There was no apparent interference. Further tests along this line are indicated.†

Summary and Conclusions. 1. Zephiran and merthiolate 1:10,000 and sodium sulfathiazole 1:500 in normal rabbit serum had no virucidal effect upon Western equine and St. Louis encephalitis viruses as determined through serial virus dilutions under the holding conditions and dilution of a standard neutralization test.

2. Mercuric cyanide 1:10,000 and phenyl mercuric borate 1:50,000 in serum under closely comparable conditions did not affect either of the above viruses or the Lansing mouse-adapted poliomyelitis virus.

3. Phenyl mercuric borate 1:50,000 has been used over a period of one year in over 1,000 serum specimens which have been subjected to diagnostic tests. There has been no apparent interference. Many of these sera have been used repeatedly in neutralization tests against several neurotropic viruses and in experimental *in vitro* tests including mumps complement fixation tests. This agent has greatly reduced difficulties previously encountered due to contamination of sera during collection and routine handling.

Phenyl mercuric borate is therefore recommended for routine use as a bacteriostatic agent for serum specimens to be used in *in vivo* diagnostic tests for the neurotropic viruses. Further experimental trial is required before it can be recommended for all types of *in vitro* tests.

† Since writing this approximately 300 sera containing phenyl mercuric borate have been tested for complement fixing antibodies to the Western equine or the Japanese B viruses. In these tests and several negative and positive controls with larger amounts of the preservative no interference was detected.

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Propagation of Theiler's GD-VII Mouse Virus in Tissue Culture.

RAYMOND C. PARKER AND AUGUSTA J. HOLLENDER.
From the Connaught Laboratories, University of Toronto.

Some years ago, various attempts were made* to cultivate monkey poliomyelitis (MV) virus in fluid cultures of monkey olfactory bulbs and sympathetic ganglia. Although this work gave promise in so far as the survival of the tissue elements was concerned, the results were otherwise disappointing. It was decided, therefore, to undertake the cultivation of other neurotropic viruses, preferably ones that could be carried in mice, in order to gain experience that might be applicable, eventually, to the cultivation of human poliomyelitis. Jungeblut and Sanders[1] reported the propagation in mouse embryo brain cultures of the SK monkey poliomyelitis virus that had previously been transmitted to mice by intermediary passage through cotton rats. The culture medium used by them consisted essentially of ox serum ultrafiltrate. The present communication deals with the propagation of Theiler's GD-VII virus[2,†] of mouse encephalomyelitis under a wide variety of special conditions.

Materials and Methods. After the virus had been carried for 20 culture passages, during which time several different sets of con-

* Unpublished experiments carried out in association with Dr. Howard A. Howe and Dr. Geoffrey W. Rake.

1 Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1940, **72**, 407.

2 Theiler, M., *J. Exp. Med.*, 1937, **65**, 705; Theiler, M., and Gard, S., *J. Exp. Med.*, 1940, **72**, 49, 79.

† The virus was supplied through the courtesy of Dr. Max Theiler of the Laboratories of the International Health Division, The Rockefeller Foundation, New York.

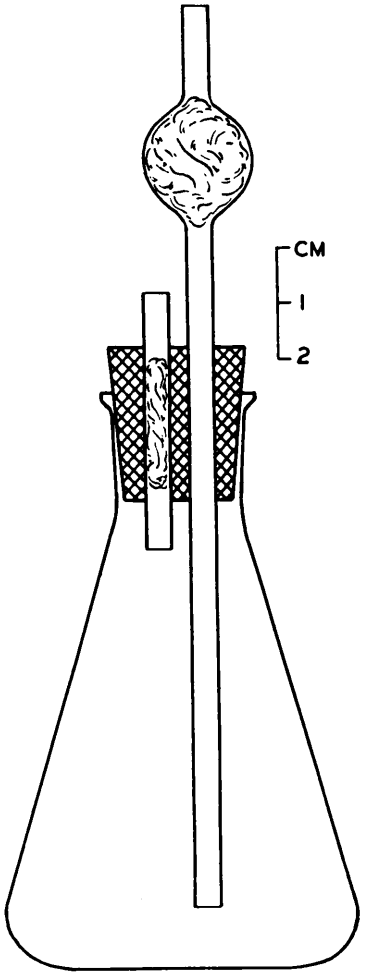

FIG. 1.

