COMPARATIVE STUDIES ON THE VIRUSES OF VESICULAR STOMATITIS AND EQUINE ENCEPHALOMYELITIS (1)

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Since Andervont and Theiler (2) induced encephalitis in white mice by means of intracerebral injection of the viruses of herpes and yellow fever respectively, these animals have been more widely employed by investigators for the experimental transmission of still other viruses.

In this paper we present an elaboration of earlier studies (3, 4) of the properties of vesicular stomatitis virus, and a similar investigation of the biological reactions of another virus inducing disease in horses, equine encephalomyelitis, in which the use of the mouse as a test animal has proved advantageous. We are indebted to Miss B. Howitt of the George Williams Hooper Foundation of the University of California for a specimen of the latter virus which was discovered by Meyer, Haring, and Howitt (5).

VIRUS OF VESICULAR STOMATITIS

The virus of vesicular stomatitis procured from horses and cattle and transferred to the pads of guinea pigs retains its dermatropism during continuous pad to pad passage (3, 4). As will be shown in this article, we have found that the virus also possesses neurotropic properties as determined by the results obtained through the employment of different routes of infection in white mice, guinea pigs, rats, rabbits, and monkeys.

Methods and Materials.—Two strains of vesicular stomatitis virus (Indiana and New Jersey) (1 a, 4) were available, samples being sent us through the kindness of Dr. W. E. Cotton of the United States Bureau of Animal Industry, who had propagated the strains in guinea pig pads for several years. Before use in our experiments, they were carried through twenty consecutive passages in the pads of guinea pigs. The material used as inoculum for animal or cultural tests con-
sisted of a 1:10 suspension of virus-infected tissue in hormone broth at pH 7.5. Unless otherwise stated, the suspension was filtered through Seitz' discs and the filtrate shown to be bacteria-free by inoculation of leptospira medium and on blood agar incubated under aerobic and anaerobic conditions.

Effects on White Mice

Intracerebral Injection.—After intracerebral injection of 0.03 cc. of filtered vesicular stomatitis virus, irrespective of its origin, that is whether derived from affected pads of guinea pigs, brains of mice, rats, guinea pigs, and monkeys, or from tissue cultures, the mice succumbed rapidly and uniformly to fatal encephalitis. Within 30 to 40 hours after injection of either the Indiana or New Jersey strain, the animals exhibited pronounced hyperesthesia, ruffling of the hair, tremors, circus movements, ataxia, and weakness of the legs. The weakness usually progressed to spastic paralysis of the posterior extremities, associated with generalized involuntary muscular contractions. In the early passages death occurred within 48 to 72 hours after inoculation, but after fifteen consecutive brain to brain transmissions, the animals lived only 24 hours after inoculation. Blood obtained by means of cardiac puncture at the height of reaction failed in every instance to yield bacterial growth in culture medium.

The gross pathological changes consist of edema of the brain with congestion and an occasional, small, focal hemorrhage. The histopathological lesions are either absent in the meninges or noted as infiltrations, here and there, by a few monocytes. The brain itself exhibits general edema and inconspicuous, diffuse, monocytic reaction. The neurones reveal various stages of degeneration. The characteristic lesion is the pronounced necrosis of the Purkinje cells and also of the nerve cells of the motor nuclei in the brain stem. The spinal cord shows corresponding changes: the membranes are practically normal; the cord itself is edematous and many nerve cells are degenerated. While the nuclei of most neurones contain acidophilic granular material, inclusion bodies are not detectable. On the other hand, as we shall soon describe, mice infected by intranasal instillation of the virus, with a resultant more protracted clinical course, frequently reveal characteristic intranuclear inclusions. It is noteworthy that the infiltrative or productive lesions are less manifest in animals having a fulminating type of infection; the predominant change then is the extensive and marked destruction of neurones.

1 Throughout the experiments reported in this paper, the Rockefeller Institute strain of white mice was employed.

 Ether anesthesia was used in all operations on animals.
We have observed more recently that while the dermotropic virus does not noticeably affect the kidney and liver of guinea pigs, neurotropic strains injure these organs in all mice and other experimental animals. Granular degeneration of the cells lining the renal tubules, especially those of the convoluted type, occurs to a greater or lesser degree. One also finds granular degeneration in the parenchyma of the liver and in more advanced cases, isolated, small areas of necrosis of liver cells and punctiform hemorrhages. The spleen and other organs are not, as a rule, affected.

