MEG.

STUDIES ON THE NEUROTROPIC VIRUS OF HORSESICKNESS III. THE INTRACEREBRAL PROTECTION TEST AND ITS APPLICATION TO THE STUDY OF IMMUNITY.

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In 1931 Sawyer and Lloyd[1] reported an intraperitoneal protection test in mice in connection with their work on the virus of Yellow Fever. This test has for its basis the finding that if mice are given an intraperitoneal injection of a massive dose of Yellow Fever neurotropic virus and at the same time, an inert substance like starch in 2% solution is injected intracerebrally to cause a traumatic injury of the brain, a fatal specific encephalitis will ensue. If however, the virus is mixed in vitro with mouse serum prior to injection, the neutralized mixture is incapable of setting up the disease in the majority of instances. This test has been applied extensively to the study of immunity[2,3,4] with considerable success, but in reading the reports of workers who have used it one cannot be other than impressed by certain limitations. Every mouse which receives the same infecting dose of virus intraperitoneally does not die and when the mortality is 100% the deaths are spread over a number of days (5 - 10). The varying susceptibility of individual mice to infection by the intraperitoneal route necessitates the use of comparatively large numbers for each serum or virus dilution in titration experiments, and it appears to be difficult to compare quantitatively the potency of different samples of anti-sera with any degree of accuracy. Furthermore, it appears that the interpretation of results, depending as it does upon the statistical survey of the percentage of mice which survive injection, and the percentage which die within a period of from 4 to 10 days, is sometimes open to question.

In the case of Horsesickness the adaptation of the

method is quite unsuitable because the injection of even thousands of infective doses of virus intraperitoneally with or without simultaneous injury to the brain cannot be relied upon to set up infection in anything approaching a constant percentage in mice. Consequently it became essential to develop a technique based upon intracerebral injection. This has been done and the test has proved invaluable in the study of many problems associated with immunity.

While the work was in progress Max Theiler[5] reported his "Yellow Fever protection test by intracerebral injection". It was immediately apparant that the nature of the neurotropic virus of horsesickness permitted modifications capable of rendering the test more accurate and delicate.

It has been shown previously[6] that the susceptibility of different mice of the strain in use at Onderstepoort is remarkably constant, that 100 % of mice which receive a certain infective dose of virus intracerebrally will die, and that with ease the titre of a stock virus suspension may be determined to two fold limits. In addition the titre of a suspension of infective brain made up in 10% saline remains constant for a period of almost six months when stored in the refrigerator at a temperature just above freezing. Therefore the first essentials of an accurate test are fulfilled, namely :-

1. A test animal of constant susceptibility which is easily infected, and in which a clear cut reaction ensues, i.e. survival or death.

2. A stable antigen permitting comparison between results obtained in different experiments conducted at different times.

3. An antigen permitting titration to fairly narrow limits with constant accuracy.

For completion of the test it only remained to be shown that the sera of susceptible horses do not contain virus neutralising substances, that the development of immunity is followed by the appearance of detectable virucidal anti-bodies in the serum, and that the action of these anti-bodies is specific. From the data given below it will be seen that these criteria have been fulfilled and that it was possible to elaborate a suitable technique for quantitative in vitro neutralization.

I. The Intracerebral Neutralization Test.

Technique.

A. Preparation of Antigen. Mice are sacrificed in extremis after intracranial injection of neurotropic fixed virus, the brains carefully removed with aseptic precautions, and placed in sterile 50 c.c. centrifuge tubes fitted with corks. After the brain substance has been macerated with a glass rod or scalpel the tubes are placed in the freezing chamber of a refrigerator overnight. Next morning an emulsion in 10% serum saline is prepared in the usual manner. The process of preliminary freezing and sudden thawing has been adopted because undoubtedly it aids in cellular fragmentation and facilitates thorough emulsification of the brains. The virus suspension is then centrifuged at 2,500 revolutions per minute for 15 minutes, the supernatant liquid carefully decanted into a second tube and again spun before being decanted into a sterile bottle fitted with a rubber cork and stored at 4°C. With the passage of time a fine precipitate settles out to the bottom of the bottle but this may be left quite undisturbed when removing samples as required and causes no detectable decrease in the virus titre.

It is realised that it would be preferable to remove

all tissue, debris and possible aggregations of virus particles by filtration but at the time the value of the Seitz filter was not appreciated and Gradocol membranes were not available so very thorough emulsification and centrifugation was relied upon for the production of a homogeneous suspension. In passing it may also be mentioned that very thorough trituration of the brain material with sand and glass particles and prolonged shaking in a mechanical shaker with beads appears to have no beneficial effect upon the ultimate product.

All manipulations are carried out with strict aseptic precautions. Antigens have been stored for six months at 4° without the addition of any preservative and at no time has any difficulty been experienced with bacterial contamination. The stock antigen has been prepared usually to comprise an approximate 2% suspension of brain substance, but the actual concentration appears to be of no importance provided it is not so high that an extensive deposit subsequently forms. The virus titre is then determined in two stages :-

1. A preliminary rough titration by serial ten fold dilution.

2. A final accurate titration by two fold dilution under the conditions used for in vitro neutralization.

This may be illustrated best by reference to the results obtained in an actual experiment. Four mouse brains representing passage generation 50 of one strain of virus were emulsified by the technique detailed above. A rough titration of the virus content of the antigen was run in mice by the ten fold dilution with the following result :-

Table I : Fate of Mice injected with the ten fold dilutions of virus.

	Virus dilutions.					
	1:10	$1:10^2$	$1:10^3$	$1:10^4$	$1:10^5$	$1:10^6$
Death of mice	4.5	4.5	4.5	5.6	0.0	0.0

Note: In this and all future tables the numeral indicates the day after injection on which the mouse died, thus 4.5 - two mice injected of which one died on the 4th and one on the 5th day after injection.

0 means survival.

X means death due to some cause other than horsesickness, usually injury to brain at time of injection.

Dose 0.05 c.c. intracerebrally.

From the figures it is seen that the infective titre of the stock emulsion is possibly above a dilution of $1:10^4$ but below $1:10^5$. Therefore a suspension consisting of 1 part of the stock antigen to 100 parts of diluent could be expected to contain in 0.05 c.c. at least 100 minimal infective doses of virus. For a neutralization test antigen and anti serum would be mixed in equal proportions so that for the fine titration, the dilutions indicated below were mixed in equal quantities with saline before injection.

Table II.

Fate of mice injected with dilutions of antigen consisting of one part of stock suspension to 100 parts of diluent then mixed as with saline.

	Dilutions.						
	Antigen	1/10	1/20	1/40	1/80	1/160	1/320
Death of Mice	4,5,5	5,5,6	4,5,6	5,6,0	0,0,0	13,0,0	0,0,0

It is seen therefore that the stock emulsion 1 to 100 produces an antigen which after mixture with an equal quantity of anti serum would contain in 0.05 c.c. approximately 40 minimal infective doses since two out of three mice injected with the 1:40 dilution died, and injection of the 1: 80 dilution resulted in no mortality.

