STUDIES ON THE NEUROTROPIC VIRUS OF HORSESICKNESS.

by

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ADDENDA.

1. 1933. ALEXANDER, R.A. Preliminary note on the infection of white mice and guinea pigs with the virus of Horsesickness. Journ. of S.A. Vet. Med. Ass. 4, 1, 1 - 9.

2. 1934. ALEXANDER, R.A. and DU TOIT, P. J. The immunization of Horses and Mules against Horsesickness by means of the neurotropic virus of Mice and Guinea Pigs. Onderstepoort Journ. of Vet. Sc. and An. Ind. 2, 2, 375-391

MEG.

STUDIES ON THE NEUROTROPIC VIRUS OF HORSESICKNESS.

NEUROTROPIC FIXATION

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The problem of the control of horsesickness is one which has presented many peculiar difficulties to those who been actively engaged in its solution. It is outside the scc of this article to enumerate those difficulties but not the lea has been the lack of an easily procurable, highly susceptible small laboratory animal, on which to carry out intensive research work on a sufficiently comprehensive scale. All the work had to be conducted on either horses and mules with the result that the almost prohibitive cost effectively limited the scope and speed of research. Consequently the progress made, and that was considerable, must always remain a monument to the patience and keen powers of observation and deduction of those, notably Theiler, who made the problem their own.

In 1910 Theiler[1] reported the susceptibility of the dog to African Horsesickness, but this animal subsequently proved of little value for experimental purposes. In 1932 Nieshulz[2] reported the susceptibility of the white mouse to the virus. Indepently and concurrently this was confirmed at Onderstepoort[3], and in addition the full susceptibility of the guinea pig was recorded. These observations immediately opened up a wide field for research into many problems connected with the disease. The results accumulated from this work form the basis of this report.

For the sake of completeness but also to illustrate the technique and methods finally adopted it is essential to detail the peculiar features of the neurotropic fixation of the virus in mice, guinea pigs and rats even though several publications on this subject have appeared in recent months. 4,5.

A. Fixation in Mice.

The credit for the selection of the white mouse as a potential experimental animal must be given to Max Theiler, who in 1930 published his "studies on the action of Yellow Fever in mice" (4). The important and far reaching results of his work prompted the application of similar methods to the study of horsesickness.

A short preliminary account has been published previously (3) on the establishment of a strain of virus, strain O, in mice. Since that initial fixation no less than seven strains from different sources have been fixed with comparative ease so that an account of the procedure now adopted as a routine may be of interest.

Technique. For all this work the most suitable syringe appears to be the 1 c.c. "Agla" tuberculin syringe manufactured by Messrs. Burroughs Wellcome. Possessing no metal parts whatever, it may be dropped into boiling water in a sterilising dish, without being dismantled with perfect safety. Being hand made and calibrated the graduations are exceedingly accurate; for dilution and titration work these syringes are preferred to pipettes. The most suitable needles are B.W. & Co. No. 20, which are conveniently short and strong and are sufficiently fine.

The use of young mice (approximately two months old) is strongly advised, in preference to full grown adults. At that age the skull is still cartilagenous and soft, so that, for injection, the needle may be inserted directly into one of the cerebral hemispheres. When adult mice are used intraeerebral injection can be carried out only by inserting the needle through the dorsal portion of the ~~foramen~~ foramen magnum, and some mortality occurs proba ly as a result of injury to the nerve

roots or vital centres of the brain stem.

A convenient and safe dose has been found to be 0.05 c.c. This volume is represented by a single division of the graduations on the syringe so that it may be injected constantly with great accuracy, and for this reason is preferred to the 0.03 c.c. advocated by American workers. Experience with many thousands of mice has shown that this injection is perfectly safe and after a little practice the mortality as a direct result of injection should not be greater than .5%. It has been found quite unnecessary to use a general anaesthetic, in fact the use of a general anaesthetic would so delay the operation as to make it a burden. Mice stand the injection well. For a few minutes they appear to be dazed but after about 15 minutes the majority will be seen running about the box or feeding.

Careful disinfection of the site of injection also has been found quite unnecessary provided no more than 10 mice are injected with each needle before sterilization. Lack of local disinfection may be expected to favour the chance of bacterial contamination but many thousands of mice have been injected without a single loss from abscessation of the brain.

An an initial source of virus preference is given to defibrinated blood withdrawn from a horse at the height of the febrile reaction to horsesickness. Infective blood, to which calcium citrate or calcium oxalate-phenol-glycerine has been added as an anticoagulent or preservative may be used. Such material is not entirely satisfactory since these substances are decidedly toxic when injected intracranially. This toxicity necessitates dilution of the blood to render injection safe and this minimizes the chance of rapid establishment of the virus. Nevertheless a strain of virus which had been stored for 12 months in oxalate-carbol-glycerine preservation has been

'fixed' with no great difficulty.

For routine subinoculations the supernatant fluid obtained after centrifuging suspensions of brain material in saline has been used. It is preferable to sacrifice mice when in extremis to obtain infective material rather than to allow them to die, since autolysis sets in fairly rapidly and such brains are difficult to remove and to handle with complete asepsis. To remove the brain from the mouse onto a board, abdomen downwards and pour a little 95% alcohol over the entire head and body as a disinfectant. Reflect the skin cranially from a point behind the withers and lay the entire skull bare. Again disinfect with spirits. All instruments must be sterilised by boiling. Insert the point of a pair of fine straight scissors into the foram magnum and snip the bones of the skull to a point just behind and above the eye. With the point of the scissors lift up the roof of the skull, push the scissors through the medulla and under the brain which may then be lifted out intact since the majority of the nerve roots will have been severed. Place the brain in a sterile centrifuge tube fitted with a cork or a cap, and macerate it with any sterile instrument such as a glass rod. Add the requisite amount of saline and make a fine emulsion by repeatedly drawing the brain substance into and forcing it out of a syringe. Centrifuge for a few minutes at about 1500 revolutions per minute to deposite gross particles and the opalescent supernatant fluid is ready for injection. It is undesirable to use a brain emulsion more concentrated than 1 part to 10 parts of saline, since in higher concentration the brain substance is decidedly toxic.

Fixation of a strain. For the first generation in

in mice not less than 10 individuals should be injected intracerebrally since it may happen that not more than 20% will show any symptoms and the actual mortality may be nil. As soon as a mouse is noticed to be ill, i.e. remains huddled up in a corner by itself or is noticed to have difficulty in running about owing to a paresis of the hindquarters subinoculate the brain into 10 other mice. In the second generation in mice, rather more than 80% can be expected to show fairly typical symptoms, and of this number few will recover. In the third generation the mortality is usually 100% and from then on the number of mice used to carry on a strain is purely a matter of individual choice. For each passage five mice have been found to be a satisfactory number. This number may seem excessively large but it must be borne in mind that in later generations death occurs suddenly so that it is not unusual to arrive in the morning to find three or four mice, which appeared perfectly well the previous afternoon dead, and partly decomposed.

In fixing a strain of virus the point to be stressed is that in the early generations large numbers of mice should be injected, so as to be sure of infecting several individuals. Then as the virus becomes adapted to neurotropic propagation the number used for passage may be decreased. To illustrate the procedure full details of the injections carried out for the neurotropic adaptation of one strain of virus is given.

Generation I.

6.2.33. 12 mice injected intracerebrally with 0.05 c.c. defibrinated blood from a reacting horse diluted aa with 0.85% saline.

18.2.33 1 mouse found dead in box, the brain having been eaten by the others.

18.2.33. 2 mice a peared definitely sick. Brains pooled and subinoculated into 5 mice of which 2 were in extremis on 23.2.33 and were passaged, 1 died on 23.2.33, and 2 subsequently a peared sick but recovered.

Generation II.

24.2.33 1 mouse found dead in box and discarded.

28.2.33 2 mice definitely sick, showing posterior paresis. Subinoculated into 5 mice of which 2 died on 6.3.33, 2 were in extremis and passaged on 6.3.33, and 1 remained in normal health for 4 weeks.

2.3.33 1 mouse died and was discarded.

6.3.33 Remaining mice discharged.

The third generation of mice all died between the 5th and 8th day and from then onwards the mortality in each generation was 100%

It will be seen that it is a comparatively simple matter to establish a strain of horsesickness in mice by cerebral passage. It is necessary to exercise patience in waiting for signs of illness in the first generation since the period of incubation may be prolonged up to 3 weeks or more. Once the strain is established it may be maintained indefinitely by brain to brain passage.

Routes of Infection.

In addition to the infection by intracerebral injection, several attempts were made to ascertain whether the disease could be transmitted by the subcutaneous, intramuscular, intraperitoneal or intravenous routes.

