Onderstepoort Journal of Veterinary Science and Animal Industry, Volume 2, Number 2, April, 1934.

The Immunization of Horses and Mules against Horsesickness by means of the Neurotropic Virus of Mice and Guinea-Pigs.

R. A. ALEXANDER, B.Sc.Agric., B.V.Sc., Empire Marketing Board Research Fellow.

P. J. DU TOIT, B.A., Dr. Phil., Dr.Med.Vet., D.Sc., Director of Veterinary Services and Animal Industry, Onderstepoort.

In December, 1932, Nieschulz reported that he had succeeded in infecting white mice with the virus of horsesickness by the intracerebral route. Independently and concurrently this work was confirmed at Onderstepoort (Alexander, 1933), but in one important respect the conclusions differed from those arrived at by Nieschulz. Whereas Nieschulz maintained that the virus was not attenuated and that its character was not altered by passage through the mouse, our experience with a greater number of horses showed that not only was there an attenuation of the virus for the horse, but that, after even a limited number of generations, the change was so marked that there existed a distinct possibility of developing a safe and simple method of immunization against horsesickness in a manner similar to that applied to yellow fever by Sawyer and his co-workers.

With this object in view the virus has been passaged serially in mice, so that, at the time of writing, one strain is in its 89th generation, a second antigenically different strain is in its 78th generation, and a third in its 64th generation. In addition to the propagation in mice the first two of the above-mentioned strains have been maintained in guinea-pigs, in which animal they have been passaged 38 times.

As reported previously, in both these animals the virus adopts exclusively neurotropic characters. After 2 to 4 subinoculations a mortality of 100 per cent. is produced, and there is a progressive acceleration of the course of the disease. From time to time susceptible horses and mules have been injected with emulsions of virulent brains in order to ascertain the degree of attenuation for equines. The results obtained form the basis of this report.

Virus Used.—In the experiments reported two antigenically different strains of virus have been used.

(1) Strain O (ordinary virus, known as O virus), an extremely virulent strain isolated some 30 years ago by Sir Arnold Theiler and maintained by periodical subinoculation into horses. Generation 193 was used in the form of whole blood drawn from a reacting horse (20319) at the height of the febrile reaction into oxalate-carbol-glycerin solution as an anticoagulant and preservative. Stored in a cool room in this way the blood is known to retain full virulence for years.

(2) Strain 20449 obtained in 1932 from a fatal case of horse-sickness that occured in an animal that had been hyperimmunized some months previously against strain O by the transfusion of 10 litres of virulent blood. It has been shown experimentally that this strain breaks down the immunity set up by O virus, and conversely horses immunized against 20449 strain always react to a subsequent intravenous injection of O virus.

Behaviour of the Strains in Mice and Guinea-Pigs.—At this stage it is unnecessary to describe and contrast the behaviour of these strains in mice and guinea-pigs. It is sufficient to state that in the case of O virus the course of the disease in mice has gradually decreased from 5-7 days to 4 days after 75 passages, and that 0·05 c.c. of a 1-100,000 dilution of fresh brain represents approximately 1 lethal dose. In guinea-pigs death usually occurs in 6-8 days, and the infective titre of the brain is about 1/10 that of mice.

Strain 20449 invariably kills 100 per cent. of injected mice on the 4th day, and emulsions of fresh brains have frequently proved infective in a dilution of 1:1,000,000. In guinea-pigs the course of the disease is somewhat longer than that produced by O. virus, but the infectivity appears to the approximately the same.

Preparation of Virus Emulsion for Injection.

Infected mice and guinea pigs are etherized *in extremis*, the brains removed with complete aseptic precautions, and placed in sterile 50 c.c. centrifuge tubes fitted with sterile corks. The brains are then frozen overnight in the freezing chamber of an electric refrigerator and next morning rapidly thawed in a water bath or incubator at 37° C. to cause the disintegration of as much cellular material as possible. The amount of fluid necessary to give the desired concentration (usually 4 per cent.) is introduced, and the brain material is macerated by rapidly drawing it into, and forcing it out of, a syringe fitted with a fine nozzle. The resulting emulsion is then centrifuged for 20 minutes at ±1,500 revolutions per minute, and the turbid supernatant fluid free from gross particulate matter is used for injection. In the text this fluid is referred to as " virulent brain emulsion ".

For emulsification serum of a normal susceptible horse diluted 1:10 with 0·85 per cent. saline was used, since it has been found to have several decided advantages over saline alone.

In those experiments where various dilutions of infective brain material were used, a stock emulsion representing a dilution of 1 part of brain substance to 25 parts of 1:10 serum-saline was made, and the requisite dilutions in serum-saline were prepared from this stock.

Horses and Mules Used.

The horses and mules used were obtained from various horse dealers, who purchase their animals in districts where horsickness normally does not occur. These dealers have supplied this Institution for many years, and experience has shown that only on rare occasions have immune animals been included in a batch. The percentage of such animals which were immune as a result of recovery

from a natural attack of horsesickness has never exceeded 2 per cent. Consequently it must be pointed out that although it is possible for an immune individual to be drafted into an experiment, duplication of each experiment will certainly prevent the drawing of erroneous conclusions from the use of insusceptible animals.

