appears not to have been emphasized before. As would be expected, the effect of digoxigenin completely disappears in 24 hours. At 3 hours, however, digoxigenin causes an increased tolerance for ouabain. Further investigation is needed to determine the significance of this finding.

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Cultivation of Virus of Equine Encephalomyelitis in Serum-Ultrafiltrate and Buffered Salt Solution (Simms).*

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Propagation of a virus in protein-free serum-ultrafiltrate cultures was first accomplished with the agent of lymphogranuloma venereum.[1] The same technic has since been successfully applied to St. Louis encephalitis[2] and a murine strain of poliomyelitis.[3] In the previous studies emphasis was placed upon the stability of the serum-ultrafiltrate cultures as manifested by consistent pH's ranging approximately between the neutral point and 7.6, by persistent viability of tissue cells for long periods of time and proportionate maintenance of high viral potencies. In 2 of the 3 viruses mentioned (*Lymphogranuloma venereum* and St. Louis encephalitis) it was noted that better growth was obtained at room temperature (approximately 20-30° C.) than in the incubator at 37° C. In the case of the third virus, that of murine poliomyelitis, the unusual level of potency of 1 to one billion was obtained with the clear supernatant fluid of cultures kept at 37° C.

The present communication deals with the application of the serum-ultrafiltrate technic to the cultivation of the virus of equine encephalomyelitis (Western and Eastern strains).

The virus of equine encephalomyelitis apparently can be grown

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1 Sanders, M., *J. Exp. Med.*, 1940, **71**, 113.

2 Molloy, E., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 563.

3 Jungeblut, C. W., and Sanders, M., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 375.

at will in Tyrode fluid preparations[4, 5] and on the chorioallantoic membrane of the developing hen's egg.[6] Potencies of 10^{-5} for the flask cultures and 10^{-6} for the chorioallantoic preparations have been reported. In more recent work[7] titers as high as 10^{-8} and 10^{-9} have been obtained from whole developing egg preparations (minced embryo plus chorioallantois). The highest titers occurred when the Eastern strain was used.

Materials and Methods. Tissue cultures were prepared in rubber stoppered 50 cc Erlenmeyer flasks by adding minced embryonic chick tissue (with eyes, beak and limbs removed) to 10 cc of serum-ultrafiltrate diluted 1 in 3 with Simms' salt solution.[1] In order that the effect of the serum ultrafiltrate on the virus might be evaluated, similar preparations were made which contained only the buffered salt solution. Both types of cultures were inoculated with 0.1 cc of a 1:10 mouse brain virus suspension (Western strain) and parallel series of each type maintained at room temperature and at 37° C. Passages were made by transferring 0.1 cc of the clear supernatant fluid every 3 days. After intervals of 3, 6, 10, 15, 20, or 30 days incubation, potency tests of various culture generations were carried out by intracerebral inoculation of groups of 4 mice (12 to 15 g) with 0.03 cc of serial tenfold dilutions of the supernatant fluid of the culture (Tables I, II, III, IV). The endpoint in these titrations was taken as the last dilution causing characteristic symptoms and death in 50% of the inoculated mice. The identity of the virus was assured by two neutralization tests with a known antiserum, carried out with the 5th and 28th culture passages.

TABLE I.
Equine Encephalomyelitis (Western Strain) in Serum-Ultrafiltrate Chick Tissue Cultures Maintained at Room Temperature (20-30°C).

Culture generation	Final dilution activity of culture fluid after varying periods					
	3 days	6 days	10 days	15 days	20 days	30 days
7	10^{-5}					
13	10^{-6}					
						at least
20	10^{-6}	10^{-5}	10^{-4}	10^{-5}		10^{-2}
24	10^{-5}					10^{-2}
30	10^{-6}	10^{-6}	10^{-5}		10^{-3}	10^{-1}
33	10^{-7}	10^{-5}	10^{-4}	10^{-3}		10^{-2}
35	10^{-6}	10^{-6}	10^{-5}	10^{-4}		

Blank space—no titration done.

4 Cox, H. R., Syverton, J. T., and Olitsky, P. K., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 896.

5 Olitsky, P. K., Cox, H. R., Syverton, J. T., *J. Exp. Med.*, 1934, **59**, 159.

6 Higbie, E., and Howitt, B., *J. Bact.*, 1935, **29**, 399.

7 Beard, J. W., Beard, D., and Finkelstein, H., *J. Immunol.*, 1940, **38**, 117.

TABLE II.
Equine Encephalomyelitis (Western Strain) in Serum-Ultrafiltrate Chick Tissue Cultures Maintained at 37°C.

