Stability of Pleuropneumonialike Organisms to Some Physical Factors\textsuperscript{1,2}

P. F. Smith and Shogo Sasaki\textsuperscript{3}

Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

Received for publication October 16, 1957

The increased significance of pleuropneumonialike organisms has spurred many laboratories to engage in programs relating these organisms to both human and animal diseases. This has resulted in the need for mass production of antigens for diagnostic purposes and in the need for maintenance of rather extensive stock culture collections. Problems of handling encountered in such undertakings require a knowledge of the effects of physical factors on the viability of these organisms.

No quantitative information has been published concerning the effects of physical environment on survival of pleuropneumonialike organisms. Several reports have been published showing qualitatively that 50 per cent glycerol is not particularly deleterious to rat strains (Beeuwkes and Collier, 1942; Findlay et al., 1939; Woglom and Warren, 1938a, b), murine strains (Sabin, 1938; Edward, 1940), and porcine strains (Switzer, 1954) and that the organisms vary in their resistance to temperatures above 37 C but survive for weeks at refrigerator temperatures (Sabin, 1938; Switzer, 1954; Bridre and Donatien, 1925; Edward, 1940; Findlay et al., 1939; Warren and Sabin, 1942; Woglom and Warren, 1938a, b). In the course of experimentation on the nutrition and metabolism of these organisms, it became necessary to obtain quantitative information to facilitate the handling of the resting cell suspensions. The study reported here suggests that pleuropneumonialike organisms are more stable to a variety of physical effects than heretofore has been assumed.

Materials and Methods

There were 5 strains selected as representative of different types of pleuropneumonialike organisms: 07, 39, 48, and Campo strains isolated from human beings and J strain from chickens with chronic respiratory disease. The medium employed for the production of resting cells was the broth described by Morton et al. (1951) supplemented with Difco PPLO serum fraction (Smith and Morton, 1951). Resting cells were prepared in the following manner: 10 ml amounts of medium were seeded with agar blocks containing colonies and incubated for 48 hr at 37 C. These cultures served as inoculum for 200 ml amounts of the medium, a 10 per cent inoculum being used. After 48 hr incubation, the organisms were sedimented under sterile conditions at 12,000 rpm in the Servall angle centrifuge\textsuperscript{4} for 5 min, the supernatant decanted, and the tubes drained. Cells were resuspended in different media and the turbidities adjusted to arbitrarily chosen readings in a Klett-Summerson photometer,\textsuperscript{5} using a 420 nm filter. Assays for turbidity were also made in the Klett-Summerson photometer. Quantitative determinations of viable organisms were accomplished by the viable cell count method described by Smith (1956). All dilutions were made in saline.

Sonic oscillation was carried out in a 9 kc Raytheon magneto-constriction oscillator\textsuperscript{6} cooled with circulating ice water. A 20 ml suspension of a given strain was placed in the oscillator and 5 ml samples removed at 10, 20, and 30 min intervals. Turbidity readings and viable cell counts were made immediately and the remainder of the aliquot replaced in the oscillator. Because of difficulties due to variation in the output of the oscillator, all tests on a given strain were performed in one day. Comparison of different strains by turbidity measurement alone in a few different suspending media on the same day was performed to check the validity of comparisons of viable cell recoveries performed on different days.

Freezing and thawing was performed by alternate immersion of suspensions of organisms in a mixture of solid carbon dioxide and ethylene glycol and in warm water. In effect, rapid freezing and thawing occurred. Samples were removed after given numbers of cycles and assayed for viable cells.

Determination of the effect of freeze-drying was accomplished by shell freezing 2 ml amounts of suspensions in which the viable organisms were determined prior to freezing. Organisms were suspended in distilled water and in the liquid culture medium. The or-

\textsuperscript{1} This work supported in part by a contract (Nonr 551(04)) between the Office of Naval Research and the University of Pennsylvania, Philadelphia, Pennsylvania.

\textsuperscript{2} Aided by National Science Foundation grant G-3026.

