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STUDIES ON THE NEUROTROPIC VIRUS OF HORSESICKNESS II. SOME PHYSICAL AND CHEMICAL PROPERTIES.

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In studying what have appeared to be some of the most important chemical and physical properties of the neurotropic virus of Horsesickness by far the greater portion of the work has been carried out with mouse adapted virus and the strain chiefly used has been that known locally as 20449. From time to time the results have been repeated and confirmed on one or more of the other strains that have been fixed so there is as yet no reason to believe that the properties of one strain of virus, other than the antigenic properties, are not identical for all strains. Prohibitive cost has prevented any attempt to confirm the results on viscerotropic virus of equine origin but as opportunity permits this aspect will gradually be dealt with.

Keeping Qualities.

Ever since the aetiological association of Horsesickness with an ultra visible virus in the blood of reacting animals has been recognised, it was known that the infective agent posesses remarkable keeping qualities. Theiler reported that undiluted blood, or virus diluted with glycerine may still be infective after 4 years, that putrefaction does not always destroy the infectivity and that a considerable degree of thermo stability was evident. On the other hand it is a common experience to find infective blood, stored in oxalate-carbol-glycerine as a preservative (aa 500 c.c. water, 500 c.c. glycerine, 5 gm. calcium oxalate, 5 gm. carbolic acid) to be completely

avirulent after a few months at room temperature. The reason for this deterioration has not been apparent but certain factors have been established in connection with the viability of neurotropic virus.

In the earlier experiments a high dilution of infective brain suspension (say 1 : 10,000) in 10% serum saline was stored under the conditions being investigated and survival of the virus merely determined by injection into mice. In the later experiments the infective titre of the suspension was determined initially by serial dilution and compared with the titre after storage. In this connection it is necessary to state that the infectivity of a given virus emulsion which has been carefully prepared and thoroughly centrifuged may be titrated accurately to two fold limits as will be seen from the following table.

Table I. Accuracy of virus Titration.

Emulsion of 4 brains = generation 60, strain 20449, titrated on different occasions by serial dilution in 1: 10 serum saline.

Dilutions

	1/10	1/20	1/40	1/80	1/160	1/320
A	4.4	5.5	5.5	4.5	5.0	0.0
B	4.4	4.5	6.6	5.5	6.0	0.0
C	4.5	4.5	4.6	5.6	6.8	0.0

Note: In this and all further tables the numerals indicate the day after injection on which death of the mouse occurred; 0 = survival. x = death due to brain injury.

From this it is apparent that the infective titre of any given emulsion of neurotropic virus which has been carefully prepared may be determined within fairly narrow limits. It is true that the limits are not sufficiently narrow to satisfy a chemist or a worker on say bacterial

toxins but they are sufficiently accurate for all the purposes of this study.

At this point it is necessary to digress to mention the harmful effect of physiological saline (0.85% sodium chloride in distilled water) as a vehicle and as a diluent. When a virus suspension was made in saline & serial dilutions also were made in saline it was found that the titre of the serum stock solution varied considerably on different occasions, odd mice died which had been injected with a dilution apparently beyond the infective titre and mice survived which apparently had ~~revived~~ received several infective doses. These variations were beyond the limits of experimental error. No adequate explanation can be offered but the irregularities may stand in some relation to the aggregation of virus particles into clumps by the sodium or chlorine ions. Further, on storage in saline alone there is a decided tendency for the virus to become inactivated even at low temperatures. When 10% normal serum is added to the saline the result of titration is typically exemplified by the figures given above and the ~~vi~~ viricidal effect disappears. Consequently for all work except routine subinoculations which are usually carried out within a few hors after preparation of the suspension, 10% serum saline is used as a vehicle and diluent.

A. <u>The effect of temperature on viability.</u>

1. <u>Storage at + 4°C.</u>

<u>Technique</u>. A stock suspension of several infective brains was made in 1 : 10 serum saline, the concentration of brain substance being approximately 1 : 50. This was centrifuged for 30 minutes at 2,500 revolutions per minute decanted into a sterile bottle fitted with a rubber cork

and placed in an electric refrigerator immediately below the freezing chamber, where the temperature remained constantly between 3 and 5°C. A fairly thick deposit gradually collected at the bottom of the bottle but when samples were withdrawn, this was left undisturbed. From time to time serial dilutions were made of 1 c.c. amounts in serum saline and injected into mice with the following results. Mouse generation 62, strain 20449 was used, and the interval is given in days after preparation of the suspension.

Table II. see over.

From the above figures it is apparent that after storage for 173 days at 4% there is very little decrease in the infectivity of a given suspension of neurotropic virus. It is true that there was a tendency for the course of the disease to become slightly longer, and eventually 0.05 c.c. of the 1:160,000 dilution simetimes contained less than 1 minimal infective dose but the fluctuations are within the limits of experimental error. It may be claimed therefore that at 4° a suspension of neurotropic virus in serum saline is capable of retaining its full virulence for a period of at least six months. Unfortunately the experiment had to be discontinued at this stage owing to depletion of the supply of virus. In the course of other experiments this obdervation has been confirmed on several occasions.

Another virus suspension originally diluted 1:100,000 but not accurately titrated was found to be fully virulent after 18 months storage under similar conditions, and another sample was active after having been kept frozen for four months.

Table II.

The Influence of storage at 4°C on viability of the virus.

Date	Interval days	Dilutions.					
		1 : 10,000	1 : 20,000	1 : 40,000	1 : 80,000	1 : 160,000	1 : 320,000
15.9.33	8	5,5	5,5,	5,5	4,5	5,0	0,0
26.9.33	19	4,5	5,6	5,6	5,0	6,0	0,0
13.10.33	36	4,5	6,6	5,5	5,6	6,0	0,0
4.11.33	58	4,5	4,6	5,5	5,0	7,0	0,0
30.11.33	84	5,5	5,5	4,5	6,0	10,0	0,0
20.12.33	104	5,6	5,6	5,6	5,0	0,0	0,0
17. 1.34	132	4,5	5,x	5,x	5,6	6,0	0,0
25.1,34	140	4,5	4,5	5,x	5,6	0,0	0,0
2. 2.34	148	4,5	x,4	4,5	4,5	6,x	0,0
9. 2.34	155	5,6	4,4	5,5	5,6	6,0	0,0
27. 2.34	173	5,6	5,6	5,6	6,6	0,0	0,0

2. Storage at room temperature and at 37°C.

Several attempts were made to run a similar series of experiments with emulsion stored at room temperature and at 37°C. These were consistently unsuccessful because in spite of the greatest care bacterial contamination prevented the intracerebral injection of mice. The results obtained when a bactericide was added are included in the results under that heading. For future reference, however, it is important to note that no decrease in titre was observed after 48 hours at 37°C.