1. Subcutaneous. On 21.3.33 10 mice were given a subcutaneous injection of 0.5 c.c. of a 4% saline emulsion of infective brain representing generation 33, strain 20449. The infectivity of the emulsion was controlled by intracerebral injection of 5 mice with 0.05 c.c. of a ten fold dilution in saline of the same emulsion; all the control mice were dead by the sixth day. The 10 mice

were kept under observation for 28 days, without showing any symptoms of ill health. As an immunity test they were then given an intracerebral injection of 0.05 c.c. of a 1% emulsion (generation 38, strain 20449); 3 died on the seventh day, 6 on the eighth day and 3 on the ninth day after injection, whereas of 5 control mice 2 died on the sixth day and 3 on the seventh day.

This experiment was repeated on two occasions using a 10% brain emulsion of mouse generations40 and 59 of the same strain for subcutaneous injection. Not one of the mice were infected. The slightly lenghtened course of the disease on immunity test as compared with the controls injected intracerebrally was apparent in each experiment. This may be taken to indicate that a slight degree of immunity was conferred but that this was insufficient to ~~avert~~ arrest the development of the virus introduced directly into the brain.

2. Intramuscular. A total of 30 mice were injected intramuscularly with 0.5 c.c. amounts of the same virus suspensions used for the subcutaneous tests above. Three mice died within five days of injection but in each case death was due to some cause other than horsesickness because subinoculation of the brains failed to reveal the presence of any virus.

On immunity test intracerebrally all the mice died but again a slightly prolonged course of the disease seemed to indicate that some immunity had been set up.

3. Intraperitonial. Since the infection of mice by the intraperitoneal route, together with the simultaneous traumatic injury of the brain by the intracerebral injection of a 2% starch solution forms the basis of the intraperitoneal protection test in Yellow Fever elaborated by Theiler, it was considered important to determine whether mice could be

infected with neorotropic horsesickness virus by this method. Of a large number of experiments carried out to clear up this point a representative few are tabulated below.

Exp.	Virus Used: Generation	Virus Used: Strain	No. of Mice	Intraperitoneal Injection	2% Starch intracerebrally	Deaths	Intracerebral Immunity Test: Deaths	Intracerebral Immunity Test: Contr
1	33	20449	8	0.5 c.c. of 4%	−	x6,6	8,8,8,9,0	5,6,6
			8	emulsion	+	x,6,6,6,7,8	10,0	
2	55	20449	6	0.5 c.c. of 4% emulsion	−	nil	all died	-
			16		+	x,4	all died	-
3	58	20449	10	0.5 c.c. of 10% emulsion	+	nil	all died	
4	59	20449	5	0.5 c.c. of 20% emulsion	−	nil		
			5	ditto	+	6,16	5,6,7	4,5,5,
			5	0.5 c.c. of 10% emulsion	−	5,6	-	
			5	ditto	+	6	-	
			5	0.5 c.c. of 5% emulsion	−	nil	-	
			5	ditto	+	7.	-	
			5	0.5.c.c. of 2.5% emulsion	−	nil	-	
			5	ditto	+	nil	-	
5	62	20449	5	0.5 c.c. of 10% emulsion	−	nil		
			5	ditto	+	6,6,7	4,7	4,4
			5	0.5 c.c. of 5% emulsion	−	nil	-	
				ditto	+	nil	-	
6	142	20449	5	0.5 c.c. of 5% emulsion	−	17.17	4,4,5	3,4,4,
			5	ditto	+	14	4,4,4,5	3,4,4,
7	40	0	5	0.5 c.c. of 10%emulsion	+	nil	-	
			5	ditto	−	nil	-	

Note: @ The numerals indicate the day after injection on which death of a mouse occurred.
o = survived after intracerebral injection.
x = death at time of injection or due to some other cause other than horsesickness.

A consideration of the results indicates that after intraperitoneal injection of 0.5 c.c. of an infective emulsion of high concentration (10%) an occasional mouse will become infected and will die. When a lower concentration of virus is used it is rare for a mouse to become infected, since none of those used in the experiments died. When the brain is injured simultaneously by the injection of 2% starch solution a greater proportion of mice become infected, particularly when heavy concentrations of brain emulsion (10%) are used. Also an occasional mouse becomes infected when concentrations lower than 10% are used. These observations hold good for a strain of virus which has been neurotropically 'fixed' by 142 passages through mice.

Consequently the uncertainty of setting up the disease by this route with or without injury to the brain makes it quite unsuitable for all practicaly purposes. From an academic point of view it is interesting because it indicates that injury to the brain sets up a 'locus minoris resistiae which serves as a starting point for the multiplication of the virus presumably transported by the blood stream.

Of a limited number of mice subjected to immunity test two survived and the majority succumbed after a lengthened period of incubation, which again indicates that some immunity had developed.

4. <u>Intravenous.</u> The results obtained with the injections given intraperitoneally showed that infection was most likely to be initiated when a large amount of virus was circulating in the blood stream, as a result of the rapid absorption from the peritoneal cavity of a heavy concentration of infective brain emulsion. If this is true, more regular results should be obtained by the injection of large amounts of virus directly into the blood stream. Unfortunately brain

emulsion in a concentration higher than 0.1% is highly toxic when given intravenously in 0.3 c.c. doses. Of a large number of mice injected intravenously, with and without the injection of starch intracerebrally, infection was set up in only a few instances, and the percentage of deaths as a direct result of injection was excessively high.

5. Infection by direct or indirect contact, or per os: In various experiments it is common for healthy mice to be housed in the same box with infected mice in various stages of the disease. In addition the healthy mice frequently devour the dead ~~mice~~, the first portions eaten being the tongue and brain. At no time has there been any indication of the spread of the disease so that it must be concluded that infection cannot take place by direct or indirect contact or by the ingestion of infective material.

Conclusions. By the subcutaneous or intramuscular injection of virus it has not been possible to infect mice. The probable reason is that slow absorption results in a concentration of virus in the blood insufficient to penetrate the barrier between the vascular system and the cells of the nervous ~~mucous~~ system in which multiplication occurs. Injected mice develop a low grade immunity which is insufficient to protect against the intracerebral injection of some hundreds of minimal ~~immunal~~ infective doses of virus.

By intraperitoneal injection, infection may be initiate particularly when the dose is massive and the brain is injured traumatically by the intracerebral injection of 2% starch solution. A greater concentration of virus probably can be produced in the blood by intraperitoneal injection than by injection directly into the blood stream since 0.3 c.c. of a 0.1% emulsion of infective brain is often toxic intravenously

yet 0.5 c.c. of a 10 % emulsion is readily tolerated in the peritoneal cavity from which absorption is rapid.

It must be concluded therefore that the only satisfactory method of infecting mice is by direct intracerebral injection. This finding together with the observation that the disease is not transmissible by direct or indirect contact or per os, renders the mouse particularly suitable as an experimental animal since the risk of accidental infection is reduced to a minimum.

Susceptibility of different strains of mice.

A very real obstacle encountered in this work has been the difficulty of obtaining a regular supply of mice since it has been essential to work in large numbers to accumulate statistical data. Practically all the mice used were obtained from our breeder, Mr. Buchannan of Pretoria North, to whom I am indebted for his efforts to maintain a regular supply of uniform age and size. The strain was originally imported from Natal and has been in-bred on an extensive scale from that time. At no time has any variation in susceptibility been observed. Preference has been given to the use of young mice about two months old because of the ease of handling, housing and injecting, but there does not appear to be any difference in susceptibility between young and adult mice. Usually young mice die about 12 hours earlier than adults but this does not represent any difference in susceptibility to intracerebral injection.

No opportunity has presented itself of comparing the relative susceptibility of the various recognised breeds or strains of mice but brown, grey, black, pubald and the ordinary wild mice have been used and all appear to be equally susceptible.

The Disease in Mice.

Symptoms. After the first shock due to the injection has passed off, the mice appear perfectly normal. Careful daily observation will show that the first sign of illness is a disinclination to feed, the food being either covered up with saw dust or scratched out of the receptacle onto the bottom of the box. An affected mouse will then show a roughened coat and will remain huddled up in a corner away from its fellows. At first it is able to walk and run about quite normally when roused, but gradually it will be noticed that the hind legs appear to be unable to support the body. This initial paresis rapidly becomes more noticeable and spreads cranially so that the mouse eventually can only attempt to drag itself on its abdomen with its hind legs stretched out behind. Eventually it is unable to move at all and remains stretched out in a commatose condition until death, which may occur some hours or even a day later. This picture is exhibited by mice which have received an injection of diluted material representing say 10 to 20 immunal infective doses. When an infection representing severel hundreds of infective doses is given the picture is usually somewhat different. Such mice at first are disinclined to feed and appear nervous and excited. They move about the box in a restless, aimless fashion and at any moment may commence a mad stampede, which may last up to 15 seconds or more. This mad rushing round the box may terminate in a convulsive fit which usually is fatal, the mouse then dying with muscles rigidly ~~turned~~ fixed. Usually, however, after the initial period of restlessness a paresis gradually develops which appears to progress from behind forwards, so that progression is at first

difficult and later impossible, after which coma and death rapidly follows.

