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The progression of herpes simplex virus to the central nervous system of the mouse

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The mechanisms by which a virus may penetrate from the periphery of an animal to the central nervous system have interested pathologists for many years. There exists a voluminous literature upon the subject which has been comprehensively reviewed from time to time (Craigie, 1937; Sabin, 1939; Faber, 1953; Rhodes, 1953; Wright, 1953; Burnet, 1955). Although most of the work concerned poliomyelitis virus, the spread of herpes simplex virus to the central nervous system of the rabbit was also extensively studied.

As a result of studies with herpes virus in rabbits two rival hypotheses were formulated; these profoundly influenced our concepts of how other viruses and even toxins might reach the central nervous system. In the first (Goodpasture & Teague, 1923), virus was supposed to ascend from the periphery by way of the axon cylinder and in the second (Marinesco & Dragonesco, 1923), by way of the tissue spaces between the axons. Both hypotheses satisfactorily explained the early localization of lesions to those segments of the central nervous system anatomically connected with the site of inoculation.

A third hypothesis, originally suggested by Doerr & Vochting (1920) and later reconsidered by Field (1953), was that virus invaded the central nervous system from the blood-stream. Following the demonstration that viraemia occurred in both experimental and in naturally acquired poliomyelitis (Horstmann, McCollum & Mascola, 1954; Melnick, 1945; Ward, Horstmann & Melnick, 1946; Wenner & Kamitsuka, 1957) this third hypothesis became important. But although it explained how virus could reach the central nervous system, it did not explain the localization of tissue damage to a few segments of the cerebrospinal axis. A further difficulty was that paralysis in poliomyelitis was not commonly associated with those segments adjacent to the *area postrema* or to the other weak points in the haematoencephalic barrier (cf. Bodian, 1952). But when it was recognized that localized paralysis in poliomyelitis was sometimes precipitated by injections of diphtheria prophylactics and the like (Martin, 1950; McCloskey, 1950) it was suggested that a reflex mechanism might in some way predispose to a local invasion of the nervous system from the blood-stream (Burnet, 1950). That such localized reflexes occurred was shown by Field, Grayson & Rogers (1949, 1951).

Despite the large volume of published work on the means by which viruses reach the central nervous system from the periphery, little of the earlier work appears satisfactory. First, few studies were made using enough experimental animals to give confidence in the conclusions drawn. Secondly, too much reliance was often

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placed on results obtained using only one criterion for assessment (e.g. development of paralysis, the presence or absence of infective virus or the presence or absence of histological change) and, thirdly, in many instances excessive preoccupation with one particular process prevented enough attention being paid to others. These criticisms do not apply to the more recent studies (e.g. Wenner & Kamitsuka, 1957).

It was originally intended to make a full study of events occurring in an experimental system in which herpes simplex virus passed from the periphery to the central nervous system of the mouse, using large numbers of animals and several different quantitative criteria for assessment. It was not possible to carry this out fully and the work was shelved. But recently the interesting studies of Johnson (1964*a*, *b*), Platt (1964) and Yamamoto, Otani & Shiraki (1965) have prompted the belief that these incomplete findings may now be of some value.

MATERIALS AND METHODS

Virus

Strains

The principal strain of virus used was HFEM whose characteristics have been described elsewhere (Wildy, 1955). It was maintained by intracerebral passage in mice. The strain Nag was originally isolated at the Walter and Eliza Hall Institute, Melbourne, from a case of herpes labialis and was maintained by passage on the chorioallantois.

Suspending fluid

All operations in which it was desired to preserve the infectivity of virus were carried out at 0° C. using broth containing penicillin (12·5 units/ml.) and streptomycin (5 μg./ml.).

Egg techniques

Conventional egg techniques were used (Beveridge & Burnet, 1946) and require no further description.

Maintenance and preparation of inocula

Stock suspensions of strains were kept in ampoules at −70° C. Inoculating suspensions were made from these by Nagler's (1946) method in which eggs at the 8th day of incubation were inoculated by way of the yolk sac with 10^{-2} dilution of stock virus. After incubation at 35° C. for 3 days, the amniotic fluid was collected, clarified by centrifugation and either used immediately or stored at −70° C. until required.

Recovery of virus

Virus was extracted by grinding tissues with alundum. The procedure was standardized by grinding with a standard quantity of alundum, which had been damped with broth, making one hundred circular movements. One ml. broth was then added and the whole ground a further 20–30 times. The resulting suspension

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was centrifuged at 2500 r.p.m. for 10 min. and usually titrated immediately, but occasionally the supernatant fluid had to be stored in ampoules at -70° C. before it was titrated.

Titration of infective virus

Infectivity titrations were made on the chorioallantois using the method of Burnet & Lush (1939*a*) and were expressed in terms of the pock unit (pock u.) which causes one lesion on the chorioallantois. For most purposes, tenfold dilutions were used and each inoculated on to four eggs, but in some experiments, where large numbers of titrations had to be made, it became impracticable to use more than two eggs per dilution. In such cases, samples of material were always set aside in ampoules and stored at -70° C. Where the eggs produced dissimilar results, the titration was repeated.

Sera

Specific neutralizing antisera were prepared in young adult rabbits as previously described (Wildy, 1954). Three graded weekly doses were given intradermally; (*a*) 2×10^5 pock u. virus inactivated with 0·01 % formalin; then (*b*) 2×10^2 pock u.; and (*c*) 2×10^5 pock. u infective virus. By the 4th week, all rabbits possessed strongly active neutralizing antisera as measured by the method of Burnet & Lush (1939*a*).

Mice

Strains

Most of the work done was in Australia using the Hall Institute strain of mice. For the experiments made in England, a strain which had originated from the Glaxo stocks (strong albino/2/G) was used. Both strains were used during the 5th week of life and both behaved similarly towards herpes simplex virus.

Injection and inoculation procedures

Conventional methods were used for inoculation by the intracerebral, intraperitoneal, intravenous, intranasal, intratesticular and corneal routes. Intraspinal inoculation was made by the method of Habel & Li (1951). To avoid artificial tensions in the tissues, intradermal inoculation was made by multiple pressure in a standard way. A drop of undiluted virus suspension was placed on the sole of one hind foot of the anaesthetized mouse and a blunt needle was pressed firmly through the drop fifty times. The sole was then rubbed firmly with the shank of the needle five or six times. If the skin was visibly broken, the mouse was discarded.

Taking tissue samples from mice

The method of removing pieces of tissue was mostly straightforward. However, the method used for removing samples from the central nervous system requires mention. After killing the mouse, it was pinned out in the prone position, and the whole of the dorsal skin was then removed. The skull cap was removed aseptically and the lumbar vertebrae divided. A syringe fitted with a wide bore needle was charged with saline and the needle inserted into the spinal canal. Moderate pressure

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on the plunger forced the whole spinal cord through the foramen magnum. The entire central nervous system was then removed and divided into three portions. The cord was divided midway between the cervical and lumbar enlargements and the medulla oblongata was removed from the base of the brain. These portions will be referred to as the lumbar cord, cervical cord and brain. The sciatic nerve was then removed after splitting the gluteal muscles. The method is easy and quick, enabling large numbers of mice to be handled, but its main disadvantage is that the dorsal root ganglia are left behind in the carcass.

