

THE ISOLATION OF VIRUS IN MICE FROM CASES OF  
HORSESICKNESS IN IMMUNIZED HORSES.

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Since it was first shown (Nieschulz, 1932; Alexander, 1933) that white mice are susceptible to horsesickness virus by the intracerebral route, numerous strains of the virus have been fixed neurotropically. From infective blood drawn at the height of the disease in non-immune horses virus isolation is comparatively simple. Suckling mice, about four days old, are now used owing to their greater susceptibility (Weiss, 1949). The incubation period in the first generation is usually from five to eleven days, as against two to four weeks in adult mice. Thereafter fixation in adult mice presents no difficulty.

In the enzootic horsesickness areas of South Africa it is customary to immunize horses annually with a polyvalent neurotropic vaccine prepared in mice. (Alexander *et al.*, 1936.) Although the immunity produced by a single injection of vaccine is known to persist for at least eight years (Alexander, 1951a) annual inoculation is encouraged with a view to the production of the widest possible polyvalent immunity. Such immunized horses usually contain serum antibodies to high titre against the eight virus strains incorporated in the vaccine. A small percentage of breakdowns to natural infection in the field does occur. Isolation of the virus strain from such cases is of considerable importance, and the submission of blood samples is encouraged. From six such samples received during the last horsesickness season in no single instance was a virus isolated directly in mice. Mulligan (1938) has reported the same difficulty in Kenya. Apparently the serum antibodies in the blood from a reacting immune horse interfere in some way with adaptation of the virus to the mouse which is susceptible only to direct intracerebral injection.

This difficulty may be overcome by subinoculating the blood sample into a susceptible horse and from virus circulating in the blood taken at the height of the febrile reaction in the horse the mouse can be infected intracerebrally. Even though susceptible horses may be available the method is expensive.

After several unsuccessful attempts to activate these blood virus mixtures, "neutral" for the mouse, attention was directed to substitution of the susceptible horse by other less expensive laboratory animals that are susceptible to horsesickness virus. Success has been achieved with the ferret and the indications are that the dog may also be used.

The experimental work connected with this investigation forms the basis of this report.

MATERIALS AND METHODS.

*Strains of Virus.*—Three strains of virus isolated from immunized horses were used in this work.

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1. Strain 1513 was obtained from a horse, No. 1513, which was reported sick on 12 March, 1951, while running on the Institute's farm Kaalplaas. The horse, which died three days later after having shown symptoms of acute horsesickness, had been immunized with the polyvalent vaccine the previous three consecutive years. Blood was collected in O.C.G.\* just before death. After several unsuccessful attempts to adapt this virus to both suckling and adult mice, 10 ml. of blood in O.C.G. were injected intravenously into a susceptible horse, 1869, which reacted on the seventh day after injection and died two days later from peracute horsesickness. Blood was collected in O.C.G. from this horse when *in extremis*. Using this blood, adaptation of the virus to suckling mice was accomplished with ease, the first mouse dying on the fifth day after injection.

For convenience in the text virus from the immune horse 1513 is referred to as N1513 (N = neutral for mouse brain) and virus from the susceptible horse 1869 is indicated as A1513 (A = active for mouse brain).

2. Strain 1408 was obtained from a horse, No. 1408, which contracted horsesickness on 30th April, 1951, while running on Kaalplaas and subsequently recovered. This horse, too, had been immunized three times. As attempts to adapt this virus directly to mice from O.C.G. blood failed, a susceptible horse, 1695, was given intravenously 20 ml. of blood. This horse reacted on the fifth day and died three days later. Virus from this reacting susceptible horse was adapted to mice; the first death amongst the suckling mice occurred on the sixth day.

A similar distinction is made with this virus; N1408 refers to virus from the immune horse and A1408 to virus from the susceptible horse.

