SURVIVOR CURVES OF BACTERIA EXPOSED TO SURFACE-ACTIVE AGENTS

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Reports on bacterial death studies since the basic work of Krönig and Paul (1896) have generally recognized that the death process of bacteria exposed to unfavorable environmental conditions follows a uniform and consistent course. The constancy of the rate of death is the outstanding feature; the term, "constant death rate," means that the number of bacteria that die per time unit is a constant percentage of the number of living organisms at the beginning of this time unit (Rahn, 1945). The order of death of organisms may be determined by computation of the value of the death rate constant, K, in the formula:

\[ K = \frac{\log b - \log B}{t} \]

where \( b \) equals the number of organisms at the beginning of the time unit; \( B \) equals the number of organisms at the end of the time unit; and \( t \) equals the time unit, i.e., the period of exposure. If the order of death is logarithmic, as has generally been conceded to be true in bacterial death, the value of \( K \) remains relatively constant throughout the exposure period. Although differences in opinion as to the interpretation to be attached to the form of the survivor curve exist, most workers have assumed that the phenomenon was due to fundamental chemical and physical factors involving some basic process analogous to a monomolecular reaction (Madsen and Nyman, 1907; Chick, 1908; Lee and Gilbert, 1918; Watkins and Winslow, 1932; Rahn, 1945).

In the course of studies on the bactericidal properties of the quaternary ammonium compounds, the observation of discordant results with phenol coefficient methods led the authors to employ a plate count, survivor curve method of germicidal evaluation. Aberrant disinfection velocity curves were reported by one of the authors (McCulloch, 1947) for various quaternary ammonium compounds. The theory was advanced that these cationic agents tend to promote clumping or agglomeration of the exposed test organisms, reducing the plate count in earlier minutes of exposure without necessarily reducing the number of viable organisms. The clumping of treated organisms, with their adherence to glass surfaces, had been observed experimentally (McCulloch and Migaki, 1947).

The following studies were conducted to investigate some of the factors other than a clumping phenomenon which might appreciably influence the death rate

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1 Scientific paper No. 846, Agricultural Experiment Station, Institute of Agricultural Sciences, State College of Washington.

2 Died, December 1, 1948.
of organisms exposed to quaternary ammonium compounds and be responsible for the observed atypical type of survivor curve. Factors considered for this report were the possible presence of unusual variations in resistance among individual cells exposed, the effect of the age of exposed cells on their relative resistance, and the possible exhaustion of active free detergent in the exposure test suspension.

EXPERIMENTAL DATA

Determination of Survivor Curve Patterns of Organisms Exposed to Cationic, Anionic, and Nonionic Surface-active Agents

Materials. Laboratory cultures of Micrococcus pyogenes var. aureus, Escherichia coli, Pseudomonas aeruginosa, and spores of Bacillus subtilis were used. The representative detergents selected were classified in three categories: cationic—"hyamine 1622," "rococal," "emulsept," "teramine"; anionic—"dref," "tide," "santomerse 3," "syntex A," "cronite D-40"; nonionic—"S.T. 37," "triton 100," "triton X50." The Food and Drug Administration broth medium (Reuhl and Brewer) was used to culture the test organisms. The medium used for plating was phenol red agar base (Difco) to which had been added 1.0 per cent glucose. In experiments involving either P. aeruginosa or B. subtilis, a film of 2.0 per cent agar was poured over the solidified medium in the plates to prevent surface growth of spreaders. Standard 99-ml milk dilution bottles containing sterile distilled water were used routinely. The first dilutions of a bacteria-detergent mixture were always made in blanks containing 10 per cent sterile evaporated milk, since it was found that this concentration of milk was able to provide extensive surface for competitive absorption and to neutralize the action of the surface-active agent against the organisms.

