EXPERIMENTAL ENCEPHALITIS

Some Factors Affecting Infection with Certain Neurotropic Viruses

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The production of encephalitis by experimental methods involves innumerable factors, many of which are well recognized, although poorly understood. Most viruses have a limited host range, affecting particular genera and even species, leaving others unaffected. Even within a given species of animals certain strains and varieties may be much more susceptible than others.

In addition to intrinsic host factors the strains of virus used introduce fresh variables. Virus isolated at different times may differ widely in virulence. Or, a given strain may possess apparently unique properties, as for example the Lansing strain of poliomyelitis shown by Armstrong to be capable of infecting cotton rats,[1] and the variety of herpes shown by Doerr and Hallauer[2] to pass directly into the spinal cord after intravenous injection in rabbits. These spontaneous variations in freshly isolated viruses must be sharply distinguished from artificially produced change due to repeated animal passage. Such treatment may alter the pathogenic properties of the virus and is generally known as fixation. The first and perhaps most widely studied example of such change is so called fixed rabies virus. The influence of fixation on poliomyelitis and equine encephalomyelitis viruses is discussed by us elsewhere.[3]

The present communication deals with certain experimental conditions which influence infection with some neurotropic viruses.

Material

Several viruses have been used. The strains of equine encephalomyelitis were those used in previously published papers.[4] The herpes virus was the HF strain of The Rocke-

[1] Armstrong, C., *Pub. Health Rep., U. S. P. H. S.*, 1939, **54,** 1719.

[2] Doerr, R., and Hallauer, E., *Z. Hyg. u. Infektionskrankh.*, 1936, **118,** 474.

[3] King, L. S., *J. Am. Med. Assn.*, 1939, **113,** 1940.

[4] King, L. S., *J. Exp. Med.*, (*a*) 1938, **68,** 677; (*b*) 1939, **69,** 675; (*c*) 1939, **69,** 691; (*d*) 1940, **71,** 95; (*e*) 1940, **71,** 107.

feller Institute, furnished by Dr. Joseph Smadel. The pseudorabies virus was obtained from Dr. R. E. Shope, who received it originally from Aujeszky. Of St. Louis encephalitis virus, three strains were employed, one (strain 3) furnished by Dr. L. T. Webster, and two by Dr. Margaret Smith (strains H and J).

For the virus of St. Louis encephalitis, purchased Swiss mice were employed. With other viruses, the mice were taken from the highly inbred colony of this Department of The Rockefeller Institute. In any single experiment only animals of the same sex and age were used. Adult mice were 10 to 14 weeks in age. Infant animals were 10 to 12 days old. All operative procedures were conducted under ether anesthesia.

The viruses used were in the form of brain emulsions. The brain of an infected animal was removed aseptically, and ground with buffered saline to make a 10 per cent suspension of brain tissue. The supernatant after light centrifugation is designated at 10^0 dilution of the virus. Serial tenfold dilutions were then made and injected as indicated in the tables.

In the tables, the fate of the several animals inoculated with each dilution is shown. 0 indicates that the test animal survived. The numbers indicate the number of full days which elapsed before death. Thus 3 means the mouse lived at least 72 hours after inoculation but died in less than 96 hours. Other numbers indicate a corresponding lapse of time.

OBSERVATIONS

In the present paper attention is directed to the production of encephalitis, with its typical clinical and pathological features. The term "infection" is used throughout only in the sense of manifest encephalitis, that is, obvious involvement of the central nervous system. Inapparent or latent infection, with or without the production of immunity, is not considered.

Retardation of Infection

1. Effect of Distance of Inoculation Site from Brain.—The virus of pseudorabies possesses certain properties which make it suitable to the study of this problem. This virus causes a natural disease among swine and cattle, for which reference may be made to the papers of Shope.[5] In laboratory animals, as demonstrated by Hurst[6] and others, the virus multiplies at the site of inoculation and then invades the local peripheral nerves. When the virus has reached the spinal ganglia, nerve cell irritation is shown by itching. The animal scratches the site of inoculation or other skin area supplied by the same nerve distribution. When the virus reaches the brain and attains a sufficient concentration, the animal succumbs.

The time elapsing until death varies directly with the distance of the site

[5] Shope, R. E., *J. Exp. Med.*, (*a*) 1931, **54,** 233; (*b*) 1935, **62,** 85; (*c*) 1935, **62,** 101.

[6] Hurst, E. W., *J. Exp. Med.*, (*a*) 1933, **58,** 415; (*b*) 1934, **59,** 729; (*c*) 1936, **63,** 449.

of inoculation from the medullary centers. This was shown in rabbits by Hurst.[6b] In mice more accurate studies can be made by the use of large numbers of animals.

An undiluted 10 per cent suspension of infected rabbit brain was used to inoculate 50 mice, 25 of which were injected in the pad of the hind foot and 25 in the pad of the fore foot. Observations were made every 2 to 3 hours during the day and 3 or 4 hours during the night, and the time at which the animal succumbed was noted.

The intervals between inoculation and death are shown in Fig. 1. It can be seen that the death rate follows a fairly smooth curve showing a linear relationship. The onset of itching marks the time at which virus reaches the spinal ganglia, but it is difficult to make precise observations on this point. Consequently only the time of death was recorded. It can be seen that the animals inoculated in the fore foot succumbed consistently 12 to 14 hours earlier than those inoculated in the hind foot. Assuming that the time required to reach the spinal ganglia of both limbs is the same, this interval of 12 to 14 hours must represent the time required for virus to traverse the length of the spinal cord. It must be emphasized that studies of this nature are applicable only to such viruses as strictly follow the direct nerve course from the site of inoculation to the central nervous system.

2. Section of the Sciatic Nerve.—With a virus which so regularly follows nerve paths as does pseudorabies, studies on sections of the nerve path are of interest. Hurst performed this experiment in rabbits and found that the animals died without showing itching of the inoculated part. He concluded that virus prevented by nerve section from travelling up the local nerve was disseminated into the body through the blood and attained the nervous system by other nerve paths after having been deposited in a secondary focus.