ditions were tested by comparative experiment, a routine set of procedures was chosen for all subsequent work. These procedures will now be outlined. For mouse embryo brain cultures, the supporting medium (3 cc) is prepared in 125 cc Erlenmeyer flasks and consists either of ox serum ultrafiltrate (⅓)[3,‡] or of heated (1 hr at 56° C) rabbit serum (⅓), combined with Simms' X7 balanced salt solution (⅔).[3] Before the tissue is added, the cell-free medium is adjusted to pH 7.1 or 7.2 by gassing for 1-2 min with a mixture of 8% CO_2, 21% O_2 and 71% N_2. For this purpose, the flasks are closed with 2-holed rubber stoppers each fitted with a long inlet tube surmounted by a cotton filter bulb and a short outlet tube partially filled with loose non-absorbent cotton (Fig. 1). After the tissue and the virus inoculum have been added, the special gassing device is replaced by solid rubber stoppers. As an indicator of pH, all cultures contain 0.001% phenol red.

The tissue component of the medium is prepared by mincing the brain on a glass plate. The mincing is done with cataract knives, with curved scissors, or with halves of razor blades held in hemostats. The tissues are chopped to extreme fineness. One large brain is required for each flask.

If the virus inoculum is prepared from brain material that has been preserved in 50% glycerine in saline, the tissues are chopped into coarse fragments and washed 3 times in great excess of glucosol (Tyrode's solution without bicarbonate). It is then ground in a mortar with quartz sand (60-80 mesh) and made up to 10^{-1} (1:10) with distilled water. This dilution is allowed to stand for 1 hour in an ice bath before the second dilution is prepared, as before, in distilled water. The third dilution (10^{-3}) and all higher dilutions are prepared in beef broth containing 10% serum ultrafiltrate. The 10^{-3} dilution is ordinarily used for the initial inoculation of the culture series; this and higher dilutions are used for mouse (Swiss strain) titrations to determine the potency of the original virus. For each culture, the inoculum is 0.3 cc; for each mouse, the intracerebral injection is 0.03 cc. Three mice are injected at each of several dilutions.

After 3-4 days incubation at 35° C, the culture virus is transferred to fresh brain cultures, and mouse titrations are made to test its killing power. Before the mouse titrations are made, and before the subcultures are infected (with 0.3 cc of undiluted culture material), the old culture mixture is centrifuged for 5-10 min at 2000 rpm in an angle head, in order to eliminate the tissue particles. Unlike the rabies virus cultivated under simi-

‡ The ox serum ultrafiltrate was supplied by William R. Warner & Co., Inc., New York, N.Y.

[3] Simms, H. S., and Sanders, M., *Arch. Path.*, 1942, **33**, 619. See also: Sanders, M., and Molloy, E., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 327; Sanders, M., *J. Exp. Med.*, 1940, **71**, 113.

lar conditions,[4] the GD-VII virus is not so intimately bound to the culture tissues that it must be released by grinding.

Experiments and Results. The first experiment with the GD-VII virus was made with embryo mouse brain tissue and with ox serum ultrafiltrate as ⅓ of the medium (3 cc). This series gave positive mouse titrations through 15 culture passages (52 days), after which time it was lost as the result of bacterial contamination. But the culture virus did not kill mice in dilutions higher than 10^{-2} and 10^{-3}. The original virus inoculum was prepared from mouse brain material of the 129th mouse passage that had been preserved in glycerine for 135 days. The cultures were prepared in 125 cc Erlenmeyer flasks, were attached to a manifold in the incubator and gassed continuously with a mixture containing 8% CO_2; and, supernatant fluid from the unground cultures was used for serial transfers and for mouse titrations. Simultaneously, a sister series gave positive mouse titrations through 9 passages at dilutions of 10^{-4}. In this series, the culture medium (4 cc) consisted of ox serum ultrafiltrate (⅓) and Simms' salt mixture (⅔) and was prepared in 50 cc Erlenmeyer flasks that were incubated with closed stoppers after an initial pH adjustment with an appropriate gas mixture.

A series of 7 short experiments (none of more than 5 passages) was then made, but all gave negative results. In 4 of these experiments, the medium consisted partly of rabbit serum; in 3, both rabbit serum and ox serum ultrafiltrate were used in duplicate series of cultures, for purposes of comparison. In each of the 7 experiments, the original virus inoculum, whether from fresh mouse brains, from mouse brains preserved in glycerine, or from mouse brains kept frozen with CO_2-ice, killed mice in dilutions as high as 10^{-6}. In some of the experiments, the tissues were ground at transfer and for mouse titrations; in others, supernatant fluid from unground cultures was used. Certain of the experiments were gassed continuously; others were not. But the results were uniformly negative.

