STUDIES ON THE DISSEMINATION OF RABIES VIRUS
IN RATS FOLLOWING FOOT-PAD INOCULATION

by

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Approved by:

[Signature]
Major Professor
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INTRODUCTION

Rabies is one of the oldest diseases mentioned in history and has been well known since the time of Aristotle (Mutyra, Marek and Manninger, 1946). But the exact mode of progression of the rabies virus from surface wound to the spinal cord or brain has remained a matter of controversy, at least since the days of Pasteur (1889).

The theory of dissemination of rabies virus by neural pathway is based upon the experiments of DiVestea and Zagari (1889) and has been confirmed by Huygelen and Mortelmans (1959) and Schindler (1961). On the other hand Krause (1957) Borodina (1959) and Field (1951) entertained the possible role of blood in the dissemination of the rabies virus. However, Huygelen (1960) and Dean et al. (1963) demonstrated the possibility of infection by both routes. It still remains an important question, whether the virus normally travels by way of the blood stream or by the neural route or it may use a combination of both.

An attempt has been made in this investigation, to study the mode of dissemination of the rabies virus in neurectomized rats following foot-pad inoculation in order to determine whether the virus reaches the brain through blood or along the nerves or both.

REVIEW OF THE LITERATURE

Huygelen and Mortelmans (1959) determined quantitatively the virus content of the different portions of the Central Nervous System of guinea-pigs, inoculated intramuscularly in the hind leg
with the Flury strain of rabies virus. Their results were in accordance with the theory of the dissemination of rabies virus by the neural pathway.

After intramuscular inoculation in the hind leg, the virus was found at first in the lumbosacral cord, where it multiplied and spread toward the brain. The animal died before the concentration of virus in the brain was as great as in the lumbosacral cord. This variation of virus concentration was parallel with the symptoms of ascending paralysis observed in guinea-pigs after inoculation of Flury virus in the hind leg. Ascending paralysis was not observed when the virus was injected in the anterior part of the body; in the foreleg or in the masseter muscles. Virus could not be detected in the liver during the incubation period, and this finding did not agree with the observations of Krause (1957). Their results however depended greatly upon the quantity and titer of the Flury strain used in the experiments, whereas the fixed virus used by Krause (1957) had a much higher titer, so that very small amounts of virus could still be detected by him in the organs sometime after the inoculation and the subsequent dispersion throughout the experimental animal's body. The results obtained by Huygelen and Mortelmans (1959) indicated that during the incubation period the virus content of some parts of the Central Nervous System may reach a very high titer before causing clinical symptoms.

Dean et al. (1963) investigated the spread of fixed and street strains of rabies virus from the site of injection to the Central Nervous System and salivary glands in various animal
species. Their studies supported and extended the observations of DiVestea and Zagari (1889). The results indicated that rabies was usually transmitted from the site of exposure to the Central Nervous System via the peripheral nerves. However, routes other than nerve transmission could occur in young animals, in highly susceptible species, or in animals whose resistance had been altered by trauma and shock.

They also confirmed the impressions of Pasteur (1889) that blood-borne transmission was possible. Blood-borne infection in nature is probably the exception rather than the rule. Air-borne infection was occasionally possible as reported by Remlinger and Bailly (1938), Constantine (1962). Results with Fluorescent Antibody Technique generally paralleled those of Kligler and Bernkopf (1943) and Schindler (1961), who used mouse injection tests to demonstrate virus in nerve tissue after intramuscular and foot-pad injection respectively.

Schindler (1961), in discussing the "Studies on the pathogenesis of Rabies", said that it still remained an important question whether the agent causing this disease reaches the Central Nervous System (CNS) via the blood or along the nerves. Rabies virus was also demonstrated in the blood, but not with the same regularity as poliomyelitis virus. During the past few years it became possible to cultivate rabies virus in vitro in non-nervous tissue Kissling (1958), Sulkin et al. (1959), Atanasiu and Lepine (1959), Kaplan, Korsek and Koprowski (1960). The virus was present in the body outside the CNS (Sulkin et al. 1959, Schindler 1959). In the literature there were few indica-
tions that rabies virus invades the CNS via blood stream. Furthermore, the finding of virus in the blood of animals did not prove that it invaded the CNS via the blood stream. (Verlinde 1960, Wenner et al. 1960). From the experimental observations it could be concluded that rabies virus travel along or in the nerves from the periphery to the Central Nervous System and vice-versa.

In 1960, Huygelen investigated the dissemination of rabies virus following intramuscular inoculation of the Flury strain into the muscles of the foreleg of the guinea-pigs. The virus was recovered from the cervicothoracic cord on the 4th day. From the 6th day on, the virus spread to the brain and lumbosacral cord. There was a close parallelism between virus distribution and clinical symptoms; the first sign of paralysis being observed in the inoculated leg. Recently Krause (1957) emphasized the role of blood in the dissemination of rabies virus. In several instances he recovered virus from the blood some time after inoculation. Huygelen (1960) observed that following an inoculation a large amount of virus is undoubtedly resorbed by the blood and disseminated throughout the body, but does this fact involve that it is this part of the virus, taken up by the blood, which is actually responsible for the establishment of the infection in the nervous tissues?

