STUDIES ON THE LIFE AND DEATH OF BACTERIA

I. The Senescent Phase in Aging Cultures and the Probable Mechanisms Involved

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The phenomenon of growth and death of bacteria in ordinary culture media has been studied in considerable detail. The data obtained in such studies show that growth follows a definite course, represented frequently by a curve constructed by plotting the logarithms of the numbers of organisms against time. This logarithmic growth curve has been divided into four main parts: the lag, logarithmic growth, maximum stationary, and death phases. After reaching the maximum stationary phase, the curve is frequently shown tapering off until it meets the abscissa, indicating sterility of the culture in a few days or weeks. However, a few of the more recent writers (Gay, 1936; Topley and Wilson, 1936) have made suppositions to the contrary. Gay states: "It is very probable that many cells may be unable to multiply but are still able to carry on other metabolic functions." He further suggests that "The number of viable organisms may also show repeated irregular increases possibly indicating spurts in multiplication. The long period of slow decline has not been studied as intensively as it probably deserves."

During the course of our investigations on factors affecting bacterial growth, certain observations suggested that a closer quantitative examination of death and death rates in bacterial cultures might be desirable. The period of prolonged survival and the probable mechanisms involved proved to be of particular interest.

I. THE SENESCENT PHASE

Methods

Sarcina lutea and *Serratia marcescens* were grown in flasks containing 1000 ml. of ordinary nutrient broth. The flasks were incubated at room temperature (approximately 24°C.), stoppered with cotton, and capped with lead foil to prevent excessive evaporation. The volume of the medium was kept constant by the periodic addition of sterile, double-distilled water. For a period of two years, duplicate plate counts of the number of cultivable bacteria were made at various intervals. During the 13th month of incubation, counts were made every other day for three weeks to determine any minor changes or fluctuations in the number of cultivable bacteria during this period.

Cultural characteristics

During the prolonged period of incubation the reaction of the aging cultures of *S. lutea* and *S. marcescens* became alkaline, reaching pH 8.49 and 8.12, respectively, as measured by the glass electrode. This is striking, especially in the case of *S. marcescens* whose limit of reaction is usually considered to be pH 8.0. When the reaction of one-year old *S. marcescens* cultures was adjusted to pH 7.0, the count rose in two weeks from 7,500,000 to slightly over a billion cells per ml., thus slowly approximating the number of cells found during the maximum stationary phase.

Microscopic examination of the aging cultures showed intact cells, a considerable amount of debris, and "ghost" forms. The cells were typical in shape and grouping and gave characteristic reactions to the gram stain. As is usually the case with dead cells, most of the cells of *S. lutea* stained gram-negative. The physiologic reactions of transplants of these cultures in litmus milk, gelatin, citrate medium, and in tryptophane, nitrate, and sugar broths remained constant. In the case of *S. marcescens*, there was definite evidence of variation when the cultures were plated out. The colonies ranged in color from white to uniformly deep red, and in morphology from the tiny, glistening to the spreader type.

That there are differences in resistance to adverse environmental conditions of cultures 3 to 6 hours old as compared with those 1 to 7 days old has been shown by Sherman and Albus (1923). Hence it was thought desirable to determine roughly whether or not such a phenomenon had occurred in these aging cultures.

The methods of these investigators were followed in subjecting the two-year old cultures of *S. lutea* and *S. marcescens* to $HgCl_2$, phenol, and high temperatures. The action of $HgCl_2$ and of phenol was tested by allowing the organisms to remain in contact with the chemical for definite time intervals after which one loopful of this suspension was transferred to a tube of nutrient broth. If no growth occurred in the broth within 48 hours, it was concluded that the organisms had been killed by the chemical agent. Young eight-hour old cultures of these organisms were run as controls.

The results, recorded in table 1, accord in general with those reported by Sherman and Albus in that they indicate physiologic differences between young and old bacterial cells. A repetition of the experiments gave similar results.