Nasal Instillation.—Recently Webster and Fite have succeeded in transmitting a fatal encephalitis to white mice by means of nasal instillation of louping ill virus (6). Vesicular stomatitis virus also induces regularly a lethal encephalitic infection in white mice. This may be effected by the intranasal instillation of 0.04 cc. of filtrate by means of a tuberculin syringe fitted with a blunt needle, care being taken to avoid contact with the nasal tissues. The series of infections has been carried through twelve passages with the Indiana, and six with the New Jersey strain of the incitant, the brain of nasally infected mice being employed as inoculum in each transfer. All of fifty-four animals exposed to the virus in this manner succumbed within 5 to 8 days.

The symptoms of the infection are similar to those occurring after intracerebral injection, with the exception of a more prolonged incubation period, namely, 4 to 6 days. The pathological changes in the brain, cord, liver, and kidney are also similar, although inflammatory lesions, such as localized mononuclear infiltrations of varying degree, occur in the vessel sheaths and spaces, and as nodular accumulations in the gray and white matter of the central nervous system. The striking lesion consists of intranuclear inclusion bodies. From one to three or four such structures can be seen usually located in the nerve cells of the hippocampus and of the anterior gray matter of the cord. They are 1 or 2 microns in diameter, acidophilic, regular in outline, flat, refractive, and while they resemble the inclusions of Borna's disease, minute study reveals their difference from the latter. The bodies lie in sac-like nuclei which are somewhat swollen and have a darkly stained basophilic membrane, and are often in juxtaposition with the nucleoli. The most effective stain for their demonstration is phloxin-methylene blue (7).

It appears, therefore, that the virus of vesicular stomatitis is highly potent when applied to the uninjured nasal mucosa which is as sensitive to inoculation as is the traumatized brain or pads of animals. Infection was induced in white mice by this method with material
from the twenty-sixth generation of tissue cultures (1 b) containing the virus, and with filtrates of affected monkey and guinea pig brain in dilutions respectively up to $10^6$ and $10^7$.

Other Routes of Infection.—White mice were also found to be susceptible to subcutaneous injection of either the Indiana or the New Jersey strain of this virus. Of thirty-six animals so inoculated with 0.5 cc. of filtrates of active mouse brain tissue, of a titer of $10^6$ or $10^7$, thirty-three succumbed to the characteristic experimental encephalitis within 6 to 10 days. Introduction of mouse brain virus into the plantar tissues of mice was similarly effective, although the mouse, unlike the guinea pig, fails to show pad lesions.

Of eighteen white mice receiving intramuscularly 0.5 cc. of filtrate of mouse brain virus of either strain, of a titer of $10^6$ or $10^7$, fifteen developed the experimental disease and died from 8 to 12 days after inoculation.

The mice were also infected by feeding for 6 consecutive days with bread soaked in 5 per cent suspension of active mouse brain ground in either hormone broth or in milk. Of eight mice thus exposed to the incitant, five developed encephalitis and died 7 to 14 days after the first feeding. When virus is ingested in this manner, however, it may enter the nasal passages, either directly by inhalation, or indirectly by regurgitation, and thus produce infection. That this is possible is indicated by the high and uniform sensitivity of the uninjured nasal mucosa to the incitant, as opposed to the irregular and delayed action after artificial feeding of the virus (8).

Only infant mice proved susceptible to intraperitoneal injection of the virus: ten of sixteen animals aged less than 14 days were thus successfully infected. On the other hand, adult mice were insusceptible. Thirty-four adult white mice which received intraperitoneally 1 or 2 cc. of filtered mouse brain virus, of a titer of $10^4$, failed to be affected. When a simultaneous injection of 0.03 cc. of 1 per cent sterile, soluble starch solution was given into the brain, only three of forty mice developed the experimental disease, followed by death 8 or 9 days after inoculation. We cannot, however, disregard the factor of subcutaneous leakage of the virus during the inoculation, in the three animals which succumbed. Adult white mice are therefore not generally subject to infection by intraperitoneal injection.