The death on the 13th day of one mouse which received the 1: 160 dilution was due to horsesickness as shown by subinoculation. Such deaths which appear to be quite out of

place do occur when material just above or just below the limit of infectivity is injected. Inaccuracies in technique may be responsible for the apparent errors but a considerable experience has shown that when consideration is paid only to those mice which die within a period of three days of the average resulting from the injection of 100 M.I.D. of virus a clear cut picture is obtained. It is submitted that the inclusion of this time factor in the interpretation of results has a definite precedent in other neutralization work and is permissible.

In recording deaths every mouse which succumbs during the critical period is assumed to have died of horse-sickness. This may be a source of some slight error but it is reduced to a minimum by using healthy mice which have been accustomed to being housed under the conditions of the experiment.

B. Collection of Anti-sera. Blood is drawn either into large test tubes or Erlenmeyer flasks fitted with a side arm, and the serum is decanted the following day after contraction of the clot. Sera have been stored in the refrigerator usually without preservative, but 0.7% tricresol-ether has been used without having any detrimental effect upon its viricidal property. Sera were not filtered but were thoroughly centrifuged before use.

C. The neutralization test proper. After a considerable amount of preliminary work on the most suitable method of carrying out the test it was decided to use falling dilutions of the anti serum under investigation against a constant antigen. Quite arbitrarily a dilution of antigen was selected to contain in 0.05 c.c. after mixture with an equal volume of normal serum or saline between 40 and 100 M.I.D.

the exact titre being determined at the time of the test.

Monax agglutination tubes (length 6.5 cms, bore 0.75 cm) have been used, the tubes being selected to have a uniform bore. Aseptic precautions have been adopted throughout i.e. all tubes are sterilized and are fitted with cotton wool plugs and freshly boiled and cooled syringes are used for all measurements and dilutions.

To each of a series of tubes placed in a row in a rack 1 c.c. of saline is added. Two fold dilutions of the serum are then prepared by serial dilution in the usual manner. For this it has been found most convenient to use syringes to each of which a long fine needle (B.W. & CO. No. 205) is attached. The 1 c.c. of fluid then may be forcibly squirted into the diluent without producing any foam since the point of the needle may be placed just below the surface. Only the point of the needle is allowed to touch the fluid before a fresh syringe is used for thorough mixing and transfer.

The 1 c.c. of antigen is then forcibly squirted into each tube containing serum dilution to ensure rapid and thorough mixing. The mixtures are ~~then~~ incubated for two hours at 37°C and stored overnight in the refrigerator. There is some evidence to show that the union between virus and antibody is somewhat loose at low temperatures, so, before injection next morning, all tubes are incubated at 37°C for a further period of one hour.

It is preferable to use at least three mice for the injection of each dilution. Unfortunately a serious shortage of mice has made it necessary to use only two mice in a number of experiments.

It will be noticed that the serial dilutions of the anti sera are made in saline and not in serum-saline. This procedure was adopted for the sake of simplicity after it has

been ascertained that 5% serum saline had no detrimental effect upon the virus - 5% being the minimum concentration possible in a mixture of equal parts of saline and antigen.

With each set of neutralization tests a control of the infective titre of the antigen was run by making serial dilutions in 1 c.c. amounts and adding to each 1 c.c. of saline to take the place of the anti serum dilution.

Experimental.

Since the antigen is the most important factor in the neutralization tests about to be described the following results from a series of different experiments have been collected together to show that by the requisite dilution of a stock suspension of virus a constant antigen can be prepared as required. Further it was of importance to show that incubation for two hours at 37°C and storage overnight in the refrigerator followed by a further one hour at 37° had no detrimental effect upon the virus.

An approximately 4% emulsion of four brains representing mouse passage generation 62 of strain 20449 was roughly titrated in mice as follows :-

Table III.

Rough Titration of Stock Antigen in Mice.

	Virus Dilutions.						
	1:4	1:4 x10	$1:4x10^2$	$1:4x10^3$	$1:4x10^4$	$1:4x10^5$	$1:4x10^6$
Death of Mice.	4,4	4,5	5,5	6,0	0,0	0,0	0,0

From this titration it would be expected that a 1 : 40 dilution of the stock antigen would contain in 0.05 c.c. approximately 100 M.I.D. of virus. This dilution was made up from time to time and tested with the results shown in Table IV. Prior to injection all dilutions were incubated under the exact conditions of an in vitro neutralization test.

TABLE IV.

Accurate Titration of Antigen.

Date	Interim days	Virus Dilutions. A	1:10	1:20	1:40	1:80	1:160	1:320
15.9.33	8	4,4	5,5	5,5	5,5	4,5	5,0	0,0
26.9.33	19	-	4,5	5,6	5,6	5,0	6,0	0,0
13.10.33	36	4,4	4,5	6,6	5,5	5,6	6,0	0,0
27.10.33	50	-	5,0	5,0	6,8	0,0	0,0	0,0
4.11.33	58	-	4,5	4,6	5,5	5,0	7,0	0,0
30.11.33	84	4,4	5,5	5,5	4,4	6,0	0,0	0,0
14.12.33	98	4,5	5,5	5,5	6,0	5,0	0,0	0,0
20.12.33	104	5,5	5,6	5,6	5,6	5,0	0,0	0,0
29.12.33	113	4,4	4,4	4,4	5,0	4,4	5,0	0,0
17.1.34	132	4,5	4,5	5,x	5,x	5,6	6,0	6,0
25.1.34	140	3,4	4,5	4,5	6,x	5,6	0,0	0,0
2.2.34	148	4,4	4,5	4,x	4,5	4,5	6,0	0,0
9.2.34	155	4,4	5,6	4,4	5,5	5,6	0,0	0,0
27.2.34	173	4,4	5,6	5,6	5,6	6,0	0,0	0,0
14.3.34	188	4,5	5,5	6,6	5,0	0,0	0,0	0,0

Note : A = Antigen, i.e. 1:40 dilution of stock suspension.

A consideration of the figures shows that from a stock suspension stored without preservative at ± 4°C an antigen of remarkably constant infectivity may be made up. It is true that some slight variation occurs up to the 5th month but this variation is well within the limits of experimental error. One particular titration, that carried out on the 50th day, showed a marked deviation but obviously this must have been due to some gross technical error. After the 5th month there is a definite and decided tendency for the titre to decrease. If incubation and overnight storage as indicated has any detrimental effect it is sufficiently constant to make it negligible for comparative purposes.

Concurrently with the experiment detailed an attempt was made to use desiccated powdered brain as the source of virus for the preparation of antigen. It would be superfluous to give the figures but it may be stated that the variations in titre were so great, probably due to inaccuracies in weighing, and imperfect mixing, powdering and sampling the dried brains that its use in preference to virus stored in fluid form could not be contemplated.

From this experiment which has been confirmed repeatedly it is concluded that by the method described an antigen of constant titre may be made up as required. This antigen will keep unaltered for a period of approximately five months after which there is a decided tendency to deteriorate.

Having demonstrated the possibility of preparing a suitable antigen it only remained to be shown that immune serum is capable of neutralizing the virus and that any neutralization in vitro is specific. That this is the case is clearly shown by reference to the figures given in Table V.

Table V.

In vitro neutralization of virus.