A. THE IMMUNIZATION OF HORSES.

The first experiment to ascertain the antigenic value of mouse neurotropic virus was carried out on two horses. The entire emulsion of the brain of one mouse, generation 5, strain 20449, was injected subcutaneously into horse 20337 on 22/11/32. There was no local reaction at the site of injection, but a mild systematic reaction characterized by slight fever commenced on the 6th day after injection and lasted for 5 days. The horse recovered rapidly and at no time would it have been possible for a clinical diagnosis of horsesickness to have been made.

Subsequently, on 6/12/32, an emulsion of the pooled brains of 9 mice, generation 8, strain 20449, was made in 100 c.c. serum-saline; 50 c.c. were injected subcutaneously into the same horse; and 50 c.c. intravenously into a 2nd horse (20334). Horse 20337 did not react. Horse 20334 developed a fairly severe febrile reaction which lasted from the 5th to the 10th day, accompanied by marked oedema of the supraorbital fossa (dikkop) and recovered.

Fourteen days later (20/12/32) both horses were given 5 c.c. of the homologous strain of virulent blood intravenously as an immunity test; neither horse reacted. That this blood was fully virulent was shown by the fact that a control horse commenced to react on the 3rd day after injection and died on the 6th day.

In passing it may be mentioned that on 3/1/33 both the above horses were given 5 c.c. of virus, strain O, intravenously. Horse 20337 reacted and died on the 6th day; horse 20334 underwent a very severe reaction, but eventually recovered.

From this experiment it must be concluded that during the process of transformation from viscerotropism to neurotropism there is a marked alteration in the virulence of the virus for horses. Consequently utilization of this process of natural attenuation encouraged the hope that after complete " fixation " the neurotropic virus might serve as a vaccine, since there did not appear to be any decrease in antigenic activity.

To confirm the conclusions drawn from this initial experiment on 2 horses, a second experiment was commenced, with the object of—

(*a*) ascertaining whether even a limited number of passages through the brains of mice and guinea-pigs would attenuate the virus to a consistently safe level;

(*b*) determining the most suitable route for the injection of equines, i.e. subcutaneous or intravenous;

(*c*) comparing the relative efficacy and degree of attenuation of the mouse and guinea-pig adapted viruses respectively.

The injections given and the results obtained are indicated below:—

20492.	28/12/32.	Subcut. 50 c.c. emulsion 3 mouse brains, generation 12 and 13, strain 20449. Severe reaction commenced 6th day. Died 11th day. Dikkop horsesickness.
20493.	28/12/33.	Intravenously 50 c.c. emulsion 3 mouse brains, generation 12 and 13, strain 20449. Slight febrile reaction from 6th to 11th day. Recovered.
	*16/1/33.	Intravenously 5 c.c. virulent blood strain 20449. No reaction.
	25/4/33.	Subcutaneously 10 c.c. 1/25 dilution 1 brain mouse, gm. 29, strain O. Severe reaction from 7th to 14th day. Recovered.
		(NOTE.—H. 20493 had an accident and had to be destroyed before the application of an immunity test to O virus.)
20494.	28/12/33.	Intravenously 50 c.c. emulsion 1 guinea-pig brain, generation 3, strain 20449. Mild reaction from 6th to 11th day. Recovered.
	*16/1/33.	Intravenously 5 c.c. virulent blood strain 20449. No reaction.
	13/2/33.	Subcutaneously 50 c.c. emulsion 2 guinea-pig brains, generation 5, strain O. No reaction.
	*3/3/33.	Intravenously 5 c.c. virulent blood strain O. No reaction.
20495.	28/12/32.	Subcutaneously 50 c.c. emulsion 1 guinea-pig brain, generation 3, strain 20449. No reaction.
	*16/1/33.	Intravenously 5 c.c. virulent blood strain 20449. No reaction.
	30/1/33.	Subcutaneously 15 c.c. emulsion 1 mouse brain, generation 13, strain O. Severe reaction from 3rd to 13th day. Recovered.
	*20/2/33.	Intravenously 5 c.c. virulent blood, strain O. No reaction.
20496.	30/12/32.	Subcutaneously 15 c.c. emulsion 3 mouse brains, generation 7, strain O. Severe reaction commenced 3rd day. Died 9th day.
20497.	30/12/32.	Intravenously 15 c.c. emulsion 3 mouse brains, generation 7, strain O. Reacted from 4th day. Died 8th day.
20502.	18/1/33.	Intravenously 10 c.c. emulsion 1 guinea-pig brain, generation 5, strain 20449. Mild fever reaction from 8th to 14th day.
	*10/2/33.	Intravenously 5 c.c. virulent blood strain 20449. No reaction.
	2/3/33.	Subcutaneously 10 c.c. 1/500 dilution 1 mouse brain, generation 16, strain O. Severe reaction from 5th day. Died 9th day.