The duration of the disease varies considerably. In the early passages individual mice may be ill for several days. In the later passages it is not uncommon for 10 mice in a box to appear perfectly normal in the late afternoon yet next morning more than half will be found dead and the remainder partially paralysed or somatose. After a particular virus has become 'fixed' neurotropically the duration of symptoms is seldom longer than 6 hours, and every mouse which receives a certain infective dose of virus will die.

As serial passage progresses the virulence for mice appears to increase markedly, as indicated by a gradual decrease in the average time between injection and death. This is clearly illustrated by reference to the figures given in Table II, in which is given the average interval between injection and death for the first five generations in mice, and then the average interval for each successive five generations. The figures include data obtained from four different strains of virus being passaged with a view to ultimate vaccine production. The origin of and difference between these strains is discussed later.

Table II.

It will be noticed that the interval between injection and death decreased rapidly for the first few generations but subsequently the disease became very much more gradual. After approximately 80 passages the course of the disease had reached what is believed to be a constant minimum, and during the next 60 passages there was little or no change.

The figures given show a slight fluctuation but this is well within the bounds of experimental error and partly may be explained by the use of mice of different average ages.

In the case of 3 of the strains investigated the majority of deaths occur on the third day after injection, in fact, it has been possible to subinoculate regularly on the third day. In the case of the fourth strain (Cyprus) neurotropic fixation appeared to have been more gradual and death can always be anticipated 12 - 24 hours later. No opinion can be expressed on the possibility of the course of the disease ultimately becoming equal for all strains.

The ~~disease~~ decrease in the course is accounted for both by a decrease in the incubation period and the duration of detectable symptoms. In the early generations it was common for mice to show symptoms of paresis and paralysis for one or more days and to be comatose for 12 hours before death; in the later generations death usually occurs within 12 hours of the onset of visible symptoms.

It must be pointed out that the figures given above were obtained from the records of mice which were each injected with many hundreds and even thousands of ~~immunal~~ minimal infective doses. The period of incubation following injection with say 10 infective doses is usually about 24 hours longer but there is little difference in the interval between onset of symptoms and death.

At this stage it is of interest to note that the increase in virulence for the mouse (the ~~disease~~ decrease of the period of incubation being taken as the index) appears to have run parallel with a ~~disease~~ decrease in virulence for the horse. Strain O, which was the most refractory to neurotropic

fixation become attenuated for horses more slowly than the other strains. It has not been possible to correlate period of incubation in the mouse directly with safe attenuation for the horse.

Identification of the disease transmitted to mice.

At the commencement of this work it was essential to determine that the fatal condition being transmitted to mice actually was the result of infection with the virus of horsesickness. Without entering into details at this stage this was proved without any doubt in the following manner.

1. Susceptible horses were injected subcutaneously and intravenously with infective mouse brain emulsion. Typical clinical Horsesickness was produced which was not always fatal. Blood taken during this febrile reaction reproduced the original condition on intracerebral injection into mice. Those horses which recovered were found subsequently to have developed a solid immunity to the homologous strain of virus.

2. Immune horses did not react to the injection of infective mouse brain emulsion.

3. Serum of the immune horses was found to be capable of specifically neutralysing the virus contained in mouse brain emulsion, in vitro.

From these tests combined with repeated demonstration of bacterial sterility of the brains it was concluded that the virus of horsesickness certainly had been transmitted to and propagated in mice.

Post mortem Appearances.

Post mortem examination reveals no ~~pathoquomonic~~ pathognomonic lesion

Fairly consistently there appears to be a slight tumor splenis but otherwise the internal organs appear to be perfectly normal. Examination of the brain shows a definite and often marked engorgement of the meningeal vessels, but there is no macroscopic change in the brain substance.

Histology of the Brain. The microscopic changes in the brain, cord, and ~~mucous~~ nervous system have not been worked out. It is considered that a correct interpretation of any lesions can only be dealt with by an experienced neuropathologist so that this aspect of the disease in mice will have to be dealt with by some other worker.

Localization of the virus in mice. During the course of serial passage of the various strains of virus abundant material was available for the determination of the localization and concentration of the virus in the organs of mice.

Brain. It has been shown above that mice can be infected with certainty only by direct intracerebral injection. Consequently it occasioned no surprise to find that the central nervous system was the site of the highest concentration of virus. Emulsion of brain material obtained from mice sacrificed when moribund have been found to be infective in dilutions up to 1 in ten million (dose 0.05 c.c.) This is an extreme, but constantly, strain 20464 B is infective in a dilution of 1 : 1,000,000; strain 20449 in a dilution of 1: 500,000 and strain 0 in a dilution of 1 : 50,000. This variation in the virus content of the brains of mice infected with different strains is curious but has been observed constantly and is of practical importance from the

point of view of vaccine production.

Virus may be detected in the brains of mice two days after injection, i.e. before the onset of any clinical symptoms but a marked and rapid increase occurs in the later stages of the disease. The virus ~~taken~~ titre of partly autolysed brains removed several hours after death does not appear to decrease but there is no evidence of increase after death.

Spinal Cord. ~~The~~ distal 1/3 of the spinal cord of groups of mice has been removed at various stages in the course of the disease. Intracerebral injection of suspensions into other mice has shown that as the disease progresses so the concentration of virus increases centrifugally. At no time however has the concentration in the cord been found to equal that in the brain.

Nerves. In the later stages of the disease virus may be demonstrated easily in the peripheral nerves notably the sciatic. The concentration was never found to be high but this is probably due to the difficulty experienced in adequality ~~underlying~~ emulsifying the somewhat fibrous nervous tissue under strictly aseptic conditions.

Liver, Spleen, Kidneys. On numerous occasions these organs have been carefully removed, thoroughly ground up with sand in ~~immunel~~ minimal quantities of saline and the supernatant fluid, after centrifuging, injected into mice. An occasional mouse that received material from each of these organs has died and subinoculation demonstrated that death was due to horsesickness, but so many negative results were obtained that it must be concluded that little or no virus is present in the internal organs. When it is present it is there apparently by accident and association with nervous tissue cannot be excluded.

Blood. The blood of mice at any stage after intracerebral injection was never found to be infective. This does not exclude the possibility of virus circulating in the blood stream shortly after intracerebral injection.

Adrenals. Theiler, in his work on Yellow Fever mentions a relatively high concentration of that virus in the adrenals of mice. This finding could not be confirmed in the case of Horsesickness.

Discussion. The susceptibility of the mouse to the virus of Horsesickness has been demonstrated and confirmed. This susceptibility presents several peculiar features which merit special attention.

Horsesickness, in equines, is of the nature of a septicaemia, and is characterised by a concentration of virus in the blood and those organs which are richly vascularised, notably the spleen and liver. No data are available as to the concentration of virus in the brain but it is generally accepted not to be high. Clinically there is no record of the symptoms in horses being correlated in any way with nervous derangement. In the mouse the disease assumes neurotropic characters exclusively. The virus at death is concentrated in the nervous system, in fact its rare demonstration in any organ cannot be disassociated from the nervous system and the only certain method of infection is by direct intracranial injection. In addition the clinical picture exhibited by an affected mouse is consistent with that of an animal suffering from an acute nervous affection. Consequently the metamorphosis from exclusive viscerotropism in one susceptible animal to

to neurotropism in another is a phenomenon for which no adequate explanation can be advanced in the light of the present state of our knowledge of biology.

A similar change has been demonstrated to occur in the case of the virus of Yellow Fever, a disease which has many features in common with Horsesickness. It still remains to be seen whether other viruses will exhibit the same characteristic.

B. Neurotropic fixation in Guinea Pigs.

After the demonstration of the susceptibility of the mouse, and after the possibility of utilising the neurotropic virus as the basis for a method of immunising horses had been appreciated, a search was made for a larger experimental animal to provide a more copious supply of virus. The guinea pig was selected, and as previously reported (3) its susceptibility was established immediately. This finding has been confirmed by Nieschulz.(5)

Technique. The intracerebral injection of guinea pigs is rather more difficult than the similar operation in mice. After clipping the hair at the back of the head and neck the guinea pigs should be deeply anaesthetised with ether by placing them in a bell jar containing a pledget of cotton wool soaked in ether. When anaesthesia is sufficiently deep, instruct an assistant to hold the guinea pig in the dorsal position with the abdomen pressed tightly against the bench as a means of firm control. Swab the site of injection with spiritus as a disinfectant, grasp the head in the left hand and sharply flex it on the neck. The upper border of the foramen magnum can then be felt and the needle of the syringe can be inserted through its dorsal portion directly

into the brain. The point of the needle must not be directed downwards as experience has shown that the injection of fluid into the medulla or the brain stem is frequently followed by gross incoordination of movements with subsequent death, or alternately sudden death from shock.