3. Strain Fourie was obtained from an immunized horse which contracted horsesickness in the Vryheid district. The blood was injected into suckling mice but as no deaths occurred which could be attributed to horsesickness, a susceptible horse, 1608, was given 5 ml. intravenously. This horse reacted on the ninth day and died on the twelfth. Blood was collected from this animal and from it a virus was isolated in suckling mice. Here again a supply of A Fourie and N Fourie was collected.

It is emphasized that, unless otherwise stated, in this work all the viscerotropic virus from the horses, whether "active" or "neutral", is contained in an O.C.G.-blood mixture.

*Sera*.—Serum was collected from horses 1513 and 1408 during the course of the breakdown reaction. These two sera were inactivated at 56° C. for 30 minutes, "Merthiolate" † 1:10,000 added as preservative and stored at 4° C.

Homologous anti-sera to the attenuated neurotropic vaccine virus strains (KA, 1180, A501, VH, Vryheid, 114, L, OD) were available in the form of freeze dried material stored at -18° C. Fully susceptible horses received a single subcutaneous injection of 5 ml. of a 10 per cent brain emulsion of mice sacrificed *in extremis* after infection with the particular virus strain. Serum was collected one hundred days after injection.

Anti-serum to strain 1513 was prepared by the hyper-immunization of rabbits with virus which had undergone 37 serial intracerebral mouse passages. The rabbits received 1 ml. of freshly prepared 10 per cent mouse brain emulsion twice

\* O.C.G.=Water, 1,824 parts; Glycerine, 500; Potassium oxalate, 5; Carbolic Acid, 5; and is used as with blood.

† Methiolate=Sodium ethyl mercuri thiosalicylate, Eli Lilly and Co.

weekly for four weeks, were rested for one week then given one more injection and bled for serum one week later. After inactivation Merthiolate was added as preservative and the serum was stored at 4° C.

*Neutralization Test.*—The method used was that described by Alexander (1935) in which serial twofold dilutions of the serum are mixed with a constant amount (100 LD<sub>50</sub>) of virus. The range of dilutions of serum tested was from  $\frac{1}{2}$  to 1/512. Each serum-virus mixture was injected intracerebrally in groups of four adult mice. Mice dying from the fourth to the tenth day were included in the death score and the 50 per cent end-points were calculated by the method of Reed and Muench.

*Adaptation to Mice.*—As the O.C.G.-blood mixture is toxic when injected intracerebrally in mice, it was diluted 1/10 with normal horse serum-saline. A dose of .03 ml. was given to suckling mice. For serial passage a 10 per cent emulsion of brains obtained from mice sacrificed *in extremis* was used. This emulsion required clarification by centrifugation at 3,000 r.p.m. for at least 15 minutes to avoid shock. The first three generations were passaged in suckling mice and subsequently mice about two months old were used. All infective brain material was stored at -18° C.

*Ferrets.*—These were from the stock kept at the Institute and were housed under conditions which unavoidably exposed them to possible *Culicoides* attack. This apparently did not influence the experimental results. To collect virus from the ferret, blood was withdrawn by cardiac puncture into O.C.G. under ether anaesthesia. When spleen was used as a source of virus, pieces of spleen were macerated in a Waring blender with an equal amount of serum-saline and lightly centrifuged. The undiluted supernatant fluid was used to infect mice.

It should be noted that considerable care must be exercised in obtaining a true picture of the rectal temperature of ferrets since they struggle violently when handled and this causes a marked elevation. Considerable experience has shown that the most satisfactory procedure is to hold the ferret by the tail only and to insert the thermometer when suspended head down in this way. The normal temperature fluctuates between 101 and 103° F.

*Serum-Saline.*—All dilutions were made in 10 per cent horse serum saline. The donors of the serum were horsesickness susceptibles maintained at this Institute under horsesickness-free conditions. The serum was inactivated at 56° C. for 30 minutes and Merthiolate added as preservative in a final concentration of 1 in 10,000.