3. Thermal range of inactivation.

Technique. For this work a 1 : 10,000 dilution of infective brain was sealed off in ~~their~~ thin glass phials in 0.5 c.c amounts. These were emersed in a large water bath at the indicated temperature for the time specified, after which they were quickly dropped into cold water and then the injections were made. It is not, however, known how long it took the temperature of the suspension to attain that of the water bath but since emersion was complete, the phials were made of very thin glass, and only ½ c.c. amounts were used this ~~long~~ lag period must have been reduced to a minimum. To obviate any error due to coagulation of the serum proteins the suspensions were made in saline alone. This procedure may seem illogical at first sight since it has been shown above that saline is definitely virucidal. This harmful effect however was eliminated by making the time of exposure to heat not longer than 15 minutes, by carrying out the injections immediately after the conclusion of the experiment ~~concerning~~ commencing with that material which had been exposed to the highest temperature, and by using only freshly prepared suspensions.

Table III.

Table III.

Effect of Heat on Virus.

Time Minutes	Temperature °C.												
	30°	35°	40°	45°	50°	52.5°	55°	57.5°	60°	65°	70°	75°	80°
15	4,4	-	4,4	3,4	4,4	-	0,0	-	0,0	-	0,0	-	0,0
15	-	3,4	-	-	3,4	4,5	6,6	5,5	0,0	-	-	-	-
10	-	3,5	4,4	4,4	0,0	-	0,0	-	0,0	0,0	-	-	-
10	-	3,4	4,5	4,4	4,5	-	0,0	-	0,0	0,0	-	-	-
10	-	-	4,4	4,4	4,4	4,5	6,6	-	0,0	0,0	-	-	-
10	-	-	-	-	3,4	3,4	4,4	4,6	0,0	0,0	-	-	-
5	-	-	4,4	4,5	4,4	4,5	4,4	-	0,0	0,0	-	-	-
5	-	-	-	-	3,x	3,4	3,4	0,0	0,0	0,0			

These experiments show slight ~~descriptions~~ discrepancies which cannot be due to gross experimental error. They indicate that there is not a sharply defined thermal death point of the virus but that inactivation occurs progressively with the rise in temperature, as indicated by a significant prolongation in the course of the disease produced by virus exposed at the higher temperatures. A temperature of 60° sufficed to destroy all virus even after exposure for as short a time as 5 minutes. After 10 and 15 minutes exposure at 57.5° some virus was definitely capable of survival. On the other hand complete inactivation was found on two occasions to have occurred after 10 and 15 minutes at 55°.

It may be concluded therefore that exposure to a temperature of 60° is sufficient to destroy neurotropic virus while exposure to temperatures below 55° for 15 minutes will not be destructive.

B. <u>The effect of Hydrogen Ion concentration.</u>

The effect of pH upon the virus contained in a given emulsion is of importance because inter alia information on this point is essential in all investigations into the determination of the electrical charge by cataphorisis, in all attempts at purification by the method of absorption and dilution, and in gaining an insight into factors associated with longevity.

<u>Technique</u>. As Buffers the following have been used :-

(a) Serum-saline 10%. The buffering effect was satisfactory but it was thought that ~~evaporation~~ coagulation and precipitation of the serum proteins within their iso-electric range might be responsible for mechanical removal of virus.

(b). Tenth molar phosphate buffer. This buffer was entirely satisfactory but its limited range was inadequate for a comprehensive investigation.

(c) B.D.H. Universal buffer prepared according to the formula of Prodeaux and Ward. This buffer appears to fulfil all the requirements for an investigation of this type. The range is wide, the buffering effect is powerful even when diluted 1 : 5 with distilled water, and in this dilution intracerebral injection is tolerated well. Used in the concentration advocated by the manufacturers it is toxic and must be diluted.

For preliminary tests B.D.H. universal indicator was used for pH determinations. For the results quoted all readings were made ~~electionatrically~~ electrometrically using a Cambridge potentiometer and ~~given~~ quin-hydrone electrodes. The readings were found to be exceedingly accurate and constant on the acid side of normality but those obtained for the ~~buffer~~ upper limits of alkalinity cannot be regarded as other than approximate.

Buffer was distributed in 50 c.c. amounts in a series of sterile bottles. Using N/5 NaOH or HCl the pH of each was adjusted approximately to the point required. Concentrated stock brain suspension known to be infective in a dilution of 1 : 1,000,000 was used and was rapidly added to each bottle in amounts sufficient to produce a final concentration of 1 : 10,000. The pH was then determined potentiometrically. This procedure was adopted to ensure practically instantaneous exposure of the virus to the particular pH, thus eliminating any possible errors due to the action of gradually increasing acidity or alkalinity. The bottles were then allowed to stand overnight in the

ice box. As the exact amount of acid or alkali added to each was known it was a simple matter rapidly to readjust the pH to normality before injecting mice.

The results, tabulated below, represent different series of experiments carried out with three different suspensions of the same strain of virus (20449).

Table IV.

Effect of pH upon infectivity of Neurotropic Virus.

pH.	Serum saline buffer	Phosphate buffer	B.D.H. Universal buffer
± 10 +	-	-	6,6
± 9.5	-	-	5,5
9.0	-	-	5,6
8.0	-	-	5,6
7.74	4,4,5	-	-
7.69		-	5,5
7.49		-	4,4
7.31	-	4,5	-
6.64	-	-	5,5
6.56	5,5,5	-	-
6.50	-	-	4,5
6.46	-	5,5	-
6.25	-	5,6	-
6.13	4,5,5	-	-
6.01	-	-	6,6
5.98	-	5,5	-
5.96	-	6,0	-
5.91	-	0,0	-
5.87	0,0,0	-	-
5.86	-	-	5,0
5.46	0,0,0	-	-
5.39	-	0,0	-
5.18	0,0,0	-	-
5.13	-	0,0	-
5.08	-	-	0,0
4.71	0,0,0	-	-
4.51	-	-	0,0
4.15	0,0,0	-	-
4.03	-	-	0,0
3.98	0,0,0	-	-
3.71	0,0,0	-	-
3.32	-	-	0,0
3.08	-	-	0,0

The figures shown are exceedingly interesting. They indicate

(1) None of the constituents of the three buffers have any effect upon the stability of the virus per se.