RESULTS

Virulence of virus inoculated intracerebrally

The first experiments were designed to evaluate the virulence of the virus to be used and to discover a method of peripheral inoculation which regularly leads to paralysis. The strain HFEM was already known to be highly virulent for mice when inoculated intracerebrally; one LD 50 $\approx$ 1 pock u. (Wildy, 1955). This conclusion was strengthened by determining the number of pock u. which constitute one LD 50 and comparing this value with the number required to infect 50 % of mice (one ID 50). Tenfold dilutions of virus were each inoculated on the chorio-allantois of four eggs and into the brains of four mice in 0·05 ml. volumes. Pocks were counted after 48 hr. incubation at 35° C. The mice were examined daily for 14 days and the number of days elapsing between inoculation and death from encephalitis noted. On the 15th day, all surviving mice were challenged with 100 pock u. strain HFEM, which is sufficient to kill the normal mouse. These were then examined daily for a further period of 14 days. Mice which survived this challenge were regarded as having been immunized by the first inoculation, and those that did not were regarded as not having been infected by it. The results obtained with strains HFEM and Nag using Hall Institute mice and Glaxo mice show three points (Table 1). First, with both viral strains the ID 50 is close to one pock unit. Secondly, whilst strain Nag killed no mice even when 116 pock u. were inoculated, the LD 50 for strain HFEM was close to the ID 50 and thirdly, both mouse strains were about equally susceptible to strain HFEM when inoculated intracerebrally.

Virulence of HFEM virus inoculated intraspinally

It was of particular interest for us to know the virulence of virus when inoculated intraspinally, rather than intracerebrally, because it was the lumbar cord which was first invaded by virus in most experiments to be described. Unfortunately, the volumes of virus inoculated intraspinally, though of standard size, were so small that they were difficult to measure. For this reason virulence of virus inoculated in this way was not compared with egg titrations; falling dilutions of virus were inoculated intraspinally into groups of mice and the infectivity (ID 50, based as before on the results of inoculation and subsequent challenge) was compared with the ability to produce paralysis (PD 50) and with the LD 50. In two experiments carried out with different seeds of strain HFEM, it was found that the ID 50 and the PD 50 lay close together but that about ten times as much virus was required to constitute the LD 50 when inoculated intraspinally (Table 2).

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Table 1. *Virulence for mice of virus inoculated intracerebrally*

Virus strain	Dilution	Chorioallantoic inoculation eggs (mean no. pocks)	Mouse strain	Intracerebral inoculation mice
HFEM	10^{-5}	0·0	Hall Institute	I, S, S, S
	10^{-4}	0·25		7, 8, S, S
	10^{-3}	2·14		7, 9, 10, I
	10^{-2}	26·5		4, 5, 6, 7
Nag	10^{-5}	0·0	Hall Institute	S, S, S, S
	10^{-4}	0·25		I, I, S, S
	10^{-3}	18·0		I, I, I, S
	10^{-2}	116·0		I, I, I, I
HFEM	10^{-7}	0·0	Glaxo	I, S, S, S
	10^{-6}	0·75		6, 7, 10, 13
	10^{-5}	7·3		6, 6, 8, 11

Results of inoculating dilutions of virus in parallel on the chorioallantoic membrane and intracerebrally into mice. Last column figures, day of death after incoculation; S, mouse survived 14 days but susceptible to challenge with 100 pock u. HFEM; I, mouse survived 14 days and immune to challenge.

Table 2. *Virulence for mice of strain HFEM inoculated intraspinally*

	Dilution	Day of paralysis	Day of death and Result of challenge
Expt. 1	10^{-7}	6, n.p., n.p., n.p.	I, S, S, S
	10^{-6}	7, 7, n.p., n.p.	I, I, I, I
	10^{-5}	3, 4, 4, 4	5, 6, I, I
Expt. 2	10^{-5}	n.p., n.p., n.p., n.p.	S, S, S, S
	10^{-4}	3, 8, 9, 10, n.p.	6, 10, I, I, I
	10^{-3}	3, 3, 8, n.p., n.p.	6, 6, I, I, I
	10^{-2}	2, 3, 3, 5	6, 5, 5, 6

Results of inoculating tenfold dilutions of two different seeds of HFEM intraspinally. Middle column figures, day after inoculation upon which paralysis was noted; n.p., not paralysed within 14 days. Third column refers to the same mice: symbols as in Table 1.

Table 3. *Virulence of virus inoculated peripherally*

Route of inoculation	Method	Inoculum size	Proportion of mice developing paralysis	Proportion of mice which died
Intravenous	Syringe	$2{\cdot}3 \times 10^6$ pock u.	0/4	2/4
Intraperitoneal	Syringe	$2{\cdot}3 \times 10^8$ pock u.	1/4	1/4
Intratesticular	Syringe	$2{\cdot}3 \times 10^6$ pock u.	0/4	0/4
Corneal	Scarification	$4{\cdot}6 \times 10^7$ pock u./ml.	0/4	0/4
Intradermal	Multiple pressure	$4{\cdot}6 \times 10^7$ pock u./ml.	1/10	1/10

Results of inoculating strain HFEM into mice in various ways.

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Virulence of HFEM virus inoculated peripherally

Plainly, strain HFEM was highly virulent when inoculated into the central nervous system; this was not the case when it was inoculated peripherally (Table 3). In several experiments large doses of virus were inoculated by various routes. No effects followed inoculation on the cornea or into the testicle; when large amounts of virus were inoculated intravenously, paralysis did not occur but two of the four mice died (the post-mortem findings are mentioned later). One of four mice inoculated intraperitoneally with a massive dose of virus and only one of ten mice inoculated intradermally became paralysed and died. The one mouse which became paralysed is of interest because it showed that HFEM might be used to investigate the path of viruses to the central nervous system of mice. The mouse, which had been inoculated on the sole of the right forefoot, developed paralysis in the right arm 6 days later. This rapidly progressed and on the following day the animal died. The infected forefoot and portions of the central nervous system were titrated for infective virus with the following result: forefoot, 7400 pock u.; lumbar cord, 240 pock u.; cervical cord, 1280 pock u.; cerebellum, 1200 pock u.; cerebral hemispheres, 100 pock u.

