THE PENETRATION OF LUMINOUS BACTERIA BY THE AMMONIUM SALTS OF THE LOWER FATTY ACIDS.

PART I. GENERAL OUTLINE OF THE PROBLEM, AND THE EFFECTS OF STRONG ACIDS AND ALKALIES.

BY SAMUEL E. HILL.

(From the Physiological Laboratory, Princeton University, Princeton.)*

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In the experiments of Jacobs[1] on hemolysis by the ammonium salts of the lower fatty acids, hemolysis is used as a measure of penetration. It is concluded that only the molecules of ammonia and of fatty acid, formed by hydrolysis of the salt, are effective in penetrating the cell membrane. These reunite within the cell to form the salt, cytolysis resulting from the increased osmotic pressure.

It was thought that some of these studies might be repeated, using luminous bacteria as the experimental material. The problem as originally conceived was a study of the rates of cytolysis of luminous bacteria by the ammonium salts of formic, acetic, propionic, butyric, and valeric acids, using solutions of all pH values at which measurements of the time of cytolysis could be made. Cytolysis would be detected by the disappearance of the light and would indicate penetration. As the work progressed it became apparent that disappearance of the light did not always indicate cytolysis but under the conditions of the experiments did indicate penetration, and numerous modifications of the original procedure were suggested. Before attempting

* The experiments described herein were carried out in the physiological laboratory of Princeton University and in the laboratory of the United States Bureau of Fisheries. I wish to acknowledge my indebtedness to Mr. Henry O'Malley, Commissioner of Fisheries, and to Mr. Elmer Higgins, Director of the Bureau of Fisheries Laboratory at Woods Hole, for placing the facilities of the laboratory at my disposal. I am also deeply indebted to Prof. E. Newton Harvey of Princeton University, under whose direction this work was done, for constant advice and guidance.

[1] Jacobs, M. H., Harvey lectures, 1927, xxii, 146.

analysis of the data obtained with the ammonium salts it was therefore necessary to answer a number of questions bearing on these points, as follows:

Are luminous bacteria cytolyzed as erythrocytes are by water, hypotonic non-penetrating salt solutions, and by penetrating solutions of all concentrations? Is disappearance of the light coincident with cytolysis? May disappearance of the light be used as a criterion of cell penetration? May the presence of the ammonium salts within the cell be responsible for the extinction of the light, independently of osmotic effects? Are the bacteria injured by the activity of hydrogen and hydroxyl ions in the range of pH values covered by the ammonium salts? Is the point of injury the same, whether hydrogen or hydroxyl ion activity is exerted on the outer surface of the cell membrane or on the protoplasm itself? May salt injury and acid and base injury be separated from the osmotic effects of the ammonium salts?

These questions will be considered under subsequent headings.

I.

Experimental Methods.

The organism studied was *Bacillus Fischeri* (Beijerinck, Migula), a marine form isolated from herring by C. S. Shoup at Woods Hole during the summer of 1927 and since maintained in pure culture in my laboratory. The bacteria were grown in Petri dishes, using the calcium carbonate buffered media described in a previous paper.[2] The cultures were used when approximately 24 hours old, the bacteria being brushed from the surface of the medium and collected in M/2 NaCl.

A single drop of bacteria suspended in M/2 NaCl was added to 10 cc. of solution, the tube vigorously shaken, and watched until the light disappeared. In some early experiments the attempt was made to use a standard low intensity of light as the end-point of the reaction. The results thus obtained were so erratic and unreliable that these experiments were discarded and total disappearance of the light taken as the end-point. The eye is sensitive to light of so low an intensity that measurements are difficult, and Reeves[3] has shown that this threshold varies from day to day. In order to minimize errors due to this variability as well as to variability in the bacteria, experiments were always run in multiple. No measure-

[2] Hill, S. E., *Biol. Bull.*, 1928, lv, 143.

