

AFRICAN HORSE-SICKNESS LIVE-VIRUS TISSUE CULTURE VACCINE (*)

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SUMMARY

Both monovalent and polyvalent tissue culture vaccines of African horse-sickness virus were successfully produced using 2 cell lines, monkey kidney stable (MS) cells and baby hamster kidney (BHK) cells. Titers of freeze-dried tissue culture vaccines were higher than those of vaccines made from affected mouse brains prepared in the same manner.

Protective antibodies were produced in vaccinated horses with monovalent and polyvalent tissue culture vaccines prepared from MS cell cultures, and no adverse postvaccination reactions were observed among them.

The stability of undiluted African horse-sickness virus grown in tissue culture medium was much better at 4 C. than at -25 C. However, if the virus was diluted with lactose-peptone-buffer solution, the virus was stable at -25 C. Various conditions to obtain higher yields of virus in tissue cultures were investigated. Growth patterns of the virus on MS and BHK cells were compared.

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Introduction

Since Alexander¹ demonstrated that African horse-sickness virus could be adapted to mice by serial intracerebral passage, mouse-adapted neurotropic strains of the virus have been widely used as vaccines and have been considered best for vaccine production.

Attempts have been made by several workers to find better hosts among various cell cultures but with little success. However, Mirchamsy and Taslimi¹¹ recently adapted virus to primary cultures of hamster kidney cells.

Recently, Ozawa and Hazrati¹³ demonstrated that the virus could be adapted to cell lines such as MS and BHK cells, producing cytopathic changes, and that all neurotropic strains could be adapted to MS cells, yielding high titers of the virus.

The purpose of this study was to explore the usefulness of these cell lines for African horse-sickness live-virus vaccine production and to determine factors essential for constant high yields of virus.

Materials and Methods

Virus.—Six antigenically different mouse-adapted neurotropic vaccine strains (A501, OD, L, Vryheid, VH, and 114) were obtained from the Onderstepoort Veterinary Institute. A mouse-adapted, neurotropic, attenuated, Iranian strain (S2)⁸ was also used. The 100th passage of each virus in mouse brains was stored at -25°C ., and a few additional passages were made in mice to prepare fresh seed virus to adapt to cell cultures. The 4th passage in suckling mice of an Iranian strain (10/60)¹⁵ isolated from an infected horse was used as a nonattenuated Asian strain. The identity of these strains was confirmed by serologic tests with homologous antisera.

Cells.—Monkey kidney stable cell line and baby hamster kidney cell line were used. The source of these cell lines has been described previously.¹³

Nutrient Mediums.—Medium for growing MS cells (YLE) consisted of Earle's solution¹⁴ with 0.5% lactalbumin hydrolysate, 0.005% yeast extract, 10.0% calf serum heated at 56°C . for 30 minutes, 100 units of penicillin per milliliter, 100 micro gramme of streptomycin per milliliter, and 0.0015% phenol red. The final pH was approximately 7.4.

For BHK cells, Hanks' balanced salt solution⁷ served as the base for the nutrient medium (YLH). The rest of the constituents were the same as for the aforementioned medium. The final pH was approximately 7.0.

Maintenance Mediums.—Unless specified, maintenance medium for MS cells consisted of YLE with 2% calf serum and for BHK cells, YLH with 2% calf serum.

In some experiments, polyvinylpyrrolidone* (PVP) molecular weight 700,000 was used in place of calf serum. This was found to serve as a growth factor by Katsuta *et al.*¹⁰

A special medium, designated Oz. medium, consisted of NaCl, 7.5 Gm.; KCl, 0.4 Gm.; CaCl₂, 0.2 Gm.; MgSO₄. 7H₂O, 0.2 Gm.; glucose, 3.0 Gm.; lactalbumin hydrolysate, 2.5 Gm.; yeast extract, 1.0 Gm. dissolved in 1,000 ml. of double distilled water; phenol red, 0.002%; penicillin, 100 units/ml.; and streptomycin, 100 micro gramme/ml. The final pH was approximately 7.4.

Subcultivation.—Cell lines were subcultured approximately every 4 days. The cell sheet was rinsed once with 0.25% trypsin in phosphate-buffered saline⁴ (PBS) solution minus magnesium and calcium ions (PBS-), covered with the same trypsin solution and incubated at 37 C. for 5 to 10 minutes. An equal volume of cold nutrient medium was added to the cell suspension. After pipetting several times the cell suspension was centrifuged at 200 g for 5 minutes. The sediment was resuspended in the appropriate nutrient medium, and the final concentration, approximately 1.6×10^5 cells/ml., was made by diluting with the nutrient medium.

Inoculation.—Monolayers of cells subcultured 3 to 4 days previously were used. The nutrient medium was removed, and the cultures were washed with PBS- only for the 1st passage. For the 1st virus passage in tissue cultures, the supernatant fluid of infected mouse brain suspension in maintenance medium (1:10 dilution) was employed after centrifugation at approximately 2,000 g for 10 minutes. All infected cultures were incubated at 37 C. for 30 minutes before fresh maintenance medium was added.

Subpassages of the virus were made whenever cytopathic changes became widespread.

Titration of Virus in Mice and in Monkey Kidney Stable Cells. — In the preliminary experiment, YLE with 2% calf serum was found to be the best diluent for the virus. Tenfold serial dilutions of the viruses were injected intracerebrally into mice aged 5 to 6 weeks, using 5 mice per dilution. Each mouse was given 0.05 ml. of inoculum. The mice were kept under close observation for 2 weeks.

Titration in MS cells were made in ordinary roller tube cultures prepared by seeding 1.5 ml. of cell suspension (approx. 160,000 cells/ml.) in the nutrient me-

* Obtained from Kyorin Pharmaceutical Co., Motomachi, Nihonbasi, Tokyo, Japan.

dium. After 3 to 4 days' incubation, the growth medium of prepared cell cultures was discarded.

In most of the experiments, 4 tube cultures were inoculated with 0.1 ml. of each dilution, and were incubated at 37 C. for 30 minutes before 1.5 ml. of the maintenance medium was added. The final reading of cytopathic effect (CPE) was made 10 days after inoculation.

Since preliminary studies had shown that virus grown in BHK cells had nearly the same titers in both MS and BHK tube cultures, all titrations of virus grown in BHK cells were made in MS tube cultures.

Tube cultures which developed CPE involving more than 50% of the cell sheet were considered to be positive.

Virus LD50 in mice and TCID50 were calculated by the Reed and Muench¹⁶ method.

