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ISOLATION OF BACTERIA FROM VIRUS AND PHAGE BY A SERIAL DILUTION METHOD

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Two schools of thought exist regarding the nature of viruses. The one considers viruses to be distinct entities, wholly unrelated to bacteria; the other believes that viruses may be a phase of the cultivable bacteria associated with virus diseases.

In previous reports methods have been described by which it is possible to obtain growth of bacteria from materials which appear to be sterile when examined by the usual methods of cultivation on agar plate and in dextrose broth. By the use of deep tubes of dextrose-brain broth, and less often by other methods,[1] I have been able to isolate streptococci from material obtained from persons who died of epidemic poliomyelitis,[2] from persons, foxes and horses that died of epidemic encephalitis [3] and from animals which had received injections of poliomyelitic and encephalitic viruses.[4] From the results of these and other studies I have considered the streptococci isolated as factors of etiologic importance rather than as secondary invaders or as accidental contaminants. Other investigators have also reported evidence indicating the probable importance of streptococci in poliomyelitis and encephalitis.[5] I wish now to report

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1. Rosenow, E. C.; Towne, E. B., and Wheeler, G. W.: J. A. M. A. **67**:1202, 1916.

2. Rosenow, E. C.: J. A. M. A. **91**:1594, 1928; **94**:777, 1930; abstracted, J. Bact. **19**:27, 1930.

3. (*a*) Rosenow, E. C.: J. Infect. Dis. **48**:304, 1931; (*b*) Proc. Soc. Exper. Biol. & Med. **31**:285, 1933. (*c*) Rosenow, E. C., and Schlotthauer, C. F.: Proc. Staff Meet., Mayo Clin. **12**:631, 1937.

4. (*a*) Rosenow, E. C.: Proc. Soc. Exper. Biol. & Med. **27**:444, 1930. (*b*) Rosenow, E. C., and Schlotthauer, C. F.: Proc. Staff Meet., Mayo Clin. **12**: 825, 1937. Rosenow.[3a]

5. Cooper, M. L.: Am. J. Dis. Child. **42**:702, 1931. Eberson, F.: J. Lab. & Clin. Med. **18**:565, 1933. Evans, A. C., and Freeman, W.: Pub. Health Rep. **41**: 1095, 1926. Hektoen, L.: Boston M. & S. J. **176**:687, 1917. Hektoen, L.; Mathers, G., and Jackson, L.: J. Infect. Dis. **22**:89, 1918. Mathers, G., and Howell, K.: ibid. **21**:292, 1917. Kolmer, J. A.; Brown, C. P., and Freese, A. E.: J. Exper. Med. **25**:789, 1917. Mathers, G.: J. A. M. A. **67**:1019, 1916. Mathers, G., and Tunnicliff, R.: ibid. **67**:1935, 1916. Nuzum, J. W., and Herzog, M.: ibid. **67**:1205, 1916. Richardson, R., and Mellon, R. R.: Proc. Soc. Exper. Biol. & Med. **29**:451, 1932. Robertson, H. E., and Chesley, A. J.: Arch. Int. Med. **6**:233, 1910. Weil, A.: Proc. Soc. Exper. Biol. & Med. **30**:1243, 1933; Arch. Neurol. & Psychiat. **31**:1139, 1934. Rosenow, Towne and Wheeler.[1]

the isolation, by a modification of the methods previously employed,[6] of streptococci from filtered and unfiltered viruses and of the respective organisms from cultures of various bacteria which had been lysed with phage.

The extension of the method is the use of serial dilution of the inoculum in dextrose-brain agar and dextrose-brain broth. When this is carried out in multiples of ten, it may be frequently observed that although little or no growth occurs in the first or second tube, at still higher dilutions growth is shown. Absence of growth in the low dilutions may be due, it is thought, to inhibiting substances in the inoculum which are rendered inactive by serial dilution.