## EXPERIMENTAL.

### 1. Attempted activation of "Neutral" Viruses in O.C.G.-Blood Mixtures.

The result of the injection of blood from reacting immune horses into susceptible horses showed that failure to isolate the virus in mice was not due to absence of viable virus but was probably due to the presence of antibodies which had developed in response to immunization. According to McKee and Hale (1946) among the methods used to reactivate neutralized serum-virus mixtures have been dilution, centrifugation and blind passage. As it was considered that the viruses N1513, N1408 and N Fourie were in a state neutral for the mouse, these three methods were investigated with a view to activating sufficient virus to enable adaptation to mice to take place.

1. *Centrifugation.*—The infective O.C.G.-blood mixture containing N1408 was centrifuged in an angle-head centrifuge for half an hour at 3,000 r.p.m. to remove gross particulate matter and the supernatant centrifuged for half an hour at 15,000 r.p.m. thus applying a centrifugal force sufficient to sediment the particles of horsesickness virus (Polson and Alexander, 1951). The sediment obtained was resuspended in 10 per cent serum-saline and again centrifuged for half an hour at 15,000 r.p.m. The sediment was resuspended in serum-saline and this was used to infect suckling mice. In this manner it was hoped that sufficient antibody would be removed to allow adaptation to mice to take place.

The attempt was unsuccessful.

This procedure was repeated with N Fourie with a similar negative result.

2. *Dilution.*—Infective blood containing N1513 was injected into families of suckling mice in the following dilutions: 1/100, 1/500, 1/1,000, 1/10,000. No deaths occurred in any of the mice which could be attributed to horsesickness. A1513 was subsequently titrated in suckling mice and the infective titre proved to be only 100 LD<sub>50</sub>. In view of the slight dilutions which such low titres would allow, further attempts at activating virus by this method were abandoned.

3. *Blind Passage.*—McKee and Hale (1946) distinguish degrees of neutralization in an influenza virus-serum mixture. In an apparent neutral mixture the virus and antibody are present in such proportions that mice are protected against death on the first but not subsequent passage. In an absolute neutral mixture, on the other hand, the ratio of virus and antibody is such that no deaths occur on blind serial passage.

Two families of suckling mice were injected intracerebrally with a 1/10 dilution of horse blood containing N1513. Brains from groups of apparently healthy mice were harvested on the fifth and seventh day for passage into further suckling mice. The second passage mice and remaining mice of the first passage were observed for several weeks but no mortality occurred.

From these three experiments it did not appear possible to adapt the viruses N1513, N1408 and N Fourie to mice even if techniques involving centrifugation, dilution or blind passage are used.

## 2. *Primary Isolation in Animals susceptible to Viscerotropic Virus.*

Following the above failures attempts were made to propagate these "neutral" viruses in an animal other than the horse but which is also susceptible to viscerotropic virus and thence to proceed with neurotropic adaptation in the mouse.

### (a) *The dog.*

Theiler (1906) and Bevan (1911) reported the susceptibility of the dog to horsesickness virus. Piercy (1951) reporting an outbreak of horsesickness in a pack of hounds, fed meat from horses which had died from horsesickness, noted that out of 35 dogs, 31 became sick and seven died.

Two groups of two dogs were given intravenously 1 ml. of N1513 and A1513 respectively. One of the dogs (No. 2) injected with N1513 showed only a doubtful febrile reaction and was discarded from the experiment. The other three all reacted and were bled into O.C.G. at what was thought to be the height of the reaction. The details are given in Table 1.

TABLE 1.  
*Behaviour of "Neutral" and "Active" Virus in Dogs.*

Dog.	Virus.	Reaction in Dog.	Adaptation to Mice.
1	N1513	Sick 4th day. Temperature reaction for 4 days	Unsuccessful.
2	N1513	No obvious reaction. Temperature 103·8 afternoon 6th day	Not attempted.
3	A1513	Sick 3rd day. Temperature 104·6. Duration temperature 4 days	Successful. First mouse died 9th day.
4	A1513	Slight temperature on 3rd day and 6th day...	Unsuccessful. Bled 4th day.