The results of the experiments proved that both blood and nerves may play a role in the dissemination of the rabies virus. The actual pathway of street virus after natural infection remained unknown. Further work is required to demonstrate whether
the virus normally travels by way of the blood stream or by neural route, or, whether it may use a combination of both, as it had been observed in the experimental poliomyelitis virus infection. (Bodian 1954)

Field (1954) observed that following corneal inoculation of fixed rabies with considerable regularity on the sixth to the eighth day the outstanding early clinical features were hypotonia and disturbance of equilibration most marked in the hind limbs. Infiltration of the ipsi- and contralateral ganglia recurred early, and in diminishing degree spread to the other sensory ganglia.

The microglia react early in the pons, cerebellum and cerebrum and presented distinct alterations at a time when the nerve cells seemed normal or only minimally affected. This early microglia reaction suggested a rapid interstitial diffusion of virus in the neuraxis independently of nervous connections. The results obtained by Field (1954), like those of Goodpasture (1925), afford no unequivocal evidence for axonal transmission of rabies virus from the cornea to the pons. No direct evidence of axonal transmission was found.

The concept of axonal transmission of rabies was enunciated by Duboue in 1879 (quoted by Gamaleia 1887). It was at first received with considerable caution, even by DiVestea and Zagari (1889), who were generally credited with the provision of an experimental basis for the theory. The position had been fairly summed up by Webster (1942) who concluded that the exact mode of progression of virus from surface wound to cord or brain remains to be determined.
Borodina (1959) injected white mice intracerebrally with 3 strains of street rabies virus and could find the virus in the blood of the experimental animals at early periods after inoculation (50 minutes - 54 hours). The results were checked by a combination of biological and morbid anatomical investigations.

The early appearance of street rabies virus in the blood of infected mice did not depend on the strain of the virus in question nor in its concentration in the infective material. At a time when the clinical picture of rabies was well marked no virus could be found in the blood of injected mice. (Borodina 1959)

In the last decade, however, the majority of studies afforded evidence for the presence of viremia in rabies. Some authors believed that in the experimental rabies the virus circulates in blood only for a short time. Wong et al. (1951) were inclined to suggest that viremia could be observed in the first hours after the infection of the experimental animals. In 1954, Regan and Brunker isolated rabies virus from blood samples collected 90 hours after infection of cats per rectum.

Field (1951) observed that inoculation of the masseter muscle of the rabbit with fixed rabies virus did not lead to an early paralysis of the masticatory muscles of the same side. Severe lesions occurred in the dorsal root ganglia of both sides and were much less intense in the lumber, than in the cervicoles region. No lesion was found in the corresponding motor nucleus of the fifth nerve, even when there was already evidence of widespread involvement of the nervous system.
In conclusion, he stated that the experimental results offered no support for belief in the axonal transmission of rabies virus from the inoculated masseter muscle to the pons. The infection was disseminated either by blood stream or by the cerebrospinal fluid.

Bartos (1957) studied the question of viremia in rabies by inoculating 20 rabbits intraperitoneally with street rabies virus, and 20 minutes later the lingual and buccal mucosa was sacrificed in order to bring blood into direct contact with the nerve endings; 17 developed rabies 12-64 days, and the remaining 3 died from other causes. In a similar experiment with 20 rabbits, part of the ear was amputated and incisions were made into the thigh muscles; 14 rabbits developed rabies after 20-90 days, 5 died from other causes and 1 survived. Four of twenty animals died of rabies after 23-45 days in a control group, in which no incisions were made.

In further experiments 5 dogs were infected intraperitoneally with rabies virus and the saliva was collected in swabs fixed inside the mouth. By the inoculation of guinea-pigs with saline extracts of swabs removed at interval, it was possible to demonstrate the presence of rabies virus in the saliva between 2 and 17 hours after infection; the virus was also found in the saliva 2-8 hours after subcutaneous infection.

These results indicated that a viremia occurred after inoculation of rabies virus, and that virus was eliminated in the saliva during this phase.

Goodpasture (1925) injected rabbits by inoculating them with
street rabies virus into the right masseter muscle, and observed that "from an anatomical standpoint there is no conclusive evidence that the virus enters the brain through nerves." But the clinical course of the disease on the whole, may be best explained on the theory of a neural transmission of the virus from the site of inoculation, an idea founded upon much careful anatomical and experimental work.

Histologic evidence based upon the presence of exudate, Negri bodies and degenerative changes in ganglionic cells indicated that the virus, when it entered the Central Nervous System, spread with great rapidity. (Goodpasture 1925) In the conclusion he said, "It is believed that the rabies virus enters the Central Nervous System through nerves supplying the inoculated muscle."