Serum	Serum Dilutions.							
	½	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Immune Horse	0,0	0,0	4,0	6,0	5,5	4,5	-	-
Immune Horse	0,0	0,0	0,0	6,0	0,0	5,5	5,6	4,5
Sheep	4,5	5,5	4,5	4,4	-	-	-	-
Susceptible fowl	4,4	4,5	5,5	4,5	-	-	-	-
Susceptible Horse (Aged)	5,5	4,5	4,5	4,6	-	-	-	-

Note: The antigen used was that which was titrated for infectivity on 4.11.33 in Table IV above.

1:40 = 10 M.L.D.

It is seen that the serum of the immune horse possesses the property of neutralizing neurotropic horsesickness virus in vitro, the intracerebral injection of mice being used as the index of loss of infectivity. Serum of a sheep, a susceptible foal and an aged susceptible horse did not possess this property. It may be mentioned that after a considerable experience there is no reason to believe that the neutralizing action is not entirely specific.

A consideration of the figures in Table V indicates further that a sharply defined end point exactly indicating the neutralizing titre of a particular serum cannot be anticipated. More accurate results are obtained from the use of larger numbers of mice but unfortunately the available supply was strictly limited.

At one time it was thought that better results would be obtained from the addition of complement in the form of fresh guinea pig serum to each dilution. This has been tried on several occasions but it was apparent that the results did not justify the additional complication as a routine measure. At all events the results reported below will indicate that the method described clearly demonstrates differences in neutralyzing power between samples pf sera, which enables a quantitative comparison to be drawn.

Keeping Qualities of Anti-sera.

Frequently it is neither convenient nor possible to carry out neutralization tests with immune sera immediately after collection. Consequently it became necessary to ascertain whether storage in the refrigerator at 4°C with or without the addition of 0.7% phenol ether as preservative has any detrimental effect upon the viricidal antibodies. A sample of serum obtained on 14.12.33 from a horse(20545) that had been immunized by the injection of neurotropic virus 177 days previously was divided into two portions, to one of

which preservative (0.7% phenol ether) was added. Neutralization tests were carried out with both these samples on 20.12.33 and 22.8.34, i.e. six days and 251 days after bleeding respectively. The results are shown in tabular form below.

Table VI.

Keeping Qualities of Anti-sera.

Date	Interval	Preservative	Serum Dilutions 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
1933	days											
20/12	6	—	-	-	0,0	6,0	0,0	0,0	6,6	6,6	5,6	5,6
20/12	6	+	-	-	0,0	0,0	0,0	6,0	6,0	5,6	5,5	4,6
1934												
22/8	251	—	-	-	3,0,0	6,0,0	0,0,0x	0,0,0x	4,5,0	3,4,5,0	3,4,5,0	4,4,4,5
22/8	251	+	-	-	0,0	0,0	0,0	0,0	4,6	3,5	4,0	4,5

Virus Titration of Antigen.

Antigen	Date	Virus Dilutions. A	1:10	1:20	1:40	1:80	1:160	1:320	1:640
Generation 82, Strain 20449	20.12.343	5,5	5,6	5,6	5,6	5,0	0,0	0,0	0,0
Generation 120, Strain 20449	22.8.34	4,5	4,5	4,5	6,6	6,0	0,0	6,0	0.0

It is seen that Horsesickness anti sera possesses remarkable keeping qualities since no apparent deterioration was detectable after 251 days storage at a temperature just above freezing. This is indeed fortunate because in the ensuing work it was necessary frequently to store samples of anti sera for more than 200 days to permit of reliable quantitative experiments by neutralization tests against a single antigen preparation. In addition 0.7 % phenol ether as a preservative has no detrimental effect upon the viricidal antibodies.

II. The Application of the test to the study of immunity: The elaboration of a suitable technique for the quantitative determination of the antibody content of serum paved the way for an intensive study of the development of immunity in equines. When the work was commenced the interesting nature of the results to be obtained was not anticipated so that samples of sera were not collected as frequently or as regularly as could have been wished. Further, the slow production of antibodies and the enormous accumulation of samples of sera has effectively prevented corroboration of every result by duplicate series of experiments, but confirmatory tests have been included at sufficiently frequent intervals to indicate that the results detailed are not open to criticism on the grounds of accuracy.

A. The antibody content of the serum following immunization. On 23.6.33 two horses (20545 and 20570) were given a subcutaneous injection of 10 c.c. of a 0.1% suspension of infective mouse brains representing passage generation 51 of strain 20449. The first horse 20545 developed a mild febrile reaction from the 19th to the 23rd day after injection; the second horse (20570) showed no febrile disturbance. In neither animal did any clinical symptoms of Horsesickness develop. Prior to injection a sample of serum was collected from both horses but unfortunately at the time the value of the neutralization test was not appreciated so that bleedings at regular intervals were not commenced until about two and a half months later. This was an unfortunate omission but it will be seen that the defect has been remedied in subsequent work. With the sera neutralization tests were carried out the results being given in tabular form in Tables VII and VIII. The titrations

of infectivity of each antigen employed are given in Table IX, from which it will be seen that unit volume of anti serum was required to neutralize approximately 100 M.I.D. of virus (between 80 and 160).

Table VII.

Table IX.

Titration of virus content of Antigen used in neutralization experiments Tables VI and VII.

No.	Date	Virus Dilutions.							
		A	1:10	1:20	1:40	1:80	1:160	1:320	1:640
1	15/9/33	4,4	5,5	5,5	5,5	4,5	5,0	0,0	0,0
2	26/9/33	-	4,5	5,6	5,6	5,0	6,0	0,0	0,0
3	30/11/33	4,4	5,5	5,5	4,4	6,0	0,0	0,0	0,0
4	20/12/33	5,5	5,6	5,6	5,6	5,0	0,0	0,0	0,0
5	25/1/34	3,4	4,5	4,5	6,x	5,6	0,0	0,0	0,0
6	2/2/34	4,4	4,5	4,x	4,5	4,5	6,0	0,0	0,0
7	9/2/34	4,4	5,6	4,4	5,5	5,6	0,0	0,0	0,0
8	27/2/34	4,4	5,6	5,6	5,6	6,0	0,0	0,0	0,0
9	20/4/34	4,4	4,5	4,5	4,5	5,0	0,0	0,0	0,0
10	27/4/34	5,5	5,5	4,4	4,4,5	4,5,0	0,0	0,0	0,0
11	8/5/34	-	5,6	6,x	5,0	5,6	0,0	0,0	0,0
12	8/6/34	-	4,4	4,5	4,4	6,0	0,0	0,0	0,0

Note: Titration Nos 1 - 8 were carried out with mouse passage 62 strain 20449
Nos 9 - 12 with mouse passage 120 of the same strain.

Table VII.

Antibody content of serum Horse 20645.

