Poliomyelitis. IV. Some Cultural and other Characteristics of Chick-Embryo-Adapted Type II Strain of Poliomyelitis Virus. (20821)

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The adaptation of the MEF1, Type II (Lansing) strain of poliomyelitis virus to the developing chick embryo has been reported recently by Roca-Garcia and his associates (1), and by Cabasso and coworkers(2). This adaptation followed the serial propagation of the virus in suckling hamsters by Moyer and coworkers(3). It seems to have been made possible by the continuous passage of the virus in this host, which resulted in a remarkable increase of virus mouse titers. Proof of the identity of the chick-embryo-adapted virus as Type II poliomyelitis was derived from cross-neutralization tests in mice with known standard-type immune sera. Furthermore, evidence of reduced virulence for primates was shown with both hamster and chick-embryo-adapted viruses by intracerebral inoculation into rhesus monkeys(1-3).

This communication presents the results of studies undertaken to determine the optimal conditions for development of the chick-embryo-adapted virus in this foreign host. Particular reference is given to the temperature and length of incubation, route of inoculation, age of embryo and invasiveness of the virus for the various embryonic tissues.

Materials and methods. Virus strains. The strains of virus used were identified previously as MEF1-A and MEF1-B(2). The passage levels of both lines are given in the descriptions of the individual experiments. *Chick embryo inoculation.* Seven-day-old White Leghorn chick embryos were used unless otherwise specified. Inoculation was usually made via the allantoic cavity, although in some experiments, the yolk sac, amniotic sac, and chorioallantoic membrane (CAM) routes were also used. The inoculum consisted of a 20% chick embryo tissue suspension in hormone broth and the following amounts were routinely injected: 0.25 ml in the allantoic cavity, 0.4 ml in the yolk sac, 0.2 ml on the CAM, and 0.1 ml in the amniotic cavity. In most experiments, the whole embryo was harvested and made into a 20% suspension in hormone broth prior to titration in mice. However, in some cases the embryo was decapitated, heads and bodies being separately titrated in mice; in others, the CAM, allantoic fluids and other embryonic parts were also harvested separately for titration in mice. *Mouse titrations.* Three-week-old Swiss albino mice were used for the titration of infected chick embryo fluids or tissues. The latter were extracted in the undiluted form by means of Ten Broeck grinders and the extract was centrifuged at 2,000 rpm for 20 minutes. Serial 10-fold dilutions were then made from the undiluted, centrifuged extract and 4 to 6 mice were inoculated intracerebrally with 0.03 ml volumes of each dilution. Inoculated mice were observed for paralysis and death for a period of 21 days. Virus LD_{50} titers were calculated by the Reed and Muench method(4).

Experimental. Effect of length of incubation on multiplication of chick-embryo-adapted virus. Two experiments were carried out with the MEF1-A strain of virus, using the 3rd and the 24th chick embryo passage levels. Since the results were practically identical only the experiment made with the 24th passage virus is described. Fifty-six, 7-day-old embryos were inoculated with the virus via the allantoic cavity. After sealing with collodion, the embryos were incubated at 36°-37°C. On each of the 9 days, beginning 2 days after inoculation, 6 live embryos were harvested; whole embryos and allantoic fluids were pooled separately. The undiluted embryo pool extract and the allantoic fluid pool from the daily harvests were each titrated in mice by the method described above. The results, Table I, seem to indicate that a post-injection incubation period of 4 to 7 days gives optimal virus titers in the whole embryo extract with maximal titers at 4 and 5 days.

TABLE I. Effect of Length of Incubation on Multiplication of MEF1 Chick-Embryo-Adapted Virus.

Post-inj. incubation, days	Mouse LD_{50} titers: MEF1-A24 Whole embryo	MEF1-A24 Allantoic fluid	MEF1-B24 Whole embryo	MEF1-B24 Allantoic fluid
2	$10^{-3.0}$	$10^{-1.0}$	$10^{-4.41}$	$10^{-4.0}$
3	$10^{-3.0}$	$10^{-2.21}$	$10^{-4.27}$	$10^{-3.74}$
4	$10^{-4.76}$	$10^{-2.33}$	$10^{-5.50}$	$10^{-1.17}$
5	$10^{-5.0}$	$10^{-1.50}$	$10^{-6.50}$	$10^{-1.20}$
6	$10^{-4.33}$	*	$10^{-5.0}$	$10^{-1.20}$
7	$10^{-4.0}$	$10^{-0.5}$	$10^{-4.76}$	$10^{-1.0}$
8	$10^{-3.63}$	$10^{-1.0}$	$10^{-5.30}$	$10^{-1.0}$
9	$10^{-2.33}$	$10^{-1.33}$	$10^{-2.50}$	*
10	$10^{-2.25}$	$10^{-0.}$	†	†

* Not detectable.
† Not done.