METHOD

The mediums were prepared from dehydrated broth or meat extract and peptone in the usual concentrations, to which 0.2 per cent dextrose and 0.1 per cent decolorized fuchsin (Andrade's indicator) (and in the case of agar, 0.25 per cent agar) were added. The reaction was adjusted to p_H 7. Approximately 18 cc. of the medium was placed in each of tall tubes (20 by 1.5 cm.) and to each tube two or three pieces of fresh calf brain, about 3 cc. in volume, were added before autoclaving (20 pounds [9 Kg.] for twenty minutes). Cultures were made under sterile conditions, either in a hood equipped with a glass shield and a copper roof, which radiates heat from the Bunsen burner, without change of air, and in which the air and the walls were sterilized with a fine spray of a solution (1 : 1,000) of mercuric chloride or a saturated solution of phenylmercuric chloride, or in a hot air sterilizer equipped with a glass shield and in which the air was sterilized by heat. All materials used in making the cultures and the mediums placed in the hoods were sterilized immediately before.

The agar was melted in the autoclave at several pounds' pressure and was cooled to about 40 C. before inoculations were made in it. Inoculations were made as a routine with graduated pipets having a total capacity of 3 cc., each plugged at one end with a cotton plug and fitted with a rubber bulb having a total capacity of 3.7 cc., which was reduced to approximately 2 cc. by placing cotton on the inside. Two cubic centimeters of the inoculum was placed in the first tube of medium. After the contents of this tube had been thoroughly mixed by means of the pipet without bubbling air through the medium, 2 cc. of the mixture was transferred to the next tube. Like transfers of 2 cc. each were made in succession to six additional tubes; then 0.2 cc. amounts were transferred successively to each of six additional tubes, making the final dilution of the original inoculum 10^{-20}.[7] As a routine, the same pipet was used for only three dilutions, a new one being then substituted. In general, it took from three to five minutes to make the fourteen dilutions. Serial dilution cultures of certain inoculums were made in duplicate at the same time by from one to four persons, always with essentially the same results. Control inoculations, made by using a separate pipet for each dilution, also gave comparable results. The pipet method of making inoculations and of mixing was

6. Rosenow, E. C.: Proc. Staff Meet., Mayo Clin. **10**:410, 1935.

7. I wish to state clearly that I am fully aware of the growth to be expected from mathematical relationship after successive tenfold dilutions. Nevertheless, growth has been observed in dilutions which cannot be explained on this basis. The significance, if any, of this growth is under investigation.

checked in various ways. Primary dilutions were made in sterile dextrose-brain broth, and 2 cc. of each dilution was transferred immediately by the separate pipet method to the dextrose-brain agar, likewise by the drop method. In the latter instance mixing was done by inverting the tubes containing the medium inoculated instead of by means of the pipets, always with essentially comparable results. Control dilution cultures in dextrose-brain agar were made at intervals throughout the period of study with sterile water, saline solution, broth, blood and filtrates of sterile material.

All tubes containing inoculated mediums were incubated at 35 C. Growth in tubes yielding positive results usually occurred within twenty-four hours, but the tubes were examined daily, and stained films were made repeatedly for from five to ten days before the tube cultures were discarded. The cultures of poliomyelitic and equine encephalomyelitic viruses were made of 5 per cent emulsions of fresh cord and brain in saline solution or after the material had been kept in glycerol for from several days to several years. Like cultures were also made with Berkefeld N and Seitz filtrates of the centrifuged emulsions.

RESULTS

The incidence of growth and the numbers of colonies in the dilutions, especially in the higher dilutions, varied between wide limits but were far greater in dextrose-brain agar than in corresponding dilution cultures in dextrose agar without brain tissue. Of 147 serial dilution cultures made in dextrose-brain agar with emulsions of 11 different strains of poliomyelitic and 31 different strains of equine encephalomyelitic viruses, streptococci were obtained in 102. Cultures made in dextrose-brain broth in the usual way and by serial dilution gave comparable results. Cultures made in dextrose broth yielded streptococci only rarely, while blood agar plate cultures never yielded streptococci. Of the 147 serial dilution cultures in dextrose-brain agar, there was growth of streptococci in 83 in the dilution 10^{-1}, in 76 in the dilution 10^{-2}, in 52 in the dilution 10^{-4}, in 47 in the dilution 10^{-6}, in 39 in the dilution 10^{-8}, in 20 in the dilution 10^{-10} and in 19, 17, 14, 12 and 18 respectively, in the dilutions 10^{-12}, 10^{-14}, 10^{-16}, 10^{-18} and 10^{-20}. In 19 instances there was no growth in the dilution 10^{-1} when one or more higher dilutions yielded streptococci (fig. 1). In contrast in only 4 of 29 serial dilution cultures in dextrose agar, representing emulsions of 28 different strains of viruses, was there growth of streptococci when the dilution was 10^{-1}, and in only 1 culture was there growth when the dilution was 10^{-2}. All cultures in higher dilutions remained sterile.