(2) virus is able to withstand alkalinity but is unstable in an acid medium.

(3) a pH of 5.96 is the maximum degree of acidity which can be tolerated.

(4) below a pH of 5.96 a minute change is sufficient to cause complete inactivation.

There is a single discrepancy in the figures. It will be noticed that every mouse which was injected with virus acidified to a pH of less than 5.96 survived with the single exception of one mouse that received material stored at pH 5.86. Death of this mouse was due to Horsesickness since subinoculation into other mice reproduced the typical condition. No explanation can be offered but it is significant that 5 mice which received virus exposed to pH 5.87 and 5.91 all survived in other experiments.

The extreme sensitivity of the virus to hydrogen ion concentrations immediately below a pH of 6.0 is so remarkable that an explanatory note is called for. The minute differences in pH were obtained by chance, in fact in the experiment with the phosphate buffer they were the result of adding the same number of drops of acid to equal volumes of fluid. It will be admitted readily that the actual figure on the scale alotted to the pH may be open to question but the sensitivity of the potentiometer is such that the differences noted must be correct. Then in two different experiments a pH of 6.01 was found to be not destructive, while pH 5.87 resulted in inactivation. This clearly emphasizes the remarkable sensitivity at or about the critical point which must be placed at ~~first~~ just below pH 6.0.

As pointed out above virus was maintained at the pH indicated usually from one afternoon until the following morning. Subsequently it was found that an infective suspension acidified to a pH of approximately 5.0 had been

inactivated when brought back to the ~~initial~~ neutral point 3 minutes later.

It is concluded that the neurotropic virus of Horsesickness is exceedingly sensitive to a change of pH to the acid side of neutrability. Above pH 6.0 it is not affected but changes below that figure, so small as to be quite indetectable by colorimetric methods, may be virucidal. On the alkaline side of neutrability the virus is stable.

The effect of desiccation. It has been stated frequently that the virus is inactivated by desiccation. The conditions under which drying was accomplished are not clear but as the result of recent work it may be stated conclusively that dehydration does not destroy the virus. On the contrary desiccation has proved a convenient means of preparing infective material for prolonged storage.

Technique. Phosphorus pentoxide, commercial concentrated sulphuric acid, and anhydrous calcium chloride have all been used as drying agents. Latterly in view of the harmful effect of an acid pH upon the virus, calcium chloride has been used conclusively.

The material to be dried is placed in a Petri dish in a large exsiccator over the drying agent, and exhausted. For rapid drying a large exciseator, a large quantity of dissiccant and a very efficient pump capable of reducing the pressure to about 3 m.m. of mercury is essential. Practically all the moisture can be removed in this way in 24 hours and invariably dehydration to a constant level will be produced in 48 hours.

For drying in the frozen state the technique described by Sawyer, Lloyd & Kitchen[2] has been used with success. It was found that the efficiency of sulphuric acid as a

drying agent decreased with the temperature and for low temperature work only phosphorus pentoxide and calcium chloride were used.

Experimental. On 28.4.33 five mouse brains representing generation 29, strain O, were pooled and carefully macerated so as to form a homogeneous mixture. This brain material was divided into three approximately equal parts which were disposed of as follows :-

A. Placed in a weighed centrifuge tube so that the weight might be obtained by difference. Sufficient 10% serum saline was added to produce a 4% suspension which was thoroughly emulsified and centrifuged. The infectivity of the supernatant was then determined by serial dilution as shown below.

B. Placed in a petri dish and dried over calcium chloride at room temperature. The desiccator was evacuated twice a day and each morning the dry brain substance was carefully powdered. Drying was continued for 6 days by which time it was assumed that a constant level had been attained. A sample of the fine powder was then taken, the weight obtained accurately by transference to a weighed centrifuge tube previously dried in a hot air oven and a 4% suspension worked up in serum saline. The supernatant after centrifuging was then titrated.

C. Treated as B except that desiccation was carried out initially in the frozen state. The desiccator was stored in an electric refrigerator set to run at ± 4°C during the entire process of drying.

The results of the titrations are given in tabular form below, mice being injected with 0.05 c.c. intracranially

Table V. The Effect of Desiccation frozen and at room temperature.

A. Fresh Material		B. dried at room temperature		C. Dried frozen	
Dilution	Result	Dilution	Result	Dilution	Result
1 : 25	7.7	1 : 200	6.8	1 : 200	6.7
1 : 100	6.7	1 : 500	6.6	1 : 500	6.7
1 : 1000	7.8	1 : 1000	6.7	1 : 1000	7.8
1 : 10,000	6.7	1 : 10,000	6.8	1 : 10,000	6.8
1 :100,000	8.0	1 :100,000	0.0	1 :100,000	10.0
1 :1,000,000	0.0	1 :1,000,000	0.0	1:1,000,000	0.0

It is seen that the infective titre of the fresh material and the dried material remained practically the same, i.e. between 1 : 10,000 and 1 : 100,000. Some virus must have been destroyed during the process of drying because dilution is based on the actual weight of the brain substance emulsified and does not take into account the loss in weight from the removal of moisture. Further, there is no significant difference in the titre of the material dried at room temperature and in the frozen state.

The fact that virus is not destroyed by desiccation has been confirmed, using virus of equine origin as opposed to neurotropic virus, in the following manner. The spleen of a horse destroyed in extremis was removed with aseptic precautions and pulped in a Latapie mincer. About 100 grams of the pulp was desiccated in vacuo over sulphuri

acid at room temperature. Of the dried powder 1.7 grams was extracted with 50 c.c. of saline and 5 c.c. of this injected into a susceptible horse subcutaneously produced a typical reaction with death on the 7th day.