Table 4. *Modification of the results of intradermal inoculation by injecting 0·1 ml. 10 % sodium chloride 24 hr. beforehand*

Inoculum concentration (pock u./ml.)	Mice pretreated with 10 % sodium chloride		Control mice	
	Vesicle formation	Onset of paralysis	Vesicle formation	Onset of paralysis
$4{\cdot}6 \times 10^3$	6, 7, –, –	9, 11, –, –	–, –, –, –	–, –, –, –
$4{\cdot}6 \times 10^5$	6, –, –, –	11, –, –, –	–, –, –, –	–, –, –, –
$4{\cdot}6 \times 10^7$	3, 3, 3, 6	7, 7, 7, 7	7, –, –, –	–, –, –, –
$4{\cdot}6 \times 10^9$	3, 3, 3, 3	7, 7, 7, 7	3, 3, 3, –	7, 8, –, –

Figures indicate day after inoculation upon which vesicle formation or paralysis was first noted. – indicates negative findings up until 14 days.

Virulence of HFEM virus for mice previously treated with hypertonic saline

Olitsky & Schlesinger (1941) found that the incidence of paralysis was greater when herpes simplex virus was inoculated into an area of skin which had previously been injected subcutaneously with a small quantity of 10 % saline. This effect was well shown when four dilutions of virus were each inoculated by multiple pressure into the soles of four normal mice and of four mice which had 24 hr. previously been given a subcutaneous injection of 0·1 ml. 10 % saline (Table 4). The amount of virus required to give local vesicular lesions and paralysis after such pretreatment was about one-hundredth of that required for the control mice.

It appeared, therefore, that this technique (the *standard procedure*) of inoculating concentrated virus by multiple pressure into the skin of the sole of a hind foot previously treated with hypertonic saline provided a satisfactory system for studying the route by which a virus invades the central nervous system from the periphery.

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The histological changes due to infection of the central nervous system with strain HFEM

It had been expected that the central nervous system of mice ill with herpetic encephalomyelitis would show well marked histological evidence of infection, but disappointingly only slight histological changes were found. Ten mice were infected intracerebrally with doses of virus ranging from 2×10^2 to 2×10^6 pock u. strain HFEM and each was killed when showing advanced clinical encephalitis 2–8 days later. The brains were removed immediately and fixed whole in 10% formol saline. Sections were cut at various levels, stained with haematoxylin and eosin, and thoroughly examined. Varying degrees of leptomeningitis and small intracerebral haemorrhages at the site of incoculation were noted. In all mice chromatolysis and nuclear ballooning was observed in some nerve cells but no inclusion bodies were seen, and there were no perivascular cellular infiltrations. Because of the slight and indefinite nature of the changes, it was not possible to correlate intensity of reaction with dose of virus nor with the interval since inoculation.

The spinal cords of 20 mice were similarly examined between 2 and 6 days after peripheral inoculation by the standard procedure. The histological picture was again indefinite. Similar nuclear changes were observed in some anterior horn cells and after 4 or 5 days in some glial cells. Two mice which had been inoculated by the standard procedure were killed 4 and 6 days later, and after decalcification were sectioned in such a way as to cut through the posterior root ganglia. No histological abnormality whatever was found. For these reasons histological methods have not been used.

Distribution of infective virus after peripheral inoculation

Twenty-four mice were inoculated by the standard procedure in the sole of the left foot with a suspension of strain HFEM containing $2{\cdot}3 \times 10^8$ pock u./ml. At daily intervals for 5 days four mice were taken at random and killed. Infective virus was titrated in the foot, sciatic nerve, lumbar cord, cervical cord and brain. The remaining group of four mice was observed for 14 days and in all cases paralysis ensued, appearing first in the inoculated limb at the 6th (2), 7th and 9th days after infection. The total amount of infective virus in the foot had reached a high value 24 hr. after infection (Table 5). There was a slight increase in the next 24 hr. which was followed by slow decline in the total amount of infective virus present. Virus was found in the central nervous systems of three of four mice on the second day, and as expected, was first demonstrated in the lumbar cord. On succeeding days it was found in the cervical cord and brain, suggesting that it had progressed up the cerebro-spinal axis. Viraemia was demonstrated in two of four mice on the 2nd day, and thereafter low levels were regularly found.

These results suggested that virus had entered the central nervous system by way of the sciatic nerve, which was found to contain infective virus from the second day. But it was possible also that virus had passed via the blood to the lumbar cord by a mechanism such as was suggested by Burnet (1950), or by other routes such as by the lymphatic vessels or even by simple extension through other tissues

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which were not examined in this experiment. Accordingly the possible roles of the blood, the lymphatic system, other intervening tissues and the peripheral nerve were investigated as potential transporters of virus.

Table 5. *Distribution of infective virus in various situations after inoculation of virus intradermally into the sole of the right hind foot which had been pretreated with* 10% *sodium chloride*

Day	Right foot	Right sciatic nerve	Lumbar cord	Cervical cord	Brain	Blood
1	5·20	0·95	< 0	< 0	< 0	< 0
	5·65	< 0	< 0	< 0	< 0	< 0
	5·70	< 0	< 0	< 0	< 0	< 0
	4·00	< 0	1·08	< 0	< 0	< 0
2	4·00	1·62	0·00	< 0	< 0	1·15
	6·36	—	0·73	< 0	< 0	< 0
	6·15	2·19	0·70	< 0	< 0	< 0
	6·36	2·00	< 0	< 0	< 0	0·90
3	4·78	3·30	3·00	2·00	1·04	0·48
	3·60	4·90	4·56	1·04	0·90	0·48
	6·23	5·70	2·55	1·67	0·85	0·30
	0·95	5·85	3·90	1·95	0·70	0·85
4	5·95	4·59	3·60	1·23	1·00	0·90
	4·94	4·51	—	0·70	< 0	0·60
	4·36	6·00	7·60	5·30	1·21	0·70
	4·38	1·96	1·23	0·00	< 0	0·70
5	6·08	2·25	3·30	1·65	0·95	0·70
	1·30	1·10	0·90	1·63	0·00	—
	4·82	3·00	2·30	1·98	< 0	0·48
	< 0	1·85	1·20	0·90	< 0	—

Results of inoculating a suspension of strain HFEM containing $2{\cdot}3 \times 10^8$ pock u./ml. by multiple pressure. Values = $\log_{10}$ pock u./0·1 ml. organ extract; –, not done.

Role of the blood

Two series of experiments were done to determine the role of the blood as a vehicle for the carriage of virus to the central nervous system; first, the effects of an artificial viraemia were observed and secondly, an attempt was made to evaluate the importance of the viraemia that occurred after peripheral inoculation in the subsequent chain of events.

Effects of artificial viraemia

Viraemia regularly occurred after standard peripheral inoculation (Table 5), yet virus inoculated intravenously killed mice without producing paralysis (Table 3). The next points examined were the rate at which virus disappeared from the circulation and the fate of virus that had been inoculated intravenously.