Injection through the foramum has been practised in preference to the method advocated by Nieschulz, of forcing the needle through the skull at a point slightly behind the ear, since the number of accidents was found to be considerably less.

The dose of fluid injected may be made as large as 0.4 c.c. provided it is given slowly but 0.25 c.c. doses have been given as a routine since this amount is tolerated well.

For the rest the technique is identical with that used for the infection and subinoculation of mice.

The Disease in Guinea Pigs.

For the original experiments on guinea pigs mouse passage virus (generation 7, strain 20449) was used. It will be seen from reference to table II above that at that time the strain had been adapted to neurotropic multiplication only partially, since injected mice on an average did not die until the fifth day. No attempt has been made to fix a strain of virus in guinea pigs, using infective blood from a horse as the inital source of infection. According to Nieschulz that virus produces no demonstrable reaction in guinea pigs, and it was found far simpler to passage a given virus for a few generations in mice and then to transfer it to guinea pigs.

Symptoms. The disease in the guinea pig is of particular interest because it afforded an opportunity of determining whether the development of the virus in the central nervous system is accompanied by any general febrile reaction.

Under the conditions of maintenance of small animals for this work it was found that ~~[illegible]~~ 101·8° at 8.30 in the morning and ~~[illegible]~~ 103·0° at 4.30 in the afternoon could be regarded as the upper limits of the normal daily fluctuation in temperature of the average healthy guinea pig. This range has been laid down after careful recording of the temperature of a large number of animals over a period exceeding 18 months. In addition it was found that as a direct result of the shock of intracerebral injection of either brain emulsion or an inert substance like starch the body temperature may rise or fall 2° or 3° but within 24 hours it will have returned to normal. If the brain is injured during the operation the temperature within 24 hours drops well below normal so that an early maintained subnormal temperature is a bad prognostic sign requiring rejection of the animal from the experiment.

Selected temperature charts of four guinea pigs representing different stages in the serial passage of strain 20449 are shown in Charts I - IV below to illustrate the febrile reaction.

Chart I

Inoculation 7 in guinea pigs

← intracerebrally 0·25 cc brain emulsion

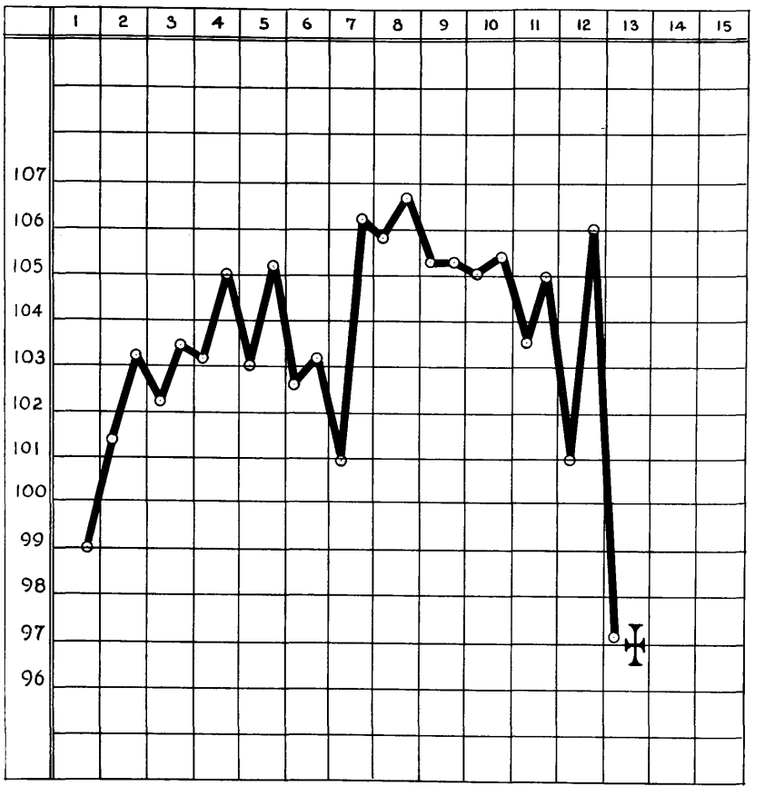


Chart II

Inoculation 7 in guinea pigs

← intracerebrally 0·25 cc brain emulsion

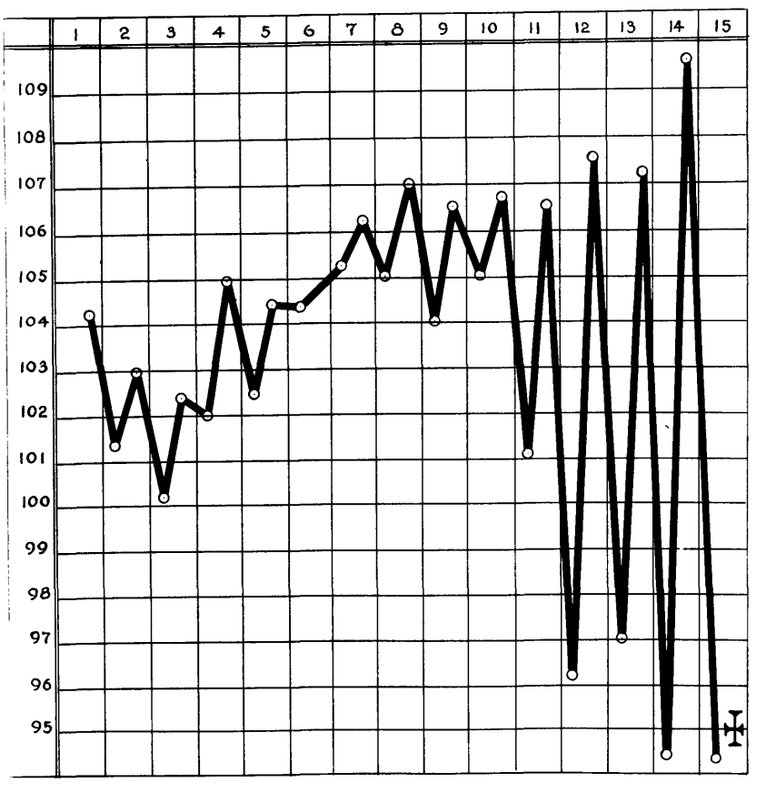


Chart III

Immunised 16 in guinea pigs

← intracerebrally 0·25 cc brain emulsion

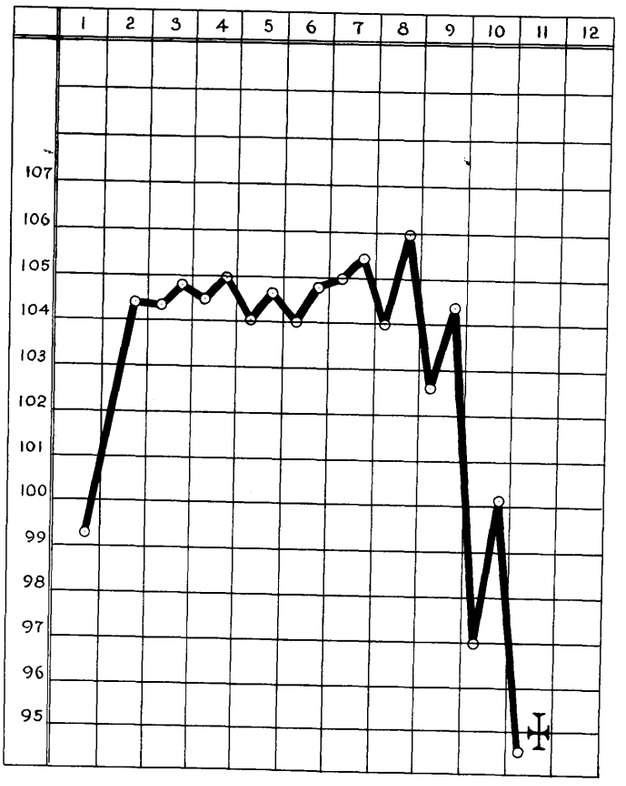
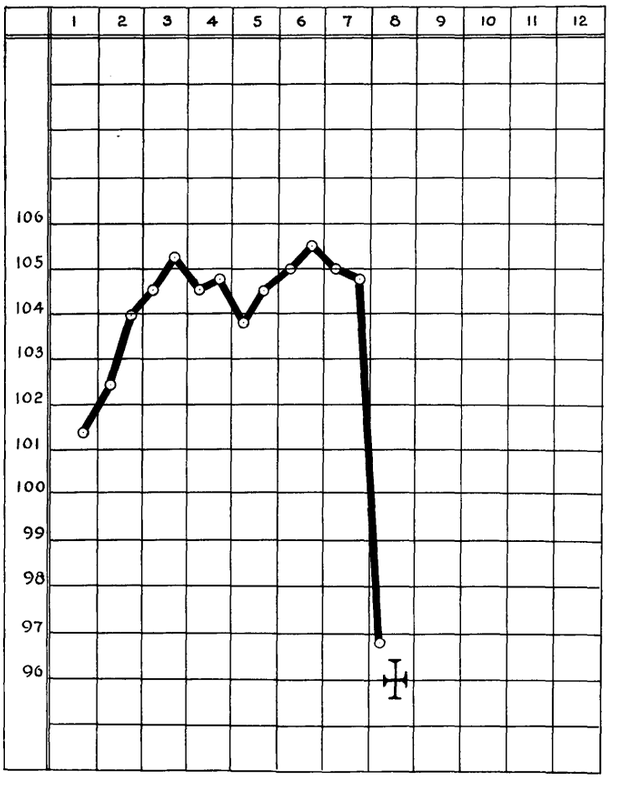