Date of Test	Date Serum taken	Interval after immunization	Serum Dilutions. 1 : 2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
15. 9.33	23. 6.33	0 days	4,5	4,4	4,6	5,5	-	-	-	-	-	-
15. 9.33	1. 9.33	70 "	0,0	0,0	0,0	5,5	5,5	-	-	-	-	-
26. 9.33	22. 9.33	92 "	0,0	0,0	0,0,0,0	5,000	0,0,0,0	5,5,5,0	5,5,5,0	4,5	4,5	4,4
30.11.33	15.11.33	148 "	-	0,0	0,0	0,0	0,0	5,0	4,5	4,5	-	-
20.12.33	29.11.33	162 "	-	-	0,0	6,0	0,0	5,6	6,6	5,6	5,6	5,5
20.12.33	7.12.33	170 "	-	-	0,0	0,0	6,0	6,0	5,6	4,5	6,0	6,6
20.12.33	14.12.33	177 "	-	-	0,0	0,0	0,0	6,6	5,5	5,6	6,6	5,5
25. 1.34	21.12.33	184 "	-	-	0,0	0,0	0,0	0,0	0,0	4,4	4,4	4,5
25. 1.34	12. 1.34	206 "	-	-	0,0	0,0	0,0	4,0	0,0	5,6	5,0	4,5
9. 2.34	1. 2.34	225 "	-	-	0,0	0,0	0,0	0,0	7,0	4,6	5,5	4,5
27. 2.34	15. 2.34	239 "	-	0,0	0,0	0,0	0,0,0	0,0,0	6,6,0	4,5,0	4,5,6	-
14. 3.34	28. 2.34	252 "	-	0,0	4,000	4,000	5,000	4,5,5	4,5,5	4,5	4,4	-
27. 4.34	22, 3,34	274 "	0,0	0,0	0,0	5,0	5,5	5,5	6,5	4,5	-	-
8. 5.34	17. 4.34	300 "	0,0	0,0	5,0	0,0	4,0	4,6	4,4	4,5	-	-
8. 6.34	8. 5.34	321 "	0,0	0,0	4,0	0,0	4,0	4,4	3,4	3,4	-	-

Table VIII.

Antibody content of serum Horse 20670.

Date of Test	Date Serum Taken	Interval after immunization	Serum Dilutions.									
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
26. 9.33	23. 6.33	9 days	5,5	3,5	4,4	4,5	-	-	-	-	-	-
26.9.33	22. 9.33	92 "	0,0	0,0	0,0	0,0	0,0	0,7	-	-	-	-
30.11.33	16.11.32	148 "	-	0,0	0,0	0,0	0,0	5,0	5,0	5,5	-	-
20.12.33	29.11.33	162 "	-	-	0,0	0,0	0,0	0,0	5,6	5,6	5,5	5,5
20.12.33	14.12.33	177 "	-	-	0,0	0,0	0,0	0,0	5,6	5,6	5,6	5,6
2. 2.34	21.12.33	184 "	-	-	0,0	0,0	0,0	0,0	5,6	5,5	4,5	4,4
2. 2.34	12. 1.34	206 "	-	-	x,0	x,0	0,0	5,6	5,5	5,5	4,5	4,4
9. 2.34	1. 2.34	225 "	-	-	0,0	0,0	0,0	5,0	4,5	4,5	4,5	5,5
27. 2.34	15. 2.34	239 "	-	0,0	0,0	0,0	0,0,0	0,0,0	4,6,6	4,6	4,5	-
20. 4.34	28. 2.34	252 "	-	0,0	0,0	5,0,0,0	6,0,0,0	5,5,6,0	4,5,6,0	4,5,5,5	4,4,5,5	-
27. 4.34	22. 3.34	274 "	0,0	0,0	0,0	5,0	0,0	4,4	5,5	4,5	-	-
8. 5.34	17. 4.34	300 "	0,0	0,0	0,0	0,0	0,0	5,6	5,5	5,6	-	-
8. 5.34	8. 5.34	321 "	0,x	0,0	0,0	5,6	5,0	4,5	4,5	4,4	-	-

A critical survey of the results indicates ~~that a sharply~~ that a sharply defined end point was seldom or never obtained in the titrations but the figures clearly indicate the rise in antibody content and its tendency to fall subsequently. An insufficient number of mice were used for the injection of each dilution to permit of a ~~sterility~~ strictly accurate conception being obtained of the viricidal potency of each sample of anti serum but it is believed that the figures underlined represent the highest concentration of serum just incapable of inactivating the test dose of antigen. It is seen that particularly with horse 20545 the rise in antibody content was rather slow since by the 70th day a concentration of 1 :8 was required completely to neutralize the antigen used. At no time did the antibody rise to a particularly high level but the peak was reached approximately six months after immunization. After the eighth month there was a decided tendency for the antibody content to fall.

As soon as the somewhat slow development of immunity was appreciated it was decided to confirm the finding by weekly tests on the sera of animals immunized by the serum-virus method. To overcome errors due to idiosyncrasies of individual horses two groups of three were selected from the survivors of a batch of animals being immunized, for the routine mass production of hyperimmune serum. Group A were hyperimmunized 69 days after immunization and consequently had to be discharged from the experiment; Group B was retained and it is proposed to continue tests of the pooled sera at intervals for at least the next two years. All the horses received the first immunizing injection (5 c.c. N. virus plus 400 c.c. serum) on 31.10.33, and the second (5 c.c. O virus plus 400 c.c. serum) on 3.11.33. The

reactions varied in severity from "very mild" to "severe". The results are tabulated in Tables X and VI, the antigen titrations being given in the corresponding Tables X. A and XI. A. In each group the sera from the three horses were mixed in equal quantities for the neutralization tests.

Table XI. (Continued).

Date of Test	Date Serum taken	Interval after immunization days	Serum Dilutions. 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
13.6.34	14.3.34	134	-	-	x,0,0,0	0,0,0,0	7,0,0,0	6,7,6,0	5,6,7,0	6,6,6,6	6,6,6,6	5,6,6,6	-
"	28.3.34	148				serum injected							
"	10.4.34	170	-	-	0,0	0,0	0,0	0,0,0	6,0,0	5,6,0	6,6,0	5,6,6,	5,6,6

Table XI. A.

Virus Titration.

Date	Virus Dilutions A	1:10	1:20	1:40	1:80	1:160	1:320	1:640
16. 5.34	5,5	5,5	5,5	5,6	5,7	6,0	0,0	0,0
23. 5.34	5,6	6,6	5,5	5,6	6,7	0,0	0,0	0,0
30. 5.34	5,5	5,5	6,6	6,x	5,0	0,0	0,0	0,0
6, 6.34	5,5	5,6	6,7	6,7	0,0	6,0	0,0	0,0
13. 6.34	5,5	5,5	5,6	5,0	6,6	8,9	0,0	0,0

Table X.

Titration of antibody content of pooled sera from horses Group A (20749, 20751 and 20767).

