THE YIELD OF RABIES VIRUS IN THE CHICK EMBRYO

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Virus material to be used for the preparation of rabies vaccine should contain virus in high concentration, be free of contaminating viruses and bacteria, and be as easily prepared and inexpensive as possible. So far, tissue cultures and chick embryos have been found to be so poor in virus as to preclude their successful use for this purpose (1–3). Animal brain has therefore been the material of choice for vaccine preparation (2), although in other respects tissue cultures or chick embryos would be more desirable. This paper reports attempts to increase the yield of rabies virus in the chick embryo.

Kligler and Bernkopf (3–5) have successfully cultivated rabies virus in young chick embryos. They found that, after inoculation on the chorioallantoic membrane a fixed strain of virus (Pasteur) would invade embryos younger than 8 days, whereas it did not migrate into older embryos. After repeated passages in the egg its virulence increased so that it would also invade 10 day old embryos. The inoculated eggs were kept at 37–38.5°C. and the virus content of the embryos was titrated at certain intervals by intracerebral inoculations in mice. The virus multiplied slowly, reaching a maximum titer of 10^{-3} to 10^{-4} in 7 to 9 days. The virus was passed serially by this method for 47 passages (3).

Dawson (6, 7) reports the successful propagation of rabies virus by intracerebral inoculation in 13 day old embryos for 65 passages. The embryos were harvested after 6 days' incubation and passages made. This worker was interested primarily in modifying the pathogenicity of the virus by egg passage and did not report on the amount of virus in the embryos. In later passages the virus had acquired the ability to invade the embryo after inoculation on the membrane.

In recent work in this laboratory (8), it was found that vesicular stomatitis virus will reach a higher concentration in embryonated eggs incubated at 35–36°C. than at 39–40°C. It was therefore decided to try the low temperature of incubation with rabies virus to see if a good yield of virus could be obtained that way.

Materials and Methods

Two strains of virus were used in the preliminary phase of this work, both obtained through the courtesy of Dr. J. Casals. One was the old Pasteur strain, which had

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had a very large number of mouse brain passages. The other, called 15811, had been isolated and passed 114 times in the mouse brain. It was fixed in its virulence, although still more invasive than the old Pasteur strain. The viruses were inoculated intracerebrally into mice. When prostrate the mice were killed and the brains harvested and ground to make a 1 per cent suspension (10^{-2} dilution of brain by weight), which was used for inoculating the embryos.

The eggs used were from Rhode Island Red chickens and were incubated at 38.5°C. for 7, 8, and 13 days, as explained in the text. 3 to 4 dozen eggs were inoculated simultaneously, each egg receiving 0.03 cc. of the virus dilution on the chorioallantoic membrane or into the cerebrum of the embryo. A 1 cc. tuberculin syringe and No. 27 needle were used. The opening in the shell through which the inoculation had been made was covered with Scotch tape and the eggs were incubated at 35–36°C., or in one case at 39–40°C. After the desired incubation period the embryos were harvested and tested for virus. 3 to 5 whole embryos were pooled for each titration and enough diluent added to make a 10 per cent suspension by weight. The suspensions were prepared in a Waring Blendor with a metal chamber that was sterilized by autoclaving before use.

In the preliminary work isotonic saline, buffered with phosphate to about pH 7.4, was used for suspending and diluting the virus. Later, distilled water was employed for making the first two dilutions, 10^{-1} and 10^{-2}, and the higher dilutions were made in a mixture of equal parts of water and buffered saline, containing 10 per cent horse serum. This latter procedure was chosen in an attempt to make the extraction of virus more complete. Ordinary means of mechanical grinding will not effectively disrupt cell structure, and the hypotonic effect of water was therefore used in the later experiments to obtain a more thorough disintegration of the tissues.

Immediately after grinding, the 10 per cent suspension was further diluted 1:10 in water to make a 1 per cent suspension (called 10^{-2}). This was kept on ice for 30 to 90 minutes and then further diluted in 10 per cent horse serum and injected. All manipulations between the harvesting of the embryos and the injections were performed in the cold.

Three to 4 weeks old mice of this Department's stock were used to titrate virus activity. In view of reports that different strains of mice are almost equally susceptible to intracerebral inoculation (9), no attempt was made to compare the susceptibility of these mice with that of other stocks. 3 mice were used for each virus dilution, and they were subsequently observed for about 3 weeks and typical deaths noted. The 50 per cent mortality end point was calculated for each titration according to the method of Reed and Muench (10).

EXPERIMENTAL

In preliminary studies the finding of Kligler and Bernkopf that rabies virus will invade the 6 day embryo after inoculation on the membrane was confirmed. The strain 15811 was detected in the embryo 5 days after inoculation, and on the 7th and 9th days the embryos titered about 10^{-5}. The Pasteur strain was not found after such inoculation in the dilutions tested (10^{-3} to 10^{-8}).

After intracerebral inoculation in 13 day embryos the 15811 virus was re-

covered after 48 hours and reached a titer of $10^{-4.5}$ in 6 days. The Pasteur strain reached a titer of 10^{-5} in the same time.

At this stage it was decided to try intracerebral injections in young embryos. 8 day embryos were used and cerebral inoculations found to be entirely feasible, the mortality *post operationem* being about the same as when 13 day embryos were used. The operation is simple in young embryos, as their heads are easy to locate. The same quantity of suspension, 0.03 cc., was inoculated.

