STUDIES IN RODENT POLIOMYELITIS

II. Cultivation of the Murine Strain of SK Poliomyelitis Virus*

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Following isolation of a mouse-paralyzing agent by passage of the New Haven (SK) monkey poliomyelitis virus through cotton rats to white mice the successful cultivation *in vitro* of this virus was described (1). The initial studies of the culture virus were, of necessity, limited to an investigation of the basic conditions which permitted serial subcultivation. It appeared from this work that the murine virus grew well in embryonic mouse brain-serum ultrafiltrate cultures at 37°C., and that the infectious agent could be effectively subcultured by transfer of the supernatant fluid at 3 day intervals.

The present communication deals with the results of uninterrupted maintenance of the mouse virus through 200 successive culture generations. This work includes observations on the importance on virus growth of cellular components in tissue culture; the effect of pH on virus propagation and viability *in vitro;* the rate of propagation of the virus in tissue culture; and, the effect of prolonged cultivation *in vitro* on the infectious properties of the virus. In addition to studying the conditions underlying virus propagation *in vitro*, certain properties of the cultured virus were also investigated. Thus, the size of the murine tissue culture virus was determined by ultrafiltration; the specificity of the cultured murine virus was studied with special reference to any relationship that might exist between lymphocytic choriomeningitis virus and murine poliomyelitis virus; and, finally, an effort was made to determine whether the culture virus could be used in interference experiments to block poliomyelitis virus in monkeys with the same measure of success which had attended similar previous experiments in which mouse passage virus was employed.

The Importance on Virus Growth of Cellular Components in Tissue Culture

The same type of fluid tissue culture, *i.e.* minced tissue suspended in ox serum ultrafiltrate,[1] as had been previously found successful for the propaga-

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tion of several other viruses (2), was employed throughout this work. Since details of the method have already been published (2, 3), it is only necessary to emphasize here that such culture media, carefully prepared according to directions, provide a stable, physiological environment for the maintenance of viable cells and for the production of high levels of virus potency.

In previous experiments an attempt had been made to determine which cells would provide the best substrate for propagation of the virus. When three different types of tissue were used, *i.e.* embryonic mouse brain, embryonic guinea pig brain, or whole chick embryo, the titers of the different preparations in the 6th culture generation were 10^{-8}, 10^{-4}, and 10^{-1}, respectively.[2] Embryonic mouse brain was therefore adopted as the routine medium for all subsequent work. Further confirmation of the earlier finding that chick tissue is an unsuitable medium for murine virus growth was obtained as the result of our failure, on six different occasions, to recover active virus from inoculated chorioallantoic membranes. At no time could virus be demonstrated beyond the 2nd egg passage. When virus was present in earlier passages the infectivity of individual tissues did not differ from that of the chorioallantoic membrane.

On the basis of the above findings it appeared that the murine virus represented an agent with decided predilection for the tissues of its most susceptible host, *i.e.* the white mouse. The question arose whether such predilection was broad enough to embrace all mouse tissues or whether a special affinity could be demonstrated between virus growth and nervous tissue, which is the site of the essential lesion in the paralyzed mouse.

In an effort to clarify this point, a tissue culture medium consisting of whole mouse embryo from which the brain had been removed was inoculated with murine virus of the 119th mouse passage. At the same time a routine medium containing embryonic mouse brain substrate was inoculated with the same virus. These cultures were run in parallel series and transferred every 3 or 4 days on the same type of media. Titrations of the 19th subcultures gave titers of 10^{-5} for the whole mouse embryo culture minus brain, and of 10^{-6} in the case of the routine media. On repeating titrations with the 26th subcultures, the titers were 10^{-4} and 10^{-6}, respectively. A comparison of the rate of virus propagation in the two media showed that the titer in the series with whole mouse embryo, minus brain, on the 5th day was 1:10, on the 6th day 1:10,000, and that a titer of 1:100,000 was not attained until the 7th day. By contrast the titer of the embryonic mouse brain culture reached 1:10,000 in 4 days, and in 5 days the maximum level of 1:1,000,000 was obtained.

[2] These particular titers had been established with a dilution technique in which serial dilutions were made with a single pipette. In all subsequent titrations separate pipettes were employed. With the latter technique no titers higher than 10^{-6} were observed.

While the difference in final culture titers was therefore not too impressive, a suggestive retardation in the growth of virus in cultures which contained no mouse brain was readily recognizable in these experiments.

Since not all central nervous system tissue had been eliminated from the whole embryo mouse cultures, the possibility was considered that small amounts of remaining spinal cord tissue might account for the relatively good yields of active virus in these cultures. Therefore, at the 26th subculture the virus was transplanted to cultures which contained whole mouse embryo but neither brain nor spinal cord. After eleven serial passages in this type of medium the culture titrated 10^{-4} as compared to 10^{-6} dilution activity for the routine embryonic mouse brain preparations.

It would thus appear that in the absence of central nervous system tissue in cultures, virus potency is definitely reduced, but not abolished. Evidently non-nervous embryonic mouse tissue is capable, to some extent, of maintaining a certain basic level of virus growth.