\textsuperscript{3} Rockefeller Foundation Fellow. Present address: School of Medicine, Keio University, Shinanomachi, Shinjuku-ku, Tokyo, Japan.

\textsuperscript{4} Ivan Sorvall, Inc., New York, New York.

\textsuperscript{5} Klett Manufacturing Co., New York, New York.

\textsuperscript{6} Raytheon Corporation, Waltham, Massachusetts.
organisms were lyophilized and immediately resuspended in 2 ml of distilled water. Following appropriate dilution, plate counts were made.

The effect of osmotic shock was tested by (1) dropping 0.01 ml of a saline suspension containing 10^8 to 10^9 organisms per ml into 10 ml aliquots of distilled water and sucrose solutions with molarities varying from 0.001 to 5.0 and (2) by placing 0.01 ml of organisms suspended in glycerol (0.5 to 0.005 M) and sucrose (2.5 to 0.5 M) into 10 ml distilled water. The turbidity of both suspensions of organisms and solutions was preadjusted to 37°C. Immediately before and following the addition of organisms, the suspensions were mixed and samples removed for viable counts.

Experiments conducted to determine the survival time in different media and at different temperatures were carried out by placing suspensions containing a known number of organisms in a 50°C water bath, in a 37°C incubator, at room temperature, which varied from 22 to 25°C, and in a refrigerator where temperature varied between 3 and 5°C. Samples were removed at time intervals appropriate with length of survival and were used for making viable cell counts.

One group of surface active compounds, the bile acids, cholic, desoxycholic, and lithocholic acids, were tested at two different concentrations in the growth medium.

**Results**

**Effect of sonic oscillation**. Subjection of suspensions of 4 strains containing 10^9 organisms per ml to sonic oscillation for 30 min resulted in approximately a 2 log drop in numbers of survivors or a 99 per cent killing. As can be seen in figure 1, the destruction of the organisms can be fitted to a straight line function when log number of survivors is plotted against time. Strain 39 in all suspending media appeared to possess slightly more resistance to killing than the other 3 strains. No difference in the effect of sonic oscillation could be demonstrated by suspending the organisms in M/15 pH 7.0 phosphate buffer, M/15 pH 7.5 tris(hydroxymethyl)aminomethane buffer, Difco PPLO serum fraction, saline, or distilled water. Typical results of the effect of different suspending media are shown in figure 2 with strain 07. The variation apparent in the figure could be accounted for entirely by variation in the output of the sonic oscillator. The use of acid buffered suspending media resulted in rapid loss of viable cells. However, there was no noted decrease but rather an increase in turbidity. It was found that if such suspensions were adjusted to an alkaline pH after sonic oscillation, the turbidity dropped to values equivalent to suspensions vibrated in alkaline buffers. This result conforms to those obtained for Azotobacter vinelandii and Escherichia coli (Rotman, 1956). In nonacid media considerable reduction of turbidity occurred during sonic oscillation. In a typical case, the Klett readings dropped from 320 to 120 or lower. However, it was found that turbidity measurements could be related to the viable count in sonicates only with readings between 250 and 150 on the Klett photometer. With measurements beyond this range, a straight line function was not obtained. Little change in pH was noted during oscillation. When distilled water was employed as the
Effect of water, distilled water. Generally it was found that oscillation for 20 min resulted in almost complete destruction of the cells.

Effect of freezing and thawing. Table 1 shows the effect of number of cycles of freezing and thawing on survival in different suspending media. In the case of distilled water, a 2 log drop in viable count occurred after 2 cycles, a 4 log drop after 5 cycles and a 6 log drop after 10 cycles. In contrast, after 10 cycles in saline, only a 2 log drop in numbers of survivors was found.

Effect of freezing and thawing. Lyophilization of 5 strains in distilled water and in fresh culture medium was carried out. Only 1 strain, Campo, survived freeze-drying in distilled water, but exhibited a 3 log decrease in numbers of viable organisms. All 5 strains survived lyophilization in the culture medium. The Campo strain showed a 1 log decrease, strains 07 and J a 2 log decrease and strains 39 and 48 almost a 4 log decrease in viable count, starting with approximately $10^{10}$ organisms per ml.