Desiccated infective mouse brains have been stored in sealed tubes separated from calcium chloride by a cotton wool plug wedged into a construction in the tube. The tubes have been stored in a refrigerator and whenever virus was required the material was found to be fully virulent. Up to the present injections have been made 15 months after desiccation.

It must be concluded therefore that desiccation not only does not destroy the virus of Horsesickness but provides a valuable means of preparing infective material for storage.

The Effect of Preservatives.

In previous publications attention has been directed to the use of virus attenuated by serial passage through mice for the immunization of equines. Since the essential component of this vaccine is a living antigen it became essential for commercial production and for use under conditions outside the laboratory to devise some means of procuring a maintaining bacterial sterility. Careful technique could be relied upon for the preparation of a final product with a minimum of bacterial contamination, so the problem resolved itself into the discovery of an agent essentially bacteriostatic rather than bactericidal in action, but exerting a minimal harmful effect upon the virus over a fairly wide range of temperature. Oxalate-carbol-glycerine had been used previously for the preservation of virulent blood with a considerable measure of succes

so that its efficiency was investigated together with that of phenol and phenol-ether, tricresol and tricresol-ether and ether alone. These substances were selected because of their value for the preservation of anti-sera and because, in the presence of serum, they are responsible for a minimum of precipitation.

Oxalate-carbol-glycerine was prepared by dissolving 5 grams of calcium oxalate and 5 grams of phenol in a mixture of 500 c.c. of glycerine and 500 c.c. distilled water. Phenol ether and tricresol ether was prepared by mixing equal parts by volume of the chemical with pure ether (Merck's The preservative was added to an infective brain suspension in 10% serum saline to produce the concentration indicated. After being filled the containers were corked with sterile corks and were sealed with wax before storage under the conditions cited in the tables below. After uncorking for the removal of samples fresh corks were used. The bacterial sterility test was carried out by seeding 0.5 c.c. amounts into broth and onto large agar slants to obtain some idea of the degree of infection; incubation at 37°C for three days.

Table VI.

From the data given above it will be seen that the bactericides used were remarkably effective but after storage at room temperature (16 - 20°C) even for as short a period as 49 days Oxalate-carbol-glycerine was the only one which had little or no viricidal effect. Both phenol and tricresol in a concentration of 0.5% destroy the virus, and tricresol-ether is considerably more viricidal than phenol ether in a concentration of 0.7%. Tricresol and

Table VI.

Effect of Preservatives at Room Temperature.

Virus used = Mouse generation 47, strain 20449, made up 1 : 500 in 1/10 serum saline.

Preservative	Concentration	Storage Temp.	Storage Days	Sterility Test	Virus Titration - Mice. Emulsion	1 : 10	1 : 100	1 : 1000	1 : 10,000	Remarks
Phenol	0.5%	Room	10 days	Sterile	5,5	4,4	5,6	6,0	0,0	Slightly turbid
			29 "	"	8,9	8,0	0,0	0,0	-	ditto
			49 "	"	0,0	0,0	0,0	0,0	-	Turbidity increased.
Phenol-ether	0.7%	Room	10 days	Sterile	4,4	4,5	5,6	0,0	0,0	Clear.
			29 "	"	4,5	5,5	0,0	0,0	-	
			49 "	"	5,7	6,7	7,0	0,0	-	Slight deposit.
TRicresol	0.5%	Room	10 days	Sterile	9,0	0,0	0,0	0,0	0,0	Markedly turbid.
			29 "	"	,4,4	0,0	0,0	0,0	-	
			49 "	"	0,0	0,0	0,0	0,0	-	
Tricresol-ether	0.7%	Room	10 days	Sterile	4,5	4,5	5,6	7,0	0,0	Clear
			29 "	"	5,5	4,6	7,0	0,0	-	
			49 "	"	0,0	0,0	0,0	0,0	-	Slight deposit.
Oxalate-carbol-glycerine	10%	Room	10 days	Sterile	3,4	4,4	5,6	6,0	0,0	Fine deposit.
			29 "	"	5,5	5,5	5,0	6,0	-	
			49 "	"	4,4	4,4	5,6	0,0	-	
Ether[x]	0.7%	Room	10 days	Heavy infection	5,5	5,6	5,6	5,0	0,0	Slight turbidity.
Controls No preservative		4°C	10 days	Infected	4,4	5,5	6,0	6,0	0,0	Clear
			23 days	"	4,4	4,5	4,6	0,0	-	Slight deposit.
			49 days	"	4,5	4,5	6,0	6,0	-	Slight deposit.

x Note. A crack in the bottle was not noticed. All ether escaped and putrefaction followed. It is to be noted that in spite of heavy bacterial contamination this did not interfere with the injection of mice.

phenol alone cause marked turbidity and precipitation but in combination with ether this effect is much diminished.

In order to determine the effect of storage at various temperatures the entire experiment was repeated. The temperatures selected were those to which a commercial vaccine would be subjected, viz. ± 4° - 6°, being the temperature of the refrigerating storage chamber, 26° - 29° being that of a hot summers day and 37° was included since it would probably be necessary to incubate vaccine for 24 hours after bottling to ensure destruction of any bacterial contaminants. Possibly the experiment is open to criticism since the virus suspension used was not artificially contaminated by the addition of a bacterial culture, but it must be stated that the container was left exposed to the air of the room unstoppered before the various bottles were filled and the preservative was added. Sterility test of the control after 48 hours showed that some contamination had been packed up. The results are given in tabular form below.

Virus used was generation 65 in mice strain 20449. Since the emulsion used was infective initially only up to a dilution of 1 : 100 a titration was not carried out at each test, 0.05 c.c. of a 1 : 10 dilution in serum saline alone being injected intracerebrally into mice to determine survival of the virus.

Table VII.

The experiment confirms the bactericidal and bacteriostatic properties of the substances used. Both phenol and tricresol in a concentration of 0.5% is viricidal at 4°C, 27°C and 37°C. Combined with ether,

Table VI.

Effect of Preservatives.