Rate of disappearance of virus from the circulation after intravenous inoculation. A known amount of virus was injected into the tail veins of pairs of mice and at intervals the mice were bled. The blood was defibrinated by whipping, a procedure which had been shown not to alter the expected infective titre when virus and freshly drawn blood were mixed. The samples were at once titrated for infective

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virus. The geometric mean of values was obtained for each pair of samples and from this, the total circulating virus was calculated on the assumption that the blood volume was 2·0 ml. Virus was removed very rapidly from the bloodstream in the first few minutes (Fig. 1); thereafter steadily decreasing amounts of infective virus were found up to the fourth hour and subsequently no virus was detected up to the 8th hr. of the experiment.

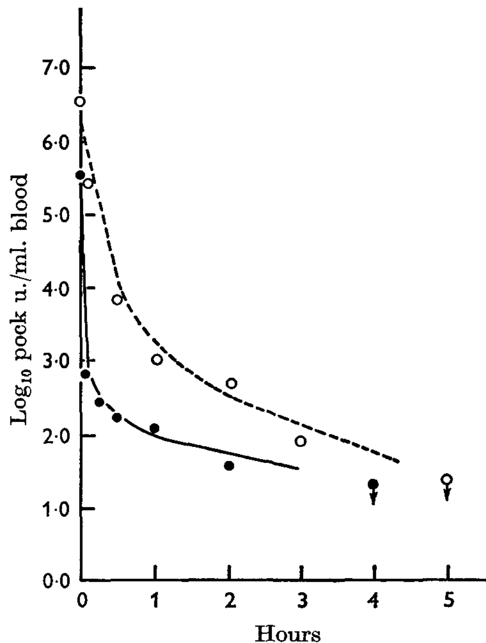

Fig. 1. The rate of disappearance of virus from the circulation of the mouse after intravenous inoculation of suspension of HFEM. Two experiments are shown.

Fate of intravenously inoculated virus. The common macroscopic appearances after intravenous inoculation of virus were varying degrees of congestion of the lung and of fatty change in the liver. Haematoxylin and eosin stained sections of lung showed gross congestion with haemorrhage into the alveoli; there was no evident increase in the number of inflammatory cells and no cytopathic changes were seen. The bronchi appeared normal with some serous secretion in their lumina. Sections of liver showed some evidence of fatty change and perivascular cells, lymphocytes, fibroblasts and small round cells. Sections of kidney, spleen and pancreas showed no abnormality.

One striking feature of these experiments was that unless massive inocula were given intravenously ($2{\cdot}5 \times 10^8$ pock u.), virus was seldom found in the brain. The possibility that virus was effectively removed from the circulation by the lungs so that very little reached the capillaries within the central nervous system was tested. A small inoculum was injected intravenously into each of 56 mice, 28 of which had immediately beforehand been given a traumatizing intracerebral injection of sterile normal saline. At daily intervals, four mice from each group were taken at random and their organs examined and scored for gross abnormalities. Separate

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pools were made of the brains, the livers and lungs from each group and these were examined for infective virus.

Three points were noted (Table 6). First, no virus was detected in the brains of mice that had only received virus intravenously, but those that had also been subjected to cerebral trauma showed a steady exponential rise in the concentration of infective virus in the brain from the first day onwards. Secondly, small amounts of virus were detected in the lungs of the former group on the 2nd and 3rd days, whilst in the latter small quantities were detected from the 2nd to the 7th days. Similarly in the livers of the former group a considerable amount of virus was detected on the 2nd day, whilst in the latter virus was much more persistent. Thirdly, the gross appearance of the livers was broadly similar with both groups of mice, but the congestion in the lung was much more intense in the group of mice which had been treated with intracerebral saline. It is interesting that in both groups congestion of the lungs was maximal on the 5th day of experiment, thereafter it disappeared.

Table 6. *Distribution of infective virus in various situations and gross pathological findings after intravenous inoculation of virus in mice, some of which were also given* 0·05 *ml. sterile physiological saline intracerebrally*

	Mice given intravenous virus only					Mice given intravenous virus and 0·05 ml. saline intracerebrally				
	Gross pathology		Content infective virus			Gross pathology		Content infective virus		
	Lung	Liver	Lung	Liver	Brain	Lung	Liver	Lung	Liver	Brain
1	–	±	0	0	0	–	+	0	3	1
2	–	+	7	125	0	–	+	3	8	25
3	–	+ +	3	0	0	–	+	1	14	72
4	±	+	0	0	0	±	+	2	30	106
5	+	+	0	1	0	+ + +	+	6	4	6,670
6	±	+	0	1	0	+ +	+	2	235	1,825
7	–	+	0	0	0	–	+	1	0	47,500

All mice were inoculated with 7×10^3 pock u. strain HFEM. Figures = total infective virus detected (pock u./0·1 ml.) in pools made from four mice. Under gross pathology: –, within normal limits; lung: +, one quarter lungs congested; + +, one-half lungs congested; + + +, three quarters lung congested. Liver: +, pale fatty liver; + +, waxy liver. Signs represent average findings per mouse.

It seems then that virus inoculated intravenously and certainly present in the cerebral vessels did not infect the central nervous system in the absence of trauma. It also appears that virus multiplied in one or more extraneural situations, because although the virus demonstrated in the lung may have been contained in the blood, that in the liver was probably multiplying there, since we had the additional information that perivascular inflammation occurred there. The fact that more virus was regularly found in these two sites when multiplication was occurring in the brain suggests that some at least was due to spill-over from the brain. The congestion of the lung which has constantly been found to be greatest at about the fifth day is of interest and cannot be easily explained.

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Evidence excluding blood transport of virus to the central nervous system

Data already given were not sufficient to exclude the possibility that blood played a part in carrying virus to the central nervous system and two series of experiments were made to eliminate it. Attempts were made to determine whether detectable amounts of virus reached the cerebral vessels after peripheral inoculation, and the effect of circulating herpes neutralizing antibody was tested.

Test for virus circulating to the brain. A number of mice were inoculated by the standard procedure on the sole of the left hind foot. At daily intervals, a group of mice was given an intracerebral injection of sterile physiological saline. These were then kept for a further 2 days to provide an opportunity for virus localizing in the brain to multiply and the mice were then killed. The concentrations of infective virus in the brain, the cervical and lumbar cords and the sciatic nerves

Table 7. *Distribution of infective virus in various situations after inoculation of virus intradermally by multiple pressure into the pretreated sole of the hind foot of mice, which were also given* 0·05 *ml. sterile physiological saline intracerebrally*

Day	Right sciatic nerve	Lumbar cord	Cervical cord	Brain
3	< 0	1·30	0·47	< 0
	< 0	< 0	< 0	< 0
	< 0	< 0	< 0	< 0
4	0·00	< 0	< 0	< 0
	—	> 2·0	> 2·0	< 0
	< 0	0·00	< 0	< 0
	> 2·0	> 2·0	2·00	1·48
5	1·18	< 0	0·00	< 0
	1·53	1·93	1·48	0·30
	1·78	0·78	< 0	< 0
	2·06	1·63	< 0	< 0
6	> 2·0	> 2·0	> 2·0	1·18
	1·64	> 2·0	1·54	0·00
	1·49	> 2·0	> 2·0	> 2·0
7	> 2·0	> 2·0	> 2·0	> 2·0
	> 2·0	> 2·0	1·08	> 2·0
	> 2·0	1·40	1·11	< 0
	> 2·0	> 2·0	> 2·0	> 2·0

Results of inoculating a suspension of strain HFEM containing $2{\cdot}7 \times 10^6$ pock u./ml. by multiple pressure into the sole of the right foot which had been pretreated with 10 % sodium chloride. At intervals the mice were given 0·05 ml. sterile saline intracerebrally and 2 days later they were killed and their organs titrated for infective virus.