In the present experiment the right sciatic nerve of 40 mice was sectioned. 20 of them were inoculated in the pad of the same leg; the remaining 20, serving as controls, were inoculated in the pad of the unoperated hind leg. Contrary to the observations of Hurst in the rabbit, all the mice showed itching, indicating attainment of the appropriate spinal root ganglia by the virus by way of local nerves. However, the animals inoculated in the denervated leg died significantly later (averaging about 15 hours) than did the controls. This result, shown in Fig. 2, must be interpreted as follows: The virus, unable to travel up the cut sciatic, invaded other nerves of the leg which may include the sympathetic nerve supply and perhaps, through spread of the inoculum, other somatic nerves. Due to the section of the sciatic nerve fibers the total mass of the available nerve pathway was re-

duced, with consequent retardation of the rate of infection. Hence it is clear that number of available nerve fibers plays a rôle in the speed with which infection occurs.

3. Effect of Inflammation.—When various agents are injected into a site of acute inflammation their dissemination through the body may be sharply reduced. This has been shown for many bacteria as well as proteins and dyes. The whole subject has been most recently discussed by Menkin.[7] It seemed of interest to determine whether inflammation would affect the action of pseudorabies virus injected into the inflamed area.

Inflammation was induced in the hind pad of 30 animals by injection of a mixture of turpentine, ether, and olive oil. 6 hours later when the inflammatory action was well advanced, virus was injected into the inflamed site. At the same time an equal number of controls were similarly inoculated with virus alone. Two concentrations of virus were used: an undiluted 10 per cent suspension (10^0 dilution) and a hundredfold dilution (10^{-2}) of the same stock suspension. The quantity of inoculum was 0.05 cc. in all cases. The results are seen in Fig. 3. In the control group the lesser concentration of virus caused death significantly later than the strong concentration. In animals injected into the inflamed areas, there was a significant slowing of the rate of infection, distinctly observable with the 10^0 inoculum but much more pronounced with the diluted virus. Here not only was the rate of infection much slower but the morbidity was significantly less.

It is obvious that with this virus, inflammation exerts a definite retarding influence on the course of infection; but the nature of the infection was not altered, that is, all animals—experimental as well as control—showed itching at the site of inoculation. Only the rate of infection was affected.

It was necessary to compare these findings with another virus whose mode of action is quite different. Unmodified equine encephalomyelitis virus, unlike pseudorabies, does not invade the nervous system through the local peripheral nerves.[8] With this virus over a wide range of dilutions, adult mice were injected in the hind pad which had previously been inflamed by the same irritant mixture, together with an equal number of controls. As seen in Table I, the inflammation did not cause any diminution in the rate or time of infection. Where the virus becomes disseminated through the body before the nervous system is involved, as in equine encephalomyelitis, no inhibitory or retarding effect of inflammation could be detected.

[7] Menkin, V., *Physiol. Rev.*, 1938, **18**, 366.

[8] Hurst, E. W., *J. Path. and Bact.*, 1936, **42**, 271.

Facilitation of Infection

1. Brain Trauma.—There are reports in the literature that with certain viruses brain trauma may facilitate infection. This has been interpreted as a mechanical disruption of the blood-brain barrier whereby the viruses find greater ease of entrance into the nervous system. The subject has been discussed in a previous paper.[3] The most striking examples of this phenomenon in the literature are furnished by the viruses of yellow fever[9] and St. Louis encephalitis.[10] In an attempt to study this problem systematically, experiments were first carried out with the viruses of equine encephalomyelitis. These results, already published,[4d] showed that brain trauma had no effect on infection. Attention was next directed to the virus of St. Louis encephalitis.

TABLE I

Unmodified Equine Encephalomyelitis Virus: Subcutaneous Inoculation into an Area of Inflammation in Adult Mice

Dilution	Injection into inflamed area	Control
10^{-6}	6, 11, 0, 0	5, 0, 0, 0
10^{-5}	5, 7, 8, 0	7, 7, 7, 12
10^{-4}	5, 5, 9, 0	5, 6, 0, 0
10^{-3}	5, 5, 6, 9	5, 7, 0, 0
10^{-2}	5, 7, 0, 0	6, 7, 0, 0

6, 7, 8, etc. = mouse died after 6, 7, 8, etc., days.
0 = mouse survived.

Three strains of the virus were utilized. Following the procedures of Webster and Clow,[10] animals were injected intracerebrally with 0.03 cc. of a 2 per cent starch solution. Immediately thereafter, the animals were given an intraperitoneal injection of 0.3 cc. of virus over a wide range of dilutions. Control animals were equally injected intraperitoneally. At the same time the titer was determined by direct intracerebral injections, furnishing a quantitative estimation of minimal lethal dosage.

This procedure was followed with three different strains of virus with the uniform results shown in Table II. It will be observed first that intraperitoneal inoculation was not especially effective in inducing the disease; and second, that the brain trauma produced by injection of starch intracerebrally did not change the infective titer. Similar results were obtained when the traumatizing agent was a hot needle stab in the brain. These results, therefore, do not confirm the findings of Webster and Clow.

[9] Sawyer, W. A., and Lloyd, W., *J. Exp. Med.*, 1931, **54**, 533.
[10] Webster, L. T., and Clow, A. D., *J. Exp. Med.*, 1936, **63**, 433.

2. Effect of Glycerine.—The observation was made with the fixed strain of equine encephalomyelitis virus[4 d] that while brain trauma had no influ-

TABLE II

Brain Trauma as Influencing Infection with St. Louis Encephalitis Virus

Dilution	Strain 3 (Webster)			Strain J (Smith)			Strain H (Smith)		
	Intraperitoneal (0.30 cc.)		Intracerebral (0.03 cc.)	Intraperitoneal (0.20 cc.)		Intracerebral (0.03 cc.)	Intraperitoneal (0.30 cc.)		Intracerebral (0.03 cc.)
	Brain trauma	Control		Brain trauma	Control		Brain trauma	Control	
10^{-8}			0, 0						
10^{-7}			0, 0			0, 0			0, 0
10^{-6}			5, 0			0, 0			3, 5
10^{-5}			5, 5			4, 0			4, 5
10^{-4}	0, 0, 0, 0	0, 0, 0, 0		0, 0, 0, 0	0, 0, 0, 0	3, 4	0, 0, 0, 0	0, 0, 0, 0	3, 4
10^{-3}	0, 0, 0, X	0, 0, 0, 0		0, 0, 0, 0	0, 0, 0, 0	4, 4	0, 0, 0, X	0, 0, 0, 0	3, 4
10^{-2}	0, 0, 0, 0	0, 0, 0, 0		0, 0, 0, 0	0, 0, 0, 0	3, 4	0, 0, 0, 0	6, 0, 0, 0	
10^{-1}	5, 5, 6, 0	7, 0, 0, 0		11, 0, 0, 0	0, 0, 0, 0		4, 0, 0, 0	6, 7, 8, 0	
10^{0}				5, 6, 10, 0	5, 7, 0, 0		4, 4, 6, 8	4, 5, 0, 0	

3 = mouse died between 72 and 96 hours after inoculation.