The Effect of pH on Virus Propagation and Virus Viability

In evaluating the influence of a single environmental factor upon virus propagation *in vitro*, other variables must, of course, be controlled. Thus, in studying the relationship between pH of culture and virus growth all other conditions relating to type of tissue, media, and air space were kept uniform. The cultures, therefore, consisted of rubber-stoppered 50 cc. Erlenmeyer flasks, with 10 cc. of serum ultrafiltrate, and enough embryonic mouse brain substrate to make a tissue-fluid ratio of about 1:300. The first test endeavored to show what effect slight pH changes would have on virus growth.

The data (Table I) suggest that a pH adjustment[3] within a comparatively narrow range is followed by only a small (tenfold) reduction in virus potency. However, it was found that two practices, not uncommon in handling fluid cultures, produce a much more serious effect upon virus growth. Thus, when cotton plugs were substituted for rubber stoppers, the pH rose to about 9, and only traces of virus could be demonstrated. Also, removal of the rubber stopper daily for about 30 seconds, without adjusting the pH after replacing the stopper, caused an appreciable decrease in virus harvest.

As a result of this experience, the pH of all cultures is now adjusted as routine at the time the culture is made and thereafter whenever it becomes necessary to open the flasks. 5 per cent CO_2 is used for this adjustment, since the method is convenient and leads to no extreme pH changes. The effect of a controlled pH is well reflected in a comparison of titers of adjusted and non-adjusted cultures. When early, non-adjusted culture generations (79th, 80th, 84th, and 87th) were tested in mice, potencies of 10^{-5} were obtained;

[3] All pH determinations were made by the Beckman potentiometer.

following the institution of routine CO_2 adjustment, however, potencies of 10^{-6} were routinely found (97th, 98th, 103rd, and 113th culture generations). It may be noted that the pH of non-adjusted cultures is approximately 7.8 to 8.0, whereas after the introduction of CO_2 the pH lies between 7.4 and 7.6.

Following these preliminary tests the next experiments were planned to investigate the influence of drastic changes in pH on the murine virus while it was actively growing, as well as on virus contained in a cell-free medium.

TABLE I

*Effect upon Virus Potency of Varying the pH of Tissue Cultures**

Culture adjusted with:	pH at time of testing	Virus potency by mouse test
Air-CO_2 mixture (1 per cent)	7.8	10^{-5}
" " " (5 " ")	7.4	10^{-6}
" " " (5 " " saturated)	7.2	10^{-5}
Non-adjusted	7.8	10^{-5}
Rubber-stoppered culture opened daily (non-adjusted)	8.2	10^{-4}
Cotton-plugged culture (non-adjusted)	9.19	10^{-0}

* Cultures inoculated with 97th culture generation and incubated for 3 days at 37°C.

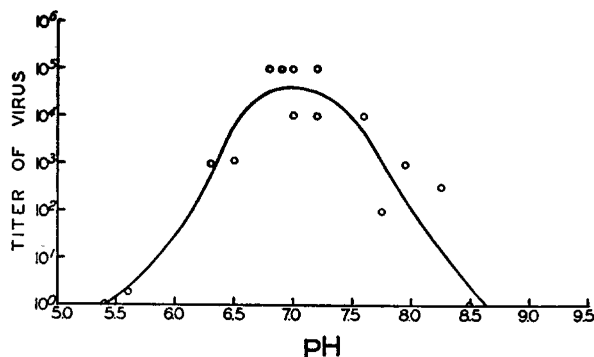

CHART 1. Relationship between pH and infectivity of murine culture virus in cell-free suspension.

In the first experiment, virus inactivation at various points of pH was studied while the virus was actively growing in the presence of living cells. Accordingly, a series of 50 cc. Erlenmeyer flasks containing the usual amount of embryonic mouse brain was seeded with 0.5 cc. of the 140th generation of culture virus. After receiving the virus inoculum, the freshly made cultures were adjusted to varying pH levels in the manner described and incubated for 3 days at 37°C. At the end of that period the infectivity of the cultures was determined by mouse test. The results are recorded in Chart 1. As will be

seen, the relationship between pH and virus infectivity is a simple one. Optimum potency prevails at about pH 7.0. A gradual decline of potency on both sides of the neutral point is easily recognizable, with no definite favoring of either the acid or the alkaline range. This fact was rather surprising since previous experience had shown that the murine passage virus was distinctly acid-resistant. It should be noted in this respect, however, that on the earlier occasion HCl had been used as the acidifier; also, that animal passage virus may be better protected by tissue than culture virus.

In the next experiment, a number of routine cultures, after incubation for 3 days at 37°C., were pooled and passed through sterile Reeve Angel No. 226 paper in order to obtain a cell-free virus suspension. Uniform volumes of 10 cc.

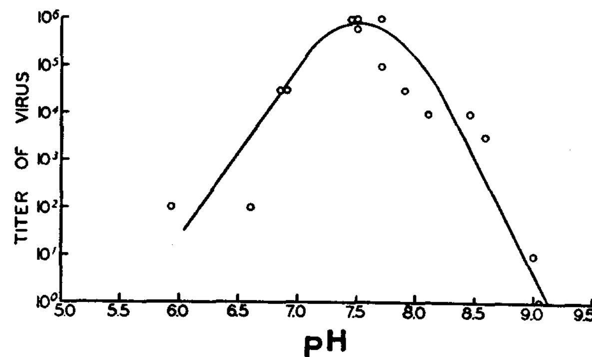

CHART 2. Relationship between pH and infectivity of murine tissue cultures.

of this material were then distributed over a series of 50 cc. Erlenmeyer flasks. The original pH of the pooled cultures was 7.4 and their potency, after paper filtration, titered 10^{-5}. The pH of each 10 cc. sample was then lowered by passing increasing concentrations of CO_2 through the fluid; alkalinization was obtained by decreasing the percentage of CO_2 or by adding sodium bicarbonate in physiological concentration. All flasks were then placed in the incubator for 3 days. At the end of this period the pH of each flask was again measured with a glass electrode and the virus content determined by mouse test. As may be seen from Chart 2, the relationship between pH and virus infectivity is again a simple one in that a single optimum occurs at pH 7.5 with a gradual decline on both sides of this point. However, a slight shift in the curve to the alkaline side is noticeable.