Wong and Freund (1951) observed that the mechanism by which rabies virus reached the blood stream was not known. It was possible that it entered the blood vessels damaged by the injection, but it seemed more probable that it reached the blood stream via the cerebrospinal fluid. The latter possibility was supported by the findings of Schaeffer and Muckenfuss (1940) on the distribution of India ink following intracerebral injection in monkeys.

It may be assumed that the rabies virus, having reached the cerebrospinal fluid, passes through the arachnoid villi into the blood stream. It is of interest that a virus as large as the fixed rabies virus, which had been estimated by Galloway and Elford (1936) to be 0.1 - 0.21 micron in size, could pass through the arachnoid villi. The assumption that after intracerebral
injection the virus would be readily demonstrable in the blood of rabbit taken from the internal jugular vein, was not confirmed. Fixed rabies virus was recovered from the blood stream of the mouse and rabbit during the first 3 hours following intracerebral inoculation.

Regan et al. (1954) injected young dogs with rabies virus by rectal instillation. Electron micrographs of erythrocytes from the dogs exposed to rabies virus, contained virus-like particles or inclusion-like bodies. These were found in the 90 hour bleeding and the blood from this bleeding did not produce disease in mice inoculated intracerebrally. They concluded that virus was present in the blood during this 90 hour period, although the puppies showed no evidence of rabies after a 30 day observation period.

On the application of the Indirect Fluorescent Antibody method to detect rabies virus, Topleninova (1961) observed that this method made it possible to detect virus in the brain cells of white mice. Rabies virus could be detected by means of FAM considerably earlier than with the standard diagnostic methods. However, typical Negri bodies, even when virus is present in the test material may not be detected in about 10 percent of the cases.

Goldwasser et al. (1959) studied the salivary gland and brains of various animal species by FAM. Brains and salivary glands were subjected to mouse inoculation (biological) test for isolation of the virus. Of 121 animals investigated 94 proved to be non-rabid on histological examination and biological
testing, negative findings by fluorescent staining were also obtained in all 94. They concluded that from the results of their study, that the speed and efficiency of Fluorescent Antibody Technique for determining the presence of rabies virus in the salivary gland of biting animals was of value. In their opinion, "considerably wider application of this method is needed, before it would be possible to determine whether the standard methods for the diagnosis of rabies in use today------could be replaced by Fluorescent Antibody Techniques."

Goldwasser and Kissling (1958) indicated that in Fluorescent Antibody staining, it was not possible to differentiate brain smears, of fixed virus infected mice with that of street virus brain smears, except that in later cases larger fluorescent bodies appeared, apparently representing the Negri bodies. Many strange and bizarre forms of fluorescent staining material were found in smears of both fixed and street virus. It seemed probable that these were due to artificial distortion of the antigenic material in making impression smears. However, he presented evidence that Negri bodies were of viral origin.

It had long been assumed that Negri bodies contain virus particles and cytochemical studies by Wolman (1952) presented indirect evidence to this hypothesis. The major part of Negri bodies, however, were thought to be composed of a matrix derived from the cytoplasm of degenerating cells (Johnson 1952). The diagnosis of rabies is based upon the demonstration of Negri bodies in brain smears of section. However, inclusion bodies could not be demonstrated in approximately 10 percent of infected
animals and under certain conditions this rose to 28 percent.

McQueen et al. (1960), while evaluating the fluorescent antibody test in the diagnosis of rabies, observed that the standard mouse inoculation was considered as the definite criterion of the presence or absence of infection. The Fluorescent Rabies Antibody (FRA) technique was in complete agreement with mouse inoculation on total of 70 examinations.

MATERIALS AND METHODS

Selection of Animals

Eighty white rats, 4-5 months old of either sex were randomly selected from the animal breeding colony of the department of Pathology. All white mice used in this investigation were 15-20 days old and were obtained as required from the same colony.

Preinoculation Procedure

Five rats of either sex were selected from the lot and kept under observation for 28 days and were then sacrificed for the following studies.

Rat brain was removed aseptically. Impression smears were prepared from the Ammon's horn and were examined for the presence of rabies virus by Seller's stain, mouse mortality, and Fluorescent Antibody Microscopy (FAM). Brain was ground in sterile mortar with pestle and emulsified with 10 percent horse serum in distilled water and 0.03 ml of this emulsion was injected intra-
cranially into each of 5 mice. This emulsion was tested for bacterial sterility. These mice were observed for 28 days and then sacrificed and examined for the presence of rabies virus.

Eighty rats were divided into ten groups consisting of eight rats each. Fifty rats were neurectomized and the remainder were used for control purposes.