In contrast, there was little variation in the allantoic fluid titers, which remained rather low. These low titers may well be due to virus residue from the inoculum rather than to active multiplication of the virus in the surrounding CAM with subsequent virus accumulation in the fluid. A similar test was carried out with the 24th passage level of MEF1-B virus. Its results also summarized in Table I, are strikingly similar to those obtained with the MEF1-A 24 virus, with somewhat higher LD_{50} titers.

Effect of incubation temperature on multiplication of the virus. Duplicate experiments were made with the 26th and the 28th chick embryo passage levels of the MEF1-A virus. The results were so similar that only the latter is described. Forty 7-day-old embryos were each inoculated via the allantoic cavity with the virus suspension. After sealing, they were divided into 4 groups of 10 embryos each, and each group was placed in a separate incubator at 32°C, 35°C, 37°C or 39°C for 5 days. Whole embryos were then harvested from each group separately, ground as described above and titrated in mice. The results, Fig. 1A, seem to indicate that optimal virus titers can be obtained at 5 days by holding the chick embryos at either 35°C or 37°C.

Effect of route of inoculation on multiplication of the virus. Following is the description of one of the experiments comparing the virus titers obtained by different routes of inoculation. Fifty-six 7-day-old embryos were divided into 4 groups of 14 embryos each. They were inoculated with the 28th MEF1-A chick embryo passage virus, the first group in the allantoic cavity (a), the second in the amniotic sac (b), the third in the yolk sac (c), and the fourth on the CAM (d). After sealing, the embryos were incubated at 36°C-37°C, for 5 days. Whole embryos were harvested from each group separately. In addition, CAM's were harvested from groups *a* and *d*. Suspensions of the 6 pools of tissues were titrated in mice, Fig. 1B. It seems that the ability of the virus to invade the embryo proper and to multiply in its tissues is equally good regardless of the route of inoculation. Slightly higher titers were consistently obtained, however, when the allantoic cavity and the yolk sac routes were used. In contrast, multiplication of the virus in the CAM seemed to be of low grade or even non-existent.

Effect of age of the embryo on multiplication of the virus. Groups of 10 to 20 embryos each, varying in age from 5 to 12 days, were all inoculated on the same day, via the allantoic cavity, with the 17th MEF1-A chick embryo passage virus. They were incubated at 36°C-37°C for 5 days, then whole embryos from each group were harvested separately. Embryonic suspensions were titrated in mice in serial, 10-fold dilutions, Fig. 1C. Optimal virus titers were obtained with 6- to 9-day-old embryos. A sudden and drastic change in susceptibility to the virus seems to take place in embryos 10 days of age or older, for their virus titers were very low or insignificant.

Virus distribution in the infected embryo and the effect of inoculum dilution. At frequent intervals pools consisting of infected whole embryos or of separate heads and bodies were titrated in mice. This was done to determine whether the virus multiplied primarily in nervous tissue and whether multiplication also occurred in other embryonic tissues. Effect of inoculum dilution on ultimate virus titers was also investigated. A typical experiment is described as follows: A group of 20 7-day-old embryos was inoculated with the 28th MEF1-A chick embryo passage virus via the allantoic cavity. After 5 days incubation whole embryos were harvested from 10 of the eggs and pooled. The other 10 embryos were