Preservative	Temperature	Interval days.	Sterility	Mouse Infectivity	Interval days	Sterility	Mouse Infectivity	Interval days	Sterility	Mouse Infectivity	Interval days	Sterility	Mouse Infectivity.
Phenol 0.5%	4°C	17	Sterile	0,0	32	Sterile	7,0	83	Sterile	0,0	192	Sterile	0,0
	27°C	17	"	4,5x	32	"	0,0	83	"	0,0	192	-	-
	37°C	17	"	0,0	32	-	-	83	-	-	192	-	-
Phenol-ether 0.7%	4°C	17	Sterile	4,4	32	Sterile	5,5	83	Sterile	5,7	192	Sterile	0,0
	27°C	17	"	4,4	32	"	6,6	83	"	0,0	192	-	-
	39°C	17	"	0,0	32	-	-	83	-	-	192	-	-
Tricresol 0.5%	4°C	17	Sterile	0,0	32	Sterile	0,0	83	Sterile	0,0	192	Sterile	0,0
	27°C	17	"	0,0	32	"	0,0	83	"	0,0	192	-	-
	37°C	17	"	0,0	32	-	-	83	-	-	192	-	-
Tricresol-ether 0.7%	4°C	17	Sterile	3,4	32	Sterile	5,9	83	Sterile	5,5	192	Sterile	0,0
	27°C	17	"	4,0	32	"	5,0	83	"	0,0	192	-	-
	37°C	17	"	0,0	32	-	-	83	-	-	192	-	-
Oxalate-carbol-glycerine 33.3%	4°C	17	Sterile	4,4	32	Sterile	4,4	83	Sterile	5,5	192	Sterile	5,7
	27°C	17	"	0,0	32	"	0,0	83	"	0,0	192	-	-
	37°C	17	"	0,0	32	-	-	83	-	-	192	-	-
Control No preservative	4°C	17	Infected	4,4	32	Infected	4,4	83	Infected	4,5	83	Infected	4,6.

x
Note. The survival of virus for 17 days at 27°C and its presence after 32 days at 4°C in the presence of phenol are discrepancies which cannot be explained.

however, the destructive effect particular of phenol ether i is less marked than tricresol ether at the lower temperatures but at 37°C all virus is destroyed within 17 days. Oxalate-carbol-glycerine even after 192 has little or no effect upon the virus when kept at 4°C but at 37° it is destructive within 17 days. This indicates that temperature has a marked bearing upon vaccine preservation and the work definitely excludes the possibility of using any of these substances for commercial production since the vaccine/in all probability would be inactive by the time it reached the user after despatch by post or by rail. A further experiment was therefore devised to determine whether ether alone would prove satisfactory. Serum saline emulsions of the four strains of virus to be used in the proposed quadrivalent vaccine were prepared under conditions which would apply to vaccine production in bulk. Ether was added to produce a concentration of 2%, the bottles were corked and sealed, incubated for 18 hours at 37° and then placed in an incubator at 30°, i.e. approximately the maximum temperature to which the vaccine would be exposed. Serial dilutions of the emulsions were injected into mice as indicated below.

Table VIII.

It will be noticed that after 15 days at 30°C there was very little decrease in the virus titre of the suspension and the 2% ether was capable of maintaining bacterial sterility. After 30 days a slight decrease in virus content had taken place but bacterial sterility was maintained. After 91 days, however, the decrease in virus content was marked and slight bacterial multiplication had taken place since two out of the four bottles showed slight infection represented by the development of

Table VIII.

The Efficiency of Ether as a Preservative.

Interval in days	Temp. °C.	Filtration in Mice: Generation 101, Strain 20464 A.				Sterility Test	Generation 92, Strain 20464 B.				Sterility Test	Generation 124, Strain 20449				Sterility Test	Generation 101, Strain 0				Sterility Test.
			1/10	1/100	1/1000			1/10	1/100	1/1000			1/10	1/100	1/1000			1/10	1/100	1/1000	
1	30°	4,4	4,4	4,4	-	Sterile	4,4	4,5	4,5	-	Sterile	4,5	5,5	5,5	-	Sterile	6,7	6,0	0,0	-	Sterile
15	30°	-	4,5	6,0	9,0	Sterile	-	5,x	5,6	6,0	Sterile	-	4,5	5,5	4,0	Sterile	7,8	8,0	0,0	-	Sterile
30	30°	4,4	4,5	5,0	-	Sterile	4,4	4,5	4,0	-	Sterile	4,4	5,5	5,0	-	Sterile	5,7	0,0	x,0	-	Sterile
91	30°	5,0	6,0	0,0	-	Sterile	6,0	0,0	0,0	-	Slight Infection	4,5	5,0	0,0	-	Sterile	0,0	0,0	0,0	-	Slight Infection

between 200 and 500 colonies on the large agar slants seeded with 0.5 c.c. of fluid. This infection did not interfere with the intracranial injection of the mice and no putrefaction was apparent.

Concurrently with the above experiment horses were injected to determine whether any alteration in antigenic property resulted ~~after~~ storage under the conditions investigated. The virus emulsions were mixed in equal proportions and 10 c.c. of the mixture were injected subcutaneously with the following results.

Table IX.

It is seen that the attenuation for horses was not affected in anyway. For the majority of immunity tests O virus was used, since it is known that a horse is able to respond to the antigenic stimulus of different strains of virus injected simultaneously, so that the strain represented by the smallest virus concentration was taken as the index of immunity. Obviously the immunising property of the virus was not altered by storage at 30°C in the presence of 2% ether. Unfortunately horses were not available for a test of the material stored for 91 days, but it would be unreasonable not to assume that so long as the virus remains infective its immunising property will remain unchanged.

In a further series of experiments carried out in conjunction with other investigations it was found that extraction of desiccated infective brain with pure ether in a Soxhler ~~socklet~~ apparatus for 6 hours did not destroy the virus and that storage of suspensions with 2% ether added did not affect the titre after five months at 4°C.

Table IX.

Immunization of horses with Virus stored with ether as a Preservative.

Horse	Date Injected	Period of Storage	Result	Immunity Test		
				Interval	Virus Used	Result
20943	19.4.34	1 day	Slight fever. Day 6 - 10.	48 days	0 + 20449	No reaction.
20944	19.11.34	1 day	Slight fever. Day 6 - 9.	40 days	0 virus	No reaction.
20910	3. 5.34	15 days	Fever. Day 8 - 13.	80 days	0 virus	No reaction.
20916	3. 5.34	15 days	No reactions	86 days	0 virus	No reaction.
20917	19. 5.34	30 days	Fever. Day 8 - 13.	65 days	20449	No reaction.
20918	19. 5.34	30 days	No clinical reaction	70 days	0 virus	No reaction.