It is apparent from this study that the optimum range of virus infectivity in tissue culture coincides with the optimum pH for cell viability *in vitro*. When cells were not present, no extreme variation in pH tolerance for the virus could

be detected, as had been noted by Taylor, Sharp, and Beard for the virus of equine encephalomyelitis (4).

Rate of Propagation of the Murine Virus in Tissue Culture

Although passage of culture fluid every 3 days and incubation at 37°C. had yielded satisfactory titers, it was desirable to determine whether the 3 day period was optimal and, if so, for how long the culture maintained its high level of potency. Flasks containing 10 cc. of serum ultrafiltrate and embryonic mouse brain were inoculated with 0.5 cc. of culture virus (titer 10^{-6}), and titrations by mouse test were carried out at various intervals after incubation at 37°C. The data have been brought together in Chart 3. This experiment

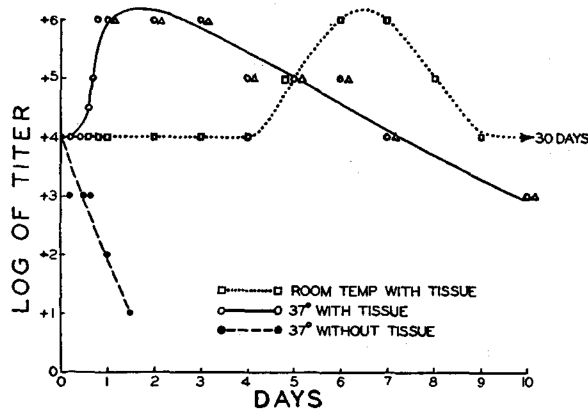

CHART 3. Rate of propagation of the murine virus in tissue culture. Large inoculum (about 17 million mouse-paralyzing doses).

shows that the murine virus propagates at a surprisingly fast rate. As early as 15 hours after inoculation there was an appreciable increase in potency from the point of original dilution activity (10^{-4}), and by 19 hours the optimum of 10^{-6} had been reached. This level of infectivity was maintained for about 3 days. Thereafter a gradual decrease in potency occurred so that by the 10th day the culture had dropped to a titer of 10^{-3}. Similar preparations maintained at 37°C., but without cells, showed a rapidly progressive decline in potency, indicating that virus deteriorates quickly under conditions which preclude its growth. When the experiment was repeated at room temperature, results essentially similar to the 37°C. curve were obtained, except that the lag period extended to 4 days; this lag period was then followed by a more gradual increase in potency to 10^{-6} on the 6th day. The optimum level was fully maintained for 24 hours with a subsequent gradual decrease to the original point of infectivity on the 9th day. The residual 10^{-4} dilution activity remained unchanged for 30 days.

If one computes the number of mouse-paralyzing doses used as inoculum in the above experiment, an approximate figure of 17 million is reached. Since the optimum dilution activity is only 10^{-6} for the whole culture, comparatively little actual increase of potency seems to have occurred in the culture. A new series of routine cultures was therefore inoculated with virus, but this time a very small dose was used as the inoculum. According to the data in Chart 3, a culture maintained for 4 days at 37°C. should have had a dilution activity of 1:100,000. A routine culture was therefore taken from the incubator after 4 days and diluted to that level. Freshly prepared culture media were then inoculated with a volume of 0.5 cc. of this dilution. A titration of

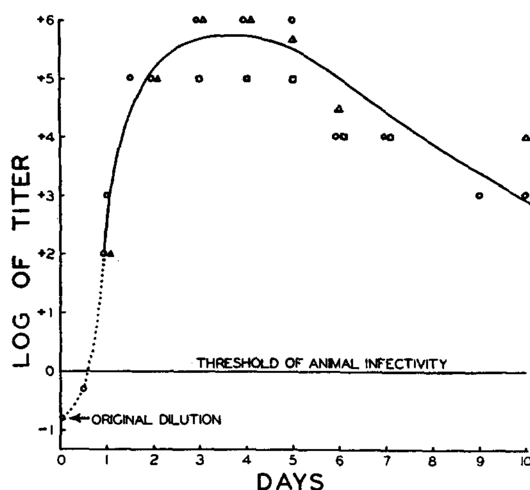

CHART 4. Rate of propagation of the murine virus in tissue culture. Small inoculum (about 1.7 mouse-paralyzing doses).

the source material revealed that the end point dilution had actually been used; in other words, the inoculum in this experiment was approximately 1.7 paralytic mouse doses. Periodic titrations of the incubated cultures (37°C.) failed to disclose the presence of any virus before 24 hours (Chart 4). Since the original inoculum had been diluted beyond the point of possible infectivity, this finding was not unexpected. In keeping with the slower rate of propagation of minimal doses of virus, the lag period was somewhat longer than when large amounts of virus had been used as seeding material. The optimum level of 10^{-6} was not obtained until the 3rd day, remained at this height for approximately 2 days, and then gradually decreased to 10^{-3} in 10 days. Thus, while the two curves are almost identical from the point of view of contour and levels, there was naturally a much greater objective virus increase in the second experiment than in the first.

