STUDIES ON THE PNEUMOCOCCUS.

II. Dissolution of Pneumococci at Varying Hydrogen Ion Concentrations. Effect of Temperature, Previous Killing of the Organisms, and Fresh Human Serum on the Phenomenon. Behavior of Other Organisms.

By FREDERICK T. LORD, M.D., and ROBERT N. NYE, M.D.
(*From the Research Laboratory of the Massachusetts General Hospital, Boston.*)

(Received for publication, December 16, 1921.)

In a previous publication[1] we have called attention to the dissolution after incubation of living washed pneumococci, suspended in standard solutions of known hydrogen ion concentration. There is clearing of the suspensions from disintegration of the organisms within the range of pH of about 5.0 to 6.0 within an hour, and diminishing density with longer incubation in the more alkaline side of the scale. In discussing the phenomenon, we suggested that an enzyme derived from the organisms might be responsible for their dissolution. Further experiments have shown that the phenomenon is constantly observed with all the strains of pneumococci tested, including examples of the types commonly known as Types I, II, and III.

Avery and Cullen[2] have since demonstrated the presence in the pneumococcus of an erepsin-like enzyme, a lipase, an invertase, an amylase, and an inulase, with an activity within a zone of hydrogen ion concentration which bears a striking correlation to that of the biologic activity of the bacterial cell.

To obtain further information on the dissolution phenomenon, it seemed desirable (1) to suspend the organisms in another solution than the standard, (2) to determine the effect of temperature on the phenomenon, (3) to observe the behavior of dead as well as living pneumococci, (4) to test the effect of fresh human serum on the dissolution of pneumococci, (5) to compare the rate of dissolution of pneumococci

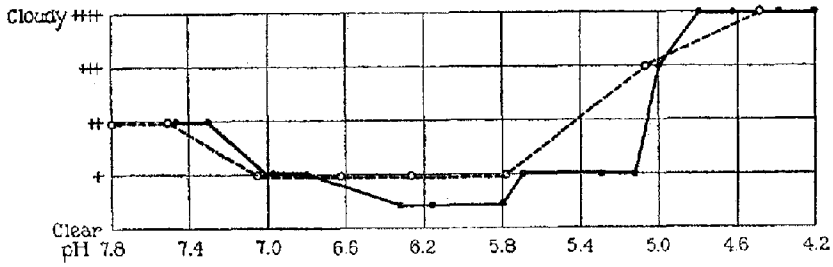
[1] Lord, F. T., and Nye, R. N., *J. Exp. Med.*, 1919, xxx, 389.

[2] Avery, O. T., and Cullen, G. E., *J. Am. Med. Assn.*, 1920, lxxiv, 1668; *J. Exp. Med.*, 1920, xxxii, 547, 571, 583.

in a fresh standard solution at pH 6.1 with that in a standard solution of the same pH in which the organisms had previously undergone dissolution, and (6) to do comparative dissolution tests on pneumococci and other organisms.

1. Dissolution of Pneumococci in Nutrient Broth at Varying Hydrogen Ion Concentrations. Comparison with Dissolution in Standard Solutions.—To observe the effect of suspending pneumococci in a medium in which the conditions are suitable for growth and multiplication at the proper range of pH, a double strength meat infusion broth was made and the pH and tonicity were adjusted as shown in the preceding article.[3]

To each of eight tubes containing 0.2 cc. of approximately isotonic ($\Delta = 0.580 - 0.748$°C.) standard solution at varying hydrogen ion

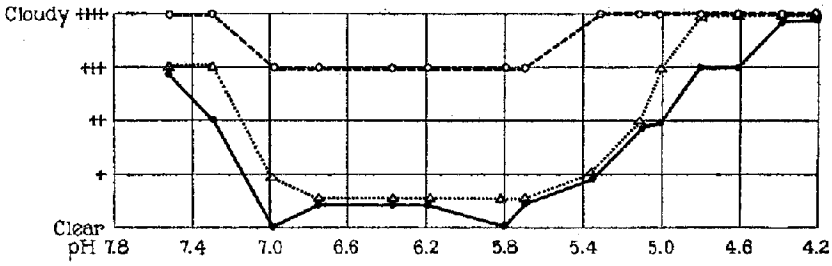


TEXT-FIG. 1. Dissolution of pneumococci in nutrient broth and standard solutions at varying hydrogen ion concentrations. Comparison of the dissolution of Pneumococcus Type I at the end of 24 hours at varying hydrogen ion concentrations in isotonic bouillon (broken line) and in isotonic standard solutions (solid line).