* Denotes immunity test.

20503.	18/1/33.	Intravenously 10 c.c. emulsion 1 guinea-pig brain, generation 5, strain 20449. Mild fever reaction from 9th to 17th day.
	*10/2/33.	Intravenously 5 c.c. virulent blood, strain 20449. No reaction.
	2/3/33.	Subcutaneously 10 c.c. 1/500 dilution 1 guinea-pig brain, generation 6, strain O. Severe reaction from 5th day. Died 10th day.
20538.	22/2/33.	Subcutaneously 50 c.c. 1/100 dilution 1 guinea-pig brain, generation 5, strain O. Severe reaction from 2nd day. Died 6th day.

Results and Conclusions.

(1) There appears to be no significant difference in the results obtained from the injection of the virus emulsion subcutaneously or intravenously. In the first instance (horses 20492 and 20493) the subcutaneous injection produced the more severe reaction; in the second instance (horses 20494 and 20495) the more severe reaction was a result of the intravenous injection; in the third instance (horses 20496 and 20497) both the injected animals died. Consequently it was decided that for the sake of ultimate simplicity all injections in the future would be given by the subcutaneous route.

(2) The virus appears to be attenuated more rapidly by serial passage through guinea-pigs than through mice. Whether this attenuation is merely more rapid at the commencement of passage but eventually would attain the same level in both animals after prolonged subinoculation, it is not possible to say at this stage.

(3) The two different strains of virus used did not become attenuated for horses at the same rate as indicated by the fact that horses 20494 and 20495 survived an injection of material consisting of the 3rd generation of strain 20449 in guinea-pigs, while horse 20538, which was injected with emulsion representing the 5th generation of strain O in guinea-pigs, died after an incubation period and duration of illness which indicated that little or no decrease in virulence had taken place. In this connection it is interesting to bear in mind that strain 20449 was obtained from a naturally contracted case of horsesickness, while strain O, which had been passaged for 193 generations in horses before being transferred to guinea-pigs, possibly had become " fixed " for horses.

(4) Apart from the definite attenuation of the virus strains, the most gratifying and most important conclusion that may be drawn from the whole experiment, is that decrease of virulence is not accompanied by a simultaneous loss of any antigenic power. This is clearly illustrated by the observation that every horse which survived an injection of guinea-pig or mouse virus was shown subsequently to be solidly immune to the homologous strain.

---

* Denotes immunity test.

(5) As regards the immunity produced by one strain of virus (20449) against a second (strain O) no definite conclusion can be drawn, since horses which had survived the injection of 20449 neurotropic virus received material representing different generations of strain O, i.e. at different levels of attenuation. It seems probable therefore that if a cross-immunity does exist, it is at most only partial.

The results obtained from the above experiment were so encouraging that it was decided to carry out a further experiment on five horses to determine the degree of attenuation after several additional passages through guinea-pigs and also to ascertain the effect of injecting various dilutions of emulsion.

Three guinea-pigs, constituting generation 10, strain 20449, were destroyed in extremis and a 5 per cent. emulsion of the pooled brains was made in 1/10 normal horse serum-saline. The supernatant fluid obtained after centrifugation was used as the highest concentration of virulent brain, and serial dilutions in serum-saline were made from this stock emulsion. The injections made and the results obtained are tabulated below:—

Horse.	Date.	Dose.	Dilution.	Reaction.
20360	7/3/33	10 c.c. subcut.	1/20	No reaction.
20354	,,	,,	1/100	,, ,,
20371	,,	,,	1/500	Reaction commenced 8th day. Died 16th day. Dikkop.
20355	,,	,,	1/1,000	Mild febrile reaction + Dikkop from 14–17th day.
20367	,,	,,	1/5,000	Reaction commenced 7th day. Died 13th day. Dikkop.

The 3 survivors were given an immunity test of 5 c.c. of virulent blood of the same strain intravenously on 4/4/33. There were no clinical or thermal reactions. A titration of the infectivity of the emulsion for mice was carried out, 0·05 c.c. amounts of each serial dilution in serum-saline being injected intracerebrally into 2 mice.

Dilution.	*Result.*
1/20	6 : 6*
1/100	6 : 6
1/500	6 : 6
1/1,000	7 : 8
1/5,000	7 : 7
1/10,000	7 : 7
1/50,000	0 : 0

* NOTE.—The numerals indicates the number of days between injection and death of each mouse. 0 means survival.

Results and Conclusions.

Of 5 horse injected with the same dose of falling dilutions of neurotropic virus in the 10th passage through guinea-pigs, 2 died of dikkop horsesickness, 1 showed a marked clinical reaction accompanied by oedema of the supraorbital fossa, and 2 did not react. Whereas strain 20449 can be relied upon constantly to produce a reaction by the 4th day and death by the 7th day, the reactions were considerably delayed; in fact, they were of a nature which would have been expected as a result of the simultaneous injection of virus and massive doses of hyperimmune serum. This clearly indicates that a considerable decrease of virulence for horses had taken place, but after 10 generations in guinea-pigs the attenuation had not reached such a level as to permit of the injection into fully susceptible animals with perfect safety.