Neutralization Test.—All neutralization tests were performed with MS tube cultures. The serums used were inactivated at 56 C. for 30 minutes. An equal volume of the serum to be tested was mixed with 0.3 ml. of a tenfold dilution of virus in YLE containing 2% calf serum. For the control, normal serum of the same species of animal was used. After mixing them well in hemagglutination tubes, the virus-serum mixtures were incubated at 37 C. for 1 hour. One-tenth milliliter of the mixture was inoculated into each culture tube. After 30 minutes' incubation at 37 C., 1.5 ml. of fresh maintenance medium was added to each tube.

The final reading was made after 10 days' incubation at 37 C. The logarithm of the difference in titers was expressed as the neutralization index.

Growth Patterns of Virus in Culture of Monkey Kidney Stable and Baby Hamster Kidney Cells.—The procedures used were nearly the same as described in the previous report.¹³ Each culture prepared in 1-liter Roux bottles was inoculated with 1 ml. of virus and incubated at 37 C. for 30 minutes. There were slight variations in sampling intervals. The 1st sampling was made immediately after adding 100 ml. of warmed maintenance medium. All samples were kept at 4 C. and titrated simultaneously in MS cell culture tubes.

Diluents for Freeze-Dried Vaccine. -- Lactosepeptone-phosphate-buffer (LPPB) solution was prepared in the same manner as described in the previous report¹² by mixing the following 2 solutions:

a) Na₂HPO₄, 2H₂O, 30.0 Gm.; and KH₂PO₄, 0.89 Gm., in 500 ml. of distilled water.

b) Peptone, 20 Gm.; and lactose, 100 Gm., in 500 ml. of distilled water. The final pH value was 7.4.

Lactose-peptone-tris-buffer (LPTB) solution was prepared by mixing solution *b* and an equal volume of tris buffer ³ (pH 7.4).

Lactose-peptone (LP) solution was the mixture of solution *b* and equal volume of distilled water.

In some experiments, the same amount of sucrose was used in place of lactose.

In some experiments, monosodium glutamate was added to these diluents to give a final concentration of 2%.

All these diluents were sterilized by filtration, after which 200 units of penicillin and 200 micro gramme of streptomycin per milliliter were added.

Freeze-Drying Vaccine.—To each of these diluents, virus fluids and sterilized distilled water were added in various proportions. The total volume of virus fluid and distilled water added was always equal to the volume of diluent. After mixing well, 5 ml. of vaccine was dispensed into sterilized 1-oz. penicillin bottles. Immediately after bottling, the vaccine was freeze-dried for 24 hours, and then each bottle was vacuum sealed.

Reconstitution of Vaccine.—Each bottle of freeze-dried vaccine was reconstituted in 25 ml. of cooled PBS⁻.

To vaccinate horses, 5 ml. of the reconstituted vaccine was used as one dose. For titration of each vaccine, 2 bottles of freeze-dried vaccine were reconstituted in 50 ml. of PBS⁻, and tenfold dilutions were made of this material.

Horses.—Horses for the experiment were obtained from an isolated village in the mountainous area by the Caspian Sea. All horses used were approximately 12 months old and had not been vaccinated against horse-sickness. Body temperature of each horse was taken twice, at 9 a.m. and 4 p.m. daily. Any signs of abnormality were recorded.

Experiments and Results

Effect of Concentration of Calf Serum for Growth Medium and Maintenance Medium.—Nutrient medium YLE was mixed with inactivated calf serum to make final concentrations of 2, 4, 6, 8, and 10%. The MS cells were suspended in each to make the final concentration 6×10^4 cells/ml.

After 2 weeks' incubation, the monolayers were trypsinized, and the cells counted after staining with 0.1% trypan blue stain. Mathematical averages of 5 consecutive counts of cells grown in each medium are shown (Table 1).

TABLE 1—Number of Monkey Kidney Stable Cells Grown in Nutrient Medium Containing Different Concentrations of Inactivated Calf Serum.

concentration Calf serum (%)	No. of cells per milliliter of demium
2	1.4×10^5
4	2.0×10^5
6	2.8×10^5
8	3.0×10^5
10	3.6×10^5

As a result of this experiment, YLE with 10% calf serum was used as the growth medium in subsequent work. The number of seeding MS cells was increased to approximately 1.6×10^5 cells per milliliter of growth medium to obtain a monolayer in 3 to 4 days after seeding.

To find the most appropriate maintenance medium for MS cells, three different concentrations, 2.0, 1.0, and 0.5% of inactivated calf serum in YLE were tested on monolayer cultures. With 2.0% calf serum, the monolayer was in good condition even after 2 weeks' incubation. Therefore, 2.0% calf serum in YLE was used as the maintenance medium in subsequent work.

The same concentration of inactivated calf serum, 10% for growth and 2% for maintenance, was added to the medium, YLH, for BHK cells.

Effect of Virus Adsorption Time on Its Titer.—Tube cultures of MS cells were divided into 4 groups. Four tube cultures from each group were inoculated with each tenfold dilution of S2-5 (5th passage in MS cells) virus. Immediately after infection, maintenance medium was added to the 1st group of infected tube cultures. The same maintenance medium was added to the other 3 groups of infected tube cultures after 30, 60, and 120 minutes' incubation at 37 C., respectively.

The titers of the 4 groups were $10^{6.6}$, $10^{6.3}$, and $10^{6.5}$ TCID₅₀/ml., respectively.

As there was no significant difference between these titers, 30 minutes' adsorption time was used in subsequent work.

Single-Passage Growth Patterns in Monkey Kidney Stable and Baby Hamster Kidney Cells.—The 6th passage of S2 virus in MS cells was used to determine the growth pattern in MS cell cultures. The growth patterns in BHK cell cultures were determined by using S2-6 (6th passage in BHK cells) virus. The inoculums used were $10^{7.5}$ and $10^{7.0}$ TCID₅₀, respectively.

The concentration of virus in the supernatant fluids of MS and BHK cell cultures at various intervals is shown (Fig. 1).

Extensive cytopathic changes in BHK cells appeared on the 3rd day after infection and extended slowly during the following few days as shown (Fig. 3, 4).

Stability of Virus of 4 and -25 C.—The object was to determine the storage condition giving the best stability. Conditions tested were (1) supernatant fluid virus at 4 C., (2) supernatant fluid virus at -25 C., and (3) virus sediment at -25 C.

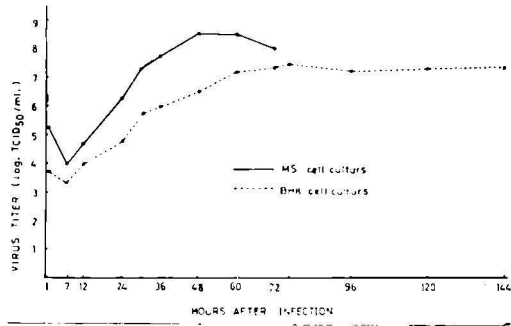


Fig. 1—Single passage growth patterns of African horse-sickness (S2) virus in monkey kidney stable and baby hamster kidney cell cultures.

