time of the second and third bleedings the level was only slightly higher in the animals with intravenous injections. The 2 injections of the antigen that followed the rest-period stimulated a sharp rise in the titers in both groups while the following injections produced a rise only in the group with intravenous injections. At the time of all of the 7 bleedings during the second course of injections the antitoxin levels were conspicuously higher in the group that received intravenous injections.

Conclusion. Antitoxin-formation in the rabbit is more rapid and abundant after intravenous than after subcutaneous injections of 25 L_f alum-precipitated diphtheric toxoid.

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Experiments on Cultivation of Virus of Infectious Avian Encephalomyelitis.*

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In connection with investigations on the virus of infectious avian encephalomyelitis (A.E.),[1] we sought to enhance antigen quantitatively for the preparation of immunizing vaccines, by cultivating the virus in developing chick embryos and in their tissues *in vitro*. The results relating to epidemiology and to additional properties of this newly discovered virus were considered of sufficient importance to be offered in the present paper.

Materials and Methods. The strain of virus employed was that kindly sent to us by Dr. Van Roekel and which was described by him[1] as well as by Olitsky.[1] The procedures followed closely those which had been employed by the latter. Ten percent infected-chick-brain suspension in broth was used to initiate the various cultures. Tests for virus, except as noted otherwise, were made by intracerebral inoculation of 0.05 to 0.1 cc of tenfold dilutions of the material to be examined into 2- or 3-weeks-old Rhode Island, or New Hampshire, Red chicks.

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1 Olitsky, P. K., *J. Exp. Med.*, 1939, **70**, 565; Olitsky, P. K., and Bauer, J. H., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 634. For earlier descriptions, see: Jones, E. E., *J. Exp. Med.*, 1934, **59**, 781, and Van Roekel, H., Bullis, K. L., and Clarke, M. K., *J. Am. Vet. Med. Assn.*, 1938, N.S. **46**, 372.

Fertile eggs of White Leghorn chickens were available. It was shown that of 11 normal, 2-week-old birds of this breed given 0.05 cc of 10^{-1} dilution of active brain intracerebrally, all developed characteristic encephalomyelitis after 11 to 16 days. Hence the Leghorn chicks are susceptible to the A.E. virus.

Cultivation Experiments. 1. *In Developing Chick Embryos.* In view of the fact that the virus is active only in avian hosts and that it has been reported[2] that the disease can be transmitted by way of the egg, the first attempts at cultivation were made in embryonated eggs as the most promising approach to the problem. Since the virus was shown to be active in nervous tissue, it was thought desirable to employ embryos of eggs of 5 or 6 days' incubation, which had been used successfully for the cultivation of rabies-virus.[3]

Parallel cultures were set up, employing 1,000 chick cerebral-test-doses of A. E. virus, one group being inoculated by way of the yolk-sac, after the manner of Cox;[4] another, through an opening over the embryo, directly into the allantois. The eggs were then incubated at 36.5°C for 6-10 days, when their contents were removed and passage made from the allantoic membrane, yolk-sac, embryonic tissue, and brain to new series of eggs. The different tissues of the 2d and 4th passages were ground in broth and decimal dilutions of 10^{-2} to 10^{-5} or 10^{-6} were injected intracerebrally into chicks. Five series of cultures were made and tested in 175 chicks, and in no instance could viral multiplication be discerned. In 2 instances, the brains of chicks hatched from such inoculated embryos were passaged in young chicks with negative results. Pathological examination of the CNS of embryos 18-20 days old (*i.e.*, 12-14 days after inoculation), or of hatched chicks failed to reveal the characteristic lesions of encephalomyelitis.

In addition, tests were made on the survival of virus injected into young and older embryos. In the former, 21 eggs with 5- to 7-day-old embryos, and in the latter, 11 eggs with 11- to 13-day-old ones were used; 0.1 cc of 10^{-1} dilution of virus (active in 10^{-5} dilution) was inoculated into the eggs through the allantois in the region of the embryo. The presence of virus in the embryos was demonstrated by pooling 2 or 3 inoculated embryos 1 hour later and injecting decimal dilutions of the suspension into chicks. After incubation at 37°C for varying periods, the embryonic brain and the other tissues were again examined for virus-content. In the series of

2 Van Roekel, H., Bullis, K. L., and Clarke, M. K., *Vet. Med.*, 1939, **34**, No. 12.
3 Kligler, I. J., and Bernkopf, H., *Nature*, 1939, **143**, 899.
4 Cox, H. R., *Pub. Health Rep.*, 1938, **53**, 2241.

young embryos, virus was recovered in dilutions up to 10^{-3} after 24, 48, and 72 hours. but not at all after 5 or 9 days; in the older ones, virus was recovered in a dilution of 10^{-4} after 48 hours, but not after 8 and 11 days. Pathological examination of the brain and cord of these embryos 11 days after inoculation failed to show any characteristic lesions.