Values indicate $\log_{10}$ pock u./0·1 ml. organ extract.

were then determined. The results (Table 7) suggested that insufficient virus was at any time circulating in the cerebral vessels to infect the brain even in spite of injury to it because infective virus was first detected as usual in the lumbar cord whence it ascended as time went by.

Experiments with neutralizing antiserum. In an attempt to prevent virus reaching the central nervous system via the blood, experiments were done using mice which

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had been given large amounts of rabbit herpes-neutralizing antibody. Preliminary experiments showed (1) that mice would tolerate large doses (as much as 1·0 ml.) of undiluted inactivated antiserum intravenously provided that it was administered slowly, and (2) that the neutralizing ability of their serum persisted for long enough to make this experimental approach practicable. This is shown by an experiment in which six mice were given different amounts of antiherpetic rabbit serum intravenously and their sera tested for neutralizing ability after 30 min. and again after 6 days. Two of the mice which were given 1·0 ml. serum possessed sufficient antibody to neutralize 99·5 % and 100 % virus by the method of Burnet & Lush (1939*a*) after 30 min. and after 6 days their sera neutralized 94 and 97 % virus. Two mice which had each received 0·5 ml. serum gave the values 98·5 and 99·5 % after 30 min. and 86 and 88 % after 6 days; whilst with two mice which had been given 0·25 ml. serum, the values were 96·5 and 96·5 % at 30 min. and 77 and 84 % at 6 days. Because the 0·5 ml. dose appeared adequate for our purpose and was well tolerated, this dose was used in the experiments which follow.

Table 8. *Effect upon development of local vesicle and paralysis and upon death of injecting neutralizing antiserum at intervals after intradermal inoculation into the sole of the hind foot which had been pretreated with* 10 % *sodium chloride*

No. days after inoculation at which mice were given 0·5 ml. rabbit antiserum	Local vesicle	Paralysis	Death
1	3, 4, 4	7, 7, 8	–, –, –
2	3, 3, 3, 3	10, 7, 7, 9	–, –, –, –
3	3, 3, 2	7, 7, 8	–, –, –
4	2, 4, 3, 3	5, 8, 7, –	7, –, –, –
5	3, 3, 3, 3	6, 7, 7, 7	7, 13, –, –
No serum given	3, 4, 3, 4	5, 7, 7, 8	–, –, –, –

All mice were inoculated with a suspension containing $2{\cdot}3 \times 10^7$ pock u./ml. strain HFEM. Figures = day upon which local vesicle, paralysis or death were first noted; –, effect not seen up to 14 days.

Effect of neutralizing antiserum on the development of paralysis

A number of mice were inoculated intradermally by the standard procedure, and at intervals groups of mice were given injections of serum. They were observed over a period of 14 days for the development of lesions and of paralysis. Except possibly for one mouse given serum on the 4th day, there is no evidence that circulating antibody prevented either the formation of local vesicles or the onset of paralysis (Table 8). It is interesting that three of the mice that were given serum on the 4th and 5th days died, although there were no signs of injury to the central nervous system.

Effect of neutralizing antiserum on the distribution of infective virus

A number of mice were inoculated by the standard method and 18 hr. later some were each given 0·5 ml. neutralizing antiserum intravenously; the remainder

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which were not so treated served as controls. At daily intervals, three mice from each group were killed and infective virus titrated in the central nervous system and the local sciatic nerve. Despite the circulating antibody, the distribution of virus in serum-treated mice was remarkably similar to that in the controls (Table 9). Taking the results of the two experiments together, it was very unlikely that circulating blood was the vehicle by which herpes virus reached the central nervous system.

Table 9. *Effect of circulating neutralizing antibody upon the distribution of virus in mice examined at intervals after peripheral inoculation by the standard procedure*

	Serum-treated mice				Control mice			
Day	Sciatic nerve	Lumbar cord	Cervical cord	Brain	Sciatic nerve	Lumbar cord	Cervical cord	Brain
1	0·60	< 0	< 0	< 0	< 0	< 0	< 0	< 0
	< 0	< 0	< 0	< 0	< 0	< 0	< 0	< 0
	< 0	< 0	< 0	< 0	< 0	< 0	< 0	< 0
2	0·90	< 0	< 0	< 0	0·00	< 0	< 0	< 0
	0·00	< 0	< 0	< 0	< 0	0·00	< 0	< 0
	< 0	< 0	< 0	< 0	1·11	< 0	< 0	< 0
3	0·48	< 0	< 0	< 0	1·08	0·30	< 0	< 0
	1·80	0·80	< 0	< 0	0·48	0·78	< 0	< 0
	0·48	< 0	< 0	< 0	< 0	< 0	<0	< 0
4	3·32	3·76	0·95	< 0	1·90	5·60	3·69	1·11
	3·48	4·60	1·60	0·60	4·00	4·30	1·00	0·85
	2·85	3·49	1·70	1·20	1·60	3·87	1·11	0·48
5	3·70	4·03	2·48	1·70	4·00	4·70	3·55	< 0
	2·48	1·89	0·48	< 0	3·67	5·60	5·23	< 0
	< 0	< 0	< 0	< 0	2·78	3·04	0·9	0·00
6	2·00	3·56	2·11	1·89	3·15	1·60	2·78	< 0
	3·76	3·46	2·00	1·75	2·60	3·43	< 0	1·08
	3·52	5·00	2·78	< 0	1·60	4·89	5·15	< 0

All mice were inoculated by the standard procedure with a suspension of strain HFEM containing $2{\cdot}3 \times 10^7$ pock u./ml. Eighteen hours later half the mice were each given 0·5 ml. neutralizing antiserum (rabbit). Values indicate $\log_{10}$ pock u./0·1 ml. organ extract.