Neurectomy of the sciatic nerve was completed as follows:

Rat was anesthetized with ether and the hair over the operation area was removed by applying Denete® depilatory lotion. An incision, one half inch long, was made in the skin on the lateral aspect of the thigh of the right hind leg just posterior to the middle third of the shaft of femur, exposing the biceps femoris muscle. The muscle was incised and the nerve was cleared of the fascia and lifted to the surface. One inch of the nerve was resected. The wound was dressed antiseptically and a nylon suture was applied.

Neurectomy of the saphenous nerve was completed as follows:

After removal of hair an incision one half inch long was made in the skin on the medial aspect of the leg just posterior to the upper half of the shaft of the tibia. The fascia surrounding the nerve, satellite artery and vein was removed. One inch of the nerve was resected, care being taken to avoid cutting the artery and vein. The wound was dressed antiseptically and a nylon suture was applied.

The wound was completely healed 5-6 days after surgery. At

* Fort Dodge Laboratories
this time, the sutures were removed. The rats were challenged by injecting 0.25 ml of National Institute of Health strain of fixed rabies virus in 10 percent horse serum in sterile water. This was inoculated into foot-pad of the neurectomized leg. The injection was made in a number of different directions without withdrawing the needle.

A normal rat was challenged with the same virus dosage as a control on the inoculation route and on the virulence of the virus. Five mice each were inoculated intracranially with 0.03 ml of the challenge virus.

Sixty minutes after challenging via foot-pad route, 1.0 ml of blood was drawn from the heart of the neurectomized rat and mixed with normal saline. Four mice were inoculated into the muscle of the thigh with 0.06 ml of the heart blood of the rat and kept under observation for 28 days. The mice, which died, were examined for the presence of virus by means of Seller's stain and FAM.

Approximately 10-12 days after neurectomy some of the challenged rats chewed their neurectomized foot probably due to lack of sensation. To control further injury to the foot a harness made of insulated wire was put on the rat, which restricted the animal from biting the neurectomized leg (Plate I, Figs. 1 and 2; Plate II). In some cases the harness caused irritation of the skin and it became necessary to remove it and treat the skin wounds.
Seller's stain, Mouse mortality and Fluorescent Antibody Microscopy (FAM)

After challenging, all the neurectomized rats were kept under observation for a period of 60 days. The brains of the rats which died before 60 days or were sacrificed after this period were examined for the presence of virus by the mice mortality test, Seller's stain and Fluorescent Antibody Technique.

The brains of the rats which died or were sacrificed were removed aseptically. Smears were prepared from the Ammon's horn and stained by Seller's stain. Impression smears were also prepared from the same area of the brain and were stained and examined by Direct Fluorescent Antibody technique using essentially the method of Cherry et al. (1960).

The brain of the rat removed by the above procedure was ground in a sterile mortar with pestle and emulsified with 10 percent horse serum in sterile water and 0.03 ml of the emulsion was inoculated intracranially into 5 mice which were observed for 28 days post inoculation. The brains of the mice which died within this period were examined for the presence of virus by Seller's stain and FAM.

Tissue Studies

The brains of the rats which died or were sacrificed were removed, fixed in formalin, sectioned and stained by Schleifstein and H & E methods. Brains were examined histologically for the presence of rabies virus. However, no effort was made to detect virus in other organs or tissues.
RESULTS

Neurectomized rats died within 60 days post challenge

Seven of fifty neurectomized rats died within a period of 13-26 days following foot-pad inoculation of rabies virus. The rats showed weakness of hind legs, tremor, irritability, aggressiveness, biting at the inoculated foot and ascending paralysis of the inoculated leg. The brains of these rats were found positive for the presence of rabies virus by Seller's stain, FAM, and mouse mortality examination. The mouse mortality on rats brain No. 5, 7, and 8 was 60 percent and rats No. 9 and 10, 40 percent; the mouse mortality on the rest of the rats was 100 percent (Table 1).

Histopathological findings on rat brain No. 5, sectioned and stained with H & E and Schleifstein respectively showed degeneration of the neurons and the presence of Negri bodies. Both extracellular and intracellular Negri bodies were observed (Plate III, Figs. 1 and 2). Extracellular Negri bodies were more abundant than intracellular Negri bodies in most of the preparations on Seller's, some preparations on FAM showed non homogeneous staining (Plate IV, Fig. 1). Many strange and bizarre forms of fluorescent staining material were encountered and these may have been due to artificial distortion of the antigenic material during the making of the impression smears.
Table 1. Passage of rabies virus in neurectomized rats which died within 60 days post challenge.