The 3 survivors, after an interval of 28 days, were solidly immune to the homologous strain of virus, again indicating no diminution of antigenic activity.

A striking feature of the experiment is the fact that the horses which received the lowest concentration of brain substance reacted most severely. If the reactions produced in horses 20355 and 20371 (1:1000 and 1:500 dilution) were interchanged, there would have been a perfect gradation from no reaction with the highest concentration (1:20) to the most severe reaction, with shortest period of incubation and earliest death, with the lowest concentration. The virus titration in mice shows that 0·05 c.c. of a 1:50,000 dilution contained less than 1 minimal lethal dose of virus for mice. Therefore the 1:5,000 dilution contained between 1 and 10 infective doses and the 1:20 dilution between 250 and 2,500 infective doses. Thus the horse (20367) which received a maximum of 2,000 mouse-infective doses reacted more severely than the horse (20360) which received probably 500,000 mouse-infective doses.

This observation appeared to be of such importance that a second experiment was planned on similar lines to confirm the unexpected finding. Unfortunately, just when it appeared likely that a similar result would be the outcome, a virulent outbreak of respiratory catarrh and pneumonia due to a streptococcal infection occurred in the stables. Several of the animals in the experiment were so severely affected that the results of the virus injections were completely obscured, and a third more comprehensive experiment was carried out as indicated below. All the animals were injected on 23/6/33 and the dose in each case was 10 c.c. of the indicated dilution of brain material subcutaneously.

Group 1.—*Strain O Virus after* 40 *Passages in Mice.*

Horse.	Dilution.	0·05 c.c. in Mice Intracerebrally.	Reaction.
20541	1/100	4 : 4	Reaction from 4th day. Died 9th day.
20542	1/100	—	Reaction from 3rd day. Died 6th day.
20543	1/10,000	5 : 6	Reaction from 3rd day. Died 9th day.
20544	1/10,000	—	Reaction from 4th day. Died 9th day.

GROUP II.—*Strain O Virus after 21 Passages in Guinea-pigs.*

Horse.	Dilution.	0·05 c.c. in Mice Intracerebrally.	Reaction.
20572	1/50	5 : 6	Reaction from 3rd day. Died 9th day.
20573	1/50	—	Reaction from 3rd day. Died 10th day.
20581	1/500	5 : 7	Reaction from 4th day. Died 9th day.
20582	1/5,000	6 : 6	Reaction from 3rd day. Died 9th day.
20583	1/5,000	—	Reaction from 4th day. Died 10th day.

GROUP III.—*Strain* 20449 *after* 51 *Passages in Mice.*

Horse.	Dilution.	0·05 c.c. in Mice Intracerebrally.	Reaction.
20545	1/100	4 : 4	Mild fever from 19–23rd day. Recovered.
20546	1/100	—	No reaction.
20569	1/1000	4 : 5	,,
20570	1/10,000	5 : 6	,,
20571	1/10,000	—	,,

GROUP IV.—*Strain* 20449 *after* 23 *Passages in Guinea-pigs.*

Horse.	Dilution.	0·05 c.c. in Mice Intracerebrally.	Reaction.
20585	1/50	5 : 6	Too wild to temperature. No clinical reaction.
20586	1/50	—	No reaction.
20587	1/500	5 : 7	Moderate reaction from 8th to 11th day. Recovered.
20588	1/5,000	6 : 7	No reaction.
20589	1/5,000	—	,,

RESULTS.

Four horses received various dilutions of O virus which had been passaged for 40 generations in mice and five horses received O virus after 21 passages in guinea-pigs. All died from horsesickness as a direct result of the injection.

Five horses received various dilutions of virus, strain 20449, after 51 generations in mice, and five horses received strain 20449 after 23 generations in guinea-pigs. Of the 10 animals eight showed no clinical or febrile reaction, one was too wild to temperature but showed no clinical reaction, one reacted mildly, and one showed a fairly severe reaction but made a rapid recovery.

At various times after recovery all the survivors were shown to be solidly immune to the homologous strain of virus.

CONCLUSIONS.

After 40 generations in mice and 21 generations in guinea-pigs O virus had not reached a level of attenuation sufficient to permit of recovery of any of the injected horses. Some attenuation probably had occurred, since the course of the disease was prolonged about 3 days beyond the time that would have been expected from the use of fully virulent virus.