Chart. IV

Immunised 26 in guinea pigs

← intracerebrally 0·25 cc brain emulsion



It will be seen that the actual period of incubation after intracranial injection is exceedingly short. In the early passages hyperthermia does not commence until the lapse of 48 hours but in the later passages the temperature is well elevated within 24 hours. There is a distinct tendency for the curve to be diphasic but this is by no means marked. The maximum rise is not particul rly high when the great normal fluctuation is taken into consideration but 106° or higher has been recorded frequently. Termination is by crisis and this, in the early passages, is interesting, as will be seen from Chart II, the record of one of the animals in the seventh passage. The morning temperature suddenly drops to a point well below normal, in fact, it may be so low that the mercury does not reach the first graduation of an ordinary clinical thermometre (recorded as NR = no reading) and, to the touch, the guinea pig feels 'icy cold'. The afternoon temperature may rise to an excessive height. This tremedous daily fluctuation was a constant feature during the progress of 'fixation' and might continue for several days before death occurred. In the later passages (chart IV) the sudden drop in temperature preceded death by 12 to 24 hours and there was little or no fluctuation in the subnormal temperature.

During the initial portion of the febrile period guinea pigs as a rule show no symptoms of ill health. They feed well and remain normally active. Gradually inappetence supervenes and movements become sluggish. Then the animal remains crouched in a corner of the cage and the head is continually nodded or jerked in a peculiar spasmodic fashion. When roused progression is unsteady, the hind-quarters swaying unsteadily from side to side. Later the

animal can attempt only to drag itself along on its belly and frequently topples over on its side making halting movements in the air with its feet. Coma gradually supervenes. The guinea pig may lie in this state on its side for several days, the respirations becoming progressively slower and more shallow until the only sign of life is a faintly beating heart. Naturally emaciation is pronounced and there is usually some lachrymation and salivation and a soiling of the anal region with green fluid faeces.

The course of the disease is clearly illustrated by the figures given in Table III, where data referring to two different strains of virus are given.

For publication Table III to be inserted here. R.A.

Table III.

A consideration of the data given shows that after a limited number of generations in mice guinea pigs may be infected easily with the partially adapted neurotropic virus, and that a mortality of practically 100% may be anticipated. With strain 20449 not a single guinea pig survived an intracerebral injection of virus which had previously been passed through seven generations in mice. Strain O was passed through nine generations in mice before being transferred to guinea pigs; in the first five generations, of 22 guinea pigs injected there were six survivers (27.3%) subsequently the mortality was 100%. It is interesting to record that each survivor subsequently proved immune to the intracerebral injection of many hundreds of infective doses of 'fixed' virus.

As the virus becomes adapted to propagation in the central nervous system, there is a progressive decrease in the period between injection and the average day on which death occurs. Moreover, as subinoculation proceeds there is

considerably more uniformity in the course of the disease , all injected animals dying within a short period of time. Passage was only carried on for approximately 40 generations. In that time it appeared that the course could not be reduced very much below seven days.

Apparently once established it is possible to carry on a strain of virus ~~independently~~ indefinitely in guinea pigs. Adaptation to the guinea pig does not appear to alter the infectivity for mice, but it has been shown that it is accompanied by a rather rapid attenuation for the horse.(7)

During the course of other work, it was necessary to transfer to guinea pigs, strains of virus which had been passaged for 71 and 84 generations in mice respectively. It was proved that by this time the virus had become so adapted to neurotropic propagation that the first generation in guinea pigs was characterised by 100% mortality and the course of the disease was as short and as regular as that seen after injection of a guinea pig fixed virus. It is apparent therefore that passage through the mouse increases the infectivity for guinea pigs.

Post mortem.

No pathognomonic lesion has been detected macroscopically. There appears to be a slight but regular tumor splenis and renal hyperaemia and in the majority of cases there is a variable catarrhal entritis. In addition the meningeal vessels are markedly engorged.

Routes of infection.

After the susceptibility of the guinea pig to the neurotropic virus had been established an attempt was made to determine the possibility of transmitting the disease by a method of injection other than directly into the central

nervous system. The results are shown in Table IV below.

In publication Table IV to be inserted here R.H.

Table IV.

In the first series of experiments it is shown that using a virus representing the second generation in guinea pigs (strain 20449), of two guinea pigs which received 0.25 c.c. of a 4% brain emulsion directly into the blood stream via the heart, one became infected and died on the 15th day, one showed a febrile reaction, recovered and was then immune to an intracerebral injection. Of three guinea pigs which received 1 c.c. of a 4% brain emulsion intraperitoneally none died but an immunity test two which survived the injection were found to have developed a solid resistance. Of three guinea pigs which in addition to the intraperitoneal injection had the brain injured by the intracranial injection of 0.25 c.c. starch none died. On immunity test one proved to be immune, one died on the 10th day after injection and one died as a direct result of mechanical injury to the brain.

This experiment indicated that a fatal infection can be set up only when a comparatively high concentration of virus in the blood is produced. As brain substance is markedly toxic when given intravenously the only means of increasing the amount of circulating virus was to make use of the rapid absorption of a massive dose of virus from the peritoneal cavity. Since traumatic injury to the brain did not appear to assist in localising infection the intracerebral injection of starch was omitted. Therefore, in the second experiment 10 guinea pigs were given 1 c.c. of a 10% brain emulsion intraperitoneally. Of these six died after a long period of incubation and a prolonged course of the

disease, and four showed no deviation from normal health during a period of 45 days. The immunity test was unsatisfactory since two of the survivors died as a direct result of injection; but one was found to be immune and one normally susceptible.

The experiment shows that the only certain method of setting up the disease is by direct intracerebral injection. But, the rather high percentage of guinea pigs which became infected by the intraperitoneal route, is interesting. In comparison with mice it indicates that the barrier between the vascular system and the nervous system is more pregnable and that when a sufficient amount of virus is circulating the central nervous system will be invaded and multiplication will gradually proceed. In other words, in the guinea pig the disease is essentially neurotropic but its character is not so sharply defined as in mice. This conception is borne out by a consideration of the localization of the virus in the body.

Localization of the virus.

Brain. As in the case of mice, the greatest concentration of virus in guinea pigs is to be found in the brain. Titration of the virus content of this organ at various stages in the course of the disease has shown that before the onset of clinical symptoms, the presence of virus may be detected by the intracerebral injection of mice but its concentration is not high, since 0.05 c.c. of a 1: 1000 dilution frequently may prove non infective. During the later stages of the disease however the concentration increases markedly and rapidly so that by the time coma supervenes it has reached its maximum. At no time does the infectivity approximate that of the brains of mice, since it is rare for a dilution greater than 1 : 50,000 to be capable of setting up the disease and for both strain 20449 and strain O a dilution of 1: 25,000

appears to be the average upper limit of infectivity. When it is realised that the brain of a guinea pig is only approximately ~~five~~ ten times the weight of a mouse brain, it will be seen that the total virus content of a mouse brain is equal to, if not greater than that of the larger guinea pig. As a source of virus therefore preference must be given to the mouse, owing to the greater ease of handling and injecting, apart entirely from economic considerations.

Spinal Cord. Before the onset of clinical symptoms it is rare for virus to be demonstrated in the spinal cord. After the onset of paralysis the cord is usually infective but the concentration of virus was never found to be particularly high and never equalled that of the brain.