concentrations and to each of a similar number of tubes containing an equal amount of approximately isotonic nutrient broth at varying hydrogen ion concentrations, 0.2 cc. of a saline suspension of living washed Pneumococci Type I was added. The two series were incubated. No change in the suspensions was observed at the end of 1 hour. At the end of 6 hours there was slight dissolution in both series at about the same range of pH; *i.e.*, at 5.78 to 7.8 in the bouillon series and 5.14 to 6.98 in the standard solutions. The result at the end of 24 hours is shown in Text-fig. 1. The dissolution in the two series was practically the same and almost complete at a pH of about 5.7 to 7.0.

[3] Lord, F. T., and Nye, R. N., *J. Exp. Med.*, 1922, xxxv, 686, Table I.

2. Effect of Temperature on Dissolution of Pneumococci at Varying Hydrogen Ion Concentrations.—Living washed Pneumococci Type I were suspended in approximately isotonic standard pH solutions at incubator and room temperature (23.2°C.) and in the ice box (12.2°C.). The series used for the test in the ice box was placed on ice and when thoroughly chilled a chilled suspension of organisms was added. The tubes were then replaced on ice. Observations were made after 1, 6, and 24 hours. No change in the density of the suspensions was noted after 1 hour. After 6 hours no change was observed in the suspensions at room and ice box temperature, but in the suspensions at incubator temperature there was slight clearing at pH 4.81 and 5.02, more marked clearing at pH 5.14 to 6.98, and slight clearing at pH 7.3 to 7.49. The appearance of the tubes after 24 hours is indicated in

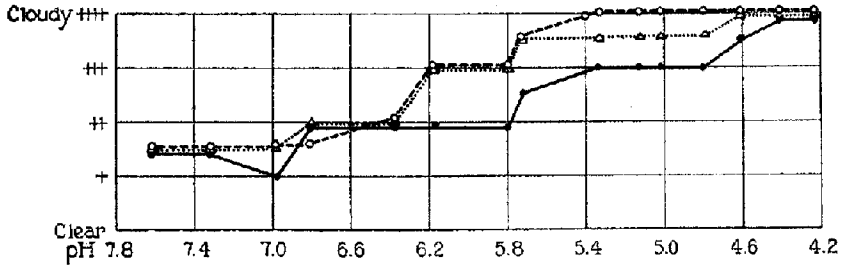


TEXT-FIG. 2. Effect of temperature on the dissolution of pneumococci. Comparison of the degree of dissolution of living Pneumococcus Type I suspended in isotonic standard pH solutions in the incubator (solid line), at room temperature (dotted line), and in the ice box (broken line). The observations were made after 24 hours.

Text-fig. 2. At the expiration of this interval dissolution had proceeded to about the same degree at incubator and room temperature and was almost complete at pH 5.33 to 6.98. Though dissolution occurs after 24 hours at ice box temperature and at the same range of pH, it is much less marked than at incubator and room temperature. After the expiration of 48 hours, however, the suspensions at ice box temperature had reached the same degree of dissolution as those at incubator and room temperature from a pH of 5.8 to 6.98 inclusive. Dissolution in the ice box was somewhat less at pH 5.02 to 5.72 inclusive.

Dissolution therefore takes place at room and ice box temperature, though much more slowly than at incubator temperature. It may be noted in this as in the preceding experiment that dissolution occurs more quickly at a certain critical range; *i.e.*, from about pH 5.14 to 6.98.

3. Effect of Previous Death of Pneumococci on the Dissolution Phenomenon. (*a*) *Dissolution of Pneumococci Allowed to Grow and Die Out.*—A glucose broth culture of pneumococci was allowed to grow and die out. At the end of about 72 hours the pH was 5.02 and a transplant was sterile. The culture was centrifuged, the sediment suspended in isotonic ($\Delta = 0.62°$C.) saline solution, again centrifuged, and resuspended in isotonic saline solution. An equal amount (0.2 cc.) of suspended organisms was added to three sets of approxi-