4 = mouse died between 96 and 120 hours after inoculation, etc.

0 = mouse survived.

X = mouse died within a few hours of the inoculation. Death not due to virus action.

Blank spaces = not tested.

TABLE III

Effects of Intramuscular Inoculation with St. Louis Encephalitis Virus

(Undiluted 10 per cent brain suspension used as inoculum)

Experiment	Treatment	No. inoculated	No. dead	No. survived	Percentage dead
Facilitation of infection by intraperitoneal glycerine	Intraperitoneal glycerine	43	36	7*	83
	None—control	40	5	35	12
Lack of facilitation by brain trauma	Hot needle stab in brain 24 hrs. before	22	2	20	9
	Intracerebral starch	23	1	22	4
	None—control	24	2	22	8

* 2 of 7 survivors showed paralysis of hind legs.

ence on infection, a marked facilitation of it was attained when glycerine was injected intraperitoneally. The same method was employed with St. Louis encephalitis virus. 0.23 cc. of 50 per cent glycerine was injected

intraperitoneally and undiluted 10 per cent virus suspension (0.25 cc.) injected intramuscularly.

With the dosage of glycerine used, death within 1 to 3 hours might result from the glycerine alone, but extensive control tests showed that mice surviving this initial period would survive indefinitely. In Table III, all deaths were due to virus action, and animals which succumbed in less than 24 hours are not included in the table. All recorded deaths were preceded by typical encephalitic symptoms, which first became manifest not less than 72 hours after virus inoculation.

Of the animals receiving the intraperitoneal glycerine 36 out of 43 succumbed with typical signs of encephalitis. At the same time, of 40 controls only 5 died. These results indicate that with St. Louis encephalitis virus, as with the fixed equine encephalomyelitis strain, intraperitoneal glycerine has a marked facilitating effect.

TABLE IV

The Effects of Glycerine and Starch on the Pathogenicity of Pseudorabies Virus

(The minimal effective dose injected into a hind pad)

Preliminary treatment	No. of mice inoculated	No. dead	Percentage dead
Intraperitoneal glycerine	25	2	10
Intracerebral starch	21	3	14
None—control	20	3	15

It was necessary to find whether with intramuscular injection, brain trauma facilitated infection. Of three groups of animals, one received intracerebral starch, the second, a hot needle stab in the brain 24 hours previously as preliminary treatment, while the third group was the control. The mortality rate in all three groups was the same. The results of these two experiments are shown in Table III.

The question then arose whether intraperitoneal glycerine would exert an action with other viruses. The same procedure was adopted with herpes virus. 25 mice were given glycerine intraperitoneally and virus intramuscularly, while 18 controls were utilized. Of the control group 3 died; of the group receiving glycerine, 5 died. All showed paralysis of the inoculated leg as the first symptom, indicating a passage of virus up the local nerve from the site of inoculation. It is clear that with herpes virus the intraperitoneal glycerine exerted no effect.

Similar experiments were carried out with pseudorabies virus. Here three groups were used, one of which received intraperitoneal glycerine, a

second intracerebral starch, while a third was a control. All groups were inoculated in the hind pad with a highly diluted virus suspension. It was

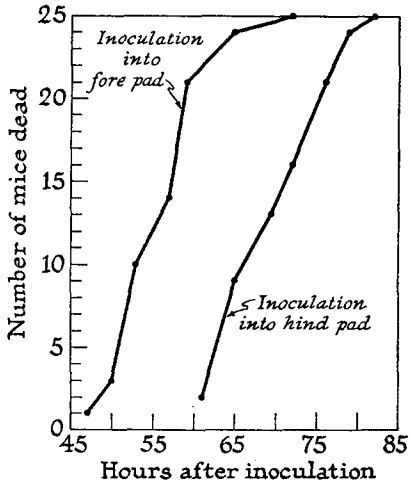

FIG. 1

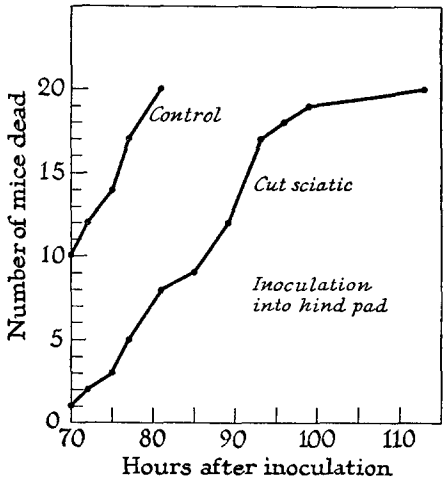

FIG. 2

FIG. 1. Pseudorabies virus. Mortality rate following inoculation into fore pad and hind pad.

FIG. 2. Pseudorabies virus. Mortality rate following inoculation into hind pad after section of sciatic nerve.