Sherman and Albus also found that mature cells may be agglutinated by acid whereas young cells are not. Several rough determinations of this kind were made on the aging cultures of *S. marcescens* and *S. lutea*, but no differences were noted between the agglutinability of the two-year old cultures and those 8 hours old.

Population studies of aging cultures

Plate counts of these aging cultures showed the number of cultivable organisms to be remarkably high. Counts of from 5,000,000 to 30,000,000 bacteria per ml. were obtained during the thirteenth month of incubation. At the end of the two-year period the plate count of *S. lutea* was 1,000,000 bacteria per ml., while that of *S. marcescens* was 1,700,000 per ml.

Plate counts of the number of cultivable cells of *S. lutea* and *S. marcescens* plotted against time are shown logarithmically in figures 1 and 2. These two curves represent the numbers of cultivable bacteria in nutrient broth cultures maintained over a

period of two years. After an early initial drop which occurs during the first few days, the curves tend to level off and maintain an almost imperceptible decline. This gradual decline, however, is probably not one of total inactivity. Frequent counts, made

TABLE 1

Effect of adverse conditions

TIME	SERRATIA MARCESCENS		SARCINA LUTEA	
	2 years	8 hours	2 years	8 hours
	Exposure to 1 per cent phenol			
minutes				
0	+	+	+	+
1	+	+	+	+
2	+	+	+	−
5	+	−	+	−
10	−	−	+	−
15	−	−	−	−
20	−	−	−	−
	Exposure to $HgCl_2$ (1:12,000)			
0	+	+	+	+
1	+	+	+	+
2	+	−	+	+
5	−	−	+	+
15	−	−	+	+
30	−	−	+	+
45	−	−	+	+
60	−	−	+	+
120	−	−	−	−
	Exposure to 55°C.			
	Cells per ml.	Cells per ml.	Cells per ml.	Cells per ml.
0	1,700,000	8,000,000	1,000,000	950,000
5	90,000	1,000	980,000	15,000
15	30	0	400,000	75
30	0	0	80,000	10
45	0	0	600	0
60	0	0	0	0

during the twelfth and thirteenth months, revealed small rises and falls in the curve, thus indicating some evidence of actual multiplication and spurts of increase during the period. We have termed this period the "senescent phase."

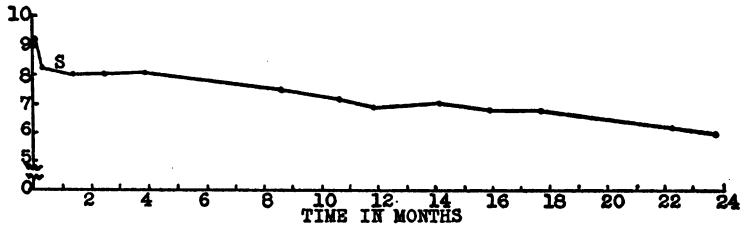

FIG. 1. GRAPH OF THE LOGARITHMS OF THE NUMBERS OF S. LUTEA DURING TWO YEARS OF INCUBATION

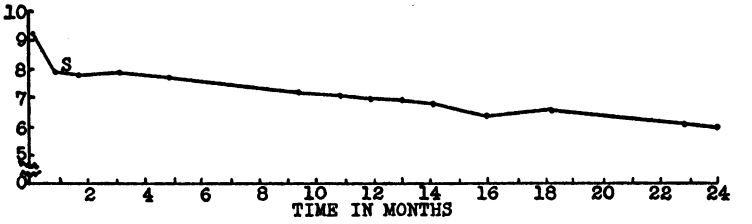

FIG. 2. GRAPH OF THE LOGARITHMS OF THE NUMBERS OF S. MARCESCENS DURING TWO YEARS OF INCUBATION

"*S*" marks the beginning of the senescent phase. The early phases of the typical growth curve do not appear because of the time scale used.