[3] Reeves, P., *Abridged Scient. Pub. Research Lab. Eastman Kodak Co.*, 1917–18, iii, 13.

ments are recorded except those made after at least 1 hour spent in light of low intensity, and at least 15 minutes in total darkness. The use of a stop watch under such conditions is obviously impossible. The timing unit was therefore a metronome adjusted to beat seconds. The experiments were run in a definite order and the results recorded on a "Sunstrand" adding machine, which possesses a type of keyboard that can be operated in the dark without error. The records were transcribed to sheets at the close of the experiment.

The pH values of the solutions were measured with either the quinhydrone or the glass electrode, and in a few cases calculated values were used, as for NaOH solutions above pH 9.0. In every case, the method of determining pH is given.

It was early found that a single suspension of bacteria could not be kept in good condition for the 4 or 5 hours necessary to run the longer experiments. In the experiments which were run in multiple, a separate culture of bacteria was used for each of the 8 or 9 parts. The suspension was aerated with a stream of air. In each case the first experiment was run from the acid to the alkaline end of the series, the second in reverse order, etc., in order to equalize any differences due to ageing of the suspension, but no definite differences were found. In all cases the average time of disappearance of the light in 8 or 9 experiments was plotted on the vertical axis and the concentration of free acid or alkali, or pH of the solutions on the horizontal axis.

II.

Applicability of Method.

Luminous bacteria are cytolyzed by water, hypotonic non-penetrating solutions, and by penetrating solutions of all concentrations. This cytolysis differs from that of erythrocytes because of the difference in shape of the cell, and because the bacillus possesses a rigid cell wall which prevents swelling. The mammalian erythrocyte, because of its peculiar shape, can increase considerably in volume without an increase in surface.[1] The surface membrane can then stretch to some extent before any great resistance is offered to osmotic swelling. With the bacillus, great mechanical resistance is at once offered to osmotic entrance of water, and the cell bursts without increasing in size. The evidence on which these statements are made is presented below.

If luminous bacteria are placed in diluted sea water, there is little change in intensity of the light until the sea water decreases to 20 per cent. Solutions from 20 to 6 per cent sea water form a series of decreasing brilliance, and after a lapse of 5 minutes no light is observed in solutions more dilute than 6 per cent sea water. Viewed in day-

light, the solutions were decreasingly turbid with decreasing sea water concentration, a marked difference being observed between the 100 cent and 6 per cent solutions. On vigorous shaking, foam developed in all of the solutions, but persisted only in those of sea water concentration below 6 per cent. This indicated the bursting of the cell membrane and the liberation in the solutions of foam-producing substances such as the cell proteins. Examined by transmitted light, the bacteria in the solutions of less than 6 per cent sea water were almost impossible to find but were easily demonstrated in the dark field. In solutions of above 6 per cent they were demonstrable also by transmitted light. Mrs. E. B. Harvey kindly measured a number of bacteria for me, finding only a slight increase in size in 20 per cent sea water.

In another experiment carefully measured quantities of bacterial suspension were added to a series of sea water dilutions as before and counts of the bacteria made in the dark field. No significant differences in number were observed even between 2 per cent and 100 per cent sea water. As before, when the solutions were examined by transmitted light it was difficult or impossible to demonstrate the bacteria in the more dilute samples.

The difficulty of demonstration of cytolyzed bacteria by transmitted light is thought to be due to the filling of the cell capsule with water, so that light passing through is not appreciably deflected from its course. With dark field illumination the surface only is concerned and the effect is therefore the same whether we are dealing with a normal cell or a cytolyzed one.

In distilled water or tap water the light disappeared in about 15 seconds. The presence of low concentrations of NaCl (up to 0.0156 M) delayed the disappearance of the light for a few seconds and in solutions of 0.0625 M and upward the light lasted for over 1 hour although greatly dimmed in 0.0625 M NaCl. The persistence of the light, undimmed, through a considerable range of NaCl dilution and its great dimming shown progressively through several dilutions, followed by sudden disappearance in slightly more dilute solutions, indicate the rupturing of the cell membrane at a critical osmotic pressure, and the presence of different degrees of resistance in the bacterial population. The least resistant were cytolyzed by 0.0625 M NaCl and the most

resistant by 0.0156 M NaCl. These differences might be individual variation or a combination of individual variation with age groups in the population. The results of microscopic examination of the NaCl dilutions were in every way similar to those of the sea water dilutions.