Date of Test	Date Serum taken	Interval after immunisation days	Serum Dilutions. 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:156	1:512	1:1024	1:2048
16. 3.34	14.12.33	14	7,0,0,0	5,6,7,0	4,6,6,6	6,6,6,0	5,6,6,6	4,5,6,8	5,5,	5,6,	-	-	-
28. 3.34	20.11.33	20	0,0,0,0	6,0,0,0	6,7,0,0	5,6,7,8	6,6,6,0	5,6,6,0	5,6	5,4	4,6	4,5	-
28. 3.34	27.12.33	27	0,0,0,0	6,0,0,0	6,6,7,x	6,6,7,x	6,6,7,0	6,6,7,7,	5,6,6,x	5,5,6,6	-	-	-
28.3.34	4.12.33	37	0,0,0,x	5,0,0,0	5,0,7,0	6,6,7,7	6,7,7,x	6,6,6,7	5,6,6,6	5,6,6,6	5,6	-	-
28. 3,34	11.12.33	41	8,0,0,0	0,0,0,0	7,0,0,0	6,6,0,0	6,7,7,8	4,6,7,0	5,6	6,6	-	-	-
5. 4.34	18.12.33	48	0,0	7,0,0,0	7,0,0,0	6,0,0,0	6,6,7,7	4,6,6,7	6,6,6,6	4,5,5,6	6,6	-	-
5. 4,34	27.12.23	57	0,0	0,0,0,x	5,0,0,x	5,0,0,0	5,5,7,x	6,6,6,x	5,6,6,8	-	-	-	-
21. 3.34	2. 1.34	63	-	0,0	0,0	6,0	7,0	6,7	6,6	6,7	6,6	-	-
21. 3.34	8. 1.34	69	-	-	0,0	8,0	0,0	8,0	5,6	6,7	6,6	4,5	5,5

Table X. A.

Virus Titration.

Date	Virus Dilutions.							
	A	1:10	1:20	1:40	1:80	1:160	1:320	1:640
16. 3.34	4,4	4,5	4,4	5,6	6,6	8,0	0,0	0,0
28. 3.34	4,5	4,6	5,5	5,6	5,7	7,0	0,0	0,0
5. 4.34	-	5,6	5,6	6,6	5,6,7	6,0,0	0,0	0,0
21. 3.34	5,5	5,x	6,6	6,6	7,7	6,7	0,0	0,0

Table XI.

Titration of Antibody Content pooled sera of Group B (20735, 20768, 20777).

Date of Test	Date Serum taken	Interval after immunisation days	Serum Dilutions. 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
16.5.34	10.11.33	14	6,6,7,7	6,6,6,7	6,6,6,6	6,6,6,6	6,6,6,6	6,6,6,6	-	-	-	-	-
"	20.11.33	20	0,0	6,0	6,0	6,6	6,6	6,6	-	-	-	-	-
"	27.11.33	27	0,0	6,0,0,0	7,0,0,0	6,0,0,0	7,7,7,0	6,6,6,6	6,6,6,7	6,6	-	-	-
"	4.12.33	34	0,0	0,0	0,0	0,0	6,6	6,7	6,6	6,6	-	-	-
"	11.12.33	41	-	-	0,0,0	6,0,0,0	6,6,0,0	6,6,6,0	6,6,6,6	4,6,6,6	6,6,6,6	6,6,6,6	-
"	18.12.33	48	-	-	0,0	6,0	0,0	6,0	6,6	6,6	6,6	6,6	-
23.5.34	27.12.33	57	-	-	x,0,0,0	0,0,0,0	6,0,0,0	6,0,0,0	6,6,6,0	6,6,6,0	6,6,6,6	6,6,6,6	-
"	2. 1.34	63	-	+	0,0	0,0	0,0	0,7	6,6	6,6	6,6	6,6	-
"	8. 1.34	69	-	-	0,0	0,0,0,0	6,0,0,0	0,0,0,0	6,6,7,0	6,6,6,0	6,6,7,6	6,6,6,6	-
"	15. 1.34	76	-	+	0,0,0,0	0,0,0,0	0,0,0,0	0,6,0,0	6,6,6,0	6,6,6,0	6,6,6,7	6,6,6,6	-
30.5.34	22. 1.34	83	-	-	0,0,0,0	0,0,0,0	0,0,0,0	6,0,0,0	0,0,0,0	6,0,0,0	6,6,6,6	6,6,6,7	-
"	29.1.34	90	-	-	0,0	0,0	0,0	0,0	x,0	7,0	6,7	6,7	-
"	5. 2.34	97	-	-	0,0,0,0	0,0,0,0	6,0,0,0	0,0,0,0	6,0,0,0	6,6,0,0	6,7,7,0	6,6,6,6	-
6.6.34	12. 2.34	104	-	-	0,0	x,0	0,0,0,0	6,7,0,0	6,7,0,0	6,6,7,0	6,6,6,6	6,6,6,6	6,6
"	19. 2.34	111	-	-	0,0	0,0	7,0,0,0	6,6,6,0	6,6,6,6	6,6,6,7	6,6,6,0	6,6	6,6
"	26. 2.34	118	-	-	0,0	0,0	0,0,0,0	7,0,0,0	7,0,0,0	6,6,6,0	6,6,6,7	6,6,6,6	6,6

Cont. over.

Results.

Group A. It is seen that 14 days after injection neutralizing antibodies were present in demonstrable amount. From the 14th day there was a slow but steady increase in titre until the 69th day when the horses were discharged.

Group B. In this group antibodies were not demonstrable on the 14th day but were present in appreciable amount on the 20th day. From this point on there was a gradual but steady rise until a peak was attained about the 83rd to the 97th day. From the 100th day there was a distinct tendency for the titre to decrease but it was maintained at a fairly high level up to the 170th day the last recorded titration.

Discussion. Again it is emphasized that the results recorded should not be scrutinized with an eye to mathematical accuracy. Rather due consideration should be paid to the difficulty of titrating a minute living entity (if evidence is given to the general consensus of opinion in regard to viruses to-day) and to the use as an indicator of a somewhat gross biological test which cannot be other than approximate unless analysed on a statistical basis. However, the rise and the tendency of the antibody content to fall is well defined so that viewed in this light the results are of singular interest. They indicate :-

1. The rise in antibody content of the serum of horses after immunization is slow.

2. After immunization by the injection of an attenuated neurotropic virus it takes about two months for the titre to reach a significant figure and the peak is not attained until the 200th day.

3. After immunization by the serum virus method

which is based upon the control in vivo by hyperimmune serum of a fully virulent viscerotropic virus the initial rise in antibody content is considerably more rapid and the peak is reached after about 100 days.

4. At no time does the titre of the anti serum obtained after immunization by either method attain a high level - unit volume of a 1:512 dilution of serum being required to neutralize ± 100 M.I.D., and this figure is approximately the same for both methods of immunization.

5. After the peak of anti body formation has been reached there is a decided tendency for it to fall but a considerable titre is maintained for 300 days and 170 days respectively (the limits of the respective tests).

6. As yet there is no data to indicate for how long the anti body content will be maintained. This aspect is being studied but results will not be available for at least two years.