TABLE 2

Representative counts of the numbers of bacteria per ml. in aging cultures

BACTERIUM	AVERAGE NUMBER OF BACTERIA PER ML.			AVERAGE REACTION OF CULTURE AT END OF ONE YEAR
	Immediately after inoculation	After 48 hours incubation	After one year incubation	
				pH
Sarcina lutea	6000	1,000,000,000	5,200,000	8.60
Serratia marcescens	8000	2,000,000,000	4,800,000	8.78
Bacillus subtilis	4000	10,000,000	300,000	8.92
Staphylococcus aureus	7000	1,800,000,000	1,300,000	8.62
*Staphylococcus aureus**	6000	2,000,000,000	750,000	8.61
Aerobacter aerogenes	5000	1,100,000,000	1,600,000	8.75
Escherichia coli	9000	1,700,000,000	300,000	8.91
Klebsiella pneumoniae	6000	2,500,000,000	775,000	8.81
Pseudomonas aeruginosa	4000	1,400,000,000	1,100,000	8.87
Rhodococcus rhodochrous	1000	700,000	430,000	7.86
Micrococcus tetragena	2000	300,000,000	2,100,000	8.83

* A non-proteolytic strain.

Table 2 gives data showing the partial results obtained in similar experiments using other bacteria for a period of one year. For the most part, the counts recorded represent an average of two to four separate cultures of each organism. The reaction of each culture at the end of this period is also indicated. Briefly stated, the results were similar to those obtained with the aging cultures of *S. lutea* and *S. marcescens*. In each case, after the early initial drop, the curve tends to level off in a manner similar to that already described for *S. lutea* and *S. marcescens*. In general, when the aging cultures were plated out, the resulting growth was very slow, thus requiring incubation periods longer than is ordinarily necessary for these species. The number of bacteria at the end of the one-year period is given in the table because in all cases this was the lowest number of bacteria per ml. at any time during the 12-months period.

II. STUDIES ON THE PROBABLE MECHANISMS INVOLVED

Speculation as to why bacterial populations are maintained over a long period of time brought up the question of whether a bacterial species could utilize cells of the same or of other species as a source of nutriment. It was not thought that this would be the sole explanation for the continued growth in the aging cultures, but it seemed desirable to know to what extent it might play a part. Consequently, experiments were conducted to test whether bacteria could utilize other bacterial cells as the only source of food.

Methods

For this purpose, pure cultures were grown on nutrient agar in 1000 ml. Blake bottles. The cells were harvested and then washed three or four times by suspending them in physiologic saline and centrifuging. An estimated 1 per cent suspension of moist cells in 1.5 per cent washed granulated agar was adjusted to pH 7.0 and autoclaved for 20 to 30 minutes. The washed cells plus washed granulated agar constituted the test medium, the only source of nutrients being the bacterial cells incorporated in the agar. Plates of this medium were then

poured in the ordinary manner and streaked with the various test organisms. Washed agar alone and ordinary nutrient agar were used as controls. Forty-eight hours after inoculation, the growth on the cell-agar medium was compared with the growth on the nutrient agar plates.

The cell-agar media used were made of the whole cells of *S. marcescens, S. lutea, Staphylococcus aureus,* and the intact as well as the disrupted cells of *Escherichia coli.* The disruption of the cells was brought about by repeated freezing and thawing of the bacterial suspension.

Results

In general, as can be seen from table 3, the organisms that are proteolytic in action grew best on the cell-agar medium. Out of twenty-one species of organisms tested, best growth was obtained with *S. lutea, S. marcescens, Bacillus subtilis, Bacillus mycoides, Bacillus anthracis,* and molds of the genera *Penicillium, Aspergillus,* and *Mucor.*

Partial but not normal growth was obtained with *E. coli* on *E. coli* cells. It seemed to make no difference whether these cells were intact or disrupted. Partial growth was also obtained with *Staphylococcus aureus* on *S. lutea* cells, *Aerobacter aerogenes* on *E. coli,* and *Pseudomonas aeruginosa* on *S. lutea* and on *S. marcescens. Corynebacterium diphtheriae* gave partial growth on *E. coli,* while *Klebsiella pneumoniae* gave light growth on all the test media.