Sucrose was almost as efficient as sea water or NaCl in preserving the luminescence, as the light disappeared in solutions of 0.0625 M and below, and exhibited a series of increasing brilliance as the sugar concentration rose. This indicated that the failure of light in diluted sea water was an osmotic phenomenon due to bursting and was not

TABLE I.

Time Required for Complete Disappearance of Light of Luminous Bacteria in Hypotonic Solutions.

No examination was made after 6 hours.

Per cent of sea water		Concentration of NaCl		Concentration of sucrose	
100.0	+	0.5 M	+	1.0 M	+
50.0	+	0.25	+	0.5	+
25.0	+	0.125	+	0.25	6 hrs.
12.5	6 hrs.	0.0625	6 hrs.	0.125	1 hr.
6.0	5 min.	0.0312	5 min.	0.0625	5 min.
3.0	1 "	0.0156	1 "	0.0312	1 "

due to the absence of the customary salts as such. This had been previously observed by Harvey[4] for an unindentified species of luminous bacteria.

In solutions of ethyl alcohol of all concentrations from molar downward the light disappeared in a few seconds as in pure water, while in presence of sea water or 0.5 M NaCl similar concentrations of alcohol were without effect. This shows that the effect of the alcohol in pure water was caused by its easy penetration, and was not due in any way to narcosis or to an attack on the cell membrane.

In solutions of glycerine from molar downward the time of disappearance of the light was scarcely distinguishable from that in pure water, being perhaps 20 per cent longer. In presence of 0.5 M NaCl

[4] Harvey, E. Newton, *Biol. Bull.*, 1915, xxix, 308.

these concentrations of glycerine were without effect on the luminescence, showing that glycerine also penetrates these cells with great readiness.

Urea did not penetrate as readily as alcohol and glycerine, the disappearance of the light occurring in M urea in about three times the time required in pure water (Table II). As before, addition of 0.5 M NaCl prevented the disappearance of the light.

It will therefore be seen that with hypotonic or with freely penetrating non-injurious substances disappearance of the light is undoubtedly coincident with cytolysis. With freely penetrating

TABLE II.

Time of Disappearance of Light in Indicated Concentrations of Urea.

[Urea]	In water	In 0.5 M NaCl
1.0 M.	41 sec.	+8 hrs.
0.8 "	38 "	+8 "
0.6 "	36 "	+8 "
0.4 "	32 "	+8 "
0.2 "	29 "	+8 "
0 "	15 "	+8 "

injurious substances the cell processes may be interfered with and disappearance of the light thus precede cytolysis, or cytolysis may not occur. Since the luminescence of bacteria is intracellular, as has been shown by Harvey[5] and others, any change in intensity of light must be due to changes within the cell. The disappearance of the light indicates cytolysis, anesthesia, the presence of some destructive substance within the cell, or cessation of function because of unfavorable environmental conditions, especially lack of oxygen.

The light may be reversibly extinguished in several ways, as follows: (a) Increase of temperature above a certain critical value, not yet determined for this species, but above 30°. This point has been determined by several investigators for other species.[6,7,8] (b)

[5] Harvey, E. Newton, The nature of animal light, Philadelphia, 1919.

[6] McKenney, R. E. B., *Proc. Biol. Soc. Washington*, 1902, xv, 213.

[7] Harvey, E. Newton, *Biochem. Bull.*, 1913, ii, 456.

[8] Morrison, Thos. F., *J. Gen. Physiol.*, 1925, vii, 741.

Decrease of oxygen pressure to a value less than 0.0053 mm. Hg.[9] (c) Presence of anesthetics in sub-lethal concentrations.[6] (d) By concentrated non-penetrating salt solutions, as by 2.5 M NaCl, the disappearance of the light in this case being due doubtless to plasmolysis.