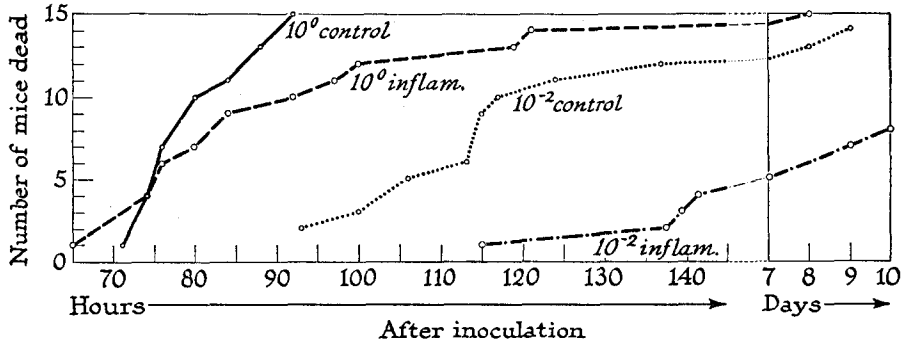

FIG. 3. Mortality rate with pseudorabies virus after inoculation into area of inflammation. The stock suspension was injected undiluted (10^0) and in hundredfold (10^{-2}) dilution.

desired to utilize only a minimally infective dose in order to determine possible facilitating results of the preliminary treatments. The results shown in Table IV indicate that with pseudorabies as with herpes no facilitation of infection could be produced either by glycerine or by intracerebral

starch. A further experiment was performed, using a concentrated virus suspension injected in the pad. A group receiving preliminary intraperitoneal glycerine was compared with a control group to determine whether the glycerine would affect the rate of infection, determined as in Figs. 1, 2, and 3. All animals, control as well as experimental, died. The rate of death was almost exactly equal in the two groups. Thus with pseudorabies virus intraperitoneal glycerine does not render a minimal dose more effective nor does it render greater the effect of a large dose.

Pseudorabies and herpes viruses act similarly, showing no effect due to glycerine, whereas St. Louis encephalitis virus is like equine encephalomyelitis virus in exhibiting a marked facilitation by this treatment.

Influence of Age

It has been known for many years that the young of some animals are more susceptible than adults. This was noted by Theiler[11] and by Andervont,[12] who worked with yellow fever and herpes virus, respectively, using mice. Work along these lines was done later by Olitsky[13] and his coworkers, particularly by Sabin and Olitsky.[14] The effect of age on infection of mice with equine encephalomyelitis viruses has been discussed in a previous publication[4d] where the findings are at some variance with those reported by Sabin and Olitsky.[14d]

The increased susceptibility of young animals seems to be especially marked in the case of mice, and to be more pronounced with certain viruses than with others. In accordance with experiments already done with equine encephalomyelitis virus, young and adult mice were tested with the virus of pseudorabies. The young animals were, as in previously published experiments, about 12 days of age, with a good coat of white hair but with eyes not yet opened. They and the adults were given the virus by intracerebral, intranasal, and subcutaneous routes. The intracerebral inoculation was utilized, first, to show the titer of the virus and, second, to determine whether increased susceptibility is restricted to peripheral inoculation or whether it applies also to intracerebral injection. The results are set forth in Table V. It can be seen that with pseudorabies virus there was a barely distinguishable difference between young and adults with the two

[11] Theiler, M., *Ann. Trop. Med. and Parasitol.*, 1930, **24**, 249.

[12] Andervont, H. B., *J. Infect. Dis.*, 1929, **44**, 383.

[13] Olitsky, P. K., Sabin, A. B., and Cox, H. R., *J. Exp. Med.*, 1936, **64**, 723.

[14] Sabin, A. B., and Olitsky, P. K., *J. Exp. Med.*, (*a*) 1937, **66**, 15; (*b*) 1937, **66**, 35; (*c*) 1938, **67**, 201; (*d*) *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 595; (*e*) 1938, **38**, 597.

modes of peripheral inoculation. This contrasts sharply with data in the literature on certain other viruses.

In order to confirm the data of Andervont[12] with herpes virus, young and adults were inoculated intracerebrally and subcutaneously as shown in Table VI. With the strain of virus used, subcutaneous inoculation in the adult was not encephalitogenic. The young, however, succumbed to high

TABLE V

Titration of Pseudorabies Virus by Various Routes in Adult and Young Mice

Dilution	Intracerebral		Intranasal		Subcutaneous (thigh)	
	Adults (0.04 cc.)	Infants (0.02 cc.)	Adults	Infants	Adults (0.10 cc.)	Infants (0.05 cc.)
10^{-7}	0, 0, 0	0, 0, 0				
10^{-6}	0, 0, 0	3, 0, 0	0, 0, 0	0, 0, X	0, 0, 0, 0	0, 0, 0, 0
10^{-5}	3, 3, 0	1, 2, 3	0, 0, 0	0, 0, 0	0, 0, 0, 0	0, 0, 0, 0
10^{-4}	2, 2, 2	1, 1, 2	0, 0, 0	0, 0, 0	0, 0, 0, 0	0, 0, 0, 0
10^{-3}	1, 2, 2	1, 1, 2	0, 0, 0	3, 4, 0	0, 0, 0, 0	3, 3, 4, 4
10^{-2}			3, 3, 0	2, 3, X	3, 3, 3, 4	3, 3, 3, 3

Symbols as in previous tables.

TABLE VI

Effects of Herpes Virus on Young and Adult Mice

Dilution	Route of inoculation			
	Intracerebral		Subcutaneous	
	Adults (0.03 cc.)	Infant (0.02 cc.)	Adults (0.05 cc.)	Infant (0.03 cc.)
10^{-5}	0, 0, 0	0, 0, 0		
10^{-4}	0, 0, 0	0, 0, 0		
10^{-3}	9, 0, 0	5, 0, 0	0, 0, 0	0, 0, 0
10^{-2}	5, 5, 5	2, 2, 4	0, 0, 0	15, 0, 0
10^{-1}			0, 0, 0	6, 0, 0
10^{0}			0, 0, 0	4, 6, 7

concentrations of virus. Here too the difference due to age was present but not marked.

The effect of age on inoculation of mice with St. Louis encephalitis virus was not investigated, since this subject is being studied by Smith.[15]

Effect of Route of Inoculation

The route of inoculation chosen may be an important determining factor in experimental encephalitis. In this respect, however, the nature of the virus must also be considered. With equine encephalomyelitis that has

[15] Smith, M., personal communication.

been freshly isolated, there is a high pathogenicity regardless of the route of injection.[4d] On the other hand when the virus has been modified by repeated intracerebral passage, there is a striking decline in effect on the nervous system when the ordinary subcutaneous, intraperitoneal, or intramuscular routes of inoculation are employed. Yet this altered or "fixed" virus remains highly active if injected into the eye or instilled into the nose.[4d]

Ease of infection by the nasal route in the face of refractoriness when other peripheral sites are used, has been observed for a number of different viruses. Inoculation into the eye as an especially favorable site has received but little attention in this relation although Burnet[16] recently reported use of the intraocular pathway in experimental poliomyelitis.