It will be seen that adaptation to mice was successful with blood from only one dog which received "active" virus. The reason for failure to isolate virus from dog No. 1 which was clinically sick when bled on the fourth day and from dog 4 bled at the same time cannot be explained.

(b) *The ferret.*

To determine whether ferrets are susceptible to viscerotropic virus, two were given each 1 ml. of A1408 intraperitoneally. One ferret reacted with a temperature of 107·8° on the morning of the fourth day after injection. That afternoon the temperature had dropped to 103·6° and from blood collected at this time virus was insolated in mice the first mouse dying on the eleventh day. The other ferret showed no definite febrile reaction but as temperatures were taken only once daily, it is possible that a short reaction was missed. No blood was collected from this ferret.

As it was apparent that ferrets are susceptible to the virus of horsesickness each of a group of four ferrets was given virus N1513 and another group N Fourie. Injections were made by the intracardiac or the intraperitoneal route (N1513) or intracardially only (N Fourie) in a dose of 1 ml. Details are shown in Table 2.

TABLE 2.  
*The Isolation of "Neutral" Virus in Mice from Ferrets.*

Ferret.	Virus.	Route of Injection.	Reaction in Ferret.	Material Collected.	Adaptation to Mice.
1	N1513	I.P.....	None.....	Spleen.... 10*	No.
2	N1513	I.P.....	None.....	Blood.... 5	No.
3	N1513	I. cardiac..	Temperature 4th to 7th day†....	Spleen.... 10	No.
				Blood.... 4	Yes.
				Blood.... 5	Yes.
4	N1513	I. cardiac..	Temperature 5th to 9th day.... Off feed 8th day.....	Spleen.... 10	No.
				Blood.... 5	Yes.
				Spleen.... 9	Yes.
5	N Fourie	I. cardiac..	Temperature 4th day.....	Spleen.... 6	Yes.
				Blood.... 4	Yes.
6	N Fourie	I. cardiac..	Temperature 4th and 5th day....	Blood.... 5	Yes.
				Spleen.... 6	Yes.
7	N Fourie	I. cardiac..	Temperature 4th and 5th day....	Spleen.... 6	Yes.
				Spleen.... 5	Yes.
8	N Fourie	I. cardiac..	Temperature 4th day.....	Spleen.... 5	Yes.

\* Number refers to days after injection that material was collected.

† Refers to days after injection. I.P. = intraperitoneal.