Culture Generation	Final dilution activity of culture fluid after varying periods			
	3 days	6 days	10 days	15 days
13	10^{-5}			
20	10^{-5}	10^{-4}	10^{-1}	0
24	10^{-4}			0
30	10^{-5}	10^{-3}	10^{-2}	10^{-1}
33	10^{-5}	10^{-3}	10^{-1}	0

TABLE III.
Equine Encephalomyelitis (Western Strain) in Salt-Solution (Simms) Chick Tissue Cultures Maintained at Room Temperature (20-30°C).

Culture Generation	Final dilution activity of culture fluid after varying periods				
	3 days	6 days	10 days	15 days	20 days
5		10^{-5}	*	*	*
6	10^{-5}	10^{-5}	10^{-3}	10^{-2}	*
9	10^{-5}	10^{-4}	10^{-3}		10^{-1}
16	10^{-6}	10^{-5}	10^{-2}	*	*
18	10^{-6}	10^{-5}	*	*	*
21		10^{-4}	*	*	*

*Discarded because of excess acid formation (pH about 5).

TABLE IV.
Equine Encephalomyelitis (Western Strain) in Salt-Solution (Simms) Chick Tissue Cultures Maintained at 37°C.

Culture Generation	Final dilution activity of culture fluid after varying periods			
	3 days	6 days	10 days	15 days
6	10^{-4}	10^{-4}	*	*
9	10^{-5}	10^{-3}	*	*
18	10^{-4}	10^{-3}	10^{-1}	*

*Discarded because of excess acid formation (pH about 5).

Throughout this study, one fact was apparent: the cultures which contained buffered salt solution alone were much less stable than those with the serum-ultrafiltrate mixture. Acid formed in many of the salt solution cultures in a short time and they had to be discarded even before inoculation of virus.‡ This was particularly true of those cultures kept in the incubator. The serum ultrafiltrate cultures, on the other hand, could be kept at room temperature for at least 10 days before being inoculated with virus. Even if the amount of tissue was somewhat increased above the normal (approximately a ratio

‡ As has been previously noted,[1] the pH of these cultures can be roughly estimated at all times since the fluid mediums contain 0.1% phenol red, an amount of dye which has been found to be nontoxic for the cells.

of tissue to fluid of 1:100) the pH of these cultures remained constantly between 7.0 and 7.5.

The highest potencies, 10^{-5} to 10^{-7} dilution activity, were obtained from the cultures kept at room temperature (Tables I, II, III, and IV). Although the serum-ultrafiltrate cultures at room temperature were only slightly more potent than similar preparations with salt solution, it is clear (Tables I and III) that more frequent titrations were possible with the former group. The greatest difference between the two types of cultures (at room temperature) seems to lie in the ability of the serum-ultrafiltrate preparations to maintain higher potencies over an extended period of time (Tables I and III). The final culture generation tested was the 35th (serum-ultrafiltrate at room temperature). This preparation attained a dilution activity of 10^{-6} after the 3rd day. The same virus content was demonstrated at the end of 6 days, and by the 10th day only a slight decrease in potency was apparent (10^{-5}). Even after 15 days, the culture fluid produced symptoms and death in 50% of the mice injected with a dilution of 1:10,000. It is interesting to note that the same type of preparation kept at 37° C (33rd subculture) reached a potency of 10^{-5} at its highest point. There was a comparatively more rapid loss of potency so that by the 10th day only a trace of virus could be demonstrated and by the 15th day no virus was present.

A preliminary experiment done with the Eastern strain of equine encephalomyelitis in serum-ultrafiltrate yielded a dilution activity of the culture fluid (4th culture generation) of 10^{-7}. The same titer was obtained after 3 days regardless of whether the culture was kept at room temperature or 37° C.

Another type of preparation which was tested with the Western strain consisted of chorioallantoic membrane of 12 to 14 day eggs in serum-ultrafiltrate.[2] The supernatant fluid of such preparations kept at room temperature regularly yielded a dilution activity of 10^{-6} (highest dilution tested).

Preparation of a vaccine. That the fluid of the preparations studied is capable of producing an immunity when made into a vaccine is evidenced by the following experiment. The clear supernatant fluid of a serum-ultrafiltrate culture (titer 10^{-6}) was treated with 0.4% formalin. Guinea pigs receiving 2 weekly intramuscular injections of 1 cc of the final neutralized product were found to be immune to 1,000 to 10,000 m.l.d. of virus. After storage for 4 months in the refrigerator, the same vaccine yielded identical results.