No appreciable growth was obtained on any of the test media with the following organisms: *Leuconostoc mesenteroides, Neisseria catarrhalis, Rhodococcus rhodochrous, Micrococcus tetragena, Streptococcus viridans,* and *Streptococcus hemolyticus.*

Relationship to proteolysis

One of the most striking reactions observed was the marked digestion of the cells in the medium by some of the species streaked upon its surface. This was particularly noticeable on the cell-agar media inoculated with *S. lutea* and *B. subtilis.* These species digested the cells in the otherwise opaque media to such

an extent that distinct, clear zones could be seen about the growing colonies. *S. marcescens* showed much smaller, although quite definite, zones of digestion. Blocks of digested and undigested areas of the cell-agar medium were removed, embedded

TABLE 3

Growth of organisms on cell-agar medium

INOCULUM	SUBSTRATE						
	S. lutea	*S. marcescens*	*S. aureus*	*E. coli*	*E. coli* (disrupted cells)	Control (nutrient agar)	Control (agar alone)
Sarcina lutea	++++	++++	+	+++	+++	++++	−
Serratia marcescens	++++	++++	−	+++	+++	++++	−
Bacillus subtilis	++++	++++	−	+++	++	++++	−
Bacillus mycoides	++++	++++	+	++++	++++	++++	−
Bacillus anthracis	+++	+++	−	++	++	++++	−
Aerobacter aerogenes	−	−	−	+	+	++++	−
Escherichia coli	+	++	−	+	+	++++	−
Klebsiella pneumoniae	++	+	−	+	+	++++	−
Pseudomonas aeruginosa	++	+	−	−	−	++++	−
Leuconostoc mesenteroides	−	−	−	−	−	+	−
Neisseria catarrhalis	+	−	−	+	−	++++	−
Rhodococcus rhodochrous	−	−	−	−	−	++	−
Micrococcus tetragena	−	−	−	+	+	++++	−
Corynebacterium diphtheriae	−	−	−	++	+	+	−
Staphylococcus aureus	++	−	−	++	+	++++	−
*Staphylococcus aureus**	+	−	−	+	−	++++	−
Streptococcus hemolyticus	−	−	−	−	−	−	−
Streptococcus viridans	−	−	−	−	−	−	−
Aspergillus sp	+++	+++	++	++++	++++	++++	−
Penicillium sp	++++	+++	++	++++	++++	++++	−
Mucor sp	++++	+++	++	++++	++++	++++	−

* A non-proteolytic strain.

in paraffin, sectioned, stained and examined. In the undigested part, the bacteria were found intact and in their characteristic groupings, while in the digested part there were fewer bacteria and those that were present appeared to be in the process of disintegration.

Additional reactions

S. marcescens, which rarely produces a metallic sheen when grown on nutrient agar, produced a very pronounced sheen when grown on its own cells. It was also observed that although *S. marcescens* produced no pigment when grown on the whole cells of *E. coli*, it again produced a deep red pigment when transferred to nutrient agar.

E. coli grew very meagerly on its own cells. However, when a sugar such as lactose was added, normal growth resulted. If the medium was made of cells of *E. coli* previously cultured on a nutrient agar medium rich in lactose it supported the growth of *E. coli* no better than did the medium made of cells propagated on ordinary nutrient agar.

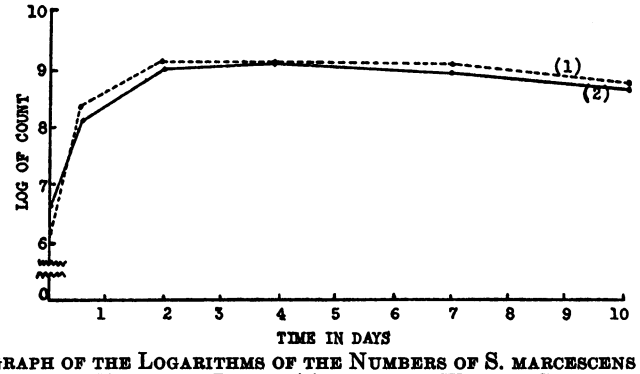

FIG. 3. GRAPH OF THE LOGARITHMS OF THE NUMBERS OF S. MARCESCENS GROWING IN ORDINARY NUTRIENT BROTH (1) AND IN A WASHED SUSPENSION OF S. MARCESCENS CELLS (2)