This conception of slow immunity development has as far as the author is aware a single precedent in the ~~titration~~ literature viz. the delayed mineralogical response in bovines to the injection of cultures of Astrococcus mycoides attenuated by continued subcultivation in artificial media as reported by Bennett (1932). From his work Bennett concluded that "the longer a virus has been maintained in artificial culture medium (at any rate in serum peptone broth) the longer it takes to produce immunity when injected subcutaneously". In making an attempt 'to apply approximately absolute terms of generations and times to this principle' he showed that 'a second generation subculture was dangerous but produced a solid immunity in less than three weeks, a seventeenth generation subculture was safe and produced a very sound immunity in three weeks and an

absolutely solid immunity in less than six weeks; a 115th generation subculture although showing some degree of protective power, did not produce a serviceable immunity in nine weeks.' These results indicate quite clearly that progressive attenuation of the virus of pleuropneumonia is accompanied by delayed antigenic response in the animal injected. Unfortunately under the conditions of field experimentation Bennett had no method of determining accurately at any particular time the relative immunity in the various groups of bovines. In the case of horsesickness the results detailed above show that an attempt has been made to accomplish this. But, it must be borne in mind that the viricidal content of the serum cannot be regarded as a true index of the degree of immunity of the serum donor since no consideration is paid to the possibility of acquired cellular resistance in addition to humoral immunity. This point is clearly illustrated by the work of Stuart and Sriharan[8] on rabies. These authors showed that the rabicidal antibody content of the serum of groups of rabbits immunized with etherized virus, living fixed virus and carbolized virus attained a maximum on the 60th day and at that time antibodies are present in the groups in the proportion 3:2:1. However, immunity tests of the serum donors by subdural injection of fixed virus failed to establish a similar mathematical relationship between the immunity produced in the different groups of rabbits, the percentage of survivors being in fact the index of immunity, in fact no significant differences could be observed. Consequently it is reasonable to assume that the same principle should hold good for horsesickness. This is borne out by the finding that in spite of the absence of detectable antibodies in the serum of horses 21 days after injection with neurotropic virus the majority of animals have developed at that time an immunity sufficient to protect against the intravenous

injection of 5 c.c. of fully virulent viscerotropic virus, although a severe febrile reaction with clinical symptoms of horsesickness has been produced after this interval. On the other hand a fairly large number of immunity tests carried out after a longer interval has shown that when antibodies are demonstrable to a substantial titre the immunity has always been solid.

If the viricidal content of the serum is not an absolute index of total immunity there appears to exist between the two a relationship which is possible on theoretical grounds and which has been established tentatively by the only critical experiments possible at present namely direct in vivo immunity tests. It still remains to be shown whether this relationship is a constant and probably this will only be accomplished after a statistical survey of the results obtained from the immunization of large numbers of animals in the field. Immunity tests in the laboratory are unsatisfactory. Either the animal is solidly resistant to the infection of virus or a fatal reaction results but in neither case is it possible to hazard any opinion as to the relative degree of resistance or susceptibility. In these comparatively rare instances where an immunity test results in a non fatal reaction or a reaction of variable severity after a lengthened period of incubation a clear interpretation of the result is ~~observed~~ obscured by a realization of the marked differences in susceptibility of different individuals to horsesickness. This unsatisfactory 'hit or miss' system of determining immunity, although it must be regarded as the ultimate criterion, has been a serious handicap particularly in the study of the efficacy of different methods of immunization. The in vitro neutralization test described appears to remedy this defect and in addition will be of immense value in effecting economies in the use of horses, and

frequently may clarify the results obtained since initial susceptibility can always be determined. The delay in appearance of immune bodies must be borne in mind and this has reduced the speed of research work to a considerable extent since it is apparent that an animal must be kept under observation for at least three months before any significance can be attached to the presence or absence of circulating viricidal antibodies.

In spite of its many limitations the in vitro neutralization test is proving the most valuable method available to-day of studying the problem of immunity production in this disease. From the point of view of technique the necessity of being forced to make use of a biological test to determine inactivation of the virus is a big handicap. Apart entirely from the necessity of having to use large numbers of mice for injection there is a delay of at least seven days before results are available and this delay is increased by the time taken up in repeating experiments when obvious errors have occurred and in carrying out confirmatory work. A vast amount of work must be recorded simply by saying that no in vitro reaction has been found to replace the biological test. Numerous attempts have been made to apply the variola-vaccinia flocculation test of Craigie and Tulloch[9] without any success. The flocculation reaction reported by Havens and Mayfield[10] in connection with their work on rabies was followed but in the case of Horsesickness flocculation when it did occur was indistinct could not always be considered entirely specific and did not seem to hold out any hope for quantitative application. Similarly the possibility of preparing an "agglutinable" or "agglutinating" antigen, following the principles described by Kahn[11] for the serum diagnosis of syphilis, was investigated with negative results.

Consequnetly at the present time only the results of intracerebral injection of mice with suitable mixtures of virus and anti serum can be considered but the urgent necessity of having a test tube (test) is appreciated only too fully in research work of this description.

B. The plurality of virus strains.

During the course of this work frequent reference has been made to the use of particular strains of virus. Details of the origin of these strains have been omitted purposely until this stage so as to enable a comparison of their antigenic differences to be made at the same time.

The strains are distinctively labelled from the laboratory number of the horse from which they were originally obtained with the exception of "O" virus in which case the letter "O" by common usage has been retained to mean 'ordinary virus'. No attempt has been made to allocate symbols as in the case of the A, B, C etc. strains of Foot and Mouth virus because such symbols, in the light of our present knowledge of the antigenic structures, cannot be other than equally meaningless. Until such time as symbols are necessary to serve as a basis for comparison between strains of virus from different institutions or countries it is proposed to retain the present nomenclature pending a rational scheme of classification.

The strains which have been investigated in these studies are :-

1. Strain O commonly termed O virus. This is the original strain extensively used by Theiler, by whom it was isolated in the first instance. The origin of this strain is somewhat obscure but it appears to be the one most commonly encountered at Onderstepoort and formed the basis of the

original serum virus method of immunization. It is exceedingly virulent and has been maintained for more than 30 years by periodical subinoculation into horses in which it is now in its 231st passage. Generation 133 is the one which has been fixed and maintained in mice.

2. Strain 20449. This strain was obtained at Onderstepoort in 1932 from a spontaneous case of the disease that occurred in an animal which had been hyperimmunized some months previously against strain O. In comparison with the result of infection with O virus, the course of the disease it produces is somewhat lengthened but the mortality also is 100%. It appears to be the strain commonly encountered after natural infection on the Institutes farm "Kaalplaas."

3. Strain 20464. On 23rd January 1933 a susceptible horse was injected with a mixture of six strains of virus obtained from spontaneous cases of horsesickness which had occurred several years previously either in the stables or on various farms in the vicinity. The horse died on the eighth day after infection and the virus contained in the blood just prior to death was subinoculated into mice by the intracerebral route. From this fixation two strains emerged which are characterized by a slightly different period of incubation and course. These strains have been labelled respectively 20464 A and 20464 B. For reasons to be noted later only the results obtained with 20464 B. are detailed.

Identification. Groups of three horses ~~each~~ were immunized against each strain by the subcutaneous injection of 10 c.c. of neurotropic virus in the form of a 0.1% saline suspension of the brains of mice sacrificed in extremis during routine passage. All of the horses were given only a single injection and the normal mild reactions

anticipated from the use of attenuated virus were produced. Details of the injections are given in tabular form below.

Table XII.

Production of Tyne Anti sera in horses.