Role of the lymphatic system

Theoretically, there exists a roundabout pathway by which virus might reach the central nervous system from the periphery by way of the lymphatic vessels (Field & Brierley, 1948). Experiments were therefore carried out to exclude this possibility. A number of mice were inoculated by the standard procedure and at intervals virus was sought in the inoculated foot, the lymphatic glands draining the area, the blood and the sciatic nerve and spinal cord. The points that emerged (Table 10) were first, that virus multiplied in the foot and appeared in the sciatic nerve and spinal cord much as was previously found (Table 5); secondly, that infective virus was demonstrated in the blood in low concentration in some mice but with less regularity than was noted before. Thirdly, only very low concentrations of infective virus were demonstrated in the lymphatic glands. Another

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experiment to be reported elsewhere, in which the distribution of herpes virus was noted at short intervals after subcutaneous inoculation of the sole of the foot, showed very little virus reaching the local lymphatic glands. These results suggested that the roundabout route from the periphery to the central nervous system put forward by Field & Brierley (1948) was a most unlikely route for this virus in mice.

Table 10. *Distribution of infective virus in the lymphatic system and nervous system at intervals after inoculation by the standard method*

Day	Foot	Inguinal gland	Popliteal gland	Iliac gland	Sciatic nerve	Lumbar cord	Blood
1	4·90	< 0	< 0	< 0	< 0	< 0	< 0
	4·00	< 0	< 0	< 0	< 0	0·00	< 0
	5·08	< 0	0·00	< 0	0·00	< 0	< 0
2	5·21	< 0	0·30	< 0	0·48	< 0	0·00
	4·84	< 0	< 0	< 0	< 0	< 0	< 0
	5·41	< 0	0·48	0·90	1·52	0·78	0·48
3	5·68	< 0	0·60	0·84	1·93	1·26	0·84
	5·77	< 0	0·30	< 0	2·20	0·70	< 0
	4·97	< 0	< 0	< 0	1·42	1·08	0·84
	4·48	< 0	0·70	0·84	0·84	0·78	0·30

Values indicate $\log_{10}$ pock u./0·1 ml. organ extract.

Table 11. *Distribution of infective virus at intervals after inoculation in the left foot by the standard procedure, including the homolateral gluteal muscles and the opposite sciatic nerve*

Day	Left sciatic nerve	Left glutei	Lumbar cord	Right sciatic nerve
1	< 0	< 0	< 0	< 0
	< 0	< 0	< 0	< 0
	< 0	< 0	< 0	< 0
2	< 0	< 0	0·00	< 0
	< 0	< 0	< 0	< 0
	1·11	< 0	< 0	< 0
3	1·08	< 0	0·30	< 0
	0·48	< 0	0·78	1·11
	< 0	< 0	< 0	< 0
4	1·90	< 0	4·30	< 0
	4·00	0·48	5·60	< 0
	1·60	< 0	3·87	0·00
5	4·00	2·11	4·70	0·00
	2·78	< 0	3·04	1·15
	3·67	< 0	5·60	< 0
6	3·15	1·65	2·00	1·78
	2·60	3·41	3·43	< 0
	1·60	< 0	4·84	< 0

Values indicate $\log_{10}$ pock u./0·1 ml. organ extract.

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The possibility of invasion by direct extension through the tissues

Very little attention has in the past been paid to the possibility that virus might invade the central nervous system by simple extension through the tissues. To be sure, there is no evidence that such an event occurs with any animal studied, but when working with the laboratory mouse, one is impressed by the nearness of the central nervous system to the periphery. To exclude the possibility that virus might penetrate via the intervening tissues, mice were inoculated by the standard procedure and at intervals infective virus was sought in the infected foot, the sciatic nerve, the muscles overlying it, the lumbar cord and the opposite sciatic nerve. No infective virus was demonstrated in the muscles overlying the sciatic nerve until the 4th day of the experiment, long after it had been demonstrated in the sciatic nerve and in the central nervous system itself (Table 11). It is interesting to note that virus was demonstrated in the contralateral sciatic nerve soon after it had been found in the lumbar cord. It seemed plain that virus was not progressing to the central nervous system by direct extension through the tissues.

The role of the peripheral nerve

Since all the evidence suggested that the peripheral nerve was behaving as a conduit for virus two approaches were tried to reinforce this evidence; (1) observation of the effect of sectioning the sciatic nerve and (2) testing for virus in dorsal root ganglia of infected mice.

Table 12. *Effect of surgical resection of the sciatic nerve upon the ability of infective virus to appear in the central nervous system*

Mice with interrupted sciatic nerve		Control mice	
Foot	Lumbar cord	Foot	Lumbar cord
4·89	< 0	4·71	1·36
4·72	0·85	3·90	2·35
4·10	< 0	4·66	1·40
3·69	< 0	3·59	2·83
4·26	< 0	4·72	2·93

Mice were inoculated by the standard procedure with a suspension of HFEM containing 8×10^7 pock u./ml. Five days later the foot and lumbar cord of each mouse was titrated for infective virus.

Values indicate $\log_{10}$ pock u./0·1 ml. organ extract.

Effect of nerve section

Several preliminary experiments had shown that interruption of the sciatic nerve by surgical section, freezing with carbon dioxide snow and intraneural injection of neutralizing antibody gave equivocal results and in some instances such procedures actually appeared to hasten the appearance of virus in the central nervous system. This might have been due to incomplete interruption of the nerve, to virus multiplying 'through' the resulting inflammatory tissue, or to a combination of both. To overcome both these objections, two groups, each of five mice, were used, one of which was used for control purposes. Mice of the other group

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were anaesthetized and their left sciatic nerves exposed. A segment of the nerve 3–5 mm. long was then resected. After suturing the skin, the mice were then left undisturbed for 3 days and except for complete flaccid paralysis of the left hind limbs of the operated mice, all were well. On the 4th day all mice of both groups were given subcutaneous injections of 10% sodium chloride into the sole of the left foot and on the 5th day after the nerve resection, all were inoculated on this site by multiple pressure. Five days later (i.e. 10 days after the operation) all the mice were killed and virus was titrated in the inoculated foot and in the lumbar cord. Infective virus was found in the feet of all mice in similar quantity (3·90–4·89 $\log_{10}$ pock u.) (Table 12); but whereas it was found in the lumbar cords of the control group in all cases (1·36–2·39 $\log_{10}$ pock u.) it was demonstrated in this situation in only one of five mice with sectioned sciatic nerves and then in smaller amount (0·85 $\log_{10}$ pock u.). It was inferred that virus did generally travel to the central nervous system by way of the sciatic nerve and that its passage had been prevented by the resection, but the objections to so simple an explanation will be discussed later.

Table 13. *Distribution of infective virus in the foot, sciatic nerve, dorsal root ganglion and lumbar cord at intervals after inoculation by the standard procedure*

Day	Foot	Sciatic nerve	Dorsal root ganglion	Lumbar cord
1	2·95	< 0	< 0	< 0
2	4·70	< 0	0·18	< 0
3	5·3	< 0	0·74	0·0
4	5·15	1·15	1·85	1·3

Results after inoculation with 2×10^6 pock u. virus. Values given are infective virus per 0·1 ml. $\log_{10}$ pock u.