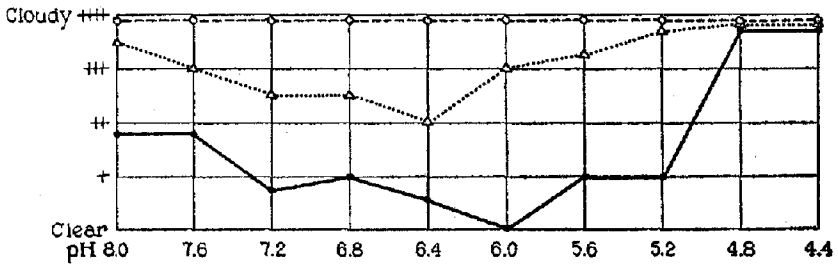


TEXT-FIG. 3. Effect of previous death of pneumococci on dissolution. Comparison of the degree of dissolution of Pneumococcus Type I allowed to grow and die out in glucose broth, suspended in isotonic standard solutions, at incubator temperature (solid line), at room temperature (dotted line), and in the ice box (broken line). The observations were made after 24 hours.

mately isotonic standard pH solutions, ranging from 7.49 to 4.23. One series was placed in the incubator, another in the ice box (11.2°C.), and the third left at room temperature (20.0°C.). Observations were made after 1, 18, and 24 hours. Dissolution slowly proceeded to about the same degree in all three sets and at about the same range of pH. The results are shown in Text-fig. 3. The degree of dissolution of dead organisms increases progressively from the acid toward the alkaline end of the scale and is in general most complete at a pH of 7.0 to 7.49. On comparison of the dissolution of living (Text-fig. 2) and dead organisms (Text-fig. 3) a difference is seen in the curves. After 24 hours the dissolution of living organisms is most marked and

almost complete within a range of pH from 5.72 to 6.98, and takes place little or not at all at the more alkaline and more acid ends of the scale respectively. Death of pneumococci allowed to grow and die out may be ascribed to acid production in the media, and the dissolution curve of organisms thus killed is different from that of organisms killed by heat (*cf.* Text-fig. 4).

(*b*) *Dissolution of Pneumococci Killed by Heat.*—To determine the effect of previous killing of the pneumococci by heat, one lot of organisms at the height of their growth in dextrose bouillon was heated to 100°C. for 5 minutes, a second lot was heated to 57°C. for 1 hour, and a third lot of living organisms was reserved as a control. Transplants from the flasks heated at 100°C. for 5 minutes and 57°C. for 1 hour were sterile. All three lots were centrifuged, washed with



TEXT-FIG. 4. Effect of heating pneumococci on dissolution. Comparison of the degree of dissolution of Pneumococcus Type II, living (solid line), killed by heat at 57°C. for 1 hour (dotted line), and killed by heat at 100°C. for 5 minutes (broken line). The observations were made after 24 hours in the incubator.

sterile isotonic saline solution, and recentrifuged. The supernatant saline solution was pipetted off and a cloudy suspension of the organisms made by adding fresh sterile isotonic saline solution, care being taken to secure suspensions of equal density in the three groups. The three lots were then mixed with standard pH solutions, placed in the incubator, and the results noted at intervals. As shown in Text-fig. 4, after 24 hours the suspensions of living pneumococci showed the usual dissolution curve, the clearing of the suspension being most marked at a pH of 6.0. No dissolution was noted at a pH of 4.8 and 4.4. Considerable, though less dissolution than at a pH of 6.0 was observed in the intervening acid end of the scale and a gradually dimin-

ishing clearing of the tubes from a pH of 6.0 toward the more alkaline end of the scale. Some dissolution of pneumococci killed by heating at 57°C. for 1 hour was observed, the general character of the curve being similar to that with the living organisms. Previous heating of the organisms at this temperature had evidently diminished their susceptibility to dissolution at the critical hydrogen ion concentrations. The pneumococci heated to 100°C. for 5 minutes showed no change in the density of the different tubes.

4. Effect of Fresh Human Serum on Dissolution of Pneumococci at Varying Hydrogen Ion Concentrations.—In view of the well established presence of antienzyme in normal serum it seemed desirable to determine its effect on the dissolution of pneumococci. For this purpose 0.5 cc. of fresh human serum was placed in each of seven sterile test-tubes. To each tube was added 2 drops of buffer solution containing equal parts of 0.5 M KH_2PO_4 and 0.5 M Na_2HPO_4. 2 drops of indicator were also added to each tube, 1 per cent aqueous sodium alizarin sulfonate being used at pH 3.6 to 5.6 inclusive and 0.01 per cent aqueous phenolsulfonephthalein at pH 6.1 to 7.8 inclusive.[4] The fluid in each tube was brought to the desired pH by the addition of drops of N HCl, 0.2 N HCl, N NaOH, or 0.2 N NaOH. The number of drops added to each tube was recorded and those receiving less than the maximum number (8 drops) were made up to volume with sterile saline solution. The comparator rack method was used in the construction of the series. Sterile precautions were used throughout and cultures from the completed series were negative.