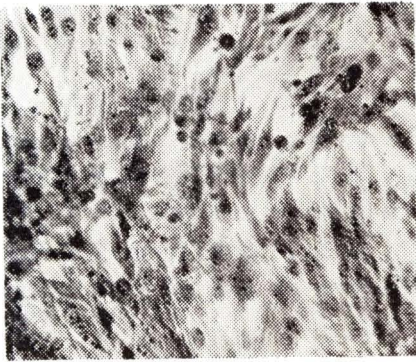


Fig. 2—Normal baby hamster kidney cell cultures. Giemsa stain; x 150.

The 5th passage of S2 virus grown in MS cells maintained in YLE with 2% calf serum was used for this experiment. When CPE became widespread, the culture bottles were vigorously shaken. Thirty milliliters of the fluid containing infected MS cells was centrifuged at approximately 500 g for 5 minutes. The pH value was approximately 6.8. One milliliter volume of the supernatant fluid was dispensed into sterilized hemagglutination tubes sealed with rubber stoppers.



Fig. 3—Baby hamster kidney cell culture infected with S2 strain of African horse-sickness virus 72 hours previously. Giemsa stain; x 150.



Fig. 4—Baby hamster kidney cell culture infected with S2 strain of virus 96 hours previously. Giemsa stain; x 150.

Half of the tubes were stored at 4 C. and the other half at -25 C. The final pH values of virus fluid stored at 4 C. was approximately 7.0.

The sediment of the infected MS cells was resuspended in 30.0 ml. of PBS-. After mixing well, 3.0 ml. of the suspension was placed in each of 10 centrifuge tubes. After centrifugation at 500 g for 5 minutes, the supernatant fluid was discarded. Each centrifuge tube was plugged with a rubber stopper and stored at -25 C. At certain intervals, the frozen cells were taken out and suspended in 1.5 ml. of sterilized double distilled water. The suspension was vigorously shaken for 5 minutes at room temperature, and then 1.5 ml. of double concentrated YLE was added to the suspension. After centrifugation at approximately 500 g for 5 minutes, the supernatant fluid was considered undiluted virus fluid.

Tenfold dilutions of the virus stored under different conditions were made in the maintenance medium and were titrated both in mice and MS tube cultures (Fig. 5, 6).

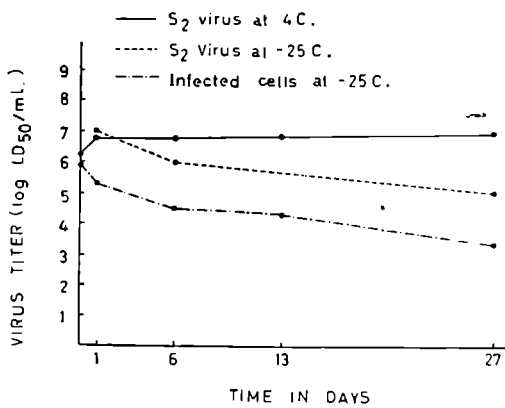


Fig. 5—Stability of virus (S₂) in supernatant fluid and sedimented cells stored at two different temperatures (titers in mice)

The titers of virus stored at 4 C. were almost constant, and virus titers in mice and in tissue cultures were nearly the same.

Titers of frozen virus either from centrifuged infected cells or the supernatant fluid markedly decreased during storage at -25 C. In a separate experiment, S₂-5 virus fluid which had the titer 10^{8.0} TCID₅₀/ml. was stored at -25 C. and titrated after 24 and 48 hours' storage. Titers were 10^{4.6} and 10^{3.5} TCID₅₀/ml., respectively.

It was also found that there was marked difference in titers of frozen virus titrated in mice and in tissue cultures. Titers of frozen virus were always higher in mice.

Stability of Virus at 37 C.—The viruses used were the 5th passages in MS cells of attenuated Asian strain, S₂, and nonattenuated Asian strain, 10/60. Virus passages were made in MS cell cultures containing maintenance medium YLE with 2% calf serum. Virus fluids were centrifuged at approximately 500 g for 5 minutes, and the supernatant fluids were dispensed into hemagglutination tubes each con-

taining 1 ml. of virus fluid. After over-night storage at 4 C. the pH values of S2 and Asian (10/60) virus fluids in hemagglutination tubes became 6.7 and 6.5, respectively.

In a preliminary experiment, it was found that the titers of both S2 and 10/60 virus did not change even after 5½ hours' incubation in a water bath adjusted at 37 C. Therefore, both virus fluids in hemagglutination tubes were kept in an incubator at 37 C. and titration of virus fluids were made at various intervals (Fig. 7). The result indicated that the neurotropic attenuated strain is more themolabile than the nonattenuated strain of the same virus. A similar result was

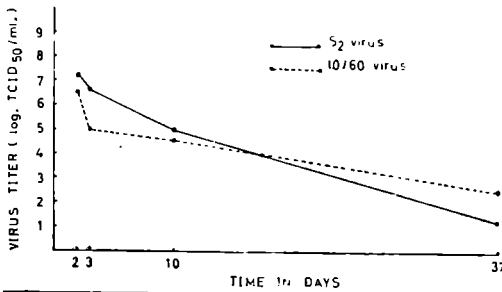


Fig. 7—Stability of S2 and 10/60 strains of African horse-sickness virus at 37 C.

The 5th passage of S2 virus was grown in MS cell cultures maintained in the following 3 maintenance mediums.

- a) YLE + PVP (polyvinylpyrrolidone, 0.1%)
- b) Special medium (Oz. medium) + calf serum (2.0%)
- c) Oz. medium + PVP (0.1%) + sodium bicarbonate (0.1%)

Virus fluids were harvested when cytopathic changes became widespread and were centrifuged at approximately 500 g for 5 minutes. The supernatant fluids were placed in hemagglutination tubes (1 ml. each), and one set of tubes was stored at 4 C. and the other at -25 C.