In the present experiments the several viruses were injected by a variety of routes. Particular attention was paid to intraocular injection and intra-

TABLE VII

Susceptibility to Reinoculation of Mice Surviving a First Injection of Pseudorabies Virus

Dilution	Control intracerebral injection	Pad injection 0.05 cc.	
		Reinoculation	Control (1st inoculation)
10^{-6}	0/3		
10^{-5}	1/3	2/20	1/21
10^{-4}	3/3	12/21	15/30

Denominator = number of mice inoculated.
Numerator = number of mice dead of encephalitis.

nasal instillation which were contrasted with at least one other mode of peripheral inoculation. Certain details call for comment.

Pseudorabies.—Pseudorabies is an example of viruses which are fatal even when minute doses are inoculated subcutaneously. Many experiments have been performed, and at times the subcutaneous route may be almost as effective as direct intracerebral injection of virus. There is considerable variation in susceptibility, however, from animal to animal.

In an experiment to test for possible immunity, animals that had, without symptoms, survived injection of a given dilution of virus (10^{-4} and 10^{-5}) were reinoculated in the hind pad, and at the same time animals previously unused were similarly inoculated as controls. Different dilutions of virus were injected intracerebrally, to determine the titer by this method. The experiment is summarized in Table VII. It shows incidentally that no

[16] Burnet, F. M., Jackson, A. V., and Robertson, E. G., *Australian J. Exp. Biol. and Med. Sc.*, 1939, **17**, 253.

immunity had been produced. The fact of interest to the present problem is that the highest dilution to kill all mice by the intracerebral route killed one-half of a large number of animals when injected in the pad. This is an extremely high mortality ratio. With the next higher dilution, containing less than one minimal cerebral lethal dose, a small proportion of mice injected in the pad succumbed to the disease. With the 10^{-4} dilution of virus, other experiments showed on subcutaneous inoculation a mortality percentage less than 50 per cent. Nevertheless, a significant proportion of all mice will succumb to pad inoculation with virus suspensions containing one to ten minimal cerebral lethal doses.

A puzzling observation of considerable interest is that inoculation of virus into the foot pad is constantly more effective than the same dilution injected subcutaneously in the thigh. A single example is shown in Table VIII, under Experiment 2 (*cf.* also Table V). The reason for this is obscure. It was thought that perhaps the tissue of the foot pad allowed greater multiplication of virus *in situ* than did the tissues of the thigh.

TABLE VIII

Titration of Pseudorabies Virus in Adult Mice Inoculated by Different Routes

Dilution	Experiment 1				Experiment 2				
								Subcutaneous, into	
	Intracerebral	Intraocular	Intranasal	Subcutaneous into thigh	Intracerebral	Intraocular	Intranasal	Pad (0.05 cc.)	Thigh (0.10 cc.)
10^{-6}	0, 0, 0				0, 0, 0, 0				
10^{-5}	3, 0, 0	6, 0, 0	0, 0, 0	0, 0, 0	2, 3, 0, 0	0, 0, 0, 0		0, 0, 0, 0	0, 0, 0, 0
10^{-4}	2, 2, 2	4, 5, 0	0, 0, 0	0, 0, 0	2, 2, 3, 3	0, 0, 0, 0	0, 0, 0, 0	5, 5, 5, 0	0, 0, 0, 0
10^{-3}	2, 2, 2	2, 2, 3	0, 0, 0	3, 5, 0		2, 3, 3, 3	3, 3, 5, 0	4, 5, 6, 0	0, 0, 0, 0
10^{-2}		2, 2, 2	2, 4, 4	3, 3, 5		2, 2, 3, 3	2, 3, 3, 4	4, 4, 4, 5	4, 0, 0, 0
10^{-1}		2, 2, 3		2, 3, 3					

An attempt was made to determine whether virus actually increased more readily in the pad. Mice were injected with a virus suspension to which India ink had been added. At intervals animals were sacrificed and the gray pad and thigh tissues excised, weighed, and separately ground up with a measured quantity of saline. The supernatant after centrifugation was diluted quantitatively and titrations of the separate tissues were performed in mice. No significant difference in rate of local increase could be detected by this method. There was consequently no evidence that the difference between pad and thigh inoculation is due to greater multiplication of the virus in the former instance. It is possible that the number of exposed and available nerve terminals is greater in the foot pad, but no attempt has been made to determine whether this is so.

Pseudorabies virus injected into the vitreous of the eye causes encephalitis in high dilutions. Here too there is variation in susceptibility from one animal to another. Some titrations, as in Table VIII, Experiment 1,

showed very little difference between the intraocular and intracerebral routes. In other experiments (*e.g.*, Table VIII, Experiment 2) there was a lesser sensitivity of the intraocular route. In the latter experiment pad inoculation was more effective than the intraocular.

Numerous experiments, only a few of which are shown in the accompanying tables, demonstrated that with pseudorabies virus the intraocular route is not significantly more effective than subcutaneous injection, provided the latter is done in the foot pad. In this respect pseudorabies virus resembles unmodified equine encephalomyelitis virus, and is in sharp contrast to the fixed equine encephalomyelitis virus.[4 d]

Herpes.—Herpes virus, injected subcutaneously into the foot pad of an adult mouse even in the form of undiluted 10 per cent brain suspension, was

TABLE IX

Titration of Herpes Virus in Adult Mice Inoculated by Different Routes

Dilution	Route of inoculation			
	Intracerebral (0.03 cc.)	Intraocular (0.01 cc.)	Intranasal (0.05 cc.)	Intraperitoneal (0.30 cc.)
10^{-4}	6, 0, 0, 0	0, 0, 0, 0, 0		0, 0, 0, 0, 0
10^{-3}	4, 5, 6, 6	7, 9, 10, 0, 0	0, 0, 0, 0, 0	7, 0, 0, 0, 0
10^{-2}	3, 4, 4, 8	5, 7, 8, 8, 14	0, 0, 0, 0, X	9, 9, 10, 0, 0
10^{-1}	3, 3, 3, 4	5, 5, 6, 6, 7	14, 0, 0, 0, 0	6, 6, 7, 7, 0
10^{0}		5, 5, 5, 5, 5	5, 6, 10, 0, 0	6, 6, 6, 7, 9

quite without pathogenic effect on the nervous system (Table VI). There was but little greater effect when it was injected intramuscularly. However, injected intraperitoneally it may prove very virulent (Table IX). This finding is not entirely constant, yet certainly the intraperitoneal route is incomparably more lethal than the subcutaneous or intramuscular. In the experiment shown in Table IX, groups of mice were inoculated in the brain, eye, nose, and peritoneum. Intranasal instillation of herpes virus is not especially effective, as Burnet and Lush[17] have already noted.