In all experiments described in this paper concentrated salt solutions have been avoided, presence of ample quantities of oxygen has been assured, and all experiments were conducted at 19 ± 1°C. The disappearance of the light under the conditions of the experiments must therefore be due to osmotic swelling of the cell or to the entrance of injurious substances. When isotonic solutions are tested the disappearance of the light may be used as a physiological criterion of cell penetration whether the failure of the light is due to osmotic swelling or to injury due to the presence of some foreign substance within the cell. The case of internal injury, distinct from osmosis, may be detected by the addition of 0.5 M NaCl to the solutions, thus preventing cytolysis. In any case, disappearance of the light is an indication of cell penetration.

With non-penetrating substances such as strong acids and bases entrance would probably follow destruction of the cell membrane. Disappearance of the light might follow injury to the membrane, and precede entrance of the destructive agent to the cell. Entrance after destruction of the cell membrane can hardly be regarded as penetration, but this case is easily detected by the behavior described in the next section.

III.

Effect of Strong Acids and Alkalies.

The experiments described below indicate that injury due to activity of hydrogen and hydroxyl ions does not take place within the range of pH values employed with the ammonium salts. Therefore any disappearance of luminescence caused by the ammonium salts must be due to osmotic swelling or to intracellular changes due to penetration of the salts, or of their hydrolytic products.

Using the non-penetrating HCl in 0.5 M NaCl, the luminescence persisted for a few seconds even in concentrations as high as 0.1 M

[9] Harvey, E. Newton, and Morrison, Thos. F., *J. Gen. Physiol.*, 1923, vi, 13.

and lasted for progressively longer periods of time as the acid mixture was diluted with 0.5 M NaCl. At pH 4.0 the light lasted for over 5 minutes, at pH 4.67 for 20 minutes, and at pH 5.46 for over 4 hours. The nature of the curve for the shorter times is shown in Fig. 1. Since none of the ammonium salts employed were more acid than pH 5.0 and since the times measured with the ammonium salts were in all instances 5 minutes or less, it is obvious that injury due to activity of hydrogen ions in the external solutions may be excluded from consideration. Similar results were obtained with NaOH, a non-penetrating base. With one series the pH values were calculated on the assumption that NaOH is completely dissociated, and in the other the glass electrode was used, corrections being applied from the measurements of pH values of ammonia in water and in 0.5 M NaCl solution. In these two series of experiments, the pH was determined after the experiment, so the glass electrode determinations are probably more accurate than the calculated values, since some carbon dioxide was absorbed from the air and some produced by the bacteria in the course of the experiment. At pH 9.0, the upper pH value for most of the ammonium salts employed, the bacteria were still luminous after 2 hours, and it was only above pH 10.0 that any immediate effects could be observed. Damage due to activity of hydroxyl ions in the external solution may also be disregarded.

An outstanding peculiarity of the behavior of luminous bacteria was noted in both HCl and NaOH, as compared with the free fatty acids and with ammonia. In 0.025 M solution of any fatty acid in 0.5 M NaCl the disappearance of the light was so rapid that the time could not be measured, while in 0.025 M HCl in 0.5 M NaCl the light persisted fully brilliant for approximately 6 seconds, then failed completely in less than 1 second. In 0.025 M ammonia in 0.5 M NaCl the light was immediately dimmed to a very low value and then lasted for some time before being finally extinguished. In 0.025 M NaOH in 0.5 M NaCl the light lasted at full value for about 4 seconds, then disappeared suddenly. This complete resistance for a certain period of time, followed by sudden complete failure, indicates that the protoplasm is protected from the strong non-penetrating HCl or NaOH until the membrane is destroyed, the destruction of the then unprotected protoplasm following almost immediately. The HCl or NaOH