Effect of osmotic shock. Heretofore it has been the supposition that pleuropneumonia-like organisms are very labile to osmotic pressure changes because of the possession of a fragile cell wall. It was of interest to quantitate the effect of osmotic shock on these organisms. By use of sucrose solutions of varying molarity and performance of the tests at constant temperature, relatively constant osmotic properties existed. As shown in table 2, no significant decrease in viable cell count was noted immediately after placing the saline suspensions of organisms in the given solutions. Conversely, preadjusting the organisms to hypertonic solutions of sucrose and glycerol followed by immersion in distilled water likewise had little effect on viable counts. Although prolonged incubation of such suspensions at 37°C resulted in rapid death of the organisms, as described later, additional storage of such suspensions at refrigerator temperatures resulted in no noticeable decrease in viable count. It is apparent that osmotic shock, in itself, was not particularly deleterious to the organisms.

Survival at various temperatures. Preliminary qualitative tests indicated that between 45 and 50°C a sharp demarcation in survival times occurred. Thus at 45°C viable organisms were found after 10 min but at 50°C few survivors were found after 2 min. Quantitative tests on survival at 50°C showed that an exponential decrease in survival occurred. The half life of the organisms at this temperature was 2 min or less with no survival after 7 to 10 min.

Table 3 shows the half-life and maximum survival time at 37°C in various suspending media. Survival was extremely poor in all media except in the culture supernatant or fresh medium. Somewhat better survival was noted in saline or alkaline buffer than in distilled water and acid phosphate buffer. A further examination of survival at 37°C was made in an attempt to elucidate the cause of such rapid death. In the course of examination of the effect of osmotic shock, it was found that sucrose solutions of high molarity permitted survival at 37°C as long as 6 hr in contrast to sucrose solutions of molar or less. As shown in figure 3 an exponential decrease in numbers of viable organisms occurred.

**TABLE 1**

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Survival Time</th>
<th>Strain O7</th>
<th>Strain J</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>×10⁶</td>
<td>2.0</td>
<td>0.9</td>
</tr>
<tr>
<td>2</td>
<td>×10⁶</td>
<td>2.4</td>
<td>0.2</td>
</tr>
<tr>
<td>5</td>
<td>×10⁶</td>
<td>2.3</td>
<td>1.0</td>
</tr>
<tr>
<td>10</td>
<td>×10⁶</td>
<td>2.4</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**TABLE 2**

<table>
<thead>
<tr>
<th>Saline Suspension Added to:</th>
<th>Viable Organisms per ml</th>
<th>Strain O7</th>
<th>Strain J</th>
</tr>
</thead>
<tbody>
<tr>
<td>No shock</td>
<td>×10⁶</td>
<td>2.7</td>
<td>2.9</td>
</tr>
<tr>
<td>Distilled water</td>
<td>2.3</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>0.01 m Sucrose</td>
<td>0.8</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>0.1 m Sucrose</td>
<td>0.9</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>1.0 m Sucrose</td>
<td>2.9</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>5.0 m Sucrose</td>
<td>1.8</td>
<td>2.1</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 3**

