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Procedures in the Isolation and Cultivation of an Organism from Berkefeld N Filtrates of Poliomyelitis Virus.

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I. *Culture Mediums.* A. *"E B" Medium* (Sheep brain after extraction with ethyl alcohol and benzol). Sheep brain, removed aseptically at the slaughter house, was washed thoroughly in sterile physiological salt solution, freed from blood and membranes, and ground finely in a meat chopper. The material was extracted once with 2 volumes of 95% ethyl alcohol overnight (12-16 hours) at room temperature (20-22°C.) and dried in air. The tissue residue was extracted in the same manner twice with 2 volumes of U.S.P. benzol. The resulting finely powdered dry material, in the proportion of 2 gm. to 10 cc. of 0.85% sodium chloride solution, was mixed thoroughly and sterilized in the autoclave at 15 pounds pressure for 20 minutes. The final pH was adjusted to 7.6.

B. *"V B" Medium* (Minced sheep brain in veal infusion broth). This substrate consisted of sterilized minced sheep brain contained in a veal infusion broth to which no peptone had been added.

Fresh sheep brain, removed aseptically at the slaughter house, was washed thoroughly several times in sterile physiological salt solution, freed from blood and membranes, and one kilo of tissue added to one liter of sterile veal infusion broth, pH 8.0, to which no peptone had been added. The mixture was coagulated thoroughly by boiling for 2-3 minutes and 0.2% glucose by volume was added. The cooled material was next passed 2 to 3 times through a meat chopper and minced. Test tubes measuring 1.5x15 cm. were filled to a depth of approximately 6 cm. (roughly 10 cc. by volume) and sterilized in the autoclave at 15 pounds pressure for 20 minutes. A second sterilization at 15 pounds pressure for 10 minutes was given on the following day, whenever the technical procedures in the preparation of the material warranted such added precaution. Frequently the culture medium was sterilized in small flasks and stored. Tubed medium prepared from these supplies was always subjected to a second sterilization at 15 pounds pressure for 10 minutes. The final pH of the medium was adjusted whenever necessary to 7.6. Owing to its natural buffering quality, the culture

medium stabilized itself during prolonged incubation at a suitable range of pH varying between 6.6 and 7.0, in the course of cultivation experiments.

II. *Tests for Sterility of Culture Medium.* The degree of heat penetration necessary for proper sterilization and ruling out the possibility of living tissue in the culture medium was determined as follows: A maximum recording thermometer was placed in the center of the material contained in a test tube and held firmly in position 2 cm. from the base. The pressure within the autoclave was raised to 15 pounds and within 5 minutes the temperature registered 118°C. After an exposure of 3 minutes to this temperature, the steam was shut off and the autoclave opened 2 minutes later. At this time the temperature recorded within the tube of medium was 96°C. The tube was allowed to cool spontaneously, and 35 minutes after the beginning of the experiment, the temperature within the tissue mass registered 68°C.

An exposure of 15 to 20 minutes at 15 pounds pressure would therefore be adequate to destroy any living tissue and to effect complete sterilization. The resistance of nervous tissue to high temperatures has been found to be considerably less than that of other tissues. Pincus and Fischer showed that an exposure to 60 or 65°C. for a few minutes was lethal.

Tests for sterility were made by incubating the culture medium at room temperature (20-24°C.) and at 37.5-38°C. for 3 to 4 weeks. As an added precaution 3-5 cc. samples of the medium were transplanted to simple and enriched culture mediums which were carefully observed for 3 to 4 weeks to rule out all possible sources of contamination.

III. *Cultivation of the Virus.* The inoculum was an active poliomyelitis virus that had been used successfully in the production of experimental poliomyelitis. *Macacus rhesus* monkeys were inoculated intracerebrally, under full ether anesthesia, with 0.2 to 0.5 cc. of a 5% saline Berkefeld N filtrate prepared from the brain and cord of a previously infected animal. After definite paralysis had occurred following a typical clinical picture of the disease, the animals were etherized and the brain and spinal cord removed with strict sterile precautions, and preserved in 50% glycerol. The glycerolated material used in the primary cultivation experiments had been stored 5 to 6 months in the icechest at a temperature of 4°C. before inoculation of the culture mediums. In the recultivation experiments the materials prepared from the brain and cord of monkeys that had been infected by means of cultures were inocu-

lated into the mediums after shorter periods of glycerolation ranging from 1 to 4 days with a maximum period of 54 days.

For primary cultivation 0.1 to 1.0 cc. of a Berkefeld N filtrate of a 5% saline suspension of the brain and cord virus was used. The first successful culture, however, was obtained with the least amount of the filtrate, and it is reasonable to assume that the larger amounts may yield more frequent positive cultures. In the re-cultivation experiments with "recaptured" virus cultures, both the filtrates and suspensions were employed separately in amounts of 1 cc.

Essentially the procedure was to inoculate the virus material into the "E B" medium and to make subplants at suitable intervals to the "V B" substrate in which the cultures were maintained by serial dilution. In a number of instances successful growth was obtained by means of the "V B" medium alone. Undoubtedly the 2 mediums exerted different effects upon growth, morphology and virulence. Reversible morphological changes were unusually striking when observed over long periods of time.