The unequivocal, uniform reaction of white mice after intracranial injection or after simple nasal instillation of the virus, even in such high dilutions as 1:10 million, points to their general suitability as experimental animals. Moreover, it is possible to obtain neurotropic strains of the virus of vesicular stomatitis, although the neurotropism

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"Titer" signifies that the test material was decimally diluted and injected intracerebrally into white mice. The figures represent the final dilutions which were capable of bringing about fatal encephalitis.
is associated with distinct, but not pronounced action on the parenchymatous tissue of the kidney and liver. In view of the high susceptibility of the uninjured nasal passages—as high in degree as is the traumatized brain—rigid precautions are necessary during experimental procedures to prevent exposure to accidental infection. The identity of the strains should be checked at monthly intervals by animal immunity tests.

Effects on Guinea Pigs

The results of inoculation of the pads of guinea pigs with the virus of vesicular stomatitis have already been described (3, 7, 9).

Intracerebral Injection.—After twenty-five consecutive transmissions of the experimental disease in pads, 0.15 cc. of filtrates of active plantar tissue was introduced intracerebrally in guinea pigs. The following symptoms were induced by both the Indiana and the New Jersey strains of the virus.

From 2 to 5 days after injection, the animals showed weakness which rapidly progressed to paresis of both posterior extremities. Tremors and circling movements were also observed. Within 2 or 3 days the posterior extremities exhibited complete flaccid paralysis, and about 75 per cent of the animals died during the paralytic stage. The survivors recovered partially; some showed only an ataxic gait, while others were left with paralyzed legs. After nineteen brain to brain passages had been obtained with the New Jersey and five with the Indiana strain of virus, the experiment was discontinued. The cerebral tissue derived from the second, fourth, and seventh passages was also inoculated, for control purposes, into the pads of guinea pigs, producing therein pronounced, characteristic vesicular dermatitis.

The gross and microscopic pathological changes in the brain, cord, kidney, and liver of guinea pigs which died of the experimental infection correspond with those observed in mice.

The guinea pig is susceptible to intracerebral injection of this virus, whether derived from guinea pig pad or brain; mouse, rat, and monkey brain, or tissue cultures (1 b). Infection was induced with these materials in dilutions up to $10^4$.

Other Routes of Infection.—Pad inoculation of guinea pigs with plantar tissue virus with simultaneous intracerebral injection of 0.12 cc. of sterile starch solution induced no encephalitis, only the vesicular plantar dermatitis. Like mice, guinea pigs are unaffected by intraperitoneal inoculation either alone or with simultaneous intracerebral injection of starch solution.

Although the guinea pig is not as sensitive to the effects of the stomatitis virus in the central nervous system as is the white mouse.
the neurotropic action of the infective agent is nevertheless clearly demonstrated by the results of the foregoing experiments.

Effects on Monkeys

*Macacus rhesus* and *Macacus cynamolus* monkeys were inoculated intracerebrally with 1 to 1.5 cc. of filtrates of 10 to 20 per cent suspensions of tissues containing vesicular stomatitis virus. The tissues employed consisted of (a) mouse brain infected with guinea pig pad virus, (b) mouse brain obtained from the twenty-fifth brain to brain passage in mice, (c) affected guinea pig pad or brain, and (d) monkey brain. The virus in guinea pig plantar tissue was least active, inducing the most protracted course of the experimental infection. The experimental disease initiated with this virus, however, was carried through at least seven brain to brain passages in *Macacus cynamolus* monkeys and five in *rhesus* animals.

Both species of monkeys react similarly. The onset of signs of infection occurs, as a rule, from 4 to 7 days after inoculation. The first symptom observed is generalized weakness which progresses to definite paresis of the limbs, frequently associated with tremors and spasticity. Paralysis of one or more limbs and of the face supervenes occasionally in the later stages but at no time are there definite signs of meningeal involvement. Salivation was noted in 25 per cent of the animals and fever occurred on 1 or 2 days at the beginning of reaction. Death ensues on the 7th to 13th day after inoculation.

Filtrates of the brain tissue removed after death of the monkeys were found, on titration in mice, to contain virus in concentrations of $10^4$ to $10^6$. The cerebral tissue of each monkey, including those of the passage series, was also injected into the pads of guinea pigs, with the production of characteristic vesicular dermatitis. Similarly, brain tissue derived from the monkeys of the final serial passage, on transfer to the brain of mice and guinea pigs, induced characteristic experimental encephalitis. It is of interest that the plantar tissues of monkeys remain free from lesions after pad inoculation of the virus.