After a slightly greater number of generations in both mice and guinea-pigs strain 20449 appeared to have become sufficiently attenuated to render its injection into horses perfectly safe. This great difference in the results obtained with the two strains of virus cannot be ascribed solely to the slight difference in number of brain to brain passages in the small animals. Either O virus is a strain which does not lend itself to rapid attenuation or after its 193 passages in horses it had become "fixed" for horses, with the result that attenuation probably will take place eventually but will take very much longer. The latter is the more acceptable explanation, since strain 20449 was originally no less virulent for horses, but, as it was isolated in its first generation in horses, it is capable of undergoing a metamorphosis from viscerotropism to neurotropism more rapidly and more completely.

In this experiment no difference could be detected in the results obtained from the injection of the highly concentrated or diluted brain emulsion. No explanation for this discrepancy can be offered. From the point of view of ultimate economic production of a vaccine, it has not been determined what concentration of brain material will constitute the most efficient antigen, nor how many doses can be obtained from a single mouse or guinea-pig brain.

B. THE IMMUNIZATION OF MULES.

The experiments carried out on horses indicate that a gradual progressive attenuation results from the serial passage of the virus through mice and guinea-pigs by the intracerebral route. After a limited number of passages the injection of a partially fixed neurotropic virus, not unexpectedly, may produce severe reactions and even some mortality in highly susceptible horses. Since mules are considerably more resistant than horses, it was decided at this stage to attempt the immunization of a few mules in the hope that some light might be thrown on the change which was taking place in the virus.

The virus strains had been passaged for a greater number of generations in mice than in guinea-pigs so that in a preliminary experiment on 10 mules the "mouse" virus was used.

The injections given and the results obtained are shown in tabular form below. It will be noticed that the animals were divided into two groups. Group I were given an initial injection of various dilutions of brain material representing mouse generation 34, strain 20449; after completion of the immunity test against the homologous strain of this virus they received various dilutions of material representing mouse generation 35, strain O. In group II the reverse procedure was adopted. The primary injections comprised various dilutions of mouse generation 29, strain O, and after the homologous immunity test mouse generation 49, strain 20449, was given.

The second table records the infectivity of the different virulent brain emulsions, as indicated by titration in mice.

TABLE I.

Mule.	Date.	Mouse gn. 34/20449.		Result.	Immunity Test 5 c.c. Virulent blood i.v.		Date.	Mouse gn. 35/0.		Result.	Immunity Test 5 c.c. O Virus i.v.	
		Dose.	Conc.		Date.	Result.		Dose.	Conc.		Date.	Result.
20597	3/4/32	20 c.c.	1/25	No reaction	25/4/33	Fever 7th to 14th day	27/5/33	10 c.c.	1/25	No reaction	16/6/33	No reaction.
20598	3/4/33	10 c.c.	1/250	Mild fever 9th to 16th day	25/4/33	Slight fever 10th to 16th day	27/5/33	10 c.c.	1/100	No reaction	16/6/33	No reaction.
20599	3/4/33	10 c.c.	1/2,500	Severe reaction 7th to 16th day	25/4/33	Slight fever 7th to 16th day	27/5/33	10 c.c.	1/1,000	Intermittent fever 9th to 14th day	16/6/33	No reaction.
20600	3/4/33	10 c.c.	1/25,000	Slight fever 7th to 13th day	25/4/33	Slight fever 8th to 17th day	27/5/33	10 c.c.	1/10,000	No reaction	16/6/33	No reaction.
20601	3/4/33	10 c.c.	1/250,000	No reaction	25/4/33	Reaction from 4th day. Died 7th day	—	—	—	—	—	—

Mule.	Date.	Mouse gn. 29/0.		Result.	Immunity Test 5 c.c. O Virus i.v.		Date.	Mouse gn. 49/20449.		Result.	Immunity Test 5 c.c. Virulent blood i.v.	
		Dose.	Conc.		Date.	Result.		Dose.	Conc.		Date.	Result.
20602	25/4/33	10 c.c.	1/25	Reacted from 10th day. Died 13th day	—	—	—	—	—	—	—	—
*20604	25/4/33	10 c.c.	1/25	Severe clinical reaction from 7th to 16th day	19/5/33	No reaction	8/6/33	10 c.c.	1/25	No clinical reaction	23/6/33	No reaction.
20605	25/4/33	10 c.c.	1/100	No reaction	19/5/33	No reaction	8/6/33	10 c.c.	1/100	No reaction	23/6/33	No reaction.
20607	25/4/33	10 c.c.	1/1,000	No reaction	19/5/33	No reaction	8/6/33	10 c.c.	1/1,000	No reaction	23/6/33	No reaction.
*20608	25/4/33	10 c.c.	1/10,000	No clinical reaction	19/5/33	No reaction	8/6/33	10 c.c.	1/10,000	No reaction	23/6/33	No reaction.

* Too wild to temperature.

† The blood used for this immunity test was obtained from a horse which contracted natural horsesickness on a farm (Kaalplaas) some 8 miles from Onderstepoort. The virus is similar to but not identical with strain 20449 and consequently the slight reactions in the mules on immunity test can be explained.

TABLE II.—*Vitrus Titration in Mice. Dose* 0·05 *Intracerebrally.*
GROUP I.