The Effect of Prolonged in Vitro Cultivation on the Infectious Properties of the Murine Virus

It had been known since the time of its isolation that the cultured murine virus is capable of infecting mice by a diversity of parenteral routes. With an apparently systemic distribution in mice, at least at certain phases of the infection, such a broad power of invasiveness was not surprising. However, since the culture virus was being maintained in a somewhat more restricted environment than the animal passage virus, it seemed desirable to investigate periodically the peripheral infectivity of tissue cultures. The first indication that mouse passage virus and culture virus, after prolonged cultivation, may differ considerably in their ability to infect mice by peripheral routes came in the 49th culture generation when a titration of culture virus by intraperitoneal injection revealed a dilution activity of only 10^{-3}, as compared to a titer of

TABLE II

Infectivity of Murine Passage Virus and of Murine Culture Virus by Various Routes of Infection

Route of inoculation	Murine passage virus	Murine culture virus					
		1st generation	2nd generation	49th generation	89th generation	92nd generation	103rd generation
Intracerebral	10^{-8}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-6}	10^{-6}
Intraperitoneal	10^{-8}	10^{-6}	10^{-5}	10^{-3}	10^{-4}	10^{-3}	10^{-4}
Intravenous	10^{-8}	10^{-4}	10^{-5}			10^{-3}	10^{-4}
Intranasal	10^{-7}	10^{-4}	10^{-5}		10^{-2}	10^{-2}	10^{-2}
Subcutaneous	10^{-7}	10^{-6}	10^{-5}			10^{-3}	10^{-3}

10^{-5} by intracerebral injection. Inasmuch as in earlier tests intracerebral and intraperitoneal titers had been of essentially the same magnitude, it became of interest to determine whether any immediate change would occur when animal virus was placed into tissue culture medium. A brain emulsion of the 108th mouse passage was tested for potency in mice by all possible routes, and 0.5 cc. of the same emulsion was inoculated into tissue culture. As may be seen from Table II, the titer of the mouse passage virus by various routes was approximately the same, *i.e.* 10^{-7} to 10^{-8}, whereas the level of potency of the culture virus in the 1st and 2nd subcultures was uniformly lower for all routes. In short, the change observed with the intraperitoneal titration of the 49th subculture had presumably already occurred somewhere between the 3rd and 49th culture generations. In returning to tissue cultures of more remote passage (89th generation), it was noted that the infectivity for mice by the intranasal route had also dropped. A test of the 92nd subculture revealed the fact that there was a general loss of virulence for all routes except the intracerebral. It appears from these data that the change in the character of the tissue culture virus, which had evidently taken place as the result of

prolonged cultivation *in vitro*, has been a gradual one (Chart 5). It would also seem that the greatest decrease in infectivity has occurred with respect to subcutaneous and intranasal inoculation. Thus the infectivity for mice by the subcutaneous route of the 100th subculture had dropped from an initial titer of $10^{-5.5}$ to 10^{-3}; as far as intranasal instillation is concerned, the decline in virulence had been equally marked, going from an early average of $10^{-4.5}$ to 10^{-2} in later passages. In comparison herewith the decrease in virulence by intraperitoneal and intravenous routes was much less spectacular (Table II; Chart 5).

Since the persistence of high intracerebral infectivity pointed toward a qualitative rather than a quantitative change in the cultures, further investigation

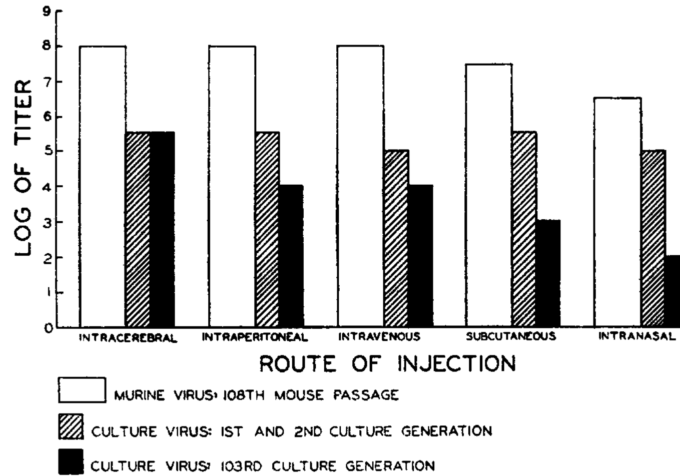

CHART 5. Infectivity of murine passage virus and of murine culture virus by various routes of injection.

was undertaken to determine whether a culture, peripherally non-infectious, would nevertheless be capable of inducing a state of resistance in mice against subsequent infection with murine passage virus. For this purpose mice were available from earlier tests which had survived the injection by different routes of varying dilutions of the 112th serial culture passage. The surviving mice numbered 104 among a total of 246 animals injected as follows:

	No. of mice injected	No. of mice surviving
Subcutaneously	71	22
Intraperitoneally	33	14
Intravenously	60	20
Intranasally	71	42
Feeding	11	6
	246	104