The microscopic lesions in the central nervous system and in the kidney and liver are comparable with those present in guinea pigs and mice, except that in monkeys there are more pronounced signs of productive inflammation; namely, invasion of perivascular sheaths and spaces with monocytes, localized areas of monocytic infiltrations in the gray and white matter of the brain and cord, multiplication of
glial nuclei and neuronophagia. Characteristic intranuclear inclusion bodies are readily found.

The observations just recorded show the neurotropic action of the vesicular stomatitis virus, now demonstrated in rhesus and cynomolgus monkeys. The course of events is not as rapid as in rodents. This may account for the more marked infiltrative lesions and for the more numerous inclusion bodies in the simians.

Effects on Other Animals

White and Hooded Rats.—These animals are susceptible to the action of the Indiana and New Jersey strains of vesicular stomatitis virus after intracerebral injection. Transmission of the experimental disease was effected through four serial brain to brain passages, the fifteen animals employed all succumbing to the disease within 7 to 10 days after inoculation. Introduction of the virus into the pads, however, leads to characteristic vesicular dermatitis of this tissue (9), as in the case of guinea pigs.

Young Chicks.—Chicks 24 to 48 hours old are unaffected after receiving intracerebrally from 0.05 to 0.12 cc. of virus filtrate. Eight different tests were made on sixteen birds.

Rabbits.—Rabbits are much more resistant to the virus, when injected in the brain, than are mice, rats, guinea pigs, or monkeys. Only three of fourteen test animals developed, within 7 to 9 days, signs of central nervous system involvement, terminating in complete flaccid paralysis of the posterior extremities. Pad inoculation is wholly without effect in the rabbit.

The employment by previous workers of the guinea pig as the experimental animal has led to the conception that the stomatitis virus is dermotropic in its action,—for of the dermal surface of the animal, only the plantar tissue has been proved to be uniformly susceptible to infection (3, 4, 9) From the results of the tests here recorded, we find that neurotropism, associated with a mild degree of viscerotropism, is a definite characteristic of the virus.

Recovery of Virus from Experimentally Infected Animals.—The employment of the white mouse as test animal, which is highly susceptible on intracerebral inoculation of the virus, has greatly facilitated its recovery. By mouse tests we have been able to demonstrate virus during the course of infection in the blood of the heart and peripheral circulation; in the submaxillary and parotid glands; in the brain, cord, and spinal fluid, and in the lung, spleen, liver, and kidney of mice, guinea pigs, and monkeys. In the monkeys it was
determined that the virus was present in the blood during the period from 24 hours after intracerebral inoculation, when the first test was made, to the onset of fever.

Cultivation of Vesicular Stomatitis Virus in Tissue Cultures.—The method of tissue culture in a medium consisting simply of minced chick embryos and Tyrode's solution has already been described (1 b). Two series of cultivation tests have now been performed, Series A with the Indiana strain and Series B with the New Jersey strain.

In Series A, filtrates of 1:10 suspensions of infected guinea pig pads or mouse brain pathogenic for mice in a dilution of $10^4$ were used to initiate the cultures. At the present time this culture is in its thirty-fifth generation and is active in mice in a dilution of $10^5$. The titer of the virus increased in this series to $10^{36}$.

In Series B, the filtrate serving to initiate the tissue cultures was active in mice in a dilution of $10^6$. The 58th generation now at hand was characteristically infective for mice in a dilution of $10^6$, and the virus increased in titer to $10^{66}$.

From these results it is evident that the virus can be cultivated in a medium consisting solely of chicken embryonic tissue suspended in Tyrode's solution. Other investigators (10) have succeeded in cultivating various viruses by this method. The fact is of interest that the virus of vesicular stomatitis was propagated with cells of the chicken, a species normally resistant to it. Vesicular stomatitis virus has a generic relationship to the incitant of foot-and-mouth disease (9, 11); and it may be possible to cultivate the latter with equal ease.