Test procedure. (1) An approximately 24-hour culture of the test organism, which had been transferred on 15 consecutive days prior to the performance of the tests, was shaken thoroughly and filtered through cotton. (2) Fresh stock solutions of surface-active agents were prepared in sterile water in volumetric flasks. (3) Ten ml of filtered culture were added to 100 ml of surface-active agent solution held in a constant temperature water bath. The mixture was immediately shaken and agitation 25 times and replaced in the water bath for the exposure period. (4) Samples of a bacteria-detergent mixture, usually of 1.0 ml, were withdrawn at given time intervals for dilution and plating. Triplicate plates were generally poured for each dilution. (5) The plates were incubated at 37 C and read after 24 hours and 48 hours. The number of survivors was determined by counting plates showing well-distributed colonies in the range of 30 to 300, taking the average, and multiplying it by the appropriate dilution factor. (6) Controls using 10 ml of bacterial culture and 100 ml of sterile distilled water were run simultaneously with all tests to establish the initial number of organisms employed in each experiment and to determine possible germicidal action of suspending waters or temperatures.

Results. The data presented in table 1 and figures 1 and 2 are typical of the results obtained using all of the agents mentioned against the given test cultures.
### Table 1

*Micrococcus pyogenes aureus exposed to 1:100,000 hyamine at 30°C*

<table>
<thead>
<tr>
<th>TIME</th>
<th>PLATE COUNT NUMBERS</th>
<th>LOGARITHMS OF PLATE COUNTS</th>
<th>K*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before exposure:</td>
<td>39,000,000</td>
<td>7.5911</td>
<td></td>
</tr>
<tr>
<td>After exposure:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 min</td>
<td>2,520,000</td>
<td>6.4014</td>
<td>1.1897</td>
</tr>
<tr>
<td>2 min</td>
<td>660,000</td>
<td>5.8195</td>
<td>0.5819</td>
</tr>
<tr>
<td>5 min</td>
<td>320,000</td>
<td>5.5051</td>
<td>0.1048</td>
</tr>
<tr>
<td>10 min</td>
<td>125,000</td>
<td>5.0969</td>
<td>0.0816</td>
</tr>
<tr>
<td>20 min</td>
<td>36,500</td>
<td>4.5823</td>
<td>0.0535</td>
</tr>
<tr>
<td>30 min</td>
<td>12,900</td>
<td>4.1108</td>
<td>0.0451</td>
</tr>
<tr>
<td>60 min</td>
<td>5,620</td>
<td>3.7497</td>
<td>0.0120</td>
</tr>
<tr>
<td>120 min</td>
<td>1,570</td>
<td>3.1959</td>
<td>0.0092</td>
</tr>
</tbody>
</table>

*K* = \( \frac{1}{t} \log \frac{\text{Initial number of organisms exposed}}{\text{Number of survivors after exposure}, (t)} \)

*Figure 1.* Survivor curves of *Escherichia coli*, *Micrococcus pyogenes* var. *aureus*, and *Pseudomonas aeruginosa* exposed to teramine at 21°C.
It is of particular interest to note that the value of the disinfection velocity constant was far from constant in any of the trials. When the same procedure was applied and phenol was used as the disinfectant against the test organisms, the survivor curves obtained were of a fairly constant, straight-line type. The data in table 1 showed the rate of bacterial reduction to be extremely rapid during the first 2 minutes, followed by a slower rate of kill which continued until the end of the tenth minute. Between the tenth and twentieth minutes the rate of kill again declined appreciably, which fact might indicate that the bacterial population was comprised of components of different degrees of resistance.

The determination of the survivor curve pattern presented by resistance spores of *Bacillus subtilis* exposed to cationic and anionic detergents revealed results of interest and possible significance. A laboratory strain of *Bacillus subtilis* was cultured on the surface of nutrient agar in a Blake bottle and allowed to incubate for a week. The growth was harvested with sterile water and shaken vigorously, and the vegetative cells were destroyed by being heated at 60 C for 30 minutes. The suspension was then poured through sterile, fine, dry silica sand; the sand was desiccated and the material stored in sterile bottles at 4 to 8 C. The spore count of this sand material was found to remain constant during the months the trials were conducted, and there was no change in the phenol resistance of the spores. Shaken with 100 ml of sterile water, 1.8 grams of the sand gave a count of 20 to 30,000,000 spores per ml. Ten ml of such a spore suspension were added to 100 ml of detergent solution in the tests.