In the comparison of ocular and peritoneal routes (Table IX), it must first be noted that the dosage for the latter was 0.30 cc., for the former, $\pm$0.01 cc. In spite of this thirtyfold difference in dosage, intraocular injection is definitely more effective. It must be concluded that with herpes, unlike pseudorabies, intraocular injection is the most favorable method of peripheral inoculation. Possible reasons will be considered subsequently.

St. Louis Encephalitis.—With this virus, too, subcutaneous pad injection

[17] Burnet, F. M., and Lush, D., *J. Path. and Bact.*, 1939, **49**, 241.

of undiluted virus suspension caused no disease, while intramuscular injection in otherwise untreated mice (Table III) showed but little activity. Intraperitoneal inoculation, in contrast with herpes, was pathogenic only with large doses, and then inconstantly (Tables II and X). On the other hand, injection into the eye (Table X) was very effective, as was instillation into the nose. These two routes are by far the most active of all forms of peripheral inoculation. The difference between these favored sites and the intraperitoneal route is vastly more pronounced than is the case with herpes virus. In all respects so far mentioned in this paper, the virus of St. Louis encephalitis seems very similar to the fixed strain of equine encephalomyelitis virus.[4d]

TABLE X

Titration of St. Louis Encephalitis Virus (Strain H) in Adult Swiss Mice Inoculated by Various Routes

Dilution	Intracerebral (0.03 cc.)	Intraperitoneal (0.30 cc.)	Intranasal	Intraocular (± 0.01 cc.)
10^{-7}	0, 0, 0			
10^{-6}	5, 0, 0			
10^{-5}	3, 5, 0			
10^{-4}	4, 4, 4		0, 0, 0	0, 0, 0
10^{-3}	3, 3, 3	0, 0, 0	0, 0, 0	8, 0, 0
10^{-2}		0, 0, 0	5, 6, 6	6, 6, 7
10^{-1}		7, 0, 0	5, 5, 8	5, 5, 0
10^{0}		6, 0, 0	5, 6, 6	5, 6, 7

Pathways from Eye to Brain

In the passage of viruses from the periphery to the central nervous system, there are three possibilities to be considered. The first is direct passage along the local nerves connecting the inoculation site and the central nervous system. The second is dissemination into the blood stream, with direct passage into brain parenchyma across cerebral blood vessels. The third is more complicated, namely, preliminary dissemination through the blood from the initial inoculation site, deposition of virus in a secondary site, with direct nerve passage to the brain from this secondary location.

Viruses have never been demonstrated in the act of so travelling up peripheral nerves, but there are valuable indirect indications that some do so. Goodpasture and Teague[18] introduced a method of histologic study, by which they showed, with herpes virus, that the portion of the brain where

[18] Goodpasture, E. W., and Teague, O., *J. Med. Research*, 1923, **44**, 139.

encephalitic lesions could first be demonstrated was directly connected with the inoculation site by specific peripheral nerves. Varying the inoculation site caused a predictable variation in the location of the centrally placed lesion. Direct axonal passage of the virus was the only logical conclusion. Another indirect method was to test appropriate portions of the nervous system for virus content, rather than for histologic damage. After peripheral injection of virus, the appearance of detectable virus in the related nerve centers prior to its appearance elsewhere in the brain, is presumptive evidence of direct nerve transmission. Obviously, the center in question must be in direct, unequivocal, anatomical relationship to the inoculation site to make such presumption valid.

Herpes.—Goodpasture and Teague showed by histologic studies[18] that when herpes is inoculated into the cornea of a rabbit, the virus passes along the fibers of the trigeminal nerve to reach the brain. But injected into the vitreous the virus passes along the optic nerve. In the experiments in mice reported in this paper, the inoculation was into the vitreous. To determine the pathway followed by the virus, serial sections were made of mouse brains when the animals were dead or moribund from encephalitis.

Examination of the stained sections demonstrated that lesions were present in the Gasserian ganglion of the side injected, and in the nuclei of the fifth nerve and the immediately adjacent tissue. The higher centers were free from histologic change. It must necessarily be concluded that herpes simplex virus eschews the ganglion cells of the mouse retina and the optic nerve, but follows the axones of the trigeminal nerve to attain the brain.

In their studies on herpes, Burnet and Lush[17] showed that following intranasal instillation the virus also passes along the trigeminal pathway, without involving the superficially placed olfactory neurones.

Pseudorabies.—The virus of pseudorabies causes such slight morphologic change in the brains of mice that histologic analysis was not deemed completely reliable. Instead, the method of testing portions of the nervous system for virus content was employed.

Mice were injected into the eyeball, and were then sacrificed at intervals. The Gasserian ganglion of each animal sacrificed, the primary optic centers (lateral geniculate bodies and superior colliculi, which were snipped off from the exposed brain stem), and the cerebral hemispheres were separately ground up in approximately 10 per cent suspension in saline, and the supernatant injected intracerebrally in test mice. At the same time, heart's blood was similarly injected to discover whether virus circulated in the blood.

The results, recorded in Table XI, demonstrate that up to 36 hours, virus could be found only in the trigeminal centers. After that, it spread to

other portions of the brain. The presence of a minimal trace of virus in the blood only at 60 hours was surprising. It has been generally believed that pseudorabies virus readily attains the blood stream, but this is not supported by the present data.

