THE CULTIVATON OF SPIROCHAETA NOVYI* WITHOUT THE USE OF TISSUE FROM ANIMAL ORGANS.

CHARLES A. BEHRENS.

(Purdue University, Lafayette, Indiana.)

In 1909 Schereschewsky[1] by deeply imbedding a piece of human tissue containing Spirochaeta Pallida in gelatinized horse serum, first demonstrated that the treponema might be cultivated *in vitro*. He was not able to obtain a pure culture of the organism, for bacteria grew along with the pallidum, nor did he succeed in reproducing syphilitic lesions by inoculating animals.

In 1910, Muhlen[2] obtained the first generation of the spirochaete by utilizing the above mentioned method. By the use of horse serum agar, he further succeeded in obtaining a culture devoid of bacteria. Muhlen's pure cultures also were evidently non-pathogenic.

During the same year, Bruchner and Galasesco[3] succeeded in cultivating "young impure cultures" by using Schereschewsky's medium. But upon inoculating rabbits with the material which also contained the original spirochaetal tissue, syphilitic lesions were produced. They were, however, unable to obtain a second generation of these organisms.

In 1911, Hoffman[4] succeeded in getting pure cultures of the spirochaete by the utilization of Schereschewsky's and Mühlen's methods. Although his cultures were morphologically typical, he, like the above mentioned experimenters, was not able to demonstrate their pathogenic properties.

Also at this time, Sowade[5] succeeded in procuring impure virulent cultures by using the gelatinized horse serum or gelatinized ascitic fluid. This inocuable material also still contained the original pallidum tissue.

In 1911, Noguchi[6] was able to cultivate pure virulent cultures *in vitro* of this organism. He accomplished this by placing a small piece of fresh sterile rabbit kidney or testicle tissue into each tube containing about sixteen cubic centimeters of serum water (one part of serum and three parts of distilled water) which was previously fractionally sterilized. A layer of sterile paraffin oil was added to each sterile tube containing this cultural medium and placed under strict anaerobiosis and incubated at 35-37° Centigrade. Under these conditions the spirochaetes which were morphologically typical and virulent were obtained and cultivated for many generations.

In the following year, Noguchi[7] cultivated for the first time the following relapsing-fever spirochaetes: Spirochaeta Duttoni, Spirochaeta Kochi, Spirochaeta Obermeieri and Spirochaeta Novyi.

In 1912, Treponema macrodentinm and microdentium[8], Treponema Refringens[9], Treponema mucosum[10], Spirochaeta Phagedenis[11], and Spirochaeta Gallinarum[12] were likewise successfully cultivated.

The media used in their cultivation was of a similar nature to that used in the pallidum cultural work. It was not essential in some instances to

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resort to strict anaerobiosis, but it was however, necessary to employ the use of sterile animal tissue in all cases. In the cultivation of pallidum, macrodentium, microdentium, mucosum, refringens, and phagedenis strict anaerobic conditions were required. Quite the opposite seems to be true for the relapsing-fever spirochaetes.

Noguchi's method of obtaining cultures of the latter is briefly as follows: A piece of sterile fresh rabbit kidney is placed in a sterile test-tube to which is added a few drops of citrated infected hearts blood of a rat or mouse. About fifteen cubic centimeters of sterile ascitic or hydrocle fluid is then added. Some of the medium is covered with sterile paraffin oil. The culture tubes thus prepared are incubated at 35-37° Centigrade. Maximum growth occurred on the fourth to the ninth day.

The use of sterile tissue being employed in all of this cultural work, of course, entails in many cases the killing of rabbits for their kidney tissue only. For this reason as well as being intensely interested along these lines in view of the fact that we attempted the cultivation of the relapsing-fever organisms three years previous to Noguchi's first publication and after obtaining cultures without difficulty by Noguchi's method, we deemed it advisable to attempt cultivation without the use of sterile tissue from animal organs. After various attempts we were finally able to obtain initial cultures without such tissue. These cultures as well as those which were obtained by the employment of the tissue medium, could be transplanted, with success, to ascitic fluid to which a small amount of sterile undefibrinated blood only, had been added.