Generation 34.	Strain 20449.	Generation 35.	Strain O Virus.
Dilution.	Course in Days.	Dilution.	Course in Days.
1/25	6 : 7	1/25	6 : 7
1/250	5 : 5	1/100	6 : 7
1/2,500	5 : 6	1/1,000	5 : 5
1/25,000	5 : 7	1/10,000	6 : 7
1/250,000	8 : 0	1/100,000	0 : 0
1/2,500,000	0 : 0	1/1,000,000	0 : 0

GROUP II.—*Generation* 29, *Strain O Virus. Generation* 49, *Strain* 20449.

Generation 29.	Strain O Virus.	Generation 49.	Strain 20449.
Dilution.	Course in Days.	Dilution.	Course in Days.
1/25	8 : 8	1/25	5 : 5
1/100	7 : 8	1/100	4 : 5
1/1,000	8 : 9	1/1,000	5 : 5
1/10,000	9 : 0	1/10,000	6 : 7
1/100,000	0 : 0	1/100,000	6 : 6
		1/1,000,000	0 : 0

RESULTS.

Group 1.—In this group the process of immunization of five animals was commenced by giving various dilutions of strain 20449, the 34th generation in mice being used. There was no mortality, and with the exception of one mule which received the 1:2,500 dilution the reactions were so mild that they might have escaped clinical recognition had a bi-daily temperature record not been kept. On immunity test, after an interval of 22 days, 4 of the mules (20597-20600) showed definite but very mild reactions and recovered; 1 mule (20601) commenced to react on the 4th day after injection and died on the 7th day. The reaction in this animal is of particular interest.

According to the titration of infectivity in mice, 0·05 c.c. of the 1:250,000 emulsion contained approximately 1 lethal dose of virus for mice, since only 1 out of 2 injected mice died and death occurred at least a day later than was anticipated. Therefore, it is not unreasonable to assume that the 10 c.c. of this dilution injected into the mule (20601) contained less than 1 mule-infective dose. If less than a single infecting dose was injected there would be no multiplication of the virus in the body and no immunity would result. Consequently the death of the animal on immunity test is easily explained, and this animal serves as a control for the virulence of the blood used.

After an interval of 32 days, the four surviving mules were injected with virulent brain emulsion comprising mouse generation 35, strain O, in dilutions varying from 1/25 to 1/10,000. There

was a slight fever reaction in one animal (20599) which had been injected with the 1/1,000 dilution. The remainder showed no clinical or febrile reaction, and all were solidly immune 20 days later.

Group II.—Immunization of five mules was commenced with the injection of various dilutions of virulent brain material comprising generation 29, strain O. The two animals which received the highest concentration reacted severely; the one (20604) recovered and the other (20602) died, the long incubation period, however, indicating that an attenuation of the virus had taken place. The remaining three mules did not react. On immunity test after an interval of 24 days, the four surviving mules proved to have developed a solid immunity.

In the light of the experience with mule 20601 in group I above, the failure of mule 20608 to react to the immunizing injection, and yet proving to be immune subsequently is of interest. According to the titration of infectivity in mice the 1/10,000 dilution used contained barely one mouse-infecting dose, since only one out of two injected mice died. The 10 c.c. given to the mules, therefore, must have contained only slightly more than 1 mule-infective dose and consequently an active immunity developed. It is unfortunate that the animal was too vicious to temperature, because the previous results have shown that often it is necessary to differentiate between a clinical and merely a febrile reaction.

In addition, it is worthy of note that the animals which reacted very severely both received the higest concentration of brain material containing a virus which had been subjected to a limited number of passages—29. This result is precisely the opposite to that obtained from the use of guinea-pig brain emulsion in horses. This observation is discussed later.

After an interval of 20 days the four mules received various dilutions of brain emulsion comprising generation 49, strain 20449. There were no reactions and 15 days later a solid immunity to the homologous strain of virus had developed.

It is difficult to put forward a convincing comparison of the results obtained in group I and group II, since adequate data have not been collected in regard to the degree of cross-immunity existing between the two strains of virus used before neurotropic fixation. But, it is interesting to note that immunization against one strain produced an undoubted immunity against the other, since the first series of immunizing injections produced reactions which were considerably more severe than those resulting from the second virus. This difference cannot be ascribed entirely to the viruses used being at different levels of attenuation by passage. Further work is in progress on the antigenic structure of the different strains of virus isolated so that a full discussion on cross-immunity will have to be reserved for a later publication.

Conclusions.

The results obtained with mules were approximately those anticipated from a consideration of the previous results obtained with horses. It is shown to be possible to immunize mules by the injection of a modified virus which has been attenuated by serial passage

through both mice and guinea-pigs. Moreover, every animal which survives an injection of a certain infective dose of neurotropic virus, irrespective of whether a demonstrable reaction or not is produced, is immune to the homologous strain as contained in virulent equine blood. The more resistant species, the mule, is able to withstand the injection of a partially fixed neurotropic virus better than horses, and it is shown that the one strain of virus (20449) is attenuated at a greater speed than the other strain (O).