<table>
<thead>
<tr>
<th>Suspending Medium</th>
<th>Survival Time</th>
<th>Strain O7</th>
<th>Strain J</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>15 min</td>
<td>60 min</td>
<td>--</td>
</tr>
<tr>
<td>Saline</td>
<td>45 min</td>
<td>120 min</td>
<td>--</td>
</tr>
<tr>
<td>pH 7 m/15 phosphate buffer</td>
<td>35 min</td>
<td>90 min</td>
<td>35 min</td>
</tr>
<tr>
<td>pH 5.6 m/15 phosphate buffer</td>
<td>--</td>
<td>30 min</td>
<td>--</td>
</tr>
<tr>
<td>Ringer’s solution</td>
<td>20 min</td>
<td>60 min</td>
<td>20 min</td>
</tr>
<tr>
<td>Culture supernate</td>
<td>&gt;24 hr</td>
<td>&gt;24 hr</td>
<td>&gt;24 hr</td>
</tr>
</tbody>
</table>
decrease in numbers of survivors occurred over a range of 5 to 2 M sucrose. The protective action of extremely hypertonic solutions of sucrose was found not to be due to viscosity since highly viscous solutions of gelatin, certain gums and gastric mucin were unable to increase survival time. Other explanations for the protective action of hypertonic sucrose could be that it brings about dehydration of the organisms thereby decreasing metabolic activity or that sucrose prevents the inhibitory action of possible toxic metabolic products and anions and cations. The addition of adenosine triphosphate, glutamine, or the lipoprotein growth factor had no effect on survival time, thus ruling out the possibility that the presence of an energy source might increase survival. Treatment of the supernate broth with Norit did not significantly affect survival time. Suspension of the organisms in balanced salt solution (Ringer's solution) likewise was of no value.

Survival at room temperature was found to be slightly longer than at 37 C when the organisms were suspended in pH 7.0 phosphate buffer or saline. For the 2 strains tested, 07 and J, half lives of approximately 2 hr were obtained while maximum survival times did not exceed 6 hr.

Table 4 shows the survival of different strains at 4 C. Considerably better survival was noted in the presence of 50 per cent glycerol. However, the maximum survival time of the organisms in alkaline phosphate buffer in most instances was equivalent to that in glycerol. Qualitative estimation of survival in distilled water and saline at 4 C indicated that survival in saline was equivalent to that in pH 6 phosphate buffer, while in distilled water a 4 log drop occurred in 3 days. The one poultry strain tested, J, was found to be considerably less stable than the 3 human strains.

**Effect of surface tension.** A detailed study of the effect of surface tension on pleuropneumonialike organisms was not undertaken. However, it is felt that it would be noteworthy to describe the effect of bile acids, the results of which were obtained during a study of the sterol growth requirement of these organisms. Three bile acids were tested in the complete medium devoid of sterols. These bile acids differ from one another in that cholic acid contains 3 hydroxyl groups on its nucleus, deoxycholic contains 2 and lithocholic contains 1. When added to the medium at 2 final concentrations, 3.0 × 10^{-4} M and 1.5 × 10^{-4} M, no growth was observed but a decrease in viable count proportional to the number of hydroxyl groups occurred. Thus, with lithocholic acid the poorest survival was noted, while with cholic acid no decrease in viable count occurred in 4 days at 37 C. Since decrease in the number of polar groups results in decreased surface tension, the effect might be the result of surface tension depression. The deleterious action of surface tension depressants on these organisms has been noted by the lethal effect of soap (Keller et al., 1952).

**TABLE 4**

<table>
<thead>
<tr>
<th>Suspending Medium</th>
<th>Strain Campo</th>
<th>Strain 39</th>
<th>Strain 07</th>
<th>Strain J</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 6 phosphate buffer</td>
<td>7*</td>
<td>&gt;28</td>
<td>4</td>
<td>21</td>
</tr>
<tr>
<td>pH 7 phosphate buffer</td>
<td>7</td>
<td>&gt;28</td>
<td>9</td>
<td>&gt;28</td>
</tr>
<tr>
<td>pH 8 phosphate buffer</td>
<td>6</td>
<td>&gt;28</td>
<td>4</td>
<td>21</td>
</tr>
<tr>
<td>50% glycerol-saline</td>
<td>17</td>
<td>&gt;39</td>
<td>20</td>
<td>&gt;39</td>
</tr>
<tr>
<td>50% glycerol-broth</td>
<td>9</td>
<td>&gt;39</td>
<td>19</td>
<td>&gt;39</td>
</tr>
<tr>
<td>Broth layered with</td>
<td>12</td>
<td>&gt;39</td>
<td>6</td>
<td>&gt;39</td>
</tr>
<tr>
<td>mineral oil</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Time in days.