The results are shown in figure 3. It is interesting to note the marked initial reduction in plate count of the exposed spores followed by negligible reduction. It is highly improbable that this observed initial reduction actually represented death of the spores to the extent indicated by the plate count numbers.

**The Possible Presence of Unusual Variations in Resistance among Individual Cells**

Past observations of abrupt breaks in survivor curve patterns of bacteria have been attributed to the presence of unusual variations in resistance of the
test organisms or to the effect of the age of cells upon relative resistance. Unusually resistant variants appearing at some stage of development of the culture could produce a decreasing value of $K$ and a survivor curve such as is shown in this report. Experiments were performed to determine whether subcultures of the survivors of different exposure periods differed appreciably in resistance from one another and from the original culture.

**Test procedure.** Colonies on the survivor plates of exposure tests were picked off and inoculated into tubes of F.D.A. broth. Approximately 24-hour cultures of these substrains were compared with the original culture for resistance to the surface-active agents. The procedure and conditions of the original tests were duplicated.

In a second series of experiments, cultures of *Escherichia coli* were exposed to sufficient quaternary ammonium compound to reduce the viable colony count by at least one million times. The survivors were inoculated into tubes of F.D.A. broth and, following incubation, were also exposed to a given concentration of quaternary ammonium compound. Exposure tests were repeated on these cultures on 6 successive days.

**Results.** The results obtained in the series gave no indication that the survivors of such exposures were more or less resistant to the surface-active agents than their parent strains. The substrains displayed the same type of survivor curve and similar degrees of resistance.

**The Possible Effect of the Age of Cells on Relative Resistance**

As previously mentioned, the age of the cells exposed to a disinfecting agent has been suggested as one factor responsible for a nonlogarithmic order of death. It is generally accepted that young cells are more sensitive to unfavorable environments than are older cells. Were the survivors curves obtained in this study due to the presence in the test culture of a small number of old resistant cells that had perhaps remained in a resting stage when transferred to a new medium, it might follow that a young culture, transferred successfully for several times at short intervals, would eliminate such old cells. The survivor curve obtained using such a culture would follow the usual constant trend.
**Test procedure.** A culture of *Escherichia coli* was successively transferred for 5 times at 4-hour intervals. With the procedure outlined above, the final subculture was tested at various ages against a 1:30,000 hyamine 1622 solution.

**Results.** The results are presented in figure 4. Although the resistance of the young cultures is less than that of the older cultures, the trends are similar, and in all instances they depart from the straight-line graph. It does not seem reasonable to attribute the phenomenon of the atypical survivor curve presented by organisms exposed to surface-active agents to the effect of age upon the relative resistance of the cells.

**The Possible Exhaustion of Active Detergent in the Test Suspension**

Another explanation proposed for the asymptotic curves was that of an exhaustion of the active detergent molecules in a bacteria-detergent mixture. The
Effectively destructive concentration of the surface-active agent may have become diminished so rapidly during the first few minutes of the exposure period that, in later minutes of exposure, there was insufficient detergent present in the solution proper to reduce the bacterial population as rapidly or as efficiently. Molecules of surface-active agents, because of their very nature, tend to concentrate themselves at interfaces and on surfaces; the fact that cationic germicides are readily adsorbed on glass surfaces and can be readily removed from solution has been demonstrated (Miller et al., 1943; Weber and Black, 1948).

Test procedure. To determine whether such a factor was responsible for the constantly decreasing death rate observed, the following procedure was devised: To 100 ml of detergent solution, 5 ml of a filtered 24-hour bacterial culture were added at each of 4 successive 20-minute intervals. The mixture was thoroughly shaken after each addition of culture, and 1.0-ml samples were removed for dilution and plating at 1-, 2-, 5-, 10-, and 20-minute intervals following each culture addition. A control run using the standard proportions of 10 ml of culture to 100 ml detergent was also made.