A series of 8 to 10 tubes of "E B" medium and "V B" medium in parallel was inoculated with the Berkefeld N filtrate and incubated at 37.5° to 38°C. Two tubes in each set were left unopened during the 3 to 4 weeks' period of observation when samples from the others were carefully withdrawn for subplants every 7 to 10 days by means of a platinum loop or a sterile capillary pipette (approximately 0.1 cc.), and for film preparations. Control tubes included Berkefeld N filtrates and suspensions of poliomyelitic monkey brain and cord inoculated into ordinary simple fluid and solid culture mediums; filtrates and suspensions from normal monkey brains and from herpes, encephalitis and neurovaccinia; and finally uninoculated "E B" and "V B" mediums.

IV. *Subplants and Film Preparations.* Tubes found to contain ordinary bacteria that might be contaminants were discarded. Generally these could be detected after 24-48 hours of incubation. Cultures that gave macroscopic evidence of growth only after 14-21 days were set aside for study and subplanting. At such times a faint turbidity changing to definite clouding of the culture medium was observed in the deeper levels of the supernatant layer of fluid in the tubes containing "E B" medium. This change was not observed regularly in the "V B" substrate. A sterile platinum loop applied to this layer of material brought away a slightly viscous fluid adhering momentarily by a fine thread as the loop was withdrawn.

Film preparations were allowed to dry on slides *without heat*

fixation and were stained by the methods of Wright, Giemsa, and Gram. The Wright stain was the method of choice because it gave the least distortion of the organism and brought out the polychromatic details of structure. *It is especially noteworthy that the first generation culture tubes containing filtrates from several strains of poliomyelitis virus showed a definite turbidity in the "E B" medium from which numerous film preparations, however, proved to be negative for growth upon careful microscopic examination. Subsequent transplants to the "V B" culture medium or to the "E B" substrate were found invariably to contain living organisms. Such positive secondary cultures were often demonstrable as early as 10 to 14 days before growth became microscopically discernible in the parent culture tubes.*

Subplants from tubes that yielded growth were made every 5 to 10 days. The second and subsequent generations were found to develop satisfactorily within one week. The unopened reserve tubes were examined after growth had occurred in the remaining series and were found to contain the identical organisms.

Transfers from the tubes showing primary growth with the inoculum were made by means of a single 2 mm. platinum loopful of material for the first 3 or 4 subplants. Thereafter a capillary glass pipette was used, containing approximately 0.1 cc. of the culture. This was done in order to effect many millionfold dilutions of the original virus under cultivation. At the same time transfers were also made into ordinary brain-heart (Bacto) infusion broth and to blood-agar plates in order to control possible contamination and the presence of ordinary bacteria.

From subplants the organisms could be grown only under strict anerobiosis on the surface of a solid culture medium prepared by adding 8 parts of "V B" medium to 10 parts of 2% veal infusion agar containing 0.4% NaH_2PO_4, having a pH of 7.6. This medium in Petri plates, or slanted in test tubes, yielded moderate growth after 7 to 10 days, and more abundant growth after 2 or more weeks, provided ample moisture was present. Successful secondary cultivation in this manner was accomplished by the use of the Boez-Olitsky apparatus equipped with a palladinized asbestos catalyzer. Subplants from these cultures failed to develop on aerobic blood agar plates and ordinary agar slants or in brain-heart infusion (Bacto) incubated aerobically and anerobically under sterile liquid albolene. All these mediums remained negative for macroscopic and microscopic growth after one month of observation.

Although successful cultivation of an anerobic organism from

poliomyelitis virus was accomplished by means of the "E B" medium in conjunction with the "V B" medium for subplants, further study will be necessary to establish the possible dependence of either one upon the other. For the recultivation of organisms from monkeys that had been successfully inoculated with the culture, the "V B" medium by itself was generally found satisfactory. However, the questions of adaptation of the strain, its multiplication, and maintaining of its virulence and infective power may be modified profoundly by the sequence of events taking place in the different culture mediums over varying periods of time. These matters must be left for future investigation.

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Agglutination of Streptococcus Hemolyticus by Rheumatoid Arthritis Sera: Thermal Characteristics of the Reaction.

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It has been reported[1, 2] that the majority of sera obtained from cases of rheumatoid arthritis agglutinate hemolytic streptococci in high dilutions at 55°C. Weaver[3] reported that scarlatinal streptococci were agglutinated at room temperature and at 37°C. by sera from cases of pneumonia, typhoid fever and erysipelas at about the same dilutions as by scarlet fever sera. Cultures heated to 60°-62°C. for one hour were no longer agglutinated by such sera. Sera similarly treated also lost their capacity to agglutinate. Tillett and Abernethy[4] recently reported that hemolytic streptococci may be agglutinated by sera obtained from various acute bacterial infections and that the agglutinability is destroyed by heating cultures to the thermal death point.

In the study of the rheumatoid arthritis reaction comparative

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3 Weaver, G. H., *J. Infect. Dis.*, 1904, **1**, 91.

4 Tillett, W. S., and Abernethy, T. J., *Bull. Johns Hopkins Hosp.*, 1932, **50**, 270.