Peripheral Nerves. The peripheral nerves only contain virus in the later stages of the disease and then only in small amounts. On several occasions an emulsion of sciatic nerve made in a minimum quantity of saline was ~~more~~ non-infective for mice even though complete posterior paralysis was evident at the time the guinea pig was sacrificed.

Liver, Spleen, Kidneys and Adrinal. At post mortem these organs were frequently extirpated and emulsions in small amounts of saline were injected intracerebrally into mice. With emulsions of each organ infection was set up on various occasions, but the presence of virus was inconstant and irregular in amount. Again association with the nervous system could not be excluded and high concentration in the adrenal could not be demonstrated.

Blood. On numerous occasions blood has been withdrawn by cardiac puncture from guinea pigs at various stages of the disease following intracerebral injection of

of passage virus. Calcium citrate was used as anticoagulant and the blood was diluted 1:3 in saline before injection into mice. The presence of virus was demonstrated in rather less than 20% of cases. It was always present in small amounts since the period of incubation in mice was lengthented and on no occasion did all of the mice injected with a particular sample of blood die. No constant concentration could be demonstrated at any particular stage of the disease, i.e. at the beginning, at the height ~~of~~ or at the end of the febrile reaction or after death. Consequently it must be concluded that virus may be found in the blood but the blood plays little ot no role in the pathogenesis of the disease. This opinion appears to be contrary to that expressed by Nieshulz(5) but is supported by the finding of Max Theiler(8) in the case of Yellow Fever.

Discussion. The course of Horsesickness in the guinea pig is similar to that in mice. After intracerebral injection the virus multiplies in the brain and spreads centrifugally but this spread is slow. Virus is more frequently found in the abdominal organs and the blood, but it is always in low concentration. Similarly, direct intracranial injection is the only certain method of initiating infection but intraperitoneal injection is more frequently successful with or without simultaneous mechanical injury to the brain than in mice. It is believed that this does represent merely a partial neurotropism in guinea pigs. The disease is neurotropic and nervous tissue is essential for multiplication of the virus but the barrier between the nervous system and the rest of the body is more easily penetrated from either side.

Since the concentration of the virus in the brains of mice is usually 10 to 100 times that in the brains of guinea pigs preference must be given to the former animal as the most convenient source of a large supply of neurotropic virus. It is possible however that for the study of immunity the guinea pig may prove ultimately to be of greater value.

C. Neurotropic Fixation in Rats.

Since it has been reported by Nieshulz that the rat is unsuceptible to the virus of horsesickness it is necessary to give in detail the steps which were taken to establish and to maintain a strain of virus in rats by serial intracerebral passage.

Technique. The technique employed was identical with that described for the infection of guinea pigs. The injection itself is somewhat more difficult and deep nacrosis is essential, because the extensor muscles of the head and neck of the rat are so powerful that the reflex extension of the head stimulated by entry of the needle into the brain frequently results in fatal laceration. Consequently in addition to deep anaesthesia the head must be held particularly firmly.

The dose injected has varied from 0.1 - 0.25 c.c. of a 4% emulsion in saline. The larger dose in large rats is perfectly safe.

Strain of Rat Used. The first successful transmission to rats was obtained with four wild brown rats captured in the stables at Onderstepoort. These were identified as Rattus ~~norwigicus~~ norvegicus. Subsequently two distinct strains of albino rats were used.

1. An ~~Introgenous~~ heterogeneous strain of white rat which has been bred promiscuously at this institute for many years. The origin is obscure.

2. A strain of white rat imported from the Wistar Institute for use in ~~institutional~~ nutritional investigations. This is the only strain being maintained to-day but it has not been possible to detect any appreciable difference in susceptibility between the two strains.

Strain of virus. No attempt was made to establish a strain of equine blood virus in ~~mice~~ rats. Mouse passage virus was used, the strain selected being that known as 20449 which was originally obtained from a case of horsesickness contracted naturally (c/f infa).

Experimental. On 28/2/33 three half grown wild rats (Rattus ~~norwiquis~~ norwegicus) that had been caught a few nights previously were given an intracerebral injection of 0.2 c.c. of a 1% saline emulsion of two mouse brains (generation 26, strain 20449). On the following day they appeared normal and had they not been distinctively marked could not have been distinguished from one control, included to exclude the possibility of any adverse effect of close confinement. This control remained in perfect health for three months. On the sixth day after injection it was noticed that the three injected rats appeared dull and listless with ruffled coats and a tendency to remain crouched in a corner of the cage. On the following morning one was found dead; the other two were moving about aimlessly in a disinterested fash Their inattention to any disturbance suggested marked impairment of vision and hearing, but on close examination no sign of paresis could be detected. ~~Lathoquation~~ Luchrymation was

profuse, the fur on the face being flattened by a profuse watery discharge. Later in the day they ceased moving about and remained twitching and shivering in a crouched attitude, obviously experiencing difficulty in maintaining a balance on their feet. The following morning (9th day) both were found comatose, lying on their sides making feeble trotting movements with their legs. They were sacrificed for transmission and an approximate 4% emulsion was injected intracerebrally into six mice and three albino rats (Onderstepoort Strain). All the mice died on the sixth day after showing a clinical picture similar to that seen during the passage of mouse and guinea-pig strains. The rats appeared perfectly normal until the seventh day when two appeared dull and listless. Two days later the third became ill and a train of symptoms developed which was similar to that exhibited by the wild rats. One became comatose and was destroyed on the 21st day after injection; the other two were moribund and were destroyed on the 23rd day.

At this stage pressure of other work prevented the maintenance of the strain in rats so that the work was suspended until a later date. Subsequently the same strain of virus was established in the following manner :-

Generation I.

24.4.33. 5 rats (Wistar strain) injected intracerebrally with 0.2 c.c. emulsion mouse brains generation 37, strain 20449.
1 died 2.5.33, 1 was destroyed in extremis for passage on 3.5.33, and 1 died on 11.5.33. The two survivors were immune to the intracerebral injection of passage virus 30 days later.

Generation II

3.5.33 3 rats injected with emulsion of brain of rat destroyed in generation I.
2 died on 2nd day from injury to brain. 1 died on 12.5.33, 1 destroyed in extremis for passage on 12.5.33, 1 died on 13.5.33, 1 on 23.5.33. The two survivors were discarded.

Generation III.

13.5.33 8 rats and 3 mice injected with emulsion of rat destroyed in generation 2. The 3 mice died on the 5th and 6th day. 1 rat died on 9th day, 2 destroyed in extremis for passage on 10th day. 2 died on 11th day and 1 on 14th day. The 2 survivors were discarded.

In the fourth generation all of 7 injected rats died, but in generation 5, one of 5 injected recovered and on immunity test 28 days later was found to have developed a solid resistance. From the sixth generation on, the mortality was 100%. The strain was only passaged 16 times but there is no reason to believe that it could not have been maintained in rats indefinitely.

The course of the disease in rats is given in detail in Table V, which shows the days on which rats died after intracerebral injection. In addition data obtained from the injection of mice with rat virus at various stages in the serial subinoculation are given.

In publication Table V to be inserted here. R.A.

Table V.

It is seen that after fixation in the rat the disease runs a fairly uniform course and that the very great majority of rats will die between the 7th and 9th day. All mice that receive a sure infecting dose of rat virus die but in the majority of cases the course of the disease is somewhat prolonged. It is believed that this is due not to a decrease of virulence for the mouse but to the presence of virus in very low concentration in rat brain.

Concentration of virus in rat brains. On six different occasions rats have been sacrificed in extremis the entire brain removed, and after thorough emulsification in 10% normal horse-serum-saline, ten fold dilution have

been injected intracerebrally into mice. The low virus content came as a very great surprise. Once the infective ~~total~~ titre reached 1 in 10,000, but in the other instances dilutions higher than 1 : 1,000 were non infective. No explanation can be offered and until this fact was realised some difficulty was experienced in determining that the virus being passaged actually was that of horsesickness.

Identification of the virus. In the first instance constant bacterial sterility of infective brain suspensions was demonstrated. It is true that occasional growth on agar and in broth was obtained but obviously this was the direct result of bacterial infection during manipulation.

On 19.6.33 a horse (20622) was given a subcutaneous injection of 10 c.c. of a 1/500 saline emulsion of two rat brains, generation 6. There was no reaction. After an interval of 16 days the horse received intravenously 5 c.c. of the homologous virus.[x] A severe reaction commenced on the 4th day and the horse died from typical horsesickness on the 9th day. Subsequent experience has shown that the failure of this experiment was due to either or both of the following causes :-

1. A subinfective dose of rat virus was given owing to the low infectivity of rat brains.

2. The interval between injection and immunity test was to short to permit of the development of a solid resistance. This is probably the correct explanation for the death of the horse.