Virus of Equine Encephalomyelitis

Meyer and his associates (5, 12) have stated that the filtrable, glycerol-resistant encephalomyelitis virus is distinct from the incitant of botulism, "forage poisoning," Borna's disease, poliomyelitis, and apparently different from that of enzootic encephalitis of the Moussu...
Marchand type. We have confirmed their studies and elaborated the findings of the prior investigators. In our experience the virus has retained its infectivity in dilutions up to 1:10 million (instead of 1:10,000) and the white mouse has proved the experimental animal of choice instead of the guinea pig.

**Effects on White Mice**

These animals react uniformly when filtered or unfiltered, centrifuged, bacteria-free suspensions of the virus are introduced into the brain or nasal passages. The suspensions employed were prepared in the same manner as the vesicular stomatitis material. They consisted of infected mouse brain tissue, active when filtered in dilutions as high as $10^5$, and when unfiltered as high as $10^7$; and filtrates of affected guinea pig pad, and tissue culture material, infective up to $10^6$ dilution. The experimental disease induced by means of nasal instillation of the virus was transferred by brain to brain passages through at least eight successive series, each of four to eight mice.

The fatal infection produced by the virus is characterized by the same clinical reaction and microscopic changes in the nervous system, liver, and kidney as are found in experimental stomatitis disease. The typical inclusion bodies are also detected in mice receiving the encephalomyelitis incitant intranasally, but not in animals injected intracranially. Moreover, precisely as in the case of experimental vesicular stomatitis, adult mice are refractory to intraperitoneal inoculation of the virus, but not infant ones. Another similarity consists in the shortening of the course of the experimental infection after twenty consecutive passages, from the usual 3 to 5 days to 2 days—transfers having been made from brain to brain every 48 hours.

As with the stomatitis virus, the incitant of encephalomyelitis produces no local dermatitis after its injection in the plantar tissues.

**Effects on Guinea Pigs**

The introduction of the virus into the brain of guinea pigs results in an infection indistinguishable from that of experimental vesicular stomatitis.

Serial pad passages at intervals of 48 hours were carried out in two sets of guinea pigs, with seventeen consecutive passages in the first and twenty-six in the second. This series of transfers was initiated with pooled, glycerolated guinea pig brain virus. As with the stomatitis virus, the encephalomyelitis virus present in the pads of the different passages induced fatal encephalitis after subdural inoculation of mice and guinea pigs.

The virus introduced into the plantar skin of guinea pigs shows vesicular reactions varying in degree and the serous exudate within the vesicles may be either blood-tinged or clear. While the plantar lesions induced by vesicular stomatitis are characterized, as a rule, by clear vesicular contents, it is known that strains
of this virus produce sometimes blood-tinged exudate. Hence there is no definite distinction between the two viruses in respect to the character of the vesicular fluid. The histopathological changes in the affected pads are, moreover, identical in the case of both incitants and the epithelial cells show the same type of intranuclear inclusion bodies.

As with the stomatitis virus, only the skin of the pad of the guinea pig shows dermatitis after inoculation with the virus of equine encephalomyelitis; and after five or six serial pad passages, the animals fail to exhibit signs of nervous involvement.

**Effects on Other Animals**

The reaction of monkeys, rabbits, and white rats to the virus after its introduction into the brain or pads is identical with that of the virus of vesicular stomatitis. Similarly, very young chicks are unaffected by intracerebral inoculation of the virus.

_Establishment of Dermotropism._—We have already mentioned that the neurotropic incitant of encephalomyelitis, after five or six guinea pig pad passages, loses its property of affecting the central nervous system following pad injection of guinea pigs and then acquires dermatropism. An experiment was undertaken in which monkeys, rabbits, white mice, and white rats were inoculated into the plantar tissues with the modified, dermatropic virus. These animals, like the guinea pigs, also failed to show nervous disturbances. Hence it is evident that just as the dermatropic stomatitis virus can exhibit neurotropism, so can the neurotropic encephalomyelitis virus act as a dermatropic agent.

_Recovery of Virus from Experimentally Infected Animals._—The virus can be recovered under similar conditions and from the same kind of tissues as in experimental stomatitis infections.

_Tissue Cultures._—The virus can be cultivated in minced chicken embryonic tissue suspended in Tyrode’s solution, in the same way as the stomatitis incitant. Fifty-three generations have been obtained to the present time; in the forty-ninth generation a dilution of the culture of $10^4$ was capable of inducing fatal encephalitis in white mice, and the virus increased $10^{14}$ times.