The 104 surviving mice were reinfected with mouse passage virus by the same peripheral routes which had previously been used for injection of the culture virus. The dose of virus was chosen so as to ensure paralysis and death among practically all of the accompanying normal control mice. The results

TABLE III

Resistance to Reinfection with Murine Passage Virus of Mice Surviving Primary Infection with Tissue Culture Virus

Route of primary infection with culture virus (112th subculture)	Dilution*	Result			Reinfection‡ of surviving mice with mouse passage virus		Result			Controls§		
		No. of mice injected	Paralyzed	Survived	Route	Dilution*	No. of mice injected	Paralyzed	Survived	No. of mice infected	Paralyzed	Survived
Subcutaneous	1:10	16	14	2	Subcutaneous	1:100,000	2	0	2	4	4	0
	1:100	30	22	8		1:100,000	8	0	8			
	1:1,000	11	9	2		1:100,000	2	1	1			
	1:10,000	12	2	10		1:100,000	10	2	8			
Intraperitoneal	1:1,000	25	13	12	Intraperitoneal	1:100,000	12	0	12	4	4	0
	1:10,000	8	6	2		1:100,000	2	0	2			
Intravenous	1:100	24	18	6	Intravenous	1:100,000	6	0	6	4	4	0
	1:1,000	18	12	6		1:100,000	6	0	6			
	1:10,000	18	10	8		1:100,000	8	2	6			
Intranasal	Undiluted	8	6	2	Intranasal	1:1,000	2	0	2	4	4	0
	1:10	25	7	18		1:1,000	18	2	16			
	1:100	20	10	10		1:1,000	10	5	5			
	1:1,000	18	6	12		1:1,000	12	4	8			
Feeding by gavage	Undiluted	11	5	6	Feeding by gavage	1:100	6	0	6	4	3	1
Totals							104	18	86	20	19	1

* The dose was 0.1 cc. for all routes except the intranasal in which 0.05 cc. was instilled.
‡ Reinfection of animals was carried out 2 weeks after inoculation with tissue culture virus.
§ Control mice were injected with murine animal passage virus.

of this experiment are given in Table III. It will be noted that of a total of 104 "immunized" mice, 86 proved insusceptible to reinfection with doses of murine passage virus that paralyzed 19 of 20 normal animals. The real difference between immunized and control mice is probably even more significant, because the fatality in the reinfected survivors was highest in those groups of animals which originally had received the smallest amount of tissue culture virus. It would therefore seem that a direct correlation exists between the

size of the antigenic dose and the degree of resistance to subsequent reinfection, in that mice which had received the larger doses of tissue culture virus showed the best protection against reinfection with highly virulent murine passage virus.

Ultrafiltration Experiments with the Murine Tissue Culture Virus

Inasmuch as nothing was known about the size of the murine virus, beyond the fact that the infectious agent passes readily through a Berkefeld W candle, it became of interest to determine the particle size of the virus by ultrafiltration. The clear supernatant fluid of virus tissue cultures suggested itself as a convenient substrate for this work, since its freedom from particulate matter obviated the necessity for preliminary purification. Moreover, by all accepted standards, the culture virus could be regarded as identical with the mouse passage virus.

The cultures were subjected to light centrifugation at about 3000 R.P.M. for 15 minutes before filtration. End points of filtrability were determined by passing the centrifuged material through a series of graded collodion membranes made in accordance with the technique introduced by Bauer and Hughes (5) for the preparation of modified Elford membranes. Filtration was carried out under positive pressure of 107 cm. mercury. During the entire process the filtrates were protected from light and were kept on ice. Precautions against bacterial contamination were observed in so far as possible. The filtrates were then injected intracerebrally into mice and the symptoms noted. When flaccid paralysis and death resulted, the specimen was considered unquestionably positive; if indeterminate symptoms, such as rough coat and awkward movements preceded death, in the absence of flaccid paralysis, the filtrate was taken as doubtful from the viewpoint of virus content; in cases in which neither symptoms nor deaths occurred in the injected mice it was assumed that the particular membranes had completely retained the virus. The results of these experiments are given in Table IV.[4]

From Table IV it would appear that the virus passes without difficulty through membranes with an A.P.D. of 30 mμ. On one occasion a positive test was obtained with a 27 mμ membrane. Inasmuch as some mice died when injected with filtrates from membranes measuring down to 25 mμ, it is possible that small amounts of virus passed through pores averaging less than 30 mμ in diameter. However, within the 25 to 27 mμ range no clear-cut paralysis was noted and it remains open to question whether the observed deaths were due to virus activity. Assuming therefore that 30 mμ represents the indisputable end point of filtration, the particle size of the SK murine virus may be placed between 10 and 15 mμ. This calculation is based upon Elford's formula

[4] We are indebted to Mr. Engle Devendorf, formerly of this Department, for preparing the membranes and carrying out the filtration.

which computes the ultrafiltration measurement of viruses as being equal to one-third to one-half the diameter of the largest retaining pore. If the murine virus is compared in size with other viruses of known particle size, it is obvious that there is no room for any possible mistaken identity with the virus of lymphocytic choriomeningitis. Of the remaining viruses which have been measured only that of Theiler's spontaneous mouse encephalomyelitis and that of monkey poliomyelitis have particle sizes corresponding with that of murine poliomyelitis virus.