These results are of the ~~intensest~~ utmost importance to commercial vaccine production. They indicate that in the present state of our knowledge, ether is the only preservative that can be used. In a concentration of 2% it is bacteriostatic rather than bactericidal at the temperatures to which the vaccine would be exposed after despatch from the laboratory. In the laboratory care must be taken to have at most only a slight infection after bottling and the vaccine must be stored at about 4°C before despatch to ensure survivial of the virus. A period of not more than 30 days should then be allowed between issue and date of injection. This period is more than adequate for all practical purposes.

Filterability of the Virus.

Very little work has been done on the filterability of neurotropic virus. Berkefeld and Chamberland candles have proved so unreliable in other studies that they have not been used. Elfords's comparatively recent work has demonstrated the many decided advantages of using 'Gradocol' membranes for ultrafiltration studies, but unfortunately these membranes were not available. However, arrangements have been made for their preparation in the near future and then a detailed investigation will be carried out. Consequently as a mere demonstration of the filter passing nature of the virus the Seitz filter has been used.

Experimental. An approximately 5% emulsion of four mouse brains representing passage generation 157, strain 20449, was prepared in 10% serum saline in the usual manner. This was diluted to make a 1% suspension

for filtration, which was carried out through single E.K. E.H. discs under positive pressure with and without the previous passage of normal serum saline to minnimise any possible errors due to virus absorption. A saline suspension of B. *prodigiosus* grown on agar slants was used as a control of the disc. Titrations of the infectivity of the original suspension and of the filtrates was carried out to determine quantitatively the filterability of the virus.

The results obtained are given in tabular form below. Filtrate B was obtained after an equal volume of normal serum saline had been forced through the disc.

Table X.

It is seen that the virus is capable of passing a Seitz E.K. disc when 50 c.c. is filtered under a positive pressure of 20 cms of mercury, but the titre of the original 1% suspension is reduced from 1:1000 to 1:100. This decrease in infectivity probably is not to be accounted for by absorption of virus particles to the disc since the previous filtration of normal serum saline had no demonstrable effect upon the infective titre of the filtrate.

This result has been confirmed in a second experiment of which it is quite annecessary to give the details of injections which were essentially similar.

In a third experiment 30 c.c. of a virus suspension, which on biological titration was found to be infective in a dilution of 1 : 80 but not in a dilution of 1 : 160 was filtered under a positive pressure of 20 cms of mercury in 5 minutes. The filtrate was not infective. In this instance using a high dilution of virus adsorbtion probably played some part.

Table X.

Filterability through a Seitz Filter.

Material	Volume Filtered	Pressure	Time	Fate of mice injected with dilutions of filtrate					
				Virus	1:10	1:100	1:1,000	1:10,000	1:100,000
Control unfiltered	-	-	-	-	4,4	4,5	4,5	0,0	0,0
A	50 c.c.	20 cm Hg	6.5 min.	4,4	4,5	4,5	0,0	0,0	0,0
B	50 c.c.	20 cm Hg	8.5 min.	4,4	4,4	4,5	0,0	0,0	0,0

Note: Bacillus prodigiosus failed to pass either filter as determined by sterility tests of filtrates on agar slants.

Effect of Light.

Attention has been focussed upon the possible effect of light upon viruses by the work of ~~Prodian~~ Perdau and Tod upon the ~~photoclynamic~~ photodynamic action of methylene blue upon bacteriophage, and the possibility of utilising this phenomenon for active immunisation. Later Kind described the harmful effect of light upon the antigenic properties of horsesickness virus inactivated by formalin. It would appear not unlikely that light may be of some practical value in future work on viruses in general, so that the limited number of observations made with neurotropic Horsesickness virus may be of importance.

A. Ultra Violet Light. It was found that exposure of an infective brain suspension to ultra violet light (220 volt 3.7 amp. Hanau quartz mercury lamp) the depth of fluid being 4 m.m., the distance from source of light 30 cms, resulted in complete inactivation of the virus in 1 minute. Up to the present it has not been possible to investigate the antigenic properties of such inactivated material on horses, but the susceptibility of mice, which had survived the ~~inactivated~~ intracerebral injection of ~~inactivated~~ irradiated virus did not appear to be decreased on subsequent immunity test.

B. Light of the visible spectrum. Infective virus emulsions, with ether added as a preservative have been exposed in colourless glass bottles in the bright diffused light of the laboratory for several weeks. A fine precipitate was gradually deposited and there was a gradual decoloration of the serum saline ~~which~~, but this also occurred in control bottles left in the dark in an unheated incubator. The virulence for mice certainly was not affected and no change could be detected in the

antigenic property for horses. The contrary could not be anticipated because immunisation appears to depend upon multiplication of attenuated neurotropic virus within the body, accompanied by antibody formation.

Light plus Methylene Blue. Technique. For this work the technique of ~~Sudlan~~ Perdrau and Tod was followed with minor modifications. As a pentolite lamp was not available as the source of light an ordinary close wound filament 6 volt 5 amp. Osram lamp housed in a suitable casing was used. The work was carried out in a dark room, mixtures being made in the very dim light which escaped from a black paper cowl placed over the lamp. No apparatus was available for the measurement of the intensity of the light. The distance from the source of light to the fluid exposed wastaken as the distance from the nearest luminous filament of the lamp to the bottom of the Petri dish used as container. During exposure the lid of the Petri dish was replaced by a thin plate of glass (cleaned photographic plate) the same plate being used for every experiment after being cleaned and washed with alcohol. The possible effect of heat was overcome by standing the dish on a thick metal block previously chilled in a refrigerator. After 45 minutes under the lamp the temperature of the fluid certainly did rise but at no time did it reach 40°C, a temperature which previous work (see above) had been shown to have no effect upon the virus.

The methylene blue used (Grûblers) was dissolved in saline to produce a concentration double that intended for use. After sterilisation it was mixed with an equal

volume of infective brain emulsion, the total volume being sufficient to produce the requisite depth of fluid in the Petri dish.