**IMMUNOLOGICAL REACTIONS OF THE VIRUSES**

_Measures._—Immunity tests were made by (a) introducing the virus into animals recovered from its effects and (b) serum neutralization. The mode of procedure in tests of the latter sort follows.
A 20 per cent suspension of virus-containing tissue in hormone broth of pH 7.5, was centrifuged at moderate speed for 10 minutes so as to clear the fluid of the grosser particles. 1 cc. of the supernatant fluid was diluted with 25 cc. of broth. Equal volumes of the diluted suspension and of the serum collected from recovered animals were mixed, placed in a 37.5°C. water bath for 2 hours, and then in an ice chest overnight. The mixtures were examined for the presence of virus by intracerebral injection into animals. In addition, neutralizing rabbit antiserum, effective against the strain used, was obtained by three successive subcutaneous injections at 5 day intervals, of 3 or 4 cc. of filtrate of guinea pig pad or brain tissues containing living virus. Ample controls were provided for each experiment.

Homologous Reactions of Vesicular Stomatitis Virus (13).—The Indiana and the New Jersey strains were found to be immunologically distinct by both the in vivo and the in vitro procedure.

Guinea pigs recovered from the effects of plantar, subcutaneous, or intracranial inoculation of tissue cultures, guinea pig pad virus, or of brain material derived from infected mice, rats, guinea pigs, or monkeys, were resistant to later pad or intracerebral injection of the homologous strain of virus, irrespective of its source as to tissue or species of animal. The rabbit antiserum, or serum secured from recovered animals, showed corresponding homologous neutralization of the virus.

Homologous Reactions of Equine Encephalomyelitis Virus.—Guinea pigs recovered from the effects of this virus were shown to be immune to a later injection of the same virus. Rabbit antiserum, prepared with encephalomyelitis material, and the serum from animals recovered from the experimental disease, also inactivated the encephalomyelitis incitant. The methods employed in all these tests were precisely similar to those in the experiments with the virus of vesicular stomatitis.

Cross-Immunity Reactions.—On repeated trials cross-immunity reactions have not been found to occur between encephalomyelitis and stomatitis viruses.

SUMMARY

We have studied certain properties, additional to those previously described (3), of the virus of vesicular stomatitis of horses, and of the characteristic biological reactions of the virus of equine encephalomyelitis.

It has been found that the virus of stomatitis, ordinarily dermotropic, can acquire neurotropism and the neurotropic encephalo-
myelitis virus can, in turn, be rendered dermotropic in its action. The neurotropism in both instances is associated with definite, although not pronounced, viscerotropism.

Both viruses can bring about a similar infection in the white mouse, rat, guinea pig, rabbit, and rhesus or cynomolgus monkeys. Of these animals, rabbits show the lowest degree of susceptibility and mice the highest, especially after intracerebral inoculation. The mouse is the best animal for work with these viruses because of the uniform and rapidly lethal encephalitis which can be induced in it. Moreover, the mouse is highly sensitive to the instillation of the viruses in the nasal passages: 1 to 10 million dilution sufficing to induce a fatal encephalitis. The uninjured nasal mucosa of mice appears, therefore, to be as susceptible to experimental infection as the traumatized brain or pads of animals.

The microscopic changes accompanying the reactions to both viruses reveal, in rapidly lethal infections, pronounced destructive lesions in the cells of the central nervous system. When the experimental disease is more protracted in its course, however, these lesions are associated with beginning productive, inflammatory reactions, consisting chiefly of mononuclear infiltrations. In the latter instances, characteristic, intranuclear inclusion bodies can be more readily observed.

Both viruses can be cultivated with facility in the medium of minced chicken embryonic tissue suspended in Tyrode's solution, although 24 to 48 hour old chicks are refractory to artificial infection.

No cross-immunity reactions occur between the two strains of stomatitis virus or between them and the encephalomyelitis strain.

The viruses are evidently similar in many biological properties. In view of the fact that the horse is the natural host for both, it is suggested that they may be generically related. They are not, of course, identical since cross-immunity between them does not exist. The absence of cross-immunity does not, however, exclude the possibility of a generic relationship, for there are at least three immunologically distinct types of foot-and-mouth disease, two of vesicular stomatitis, and two of equine encephalomyelitis (14) virus.
REFERENCES