<table>
<thead>
<tr>
<th>Serial No. of Rat</th>
<th>No. of Animals Used</th>
<th>Rat Brain Examination of Mouse Brain</th>
<th>F.A. Technique</th>
<th>Seller's Test</th>
<th>Mouse Mortality No.*</th>
<th>%</th>
<th>Examination of Mouse Brain</th>
<th>F.A. Test</th>
<th>Seller's Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat No. 4</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>5/5</td>
<td>100</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rat No. 5</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>3/5</td>
<td>60</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rat No. 6</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>5/5</td>
<td>100</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rat No. 7</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>3/5</td>
<td>60</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rat No. 8</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>3/5</td>
<td>60</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rat No. 9</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2/5</td>
<td>40</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rat No. 10</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2/5</td>
<td>40</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Number of animals dying of rabies over the number inoculated.
Neurectomized rats survived 60 days post challenge

Forty-three of 50 rats survived the observation period of 60 days indicating that in 86 percent of the rats studied, the virus did not bypass the sectioned nerves. All the rats were sacrificed after 60 days and their brains examined. These were found negative for rabies by Seller's stain, FAM and mouse mortality test (Table 2).

In regard to the mouse mortality findings on rats No. 3, 39 and 40, only 40 percent of the mice died, and in rats No. 12, 23, 24, 29 and 39 only 20 percent of the mice died. In each case, the brains of the mice were negative for the presence of virus by Seller's stain and FAM examination.

Apparently the mouse mortality test was more sensitive to detect small quantities of virus than FAM, particularly if weanling mice were used.

The neurectomized rats used for control animals were sacrificed and examined after the 60 days observation period and appeared normal except for some atrophy of the neurectomized leg. The rats kept in harness did not develop as well as the normal rats held for comparative studies. No degenerative changes were apparent on histopathological examination of the brains of the rats which survived the observation period.
Table 2. Study of rabies virus in neurectomized rats which survived 60 days post challenge period.

<table>
<thead>
<tr>
<th>Serial No. of Rat</th>
<th>No. of Animals Used</th>
<th>F.A. Technique</th>
<th>Rat Brain Seller's Test</th>
<th>Mouse No.*</th>
<th>Mortality %</th>
<th>Examination of Mouse Brain F.A. Seller's Test</th>
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<tr>
<td>Rat No. 1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>0/5</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>Rat No. 2</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>0/5</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>Rat No. 3</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>2/5</td>
<td>40.0</td>
<td>-</td>
</tr>
<tr>
<td>Rat No. 11</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>0/5</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>Rat No. 12</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1/5</td>
<td>20.0</td>
<td>-</td>
</tr>
<tr>
<td>Rat No. 13</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>0/5</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>Rat No. 14</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>0/5</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>Rat No. 15</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>0/5</td>
<td>0.0</td>
<td>-</td>
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<tr>
<td>Rat No. 16</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>0/5</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>Rat No. 17</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>0/5</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>Rat No. 18</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>0/5</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>Rat No. 19</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>0/5</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>Rat No. 20</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>0/5</td>
<td>0.0</td>
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* Number of animals dying of rabies over the number inoculated.
<table>
<thead>
<tr>
<th>Serial No. of Rat</th>
<th>No. of Animals Used</th>
<th>F.A. Technique</th>
<th>Rat Brain Test</th>
<th>Mouse Mortality %</th>
<th>Examination of Mouse Brain F.A. Seller's Test</th>
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<td>Rat No. 21</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>0/5</td>
<td>0.0</td>
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<tr>
<td>Rat No. 22</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>0/5</td>
<td>0.0</td>
</tr>
<tr>
<td>Rat No. 23</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1/5</td>
<td>20.0</td>
</tr>
<tr>
<td>Rat No. 24</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1/5</td>
<td>20.0</td>
</tr>
<tr>
<td>Rat No. 25</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>0/5</td>
<td>0.0</td>
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<tr>
<td>Rat No. 26</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>0/5</td>
<td>0.0</td>
</tr>
<tr>
<td>Rat No. 27</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>0/5</td>
<td>0.0</td>
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<tr>
<td>Rat No. 28</td>
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<td>-</td>
<td>-</td>
<td>0/5</td>
<td>0.0</td>
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<td>0/5</td>
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</tr>
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<td>Rat No. 30</td>
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<td>-</td>
<td>-</td>
<td>1/5</td>
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</tr>
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<td>Rat No. 31</td>
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<td>-</td>
<td>0/5</td>
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<td>Rat No. 32</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>0/5</td>
<td>0.0</td>
</tr>
<tr>
<td>Rat No. 33</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>0/5</td>
<td>0.0</td>
</tr>
<tr>
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<td>1</td>
<td>-</td>
<td>-</td>
<td>0/5</td>
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</table>

