A METHOD FOR OBTAINING MASS CULTURES OF BACTERIA FOR INOCULATION AND FOR AGGLUTINATION TESTS:

WITH SPECIAL REFERENCE TO PNEUMOCOCCI AND STREPTOCOCCI.

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PLATE XI.

It is often desirable to obtain bacterial cultures in mass for chemical analysis, for purposes of inoculation, or for use in agglutination tests.

In the case of many organisms, pathogenic as well as non-pathogenic, the usual nutrient agar and broth media of our laboratories furnish, under proper temperatures and life conditions, all requisites for abundant growth, and these organisms may thus readily be obtained in mass. On the other hand, certain pathogenic bacteria grow poorly or after slight initial growth die off in the usual artificial media, and it is with much difficulty that these can be obtained in sufficient quantity for chemical study or inoculation, or in the proper conditions for studies in agglutination. This is often due either to a lack of the proper nutriment, or, if this be present, to the accumulation of products of metabolism (principally acids) which either inhibit further growth or actually bring about the death of the organisms.

Often one of the prerequisites of the abundant growth of organisms in our artificial media, either with or without free access of oxygen, is the presence of some carbohydrate or allied substance available in their metabolic processes; and all bacteriologists are familiar with the differences in amount and rapidity of growth of certain organisms in media containing available sugars as compared with the growth in the same media when such sugars are absent. When, however, the acid given rise to by the action of the organisms on the sugar reaches a certain percentage, varying in the case of different organisms, cessation of

growth occurs, and even the death of the organisms may result. With certain sensitive organisms, such as the pneumococcus, the amount of sugar often normally present in ordinary meat infusion broth is sufficient to lead to the death of the organisms after a few days, from the acid produced. On the other hand, the pneumococcus will develop only slightly or not at all in perfectly sugar-free broth.

In the study of fermentations from the time of Pasteur on, it has been known that if certain non-soluble salts, particularly calcium carbonate, not positively influencing the alkalinity of the medium, be added to solutions in which fermentations are taking place, the organic acid produced by the action of the organisms on the sugars present would unite with the calcium, liberating carbon dioxide, and be neutralized, and that so long as the other requisite nutrient materials were present in sufficient amount the organisms would continue to ferment the remaining sugar. Whether there is during this period of activity a great increase in the actual number of bacterial cells depends, however, often on other factors than the mere viability and fermentative activity of the organisms concerned, as, for instance, upon the amount of free oxygen furnished the organisms during this period, and doubtless upon other nutrient conditions not so well understood.

Although this scheme of neutralization of acids formed from the sugars normally present in ordinary media has been taken advantage of by various workers, and an addition of calcium carbonate has been recommended for the removal of acid and the preservation of the viability of organisms sensitive to even small amounts of acid [in case of the pneumococcus (*Wurtz and Mosny*); the neutralization of primary acid in the case of diphtheria broth for toxin production], no attention, so far as the writer at present knows, has been given to the use of fluid media, such as meat infusion broth, to which sugar and calcium carbonate have been added, for the purpose of getting these organisms in large amounts and in sufficiently dense emulsions to be useful for large inoculations and in agglutination experiments.

Certain experiments carried on by the writer during the last

four or five years with the above and other objects in view, not pertinent to this communication, have fully demonstrated that a massive growth may be obtained and the life and active growth of even such sensitive organisms as pneumococci and streptococci may be preserved over long periods in fluid media plus sugars, by the application of this principle of neutralization. The pneumococcus, for instance, may be preserved alive and growing at 37° C. in media rich in sugar (4 % or 5 %) from which it continuously produces acid, for long periods even extending over months. In a comparatively short time the density of the growth is astonishing to one familiar with the scanty growth of the organism in the same media under the usual conditions. It is obvious that the employment of such technique may also be useful in determining some of the factors influencing the growth or cessation of growth and factors leading to the death of organisms in certain nutrient media. Such a method may further be useful in other fields of work not germane to the present communication, but of which it is the writer's intention to deal subsequently in another paper.

The writer's chief object in making this short and outline presentation of the subject at this time is the urgency of the need of a satisfactory method of obtaining suitable cultures of sensitive and poorly growing organisms, especially pneumococci and streptococci, for purposes of inoculation and for agglutination tests.

Agglutination tests with pneumococci and streptococci, when the usual broth cultures (either with or without sugars added) or emulsions from agar are employed, have in the experience of most workers been found not only technically unsatisfactory but have given varying and often contradictory results.