The appearance of virus in the dorsal root ganglia

It has already been pointed out that the method usually used for taking tissue samples left the dorsal root ganglia *in situ* in the carcass. Attempts were made to remove these structures by straightforward dissection and to titrate them for infective virus along with the sciatic nerves, the lumbar cords and the feet of peripherally infected mice. Accordingly, a number of mice were inoculated by the standard procedure and at intervals mice were killed and tissue samples taken. The dissection was found very difficult and in only four instances were the ganglia successfully removed. However, the results of titrating for infective virus which are shown in Table 13 suggested that the virus had passed centripetally via the sciatic nerve trunk and reached the sensory ganglia before it reached the spinal cord.

DISCUSSION

Some strains of herpes simplex virus are virulent for mice when inoculated intracerebrally whilst others are not (cf. Burnet & Lush, 1939*b*; Florman & Trader, 1947). The ideal host-virus combination for this study would have been one in which one infecting particle was sufficient to cause encephalitis when

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inoculated intracerebrally. The strain HFEM must have come close to this requirement for it was clear that for two mouse strains the intracerebral dose of virus required for one LD 50 was close to that required to immunize 50 % of mice so inoculated and that it was of the same order as the pock u. Moreover, the PD 50 when given intraspinally is close to the 50 % immunizing dose.

It is also well established that strains of virus when inoculated intracerebrally may be virulent for mice of any age, but, when inoculated peripherally, they may only be virulent for young mice. This was shown for herpes virus by Andervont (1929), for this and several other viruses by Lennette & Koprowski (1944) and recently it has been confirmed by Johnson (1964*b*). It was not surprising, therefore, that, using mice in the 5th week of life, we should find very few which contracted encephalomyelitis after peripheral inoculation. But using the technique of inoculating intradermally into a patch of oedema resulting from a previous subcutaneous injection of 10 % sodium chloride (Olitsky & Schlesinger, 1941) it was found possible to precipitate virtually 100 % paralysis with suspensions of virus which had readily attainable infective titres. Moreover, by using multiple pressure it was possible to infect mice without causing artificial tensions in the tissues. In fact, the host virus combination would have been nearly perfect had the infection given rise to more clearly defined histological disturbances so that we could have employed another criterion for the movement of virus. This might have been especially valuable because one of the limitations of infectivity titrations was that we did not follow the path of non-infective virus, and possibly virus may have moved intracellularly in a non-infective state. Indeed, Sanders (1953), working with mice, obtained some evidence suggesting that encephalomyocarditis virus travelled in this way.

In several experiments the distribution of infective virus was examined at intervals after peripheral inoculation. Although the high concentrations of infective virus found in one of these (Table 5) were not attained in other experiments, the distributions conformed to a general pattern in which high concentrations of virus were found from the 1st to the 5th days after infection in the inoculated foot, and steadily increasing titres successively in the sciatic nerve, lumbar cord, cervical cord and finally the brain. This strongly suggested that virus invaded the central nervous system at a focal point, possibly related to the roots of the sciatic nerve; it provided a striking contrast to the picture found by Mims (1957) with the Asibi strain of yellow fever virus which gave similar titres in different portions of the central nervous system of the infant mouse after peripheral infection. The pattern did not, however, indicate that virus necessarily passed up the sciatic nerve.

The striking result of resection of the sciatic nerve (Table 12) eliminated the hypotheses of direct extension and of lymphatic transport to the central nervous system. The results obtained were, however, in accord with the idea that virus had passed up the peripheral nerve or that it was blood-borne but had localized in some way by a reflex phenomenon. The latter hypothesis was made most improbable because neutralizing antibody did not interfere with the development of paralysis nor did it prevent the entry of virus to the central nervous system (Tables 8 and 9). Moreover, other evidence (Tables 6 and 7) suggests that virus did not easily penetrate the central nervous system from the blood and that after

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peripheral inoculation too little virus was circulating to infect even the traumatized brain. This last finding contrasted with the findings of Burnet & Lush (1938), Sawyer & Lloyd (1931) and Mims (1957) that louping ill virus and yellow fever virus readily infected the traumatized brain of the adult mouse after intraperitoneal inoculation.

Taking the results as a whole, there seems no doubt that herpes simplex virus ascends to the central nervous system of the mouse by way of the peripheral nerve, although the question of how this comes about remains unanswered.

Interest in our problem has recently revived and Johnson (1964*a*, *b*) Platt (1964) and Yamamoto *et al.* (1965) have all examined the behaviour of herpes virus in animals. Of these the work of Johnson (1964*a*) is especially interesting. Using the same strain of virus and descendants of the same colony of mice as were used here, he has made valuable contributions on how herpes spreads in the body mostly using fluorescent antibody techniques. He clearly demonstrates that after subcutaneous inoculation of 4- to 5-day-old mice, virus passes centripetally up nerve fibres to the central nervous system. Only occasional specifically fluorescing cells were found in the local lymph node. No viraemia was detected. But when the virus was introduced by certain other routes there was evidence of haematogenous spread. It is interesting that cells in the peripheral nerves supplying the inoculated area specifically fluoresced, suggesting that the virus was progressing stepping-stone fashion up the nerve. The findings of Yamamoto *et al.* were similar. We found (unpublished observations) supportive evidence for this idea inasmuch as straightforward histological techniques showed chromatolysis and ballooning of nuclei in the Schwann cells of affected sciatic nerves. It remains to be formally demonstrated that these fluorescent cells really contribute to spread by producing infective virus and are not merely silent witnesses of centripetally moving virus in the periaxonal spaces; it is possible, though improbable, that the fluorescence indicates abortive infection in which antigens are formed but not infective virus.

Most of this work was done at the Walter & Eliza Hall Institute whilst holding the British Memorial Fellowship in Virology 1953–4.

SUMMARY

1. The strain HFEM of herpes simplex virus is highly virulent for the 5-week-old mouse when inoculated intracerebrally; the LD50 and the ID50 both being close to the egg infectious unit. It is also virulent when given intraspinally.

2. When inoculated peripherally it seldom causes nervous symptoms in the normal mouse but regularly does so if inoculated into skin which has previously been given a subcutaneous injection of 10 % sodium chloride.

3. After intradermal inoculation of pretreated mice with strain HFEM, the paralysis is preceded by the appearance of infective virus in the blood, the sciatic nerve and the central nervous system. Invasion of the central nervous system is prevented by interruption of the local peripheral nerve.

4. There is no evidence that virus enters the central nervous system by way of the lymphatics or by direct extension through the tissues.

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5. After intravenous inoculation virus does not invade the central nervous system unless (*a*) massive inocula are used or (*b*) the brain is traumatized by injecting sterile saline. After peripheral inoculation insufficient virus to infect even traumatized brain seems to circulate in the cerebral vessels. Paralysis and invasion of the central nervous system are not prevented by circulating neutralizing antibody given 18 hours after inoculation.