* Number of animals dying of rabies over the number inoculated.
<table>
<thead>
<tr>
<th>Serial No. of Rat</th>
<th>No. of Animals Used</th>
<th>F.A. Technique</th>
<th>Rat Brain Examination</th>
<th>Mouse Brain Examination</th>
<th>Mortality %</th>
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<tr>
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</tr>
<tr>
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<td>-</td>
<td>0/5</td>
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</tr>
<tr>
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<td>-</td>
<td>1/5</td>
<td>-</td>
<td>20.0</td>
</tr>
<tr>
<td>Rat No. 39</td>
<td>1</td>
<td>-</td>
<td>2/5</td>
<td>-</td>
<td>40.0</td>
</tr>
<tr>
<td>Rat No. 40</td>
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<td>-</td>
<td>2/5</td>
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<td>40.0</td>
</tr>
<tr>
<td>Rat No. 41</td>
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<tr>
<td>Rat No. 42</td>
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<td>-</td>
<td>0/5</td>
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<tr>
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<td>-</td>
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<tr>
<td>Rat No. 47</td>
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<tr>
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<tr>
<td>Rat No. 49</td>
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<td>-</td>
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* Number of animals dying of rabies over the number inoculated.
<table>
<thead>
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<th>F.A. Technique</th>
<th>Seller's Test</th>
<th>Rat Brain Examination of Mouse Brain</th>
<th>Mouse Mortality No.*</th>
<th>%</th>
<th>F.A. Test</th>
<th>Seller's Test</th>
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<td>0/5</td>
<td>0.0</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control Rat on Virus</td>
<td>10</td>
<td>+</td>
<td>+</td>
<td>50/50</td>
<td>100.0</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Control Rat on Harness</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>0/50</td>
<td>0.0</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Uninoculated Control Rats</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>0/50</td>
<td>0.0</td>
<td></td>
<td>-</td>
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</tbody>
</table>

* Number of animals dying of rabies over the number inoculated.
Viremia studies

Seven of 25 neurectomized rats had sufficient virus in their heart blood 60 minutes after foot-pad challenge to infect weanling mice inoculated in the muscles of the thigh. In 13-26 days the rats died as a result of rabies. The finding of rabies virus in the mice brains was confirmed by Seller's stain and FAM examination (Table 3).

Table 3. Rabies viremia studies of rat heart blood after challenge, as determined by mouse mortality test.

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>No. Neurectomized Rat*</th>
<th>No. of Mice** Inoculated</th>
<th>No. of Mice Paralysed Or Died</th>
<th>Mice Brain Mortality %</th>
<th>F.A. Seller's</th>
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<tbody>
<tr>
<td>1</td>
<td>Rat 1V</td>
<td>4</td>
<td>0</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Rat 2V</td>
<td>4</td>
<td>0</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Rat 3V</td>
<td>4</td>
<td>4</td>
<td>100.0</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Rat 4V</td>
<td>4</td>
<td>0</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Rat 5V</td>
<td>4</td>
<td>0</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Rat 6V</td>
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<td>0</td>
<td>0.0</td>
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</tr>
<tr>
<td>7</td>
<td>Rat 7V</td>
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<td>0</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Rat 8V</td>
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<td>0</td>
<td>0.0</td>
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<tr>
<td>9</td>
<td>Rat 9V</td>
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<td>+</td>
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<td>10</td>
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<tr>
<td>11</td>
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<td>0</td>
<td>0.0</td>
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<tr>
<td>12</td>
<td>Rat 12V</td>
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<td>0</td>
<td>0.0</td>
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<td>13</td>
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<td>0</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>Rat 14V</td>
<td>4</td>
<td>4</td>
<td>100.0</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
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<tr>
<td>16</td>
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<tr>
<td>17</td>
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<tr>
<td>18</td>
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<td>19</td>
<td>Rat 19V</td>
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<td>4</td>
<td>100.0</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
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<td>+</td>
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<tr>
<td>21</td>
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<td>+</td>
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<tr>
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</tr>
<tr>
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<td>Rat 24V</td>
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<td>25</td>
<td>Rat 25V</td>
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</tbody>
</table>

* Rat heart blood inoculated intramuscular into mice, 60 minutes post challenge.
** Mice kept under observation for 28 days post inoculated I/M.
DISCUSSION

Goodpasture (1925), using street rabies virus, injected nine rabbits into the masseter muscle and allowed five to live long enough to exhibit definite clinical signs. Of these five with symptoms of nervous system involvement, three showed weakness of the forelimbs on the inoculated side first. In one case, the opposite forelimb was first affected and in one case, both forelimbs simultaneously. No hind leg paralysis was observed in any of the five animals with nervous involvement but one of the non-paralysed animals showed "general loss of muscular coordination." He concluded that following an inoculation into the right masseter muscle the anterior extremities were first affected and more often on the right side. In this investigation (using fixed virus), paralysis first appeared in the inoculated hind leg and then typical ascending paralysis was observed in the rats. These results are in accordance with the findings of Huygelen and Mortelmans (1959) who observed that propagation of the virus ran parallel with the symptoms of ascending paralysis observed in guinea-pigs after the inoculation of Flury virus in the hind leg.

Rabies, like poliomyelitis, shows a strong tendency to produce paralysis in the hind limbs even when inoculated intracerebrally (Babes 1912). Moreover, a successful intravenous inoculation commonly leads to initial posterior paralysis (Pasteur, et al. 1882).