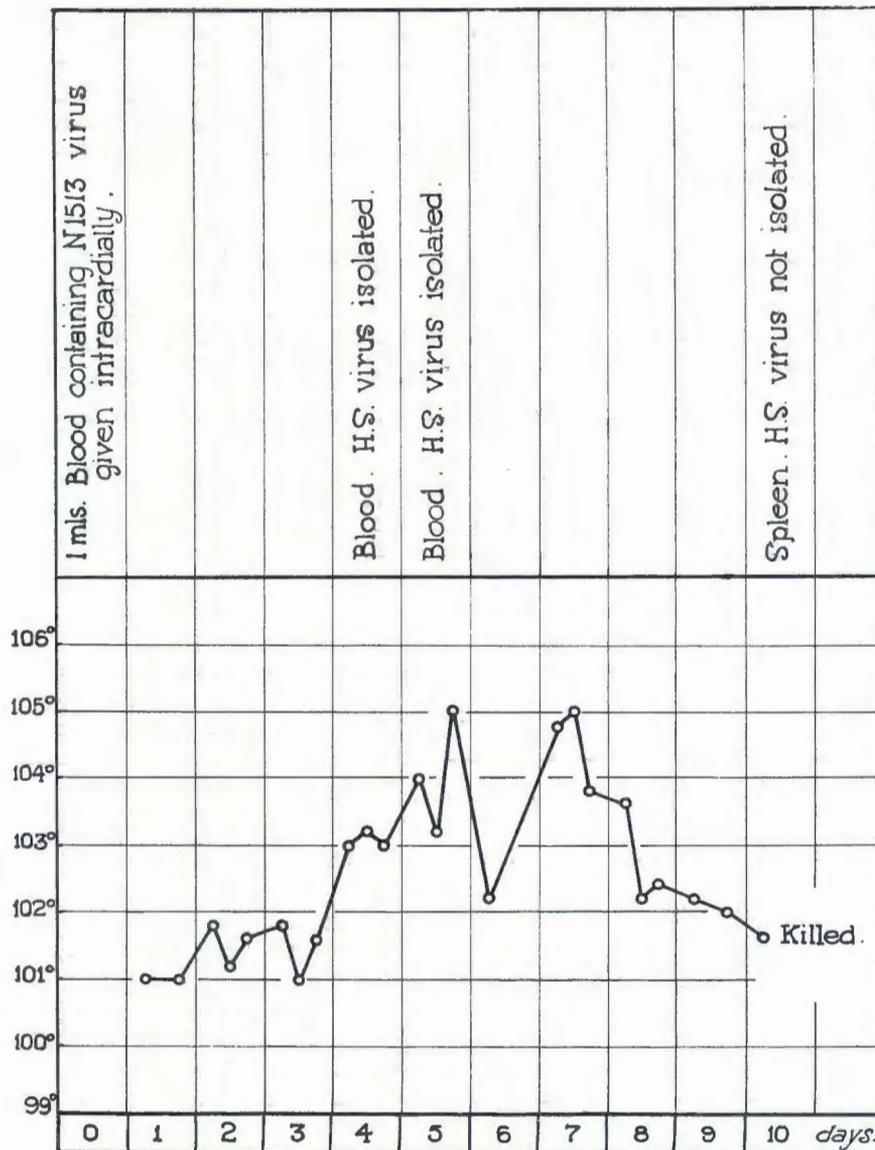
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*Result.*—While neither ferret injected intraperitoneally showed any apparent reaction, the other six injected intracardially all showed a definite temperature reaction starting on the fourth or fifth day after injection.

The duration of the febrile reaction varied from two to four days. The temperature chart of ferret No. 3 is shown in figure 1. One ferret (No. 4) was off its feed for one day. This was the only clinical symptom other than the temperature reactions.

Chart No. 1

Isolation of "neutral" virus from blood of ferret



From Table 2 it will be seen that virus was isolated from each of the six ferrets injected intracardially. Blood collected during the temperature reaction or spleen material collected either during, or the day after the reaction, was able to infect suckling mice. From one ferret (No. 6) virus was isolated on three consecutive days. From neither ferret injected intraperitoneally was virus isolation in mice successful.

The incubation period in the suckling mice injected with ferret blood or spleen varied from four to eleven days. Blood and spleen appear to be equally effective as a source of virus.

### 3. *The Effect of Vaccine Antibodies on the Adaptation of "Active" Viscerotropic virus to the Mouse.*

As it appeared probable that the serum antibodies in the immunized horses prevented the adaptation to mice of the virus in the blood of these horses, an attempt was made to demonstrate such interference experimentally.

As it was known that serum 1513, obtained from horse 1513 at the time of its breakdown reaction contained antibodies against all the eight attenuated vaccine virus strains to a high titre (*vide infra*) and also as this serum was indicative of the state of this horse's humoral immunity at the time virus N1513 was circulating in the blood, it was thought that a mixture of this serum and virus A1513 would reproduce the conditions present in the O.C.G.-blood mixture containing N1513.