On 24.11.33 a second horse (20660) received subcutaneously 30 c.c. of a 4% emulsion of two rat brains

[x] In the text virus is the term used for infective horse blood. The terms rat virus, mouse virus etc. is used to designate other infective material.

representing generation./3. A mild febrile reaction commenced on the 25th day after injection and lasted for 5 days. On 31.1.34 serum was tapped for in vitro neutrlization tests and on 5.2.24 5 c.c. of the homologous strain of virus was given as an immunity test. The horse did not react.

In vitro neutralization tests showed that the serum drawn after the febrile reaction in a dilution of 1: 128 was capable of neutralizing approximately 80 ~~immunical~~ minimal infective doses of mouse virus, whereas serum from the horse before the rat virus infection possessed no neutralising properties at all. It should be noted that the virus used for in vitro neutralization was the mouse passage strain which had been passed though an additional 27 generations in mice after transfer to the rats, before being used.

This experiment even though conducted on a single animal justifies the conclusion that the disease excitant serially transferred through successvie generations in rats actually was the virus of horsesickness.

Discussion. The susceptibility of the rat to the neurotropic virus of horsesickness has been demonstrated. However, the suitability of this species of animal for research purposes is open to doubt mainly on account of the low virus content of the infective brains. For this reason it was considered that the rat would not be of immediate practical importance in the study of the disease, and no effort was made to determine either the virus content of organs other than the brain, or the possibility of constant infection by a route other than the intracerebral.

D. Susceptibility of other animals.

(a) Rabbit. On four different occasions rabbits

have been injected intracerebrally with mouse and guinea pig neurotropic virus. In no single instance was there any febrile reaction or any deviation from normal health. It must be concluded therefore that the rabbit is unsusceptible to the virus of horsesickness.

(b) Mastomys coucha. (The multimammate mouse Several multimammate mice were captured at Onderstepoort. On cerebral injection they were found to be fully susceptibl to mouse neurotropic virus. Insufficient individuals were obtained to permit of prolonged passage, but every mouse injected died, the course of the disease being approximately 1 - 2 days longer than that observed in the common white mouse.

(c) Tatera obengula. Through the kindness of Mr. ~~Chiwys~~ Chivers, Rodent Officer, Union Department of Her a number of young Gerbilles identified as Tatera lobe were obtained in December 1933. They were left under observation for about a month to accustom them to close confinement and on 29.1.34 four were injected intracerebrally by the usual technique with 0.2 c.c. of a 1% infective brain suspension representing mouse generation 106, strain 20449. From the 5th day all four commenced to show symptoms identical with those described in rats, two were sacrifieed on 8th day for subinoculation, the remaining two died on the 9th day.

Two tatera were injected intracerebrally in the second generation. Both were sacrificed in extremis on the 7th day. The infectivity of the suspension from the poole brains was titrated in mice, and it was found that 0.05 c.c. of a 1/25,000 dilution contained approximately 1 infective dose, the mice dying on the 4th and 5th day after injection. At the same time a susceptible horse (20840) was given

20 c.c. of a 4% emulsion of the brain subcutaneously. An exceedingly mild febrile reaction accompanied by any clinical symptoms commenced on the 7th day and lasted for 6 days. After an interval of 48 days the horse was immune to 10 c.c. of homologous blood virus given intravenously.

Conclusion. Tatera lobengula is susceptible to the intracerebral injection of mouse virus, the clinical course of the disease being similar to that observed in rats. Death occurs between the 7th and 9th day and after two generations the infectivity of the virus for mice did not appear to have been altered. The virus content of the brain is not high (1 : 25,000) and this virus is attenuated for horses, since a single animal developed a solid immunity after showing only a slight febrile reaction.

Discussion. The susceptibility of the mouse to the virus of horsesickness has opened up a wide field of research into many properties of the virus which previously had to be taken on trust to a large extent. Naturally there is no reason to assume that the change from viscerotropism to neurotropism has not been accompanied by other definite alterations but at least the study will narrow the field to be explored and in time the application of the knowledge of the mouse virus to investigations on the horse will round off the whole subject.

For this study the mouse is particularly suited. The susceptibility appears to be constant, they lend themselves to cheap and easy maintenance on a large scale, they are easily infected yet the chance of accidental infection is negligible, they are easily handled and the high concentration of virus in the brain provides a voluminous source of the infective agent

associated with a relatively small amount of foreign matter.

Apart entirely from the value of the neurotropic virus as a basis for the rational immunization of horses, the mouse has proved a valuable means of studying many peculiar features of immunity production in horses, as will be seen from the work detailed ~~below~~. in later publication.

After the virus had been adapted to neurotropic propagation in the mouse the susceptibility of the rat, the guinea pig, and a species of gerbille ~~were~~ was demonstrated. The place of these animals in the scheme of investigation has yet to be determined. Their value will not lie in their utilization as a basis of vaccine production but may be associated with the intensive study of immunity production.

Summary.

1. The technique for the fixation of a strain of horsesickness virus in mice is described in detail.

2. Method of infection. Direct intracranial injection is the only certain means of setting up the disease. The subcutaneous, intramuscular, intravenous and intraperitoneal routes are uncertain and unsatisfactory. The disease is not transmitted per os or by direct or indirect contact.

3. The symptoms and course of the disease is described in detail.

4. The virus is concentrated in the brain. It spreads centrifugally and appears to be exclusively neurotropic.

5. In guinea pigs the features of the disease are similar essentially to those in mice, and its febrile nature is described.

6. The disease spreads more slowly through the guinea pig, but it is characterised by neurotropism.

7. Symptoms, course, localization of virus and methods of infection are described.

8. The susceptibility of the rat and the course of the disease is described and discussed.

9. Attention is directed to the susceptibility of Tatera lobengula, Mastomys ~~and~~ coucha, and to the unsusceptibility of the rabbit.

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TABLE II.

Course of Horsesickness in Mice during Serial Passage.