The infective brain emulsions were prepared either from desiccated material or from thoroughly centrifuged but not filtered stock suspensions in saline that had been stored at ± 4°C. Control material stored in the dark was injected after all other operations had been completed.

Experimental. As soon as it had been established that the virus was sensitive to the photodynamic action of methylene blue an experiment was run to determine the effect of irradiation on :

(a) brain suspension prepared from desiccated material, i.e. containing no living tissue material.

(b) fresh brain suspension prepared and centrifuged within a few hours of use, i.e. containing living cellular elements.

(c) brain suspension stored in the ice box for several days before use, i.e. containing nonviable cellular fragments.

Neurotropic mouse passage virus was used.

The results are given in tabular form below.

Table XI.

The results confirm those of Perdrau and Tod and show that virus is protected from the photodynamic action of methylene blue by the presence of living cellular elements or fragments of them. Even material stored for six days is capable of affording protection but this action has disappeared after 27 days. But it is worthy of note that even living cellular debris which cannot be removed entirely by centrifuging for 15 minutes at 250 revolutions per minute is incapable of protecting the virus if the source of illumination is sufficiently

Table XI.

Photodynamic Action of Methylene Blue.

Brain Suspension	Concentration	Concentration of Methylene Blue	Distance	Depth	Time of Exposure in Minutes								Control in Dark
					2½	5	10	15	20	25	30	35	
Desiccated brain	1 : 100	1:100,000	25 cms	0.2 cms	5,5,6	7,7,8	11,0,0	0,0,0	0,0,0	0,0,0	0,0,0	-	4,5.
Fresh brain	1 : 100	1:100,000	25 cms	0.2 cms	-	-	5,6,7	5,5,5	5,5,6	5,6,7,	6,7,7	7,0,0	4,5
Fresh brain	1 : 100	1:100,000	15 cms	0.2 cms	-	4,5	5,0	0,0	0,0	0,0	0,0	-	4,5
Stored 6 days	1 : 100	1:100,000	25 cms	0.2 cms	-	4,5,5	6,6,8	6,7,7	7,0,0	0,0,0	0,0,0	-	4,4,5
Stored 27 days	1 : 100	1:100,000	25 cms	0.2 cms	-	5,6	5,0	0,0	0,0	0,0	0,0	-	4,5
Stored 37 days	1 :100	1:100,000	25 cms	0.2 cms	-	5,6	0,0	0,0	0,0	0,0	0,0	-	5,5

bright. This is shown by destruction of the virus when the light was brought within 15 cms of the fluid. It is apparent, however, that in work of this description, it is preferable to exclude living cells either by using suspensions prepared from desiccated brain substance or by storing stock suspensions for about a month. Probably, as shown by Galloway with rabies virus filtration would serve the same purpose.

It was then decided to determine what concentration of methylene blue could be relied upon to produce the most constant and rapid inactivation. The various concentrations were prepared by serial dilution from a stock solution made 1 : 500, to minnimise errors due to inaccuracies in weighing out small quantities of dye. Infective guinea pig brain suspension which had been stored for 30 days after preparation was used as the source of virus. The depth of fluid was 4 m.m. and the distance from the lamp 25 cms. The results are tabulated below.

Table XII.

The results show that a concentration of 1 : 1,000,000 is capable of producing inactivation but the virus is destroyed slowly. A concentration of 1 : 1,000 is also destructive but the action is unreliable. The irregularity is probably due to the opacity of the solution preventing penetration of the light so that active virus may be picked up from the bottom of the layer of fluid after that towards the surface has been destroyed. A concentration of 1 : 10,000 to 1 : 100,000 is probably within the range of that most satisfactory for use since complete inactivation would probably take place

Table XII.

Optimal Concentration of Methylene Blue.

Concentration of Methylene Blue	Time of Exposure. Minutes.									Control in Dark
	5	10	15	20	25	30	35	40	45	
1 : 1,000	90	90	00	67	90	60	00	-	-	6,7,7
1 : 10,000	00	00	00	00	00	00	-	-	-	6,6,6
1 : 10,000	-	-	00	00	00	00	00	-	00	5,6,6
1 : 100,000	60	00	00	00	00	-	-	-	-	5,6,6
1 : 100,000	66	50	00	00	00	00	-	-	-	6,6,6
1 : 1,000,000	-	5,5	5,5	5,6	6,0	-	0,0	00	-	5,6

within 15 minutes.

Concurrently with the above experiments an additional series was run in the hope of gaining further insight into the phenomenon of inactivation. As far as possible each experiment was planned to differ from the preceding in respect of a single factor so that a direct comparison would be possible. The intensity of the light, the depth of fluid irradiated and distance from the illumination were varied while the concentration of methylene blue and the source of virus were kept constant. Methylene blue 1 : 100,000 was used to inactivate guinea pig passage virus in a concentration of 1 part of infective brain to 50 parts of saline, the suspension being stored for 18 days before use. As previously stated the electric bulb used was a 6 volt 5 amp. Osram close wound filament lamp. The results obtained are tabulated below.

Table XIII.

From the figures given it is readily apparent that the destructive action of the methylene blue is related to the intensity of the artificial light used for radiation since the time taken for inactivation varied with the distance from the light and its brightness. A great number of experiments of this nature would be necessary to determine whether the photodynamic action is directly proportionate to light itensity. Since the electric current supplied to the laboratory varies greatly and since no accurate means of measuring light intensity was available any results obtained could not be other than approximate. On the other hand it is significant that exposure to the diffuse dull light in

Table XIII.