The results were so encouraging that it was decided to run a second experiment on 10 mules with the object of—

(1) comparing the relative value of the guinea-pig and mouse viruses;

(2) comparing the production of immunity as a result of the injection of minimal doses of infective emulsion;

(3) ascertaining whether a series of injections of different strains of virus could be replaced by a single injection of a bivalent mixture, i.e. to obtain some information as to the possibility of producing a polyvalent vaccine consisting of a mixture of known antigenically different strains.

The injections given and the results obtained are shown in tabular form below. It should be stated that the different dilutions of each strain from both guinea-pigs and mice were made up separately, and after dilution the requisite mixtures were made.

A. Guinea-pig Virus.—Generation 23, *Strain* 20449 + *Generation* 21, *Strain O.*

Mule.	Date.	Dose.	Dilution.	Result.
20596	23/6/33	10 c.c.	1/50	Moderate reaction from 4th to 12th day.
*20603	23/6/33	10 c.c.	1/50	Very mild indefinite febrile reaction.
20606	23/6/33	10 c.c.	1/500	Moderate reaction from 4–14th day.
20610	23/6/33	10 c.c.	1/5,000	Severe reaction from 4-16th day. Recovered.
20609	23/6/33	10 c.c.	1/5,000	Mild febrile reaction from 7–14th day.

B. Mouse Virus.—Generation 51, *Strain* 20449 + *Generation* 40, *Strain O.*

Mule.	Date.	Dose.	Dilution.	Result.
20611	23/6/33	10 c.c.	1/100	Reaction commenced 3rd day. Died 11th day.
20612	23/6/33	10 c.c.	1/100	Severe reaction from 4–16th day. Recovered.
20613	23/6/33	10 c.c.	1/1,000	Reaction commenced 4th day. Died 12th day.
*20614	23/6/33	10 c.c.	1/10,000	Mild indefinite febrile reaction.
20615	23/6/33	10 c.c.	1/10,000	Moderate reaction from 5th to 11th day.

* NOTE.—After an interval of 28 days, mule 20603 received an immunity test of 5 c.c. of virulent blood strain 20449 intravenously, and mule 20614 5 c.c. of virulent blood strain O. neither animal showed any reaction so the remainder were turned out to grass to be exposed to natural infection.

Results.

Of the five mules which received dilutions of the guinea-pig virus all showed definite reactions, but only one (20610) reacted so severely that any doubt was entertained as to its ultimate recovery.

In group B, which received mouse virus, all the mules reacted. The reactions clinically were of a much more severe nature than in group A, and there were two deaths.

The immunity test which was carried out may be considered inadequate, but it must be borne in mind that previous work has shown conclusively that every animal which receives a sure infecting dose of virus develops a solid immunity. Hence the demonstration that immunity against both strains of virus had been induced was regarded as sufficient. During the period the mules have been at grass there has been no natural outbreak of horsesickness, so that no additional information has been collected.

Conclusions.

Again it is demonstrated that horsesickness virus is more rapidly attenuated by passage through the guinea-pig than through the mouse, but it cannot be stated whether the attenuation after complete fixation in both animals eventually would attain the same level. This point will be tested adequately in due course.

It is shown that it is possible to immunize against two strains of virus simultaneously, but when two partially attenuated strains are used the reactions produced by either strain separately are considerably less severe than the reactions produced by the injection of a mixture of equal parts of the two strains.

Attention must be directed to the fact that the two deaths and possibly the most severe reaction occurred in those animals which received the highest concentration of mouse virus, and the mildest reactions occurred in those animals which received the highest concentration of guinea-pig brain emulsion. The possible significance of this finding is discussed below.

Lastly it is clear that high dilutions of virulent brain emulsion make satisfactory antigens so long as one or more minimal infective does are injected. This point is of extreme importance from the point of view of the ultimate economic production of a vaccine in bulk.

DISCUSSION.

The experiments on mules and horses detailed above indicate conclusively that there is a profound alteration in the nature of the virus of horsesickness as a result of serial brain to brain passage through both mice and guinea-pigs. No accurate data as to the virus content of virulent equine blood have been collected, since the cost of carrying out an accurate titration in horses is prohibitive; but the alteration in the virus on neurotropic fixation represents not a decrease in infectivity, since high dilutions of brain emulsion are infective, but does represent some change in the actual nature of the virus resulting in a marked natural attenuation. The change from viscerotropism to neurotropism is not accompanied by any apparent alteration in antigenic power. Consequently the use of a neurotropic virus " fixed " for mice or guinea-pigs has been shown to constitute a rational method of immunization against horsesickness.