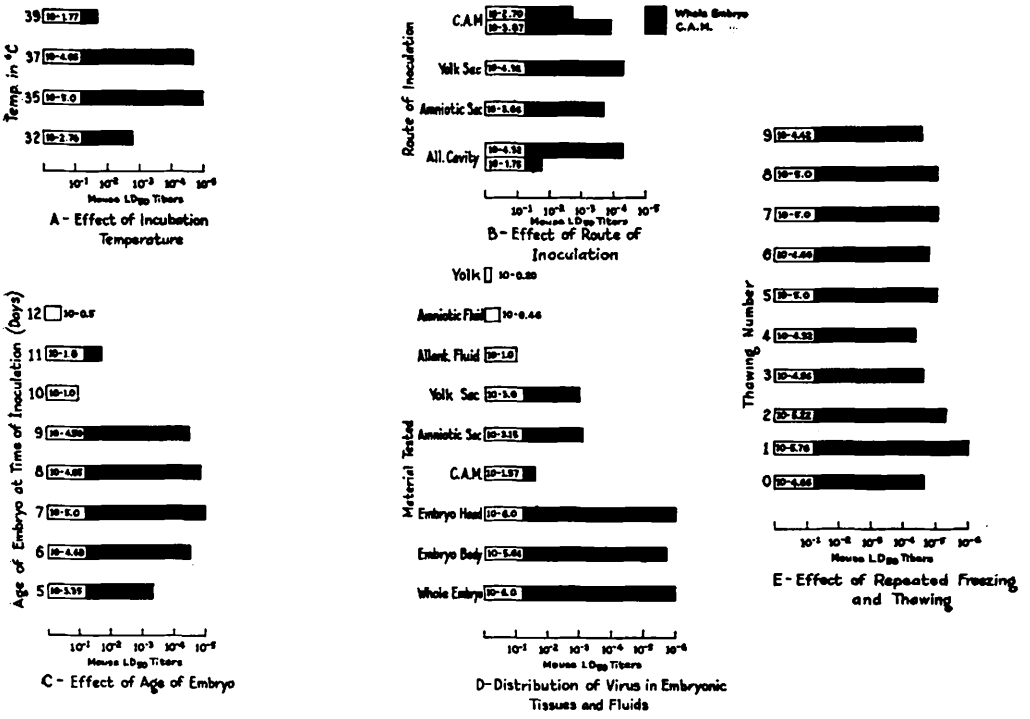

FIG. 1.
Some cultural and other characteristics of a Type II, chick embryo adapted poliomyelitis virus.

decapitated and their bodies and heads pooled separately. Suspensions from each of the 3 pools were in turn inoculated into the allantoic cavity of 20 7-day-old embryos. In addition, serial 10-fold dilutions ranging from 10^{-1} to 10^{-4} were made with the suspension of embryo heads and each dilution was inoculated into the allantoic cavity of 10 7-day-old embryos. Tissues harvested from all groups of embryos after 5 days incubation were titrated in mice. The results appear in Table II. It seems evident that the virus is uniformly distributed throughout the embryo with no particularly greater concentration in the head. In addition, there does not seem to be an optimal inoculum dilution: the greater the concentration of the virus in the inoculum, the greater the virus titer obtained. In addition to determining the virus distribution in the embryo proper, experiments were also devised to study its distribution in other embryonic tissues and fluids. In one such experiment, 20 7-day-old embryos were inoculated via the allantoic cavity with the 41st MEF1-B chick embryo passage virus, which had a mouse LD_{50} titer of $10^{-5.75}$. After 5 days' incubation the following were harvested from 10 chick embryos and the homologous materials pooled: whole embryos, CAM's, amniotic sacks, yolk sacs, allantoic fluid, amniotic fluid and yolk fluid. Tissues were pooled in their entirety whereas only aliquot parts of the fluids were combined. Heads and bodies of the remaining 10 embryos were harvested separately and pooled. Tissue pools were made into 20% suspensions and the 9 tissue and fluid preparations thus obtained were titrated in mice in 10-fold dilutions. The results, Fig. 1D, again seem to indicate that the virus is uniformly distributed throughout the whole embryo. Both allantoic sac and fluid once again were found to be relatively poor in virus content, whereas amniotic and yolk sacs had appreciable titers. Only negligible amounts of virus were found in amniotic and yolk fluids.

Effect of repeated freezing and thawing on infectivity of the virus for mice. It is well

TABLE II. Virus Distribution in Infected Embryos and Effect of Inoculum Dilution.

Chick embryo passage level	Source of inoculum	Dilution of inoculum (20% suspension)	Mouse LD_{50} titers: Whole embryos	Embryo bodies	Embryo heads
29	Whole embryos 28th passage	Undiluted	$10^{-4.37}$	$10^{-5.0}$	$10^{-4.75}$
30	*Idem* 29th passage	"	$10^{-4.5}$		
	Embryo bodies 29th passage	"	$10^{-4.0}$	$10^{-3.25}$	$10^{-4.30}$
	Embryo heads 29th passage	"	$10^{-4.48}$	$10^{-4.0}$	$10^{-4.0}$
		1:10	$10^{-4.76}$		
		1:100	$10^{-4.62}$		
		1:1000	$10^{-2.55}$		
		1:10000	$10^{-2.25}$		