TABLE IV

Ultrafiltration of SK Murine Poliomyelitis Virus through Graded Collodion Membranes

Average pore diameter of membranes	Intracerebral injection of filtrates into mice		
	Positive: paralysis and death	Doubtful: death with questionable symptoms	Negative: no deaths nor symptoms
mμ			
39	4/4		
34	3/3		
30	3/3		
30	4/4		
30			0/4
27 (3–4 hrs.)	5/5		
27 (overnight)			0/4
27		2/3	
27		2/3	
27			0/4
25		1/4	
20		5/5	
20			0/4
20			0/4
20			0/4
20			0/4
11			0/4

Numerator equals number of mice affected, denominator equals the number of mice injected.

Virus Specificity Illustrated by a Comparison of Murine Poliomyelitis Virus with Lymphocytic Choriomeningitis Virus in Tissue Cultures

On first consideration there seems little reason for suspecting any relationship between murine poliomyelitis virus and lymphocytic choriomeningitis virus. With the murine virus the incubation period is extremely short, sometimes as little as 24 hours, and the cardinal clinical symptom is flaccid paralysis. By contrast, lymphocytic choriomeningitis virus has an average incubation period of 6 to 8 days and produces in mice a syndrome of hypersensitivity and convulsions which becomes most apparent on sensory stimulation. In other

words, poliomyelitic infection is essentially referable to a lower motor neuron involvement, while the localization of lymphocytic choriomeningitis virus is preponderantly in the brain itself. However, some diversity among various strains of lymphocytic choriomeningitis virus and their widespread presence in natural hosts serve to emphasize the necessity for a strict comparison between the properties of these two viruses.

Apart from what has been said, there are other reasons which prompted this investigation. To begin with, the symptoms produced by lymphocytic choriomeningitis virus may, on occasion, range from the classical syndrome previously described to the occurrence of spastic or flaccid paralysis. Second, the guinea pig is susceptible to this virus and the same species, under certain conditions, may also be infected with the murine poliomyelitis virus (6). Third, lymphocytic choriomeningitis is not only an intercurrent disease in laboratory animals, but may also figure as an occasional contaminant in tissue cultures (7). Finally, there is some evidence (8) that lymphocytic choriomeningitis virus, when grown in tissue culture, may undergo a change so that the virus, after extended subcultivation, will cause flaccid paralysis in mice.

Since the recent report by MacCallum and Findlay (8) has stimulated and formed the basis for this part of our work, it might be well to review certain phases of their investigation. During an extended cultivation of a strain of lymphocytic choriomeningitis virus, the virus was carried for 270 consecutive subcultures in Tyrode-serum cultures, using chick substrate of varying composition. At the 66th culture generation the injected mice became lethargic and showed spastic and flaccid paralyses. Transfer of the brain of such mice to guinea pigs produced a moderate fever which was followed by cachexia and death. However, retransfer of the blood and brain from such guinea pigs to mice produced once more the typical symptoms of lymphocytic choriomeningitis without spinal involvement. When mice were injected with the 90th subculture, flaccid paralysis occurred again; however, upon transfer to guinea pigs no symptoms were noted, nor were the injected guinea pigs resistant to subsequent inoculation with the W.E. strain of lymphocytic choriomeningitis virus. On the other hand, when tissue cultures were injected into guinea pigs intracerebrally the characteristic picture of lymphocytic choriomeningitis was seen. While the symptom complex of flaccid paralysis therefore persisted in passages from mouse to mouse, conversion of the culture strain to its original state of pathogenicity was evidently accomplished by passage through guinea pigs.

The following experiment was carried out in order to see how murine poliomyelitis virus in tissue culture would react if subjected to experimental conditions similar to those employed by MacCallum and Findlay.

A group of 7 guinea pigs and a group of 15 mice were inoculated with sterile broth, both intraperitoneally and intracerebrally. The doses were 0.75 and 0.5 cc. for the guinea pigs and 0.1 and 0.02 cc. for the mice. No symptoms were noted and no fever occurred in either group of animals. By all accepted standards these particular

groups of animals could be considered free from latent infection with lymphocytic choriomeningitis virus. These animals were segregated and used in the subsequent tests. Three guinea pigs were given 0.1 cc. intracerebrally and 0.75 cc. intraperitoneally of murine tissue culture virus (110th passage). No symptoms were seen and the daily temperature variation was well within normal range for several days. One week after this injection 1 guinea pig was sacrificed. The blood of this guinea pig was injected intraperitoneally (0.15 cc.) and intracerebrally (0.02 cc.) into 3 mice; another group of 3 mice received a similar injection of cord-brain emulsion. Blood or cord-brain suspensions were also transferred to 2 guinea pigs by intraperitoneal or intracerebral injection. The whole procedure was then repeated with blood and cord-brain suspension of 1 of the 2 latter guinea pigs. Again, no symptoms were observed in any of the injected animals.

As a result of this experiment, it becomes clear that when murine poliomyelitis virus is substituted for the virus of lymphocytic choriomeningitis, and the *in vitro* experiments of MacCallum and Findlay are repeated with their original technique, entirely different results are obtained. No lymphocytic choriomeningitis virus was obtained from murine tissue culture.

Interference between Murine Tissue Culture Virus and Poliomyelitis Virus in Monkeys

In the first study of the murine virus (1) the fact was reported that mouse passage virus, when introduced simultaneously or at short intervals before or after infection with SK or Aycock monkey poliomyelitis virus, prevented the occurrence of paralysis in the injected monkeys. It now became of interest to ascertain whether the cultured murine virus would act likewise under similar experimental conditions. Three such experiments are herewith recorded.