Of 86 dilution cultures in dextrose-brain agar, made with Berkefeld N or Seitz filtrates from 9 poliomyelitic and 16 equine encephalomyelitic viruses, streptococci were isolated in 21. In 16 there was growth of streptococci in the dilution 10^{-1}, in 12 in the dilution 10^{-2}, in 7 in the dilution 10^{-4}, in 9 in the dilution 10^{-6}, in 7 in the dilution 10^{-8}, in 6 in the dilution 10^{-10}, in 6 in the dilution 10^{-12} and in 6, 4, 2 and 1, respectively, in the dilutions 10^{-14}, 10^{-16}, 10^{-18} and 10^{-20}. In 5 instances there

was no growth in the dilution 10^{-1} when one or more higher dilutions yielded streptococci (fig. 2). In contrast, all corresponding dilution cultures of filtrates of virus in dextrose agar remained sterile. All cultures of filtrates in dextrose broth and on blood-agar plates remained sterile, whereas 5 yielded streptococci in dextrose-brain broth. In altogether 190 control dilution cultures in dextrose-brain agar, made with sterile material, represented by 2,660 tubes, streptococci were never encountered.

Serial dilution cultures in dextrose-brain agar were made in 18 freshly phage-lysed cultures, representing 3 staphylococcus phages and 1 each

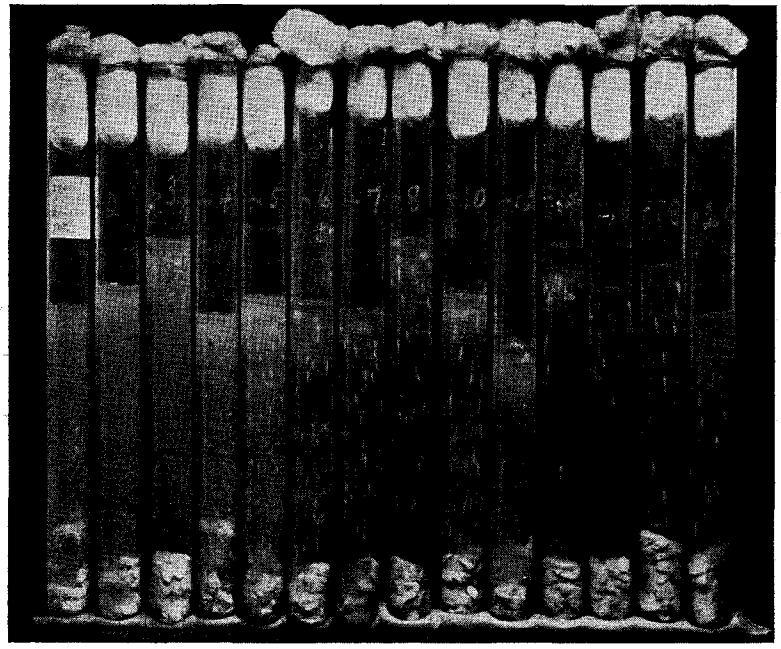

Fig. 1.—Serial dilution cultures in dextrose-brain agar of an emulsion of an adapted eastern strain of equine encephalomyelitic virus. There was no growth in the first two tubes, representing the dilutions 10^{-1} and 10^{-2}. There was a countless number of colonies in the third tube, gradually diminishing numbers from the fourth to the twelfth tubes and larger numbers of colonies in the last four tubes.

of Bacillus coli, Bacillus aerogenes, Bacillus subtilis and Bacillus Friedländer, A and B.[8] Growth that was characteristic of the organisms lysed by phage was obtained in 17 of the 18 dilution cultures. In 11 there was growth in the dilution 10^{-1}, in 17 in the dilutions 10^{-2} and 10^{-4},

8. Dr. Morris L. Rakieten, of the Long Island College of Medicine, Brooklyn, supplied cultures and phages of staphylococci, streptococci, B. subtilis, B. aerogenes, B. coli and B. Friedländer, A and B.

in 14 in the dilutions 10^{-6} and 10^{-8} and in 15, 10, 7, 9, 9 and 8, respectively, in the dilutions 10^{-10}, 10^{-12}, 10^{-14}, 10^{-16}, 10^{-18} and 10^{-20}. In 6 instances no growth occurred in the dilution 10^{-1} when growth occurred in higher dilutions. Serial dilution cultures of 11 filtrates of phage-lysed cultures, representing 7 different strains and 5 species of bacteria, remained sterile. The number of organisms and the end point of growth of the freshly lysed cultures, which showed no organisms in stained films, were usually far higher than those of the lysed culture that had stood for one or more days and those of the corresponding cultures before treatment with phage.