It therefore appears that invasion of the central nervous system takes place only by way of the peripheral nerve.

REFERENCES

ANDERVONT, H. B. (1929). Activity of herpetic virus in mice. *J. infect. Dis.* **44**, 383.

BEVERIDGE, W. I. B. & BURNET, F. M. (1946). The cultivation of viruses and rickettsiae in the chick embryo. *Spec. Rep. Ser. med. Res. Coun.* no. 256.

BODIAN, D. (1952). A reconsideration of the pathogenesis of poliomyelitis. *Am. J. Hyg.* **55**, 414.

BURNET, F. M. (1950). In Poliomyelitis following inoculations. *Br. med. J.* i, 1004.

BURNET, Sir F. M. (1955). *Principles of Animal Virology.* New York: Academic Press.

BURNET, F. M. & LUSH, D. (1938). Infection of central nervous system by louping ill virus; investigation by quantitative egg membrane technique. *Aust. J. exp. Biol. med. Sci.* **16**, 233.

BURNET, F. M. & LUSH, D. (1939*a*). The inactivation of herpes virus by immune sera. Experiments using the chorioallantoic membrane technique. *J. Path. Bact.* **48**, 275.

BURNET, F. M. & LUSH, D. (1939*b*). Studies in experimental herpes infection in mice using the chorioallantoic technique. *J. Path. Bact.* **49**, 241.

CRAIGIE, J. (1937). Poliomyelitis: virus and experimental infection. *Can. publ. Hlth J.* **28**, 421.

DOERR, R. & VOCHTING, K. (1920). Sur le virus de l'herpès fébrile. *Rev.gén. Ophtal.* **34**, 409.

FABER, H. K. (1953). *The Pathogenesis of Poliomyelitis.* Illinois: Thomas Springfield.

FIELD, E. J. (1953). Pathogenesis of herpetic encephalitis following corneal and masseteric inoculation. *J. Path. Bact.* **64**, 1.

FIELD, E. J. & BRIERLEY, J. B. (1948). The lymphatic connexions of the subarachnoid space. *Br. med. J.* i, 1167.

FIELD, E. J., GRAYSON, J. & ROGERS, A. F. (1949). Circulatory changes in the spinal cord resulting from peripheral stimulation. *J. Physiol., Lond.* **110**, 14*P*.

FIELD, E. J., GRAYSON, J. & ROGERS, A. F. (1951). Observations on the blood flow in the spinal cord of the rabbit. *J. Physiol., Lond.* **114**, 56.

FLORMAN, A. L. & TRADER, F. W. (1947). A comparative study of pathogenicity of four strains of herpes simplex virus. *J. Immun.* **55**, 263.

GOODPASTURE, E. W. & TEAGUE, O. (1923). Transmission of the virus of herpes febrilis along nerves in experimentally infected rabbits, *J. med. Res.* **44**, 139.

HABEL, K. & LI, C. P. (1951). Intraspinal inoculation of mice in experimental poliomyelitis. *Proc. Soc. exp. Biol. Med.* **76**, 357.

HORSTMANN, D, M., MCCOLLUM, R. W. & MASCOLA, A. D. (1954). Viraemia in human poliomyelitis. *J. exp. Med.* **99**, 355.

JOHNSON, R. T. (1964*a*). The pathogenesis of herpes virus encephalitis. I. Virus pathways to the nervous system of suckling mice demonstrated by fluorescent antibody staining. *J. exp. Med.* **119**, 343.

JOHNSON, R. T. (1964*b*). The pathogenesis of herpes virus encephalitis. II. A cellular basis for the development of resistance with age. *J. exp. Med.* **120**, 359.

LENNETTE, E. H. & KOPROWSKI, H. (1944). Influence of age on the susceptibility of mice to infection with certain neurotropic viruses. *J. Immun.* **49**, 175.

MARINESCO, G. & DRAGANESCO, S. (1923). Recherches expérimentales sur le neurotropisme du virus herpétique. *Annls Inst. Pasteur, Paris* **37**, 753.

MARTIN, J. K. (1950). Local paralysis in children after injections. *Archs Dis. Childh.* **25**, 1.

MCCLOSKEY, B. P. (1950). The relation of prophylactic inoculations to the onset of poliomyelitis. *Lancet* i, 659.

https://doi.org/10.1017/S0022172400045678 Published online by Cambridge University Press

MELNICK, J. L. (1945). Poliomyelitis in the bloodstream in the experimental disease. *Proc. Soc. exp. Biol. Med.* **58**, 14.

MIMS, C. A. (1957). The invasion of the brain by yellow fever virus present in the blood of mice. *Br. J. exp. Path.* **38**, 329.

NAGLER, F. P. O. (1946). A herpes skin test reagent from amniotic fluid. *Aust. J. exp. Biol. med. Sci.* **24**, 103.

OLITSKY, P. & SCHLESINGER, R. W. (1941). Effect of local edema and inflammation in the skin of the mouse on the progression of herpes virus. *Science, N.Y.* **93**, 574.

PLATT, H. (1964). The local and generalised forms of experimental herpes simplex infection in the guinea-pig. *Br. J. exp. Path.* **45**, 300.

RHODES, A. J. (1953). Recent advances in the study of poliomyelitis with particular reference to prevention. *Br. med. Bull.* **9**, 196.

SABIN, A. B. (1939). Portal of entry and transmission of poliomyelitis virus. *Int. Bull. Econ. med. Res. publ. Hyg.* A **40**, 1.

SANDERS, F. K. (1953). Possible multiplication cycles in neurotropic viruses. The nature of virus multiplication. *2nd Symp. Soc. gen. Microbiol.* p. 297. Cambridge University Press.

SAWYER, W. A. & LLOYD, W. (1931). Use of mice in tests of immunity against yellow fever. *J. exp. Med.* **54**, 533.

WARD, R., HORSTMANN, D. M. & MELNICK, J. L. (1946). The isolation of poliomyelitis from extraneural sources. IV. Search for virus in the blood of patients. *J. clin. Invest.* **25**, 284.

WENNER, H. A. & KAMITSUKA, P. (1957). Primary sites of virus multiplication following intramuscular inoculation of poliomyelitis virus in cynomolgus monkeys. *Virology* **3**, 429.

WILDY, P. (1954). The growth of herpes simplex virus. *Aust. J. exp. Biol. med. Sci.* **32**, 605.

WILDY, P. (1955). Recombination with herpes simplex virus. *J. gen. Microbiol.* **13**, 346.

WRIGHT, G. PAYLING, (1953). Nerve trunks as pathways of infection. *Proc. R. Soc. Med.* **46**, 319.

YAMAMOTO, T., OTANI, S. & SHIRAKI, H. (1965). A study of the evolution of viral infection in experimental herpes simplex encephalitis and rabies by means of fluorescent antibody. *Acta neuropath.* (*Berl.*) **5**, 288.

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