The above experiment was designed to decide whether the optic or the trigeminal pathway was utilized in passage from the eye to the brain. The answer is clearly in favor of the trigeminal. Whether sympathetic nerve fibers are also involved in the spread was not investigated.

St. Louis Encephalitis.—Similar experiments were performed with the virus of St. Louis encephalitis, except that blood was not tested. As shown in the second part of Table XI, this virus, unlike pseudorabies, was detectable in the optic centers before appearing in the Gasserian ganglion. It

TABLE XI

Spread of Virus from Eye to Brain after Intraocular Inoculation

Virus	Material tested	Virulence for mice of inocula tested at				
		12 hrs.	24 hrs.	36 hrs.	48 hrs.	60 hrs.
Pseudorabies	Trigeminal centers	0, 0, 0	2, 3, 0	2, 2, 2	1, 1, 1	1, 1, 1
	Optic centers	0, 0, 0	0, 0, 0	0, 0, 0	3, 6, 0	2, 3, 3
	Forebrain	0, 0, 0	0, 0, 0	0, 0, 0	2, 3, 0	2, 3, 4
	Blood	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	7, 0, 0
		24 hrs.	48 hrs.	72 hrs.	96 hrs.	
St. Louis encephalitis	Trigeminal centers	0, 0	0, 0	5, 5	7, 7	
	Optic centers	0, 0	3, 4	4, 4	3, 4	
	Forebrain	0, 0	0, 0	4, 4	4, 5	

appeared in the latter situation only after it had become generalized throughout the entire nervous system. Histologic study confirmed the view that this virus, injected into the eyeball, proceeds along the optic nerve to the visual centers of the brain. St. Louis virus does not lend itself as well as herpes to histologic analysis of its mode of spread. Webster and Clow[10] have already noted that with St. Louis virus the progression of lesions need not accurately reflect the progression of virus. Nevertheless the available evidence indicates that after introduction into the eye the virus primarily attacks the optic pathway, in contrast to herpes and pseudorabies, which utilize the trigeminal pathway. With St. Louis encephalitis virus the possible utilization of other pathways or modes of spread cannot be definitely excluded. The point under consideration here concerns only the relative preference of the different viruses for the optic as opposed to the trigeminal pathway.

Fixed Equine Encephalomyelitis.—It has previously been shown[4c] that when fixed equine encephalomyelitis virus is injected into the eyes of guinea pigs the optic pathway to the brain is followed. This was demonstrated by two methods, histologic analysis and the testing of brain portions for virus content. Similar experiments were carried out in mice. In comparison with guinea pigs, the equine encephalomyelitis virus in mice spreads somewhat more diffusely and more rapidly through the nervous system, making the exact pathways a little more difficult to trace.

The clearest evidence was obtained by the study of brain serial sections from animals killed at different intervals after inoculation. With an inoculum that regularly killed 66 to 70 hours after injection into the eye, a critical stage was observed after 48 to 49 hours. At that time there were definite inflammatory changes along the course of the optic tracts and tissues immediately adjacent, and in the lateral geniculate bodies and superior colliculi, predominantly contralateral to the inoculated side. At the same time the rest of the brain was free of lesions. In animals sacrificed earlier, there were no definite lesions at all. If examined after longer intervals, lesions were very widespread, being present not only in the optic system but in all parts of the brain, so that any differential significance was lacking. At the critical stage, however, the evidence was clear that the primary attack of the virus is along the optic pathway. This evidence was confirmed by the method of testing brain portions for virus.

As with St. Louis virus the evidence indicates a preference for the optic system as compared with the trigeminal, but does not exclude utilization of other modes of spread.

DISCUSSION

It is readily apparent that the neurotropic viruses differ widely one from another in methods of spread. Some of them invade the local nerve at the site of inoculation, and proceed to the central nervous system by means of this pathway. Pseudorabies virus does this to a superlative degree, herpes slightly less. That these two viruses are closely related biologically is maintained by Burnet and Williams,[19] and their similarities have also been pointed out by Hurst.[20] Pseudorabies is much more pathogenic on pad inoculation than herpes, but the latter virus, on intramuscular inoculation, shows the power of nerve ascension. Both of the viruses when injected into the eye choose the fifth nerve along which to travel, to the neglect of the optic nerve. Sabin[21] showed that pseudorabies virus when instilled

[19] Burnet, F. M., and Williams, S. W., *Med. J. Australia*, 1939, **1**, 637.

[20] Hurst, E. W., *Brain*, 1936, **59**, 1.

[21] Sabin, A. B., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 270.

into the nose, followed trigeminal fibers and not the olfactory; while Burnet and Lush[17] similarly demonstrated for herpes the use of trigeminal pathway and avoidance of the olfactory.

When virus injected into the eye attacks the trigeminal nerve fibers, the situation is comparable to the effects of subcutaneous or intramuscular injection in the leg when the virus ascends the local peripheral nerve. In both cases the virus involves the axone first and then proceeds towards the nerve cell body. This may be called cellupetal progression. Pseudorabies and herpes exhibit this phenomenon to a striking degree.

On the other hand, when virus similarly injected into the eye involves the optic nerve fibers the situation is reversed. The passage from the superficially placed ganglion cells of the retina backwards into the optic nerve is away from the cell body, or cellufugal. After inoculation into the eye, fixed equine encephalomyelitis and St. Louis encephalitis viruses choose this mode of progress. Sabin and Olitsky[14c] showed that vesicular stomatitis virus also follows the optic nerve.

Those viruses which attack the optic system after injection into the eye involve the olfactory pathway after instillation into the nose. In both instances infection seems to be initiated by primary attack on ganglion cells. But pseudorabies and herpes, whether in the eye or the nose, show preference for the exposed axones and avoidance of the ganglion cells.

It is not clear why one virus should attack exposed axones, while another attacks ganglion cells but leaves axones for the most part alone, when both are inoculated at the same site. The different behavior of different viruses may perhaps be correlated with the different metabolic rates of cell bodies as compared with cell processes or axones, as well as with different cytoplasmic and chemical composition of these structures. Such factors may possibly determine the tropism of a given virus, and be concerned with fundamental reactions between virus and cell.

