equal amounts of muscle toxin and pure toxin were neutralized by equal amounts of antitoxin *in vitro* and *in vivo*. This shows that the hypothetical secondary substance is not present in the mixture of muscle and toxin, although this does not preclude its production in the living muscle. It is also clear that the amount of toxin in the mixture is not increased. The mechanism of the potentiation of the toxin by the muscle is still under investigation.

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Further Studies of Agar-slant Tissue Cultures of Typhus Rickettsiæ.*

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In a preliminary note published in these PROCEEDINGS,[1] the writers described an agar-slant-tissue method for the cultivation of Rickettsiæ, both of the murine and of the classical types of typhus fever.

Further study of this method has led to modifications and standardizations which are reported herewith in order to facilitate its use in the hands of others. Preliminary experiments by Pinkerton with Rocky Mountain Spotted Fever virus indicate a possibility that the method may be successful with Rickettsiæ other than those of typhus.

The medium as now used differs from the original only in proportions of ingredients. We have found that results are most regular when the agar is prepared as follows:

A "Tyrode" solution of the ordinary formula is made up double strength throughout, except that only 1 gm. of bicarbonate of sodium is used per liter. The agar used is a Difco granular Bacto-agar.

A mixture of 150 cc. double strength Tyrode and 150 cc. of horse serum with 8 cc. of phenol red solution is filtered through a Seitz

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[1] Zinsser, Hans, Wei, H., and FitzPatrick, Florence, PROC. SOC. EXP. BIOL. AND MED., 1937, 37, 604.

filter.† To the filtrate is added 150 cc. of 4% agar in distilled water, cooled to 45°C.

The mixture is immediately put into 6 x ¾ inch test tubes—not more than 8 cc. to each tube; the tubes are closed with rubber stoppers and can be kept in the refrigerator. The initial reaction should be pH 7.4 to 7.6. This point is very important.

The Tissue. For the tunica tissue of guinea pigs which was used in the early cultures, we have now been able to substitute mouse embryo tissue. This has the advantage of greater ease of sterile manipulation, of furnishing larger amounts of tissue at smaller expense and of greater regularity of results. In general, the smaller the embryos the better. When large embryos are used the liver had best be removed.

Inoculation. For subcultures, the infectious material from a preceding tube is placed into a short and wide sterile tube (the ordinary 60 cc. centrifuge tube), together with the fresh embryonic tissue and the two are minced together with a long handled pair of scissors. This material is then laid on the agar so that about one-third of the agar surface is covered. It is important to avoid piling up the tissue.

Growth is usually determinable after 4 days and is at its maximum in from 6 to 8 days.

The eventual objective of attempts to develop a simple method of Rickettsia cultivation was to obtain quantitatively adequate amounts of the European organisms for vaccine production. The X-rayed rat technique, which continues to furnish satisfactory quantities of Rickettsiæ of the murine type, has consistently failed with the European strain. There was thus no available source of this latter type sufficient in yield to permit homologous vaccination experiments other than the louse vaccine devised by Weigl.

The cultivation method here described appears to have solved this problem. (Fig. 1.) But it was of great importance to make sure that these cultures had not dissociated into avirulent and antigenically modified forms. For this reason animal experiments were carried out.

It may be stated that the European strain now carried on agar was isolated from an infected guinea pig on Maitland medium on November 9, 1936. It was carried through 33 Maitland genera-

† Owing to the frequent alkalinity of the Seitz filter discs, we have been rapidly washing these discs with 3 changes of distilled water in a Petri dish and drying them overnight in the incubator before putting them into the holders for sterilization.