An effort was made to demonstrate the protein content of the vaccine by adding equal parts of saturated ammonium sulfate. No precipitate formed immediately, but after standing in the refrigerator

overnight, a faint precipitate could be seen at the bottom of the test tube, suggesting a very low protein content. Further studies on the immunizing capacity and quantitative protein determinations of this type of vaccine are being carried out.

Discussion. Although the potencies which have been obtained in this study are not unusually high for the equine encephalomyelitis virus, it must be remembered that in the developing chick preparation the peak of growth is reached within 9 to 13 hours for both the Eastern and Western strains.[6] In the presence of embryonal death, which occurs regularly by the end of 24 hours, there is a rapid decline of virus potency. Thus, unless the virus is quickly harvested, a poor yield is obtained. However, in the case of the serum-ultrafiltrate cultures maintained at room temperature, the danger of loss of virus due to rapid deterioration is negligible, since there is relatively little decrease in virulence even after 10 to 15 days.

Because the developing egg can be so well impregnated with the equine encephalomyelitis virus, an effective formalized vaccine has been in use for several years. However, the high degree of infectivity is somewhat nullified by the fact that the final vaccine contains as much as 40% chick embryo-tissue-extract.[7] It is therefore not surprising that occasional reactions of varying degrees are seen following the use of chick embryo-tissue-extract vaccines.[8, 9] With the growing need for human vaccination such a problem may well assume greater proportions than it has in the past.

Preliminary tests have shown that the serum-ultrafiltrate from virus infected cultures may be used for the production of a vaccine. Such a product involves a protein-free substrate, minimal manipulation in its preparation, and a good degree of effectiveness and stability. In this respect, it should also be noted that experiments with larger cultures (50 cc) have yielded amounts of virus similar to those obtained from the smaller (10 cc) cultures. There is every reason to believe that large quantities of a serum-ultrafiltrate vaccine can be produced as readily as small quantities.

Conclusions. The Western strain of equine encephalomyelitis has been successfully grown in whole minced chick embryo or chorioallantois in serum-ultrafiltrate and buffered salt solution (Simms') at room temperature and at 37° C.

The highest titers (10^{-5} to 10^{-7}) have been obtained with the clear supernatant fluid of chick and serum-ultrafiltrate cultures maintained at room temperature. The titers of these cultures can be sus-

[8] Graham, R., *J. Am. Vet. Med. Assn.*, 1940, **97**, 38.

[9] Schoening, H. W., *J. Am. Vet. Med. Assn.*, 1940, **97**, 39.

tained with little decrease for as long as 10 and 15 days.

The Eastern strain of equine encephalomyelitis has been grown with a dilution activity of 10^{-7} in whole minced chick embryo in serum-ultrafiltrate at room temperature and 37° C.

A vaccine, containing very small amounts of protein, prepared from the clear supernatant fluid of serum-ultrafiltrate cultures protected guinea pigs against 1,000 to 10,000 m.l.d. doses of virus (Western strain).

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Characteristics of a Fixed Rabies Virus Cultivated on Developing Chick Embryos.

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In a preceding paper[1] we reported the cultivation of rabies virus in developing chick embryos in 2 series through 9 and 6 successive passages respectively. Since then, one series has been carried through 16 subcultures; the second series has been passaged through 47 subcultures during a period of 18 months and is still being maintained. The purpose of this note is to summarize our observations on the behaviour of this virus to different hosts after prolonged cultivation in the developing chick embryo.

Technic. A fixed virus, presumably derived from the original Pasteur strain, was used for initiating the chick embryo cultures. The first inoculation was made by dropping 0.1 cc of a 10% saline emulsion of infected mouse brain on the allantois immediately over the embryo using the Burnet[2] technique. The same technique was used for subcultures: pieces of the inoculated allantois, or preferably the brains of the embryos of 3 to 6 infected eggs, were ground in a sterile glass mortar and enough saline added to make a 10% emulsion. Each passage material was titrated intracerebrally in mice in tenfold dilutions. In the first 12 passages, 5-day-old embryos were infected; subsequently 6-day-old embryos were used because they were more resistant to the manipulations involved. Passages were usually made 6-11, sometimes up to 14, days after the inoculation.

The infection in the chick embryo. The embryos of earlier as well

1 Kligler, I. J., and Bernkopf, H., *Nature*, 1939, **143**, 899.

2 Burnet, F. M., Sp. Rep. Ser. Med. Res. Coun. Lond., No. 220, 1936.