**DISCUSSION**

A detailed analysis of the effects of different physical factors under a great many varied conditions was not undertaken since the primary aim of this study was to determine the effects of various methods of handling resting cell suspensions. In general, none of the common handling methods for microorganisms can be considered greatly deleterious to the viability of the pleuropneumonialike organisms. The stability of the organisms in
a cool, nonacid environment to alternate freezing and thawing and to osmotic shock was found to be considerably better than has been assumed in the past. Some variation was noted among different strains, especially the poultry strain, J, which is considerably more labile to storage than the human strains tested.

The most surprising result was the extreme stability of pleuropneumonia-like organisms to osmotic shock. In no instance was there a considerable reduction in number of viable cells immediately following osmotic shock. This finding is contradictory to the effects observed in preparing specimens for electronmicroscopy (Smith et al., 1948; Morton et al., 1954). An explanation for this resistance to lysis by osmotic shock could be that the pliable nature of the limiting cell membrane of these organisms permits considerable distortion, thus preventing rupture of the cells. The difference from species of Azotobacter (Robrish and Marr, 1957) of the effects of osmotic shock may be due to a greater permeability of pleuropneumonia-like organisms to glycerol.

Pleuropneumonia-like organisms were found to be relatively more labile to temperatures above 4 C when removed from their growth environment than bacteria (Winslow and Falk, 1923). An increase of survival at these temperatures could be obtained by suspending the organisms in hypertonic sucrose not by addition of certain metabolites, control of pH, and nature and concentration of anions and cations.

With regard to the other physical factors tested, pleuropneumonia-like organisms do not differ considerably from bacteria (Porter, 1947; Mitchell, 1951). The effect of the bile acids examined conforms to the results with bacteria with which there was found to exist a simple relationship between the bacteriostatic activities of closely related bile acids and their ability to depress surface tension of the medium (Stacey and Webb, 1947).

The resistance of pleuropneumonia-like organisms to certain physical effects parallels their greater resistance than bacteria to chemical agents. These findings further support the contention that pleuropneumonia-like organisms are resistant forms having arisen phylogenetically from bacteria as a result of a deleterious environment. The "unstable" and "stable" L forms can be considered intermediate forms in the transition of a bacterium to a pleuropneumonia-like organism. The process involved in such a transition might involve first adaptive changes not fixed genetically, as in L forms, followed by genetic mutations by which the characters become permanently fixed (Mudd, 1955).

**SUMMARY**

Pleuropneumonia-like organisms were found to be more resistant to adverse physical environment than previously has been assumed. Four strains of human and poultry origin exhibited 99 per cent lysis after sonic oscillation for 20 min. No significant difference occurred among different strains or with different suspending media. Alternate freezing and thawing resulted in considerable destruction of the organisms in distilled water but not in saline. Pleuropneumonia-like organisms survive lyophilization in the culture medium but not in distilled water. No loss in viable organisms was noted after subjection to osmotic shock. The organisms survive only a few hours at temperatures above 4 C when suspended in solutions other than the culture medium. Survival at refrigerator temperatures extends over many weeks. A preliminary examination of the reason for poor survival at temperatures above 4 C did not reveal the cause. The organisms were very labile in suspending media acid in nature, regardless of temperature. The deleterious action of bile acids may be related to reduction of surface tension of the medium.

**REFERENCES**

Beeuwkes, H. and Collier, W. A. 1942 Studies on arthropo-
tropic pleuropneumonia-like microorganisms. J. Infec-
tious Diseases, 70, 1-6.

Brière, J. and Donatten, A. 1925 Le microbe de l'agalaxie-
contagieuse du mouton et de la chèvre. Ann. inst. Pas-
teur, 39, 925-951.

Edward, D. G. 1940 The occurrence in normal mice of
pleuropneumonia-like organisms capable of producing

Findlay, G. M., Mackenzie, R. D., MacCallum, F. O., and
Kliewerberger, E. 1939 The etiology of polyarthritis in
the rat. Lancet, 239, 7-10.

