rats, but this does not seem very probable, since mice were greatly preponderant over rats in the house, and from the observations of Lépine and Lorando[5, 6] and Sparrow,[7] it appears that natural transmission of typhus infection from rats to mice in the same households does not commonly occur. It must be said, however, that in general, inasmuch as rats are considerably more flea-infested than mice, and the common rat-flea, *X. cheopis,* attacks man much more readily than the common mouse-flea, *L. musculi,* rats are probably much more important than mice as reservoir of "endemic" typhus.

11850

Cultivation of *Clostridium tetani* in Unfertilized and Developing Fertilized Hens' Eggs.

KUO-CH'ING, CHEN. (Introduced by S. H. Zia.)

From the Department of Bacteriology and Immunology, Peiping Union Medical College, Peiping, China.

The employment of fertilized egg has proven of value for the cultivation of some bacteria and a number of filterable viruses. The chorio-allantoic membrane has been mostly used as the site of infection. Since the cultivation of anaerobic microörganisms in unfertilized and developing fertilized eggs has not been recorded, the preliminary results of such a study with an obligatory anaerobe, *Cl. tetani,* is herewith communicated.

Technic. A pure toxigenic strain of *Cl. tetani,* recently isolated from a patient, was used. Cultures in cooked meat tube after 48 hours' growth were used as the inoculum. Unfertilized and developing fertilized hens' eggs of different age were employed. After the position of the embryo and the air sac was identified by transillumination and marked off and the shell opposite the air sac was sterilized with tincture of iodine, a small opening was made with a carborundum disc attached to a dental drill, leaving the inner shell membrane intact, which was later cut with a small knife after resterilization with 70% alcohol and heat. A loopful of inoculum was then introduced through this opening to the center of the yolk sac, and in the case of fertilized eggs toward a point at some distance from the embryo. The shell aperture was then closed with melted hard paraffin. The eggs were then incubated at 37°C. Different batches of them were taken out and examined grossly and by

stained smears microscopically. Aerobic contaminations were excluded in every instance by absence of growth of the eggs' content on blood agar plates.

Results. At first, cultivation was done in unfertilized eggs. Fifty-four eggs were divided into 9 groups of 6 each: One group unheated, others heated in water bath at 90°C for 0.5, 1.0, 1.5, 2.0, 3.0, 5.0, 10.0 minutes respectively, and still others fully cooked by boiling for over 15 minutes. Inoculation into the heated eggs was done after they had been cooled. One egg from each group was used for examination at 2nd, 3rd, 4th, 5th, 7th, and 14th day of incubation. From the 7th day, about ⅓ of the eggs heated for from 2-5 minutes began to show some blackening and liquefaction, at the same time a foul odor was present, all of which became progressively worse. All the raw and fully cooked eggs and the remaining ⅔ of the heated eggs showed no apparent change up to 2 weeks of incution and hardly any bacilli were found microscopically. On the other hand, numerous gram-positive bacilli with terminal spores could be seen in the liquefied and blackened eggs. The number of organisms appeared to be in direct proportion to the degree of proteolysis and blackening. The outer layer, consisting of hardened egg-white, remained intact.

It is difficult to determine the optimum length of time required for heating the eggs as the results have not been consistent. Nevertheless, the experiment did suggest the heating must be sufficient to harden the outer layer of the egg-white but must not be too long to effect a complete hardening of the egg-white.

Next, an attempt was made to grow the organisms in developing fertilized eggs of different ages. The incubation of eggs before inoculation was at 38°C. Eggs with viable embryos of 4, 6, 8, 10, and 12 days of age, were seeded with the culture broth. One egg from each group was used for examination at different periods as in the first experiment. It was found that all the eggs examined showed various degrees of proteolysis and blackening on the second day of incubation. Complete liquefaction set in from the third day on. The embryos were invariably dead. Numerous gram-positive bacilli with terminal spores were found in the contents of the eggs. The eggs with 6- to 10-day-old embryo yielded the most abundant growth. The results have been consistent and therefore dependable.

From the above experiments, it can be seen that the developing fertilized eggs serve as a better medium for cultivating *Cl. tetani* than the heated unfertilized eggs. A third experiment was made to in-

vestigate whether heating the fertilized eggs would enhance the growth of *Cl. tetani*. Fertilized eggs with 6-7-day-old embryo were divided into 2 groups: one group unheated, the other heated in water bath at 90°C for 3 minutes; and a number of unfertilized eggs similarly heated was included for comparison. The results were somewhat unexpected. Heating of fertilized eggs retards instead of accelerates the growth of *Cl. tetani*. But they still gave earlier and better growth than the heated unfertilized ones.

Comment. In the cultivation of anaerobic microörganisms, it is well known that they are not as fastidious about their medium requirements as they are about the anaerobic environment. The growth of *Cl. tetani* in some of the heated unfertilized eggs may be due to a better anaerobic condition, as heating drives off the air in the egg and the hardened layer of egg-white prevents the re-entry of air from outside.

There may be two reasons for the luxurious growth of *Cl. tetani* in developing fertilized eggs. Firstly, the developing embryo consumes oxygen and thus creates a relatively better anaerobic condition, and, secondly, the presence of fresh animal tissue—the embryo—may stimulate growth. Perhaps this stimulating factor may be destroyed by heating.

The addition of animal tissue to media for the cultivation of anaerobes is not new. Von Hibler's brain medium,[1] and Holman's cooked meat tubes,[2] are notable examples. As pointed out by Holman, although the use of fresh tissue may be necessary for the growths of some of the stricter anaerobes, there is always present the disturbing possibility of contamination, not so much from without as from the animal tissues themselves.[3] The developing chick embryo has the advantage of being a living tissue free from contamination.

---

1 Hibler, E. V., *Untersuchungen die pathogenon anaeroben*, Fischen, Jena, 1908.

2 Holman, W. L., *J. Bact.*, 1919, **4**, 149.

3 a. Theobald Smith, *Cent. f. Bakt.*, 1890, **7**, 502; b. Ford, W. W., *Trans. Assn. Am. Phys.*, 1900, **15**, 389; c. Wolbach, S. B., and Saiki, T., *J. Med. Res.*, 1909, **21**, 267.