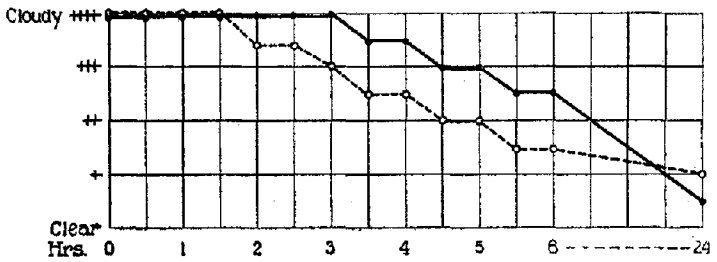
To each tube in the two series 0.2 cc. of a heavy suspension of washed living Pneumococci Type I was added. The two sets were incubated for 36 hours. The pH did not change during this interval. Macroscopic changes in density were difficult of appreciation and reliance was placed on microscopic examination of stained smears. After 36 hours, examination of stained smears from the tubes containing organisms suspended in serum at varying pH showed no apparent dissolution in any of the tubes. The organisms suspended in standard pH solutions showed the usual curve of dissolution.

Suspension of pneumococci in normal serum at varying hydrogen ion

[4] Experiments have shown that these two indicators themselves do not prevent dissolution and the inhibition is to be ascribed to the serum.

concentrations therefore prevents the dissolution of the organisms at the critical range of pH.

5. Comparison of the Degree of Dissolution of Pneumococci in Standard Solution of pH 6.19 with Dissolution in the Supernatant Fluid of the Standard Solution of the Same pH in Which Large Numbers of Pneumococci Have Been Previously Dissolved.—The most likely explanation of the dissolution of pneumococci at the critical range of pH in standard solutions seems to be the activation of an intracellular enzyme. If the enzyme is set free by the process of dissolution and exists in the fluid in which the organisms are undergoing disintegration, then dissolution of a fresh lot of pneumococci should take place at a more rapid rate in fluid in which dissolution has already taken place than



TEXT-FIG. 5. Comparison of the rapidity of dissolution of Pneumococcus Type I at pH 6.19 in the supernatant fluid of standard solution in which pneumococci have been previously dissolved (broken line), and in fresh standard solution (solid line).

in fresh standard solutions at the same pH. For the purpose of testing this hypothesis the following experiment was performed.

The contents of a liter flask of glucose bouillon culture of living Pneumococci Type I were centrifuged. The sediment was collected, washed in sterile isotonic ($\Delta = 0.620°C.$) saline solution, recentrifuged, and suspended in 2 cc. of isotonic salt solution. This suspension of living pneumococci was added to 5 cc. of isotonic standard solution of pH 6.15 and incubated for 48 hours. At the end of this time the suspension was centrifuged. In one test-tube was placed a part of the supernatant fluid thus obtained and in another an equal amount of fresh standard solution of pH 6.19. Equal amounts of a heavy suspension of living Pneumococci Type I were now added to both tubes and the result

was noted after incubation[5] (Text-fig. 5). At the expiration of ½ hour no difference was observed in the density of the two suspensions. After 2 hours the suspension of organisms in the supernatant fluid had cleared to a perceptibly greater degree than that in the fresh standard solution. Further observation over a period of 6 hours continued to show greater dissolution in the supernatant fluid. At the expiration of 24 hours both tubes had cleared, a result to be expected in suspensions of pneumococci at pH 6.19 after a sufficient interval. Observations were made by gross and microscopic examination. The pH did not change during the experiment. Repeated experiments showed the same result.

The theory was therefore confirmed that dissolution of pneumococci takes place more rapidly in standard solution of pH 6.19 in which large numbers of organisms have been previously dissolved than in fresh standard solution at the same pH.

6. Dissolution Tests with Other Organisms at Varying Hydrogen Ion Concentrations.—Tests with *Streptococcus viridans* and *hæmolyticus* and *Staphylococcus aureus*, similarly performed with washed suspensions of living organisms in standard solutions at incubator temperature, showed no dissolution.

DISCUSSION.