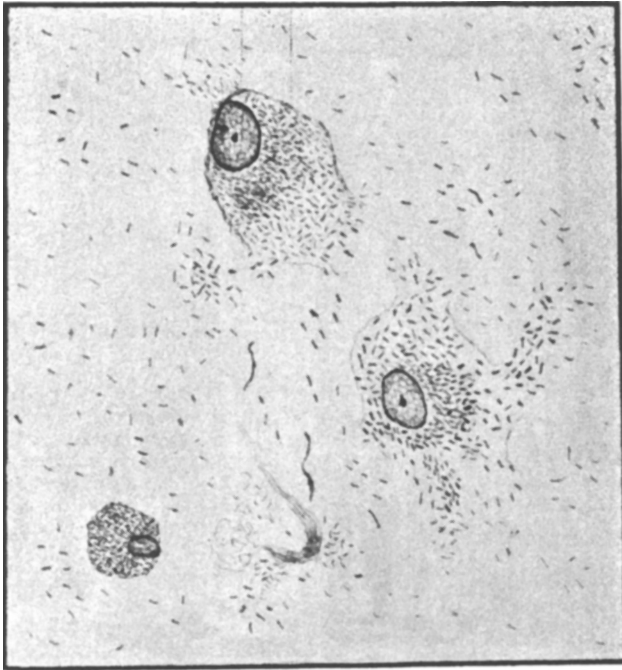

FIG. 1.
Smear on slide of Rickettsiae from 6-day agar tissue culture (mouse embryo tissue).

tions and transferred to agar tissue culture on November 8, 1937. It is now in its 12th agar generation, that is its 45th culture generation and has been out of the animal body for a period of over 15 months. With the 19th Maitland culture generation we were able to produce typical temperature reactions, specific immunity to passage virus and characteristic brain lesions in guinea pigs.

We have carried out a series of animal inoculations with agar cultures representing respectively the 5th, 6th, 7th, and 8th agar culture generations (*i. e.,* 38th to 41st generations outside the animal body). In all of these animals temperature reactions developed which, in most cases, differed from the passage virus controls only in that the incubation times were shortened to 4 or 5 days instead of the usual 6 to 9 days. In all of these animals subsequent inoculation of passage virus demonstrated specific immunity. (Fig. 2.) In a few animals in which cultures older than 10 days were used the reactions were mild—but subsequent immunity tests were nevertheless positive. Some of these animals showed, at an early stage of the fever, the scrotal swelling not infrequently observed in European

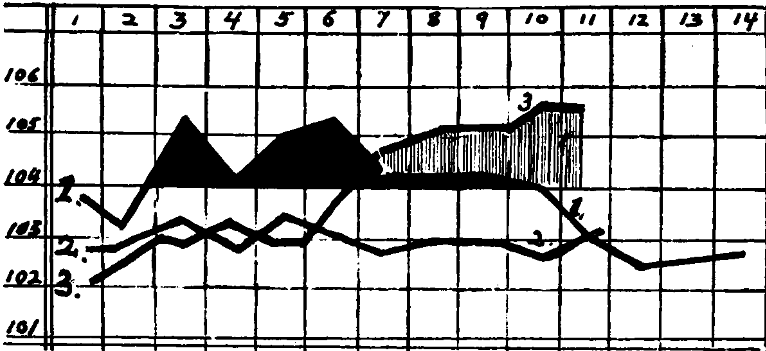

FIG. 2.

1. Temperature curve of guinea pig inoculated intraperitoneally with organisms from 7th generation agar culture. This agar strain started from 33d generation of classical European typhus Rickettsiæ on Maitland flasks. The inoculum therefore represents 40th culture generation isolated from an infected guinea pig on Nov. 9th, 1936. Culture inoculation on Jan. 3d, 1938.

2. Temperature curve of same guinea pig inoculated intraperitoneally on Jan. 31st, 1938, with passage virus, blood and brain of same strain maintained in guinea pigs.

3. One of two passage virus controls of immunity test as above.

passage virus infections when heavy inoculation is used. In one animal a testicle was removed on the fourth day when the temperature had risen to 105.8. Examination of the tunica showed a few Rickettsiæ and inoculation of tunica material into another guinea pig resulted in a severe and characteristic typhus reaction. The original animal recovered, defervescing on the 12th day and proved immune on reinoculation with properly controlled passage virus.

It is thus clear that the Rickettsiæ of the European type cultivated for 15 months through 33 Maitland generations and 8 subsequent generations on agar-tissue cultures have lost neither their characteristic infectiousness nor their capacity for specific immunization.

This encourages the hope that the method may furnish a satisfactory source of the European type of Rickettsiæ *(Rickettsiæ prowaceki)* for vaccine production.