Generation S.	Strain 20464 A			Strain 20464 B.			Strain 20449			Strain C		
	No. Injected	Death	Av. Days	No. Injected	Death	Av. days.	No. Injected	Death	Av. days	No. Injected	Death	Av. Days
1	7	3/12,1/18,2/22,1/0	16.3	7	2/12,1/13,1/18,1/21,1/22	16.3	9	1/11,1/13,1/18,1/19,1/20,2/21,3/0	17.3	10	1/7,1/8,1/9,2/10,2/11,2/13	10.2
2	10	0/4,3/5,1/6	4.5	5	3/6, 2/7.	6.4	12	7/6,4/7, 1/8	6.6	20	1/5,4/6,5/7,5/8,0/9,4/10	8.0
3	10	9/4, 1/5	4.1	5	2/4, 2/5, 1/6	4.8	15	2/5, 10/6, 3/7	6.1	15	1/5,5/6,2/7,3/8,4/9	7.3
4	8	7/5, 1/7	5.2	5	4/5, 1/6	5.2	16	3/4,3/5,0/6, 4/7	5.7	19	3/5,7/6,0/7,1/8,2/10	6.7
5	8	6/4, 2/5	4.3	6	1/4,2/5,1/6,1/7	5.4	12	4/5,6/6,2/7	5.8	16	2/5,11/6,3/7	6.1
6-10	21	3/3,15/4,3/5	4.0	21	5/4,13/5,3/6	4.9	73	3/3,15/4,34/5,12/6,0/7	5.0	68	1/4,7/5,30/6,23/7,7/8	6.1
11-15	24	6/3,19/4	3.8	25	15/4,8/5,2/6	4.5	43	13/3,16/4,11/5,2/6,1/7	4.1	72	19/4,27/5,17/6,9/7	5.2
16-20	28	8/3,19/4,1/5	3.7	25	3/3, 13/4, 9/5	4.2	64	17/3,34/4,13/5	4.1	61	16/4,23/5,16/6,6/7	5.2
21-25	30	15/3,12/4,3/5	3.6	38	2/3,27/4,9/5	4.2	51	7/3,32/4,10/5,2/6	4.1	67	7/4,21/5,28/6,11/7	5.7
26-30	50	14/3,25/4,11/5	3.9	25	1/3,9/4,15/5,1/6	4.6	31	2/3,18/4,11/5	4.3	68	3/4,25/5,33/6,7/7	5.6
31-35	45	5/3,22/4,15/5	4.3	25	7/4,18/5	4.7	45	33/4, 12/5	4.3	45	2/4,16/5,25/6,2/7	5.6
36-40	25	5/3,14/4,6/5	4.0	25	9/4,15/5,1/6	4.7	60	1/3,32/4,27/5	4.4	35	3/4,24/5,6/6,2/7	5.2
41-45	25	5/3,12/4,8/5	4.1	25	2/3,13/4,10/5	4.3	77	5/3,55/4,17/5	4.2	55	2/4,22/5,31/6	5.5
46-50	25	2/3, 16/4,7/5	4.2	25	6/3,16/4,3/5	3.9	53	5/3,35/4,10/5,3/6	4.2	57	6/4,36/5,14/6,1/7	5.2
51-55	25	2/3,13/4,10/5	4.3	25	9/3, 16/4	3.6	47	3/3, 28/4,16/5	4.3	70	14/4,31/5,25/6	5.2
56-60	25	9/3,12/4,4/5	3.8	25	10/3, 15/4	3.6	55	12/3,30/4,13/5	4.0	50	16/4,27/5,7/6	4.8
61-65	25	13/3,10/4,2/5	3.6	25	11/3,14/4	3.6	47	7/3,32/4,8/5	4.0	50	27/4,21/5,1/6	4.4
66-70	25	10/3,14/4,1/5	3.6	25	15/3,8/4,1/5	3.4	48	7/3,33/4,8/5	4.0	60	7/3,24/4,28/5,1/6	4.4
71/75	25	10/3,14/4,1/5	3.6	25	16/3,9/4	3.4	50	20/3,25/4,5/5	3.7	50	19/3,21/4,10/5	3.9
76-80	25	12/3,12/4,1/5	3.6	25	14/3,11/4	3.4	50	20/3,33/4,2/5	4.0	60	19/3,34/4,7/5	3.8
81-85	25	12/3, 13/4	3.5	25	14/3, 11/4	3.4	50	32/3,17/4,1/5	3.4	50	13/3,30/4,7/5	3.9
86-90	25	12/3,12/4,1/5	3.6	25	14/3,11/4	3.4	50	31/3,19/4	3.4	50	19/3,27/4,4/5	3.7
96-100	25	16/3, 9/4	3.4	25	16/3, 9/4	3.4	50	32/3, 18/4	3.4	25	8/3, 16/4,1/5	3.7
101-105	25	16/3,9/4	3.4	25	18/3, 7/4	3.3	50	35/3,15/4	3.3	25	9/3,14/4,2/5	3.7
106-110	25	17/3,8/4	3.3	25	19/3,6/4	3.2	25	15/3,10/4	3.4	25	8/3,15/4,2/5	3.7
111-115							25	16/3,9/4	3.4	25	6/3,17/4,2/5	3.8
116-120							25	15/3,10/4	3.4	25	8/3,14/4,3/5	3.8
125-130							25	17/3,8/4	3.3	25	9/3,14/4,2/5	3.8
130-135							25	16/3,9/4	3.4			
135-140							25	16/3,9/4	3.4			

Numerals 8/12, 27/6, mean 8 mice died on 12th day, 7 mice on 6th day after injection, etc.
0 = survival for 30 days.

TABLE III.

Course of Horsesickness in Guinea Pigs during serial passage.

Strain 20449 commencing with mouse generation 7, and strain 0 with mouse generation 9.

Generation	Strain 20449			Strain 0		
	No. injected	Death	Average	No. injected	Death	Average.
1	6 + 0	2/8, 2/12, 2/16	12.0	3 + 0	1/10, 1/13, 1/0	13.0
2.	5 + 1	1/8, 2/10, 2/11	10.0	6 + 1	1/6, 3/10, 1/15, 1/0	10.0
3.	5 + 1	1/8, 1/9, 2/11	9.0	6 + 0	1/6, 2/10, 1/13, 2/0	9.8
4.	5 + 1	1/8, 1/9, 1/10, 2/12	10.2	4 + 0	1/6, 2/10, 1/0	8.7
5.	8 + 1	4/9, 2/10, 1/12, 1/13	10.1	3 + 1	1 /8, 1/9, 1/0	8.5
6 - 10	21 + 4	2/7, 3/8, 5/9, 4/10, 3/11, 2/12, 2/14	10.0	27 + 7	1/4, 3/5, 2/6, 9/7, 8/8, 4/9	7.2
11 - 15	27 + 9	1/5, 4/6, 1/7, 10/8, 8/9, 1/11 1/12, 1/15	8.4	24 + 4	3/4, 4/6, 10/7, 2/8, 3/9, 1/10, 1/17	7.3
16 - 20	17 + 6	4/5, 1/6, 5/7, 2/8, 5/9	7.2	18 + 2	1/4, 2/5, 5/6, 10/7	6.3
21 - 25	17 + 3	1/4, 2/5, 3/6, 8/7, 1/8, 2/9	6.7	15 + 5	3/5, 5/6, 5/7, 2/8	6.4
26 - 30	17 + 3	1/5, 2/6, 11/7, 2/8, 1/9	7.0	20 + 3	1/4, 1/5, 3/6, 11/7, 4/8	6.8
31 - 35	17 + 3	5/5, 4/6, 4/7, 2/8, 2/9	6.5	12 + 2	1/5, 4/6, 6/7, 1/9	6.7
36 - 40	17 + 3	2/5, 7/6, 7/7, 1/8	6.4			
41 - 43	10 + 0	1/5, 3/6, 4/7, 2/8	6.7			

Note. 6 + 0, 17 + 3 means of 6 guinea pigs injected none died as a direct result of injection; of 17 injected 3 died as a result of injection etc.

1/6, 3/8 means 1 died on 6th day, 3 on 8th day after injection.

1/0 means 1 survived.

TABLE IV.

Exp.	Date	Virus: Source	Virus: Concentration	Virus: Dose	Method of injection	Number	Result	Interval	Immunity Test Result
I.	20.12.32	Guinea pig generation2 strain 20449	4%	0.25 c.c.	intracardiac	1	died day 15	-	-
						2	febrile reaction survived.	20 days	died day 7
	20.12.32	ditto	4%	1.0 c.c.	intraperitoneal	3	no reaction	20 days	died at injection
						4	no reaction	20 days	no reaction
						5	no reaction	20 days	febrile reaction recovered
	20.12.32	ditto	4%	1.0 c.c.	intraperitoneal plus 5.25 of 2% starch intracerebral	6	slight febrile	20 days	died at injection
						7	slight febrile	20 days	no reaction
						8	severe febrile	20 days	died day 10.
II.	30.1.33	guinea pig generation 7 strain 20449	10%	1.0 c.c.	intraperitoneal	1	died day 21	-	-
						2	died day 22	-	-
						3	died day 25	-	-
						4	died day 28	-	-
						5	died day 29	-	-
						6	died day 31	-	-
						7	survived	45 days	died at injection
						8	survived	45 days	died day 3. Operative
						9	survived	45 days	survived
						10	survived	45 days	died day 8.

Note. Immunity test consisted of intracerebral injection of 0.2 c.c. of 1% guinea pig brain emulsion.

TABLE V.

Course of Horsesickness in Rats (Strain 20449).

Generation S.	Rats: No. injected	Rats: Death	Rats: Average	Mice: No. injected	Mice: Death	Mice: Average
1.	8 + 0	1/8,1/9,1/17,2/o.	-	-	-	
2.	o + 2.	2/9,1/10,1/20,2/o.	-	-	-	
3.	8 + 0	1/9,2/10,2/11,2/14,2/o.	-	3	1/5,2/o	5.7
4.	7 + 0.	4/8, 3/9	8.4	3	2/5, 1/6	5.3
5.	8 + 1	1/7, 2/8, 1/9, 1/o	-	-	-	
6.	4 + 1.	3/8, 1/9	8.3	3	2/4, 1/5	4.3
7.	3 + 0	2/8, 1/9.	8.3	-	-	
8.	3 + 0.	1/7, 2/8.	7.7	-	-	
9.	3 + 0.	2/7, 1/8	7.3	3	2/4, 1/5.	4.3
10.	2 + 0	2/7	7.0	-	-	
11	3 + 0.	1/7, 1/8, 1/9	8.0	4	3/6, 1/7	6.3
12.	3 + 0	2/8, 1/9	8.3	5	1/4, 4/5	4.8
13.	3 + 0	1/7, 2/8	7.7	-		
14.	3 + 0	2/8, 1/9	8.3	-		
15.	4 + 0.	1/8, 2/9, 1/10	9.0	-		
16.	4 + 0	1/7, 2/8, 1/9	8.0	-		

Note. 5 + 0 means 5 rats injected, none died as a result of injection.
6 + 2 means 8 rats injected of which 2 died as a result of injection owing to traumatic injury to brain.
1/8, 2/9 means 1 rat died on 8th day, 2 rats died on 9th day after injection.
2/o means 2 rats survived for more than 30 days.