Keller, R. T., Smith, P. F., and Morton, H. E. 1952 Suscep-
tibility of pleuropneumonia-like organisms from human
7, 313-319.

Mitchell, P. 1951 Physical factors affecting growth and
death. In *Bacterial physiology*, p. 126. Edited by C. W.
York, New York.

Investigation of the cultivation of pleuropneumonia-like
organisms from humans. Am. J. Syphilis, Gonorrhea,
Veneral Diseases, 35, 361-369.

1954 Electron microscope studies of pleuropneumonia-like
68, 607-717.


Robrish, S. A. and Marr, A. G. 1957 Osmotic disruption of

Rotman, B. 1956 On the mechanism of sonic lysis of bac-

Sabin, A. B. 1938 Isolation of a filtrable, transmissible agent
with "neurolytic" properties from toxoplasma-infected

Smith, P. F. 1956 Quantitative measurement of the growth
of pleuropneumonia-like organisms. Appl. Microbiol., 4,
284-299.

Smith, P. F. and Morton, H. E. 1951 The separation and
characterization of the growth factor in serum and ascitic
fluid which is required by certain pleuropneumonia-like
Isolation of Salmonellae from Food Samples

I. Factors Affecting the Choice of Media for the Detection and Enumeration of Salmonella

W. I. Taylor, J. H. Silliker, and H. P. Andrews

Research Laboratories, Swift & Company, Chicago, Illinois

Received for publication October 29, 1957

At a recent symposium on the problems of detection and enumeration of Salmonella in foods (Dack, 1955), shortcomings of the methodologies in current use were discussed by both food analysts and public health microbiologists. It has become increasingly apparent to the food microbiologist, who finds himself handicapped by inadequate methods, that the analytical problem of salmonellae in foods possesses certain peculiarities not commonly encountered in the clinical laboratory. First, the organisms have often been subjected to physiologically debilitating processes such as freezing, desiccation, curing ingredients, and extremes of pH, heat, and osmotic pressures during the manufacture or storage of the product. Second, salmonellae in foods usually comprise an exceedingly small component of the total microbial population, and in addition, are almost invariably outnumbered by physiologically similar coliform bacteria. Examination of the methods used for the enumeration of the salmonellae and the problems posed evade the conclusion that the food bacteriologist has appropriated in toto the media and methods of the clinical laboratory in an attempt to solve problems for which they were not designed, with little consideration being given to their applicability. The results have been unsatisfactory.

The paucity of Salmonella prevents their enumeration by direct plating methods. The most probable number (MPN) technique (Hoskins, 1934) is used routinely for that purpose and involves quantitative inoculation of enrichment broth with a food sample with subsequent identification of salmonellae on a differential medium. Improvements in the enrichment broth are, therefore, of primary importance in this schema. Selenite F enrichment broth has been established as the medium of choice over tetrathionate broth. This does not preclude the possibility that there are changes in formulae which would enhance the ability of selenite F to detect salmonellae. Similarly, evaluation of the abilities of differential and selective media to facilitate recovery of Salmonella sp., specifically, completes the selection of the tools with which to continue the examination of changes in methodology. The authors intend to present in this and subsequent reports the results of some of the investigations which examine factors affecting the choice of media, and to discuss changes in methodology which have proved efficacious in detection of Salmonella sp. and their enumeration from foods.

Materials and Methods

Selenite F medium (Leifson, 1936) modified by the addition of cystine (North and Bartram, 1953) was used as the control for experiments conducted on enrichment media. Dulcitol or mannitol were substituted for lactose in the appropriate formulae tested.

Selenite brilliant green sulfapyridine medium (SBS) was prepared according to directions supplied by Dr. Stokes; when it became available commercially, the dehydrated medium was used (Osborne and Stokes, 1955).

Metabolite solutions (“metsol”) (Heinmets et al., 1954) consisted of the following metabolites in 0.2 per