Finally, 21 of the birds which had hatched out of inoculated eggs or had received intracerebral inoculation of the mentioned embryonic material, and failed to show signs of the malady for at least 40 days, were then subjected to an intracerebral test for immunity. All came down with characteristic A. E. virus infection, thus indicating the absence of active multiplying virus in the chicks and in the materials used in an amount sufficient to induce immunity.[1] At the time of the test, the fowls were 9-10 weeks of age.

2. *In Minced Embryonic Tissues in vitro.* Three series of cultures of this type were set up. In one, minced chick-embryo brain plus Tyrode's solution and 10% chicken serum served as medium. The second series was the same as the first, except that minced whole chick embryo was used in place of brain. In the last, Rivers' medium was used: minced chick embryo in Tyrode's solution. In each instance the viral suspension, as well as 2 flasks of the inoculated culture, was titrated for determination of the viral titer at the outset. (Similar titrations of 2 or 3 culture flasks were carried out at the time of transfer.) The cultures were incubated at 36.5 to 37°C and transfers into the respective media were made at 5-6 days' intervals. Each series was carried through 5 passages, so as to exclude any possible dilution factor of the original viral inoculum. The results are summarized in Table I and reveal that in the culture of whole-embryo tissue plus serum, there was a viral titer of 10^{-3} at the outset and at least 10^{-2} in the 5th subplant, although the original virus itself was calculated as being diluted to 10^{-8} in this passage. In the other 2 series the virus was detectable only through the 2d subplant; this corresponds to the point beyond which the virus is diluted out of its original activity.

Again it is clear that the avian virus has distinctive requirements for multiplication, such as are not found to exist for several others. Multiplication took place in minced whole embryos suspended in serum-Tyrode-solution mixture. The brain cultures were negative after the 2d subplant, as were those made with minced embryonic tissue in Tyrode's solution without serum.

It would appear, therefore, that the virus is rapidly lost at 37°C unless embryonic tissue and serum are present.

TABLE I.
Viral Activity in Various Minced Chick Embryo Media.

Titer of stock virus used for seeding cultures	Media	Titer of virus + media immediately after set-up	Subplants				
			1st	2d	3d	4th	5th
10^{-5}	Brain + serum	10^{-3}	10^{-3}	$<10^{-2}$	0	0	0
10^{-5}	Embryo + serum	10^{-3}	$>10^{-4}$	10^{-2}	10^{-4}	10^{-4}	10^{-2}
—	Embryo + Tyrode's solution	10^{-4}	$>10^{-2}$	10^{-2}	0	0	0

Summary and Discussion. 1. Embryonic chicks are apparently not susceptible to infection with this avian virus, although birds just hatched are. This is a strikingly reversed state from that which prevails in the case of certain other viruses,[5] which multiply readily in the undifferentiated tissues of the developing embryo but are inactive in the hatched chick. Whatever the reason may be, a fact of epidemiological significance emerges: the disease-agent is probably not transmissible by way of the egg, thus supporting the prior finding of Jones.[1]

2. The virus multiplied in minced whole-embryo tissue-cultures *in vitro* only under certain indicated conditions. The method in its present state, however, is not favorable for obtaining large yields of highly potent virus for use in immunizing procedures. No multiplication of virus was noted in this medium when chick-embryo brain was used instead of whole-embryo tissue. It is of interest in this connection that mammalian embryo-brain cultures have been found suitable for the multiplication of the neurotropic viruses of poliomyelitis[6] and of rabies.[7]

[5] For a discussion and references, see: Rivers, T. M., and Schwentker, F. F., *J. Exp. Med.*, 1932, **55**, 911; and Mackenzie, R. D., *J. Path. and Bact.*, 1933, **37**, 75.

[6] Sabin, A. B., and Olitsky, P. K., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 357.

[7] Kanazawa, K., *Japanese J. Exp. Med.*, 1936, **14**, 519; Webster, L. T., and Clow, A. D., *J. Exp. Med.*, 1937, **66**, 125; Bernkopf, H., and Kligler, I. J., *Brit. J. Exp. Path.*, 1937, **18**, 481.