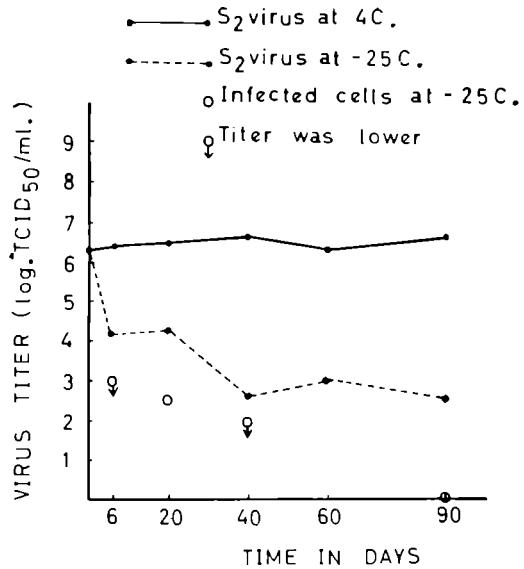


Fig. 6—Stability of virus (S2) in the supernatant fluid of infected cultures and sedimented cells stored at two different temperatures (titers in monkey kidney stable tube cultures).

obtained in the repeated experiment.

Effect of Calf Serum and Phosphate Buffer on the Stability of Virus Stored at 4 and -25 C.—

The purpose of this experiment was to determine the effect of calf serum and phosphate buffer used in the maintenance mediums on infective titers of virus during storage at 4 and -25 C.

Titers of virus grown in tissue cultures are shown (Table 2). The most rapid decrease in titer occurred with Oz. medium containing 2% calf serum and stored at -25 C. Titers of virus in mediums without calf serum decreased during storage at 4 C.

TABLE 2—Titers of S2 Strains of African Horse-Sickness Virus Grown in Monkey Kidney Stable Cells Maintained in Three Different Mediums and Stored at 4 and -25 C.

Days of storage	Medium a (YLE + PVP)		Medium b (Oz. + calf serum)		Medium c (Oz. + PVP)	
	4 C.	-25 C.	4 C.	-25 C.	4 C.	-25 C.
36					10 ^{8.5}	10 ^{5.8}
42	10 ^{9.0*}	10 ^{5.9}	10 ^{7.0}	10 ^{2.0}		
60					10 ^{7.5}	10 ^{4.7}
65	10 ^{7.7}	10 ^{3.9}	10 ^{7.5}	10 ^{1.5}		

* TCID₅₀/ml. PVP = Polyvinylpyrrolidone.

Stability of Virus in Lactose-Peptide-Buffer Solutions Stored at 4 and -25 C.—The 5th passage of S2 virus in MS cell cultures maintained in YLE with 2% calf serum was used. The titer of the virus was 10^{8.0} TCID₅₀/ml. Three milliliters of the virus fluid centrifuged at approximately 500 g for 5 minutes was added to 30 ml. of LPPB solution, and 27 ml. of sterilized double distilled water was added to the mixture. The final pH was 7.0

In the same manner, 3 ml. of the same virus fluid was added to 30 ml. of LPTB solution, and 27 ml. of sterilized double distilled water was added to the mixture. The final pH was 6.7.

These virus fluids diluted in 2 different diluents were placed in hemagglutination tubes each containing 1 ml. of the diluted virus. Virus diluted in LPPB solution was divided into 2 groups and stored at 4 C. and -25 C. Virus diluted in LPTB solution was stored at -25 C. Titers of virus kept at 4 and -25 C. are shown (Fig. 8).

Stability of Virus at Different pH Virus. The object was to determine the optimum pH range of virus fluids for storage at 4 C.

Fifteen milliliters of the supernatant fluid of S2-6 (6th passage in MS cells maintained in YLE 2.0% calf serum) virus was placed in each of five 2-oz. prescription bottles. The pH was approximately 7.0. The pH values of the fluids of 2 bottles were adjusted to 7.5 and 8.0, respectively, by adding heated

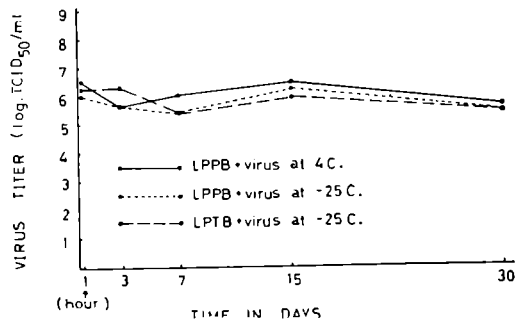


Fig. 8—Stability of S2 strain of African horse-sickness virus diluted in two different lactose peptone buffer solutions, lactose-peptone-phosphate-buffer and lactose-peptone-tris-buffer, and stored at two different temperatures.

sodium bicarbonate (7.5%). The pH values of the other 2 were adjusted to 6.5 and 6.2 with CO₂ gas. From each pH adjusted virus suspension, 1.5 ml. was immediately placed into 5 hemagglutination tubes. After sealing with rubber stoppers, all the tubes were stored at 4 C.

Titraions of each virus fluid were made in MS cell cultures 3 hours, 2 days, and 6 days later (Fig 9). The titers of virus in the fluid of pH 6.2 were much lower than other virus fluids.

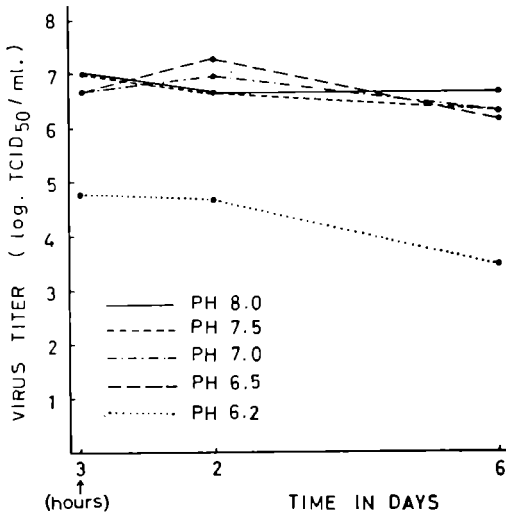


Fig. 9—Stability of S2 strain of African horse-sickness virus at 4 C. in the maintenance medium at different pH values.

Effect of pH of Maintenance Medium on Titters of Virus.—The object was to investigate the effect of pH values of maintenance medium on the infectivity end points in virus titrations.

The pH values of maintenance medium YLE with 2% calf serum added were adjusted to 5 different pH values, 8.0, 7.5, 7.0, 6.5, and 6.2, in the same manner as the previous experiment.

The results of the final reading made 10 days after inoculation with the 6th passage of S2 virus are shown (Table 3).

TABLE 3—Titters of S2 Strain of African Horse-Sickness Virus in Monkey Kidney Stable Cell Tube Cultures Titrated Using Maintenance Medium of Different pH Values

pH values of medium	In ordinary monolayer tube cultures	Double concentrated cell cultures
8.0	7.0*	ND
7.5	7.0	7.0
7.0	7.0	ND
6.5	6.7	6.7
6.2	6.7	6.3

* Log TCID₅₀/ml. ND = Not done.