In a final effort to determine whether or not the original virus material was still capable of survival in cultures, a double series was inoculated with brain material that had been preserved for over 15 months in glycerine and that was still potent enough to kill mice at a dilution of 1:100,000 (10^{-5}). Half of the cultures were made with rabbit serum and half with serum ultrafiltrate. Again, the rabbit serum cultures gave negative results; but the serum ultrafiltrate cultures yielded virus of low potency that persisted through 5 passages. At this point, still another double experiment was started in which rabbit serum and ultrafiltrate were again used in two comparable series. This time, however, the virus inoculum was prepared from the fresh brain of a paralyzed mouse that had been inoculated with the 3rd culture passage material from the experiment just described. Here again, the ultrafiltrate cultures continued to support the virus, whereas the rabbit serum cultures became negative after the 3rd passage. In the ultrafiltrate cultures, the virus attained a potency of 10^{-5} in the 5th passage.

The culture virus that was developed in this experiment was carried eventually, and without intercurrent animal passage, for 20 transfers.§ During this time, it was used for 10 separate and distinct experiments, each of which was designed to compare the effect of two variations either in the composition of the medium or in other conditions that might affect the multiplication of the virus. It was also used in 3 experiments designed to determine the interval required for maximum yield. Over the entire period, it continued to multiply to the extent that, under favorable conditions, it would kill at least 2 out of 3 mice injected intracerebrally with unground cell-free culture fluid in dilutions as high as 1:1,000,000 (10^{-6}). The results of these

[4] Parker, R. C., and Hollander, A. J., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 94.

§ Culture virus from the 10th passage was submitted to Dr. Theiler for verification of identity. On the basis of pathological findings and neutralization tests, Dr. Theiler concluded that the culture virus was indistinguishable from GD-VII.

TABLE I.
Theiler's GD-VII Mouse Virus in Fluid Cultures of Mouse Embryo Brain.*

Exp. No.	Series No.	Passages	Conditions under comparison	Temp. (°C)	Highest dilution of culture fluid that killed at least two of the three mice injected with each of the various dilutions tested at each successive passage							
					1st	2nd	3rd	4th	5th	6th	7th	8th
1	13233	1-5	R.S.†	37½	neg	neg	neg	neg	neg			
	13236		U.F.‡	"	10^{-1}	10^{-4}	10^{-2}	10^{-3}	10^{-2}			
2	13333	1-5	R.S.	"	10^{-1}	10^{-2}	neg	neg	neg			
	13336		U.F.	"	10^{-3}	10^{-4}	10^{-4}	10^{-4}	10^{-5}			
3	13437	6-13	37½°C		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-3}	10^{-3}	10^{-4}	—
	13441		35°C		10^{-4}	10^{-5}	10^{-5}	10^{-5}	10^{-6}	10^{-6}	10^{-6}	10^{-5}
4	13445	5-11	E-125§	"	10^{-3}	10^{-2}	10^{-3}	10^{-4}	10^{-4}	10^{-4}	10^{-5}‖	
	13449		E-50§	"	10^{-2}	10^{-2}	10^{-1}	10^{-3}	10^{-3}	10^{-3}	10^{-3}‖	
5	13606	12-16	Unground	35	10^{-6}	10^{-6}	10^{-5}	10^{-6}	10^{-6}			
	13610		Ground	"	10^{-6}	10^{-5}	10^{-5}	10^{-6}	10^{-6}			
6	13621	14-17	U.F.	"	10^{-5}	10^{-6}	10^{-6}	10^{-6}				
	13624		R.S.Filt.¶	"	10^{-4}	10^{-5}	10^{-5}	10^{-5}				
7	13710	14-17	R.S.	"	10^{-5}	10^{-5}	10^{-5}	10^{-5}				
	13714		R.S.Filt.	"	10^{-5}	10^{-5}	10^{-5}	10^{-6}				
8	13718	17-18	No gas**	"	10^{-6}	10^{-5}						
	13721		Gas††	"	10^{-5}	10^{-5}						
9	13823	18-20	U.F.	"	10^{-5}	10^{-5}	10^{-4}					
	13827		R.S.Filt.	"	10^{-6}	10^{-6}	10^{-5}					
10	13881	18-20	No gas	"	10^{-6}	10^{-5}	10^{-6}					
	13885		Gas	"	10^{-6}	10^{-5}	10^{-5}					

Exp. 1. Theiler's GD-VII virus (M.P. 129); from mouse brain in glycerine in 15½ months.
2. Virus from fresh brain of paralyzed mouse inoculated with 3rd culture passage material of 13236 series.
3. Culture virus from passage 5 of 13336 series; frozen in CO_2-ice 13 days.
4. Culture virus from passage 4 of 13336 series; frozen 17 days.
5. Culture virus from passage 11 of 13449 series. Virus from both series filtered through Pyrex (UF) fritted glass filters between 13th and 14th passages.
6. Culture virus from passage 13 of 13441 series.
7. Culture virus from passage 13 of 13606 series after filtration through Pyrex fritted glass filter; frozen 9 days.
8. Culture virus from passage 16 of 13606 series.
9. Culture virus from passage 17 of 13714 series.
10. Culture virus from passage 17 of 13710 series; frozen 12 days.