Field (1951), concluded that the inoculation of the masseter muscle of the rabbit with fixed rabies virus did not lead to an
early paralysis of the masticatory muscles of the same side. In five of thirteen animals, paralysis began in the hind limbs, which was contrary to the findings of Coodpasture (1925).

In this investigation the virus bypassed the sectioned nerves in seven of fifty neurectomized rats, and reached the Central Nervous System (CNS). Then the rats showed typical ascending paralysis and died within 13-26 days. The brains of these rats were positive for the presence of rabies virus, determined by FAM, Seller's stain and mouse mortality tests. These observations are contrary to the findings of Dean et al. (1963). They challenged ten neurectomized rats with the CVS strain of rabies virus and the mortality was 0.0 percent whereas mortality in this investigation was 14 percent indicating that in these cases virus did reach the CNS, using a route other than the neural. The possible role of blood in the dissemination of rabies virus in these cases may be considered. Wong and Freund (1951) observed that the mechanism by which the virus reaches the blood stream is not known. It is possible that it enters the blood stream through blood vessels damaged by the injection, but it seems more probable that it reaches the blood via cerebrospinal fluid. The latter possibility is supported by the findings of Schaeffer and Muckenfuss (1940) on the distribution of India ink following intracerebral injection in monkeys. Wong and Freund (1951) recovered fixed rabies virus from the blood of the mouse and rabbit during the first three hours following intracerebral inoculation. Field (1951), while discussing the role of the blood in the dissemination of rabies
virus, observed that following peripheral introduction the virus might reach the dorsal root ganglia, other than those in neural relationship with the part inoculated, either by the blood stream or through the cerebrospinal fluid. The ganglia are relatively easily accessible to certain colloidal dyestuffs (e.g. trypan blue) introduced into the blood stream and there is evidence that peripheral irritation enhances this accessibility in diminishing degree as one passes away from the level of irritation (Zakaraja, 1932). While rabies virus has been but rarely demonstrated in the blood, (Marie, 1905; Marie and Urbain, 1931), the fact that it may pass from an infected mother to fetus (Perroneito and Carita, 1887; Konardí, 1905) indicated that infection by the blood stream is possible even though no virus may be demonstrable by the methods commonly in use. While the cerebrospinal fluid is generally held to be non-infective, there has been some reports to the contrary (Konardí, 1922). It is possible that here again the ordinary methods for demonstration of the virus were not adequate, for the absence of virus from the cerebrospinal fluid is remarkable. Krause (1957) emphasized the role of blood in the dissemination of rabies virus and in several instances he recovered virus from the blood some time after inoculation.

Following an intramuscular injection, a large amount of virus is undoubtedly resorbed by the blood and disseminated throughout the body. Probably this virus taken up by the blood was responsible for establishment of infection in the nervous tissues of rats which died of rabies.
In this investigation, 86 percent of neurectomized rats did not die of rabies, indicating that the virus did not bypass the sectioned nerves. These results are in accordance with the theory of the dissemination of rabies by nervous pathways which is based on the experiments of DiVestea and Zagari (1889) and has been confirmed by Gamaleia (1887), Roux (1888, 1889), Bardach (1888), Helman (1889) and Bartarelli (1904).

Recent work has shown that poliomyelitis virus and similar viruses after entering the body first multiplied in tissues other than CNS, and that this multiplication was followed by viremia. It is difficult to decide whether there was a real difference between the pathogenesis of poliomyelitis and rabies or whether the apparent difference was only a consequence of the fact that during the past few years rabies had not been investigated as thoroughly as poliomyelitis.

Some time ago Burnet (1955) furnished a new argument against the assumption that neurotropic viruses move along the peripheral nerves towards the CNS. He wrote "it is quite impossible to review the literature without accepting the existence of such movement and almost equally impossible to believe in its physical reality." Another aspect of the problem was touched on by Sulkin et al. (1959). These workers investigated the muscular tissue of 27 hamsters infected intracerebrally with rabies street virus. They observed that the virus multiplied in the muscular tissue.

Schindler (1961) felt that it was necessary, when discussing the speed of movement of virus along the nerves, to take the
diameter of the nerves into consideration. Sulkin and co-workers (1959) carried out their investigation after the symptoms of rabies had appeared at a stage of infection in which the virus had multiplied extensively in the CNS. For this reason one cannot conclude that in rabies a multiplication of virus in the muscular tissue is an essential preliminary step to invasion of the CNS. Furthermore, the finding of virus in the blood of animals does not prove that it invades the CNS via the blood stream (Verlinde, 1960; Wenner et al. 1960).