The same experiment was repeated on the same day, using MS tube cultures that had been seeded with double the concentration of MS cells at the same time

as the previously mentioned MS cell cultures were prepared. They were infected with the same virus dilution and then treated in the same manner. The results of these titrations are also shown (Table 3). Maintenance medium which had pH values 7.0 or higher gave the most consistent results.

Test of Maintenance Mediums for Freeze-Dried Vaccine.—The object was to select the most suitable maintenance medium for virus growth in infected MS cell cultures prior to freeze-drying.

The following 10 different maintenance medium were tested:

- 1) Medium 199 *
- 2) Bovine amniotic fluid (BAF)
- 3) YLH + 2.0% calf serum
- 4) Oz. medium + 2.0% calf serum
- 5) Oz. medium + 10.0% BAF
- 6) Saline solution (0.85%) + 50.0 % BAF
- 7) YLE + 10.0% BAF
- 8) YLE + 2.0% BAF
- 9) YLE + 0.1% PVP
- 10) YLE + 2.0% calf serum

The LPPB solution was employed for freeze-drying the 5th passage of S2 virus cultivated in MS cells maintained in these 10 different maintenance mediums. Five milliliters of supernatant virus fluid from each group was added to 50 ml. of LPPB solution, and 45 ml. of sterilized distilled water was added.

Half the bottles of freeze-dried vaccine from each group were incubated at 37 C. for 3 days. The titers of vaccine after reconstitution in 25 ml. of PBS- per bottle are shown (Table 4).

TABLE 4—Titers of S2 Strain of African Horse-Sickness Virus in Different Maintenance Mediums After Freez-Drying

Maintenance medium No.	Before incubation	After 3 days' incubation at 37 C.
1	ND	2.7
2	3.7*	3.2
3	ND	3.6
4	4.5	4.4**
5	ND	3.5
6	ND	3.5
7	ND	3.6
8	ND	2.6
9	ND	2.8
10	4.0	3.7

* Log TCID₅₀/ml. of reconstituted vaccine. ** Used for vaccinating horses 4, 5, and 6.

* Prepared from Dried Medium 199, Difco Laboratories, Detroit, Mich.

As a result of this experiment, either Oz. medium with 2% calf serum or YLE with 2% calf serum was used for most maintenance mediums in subsequent work.

Test of Various Freeze-Drying Diluents.—The object was to determine the effect of buffer systems, sugars, and monosodium glutamate on the maintenance of infective titers of vaccines during the freeze-drying process and the subsequent incubation period at 37 C.

Diluents of 12 different combination of buffer systems and chemicals were prepared in the manner previously described. The combinations were as follows:

- 1) Lactose-peptone-phosphate-buffer
- 2) Lactose-peptone-solution
- 3) Lactose-peptone-tris-buffer
- 4) Lactose-peptone-Na glutamate-phosphate-buffer
- 5) Lactose-peptone-Na glutamate solution
- 6) Lactose-peptone-Na glutamate-tris-buffer
- 7) Sucrose-peptone-phosphate-buffer
- 8) Sucrose-peptone-solution
- 9) Sucrose-peptone-tris-buffer
- i0) Sucrose-peptone-Na glutamate-phosphate-buffer
- 11) Sucrose-peptone-Na glutamate-solution
- 12) Sucrose-peptone-Na glutamate-tris-buffer

Two batches of monovalent vaccines were prepared by using the 5th passage of S2 virus. The virus used for the 1st batch of vaccine was grown in MS cells maintained in Oz. medium with 2% calf serum. The titer was $10^{6.7}$ TCID₅₀/ml. The virus used for the 2nd batch was grown in MS cells maintained in YLE with 2% calf serum. The titer was $10^{6.3}$ TCID₅₀/ml.

Vaccine was prepared by mixing 30 ml. of each of these diluents with 27 ml. of sterilized double distilled water and 3 ml. of virus fluid.

The results of the titrations of these 2 batches of freeze-dried monovalent vaccines before and after incubation at 37 C. are shown (Table 5).

It appeared from these results that tissue culture infectivity of virus in the vaccine freeze-dried with LPTB or with LP solution was more stable than that of vaccine freeze-dried with LPPB.

There was no significant evidence that the addition of monosodium glutamate increased the stability of freeze-dried vaccine. Many of freeze-dried vaccine with sucrose lost the original shape of its pellet and formed spongelike clumps during storage.

TABLE 5—Titers of Freeze-Dried Monovalent African Horse-Sickness Virus Tissue Culture Vaccines Before and After Incubation at 37 C.

Diluent No.	Days of incubation at 37 C.				
	1st batch		2nd batch		
	0	24	0	0	37
1	4.8*	3.7	4.5		3.3
2	5.0	4.7	5.0		4.3
3	5.0	5.0	4.7		4.3
4	5.3	4.5	4.7		3.5
5	5.0	4.7	4.7		4.3
6	5.0	4.5	4.7		3.8
7	4.7	4.3	4.5		4.3
8	4.7	4.0	4.7		4.5
9	5.0	4.8	4.5		3.7
10	5.0	4.7	5.0		3.7
11	5.3	4.5	4.5		4.3
12	5.3	5.6	5.0		4.5

* Log TCID₅₀/ml. of vaccine reconstituted.

Polyvalent vaccine was prepared by using LPPB solution as freeze-drying diluent. Thirty milliliters of the solution was diluted with 12 ml. of double distilled water. Three milliliters from each of 6 strains of virus, representing antigenic groups 1 to 6, was added to the diluent. Vaccine strains used were the 5th tissue culture passage of A501, OD, L, Vryheid, VH, and 114 virus grown in MS cells maintained in Oz. medium with 2% calf serum. Their titers were 10^{5.6}, 10^{6.7}, 10^{7.5}, 10^{6.7}, 10^{7.3}, and 10^{6.7} TCID₅₀/ml., respectively. Titers of polyvalent vaccine before and after freeze-drying were 10^{6.7} and 10^{6.2} * TCID₅₀/ml., respectively. The titer of the polyvalent freeze-dried vaccine incubated at 37 C. for 24 days was 10^{5.0} TCID₅₀/ml.

Another batch of polyvalent vaccine was prepared in the same manner by using the same strains of viruses grown in MS cells maintained in YLE with 2% calf serum. Titers of the freeze-dried vaccine before and after incubation at 37 C. were similar to the 1st batch of polyvalent vaccine.

Model Vaccine Production.—Monovalent vaccines were produced with the 5th passage of S2 virus grown in MS cells maintained in YLE containing 2% calf serum and the 5th passage of S2 virus grown in BHK cells maintained in YLH containing 2% calf serum. Both supernatant virus fluids were freeze-dried, using LPTB solution in the manner described previously. The titers of virus fluids before freeze-drying, after freeze-drying, and after incubation at 37 C. for 1 week are shown (Table 6).