To 1 ml. of O.C.G.-blood containing A1513 1 ml. of the undiluted serum from horse 1513 was added. To another 1 ml. amount of A1513 an equal amount of normal heat inactivated horse serum was added. These two serum-virus mixtures were allowed to stand for 48 hours at 4° C. when each was diluted 1/5 in serum-saline and injected into families of suckling mice. To exclude the possibility of active virus being present in serum 1513 this serum was injected undiluted into a third family of suckling mice. The results are shown in Table 3.

TABLE 3.

#### *The Effect of Horse Serum 1513 on the Adaptation of A1513 Virus to Mice.*

Mixture Injected.	Days after Injection.													
	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.*
A1513 + serum 1513.....	0/6†	0/6	1/5‡	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
A1513 + normal serum...	0/7	0/7	3/4	1/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Serum 1513.....	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7

\* Mice observed for three weeks or longer.

† Mice dead/mice surviving.

‡ Death of this mouse was confirmed as horsesickness.

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Although these results indicated that serum 1513 had reduced the mortality they were inconclusive and it was decided to repeat this experiment using, as a source of virus, spleen emulsion obtained at post mortem from a susceptible horse which had succumbed to infection with A1513 virus. This spleen emulsion was first titrated in suckling mice and gave an infective titre of  $10^{-1.5}$

To each of four test-tubes .3 ml. of this spleen emulsion was added and then to each tube one of the following sera was added in .3 ml. amounts of the undiluted serum:—

- (1) Horse serum 1513.
- (2) Horse serum 1802. This was a serum obtained from a susceptible horse 3 months after a single dose of horsesickness vaccine. By means of neutralization tests against some of the vaccine virus strains this serum was shown not to contain anti-body to such high titre as horse serum 1513 which horse, as mentioned above, had been immunized three times.
- (3) Rabbit Antiserum to strain 1513.
- (4) Normal horse serum.

These four mixtures were allowed to stand for 48 hours at 4° C. and then each was injected into a family of suckling mice. The results are given in Table 4.

TABLE 4.

*The Effect of Various Sera on the Adaptation of Virus A1513 to mice.*

Mixture Injected.	Days after Injection.													
	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.
Virus 1513 + horse serum 1513.....	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
Virus 1513 + horse serum 1802.....	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	2/4	0/4	1/3	0/3	0/3	0/3
Virus 1513 + rabbit serum 1513.....	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
Virus 1513 + normal horse serum.....	0/6	0/6	0/6	2/4	0/4	0/4	0/4	0/4	1/3	0/3	0/3	0/3	0/3	0/3

It will be noted that while no mortality occurred in the mice injected with the mixtures containing horse serum 1513 and rabbit serum 1513 there were some deaths amongst the group of mice injected with the mixture containing serum 1802. Despite the fact that only half the mice injected with the control mixture died it is felt that these experiments indicate that horse serum 1513 did neutralize viscerotropic virus 1513.

A similar experiment was conducted using A Fourie virus present in the O.C.G.-blood mixture. In place of horse serum 1513 the serum obtained from horse 1408 at the time to breakdown reaction was used. As indicated later this serum was known to contain high titre antibodies against the vaccine virus strains.

As an example of a serum from a horse immunized only once serum from horse 1582 was included in the test. The mixtures were allowed to stand for 48 hours at 4° C. and each mixture was then injected into two families of suckling mice. The results are shown in Table 5.

TABLE 5.

*The Effect of various Sera on the Adaptation of Virus A Fourie to Mice.*

Mixture Injected.	Days after Injection.														
	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.
A Fourie + serum 1408.....	0/13	0/13	0/13	3/10	0/10	2/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
A Fourie + serum 1582.....	0/13	2/11	6/5	2/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
A Fourie + normal horse serum.....	0/13	3/10	2/8	5/3	0/3	1/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2

Here again the serum from the once immunized horse (1582) gave less protection (if any) than the serum from the thrice immunized horse (1408). Although no serum was available from the horse in the Vryheid district which succumbed to infection with Fourie virus and hence could not be included in the test, due to the policy of annual immunization it is very likely that serum 1408 represented a close approximation to the humoral immunity possessed by that horse.