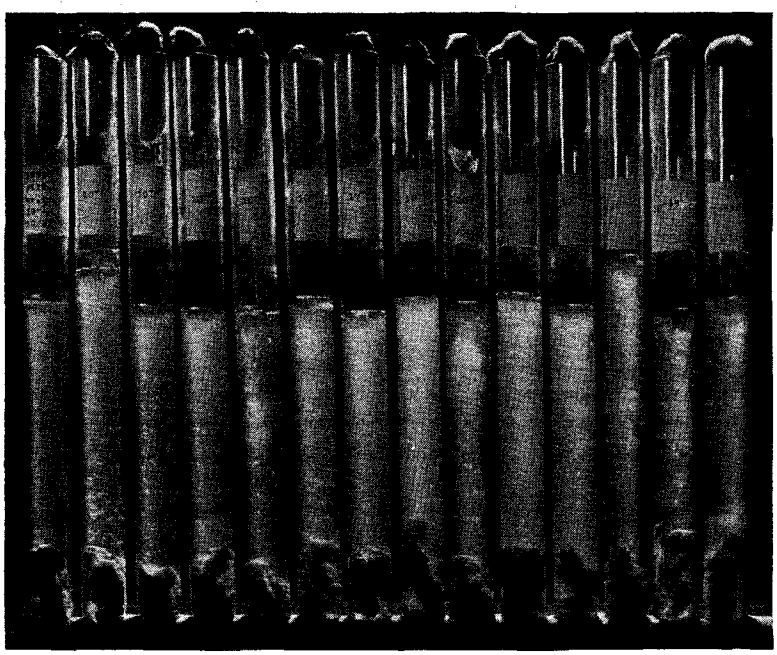

Fig. 2.—Serial dilution cultures in dextrose-brain agar of a filtrate of an emulsion of the brain of a horse that died of acute encephalomyelitis. The first tube, containing the 10^{-1} dilution of the filtrate, was sterile; in the second tube, representing the 10^{-2} dilution, there was 1 colony; in the third tube, or the 10^{-3} dilution, there were 75 colonies, and the rest of the tubes contained diminishing numbers of colonies up to the tube representing the dilution 10^{-15}. The control tube of dextrose-brain agar inoculated with sterile salt solution remained sterile.

In the case of serial dilution cultures of streptococci isolated from viruses, heavy growth occurred in the first few tubes, but growth was frequently obtained at dilutions much higher than could occur from the expected mathematical relation of the number of bacteria originally present. If the streptococci or other bacteria were first grown in chick mash medium and then cultures made by serial dilution in dextrose-brain agar, growth occurred in much higher dilutions (fig. 3) than if

the organisms were first grown in dextrose broth or dextrose-brain broth. The number of colonies and the end-point of growth in dilution cultures in dextrose agar of cultures in dextrose broth usually approximated the expected mathematical relation of the original bacteria present. The supernatant fluid from chick mash cultures of streptococci that had been centrifuged to the point where Gram-safranine stained films and blood-agar platings showed only few organisms and colonies often yielded in serial dilution cultures in dextrose-brain agar almost as many colonies, in dilutions almost as high, as did the corresponding uncentrifuged cultures. Sherman and Safford [9] reported growth of "primitive" forms of bacteria from milk, sewage and other materials in a dilution as high as 10^{-18} in dextrose-meat infusion broth.[10]

