 6 weeks.

The technique used by Dr. Novy to seal glass tubes which contained virus-infected blood undoubtedly was an important factor in the successful preservation of the virus. In a series of experiments designed to study the persistence of infectivity of the virus in glycerinated blood, he found that in blood mixtures virus remained infective for a much longer period when tubes were sealed in a flame than did virus in tubes which were closed with rubber stoppers (40). He found that virus remained active for at least 297 days at refrigerator temperature; unfortunately, in the process of opening and resealing the glass tubes the material within became contaminated with bacteria and tests were discontinued. It is possible that during the sealing process blood was coagulated on the walls of the tubes and hence by drying a partial "lyophilization" was accomplished sufficient to preserve virus activity through the years.

SUMMARY

1. A filtrable infectious agent has been recovered by passage of virus-infected rat blood collected and stored by Dr. F. G. Novy approximately 40 years ago. It has been named the Novy rat virus.
2. Although the virus has been shown to be predominately neurotropic, causing in susceptible animals the greatest amount of damage in the central nervous system, other organs of the body are involved to a certain degree.
3. The virus causes fatal infection in young rats and mice when inoculated by any route; susceptibility to extraneural inoculation of virus decreases with increasing age of the animals. When rats are inoculated by different routes, the virus may be recovered from the blood and other tissues in high concentration.
4. Young and old guinea pigs were equally susceptible to the lethal effects of the virus given intracerebrally, whereas only young guinea pigs were susceptible to intranasal instillation of the virus.
5. Rabbits and young chickens died of encephalitis following injection of the virus by the intracerebral route.
6. The virus has been cultivated in developing chick embryos, which are killed when inoculated by any route. Age of the embryo or of hatched chicks has little or no influence on the lethal effects of the virus, although some resistance to infection was observed with an increase in age, as indicated by prolongation of survival time.
7. The virus is filtrable through Berkefeld and Seitz filters; its diameter, estimated by filtration with gradocol membranes, is approximately 18 to 26 millimicrons; by electron micrography it is about 35 millimicrons.

8. The virus is most stable when suspended in solutions with a pH range of 7.3 to 9.7.

9. The virus is resistant to 0.25% formalin for 2 hours at room temperature, but is completely destroyed after 2 hours in 1.0% formalin. It is resistant to chloroform and phenol at 3 - 5°C, having remained active for 5 and 7 weeks respectively in the presence of these reagents.

10. Brain suspensions of the virus resisted heating at 56°C for 30 minutes, but were inactivated at 70°C in 10 minutes.

11. The virus can be preserved by freezing and retains its activity for several months when mixed with glycerine and stored at low temperatures.

12. On the basis of its pathogenicity for experimental animals, the immunological reactions thus far studied, and the lesions which it induces, the Novy virus is believed different from all other known viral agents, although it may be related to the eastern equine encephalomyelitis virus.

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