* This vaccine was used for vaccinating horses 7 and 8 in a subsequent experiment.

TABLE 6—Titers of Monovalent S2 Strain of Tissue Culture Vaccines Experimentally Produced with Lactose-Peptide-Tris-Buffer Solution

Virus grown in	Virus titers (log TCID ₅₀ /ml.)		
	Fluid used	Freeze-dried vaccine	After 7 days' incubation at 37 C.
MS cells	8.0	5.7*	5.3
BHK cells	6.7	4.5	4.2

* Titer of vaccine reconstituted in 25 ml. of phosphate-buffered saline solution. MS = Monkey kidney stable; BHK = baby hamster kidney.

As titers of freeze-dried tissue culture vaccines were often almost 100 times as high as those of mouse brain vaccines an attempt was made to produce a vaccine by diluting 10 times more than that already described.

One volume of the S2-5 virus grown in MS cells was mixed with 200 volumes of the same LPTB solution, and freeze-dried. Titers of the freeze-dried vaccine before incubation and after 7 days' incubation at 37 C. were $10^{4.3}$ and $10^{4.0}$ TCID₅₀/ml., respectively.

A polyvalent vaccine was produced, using the viruses grown in BHK cells. Viruses used were the 5th passages of 6 neurotropic strains, A501, OD, L, Vryheid, VH, and 114. Titers of virus fluids used were $10^{6.3}$, $10^{7.3}$, $10^{7.5}$, $10^{7.7}$, and $10^{7.5}$ TCID₅₀/ml., respectively.

Titers of freeze-dried vaccine before incubation and after 7 days' incubation at 37 C. were $10^{6.0}$ and $10^{5.5}$ TCID₅₀/ml. of reconstituted vaccine. These titers were almost as high as those of polyvalent vaccine produced previously, using viruses grown in MS cells.

Antigenicity Test of S2 Virus in Horses.—Serologic examinations of 3 horses were made by neutralization tests in MS cell cultures. They were found to be free of S2 antibody. Horses 1 and 2 were inoculated subcutaneously with the 4th passage in MS cells of virus. The inoculum was 1 ml. of supernatant virus fluid containing $10^{7.0}$ MLD₅₀ of virus titrated in mice.

Horse 3 was subcutaneously inoculated with 1 ml. of virus of the 36th passage in MS cell cultures. The inoculum contained $10^{6.5}$ TCID₅₀. The titer of the inoculum in mice was $10^{4.0}$ MLD₅₀.

A significant response in body temperatures of these 3 horses was not recorded within a month following injection. Blood samples were obtained from the horses 1, 2, and 5 months after injection, and antibody responses were exami-

ned by neutralization tests, with S2-4 virus as the antigen (Table 7). There were

TABLE 7—Antibody Response in Horses Inoculated with S2 Virus Prepared from 2 Different Passage Levels in Monkey Kidney Stable Cell Cultures

Horse No.	Passage No. of virus	Months after injection		
		1	2	5
1	4	ND	2.5	3.7
2	4	3.2*	3.7	3.2
3	36	ND	1.2	2.0

* Neutralization index against 4th passage of S2 virus in monkey kidney stable cell cultures. ND = Not done.

marked antibody responses in horses 1 and 2 that had been vaccinated with the 4th passage of S2 virus, but antibody response in horse 3 was poor.

Signs of abnormality at the site of injection were not recorded during 5 months' careful observation of these 3 horses.

In order to compare these results with a horse actually infected with Asian type horse-sickness in the field, serum was obtained from a horse recovered from horse-sickness during the epizootic season in 1963, exactly a month after the first sign of the disease was recorded. A neutralization test was made with the same virus, 4th passage of S2 in MS cell cultures. The neutralization index was 0.9.

Vaccination of Horses with Freeze-Dried Monovalent and Polyvalent Tissue Culture Vaccine.—Eight healthy horses were used for the experiment, and preinjection blood samples were obtained for serologic examination. The horses were allotted to 4 groups. The 1st group consisted of horses 4, 5, and 6. Each horse was inoculated subcutaneously with 5 ml. of reconstituted monovalent vaccine (Table 4). The inoculum contained, at the time of injection, 125,000 TCID₅₀ of virus.

The 2nd group consisted of horses 7 and 8. Each was inoculated subcutaneously with 5 ml. of reconstituted polyvalent vaccine. The inoculum contained, at the time of injection, 7,900,000 TCID₅₀ of virus.

The 3rd group consisted of horses 9 and 10. They were inoculated subcutaneously with 5 and 10 ml. of mouse brain suspension, respectively. The suspension was the supernatant fluid of a 1 to 10 dilution of mouse brains infected with 102nd brain passage of S2 virus. Ten milliliters of the brain suspension contained 126,000 MLD₅₀ of S2 virus titrated in mice. These horses were vaccinated

with brain suspension in order to compare the antibody response with horses of the previously mentioned 2 groups.

A control horse, No 11, was kept in the same stable.

After vaccination, body temperature of these horses, including the control horse, were taken twice daily, but no significant response due to vaccination was recorded during a month following injection.

Blood from horses was tested for antibody responses 48 days after injection. The results of the neutralization tests, using S2-7 virus (7th passage in MS cells), are shown (Table 8).

TABLE 8—Antibody Titration of Horse Serums Before and After Vaccination (Titrated Against S2 Virus)

Horse No.	Vaccine used	Pre-vaccination serum	Post-vaccination serum	Neutralization index
4	T. C. monovalent	6.5*	2.0	4.5
5	T. C. monovalent	6.3	2.0	4.3
6	T. C. monovalent	6.6	4.4	2.2
7	T. C. polyvalent	6.5	5.0	1.5
8	T. C. polyvalent	6.5	4.5	2.0
9	Mouse monovalent	6.3	2.0	4.3
10	Mouse monovalent	6.0	2.0	4.0

* Log TCID₅₀/ml.

Horse 5 died of severe peritonitis due to rupture of the stomach. The cause of rupture was due to an extremely severe gastrophilus infection. This gave us a chance to examine the horse for pathologic postvaccination reactions. The only finding considered to be due to vaccination was a slight yellowish edema found on the ventral muscles. There was no sign of vaccination reaction at the site of injection. The horse had developed a high titer of antibody before the accidental death (Table 8).

All the vaccinated horses were subjected to intravenous challenge with 2 ml. of mouse brain suspension 50 days after vaccination. The control horse was inoculated with 1 ml. of the same virus. The brain suspension was prepared with the 5th passage of the Asian strain (10/60) virus in suckling mice. The inoculum contained 6,300,000 MLD₅₀/ml. of virus titrated in suckling mice.