Experiment 1.—A total of 10 monkeys was used. Five control monkeys were injected intracerebrally with 0.5 cc. of a 1:100 dilution of SK monkey virus; 3 monkeys received the same dose of SK monkey virus mixed with 0.5 cc. of undiluted murine tissue culture virus; the remaining 2 monkeys received a similar mixture, except that the SK monkey virus was used in a tenfold stronger concentration, *i.e.* 0.5 cc. of a 1:10 dilution. Whereas all 5 control monkeys became paralyzed none of the 3 monkeys which had received a mixture of the same dose of monkey virus (1:100) with murine culture virus developed paralysis. Paralysis did occur, however, in both monkeys which had been injected with the heavier dose of monkey virus (1:10) together with murine culture virus. The outcome of this experiment indicates that murine culture virus will effectively interfere with SK poliomyelitis virus. At the same time there is a suggestion that the interaction between the 2 viruses may proceed on a quantitative basis.

Experiment 2.—Three control monkeys were injected intracerebrally with 0.5 cc. of a 1:100 dilution of SK monkey virus mixed with 0.5 cc. of uninoculated tissue culture medium. Three additional monkeys were similarly injected, except that

murine tissue culture virus was substituted for the uninoculated medium. In a third group, 3 animals received 6 cc. of murine tissue culture virus intravenously immediately following the intracerebral inoculation of 0.5 cc. of 1:100 dilution of SK monkey poliomyelitis virus. Whereas the 3 control animals became paralyzed, all 3 animals receiving the mixture of tissue culture and monkey virus escaped paralysis. Of the monkeys which had received the tissue culture virus intravenously following intracerebral infection with SK poliomyelitis virus, 1 animal developed paralysis, 1 remained normal, and 1 died after 18 days with questionable symptoms.

Experiment 3.—Two control monkeys were injected intracerebrally with 0.5 cc. of a 1:100 dilution of Aycock monkey virus. Two additional monkeys received intracerebrally the same dose of monkey virus mixed with 0.5 cc. of undiluted murine tissue culture virus. One last monkey was similarly injected with 0.5 cc. of a 1:10 dilution of Aycock virus mixed with 0.5 cc. of undiluted tissue culture virus. Whereas the two control animals became paralyzed, the two monkeys receiving the mixture of tissue culture virus with the smaller dose of Aycock virus (1:100) remained free from paralysis; on the other hand, the monkey injected with the heavier dose of Aycock virus (1:10) together with murine tissue culture virus became paralyzed.

In summarizing the above results it seems safe to say that effective interference can be obtained in mixture tests between poliomyelitis virus and murine tissue culture virus, provided the experimental animals are not overloaded with too large a dose of monkey virus. If one considers the total number of control and experimental animals involved in the three experiments, it is evident that all 10 control monkeys became paralyzed, whereas 8 animals failed to show any symptoms when murine culture virus was used together with monkey virus in intracerebrally injected mixtures. The limitations of such interference are also apparent from the failure of murine virus to protect against a larger dose of monkey virus. Obviously, the group of animals treated intravenously with murine tissue culture virus is too small to be properly interpreted at this time. But there is at least a suggestion that favorable results may also be secured by peripheral administration of culture virus.

At the time these experiments were run a few tests were also carried out in order to determine whether the murine culture virus *per se* had any demonstrable pathogenicity for monkeys. Accordingly, 4 monkeys were injected intracerebrally with 1.0 cc. of undiluted murine tissue culture virus. None of these animals suffered any ill effects. Temperature readings were within normal range and no abnormal symptoms of any kind were noted. Under the conditions of this test, therefore, the murine culture virus has given no evidence of being pathogenic for *rhesus* monkeys.

DISCUSSION

If one reviews the field of virus cultivation one becomes cognizant of the dearth of knowledge concerning the basic processes which take place in a tissue culture when a virus is brought together with living cells. While certain

viruses grow in the presence of chick tissue, apparently with utter disregard for species or type of tissue, other viruses with broad experimental and natural host range possess comparatively rigid cellular requirements. Our study of the behavior of the murine virus on different cellular substrates was approached with a belief that embryonic mouse tissue was the most important single factor necessary for virus growth and that variations in the amount of nervous tissue would produce little change in potency. We felt justified in making this assumption because, in the paralyzed mouse, large amounts of murine virus can be demonstrated not only in the central nervous system, but also in the blood, spleen, liver, adrenals, and lymph nodes. The seemingly systemic distribution of the murine virus was taken to indicate that the parent strain, with its highly developed neurotropism in the monkey, had been transformed into a non-selective agent in the mouse during the adaptive process. It was therefore surprising to find that the level of virus potency showed a distinct tendency to vary in proportion to the amount of nervous tissue in the culture medium, and that, in tissue culture, the murine virus displays more definite affinity for the central nervous system than in the paralyzed mouse. The reasons for this discrepancy may well be due to the fact that the apparent systemic distribution of the murine virus in the mouse is caused by its unusually high virulence.