* Unless otherwise indicated, the medium (3 cc) consisted of ultrafiltrate of ox serum (1/3) and Simms' X7 solution; the flasks (Erlenmeyer, 125 cc) were tightly stoppered, without continuous gassing, during incubation; and, transfers of culture virus were made without grinding the tissue.

† Rabbit serum, unfiltered.

‡ Ultrafiltrate of ox serum.

§ Erlenmeyer flasks of 125 cc and 50 cc capacity.

‖ 35°C for final (11th) passage only.

¶ Rabbit serum, filtered.

** Preliminary pH adjustment with gas mixtures, but cultures left lightly stoppered during incubation.

†† Cultures gassed continuously.

experiments are presented in Tables I and II, and will now be summarized.

Serum Ultrafiltrate. Of the 10 experiments presented in Table I, 4 were designed to compare the effect of ultrafiltrate with that of heated rabbit serum when each was used as the main ingredient of the culture fluid. In 2 of these experiments (Exp. 6 and 9), the rabbit serum had been filtered through Pyrex (U.F.) fritted glass filters; in two (Exp. 1 and 2), the serum was unfiltered. In Exp. 1, the killing power of the ultrafiltrate cultures remained low; and at the end of the 5th passage the titer was reduced to 10^{-2}. But in Exp. 2, which was infected with virus from a mouse inoculated with 3rd culture passage material of the 1st experiment, the titer was increased to 10^{-5} by the end of the 5th passage. By this time, however, the sister series treated with rabbit serum gave negative results in dilutions of 1:10 and higher, just as the serum cultures of the first experiment had been negative throughout. In Exp. 6, the ultrafiltrate cultures improved slightly, al-

TABLE II.
Amount of GD-VII Virus Obtained After Various Intervals of Cultivation.

Exp. No.	Culture Nos.	Passage No.	Highest dilution of culture fluid that killed at least 2 of the 3 mice injected at 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}, respectively: After 1 day	After 2 days	After 3 days	After 4 days	After 6 days	After 7 days
I	13847-56	18	10^{-5}	10^{-6}	10^{-5}			
II	13889-96	18	10^{-5}	10^{-6}		10^{-5}	10^{-4}	10^{-4}
III	13905-12	19	10^{-5}	10^{-6}		10^{-5}		10^{-4}

though this increase was probably the result of incubation at 35° C, which had already been found to be superior to 37.5° C. In Exp. 9, this improvement was lost, and the killing power of the ultrafiltrate cultures was somewhat less than that of those containing filtered serum. It should be noted, however, that in 5 other experiments presented in Table I (Exp. 3, 4, 5, 8 and 10), serum ultrafiltrate was the main component of the culture fluid. And whenever, in these 5 experiments, the temperature of incubation was set at 35° C, the culture virus reached a potency of 10^{-5} and 10^{-6}.

Rabbit Serum. Before the experiments presented in Table I were undertaken, 7 short experiments made with rabbit serum had given negative results. And the first 2 serum experiments recorded in Table I were also negative. But in Exp. 6, 7, and 9, in which heated and filtered rabbit serum was used, the culture virus reached a potency of 10^{-5} and 10^{-6}. In Exp. 7, the advantages of filtration were shown to be negligible.

Temperature of Incubation. In the 3rd experiment presented in Table I, a comparison was made of the relative effect on the multiplication of the virus of incubating the cultures at 37.5° C and 35° C, respectively. The virus yield at 35° C was invariably higher than that obtained at 37.5° C; and this slight decrease in temperature is regarded as being responsible for the general improvement in the results obtained in all subsequent experiments.

Use of Gas Mixtures. From the results obtained in Exp. 8 and 10 (Table I), it will be seen that the virus content of cultures that were gassed continuously with a mixture of 8% CO_2, 21% O_2 and 71% N_2 showed no improvement over those in which the gas mixtures were used only to adjust the initial pH of the culture medium. The pH was maintained at a relatively constant level in each instance.