The distinction of cellupetal and cellufugal progression of viruses, although clear cut, is only approximate. Injection into the eye offers any virus a choice of pathways. Injection into the subcutaneous or muscular tissue on the other hand offers no choice. If the direct nerve pathway is to be followed, only axones are available with passage towards the cell body. Under such conditions, pseudorabies and herpes quite regularly proceed along this pathway and fixed equine encephalomyelitis and St. Louis encephalitis viruses, injected intramuscularly in high concentration, will also do so in a small percentage of cases. It must be concluded that these last two viruses infect nerve cell bodies readily and in relatively low concentra-

tions, but also have a very limited ability to invade axones when the concentration of virus is high.

On the other hand, herpes virus, which readily infects axones, and does so even when a choice is offered, may also occasionally infect nerve cell bodies. Herpes instilled into the nose of mice attacks the terminals of the trigeminal nerve, but it may also occasionally infect the olfactory nerve cells and proceed cellufugally into the olfactory bulb. Histologic preparations demonstrating this unequivocally were shown to us by Slavin.[22] Consequently, the distinction of preferential cellufugal or cellupetal passage is not absolute, but represents a general trend.

In connection with intraocular injection, the high effectiveness of this route with certain viruses deserves comment. With fixed equine encephalomyelitis and St. Louis viruses, the ready availability of ganglion cells through which infection may easily be initiated offers a satisfactory explanation. A similar explanation applies to the results of intranasal instillation, where the superficially placed olfactory nerve cells offer an easy pathway. By contrast these viruses are non-infectious on subcutaneous pad inoculation where only axones are available, and only slightly effective on intramuscular injection.

Pseudorabies virus, which by choice ascends axones, is not more infectious by the ocular route than by subcutaneous pad injection. But herpes, which has many properties in common with pseudorabies, shows a markedly greater effectiveness when introduced by the intraocular route as compared with the subcutaneous or intramuscular. The availability of ganglion cells in the eye will not explain this, since herpes selects axones in preference to ganglion cells. It is possible that the number of nerve fibers per unit area, or conceivably some qualitative as well as quantitative factor, plays a part in this phenomenon. But this must be left for future study and investigation.

With the pseudorabies virus, the number of nerve fibers available for passage plays a part in the retardation and facilitation of infection. Why inflammation should retard ascension of the virus is not clear. Further work on the effect of inflammation on dissemination of viruses through the body, as well as on the interaction of virus and nerve fibers, is clearly necessary.

The results here presented on the effect of brain trauma on infection, do not agree with those of Webster and Clow.[10] These authors, using St. Louis encephalitis virus, found it much more effective by intraperitoneal

[22] Slavin, H. B., personal communication.

inoculation into normal controls than was the case in the present experiments. Two possibilities suggest themselves in explanation of the differences found. In the years intervening between the experiments of Webster and Clow and of the present author, the virus may have changed its properties by increased fixation. Its loss of infectiousness by peripheral inoculation strongly suggests that such a process may have gone on. Also, there may have been a difference in the strains of mice used for the experimental work, since all Swiss mice are not alike. Webster[23] was able to show that from a given hybrid strain, separate lines may be bred differing markedly in susceptibility to St. Louis virus.

Webster and Clow[10] found that brain trauma raised the titer of intraperitoneal inoculation. Using three different strains of virus, we have been unable to confirm this work.

There is no readily available explanation why intraperitoneal injections of glycerine should facilitate infection. Both fixed equine encephalomyelitis and St. Louis viruses exhibited this phenomenon, but neither herpes nor pseudorabies did so. The latter viruses primarily invade axones; the former do so only exceptionally. The glycerine is effective only in aiding the production of a primary encephalitis. Where the infection takes the form of ascending myelitis, there is no facilitation. The mechanism of the phenomenon will receive further study.

The increased susceptibility of young mice cannot be discussed in detail here. This subject has received particular attention in the work of Sabin and Olitsky[14] on vesicular stomatitis virus. On the basis of their work they develop the concept of a series of local barriers to infection, developing with age. They have also published results with Eastern and Western equine encephalomyelitis viruses,[14 d] which again showed the greater susceptibility of young animals, but for this they invoked a somewhat different mechanism. Some sort of generalization whereby the various instances of the phenomenon in question might be harmonized is obviously desirable. That such a scheme might also explain comparable phenomena, such as the greater penetration of the young brain by vital dyes, has already been suggested.[3]

SUMMARY

The action on mice of several neurotropic viruses was studied with reference to factors which influence infection.

With pseudorabies virus, section of the sciatic nerve with inoculation into the ipsilateral foot pad significantly retarded the speed of infection. The

[23] (*a*) Webster, L. T., and Clow, A. D., *J. Exp. Med.*, 1936, **63**, 827. (*b*) Webster, L. T., *J. Exp. Med.*, 1937, **65**, 261.

virus ascended other nerves of the leg, but at a slower rate. It would appear that the number of nerve fibers available for passage may play a rôle in the speed with which infection occurs with this virus.

When pseudorabies virus was inoculated into an area of inflammation its effects were markedly lessened. Similar experiments with unmodified equine encephalomyelitis virus which, unlike pseudorabies, does not ascend along local nerves, showed no impedance of infection.

Brain trauma did not change the rate of infection with the viruses of St. Louis encephalitis, herpes, or pseudorabies. But intraperitoneal injection of glycerine, followed by intramuscular inoculation of St. Louis virus, resulted in marked facilitation of infection, as already remarked of fixed equine encephalomyelitis virus. This phenomenon did not occur with pseudorabies or herpes.

In contrast to certain other viruses, pseudorabies and herpes viruses were only slightly more effective in young mice than in adults.

With St. Louis virus, as with fixed equine encephalomyelitis viruses, inoculation into the eye or nose was far more effective than other peripheral routes. This was not the case with pseudorabies. Herpes, however, also showed greater sensitivity of the intraocular route.

After injection into the eye, St. Louis and fixed equine encephalomyelitis viruses invaded the optic pathway, while herpes and pseudorabies avoided the optic fibers and attacked the trigeminal nerve. These phenomena are discussed in the light of cellufugal and cellupetal progression of viruses.

The similarities in the action of fixed equine encephalomyelitis and St. Louis encephalitis viruses are discussed and contrasted with herpes and pseudorabies.