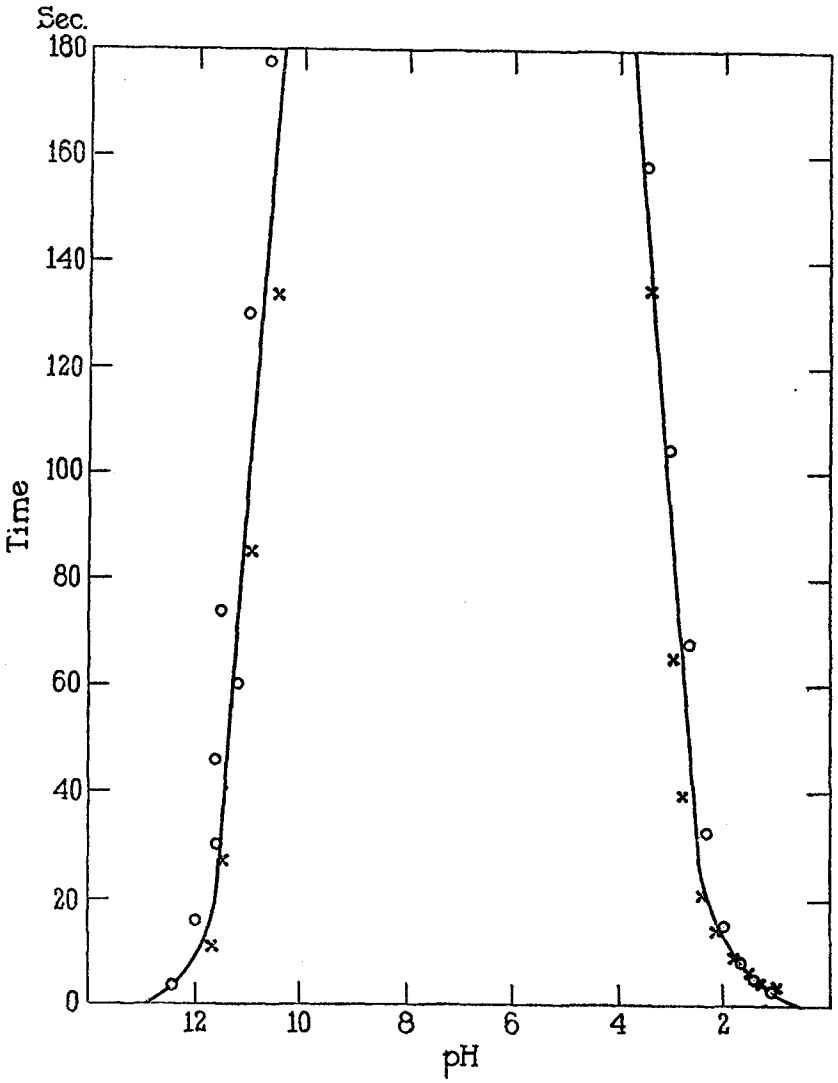

FIG. 1. Time of disappearance of light (in seconds) in solutions containing 0.5 M NaCl plus NaOH or HCl. Each circle is average of 9 experiments, each X of 10. For the circles, pH was determined with the quinhydrone electrode on the acid side, calculated on the alkaline side. For the X's, pH was determined with the glass electrode, with appropriate corrections.

must combine with some substance in the cell membrane, forming a permeable substance, or else simply etch the membrane, before entrance is possible; whereas the ammonia and fatty acid pass freely through without the necessity for first making a path for themselves.

This is similar to the entrance of NaOH into Paramecia stained in neutral red, described by Harvey.[10] In 0.002 M NaOH no change in color is seen for 8 or 10 minutes, when there is a sudden change in color of the entire animal. In 0.001 M ammonia a color change from red to yellow begins at the moment the animals are placed in the solution. A similar behavior was observed for *Elodea* leaves stained in neutral red.

SUMMARY.

It is shown that disappearance of the light of luminous bacteria may be used as a criterion of cell penetration; that luminous bacteria are cytolyzed by water, hypotonic solutions, and by freely penetrating solutions; that luminous bacteria are not injured by hydrogen or hydroxyl ions in the external solutions within the range of pH values employed with the ammonium salts and that therefore disappearance of the light in isotonic solutions of these salts must be due to penetration of the solute; and that there is a characteristic difference between the effects of strong and of weak acids and alkalies on luminous bacteria.

[10] Harvey, E. Newton, *J. Exp. Zool.*, 1911, x, 507.