Attenuation by passage through the guinea-pig occurs at a greater rate than by passage through the mouse, but no opinion can be expressed as to the ultimate level which will be attained after repeated subinoculation over a number of years. Whether the degree of attenuation in either animal will eventually reach such a point that injection into horses will be perfectly safe in every case, has yet to be determined. This point will only be cleared up by the collection of data from a large number of animals since the susceptibility of individual horses is known to vary within wide limits. At the present moment it is hoped that attenuation will reach a level which is consistently safe and that it will not be necessary to resort to the preliminary injection of a virus artificially attenuated by chemical or physical means, or to modify the reaction by the simultaneous injection of hyperimmune serum.

A consideration of the results obtained from the experiments recorded indicates that when once the anti-body producing mechanism of the animal body has been stimulated, it is comparatively easy to reinforce the initial immunity. This point was demonstrated by the mildness of the reactions produced by neurotropic virus O in animals previously immunized against strain 20449, whereas the same material produced severe reactions and even mortality in fully susceptible animals. Consequently, it has still to be decided whether the ultimate procedure will consist of a series of injections of different virus strains or a single injection of a polyvalent mixture. The possibility of the latter procedure has been demonstrated by the successful immunization of mules against two viruses by a single subcutaneous injection.

This conception of the production of a polyvalent vaccine constitutes a considerable advance over previous methods. Both the hyperimmune serum-virus simultaneous method and the formalized spleen virus method have as their basis the use of a stingle strain (strain O) which, experience has shown, is capable of immunizing against a greater number of different strains than any other virus isolated. Yet the immunity produced by these methods is not complete as evidenced by periodical breakdowns in the field; practical difficulties and cost prevented any attempt at producing either a polyvalent serum or formalized vaccine. With the neutrotropic virus method an attempt certainly can be made to immunize against all strains that are isolated, since a technique of *in vitro* neutralization* has been developed which permits of an evaluation of the antigenic structure of the different natural strains of virus which have been encountered.

In conclusion it must be pointed out that up to the present it has merely been shown that immunization by means of an attenuated neurotropic virus is possible. The work on mass production of such a vaccine is still in its initial stage, since a very large number of important points have yet to be subjected to a thorough investigation. For example, the relative efficacy of the mouse and guinea-pig adapted viruses have not been evaluated. The ease of serial transfer and the short course of the disease produced in mice gives preference to this animal for rapid passage and maintenance of a virus. On the other hand, the guinea-pig possesses a brain which is approximately 5 times the size of that of the mouse, though the

* This forms the basis of a report to be published later.

barins of moribund mice have been found to contain about 10 times more virus per unit of brain substance so that again preference must be given to the mouse for the production of large qualities of virus. In spite of this, attention must be directed to the phenomenon of high concentrations of virulent guinea-pig brain on some occasions producing very much milder reactions than low dilutions, whereas the reverse has been the experience with mouse material. No adequate explanation can be offered for the phenomenon at this stage, but the tentative suggestion is advanced that the prolonged development of the virus within the cells of the guinea-pig brain may result in the production of a non-infective antigenic substance in appreciable amount analogous to the specific poly-saccharide antigens of some bacteria.

This report therefore must be regarded as being of the nature of a progress report since, to be of any real value, work on the immunization of equines against horsesickness must be carried out on large numbers of animals because the individual susceptibility of different horses varies so greatly that a true interpretation of the relative value of different methods of immunization is only possible after the collection of adequate data from work on large numbers of animals housed and maintained under a variety of conditions.

SUMMARY.

(1) Details of the results obtained from the injection of horses and mules with neurotropic mouse and guinea pig adapted virus are given.

(2) It is shown that the virulence of horsesickness virus progressively decreases for equines as neurotropic fixation takes place by serial passage through mice and guinea-pigs.

(3) The attenuation occurs more rapidly through the guinea-pig, but it is not known whether the ultimate level will not be the same.

(4) All animals which survive an injection of one infective dose of neurotropic virus, whether or not a demonstrable reaction is produced, are immune to the homologous strain of virus. Immunity to heterologous strains of virus is at most only partial.

(5) No difference in favour of either the subcutaneous or intravenous method of injection could be determined.

(6) It is shown that the subcutaneous injection of as small a dose as 10 c.c. of a 1:10,000 dilution of infective brain emulsion is adequate.

(7) Attention is directed to the phenomenon of a high concentration of infective guinea-pig brain emulsion producing a milder reaction than a low but still infective concentration.

(8) The possibility of developing a polyvalent vaccine is discussed.

REFERENCES.

NIESCHULZ, O. (1932). Over die infectie van muizen met het virus der Zuid-Afrikaansche paardenziekte. *Tydsch. voor Diergeneesk.* Vol. 59, No. 24, pp. 1433-1445.

ALEXANDER, R. A. (1933). Preliminary notes on the infection of white mice and guinea pigs with the virus of horsesickness. *Jnl. S.A.V.M.A.*, Vol. 4, No. 1, pp. 1-9.

SAWYER, W. A., KITCHEN, S. E., & LLOYD, W. (1932). Vaccination against yellow fever with immune serum and virus fixed for mice. *Jnl. of Exp. Med.*, Vol. 55, No. 6, pp. 945-969.