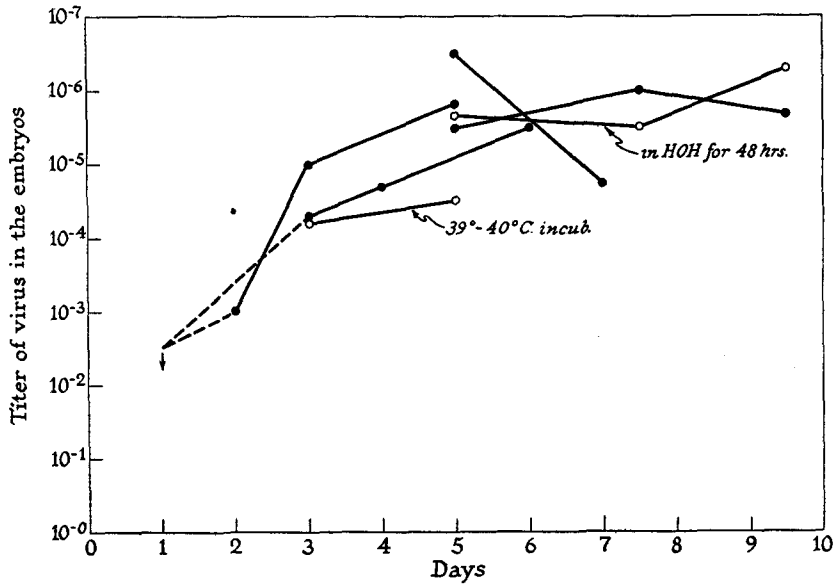

FIG. 1. The points indicate 50 per cent mortality end points obtained by titrating intracerebrally in mice the infected chick embryos harvested at different intervals after inoculation. Points connected by a line are obtained from the same experiment. The eggs were incubated at 35–36°C. except in one case in which 39–40°C. was used and this is indicated on the figure.

The 15811 virus was used because it had shown a higher degree of invasiveness in the preliminary experiments.

Fig. 1 shows the results of these experiments. All but one of the points (10^{-3} after 48 hours) were obtained from titrations where HOH rather than an isotonic diluent was used in the first two dilutions. The chart shows that a fairly high titer was regularly obtained, $10^{-6.5}$ being the highest figure found. Only two titrations were made on embryos incubated at 39–40°C. and those were low (10^{-4} and $10^{-4.5}$).

Some indication had been obtained that prolonged maceration of virus-containing tissue would give a suspension with unusually high activity. One

series was therefore set up in which embryos were harvested after 5, 7, and 9 days' incubation and the 10^{-1} suspension in HOH tested after 60 minutes and 2 days at 0°C. in each case. The prolonged maceration in HOH did not appreciably change the infectivity titer of the suspension (Fig. 1). The figures, however, show that the titer remained approximately constant from the 5th to the 9th day of infection.

One experiment only was done with the Pasteur strain inoculated intracerebrally in 8 day embryos. The technique was the same, with incubation at 35–36°C. The titers found were $10^{-5.8}$ on the 5th day, $10^{-4.4}$ on the 7th day, and $10^{-5.3}$ on the 9th day.

In order to get some direct comparison of the activity of infected mouse brains and chick embryos, 3 mouse brain pools were titrated by the same technique as was used for the embryos. The mice were infected with the 15811 virus and killed when moribund. In the first and second experiments, in which pools of 15 and 2 brains respectively were used, the titers found were $10^{-5.7}$ and 10^{-8}. In the third experiment (pool of 3 brains) two titrations were performed: one after the virus had been kept suspended in water for 1 hour, which gave a titer of $10^{-6.6}$, and the second after maceration in water for 48 hours, which gave a titer of $10^{-7.7}$. The titers found in mouse brain material were thus somewhat irregular but on the whole higher than those found in embryos.

DISCUSSION

These results show that a reasonably high concentration of rabies virus can be obtained in the chick embryo. The titers found are appreciably higher than those obtained by a different technique in the embryo (3) or in tissue cultures (1) and seem to be within the limits set by Webster (2) for material suitable for preparation of vaccine by his ultraviolet irradiation method. It is felt that the titers obtained may be at least partially due to the low temperature of incubation. The low titer found after incubation at 39–40°C. for 5 days, $10^{-4.5}$, would fit in with such an explanation.

It is quite possible that a strain which has been adapted to the chick embryo would give a higher titer of virus, but such a strain was not available. It is also possible that a still younger embryo might be more suitable.

The whole embryos were suspended and titrated. Their weight was usually about 8 to 12 gm., depending on the time at which they were harvested and on individual variation. Thus considerable quantities of rabies virus can be obtained from each egg.

SUMMARY

Rabies virus was inoculated intracerebrally in 8 day old chick embryos and the virus activity of pools of embryos titered after incubation at 35–36°C. for different lengths of time. The virus reached a titer of $10^{-5.5}$ to $10^{-6.5}$ in 5 to

to 6 days and remained at a rather constant level until the 9th day of the infection. The report by Kligler and Bernkopf that rabies virus will invade the very young embryo after inoculation on the chorioallantoic membrane was confirmed.

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