Although in these series of experiments only small numbers of mice have been used it may be concluded that sera from repeatedly immunized horses will give some protection to suckling mice injected with the virus strains A1513 and A Fourie. It is quite possible that the union between these viruses and anti-body *in vitro* under the conditions of the experiments was not as complete as that which occurred *in vivo* in the reacting horses. This may explain the failure to demonstrate absolute protection by horse sera 1513 and 1408.

As it did not appear possible to neutralize completely viscerotropic virus for the mouse there appeared to be no point in determining whether these mixtures were still active for the horse, ferret, or dog.

An interesting observation is the greater protection obtained from sera from repeatedly immunized horses as against sera from horses which had been immunized only once. This would support the present policy of annual immunization even though it is known that a single dose of vaccine confers an immunity which is probably life-long.

#### 4. Neutralization of the Attenuated Vaccine Virus Strains by Sera from "Breakdown" Cases.

To support the contention that it was the antibodies present in the O.C.G.-blood mixtures from horses 1513 and 1408 which prevented isolation of virus in mice directly from these horses, it was necessary to show that at the time of the "breakdown" antibodies were present in the serum. Neutralization tests were carried out with sera 1513 and 1408 against each of the eight neurotropic vaccine strains. The results are recorded in Table 6.

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TABLE 6.

*Titre of Sera from Horses 1513 and 1408 against the Vaccine Virus Strains.*

Virus Strain.	Serum 1513.	Serum 1408.
VH.....	32	24
L.....	112	576
KA.....	256	256
Vryheid.....	36	178
OD.....	74	1024
114.....	1024	1024
1180.....	200*	NT
A501.....	294	708

\* End point doubtful.

NT = Not tested.

From these results it is apparent that the serum of both horses 1513 and 1408 contained a considerable amount of antibody against the vaccine strains at the time of the reaction. The titres are somewhat higher than those usually found in horses which have been immunized only once but due to the practice of annual immunization in South Africa they are probably representative of a majority of horses in enzootic areas.

5. *The Neutralization of the Mouse-adapted Strain 1513 by the Vaccine Antisera.*

As a result of the somewhat paradoxical finding that a strain of virus is unable to multiply in mice presumably owing to the presence of heterologous antibodies, it appeared worth while to investigate the effect of these antibodies on a breakdown strain after it had become fully adapted to mice. Neutralization tests with mouse-adapted strain 1513 in its 37th intracerebral passage and the antisera against individual vaccine strains as well as serum from horse 1513 and the rabbit homologous serum to strain 1513 were carried out. The 50 per cent end points obtained are shown in Table 7.

TABLE 7.

*Titre of Various Sera tested against the Mouse-adapted Strain 1513.*

Serum.	Titre.
Horse serum 1513.....	408
Rabbit serum 1513.....	24
VH.....	0
L.....	0
KA.....	0
Vryheid.....	0
OD.....	128
114.....	0
1180.....	10
A501.....	0

From these results it can be seen that there is a strong neutralization by the polyvalent serum obtained from horse 1513 and also by serum OD and slight neutralization by serum 1180.

## DISCUSSION.

From the results of this work it is evident that, whereas a mixture of viscerotropic horsesickness virus and antibody present in blood from horses, which have been immunized but nevertheless developed horsesickness, may be non-pathogenic for mice, that mixture is still capable of infecting horses, dogs and ferrets.