If one finally compares the accepted theories concerning pathogenesis of poliomyelitis with the results of these experiments performed with the rabies virus, the following observations are of significance. In spite of numerous observations it has not been possible to decide with any certainty whether poliomyelitis virus moves via the blood or along the nerves towards the CNS, except in the case of traumatically induced infection; for example, after tonsillectomy or intramuscular infection, where the majority of investigators presume that the virus travels along the nerves (Sabin 1956, Gard 1958, Verlinde 1960). What has to be considered as the exception in poliomyelitis is the rule in rabies, because rabies is almost exclusively transmitted by the bite of animals. Therefore, although one cannot compare rabies with poliomyelitis absolutely, one can compare poliomyelitis induced traumatically with rabies. Then one will find, in spite of many reservations, a certain similarity in the dissemination of the two virus diseases.

In the viremia studies, the results indicated that virus was
found in the blood of 28 percent of rats under investigation. These observations are similar to those of Borodina (1959). She observed that virus could be found in blood of the experimental mice at early periods after inoculation (50 minutes–54 hours). Wong and Freund (1951) observed that viremia occurs in mice and rabbits after injection with rabies virus. It is considered a possible mechanism of infection in the occasional neurectomized animal succumbing to rabies. This observation is in accordance with the results of Bartos (1957) who showed that a viremia occurred after the incubation of rabies virus, and that virus was eliminated in the saliva during this phase.

CONCLUSIONS

Results of this investigation indicated that in 86 percent of neurectomized rats rabies virus did not reach the brain, suggesting that, it usually traveled by the nerve pathways from surface wound to CNS. However, 14 percent of rats under study did succumb to rabies indicating that the virus did bypass the sectioned nerves and reached the brain by way of other routes.

It seems probable, that blood played an important role in dissemination of the virus in these cases. Results of the viremia studies indicated that viremia occurred in rabies as in 28 percent of cases virus was found in the blood of neurectomized rats. It is likely that the virus absorbed by the blood was responsible for establishing rabies in these animals.

It is concluded that rabies virus travels principally by means of the nerve pathways, but in some cases it may also
disseminate by blood or it may progress by another route.
ACKNOWLEDGEMENTS

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APPENDIX
EXPLANATION OF PLATE I

Fig. 1. Rat showing self-multilation of neurectomized foot and leg.

Fig. 2. Harness used to prevent self-multilation.
EXPLANATION OF PLATE II

Rat showing foot healed after installing harness.
PLATE II
EXPLANATION OF PLATE III

Fig. 1. Brain of rat #7. Note extracellular Negri body. X500 (Seller's stain)

Fig. 2. Brain of rat #8 showing intracellular Negri body. X500 (Seller's stain)
EXPLANATION OF PLATE IV

Fig. 1. Brain of rat #7 showing non homogeneous Negri bodies. X500 (F.A.M.)

Fig. 2. Brain of rat #8 showing Negri bodies. X500 (F.A.M.)
PLATE IV

Fig. 1

Fig. 2
STUDIES ON THE DISSEMINATION OF RABIES VIRUS IN RATS FOLLOWING FOOT-PAD INOCULATION

by

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Rabies is one of the oldest diseases mentioned in history. But the exact mode of progression of rabies virus has remained a matter of controversy. Some workers believe in the theory of dissemination of rabies virus by neural pathway while others believe that blood plays a role in the dissemination of virus. This study was undertaken to investigate the mode of dissemination of rabies virus in neurectomized rats following foot-pad inoculation.

Fifty rats were neurectomized by removing surgically a section from the sciatic and saphenous nerves of the right hind leg. All rats were challenged with a constant dose of fixed rabies virus.

Rats were observed for a period of sixty days post inoculation. Brains of rats which died before this period or were sacrificed after 60 days were examined for the presence of Negri bodies by Fluorescent Antibody microscopy (FAM), Seller's stain and mouse mortality test. Brains of all rats were sectioned, stained by Schleifstein and H & E method for histopathologic examination. An additional twenty-five rats were neurectomized for viremia studies. Rats were challenged with fixed rabies virus via foot-pad route. After sixty minutes, blood was drawn from the heart of each rat and mixed with normal saline. This solution was inoculated intramuscularly into the thigh muscles of mice. Brains of all the mice which died or paralyzed were examined by FAM and Seller's stain for the presence of virus.

Seven of fifty neurectomized rats succumbed to rabies within 13-26 days. Brains of these rats were positive for the presence of rabies virus, and neuronal degeneration was present.
This indicated that in 14 percent of cases virus did jump the gap and reached the brain via routes other than nervous pathway. Forty-three rats did not die during the observation period. Brains of these rats were found negative for the presence of virus. The fact, that in 86 percent of rats, virus did not reach the brain or lumbar cord suggested that rabies virus mostly traveled by neural pathways. The results of viremia studies suggested that virus was present in the blood of 28 percent of rats. This indicated the possible role of blood in the dissemination of rabies virus.

It is concluded that rabies virus travels mostly by means of the nerve pathways, but in some cases it may also be blood-borne or it progresses by some other means.