Effect of Varying Light Intensity

Depth of Suspension	Distance from light	Current	Time of Exposure. Minutes.								Control in Dark
			$2\frac{1}{2}$	5	10	15	20	25	30	35	
2 m.m.	20 cms	5 Amps.	5,5,6	7,7,8	11,0,0	0,0,0	0,0,0	0,0,0	0,0,0	-	4,5,5
2 m.m.	25 cms	5 Amps	4,5,5	5,5,5	6,6,8	6,7,7	7,0,0	0,0,0	-	-	4,4,5
2 m.m.	30 cms	5 Amps	-	5,5,5,	5,5,6	5,5,5	6,7,7	7,8,0	7,9,0	-	4,4,5
2 m.m.	30 cms	4.5 Amps	-	5,5,5	5,5,5	5,5,5	5,5,6	6,6,7	5,6,7	-	4,5,5
2 m.m.	25 cms	5 Amps.	-	5,6	5,5	5,8	8,0	0,0	0,0	-	4,4,5
4 m.m.	25 cms	5 Amps	-	4,6	5,5,	7,8	4,8	0,0	0,0	-	4,5,5
2 m.m.	on bench	before window[x]	-	4,4,4	6,6,6	5,6,6	6,6,6	-	0,0,0	-	4,4,5

Note (x) : Lamp not used but dish exposed on laboratory bench about 10 feet distant from a window at midday on a bright summers day.

the laboratory in the presence of methylene blue resulted in inactivation at least not slower than exposure to the lamp at a distance of 30 cms. At this distance the lamp casts a distinctly visible bright circle of light on the bench, indicating that the diffuse daylight from which the ultra violet portion is excluded by passage through thick window glass and through the glass of the Petri dish is relatively richer in active rays than the artificial electric light. No information was available as to which portion of the spectrum was responsible for the photodynamic action of the methylene blue, and suitable filters which would enable an investigation of this point to be carried out were not obtainable in South Africa. It was decided reluctantly therefore that before a detailed study of the antigenic value of light inactivated virus was warranted a considerable amount of additional work on the many fundamental aspects of inactivation was essential. In the absence of suitable apparatus these investigations were abandoned but it is hoped to continue them in the near future. However, a single experiment on immunisation by means of virus inactivated by light in the presence of methylene blue was conducted.

Saline suspensions of neurotropic virus of mouse and guinea pig origin in a concentration of 4% were stored in the refrigerator for 12 days at 4°C. On two occasions during this time the suspensions were frozen solid in the freezing chamber and allowed to thaw rapidly in a water bath at 30°C. In spite of initial thorough centrifuging a fine deposit settled down, but only the

carefulle decanted supernatant was used for inactivitation. For this purpose methylene blue in a final concentration of 1 : 100,000 was used and the mixture was exposed for 30 minutes at a distance of 25 cms. from the lamp the depth of fluid being 4 m.m. During the course of exposure mice were injected at 5 minute intervals with the following results :-

Table XIV. Photodynamic inactivation for immunisation.

Virus exposed	Time of Exposure in minutes							Control in dark.
	5	10	15	20	25	30	35	
Mouse virus generation 68, strain 20449.	-	4,4,4	4,4,5	5,5,5	6,6,7	6,6,6	-	4,5,5.
Guinea pig virus, generation 32, strain 20449	-	6,6,6.	4,5,6.	6,6,6.	7,8,9.	0,0,0	-	4,5,6.

At the end of 30 minutes exposure the suspensions were transferred to separate bottles covered with black paper, every care being taken to exclude light until the horses were injected. Nevertheless the injections were made in a bright hall and glass syringes were used so that it is quite certain some additional exposure to light occurred.

The time of exposure (30 minutes) was selected because the previous work had shown that in the manjority of instances this was more than sufficient for complete inactivation. At the time the horses were injected the results of the tests on mice naturally were not known, so that only at a later date was it realised that whereas the guinea pig virus had been completely inactivated the mouse virus was not, although the major portion must

have been inactive since the course of the disease in the mice was ~~approximately~~ appreciably lengthened. It is extremely likely that additional unavoidable exposure to light at the time of injection resulted in total inactivation.

Horse 20715 received 10 c.c. extract of material of mouse origin.

Horse 20662 received 10 c.c. extract of material og guinea pig origin.

At no time did any febrile reaction follow in either animal, whereas it is definitely known that virus at that level of attenuation by passage invariably produces a fairly severe febrile reaction. Blood was taken at frequent intervals from both horses and after defebrination injected into mice to ascertain whether any virus could be detected in the blood stream; none of the mice died. By a technique of in vitro neutralization to be described in the following article horse 20715 (inactivated mouse virus) failed to show the presence of anti bodies in the serum after six tests over a period of six months; on immunity test it then succumbed to an injection of 5 c.c. of homologous virus. Horse 20662 (guinea pig inactivated virus) showed the gradual development of antibodies in the serum and after an interval of six months was solidly immune to the homologous virus.

This result is difficult to interpret, and no definite conclusion can be drawn from it but it indicates that the antigenic property of virus inactivated by the photodynamic action of methylene blue is not necessarily destroyed but that the origin of the virus (guinea pig or mouse brain material) is of importance. At this stage, for the reasons stated above, and because of the economic

necessity of concentrating upon vaccine production the work had to be abandoned. It is considered most unlikely that the method will be of any importance from a practical point of view in Horsesickness but the academic interests warrants additional work in the near future.

The result of this single experiment should be considered in the light of the previously recorded finding that a massive injection of highly concentrated guinea pig infective brain tends to produce a milder reaction in horses then injection of a dilute suspension, and that the reverse is the tendency with material of mouse origin.

Summary.

1. The keeping qualities of neurotropic Horsesickness virus are good. The infective titre of a suspension in 10% saline stored at ± 4°C remains practically unaltered for a period of about 5 - 6 months. The virus is gradually destroyed at higher temperatures and the thermal death range is 55 - 60°C.

2. Attention is directed to the unsuitability of isotonic saline either as a vehicle for suspensions or as a diluent. Serum saline (10%) is satisfactory.

3. The sensitivity of the virus to a slight degree of acidity is shown. A pH between 5.90 and 5.98 is virucidal. On the alkaline side of neutrality up to pH 10 the virus is stable.

4. Desiccation either at room temperature or in the frozen state has no detrimental effect. On the contrary dried infective brain material is a suitable form in which to store virus strains.

5. Phenol and tricresol in a concentration of 0.5% are virucidal. Mixed in equal proportions with ether and used in a concentration of 0.7% the virus is destroyed but considerably more slowly at low temperatures. Virus is destroyed by oxalate carbol glycerine at the higher temperatures.

6. The best preservative to use, of those investigated, is ether, which is bacteriostatic rather than bactericidal.

7. The virus is capable of passing a Seitz E.K. disc.

8. Methylene blue has a marked photodynamic inactivating effect. The antigenic value of virus inactivated by this means is not clear.

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