The medium employed by us differs considerably from that used by Noguchi. Approximately eighteen cubic centimeters of sterile Ascitic fluid are transferred to each of a number of sterile test-tubes (tubes 20 by 1.5 centimeters were used). The pipette for holding and measuring sterile fluids[13] may be conveniently used in transferring this fluid to the tubes. It is very important as Noguchi also notes that the ascitic fluid does not contain bile, but forms a loose fibrin. Specimens of this fluid which do not possess this property or which have been sterilized by passing thru a Berkefield filter are entirely worthless for this work.

Rats were inoculated with a small amount (about one-eighth cubic centimeter) of spirochaetal blood obtained from the heart or tail of an infected rat. The blood of these rats is conveniently examined by clipping off the end of their tails. When it reveals the presence of from twenty-five to one hundred spirochaetes per field, (one-twelfth oil immersion lense), before large agglutinating masses of the organisms are seen, usually requiring from eighteen to twenty-four hours after inoculation, they are bled from the heart by means of a small bulb capillary pipette[14].

The blood thus obtained is transferred before coagulation takes place to the bottom of the tubes containing ascitic fluid which have been previously warmed to a temperature of thirty-seven degrees centigrade. It is most important that whole undefibrinated blood is used. The inoculated tubes are then incubated at thirty-seven degrees centigrade. No perceptible growth occurs at twenty-five degrees centigrade.

Multiplication apparently begins in the neighborhood of seventy-two hours after inoculation and the maximum growth is reached in from six to eight days. Material for examining the cultures is obtained by introducing a capillary pipette[15] near the bottom of the tubes.

The organisms are perpetuated by transferring about one-half cubic centimeter of the cultures from the positive tubes to fresh undefibrinated blood ascitic fluid medium, preferably just before the cultures reach their maximum growth (five to seven days). These transplants are made by sucking up the material in a capillary pipette which is introduced about one inch from the bottom of the culture tube, thus avoiding a large number of so-called skeleton forms.

By the means above described we have been able to cultivate Spirochaeta Novyi and to carry the same thru six generations without the use of sterile tissue.

If the positive tubes are covered with paraffin oil and placed in the ice box (0-10° centigrade) just before the maximum growth results, successful subcultures may be made ten to twelve days later. These iced paraffin oil covered cultures will also exhibit pathogenic properties two or three weeks later. As a matter of fact, whenever motile forms are present, although the greater part of the culture has degenerated into granules and skeleton forms, they are pathogenic. It is advisable to warm the cultures in order to ascertain motion.

One hundred percent infection resulted when rats were injected with generations one to six inclusive. The period of incubation is somewhat longer in the case of culture infected rats than in rats receiving the blood type of spirochaetes. The period of incubation in the former generally is from seventy-two to ninety-six hours, while that of the latter is usually eighteen to twenty-four hours. Once the infection is established the cultural spirochaete form is identical in appearance with the straight blood type. Besides the period of incubation being somewhat longer, the disease thus induced seems also to be less fatal for out of twelve rats receiving culture material of various generations, (one to six) no deaths resulted.

In cultures approximately ninety-six hours old there are actively motile spirochaetes of various description. Some have two or three irregular curves, while others have six or eight. Degenerated skeleton and granular forms and highly refractive granules are also present at this time but more especially in tubes a week or more old. The spirochaetes may occur singly, in pairs or chains, but there is a tendency for them to form agglutinated masses. These cultures do not stain as easily nor as distinctly as the blood type by the Wright or Romanowsky methods.

SUMMARY.

Spirochaeta Novyi has been cultivated *in vitro* by employing a medium devoid of tissue obtained from animal organs.

Undefibrinated blood, ascitic fluid free from bile and capable of forming a loose fibrin are necessary for obtaining and maintaining such cultures.

The activity of these cultures is prolonged by covering the medium with sterile paraffin oil and chilling (0-10° Centigrade) just before their maximum growth (five to seven days) is reached.

These cultures are pathogenic, altho, apparently their virulence is lowered as indicated by a longer period of incubation and no fatalities.

The cultural type differs from the normal blood type of the spirochaete morphologically and in its staining properties.

This culture of Spirochaeta Novyi has been perpetuated upon ascitic fluid containing undefibrinated normal blood for six generations.

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