Effect of Grinding the Tissues. The fifth experiment presented in Table I was designed to compare the multiplication of virus in a double series inoculated with ground and unground culture material, respectively. In one series, the tissues were ground at transfer (and for mouse titrations) with Tenbroeck grinders that were kept chilled in an ice bath during the grinding. In the sister series, the material for the inoculation of the subcultures and for the mouse titrations was prepared from cell-free supernatant obtained by centrifuging the unground tissue-fluid mixture. Even after 5 passages, there was no appreciable difference in the amount of virus recovered from the two sets.

Later, after it had developed that the culture virus reached its maximum concentration much earlier than had been assumed, a comparative experiment was made with culture virus of the 22nd passage that had been frozen in the CO_2-ice box for 29 days before it was used to infect a series of 5 embryo mouse brain cultures. After 2 days' incubation, mouse titrations were made from the cell-free culture fluid after low speed centrifugation (5 min at 2000 rpm) of the unground material from the 5 cultures, and, simultaneously, a duplicate series of mouse titrations were made from the same culture material after the component tissues had been ground with Tenbroeck grinders, resuspended in the supernatant, and centrifuged again at low speed to eliminate tissue particles. The 2 sets of mouse titrations gave identical results (10^{-6}).

Size of Culture Flasks. Comparative series

were run in Erlenmeyer flasks of 125 cc and 50 cc capacity. In the larger flasks, 3 cc of medium were used, whereas 4 cc were used in the smaller flasks. The virus yield was definitely greater in the larger flasks. (Exp. 4, Table I).

Time Required for Maximum Yield. Three experiments were made in an effort to determine the length of time required to obtain the maximum concentration of virus in the culture medium. The virus used had been carried for 17 and 18 passages and was derived from the series shown in Table I. The culture medium consisted of serum ultrafiltrate and Simms' X7 solution; and the cultures were incubated at 35° C. Each experiment comprised 8 or 10 cultures. Mouse titrations were made after the 1st and 2nd day and at subsequent intervals up to 7 days. At the time of testing, 0.1 cc was removed from each culture; and the samples were pooled and diluted. The results are shown in Table II. In all 3 experiments, the greatest concentration of virus was obtained after 2 days' incubation, when the titer reached 10^{-6}. After 2 days, the concentration of culture virus diminished. But because the differences observed from day to day were not greater than tenfold, they were probably of doubtful significance.

Postembryonic Mouse Brain Cultures. Culture virus derived from 20th passage embryo mouse brain cultures (Exp. 10, Table I), and subsequently frozen at —60° C for 49 days, was propagated for 13 additional and successive passages in cultures of brain tissue from 1 day old mice (5 passages), 3 day old mice (3 passages), 5 day old mice (4 passages) and 14 day old mice (1 passage), respectively. At the end of the 5th passage in cultures prepared from the brains of 1 day old mice, the mouse titer was 10^{-6}. After 3 passages in cultures prepared from the brains of 3 day mice, the titer was reduced to 10^{-4}. In the next culture series, in which the brain tissue was obtained from 5 day mice, the titer increased to 10^{-5}. But in the final passage, with tissue from a 14 day old mouse brain, the titer dropped again to 10^{-3}. And at this point the experiments were discontinued. It seems likely, however, that the virus could have been propagated indefinitely and without loss of potency in cultures prepared from the day old brains.

Summary and Conclusions. (1) Theiler's GD-VII virus has been propagated for 20 passages (transfers) in cultures of chopped mouse embryo brain suspended in ox serum ultrafiltrate and Simms' X7 solution. After an initial series of culture passages, the cell-free culture fluid killed mice in dilutions as high as 1:1,000,000 when 0.03 cc was injected intracerebrally. Eventually, the same results were achieved when the ox serum ultrafiltrate was replaced by heated rabbit serum. (2) The virus multiplied more abundantly at 35° C than at 37.5° C; and, the yield was higher in 125 cc Erlenmeyer flasks containing 3 cc of medium than in 50 cc flasks containing 4 cc of medium. (3) No increase in virus output was ever obtained by inoculating subcultures with ground material; nor did grinding the culture tissues improve the mouse titrations. Also, no real advantage was gained by subjecting the cultures to continuous gassing with mixtures of O_2, CO_2 and N_2. (4) The concentration of virus in the culture medium was higher after 2 days' incubation than after 1 day. After 2 days, the yield diminished. (5) Culture virus from 20th passage embryo mouse brain cultures that had been frozen at —60° C for 49 days was propagated for 13 additional passages in cultures of brain tissue from 1 day old mice (5 passages), 3 day old mice (3 passages), 5 day old mice (4 passages) and 14 day old mice (1 passage), respectively.