Four days after challenge, the body temperature of the control horse rose. Two weeks after challenge, the horse developed predominant cardiac signs and died of horse-sickness. Postmortem findings and isolation of the virus from the

organs confirmed that the cause of death was African horse-sickness virus infection.

Significant temperature increases among the vaccinated horses were not recorded during the month following challenge.

These results indicate that both monovalent and polyvalent tissue culture vaccines protected horses against the challenge virus, strain 10/60.

Discussion

For testing the specificity of virus and antibody titers, neutralization tests in tissue cultures were employed because they are still considered to be more type specific than hemagglutination-inhibition or complement-fixation tests.

A maximum titer of $10^{8.5}$ TCID₅₀/ml. obtained in MS cells 48 hours after infection (Fig. 1) is believed to be the highest titer yet reported with African horse-sickness virus. In BHK cell cultures the fibroblastic cells were so densely grown one on top of another that it took more than 5 days to produce 100% CPE after infection. On the other hand, all infected MS cells produced characteristic CPE simultaneously, approximately 48 hours after infection as described in the previous report.¹³

From the results of a series of experiments on the stability of virus, it appears that undiluted virus fluids containing 2% calf serum are stable at 4 C., and that freezing of the virus fluids has a detrimental effect on the infectivity of virus. This was common with the virus grown in MS cells maintained in 3 different maintenance mediums. The freezing effect was more evident with the maintenance medium containing calf serum. After freezing, titers of virus fluids titrated in mouse brains were higher than those in tissue culture. This indicates that the effect of freezing is mainly on the protein coat of virus particles rather than on infective nucleic acid portions of the virus. Another indication which supports this theory is that the freezing effect on virus infectivity is minimized by diluting 1 volume of virus fluid in 20 volumes of lactose-peptone-buffer solution (Fig. 8).

It appears that during storage at 4 C. the virus is more stable when the pH of the fluid is maintained at 6.5 or higher and that pH values lower than 6.5 have a detrimental effect on the infectivity of the virus (Fig. 9).

There was no clear evidence that the pH value of maintenance medium is the major cause of variation in titers (Table 3). However, maintenance medium with a pH 7.0 or higher seems to give the most consistent results.

Until quite recently the use of continuously cultured tissue cells for the propagation of viruses to be used for human vaccines was out of the question. How-

ever, the report,² submitted by the Committee on Tissue Culture Viruses and Vaccines of the U.S.A., encouraged the use of established cell lines, taking every possible precaution to ensure that the cell lines used are developed from normal tissues and are nononcogenic, and that viral vaccines grown in the cell cultures are free from living cells.

The MS cell line was derived from normal monkey kidney cells and there is no evidence that MS cells are oncogenic. Centrifugation of virus fluids and subsequent freeze-drying will eliminate the possibility of contamination by living cells. This is especially ensured with MS cells, because all the infected cells develop cytopathic changes simultaneously and the usual freezing process is lethal to them.

Recently, the tumorigenic properties of BHK cells in baby hamsters have been reported by Gottlieb-stematsky and Shilo.⁶ Therefore, the use of tissue culture vaccine prepared from BHK cell cultures is not recommended unless tested in a large number of equine animals.

From results shown (Table 4), special Oz. medium and YLE both containing 2% calf serum were chosen for subsequent freeze-drying experiments. Results of these experiments indicate that constituents of maintenance medium were not major factors if the virus fluid was diluted in the proper freeze-drying diluent. A primary requirement for obtaining high titer vaccine is having fluid with high virus titer. The virus fluid, then, must be diluted more than 20 times in proper diluent such as LPTB solution to be freeze-dried. However, concentration of calf serum in maintenance medium should be limited to 2% or less, because infectivity of virus suspended in the medium containing calf serum decreased markedly by freezing at -25 C. (Table 2). Increasing serum concentration may result in decreasing the titer of vaccine.

Among freeze-drying diluents used, LPPB solution, even with monosodium glutamate, appears to be inferior to others in preserving infectivity of virus in freeze-dried vaccine (Table 5). Use of sucrose in place of lactose is not recommended, because vaccines freeze-dried with sucrose in the diluent may lose the original shape of its pellet and shrink into small spongelike clumps during storage, if freeze-drying is not sufficient.

From the result shown (Table 7), it appears that the antigenicity of S2 virus in horses decreases when the virus passage in MS cell cultures increases. The passage of vaccine strains of virus in MS cells should be minimized. It is therefore recommended that the maximum passage level of liquid vaccine seed virus be limited to the 5th passage.

Although the number of horses used for testing the antibody response after vaccination with freeze-dried vaccine was limited, the results shown (Table 8) in-

dicate that the antibody response of horses vaccinated with monovalent MS tissue culture vaccine was comparable to that obtained in those vaccinated with monovalent infected mouse brain suspension. As the polyvalent tissue culture vaccine did not contain the attenuated Asian strain of virus, the antibody titer against S2 strain was lower, but was sufficient to protect animals from the challenge virus.

Since S2 strain was proved to be safe, immunogenic monovalent vaccine is recommended for use in most of the Middle Eastern countries, where only one serologic type of horse-sickness virus is known. This is not only because polyvalent vaccines so far produced do not contain attenuated Asian strain of virus but also because animals vaccinated with polyvalent vaccine often fail to develop specific antibodies against certain components of the vaccine in spite of repeated vaccination.⁹ It is not advisable to spread attenuated strains of serologically different groups of virus in the areas that are not exposed to the danger of the invasion of these groups of virus.

Use of MS cells for production of African horse-sickness vaccine is recommended not only because 1 liter of tissue culture virus fluid can yield 20,000 to 200,000 doses of monovalent vaccine of high quality, but also because it is easier and less expensive than producing the vaccine from infected mouse brains.

Addendum

The following procedures are recommended for the production of African horse-sickness tissue culture vaccine.

ADAPTATION OF NEUROTROPIC STRAINS OF VIRUS TO TISSUE CULTURE CELLS

Neurotropic strains of virus attenuated by 100 intracerebral passages in adult mice are used as the source of virus for tissue culture passages. Supernatant virus fluids of freshly prepared 10% brain suspension in maintenance medium containing 2% calf serum are used as inoculums. The nutrient medium of cultures prepared in large flasks (preferably 1-liter Roux bottles) is discarded and the cells are infected with the virus fluids. After 30 minutes' incubation at 37 C., fresh maintenance medium, YLE or YLH containing 2% calf serum (pH 7.0), is added to them. Subpassages of virus are made whenever cytopathic changes become widespread. Sometimes, during the first passage, the pH of the maintenance medium has to be adjusted if the pH becomes lower than 6.5.