The results of our studies on the relationship between variations in pH and virus growth appear to justify the emphasis which should be placed upon minute details of the culture system. After many years of cultivating viruses, authorities are still in disagreement as to the condition of the cell which is most receptive for virus propagation. A review of the pertinent opinions, which have been expressed from time to time (9), reveals that some stress the functional activity of cells for proliferation, while others believe the resting state of the cell to be most important. The same confusion exists with regard to the optimum ratio of tissue to fluid medium. The conclusion seems inevitable that when more attention is paid to the physiology of the host cell a better understanding of the virus itself will follow.

As far as the general effect of *in vitro* environment on virus potency is concerned, experience with viruses which have been maintained outside the animal host, either in tissue cultures or in fertilized egg preparations, has shown that three general types of reactions may be expected. First, the virus may undergo no demonstrable change. This class includes equine encephalomyelitis virus (10), influenza virus (11), and the classical example of the virus of fowl plague, which was cultivated for 5 years with fully maintained virulence (12). Or, viruses may become attenuated after continued cultivation, as is true for vaccinia (13). Finally, certain viruses, as the result of prolonged cultivation, undergo profound changes which suggest an alteration in the character of the virus itself (yellow fever virus (14), lymphocytic choriomeningitis virus (8),

and pseudorabies virus (15)). As far as the behavior of murine virus in tissue culture is concerned, some attenuation has evidently taken place in that mice can no longer be infected peripherally with culture virus in doses which prove highly virulent with mouse passage virus. The fact that such mice resist reinfection with passage virus would suggest that the antigenic character of the culture virus had been preserved. Another profound change in pathogenicity is indicated by the fact that cultured murine virus has never infected guinea pigs, although these animals have proven highly susceptible to infection with mouse passage virus at certain phases of its transmission through mice (6). These findings bring to mind similar observations with the yellow fever virus. Interestingly enough, as in the case of yellow fever, cultivation of the poliomyelitis virus succeeded only after the infectious agent had been transmitted from its original host to lower animals. It is of interest, in this connection, to briefly mention the fact that preliminary experiments with the Lansing strain of murine poliomyelitis virus have given evidence of some degree of *in vitro* propagation with the type of tissue culture medium employed in this work. On the other hand, Theiler's virus has failed to grow under similar conditions of cultivation.

In evaluating the data relating to the rate of propagation of the murine virus in tissue culture, the word "propagation" should be used with circumspection. Our present knowledge does not permit us to designate the interaction between virus and cell as a process which leads to an actual numerical increase of infectious particles. To be precise, it is unknown whether multiplication of an infectious virus unit occurs within the culture, or whether the virus particle may not consist of a multiplicity of factors with individually varying antigenic and infectious capacities. The only criterion available at the moment is the measurable increase in virus infectivity for the susceptible host, which stands in direct proportion to the length of time for which the cell has been in contact with the virus. Moreover, the methods of titration in animals are themselves not too satisfactory since they are subject to tremendous variability in host response, and the customary tenfold dilution method, at best, can only be a rough approximation. These facts, although they may be self-evident, bear reassertion when one attempts to interpret data which purport to make available a virus "growth curve." Nevertheless, it has been a source of surprise that repeated experiments have yielded confirmatory results in so far as the measurable increase in virus potency is concerned. Responsible for the regularity of these results is partly the unusual stability of the murine virus and the fact that the pathological symptoms are so clearly dependable. The unusual speed with which infectivity develops in murine virus cultures imitates to a full extent the extraordinary rapidity which characterizes the progress of murine infection in the living animal.

The interference phenomenon and its mechanism, will be fully discussed

elsewhere (16). Suffice it to point out here that the observations reported in this paper strongly suggest that cultured murine virus, in principle, is also capable of blocking poliomyelitis virus in monkeys. As had previously been suspected, the interaction between the two viruses appears to be quantitative, and the available data are in harmony with this thought. Further perfection in the use of culture virus as an interfering agent in poliomyelitic infection must await the development of improved methods which permit a more abundant yield of virus in tissue cultures.

CONCLUSIONS

1. The murine virus may be grown in embryonic mouse brain-serum ultrafiltrate cultures. The virus fails to grow in embryonic chick tissue cultures or in fertilized egg preparations.
2. Some relationship can be demonstrated between the amount of nervous tissue and the infectivity of the culture.
3. Optimum titers of virus potency (10^{-6}) can be obtained by adjusting the pH of the growing culture at 7.3 to 7.6.
4. A simple pH inactivation curve for virus alone and for virus when actively growing in tissue culture has been obtained.
5. The rate of virus propagation, as determined by potency tests in mice, has been established for cultures which were seeded with large or small amounts of virus. The murine virus "grows" relatively fast. The optimum titer for a large inoculum was reached in 19 hours, for a small inoculum in 72 hours.
6. With extended subcultivation *in vitro* the cultured virus shows a loss of infectivity for mice by peripheral injection. However, potency as determined by intracerebral injection remains constant.
7. Mice surviving inoculation of culture virus by routes other than intracerebral acquire a relative resistance to reinfection with mouse passage virus.
8. The murine culture virus passes without difficulty through collodion membranes with an A.P.D. of 30 mμ. Its particle size may therefore be estimated as lying between 10 to 15 mμ.
9. On a basis of *in vitro* activity and cross infection, the murine culture virus is distinct from the virus of lymphocytic choriomeningitis.
10. Murine culture virus may be used as an interfering agent to block infection with poliomyelitis virus in monkeys. The interaction between the two viruses seems to be quantitatively limited. Such interference, with the present potency of culture virus, operates effectively only against comparatively small doses of monkey virus.

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