Results. Figure 5 shows the results obtained using oronite D-40 against Micrococcus pyogenes var. aureus. Following each culture addition, there was more than a 2.5 log drop in the survivor count at the end of the first minute of exposure. Actual exhaustion of the detergent is represented only by the very slight differences in the total plate count drop. The data seem to indicate conclusively that the concentration of free and active detergent in an exposure solution in which the proportions of bacterial culture to detergent solution are 1 to 10 is more than sufficient to exert its maximum action throughout the entire exposure period.

In a number of plate count studies it was noted that the counts of serial dilutions plated from a given sample of bacteria-detergent mixture were significantly
inconsistent, with the low dilution plates giving lower survivor counts per ml than the higher dilution plates. For example, a $10^{-3}$ plate would reveal 200 colonies or 200,000 survivors per ml, but a $10^{-4}$ plate would reveal 30 to 50 or 300,000 to 500,000 survivors per ml. To determine whether such discrepancies were due to error in the dilution technique itself, experiments were conducted substituting phenol and chlorine as the disinfectants. Plate counts of serial dilutions followed the normal pattern. Such results indicated that the process of dilution, which is necessarily accompanied by vigorous shaking and agitation, was responsible for the release of additional viable cells.

**DISCUSSION OF RESULTS**

The results obtained in the foregoing experiments indicate that the observed atypical survivor curves of organisms exposed to surface-active agents cannot be adequately explained on the basis of (1) the presence or development of unusually resistant variants among the exposed organisms; (2) the effect of the age of cells upon relative resistance; (3) the exhaustion of active detergent in the exposure test suspension.

Evidence presented seems definitely to eliminate the latter two factors. The first of these factors cannot perhaps be conclusively discounted. However, the following observations do not substantiate the explanation that the cultures tested were comprised of growth phases or substrains possessing an unusual distribution of resistance against the surface-active agents: (1) The same survivor curve trend was observed in all trials with all types of cationic and anionic detergents and against all the bacterial cultures tested. (2) Parallel tests using phenol gave reasonably uniform values of the death rate constant, $K$, indicating a normal resistance distribution against phenol. (3) The same general pattern was observed with young and old cultures. (4) Organisms surviving exposure to the detergents did not give rise to substrains more resistant than the original culture.

The data presented seem to indicate that the observed survivor curves do not represent a true index of the death rate. The surface-active agents appear to effect an agglomeration of the exposed organisms. The observation that the process of dilution and the shaking accompanying it resulted in a release of greater numbers of viable cells and, consequently, in higher plate count readings is evidence in favor of this belief. Organisms exposed to cationic agents are known to undergo alteration of their surfaces and their surface electrical charge (Dyar and Ordal, 1946; Kivella et al., 1948). A rapid initial agglomeration, a clumping of exposed cells, or the formation of "bacteria-detergent complexes" could be anticipated as a result of such alteration.

With plate count methods, which give the number of colonies developing from a known volume of bacterial suspension, the colonies appearing on the plate represent the actual number of original bacteria only when such organisms are single units. If the cells are clumped together, one colony may easily represent a number of cells. In such a case, death would become evident only after the last of the cells within the clump had been killed; the plate counts would reveal the many viable cells within an agglomerate as a single unit, since the bound
cells would give rise to but a single colony. In the later intervals of the exposure period, however, when the rate of decrease appears to be extremely slow as determined by plate count readings, the actual destruction of organisms may be assumed to be taking place at a rate more rapid than was indicated. The survivor curves of bacterial cultures exposed to a surface-active agent seem, therefore, to represent not only chemical disinfection action, but also physical phenomena that are characteristic of these compounds.

SUMMARY

The factors involved in the production, using plate count methods, of the unusual survivor curves observed with bacterial populations exposed to various surface-active agents have been investigated. It is postulated that the survivor curves observed in the studies are a resultant, not only of direct chemical disinfection, but of certain physical effects exerted by these compounds and do not provide a true index to the rate of death.

REFERENCES

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