Horse	Strain and generation of virus.	Date of Injection	Reaction.
20836(1)) 20837) 20838)	Mouse generation 73, Strain O (2)	7.2.34	No clinical or febrile reaction. Slight fever from 6th - 13th day. Moderate fever from 5th - 11th day.
20645) 20646(1))	Mouse generation 51, Strain 20449	23.6.33	Mild fever from 19th - 23rd day. No reaction.
20670)			No reaction.
20831) 20832) 20833)	Mouse generation 71, Strain 20464 B	23.1.34	No clinical or febrile reaction. No clinical or febrile reaction. Moderate fever from 21st to 33rd day.

Note. (1) Horses 20836 and 20646 were urgently required in other experiments and their sera has not been used.

(2) Strain O in addition to 73 passages through mice had been passed through six generations in guinea pigs.

Prior to injection samples of sera were obtained from each horse. At various times in different experiments these sera were used as normal controls; never could the presence of any viricidal antibodies be detected.

Subsequent to injection the horses were continually stabled except for a period of about five hours each day when they were allowed to run in a small paddock. The chance of exposure to natural infection therefore was reduced to a minimum. Moreover no natural cases of horsesickness occurred amongst a number of susceptible horses which always accompanied them.

For in vitro neutralisation tests serum was collected on the dates specified. This serum was stored separately without preservation in a refrigerator at ± 4°C. Immediately prior to use a pool for each group was made by mixture of equal quantities of serum from each horse.

Neutralization tests for each group against each neurotropic virus were carried out with the results indicated in tabular form below.

Table XIII.

Neutralization of Virus O by type sera.

For neutralization virus mouse passage generation 138 was used as antigen.

Date of Test	SERUM			Serum Dilutions.									
	Type	Date	Interval after immunization	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
4. 7.34	O	25. 5.34	107 days	-	0,0	0,x	0,x	0,0	6,6	5,6	5,6	5,5	-
15. 8.34		31. 7.34	174 "	-	-	8,0	0,0	0,0	0,0	0,0	0,0	5,5	5,5
4. 7.34	20449	22. 9.33	92 days	6,6	6,6	6,6	5,5	5,6	5,5	5,5	5,5	-	-
15. 8.34		14.12.33	177 "	5,6	5,6	5,5	5,5	5,5	5,5	5,6	5,5	-	-
4. 9.34	20464 B	11. 5.34	108 days	5,5	5,5	5,5	5,5	5,5	5,5	5,6	5,5	-	-
15. 8.34		20. 7.34	178 "	5,5	5,6	5,5	5,0	5,5	4,5	5,5	5,5	-	-

Titration of Virus Antigen.(5 c.c. in 50 c.c. of stock used)

Date	Virus Dilutions.							
	A	1:10	1:20	1:40	1:80	1:160	1:320	1:640
4.7.34	5,5	5,5	5,6	6,0	6,7	0,0	0,0	0,0
15.8.34	5,6	6,6	6,7	5,6	5,0	0,0	0,0	0,0

Result. Virus strain O is neutralized by the homologous anti serum but is not neutralized by strain 20449 anti serum. or 20464B anti-serum

TABLE XIV.

Neutralization of Virus 20449 by type sera.

For neutralization virus mouse passage generation 112 was used as antigen.

Date of Test	Serum			Serum Dilutions.									
	Type	Date	Interval after immunization	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
28.6.34	O	25. 5.34	107 days	5,6	4,5	4,4	4,4	4,4	4,4	4,4	4,4	-	-
22.8.34		31. 7.34	174 "	4,6	4,5	5,0	5,5	4,5	4,5	5,5	4,4	-	-
28.6.34	20449	22. 9.33	92 days	-	0,0	0,0,0,0	0,0,0,0	0,0,0,0	5,0,0,0	4,0,0,0	6,6,8,0	4,5,5,0	4,4
22,8.34		14.12.33	177 "	-	-	3,0,0,0	6,0,0,0	0,0,0,x	0,0,0,x	4,5,0,0	3,4,5,0	3,4,5,0	4,4,4,5
28.6.34	20464 B	11. 5.34	108 days	5,6	5,5	5,5	4,4	4,4	4,4	4,4	4,4	-	-
22.8.34		20. 7.34	178 "	4,5,0,0	4,0,0,0	0,0,0,0	6,6,0,0	5,5,5,0	4,5,6,0	4,4	4,4	-	-

Titration of Virus Antigen (0.9 :100 of stock antigen)

Date		Virus Dilutions						
	A	1:10	1:20	1:40	1:80	1:160	1:320	1:640
28.6.34	4,4	4,5	4,5	5,5	5,6	0,0	0,0	0,0
22.8.34	4,5	4,5	4,5	6,6	6,0	0,0	6,0	0,0

Result. Virus strain 20449 is neutralized by the homologous anti serum. It is not neutralized by Strain O anti serum but is neutralized by strain 20464 B anti serum though only to a decidedly lower titre. Moreover, in horses immunized against strain 20464 B viricidal antibodies capable of neutralizing strain 20449 do not make their appearance until after a lapse of 108 days and even after 178 days do not attain a high titre.

Table XV.

Neutralization of Virus 20464 B by type sera.

For neutralization virus mouse passage generation 100 was used as antigen.

Date of Test	Serum Type	Serum Date	Interval after immunization	Serum Dilutions. 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024.
6.7.34	O	25.6.34	107 days	3,4	4,4	4,4	4,4	4,4	3,4	4,4	4,4	-	-
9.8.34		31.7.34	174 "	0,0	0,0	5,8	5,5	5,0	4,5	4,5	4,4	-	-
6.7.34	20449	22.3.33	92 days	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,8	-	-
9.8.34		14.12.33	177 "	-	-	0,0	0,0	0,0	0,0	0,0	0,0	4,5	4,5
6.7.34	20464 B	11.5.34	108 days	7,0	4,7	4,6	5,4	4,5	4,4	4,4	5,4	-	-
9.8.34		20.7.34	178 "	0,0	0,0	0,0	5,0	5,0	0,0	4,4	4,0	-	-

Titration of Virus antigen (1:100 dilution of stock antigen)

Date	Virus Dilutions. A	1:10	1:20	1:40	1:80	1:160	1:320	1:640
6.7.34	5,4	5,3	4,4	5,5,5,	5,5	0,0	0,0	0,0
9.8.34	4,4	4,5	5,0	5,5	4,0	0,0	0,0	0,0

Result. Strain O anti serum is not capable of neutralizing virus 20464 B 107 days after the immunizing injection but is viricidal in a low titre after 174 days. After immunization with strain 20464 B the homologous viricidal antibodies develop more slowly and do not attain so high a titre as those contained in type 20449 anti serum.

Before the significance of the findings is discussed it is essential to detail the results obtained in a preliminary experiment on one horse designed to throw some light upon the antigenic structure of one virus strain - 20449. Commencing on 16.11.33 a horse (20659) was given repeated injections of fairly massive doses of infective brain suspensions prepared for routine subinoculation. No definite procedure was adopted, injections being made after the termination of the original febrile reaction at times when an adequate supply of neurotropic virus was available. The injections given are shown in tabular form below.

Table XVI.

Repeated injections of neurotropic virus (20659).