known that monkey or rodent-grown poliomyelitis virus can survive in the frozen state for long periods of time and may even remain viable for months when stored at +4°C. The virus also survives repeated freezing and thawing. The following experiment was performed to determine whether this characteristic of poliomyelitis virus was maintained after its adaptation and serial propagation in the chick embryo. A group of 20 7-day-old embryos was inoculated via the allantoic cavity with the 27th MEF1-A chick embryo passage virus. After 5 days' incubation the whole embryos were harvested, pooled, and an undiluted extract prepared as described under *Methods*. A portion of this extract was at once titrated in mice in 10-fold dilutions and 10.0 ml of the remainder were shell-frozen in a glass vial and stored at −35°C. Beginning the next day and every 4th day thereafter, the frozen suspension was thawed under cold running water, and a quantity of material sufficient for one virus titration was removed. The remainder was refrozen and stored. This operation was repeated 9 times. Fig. 1E shows the mouse LD_{50} titers obtained at the different thawings, and indicates that the viability of the chick embryo propagated virus exhibited surprising resistance to 9 consecutive freezings and thawings. It is worth noting that, following the first thawing, the virus titer was found to be over a full 10-fold higher than that of the fresh preparation, an observation which has been made on a great number of other preparations of this virus. It cannot be argued that freezing helps to free more virus particles from cells which remained intact, since the undiluted chick embryo extract was centrifuged prior to freezing. A more appropriate explanation may be that freezing helps disperse virus aggregates present in the fresh extract.

Effect of chick embryo propagated MEF1 virus on hatchability of infected embryos. It was repeatedly observed during the course of this study that although embryos infected with either one of the 2 strains of chick embryo adapted MEF1 virus seemed to overcome the infection around the 12th to 13th day following inoculation, very few chicks hatched out and the great majority died between the 22nd and 26th day of total incubation. The following experiment illustrates the influence of the virus on the hatchability of infected embryos: A total of 60 7-day-old embryos was divided into 3 groups of 20 embryos each and inoculated via the allantoic cavity. Group 1 was inoculated with the 65th MEF1-A passage virus, having a mouse LD_{50} titer of $10^{-5.15}$. Group 2 received the 47th MEF1-B passage virus with an LD_{50} titer of $10^{-5.34}$, and Group 3 was injected with an equal volume of sterile broth diluent. All embryos were incubated at 36°-37°C until they hatched out or died. The results, Table III, show a significant difference between the number of control and infected embryos that hatched out. It may also be observed that strain B seemed to allow fewer embryos to hatch out than did strain A. However, the difference between these may well be within the range of experimental error. It should be

TABLE III. Effect of Chick Embryo Propagated MEF1 Virus on Hatchability of Injected Embryos.

Age of embryo	Virus injected						Broth control		
	MEF1-A65			MEF1-B47					
	Alive	Dead	Hatched	Alive	Dead	Hatched	Alive	Dead	Hatched
8	20			20			20		
10	20			19	1		19	1	
11	20			19			19		
12	20			17	2		19		
13	20			17			19		
14	20			16	1		18	1	
15	20			16			18		
17	19	1		16			18		
18	18	1		15	1		18		
19	18			15			17	1	
20	18			15			17		
21	17	1		15			16	1	
22	15	2		12	3		16		
24	8	1	6	3	6	3	8	2	6
25	5	3		0	3		2	1	5
26	1	4					2		
27	0		1				0		2
Total	0	13	7	0	17	3	0	7	13
%	0	65	35	0	85	15	0	35	65

noted that the temperature of incubation (36°-37°C), though optimal for multiplication of the virus, was substantially lower than the optimal hatching temperature (38°-40°C). This may explain the fact that only 13 out of the 20 control embryos hatched out, with a delay of from 3 to 7 days over the normal hatching time of 21 days.

Summary. 1. Effects of incubation time and temperature, age of embryo and route of inoculation on multiplication of a Type 2 chick-embryo propagated poliomyelitis virus were studied. The distribution of the virus in chick embryo tissues and fluids, and its action on the hatchability of infected embryos, were investigated. 2. Optimal conditions for multiplication of the virus were inoculating 6- to 9-day-old embryos via either the allantoic cavity or the yolk sac, and incubating for 4 to 6 days at 35°-37°C. 3. The best source of the virus was the embryo proper. No greater concentration of virus was found in the head than in the body. 4. Although the embryos seemed to overcome the infection by the 12th or 13th day, relatively few hatched out. The majority died between the 22nd and 27th day of total incubation. 5. The mouse LD_{50} titers of the chick-embryo-adapted virus were essentially unaffected by 9 consecutive freezings and thawings of the same virus preparation.

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Received December 21, 1953. P.S.E.B.M., 1954, v85.