Although attempts to demonstrate that the antibody prevented adaptation of virus to the mouse were inconclusive it is still felt that this is the correct explanation. Why the virus is neutral for the mouse and not for the horse, dog and ferret is not clear. When it is realized that the infectivity for the suckling mouse of virus in blood from susceptible horses is barely 100 LD<sub>50</sub> and that it has been shown in the case of one of the strains (1513) there was a strong neutralization of this strain after it had become mouse adapted by serum obtained from the horse during the breakdown reaction it is possible that there occurs just sufficient neutralization to prevent multiplication of virus in the brain tissue of the mouse. Isaacs (1948) has reported that an influenza virus-serum mixture can be prepared which will not show multiplication of virus when injected intranasally into mice, but will produce infection in the more sensitive chick embryo.

Several examples can be quoted of homologous antibody interfering with the isolation of a virus. McKee and Hale (1946) report that the presence of antibody in the mucus from the throat of carriers of influenza virus will interfere with the isolation of virus. Melnick (1951) suggests the possibility that the many negative results, obtained in attempts to isolate virus from the blood of patients at the time of onset of paralysis in poliomyelitis, may have been due to an *in vivo* neutralization of the virus, as antibodies at this stage of the disease are known to be present in the blood. In bluetongue of sheep it has been noticed that a mild reaction often results in a fully susceptible sheep receiving infective blood taken from a natural case. In this disease antibodies appear early in the course of the disease (Alexander *et al.*, 1947) and blood collected after the appearance of clinical lesions will contain antibody as well as virus.

In two of the immunized horses (1513, 1408) which developed horsesickness and from which virus was eventually isolated it has been shown that these animals had a high level of serum antibody at the time of the breakdown to natural infection. In the case of horse 1408 this immunity was apparently sufficient to prevent a fatal outcome although the illness was acute. Horse 1513 died of acute horsesickness despite the presence of this circulating antibody. Neutralization tests on virus 1513 after it had become mouse adapted showed that besides an antigenic relationship to two of the virus strains used in the vaccine it was neutralized to a high titre by serum obtained from horse 1513 at the time of its illness.

It appears that individual immunized horses may react severely to infection with virus strains which are nevertheless closely related antigenically to strains used in the vaccine. The invading virus appears to be in a phase of "non-neutralizability". A somewhat similar observation has been noted in neutralization tests in mice where poorly neutralized virus is indicated by occasional deaths in mice receiving virus plus high concentrations of serum. From such mice virus can be recovered on passage but the recovered virus has regained its original "neutralizability" (Polson 1951).

Although it was not possible to isolate virus from the two dogs injected with "neutral" virus it is believed that this is a practical alternative method when ferrets are not available. Due to the necessity to dilute the O.C.G.-blood mixtures

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for injection into mice spleen suspensions would appear to be the material of choice for infecting mice. Failure to isolate virus from dog No. 1 may have been due to the low infectivity of dog blood.

It should be emphasized that virus was isolated from the blood of ferrets only during the febrile reaction although from the spleen it may be isolated a day after the temperature returns to normal. Insufficient ferrets were used to determine whether the route of infection of the ferret is important but it appeared that the intracardial route was more reliable than the intraperitoneal.

### SUMMARY.

It has been shown that whereas blood obtained during the breakdown reaction in immunized horses may be non-pathogenic for mice it is still capable of infecting horses, dogs and ferrets. Due to this fact it is often impossible to isolate virus directly in mice from cases of horsesickness in immunized horses.

This difficulty may be overcome by first isolating the virus in the horse or ferret from which animal mice may readily be infected. It is probable that the dog may be used in a similar manner.

Attempts to reactivate for the mouse virus present in the blood from immunized horses by various methods were unsuccessful.

Just how this virus is inactive for the mouse while active for the horse, dog and ferret is not understood, although the serum antibody produced as a result of immunization is believed to be involved.

By means of neutralization tests in mice with the neurotropic form of a virus which produced a fatal breakdown in an immunized horse it was shown that such immune horses may react severely to infection with virus which is related antigenically to strains of virus incorporated in the vaccine.

Ferrets have been shown to be susceptible to viscerotropic horsesickness virus. The disease in ferrets is mild and results in a febrile reaction with viraemia.

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