Date	Interval since last injection	Virus Dose	Concentration	Reaction.
16.11.33	-	10 c.c.	1:100	Severe fever from 6th -17th day.
8.12.33	22 days	10 c.c.	1:25	No reaction
14.12.33	6 days	10 c.c.	1:25	No reaction.
12.1.34	29 days	10 c.c.	1:50	Slight fever from 16th - 25th day.
28.2.34	47 days	10 c.c.	1:50	No reaction.

Serum was collected at approximately weekly intervals but up to the present it has been possible to run neutralisation tests against the three virus strains with only two samples which were selected haphazardly namely serum of 22/5/34, i.e. 187 days after first injection and 83 days after last injection and of 6.6.34 i.e. collected 15 days later.

The antigens used in the neutralisation tests were those used in the tests tabulated above, i.e. unit volume of the anti serum was required to neutralise approximately 100 M.L.D. of each neurotropic virus. The complete results are shown in tabular form in Table XVII.

Table XVIII.

Neutralization test using anti serum from a horse which was given repeated injections of virus strain 20 49.

Date of Test	Strain of virus Antigen	Serum collected	Serum Dilutions. 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
15.8.34	Strain 0	22.6.34	-	-	-	x,0	0,0	0,0	0,0	0,0	5,0	5,8	6,0	-
4.7.34	do	6.6.34	0,0	0,0	0,0	0,0	0,0	0,0	5,6	0,0	-	-	-	-
22.9.34	20449	22.5.34	-	-	-	-	0,0	0,0	0,0	0,0	0,0	0,0	5,0	5,8
28.6.34	do	6.6.34	0,0	0,0	0,0	0,0	0,0	0,0	0,0	5,0	-	-	-	-
9.8.34	20464 B	22.5.34	-	-	0,0	0,0	0,0	0,0	0,0	0,0	0,0	3,4	-	-
	do	6.6.34	0,0	0,0	0,0	0,0	0,0	4,0	4,0	3,0	-	-	-	-

Result. From the above results it is seen that after five repeated injections of a single strain of neurotropic virus there appeared in the serum of a horse antibodies which were capable of neutralizing not only the homologous strain but also two heterologous strains to a high titre. It is worthy of note that serum collected 83 days after the last injection was viricidal to a considerably higher titre than that collected 15 days later. It is not known when the peak of antibody production is reached nor what the titre might be.

Conclusion. Of the three strains of virus investigated Strain O and strain 20449 are distinct antigenically since the type anti sera collected before and at the peak of anti body production do not cross neutralise. This finding is directly in keeping with the origin of the strains and the results of in vivo cross immunity tests.

Strain O differs from strain 20464 B, since 20464 B type anti serum does not neutralise O antigen, but O anti serum does neutralise 20464 B antigen. These viricidal substances are produced exceedingly slowly and do not attain a high titre since a concentration of 1/4 is required to neutralize ±± 100 M.I.D. In vivo cross immunity tests could not be carried out to confirm this result because of the obscure origin of strain 20464.

Between 20464 B and 20449 there is a well marked resemblance. The type sera neutralize each virus to almost the identical titre, the differences obtained being well within the limits of experimental error and it is worthy of note that 20464 B anti serum neutralises the homologous strain to a lower titre than the heterologous strain. But there is this slight difference - whereas O anti serum shows no neutralizing action whatever against strain 20449 it definitely does neutralise 20464 B antigen though to a low titre. This last result has been confirmed by neutralization tests using as antigen a strain of 20464 B virus at a considerably different level of neurotropic fixation. (generation 81).

Note. Identical investigations to those detailed above were carried out with strain 20464 A. The results have been omitted purposely because a discrepancy occurred which requires repeated confirmation before publication. A batch of three horses were immunized by subcutaneous injection of

mouse virus, one reacted mildly and two showed no reaction. Serum collected up to 175 days after injection has failed to show any viricidal effect on either the homologous or the heterologous virus although the antigen used was readily neutralised to high titre by the serum obtained from horse 2.659 which received the repeated injections of strain 2.449. The work has been carefully checked. The horses were injected with passage virus generation 77 on a day when no other injections were made so that the type serum must be correct. Neutralization tests have been carried out using as antigen generations 81, 92 and 101 of the homologous strain but always no neutralisation of the homologous strain was obtained. It is admitted that there exists a possibility of mixing up two strains during routine passage but as such care has been taken to avoid this error and as no variation has occurred in the other strains the commission of such a mistake is not conceded readily. If the results obtained are correct, they indicate the existence of a non antigenic strain, a finding which would occasion no great surprise when viewed in the light of the results obtained with strain 2.464 B above. However, the entire experiment must be repeated and as this necessitates a delay of at least 200 days the remainder of the work is detailed in view of its possible interest. A further report will be submitted in due course.

Discussion. A plurality of strains of Horse-sickness virus is demonstrated conclusively by the work detailed. This supports the observation that has been made in the field that an animal which is solidly resistant to natural infection in one particular area may succumb to Horsesickness soon after transfer to some other district.

From the preliminary experiment reported the difference in antigenic structure between these strains appears to be quantitative rather than qualitative since the response to repeated injections of our virus strain was the production of viricidal bodies capable of neutralizing all the other strains that had been fixed neurotropically. In other words it appears that each of the virus strains investigated possess the same antigenic components which are present in vastly different proportions. The result is that a single immunizing injection produces immunity against that component which predominates but that repeated injections eventually produces immunity against all the components.

From the point of view of mass immunization in the field these findings are of great importance. From a practical and economic aspect it is almost essential to limit the process of immunization to a single injection. Consequently there must be incorporated in the vaccine every attenuated virus which possesses a different antigenic structure no matter how slight in order that an adequate immunity may be produced against the unknown number of naturally occurring strains of different constitution. This conception clarifies the whole problem of immunization and presupposes the ultimate development of a completely polyvalent vaccine which should be efficacious in all areas.

One other result merits discussion and that is the production of high titre serum by repeated injections of virus. This is in direct contrast to the finding of Lloyd and Mahaffy[11] who in their work on Yellow Fever showed that "no significant alteration was found to take place in the protecting antibody titre of the sera of five Macacus rhesus monkeys, immune to Yellow Fever after repeated subcutaneous injections of neurotropic yellow fever virus at monthly intervals". In the case

of horsesickness a significant (8 fold) increase did occur and it is not known whether the serum used represented the most potent product.

In conclusion it must be admitted that this aspect of the study of immunity to horsesickness has only just been touched upon but the results appear to be sufficiently important to merit early report in the hope that confirmatory and additional work may be launched by other investigators. Moreover, in view of the fact that antibodies are produced so slowly a comprehensive report cannot be anticipated for many years so that the publication of progress reports is excusable.

Summary.

1. The technique of the intracerebral protection test is described in detail, particular attention being paid to the preparation of a constant antigen and its keeping qualities to permit of quantitative comparison of the results of different tests.

2. The slow production of viricidal antibodies in immunised animals is illustrated, and it is shown that this rate is considerably slower after the injection of attenuated virus.

3. The relation between antibody content of the serum and total immunity is discussed.

4. The plurability of strains of horsesickness virus is demonstrated.

5. The probable antigenic structure of different strains is deecussed.

6. The production of high titre immune serum is indicated.

7. Failure to devise on in vitro test to replace the biological test at present essential for the completion of neutralization experiments is recorded.