STORAGE OF VARIOUS ANTIGENS

Freeze-Dried Antigen.—The 1st and 2nd passages in tissue cultures of vaccine strains are freeze-dried. The supernatant virus fluid is diluted at least 10 times in sterilized LPTB solution or in lactose-peptone aqueous solution. The virus fluid dispensed in sterilized small bottles or ampules is freeze-dried, and each container is sealed under vacuum. Every precaution should be taken to avoid mixing other strains of virus. Freeze-dried antigen may be stored at 4 C. or lower temperature. If the 1st passage does not produce distinct cytopathic changes, the 3rd passage may be also freeze-dried.

Every 12 months fresh freeze-dried antigens should be prepared from fresh mouse brain material.

Frozen Stock Antigen.—This is not absolutely necessary, but as a precautionary measure some of the virus fluid prepared for freeze-drying can be dispensed in hemagglutination tubes with rubber stoppers and stored at -20 to -25 C.

Liquid Stock Seed Virus.—Supernatant virus fluids containing 2% calf serum of the 3rd and 4th passages in tissue cultures are dispensed in hemagglutination tubes. They are sealed with rubber stoppers, and stored at 4 C. The final pH value of the fluids should be between 6.5 and 7.5. A portion of the virus fluid is diluted in lactose-peptone solution, dispensed into small tubes, and stored at -20 C. or a lower temperature. Every 6 months fresh liquid stock seed virus should be prepared.

Liquid Vaccine Seed Virus.—This is prepared from liquid stock seed virus either by 1 or 2 additional passages in tissue cultures. The supernatant virus fluids are dispensed in small tubes, sealed with rubber stoppers, and stored at 4 C. The final pH value should be maintained between 6.5 and 7.5. A titer of $10^{5.5}$ TCID₅₀/ml. or higher should be maintained. Every 3 months fresh vaccine seed virus should be prepared.

PREPARATION OF VACCINE

Cell Cultures.—The MS cell cultures are prepared in the manner described previously, using large flat bottles such as 1-liter Roux flasks or 5-liter diphtheria toxin culture bottles. The density of the cells over the entire surface of the bottles should be even.

Inoculation.—As soon as complete cell sheets are formed, the nutrient medium is discarded. If the pH value of the medium is between 6.5 and 7.0, the cells are infected without washing. The volume of inoculum is approximately 1/100 volume

of the medium used. The bottles of infected cultures are rocked a few times during the 30-minute absorption period at 37 C. in order to distribute the inoculum over the entire surface of the cell sheets. After placing fresh maintenance medium in each bottle, the culture is incubated at 37 C.

Harvesting.—In MS cells, complete CPE usually appears between 48 and 72 hours after infection. To obtain the highest titer, virus fluids should be harvested when almost all cells remaining on the surface of the glass have a distinct CPE. There is a slight difference of incubation period between the strains used. As the incubation periods vary according to the virus titers, stains used, density of cells, and other delicate factors, it is hard to determine the exact time to harvest various strains of virus.

Preparation of Liquid Vaccine.—After shaking vigorously, virus fluids are harvested into centrifuge bottles and centrifuged at 500 g for 10 minutes at 4 C. The supernatant fluid is harvested, paying careful attention to avoid contamination of large cell debris. For monovalent vaccine, 1 volume of the supernatant virus is mixed with 10 volumes of child freeze-drying diluent, preferably LPTB solution, and 9 volumes of distilled water. For polyvalent vaccine, N volumes of supernatant virus fluids of vaccine strains mixed in equal parts are mixed with 10 volumes of the freeze-drying diluent and (10—N) volumes of sterilized distilled water (where N is the number of strains incorporated in the vaccine).

The total volume of virus fluid and distilled water added is always equal to the volume of freeze-drying diluent containing penicillin and streptomycin.

If one wants to dilute any strain of virus more than 100 times, it is safer to store the supernatant virus fluid at 4 C., adjusting the pH value between 6.5 and 7.0, and determining the approximate titer of virus in the fluid. If the titer is approximately $10^{7.5}$ TCID₅₀/ml., the virus fluid may be diluted 200 times in freeze-drying diluent. Diluted liquid vaccine may be stored at 4 C. until freeze-dried.

Freeze-Dried Vaccine.—The liquid vaccine is dispensed in sterilized ampules or bottles and freeze-dried by overnight operation of machines. Each ampule or bottle should be vacuum sealed. The freeze-dried vaccine should be white or light pink pellet that should maintain its shape during storage in a refrigerator.

VARIOUS TESTS OF SEED VIRUS AND VACCINE

Test of Seed Virus.—Liquid stock seed virus should be tested for specificity, antigenicity, and safety.

Specificity may be tested by neutralization tests in tube cultures.¹³ The cul-

tures should be observed for 2 weeks to ensure that there is no other virus mixed within the seed virus.

Antigenicity and safety may be tested in horses simultaneously. At least 2 nonimmunized horses should be used for testing each attenuated strain of African horse-sickness virus. Blood samples should be obtained from the horses before injection. After injection, the horses are kept under close observation for 4 to 5 weeks and temperatures are recorded daily. Blood samples are drawn again, then the immunity is challenged with a non-attenuated strain of the virus. Antibody titers of these horse serums should be tested by the method described herein.

If inoculated horses are kept under comfortable conditions without work, they should not develop any severe signs of abnormality.

The use of guinea pigs in determining the potency and antigenicity of attenuated strains has been recommended by Erasms.⁵

Test of Liquid Vaccine.—Control cell cultures used for the production of liquid vaccine must be kept at 37 C. for at least 2 weeks after the preparation of cell cultures in order to detect any virus accidentally introduced with the medium. If any abnormality is found in the control cell culture, the medium should be tested by subcultivations.

Virus fluids must be carefully examined microscopically for bacterial or fungus contamination before pooling and preparation of liquid vaccine.

Test of Freeze-Dried Vaccine.—The sterility of freeze-dried vaccine is tested by inoculating 1.0 ml. of reconstituted vaccine into thioglycollate medium and incubating for 7 days at 37 C. If there is contamination, the contaminant is identified and the test is repeated. Five adult mice are inoculated intraperitoneally, each with 0.2 ml. of reconstituted vaccine, and kept under observation for 2 weeks.

Two bottles of freeze-dried vaccine are placed in the incubator at 37 C., and after 7 days the incubated vaccine is titrated in MS tube cultures in comparison with vaccine stored at 4 C. They are reconstituted in the manner described previously. The titrations are read approximately 9 days after infection. The difference in titers should be less than 0.5 log.

The minimum requirements for African horse-sickness vaccines are 20,000 TCID₅₀/dose for monovalent vaccine and 120,000 TCID₅₀/dose for polyvalent vaccine. All vaccine vials should be tested for vacuum before they are issued.

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