On the other hand, the technique recommended by Wadsworth [1] is valuable and reliable, but when a long series of organisms, as in comparative studies, is being tested against the same or various sera, much time and energy must be consumed, when following his technique, in centrifuging and preparing the organisms for the tests.

The method here proposed, which has given eminently satis-

[1] Wadsworth, *Journal of Medical Research*, 1903, x, 228.

factory and striking results in a comparative study of pneumococci and streptococci in immune sera,—results and appearances which are entirely comparable to those familiar in typhoid, dysentery, and various other agglutinations,—is simple and particularly available for the study of pneumococci and streptococci and other organisms which ferment carbohydrates, but which thrive poorly or die out rapidly in the usual media. The streptococci often, as is well known, grow in broth, either with or without sugar, in flocculi or thickly matted masses entirely useless for agglutination purposes. By the proposed method usually a fair and often a good and satisfactory emulsion may be obtained, from which agglutinative limits may easily be determined.

The medium used should be a one or two per cent peptone broth made from meat infusion, which has been brought to neutrality before boiling and coagulation. After filtration for clearing, one per cent of dextrose (or other sugar fermentable by the given organism) and one per cent of calcium carbonate are added. If the medium be acid, the latter salt will of course bring it to neutral. The calcium carbonate may then be well distributed throughout the broth by shaking and the emulsion rapidly decanted into tubes or preferably small Erlenmeyer or Florence flasks, say 100 cubic centimeters to 150 cubic centimeters in each. These are sterilized on three consecutive days at 100° C. in the usual manner. The flasks after inoculation are placed at 37° C. and are thoroughly shaken once or twice a day to neutralize the acid formed and to break up the chains and masses in the case of streptococci. The growth may be sufficient for purposes of agglutination in two days or even in one day, but as a routine up to the present time we have employed a three or four days' growth, which seems to give more uniform results and more marked agglutination.[1] About an hour before using for agglutination tests the culture should be thoroughly shaken and the calcium carbonate and larger clumps, if present, allowed to settle during this time. The sample to be tested should then be taken from the upper portion of the fluid; or the cultures, after

[1] Such cultures may then be preserved in the ice-box. We have tested them frequently in the same sera from day to day, and have found little or no change in their limit of agglutination even after weeks.

shaking, may be centrifugated for a few minutes. This centrifugalization, however, is not necessary if one remembers that a slight primary deposit may occur which is not due to agglutination.

Such emulsions, tested in the serum of pneumonia patients and in the serum of immune animals, give clumps in every way comparable to those seen in typhoid agglutinations. (See photograph, Plate XI.)

Routine agglutination tests are made by adding one cubic centimeter of the serum dilution to one cubic centimeter of the emulsion in small test-tubes. The tubes are placed at 37° C. for two or three hours—after which time the agglutinations are often practically complete—and then transferred to the ice-box to prevent growth taking place and permit of the further deposition of the clumps of agglutinated organisms, and the final control reading made after eighteen to twenty-four hours.

For routine work on patients' sera an organism should be selected which shows little or no tendency to agglutinate spontaneously or upon the addition of normal salt solution in the control. Spontaneously agglutinating cultures of course are met with among the pneumococci, and are frequent with streptococci, but even with these the limits of agglutination can be determined with much certainty if careful comparison with the control is made.

DESCRIPTION OF PHOTOGRAPH.

PLATE XI.

AGGLUTINATION OF PNEUMOCOCCI, GROWN IN CALCIUM-CARBONATE-GLUCOSE BROTH, IN PNEUMOCOCCUS IMMUNE RABBIT SERUM.

The photograph represents the stage of agglutination two hours after making the mixtures, the tubes having been in the incubator at 37° C. during this time. Tube No. 1 contains a 1-20 control of the pneumococcus culture and normal rabbit serum. Tubes Nos. 2, 3, 4, 5, 6, 7, and 8 contain the pneumococcus culture and immune serum in dilutions of 1-10, 1-20, 1-50, 1-100, 1-200, 1-400, and 1-800, respectively, as indicated on the tubes. Tube No. 9 contains a control of normal salt solution and culture. The 1-10 dilution is nearly clear, and large clumps are apparent even in the photograph at 1-200. A hand-lens shows fair clumps at 1-400, and traces of agglutination at 1-800. The controls are uniformly clouded.

Each tube contains two cubic centimeters, one cubic centimeter of the culture and one of the serum dilution, which is made up with normal salt solution. The culture used in the test was grown for four days at 37° C.