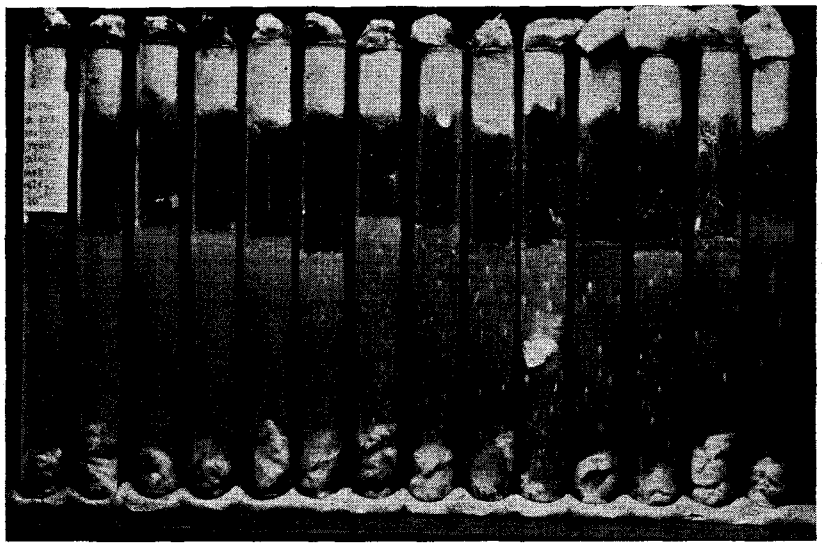

Fig. 3.—Serial dilution cultures in dextrose brain agar of a streptococcus isolated from poliomyelitic virus, after growth of the streptococcus in chicken mash medium. There was diffuse growth in the first 2 tubes, innumerable colonies in the third tube, and progressively fewer colonies with increasing dilution but not in direct proportion therewith.

Diffuse clouding sometimes occurred in one or two tubes of dextrose-brain agar which showed few or no colonies, and in dextrose-brain broth

9. Sherman, J. M., and Safford, C. E.: Science **74**:602, 1931.

10. Since this paper was submitted for publication, my attention has been called to P. B. Hadley's work with the organism of fowl cholera in which growth was obtained in serial dilution cultures in broth up to 10^{-21} (Studies on Fowl Cholera: II. The Role of an Homologous Culture of Slight Virulence in the Production of Active Immunity in Rabbits, Bulletin 150, Rhode Island State College, Agricultural Experiment Station, 1912).

when at an early stage of the growth no organisms were demonstrable, whereas later, stained films revealed pleomorphic cellular forms usually characteristic of the inoculum. Shadow forms of organisms were commonly found, and dilution cultures in dextrose-brain broth sometimes yielded growth from clouded tubes when no organisms were demonstrable microscopically. When a certain dilution showed diffuse clouding, with few or no colonies in dextrose-brain agar, the next higher dilution often revealed a larger number of colonies than the one showing clouding and than the dilution immediately preceding the latter. By the use of a special staining method [11] with which small, light, immature diplococci were demonstrable in filtrates of poliomyelitic and encephalitic viruses, my associates and I have demonstrated cellular forms in smears from these clouded areas earlier and in larger numbers than when such smears were stained by the Gram-safranine method.

Throughout this study the bacteria that grew were uniformly consistent with the material inoculated. The viruses of poliomyelitis and equine encephalomyelitis yielded streptococci, and the phage-lysed cultures yielded the stainable forms characteristic of the respective organisms under study. The streptococci at the end point in dilution cultures in dextrose-brain agar remained viable longer than those that grew in the low dilutions and possessed especially high and specific virulence. They were agglutinated specifically by the respective antiserums. In the case of equine encephalomyelitis, vaccines prepared from the streptococcus isolated protected horses against epidemic encephalomyelitis and guinea pigs and mice against inoculations of encephalomyelitic virus.[4b]

SUMMARY

By the use of the serial dilution method with dextrose-brain agar and dextrose-brain broth it has been found that growth of bacteria can occur from so-called bacteria-free viruses and phage-lysed cultures after these have been submitted to high dilution. This is true even when low dilutions and cultures in ordinary mediums remain sterile. The number of colonies in the dextrose-brain agar was not in proportion to the well established laws of dilution. Viruses and phage-lysed cultures and cultures in chick mash medium of streptococci isolated from viruses often yielded pure cultures in dilutions so high as to exclude the possibility of growth from stainable, visible organisms originally present as such. These results have been obtained consistently, in orderly sequence, not for a day but over a period of seven months, in numerous experiments performed under rigidly controlled conditions.

Of the fact that the bacteria which grew came from the inocula there can be no question, but the nature of the underlying processes responsible for these phenomena is obscure.

11. Rosenow, E. C.: Proc. Staff Meet., Mayo Clin. **10**:115, 1935.

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