The phenomenon of dissolution of pneumococci at critical hydrogen ion concentrations seems to bear a definite relation to the living organisms. It is most marked when living organisms are used in the experiment and takes place most quickly at a range of hydrogen ion concentrations which is slightly less acid (pH 5.5 to 6.7) than the limiting hydrogen ion concentration (pH 5.1) of growing glucose bouillon cultures and slightly more acid than the most acid reaction (pH 6.8) at which growth of organisms can be continued. It occurs, however, with the lapse of time throughout the whole range of hydrogen ion concentrations (pH 6.8 to 7.8) at which the pneumococci are biologically active.

[5] Both at the beginning and end of the experiment the suspensions of pneumococci in the supernatant fluid are somewhat more cloudy than the suspensions in fresh standard solution because of the presence of fine particles of pneumococci not removed by the centrifuge.

As we have already noted in our previous paper, the most probable explanation seems to be the activation of an enzyme derived from the bacteria themselves. Avery and Cullen's[2] demonstration of the presence of enzyme in bile and in phosphate solutions of pH 6.2 in which pneumococci have undergone disintegration lends support to this point of view. The fact that the phenomenon is most marked with living organisms at incubator temperature, the inhibiting effect of death of the organisms (Text-figs. 3 and 4) and of the addition of fresh human serum to the solutions, and the greater rapidity with which dissolution takes place in standard solutions in which pneumococci have been previously dissolved (Text-fig. 5) are consistent with enzymatic action. If merely the effect of acidity on ferment action is considered, the absence of dissolution at acidities greater than about pH 4.6 coincides closely with the failure of Avery and Cullen to demonstrate any appreciable ferment activity at acidities greater than pH 4.5. The less complete dissolution and change in the character of the curve of dissolution of pneumococci allowed to grow and die out in glucose bouillon (Text-fig. 3) may be due to partial destruction of the enzyme or a change in the physical state of the bacterial cell in consequence of exposure to this acidity (pH 5.02). Avery and Cullen have shown that the activity of the peptonase and lipase obtained from the pneumococcus is not appreciably diminished by exposure to pH 5.0 for 2 hours. It may be, however, at this hydrogen ion concentration, which corresponds closely with the isoelectric points and coincident precipitation optima of various biologically important proteins, that some change has occurred to diminish the permeability of the cell membrane or to change the physical state of the bacterial cell protoplasm. Less dissolution when the pneumococci are killed by heat and the absence of dissolution after heating to 100°C. may be ascribed to partial and complete destruction respectively of the enzyme at these temperatures.

Assuming that dissolution occurs in consequence of the death of the organisms and the activities of an endocellular enzyme, the disintegration may be conceived to take place most actively at that point in the scale where the largest number of organisms are most rapidly killed with, at the same time, a minimum of injury to the cell membrane which still remains permeable for the hydrogen ions and permits the activation of the endobacterial ferment.

Enzymatic action at ice box temperature is at variance with the usual temperature range of ferment action. Dissolution proceeded much more slowly at this temperature but was finally as complete as at incubator temperature.

CONCLUSIONS.

Suspensions of living pneumococci in approximately isotonic standard solutions and in approximately isotonic bouillon with pH varying from about 4.0 to 8.0 after incubation show dissolution of organisms in those solutions having a pH higher than about 5.0. Dissolution is most marked at a critical range of about pH 5.0 to 7.0. Some dissolution also takes place toward the more alkaline end of the scale. No dissolution occurs at the most acid end of the scale.

Dissolution in the standard solutions occurs at incubator, room, and ice box temperature. It is less marked at ice box temperature. Dissolution takes place in standard pH solutions with pneumococci allowed to grow and die out in glucose bouillon but unlike dissolution with living organisms is progressive from the acid toward the alkaline end of the scale. Pneumococci killed by heat for 1 hour undergo less dissolution than living organisms, the general character of the curve being similar to that with living organisms. Pneumococci killed by heat at 100°C. for 5 minutes do not undergo dissolution. The addition of fresh human serum to the suspensions of pneumococci at varying pH prevents dissolution. Dissolution of pneumococci takes place more rapidly at pH 6.1 in standard solutions in which large numbers of pneumococci have been previously dissolved than in fresh standard solutions at the same pH.

The dissolution of pneumococci under the conditions of the experiments may be ascribed to an enzyme derived from the bacteria themselves.

Other organisms such as *Streptococcus viridans* and *hæmolyticus* and *Staphylococcus aureus* do not undergo dissolution under conditions similar to those to which the pneumococcus was exposed.