Cell suspension medium

When washed, 1 per cent suspensions of autoclaved cells of *S. marcescens* and of *S. lutea* were suspended in saline and inoculated with the homologous organisms, normal growth resulted (fig. 3). Plate counts of this growth corresponded to the results obtained on the cell-agar plates. It is interesting to note that in the case of *S. marcescens* there still remained over 40,000,000 viable bacteria per ml. in the suspension, even after three months incubation.

Although these experiments indicate that not all bacteria grow

upon the cells of the same or different species, some bacteria seem to have a marked ability to use bacterial cells as a source of nutriment. No doubt these experiments illustrate only a few of many such "cannibalistic" relationships between bacterial species. This study is, therefore, being broadened to include any such possible relationships between the more fastidious bacteria.

DISCUSSION

It seems that most investigators consider that bacteria in nutrient broth cultures die or decrease in numbers in a manner similar to and governed by the same laws which determine the manner or rate at which they die when subjected to such unfavorable environments as disinfectants. Apparently little or no effort has been made to learn the fate of the culture after the rapid drop in numbers following the maximum stationary phase. Most workers seem to have concluded that the decline continues quite steadily until the culture reaches sterility. This may be the case when bacteria are grown in media containing a fermentable sugar or when bacteria are acted upon by strong disinfectants.

The results of this investigation indicate that the period of death is not necessarily one of regular decrease when bacterial cultures are grown in ordinary nutrient broth. This is evident from the small rises and falls in the curve which probably indicate spurts in multiplication. In general, however, the curve secured when the logarithms of the numbers of bacteria are plotted against time shows a slow, almost unnoticeable decline. With such data as evidence, it seems that we are here presented with a period in the life of bacterial cultures which has not had its due share of recognition. We consider it a distinct phase in the culture cycle of bacteria and suggest that it be designated as the "senescent phase." This is in accord with the terminology used in connection with the other parts of the growth curve. Whether all bacteria possess this phase will require further investigation.

Any discussion as to how the senescent phase is maintained

would, as yet, have to be more theoretical than factual. The maze of interrelated factors makes the solution particularly difficult. In accord with general conceptions, the criterion of death in such experiments as these will, of necessity, have to be the inability of cells to reproduce when placed in a favorable environment, as, for instance, when transferred to agar plates for the purpose of counting. It is, furthermore, possible that cells may be able to carry on other metabolic functions but be unable to reproduce. Then too, the "resting" stage, studied by Quastel (1926) and Kendall (1930), may be an important factor.

Regardless of the attacks made against Chick's (1908) theory of variable resistance of individual cells in regard to the death rates of bacteria as influenced by disinfectants, such a possibility must also be considered. This is likewise suggested by what Sherman and Albus (1923) have termed "physiological youth." According to these authors the physiologically young bacteria are more sensitive to adverse conditions than the old. As the cultures age, the remaining cells may become old and are thus able to withstand the adverse conditions of alkaline reaction, accumulation of waste products, and other detrimental environmental conditions which ordinarily kill those organisms which are physiologically young. The possibilities that the cultures are undergoing variation and that the medium is acting as a selective agent must also be considered. This may partially explain the occasional spurts in multiplication during the senescent phase.

Although perhaps of less significance than originally supposed, many writers still consider starvation to be one of the causes of death of bacteria in old cultures. Therefore, we were prompted to ask ourselves if the dead organisms in aging cultures could be of sufficient nutritive value to serve as a source of necessary food elements to aid in maintaining the senescent phase. One can readily imagine that due to long standing the dead cells remaining in the culture autolyze and liberate some nutriment which is used by the remaining viable cells. It might further be supposed that dead bacterial cells contain all the constituents necessary for the growth of new cells of the same species pro-

viding, of course, that the living cells possess enzymes necessary to utilize the dead cells. The data here presented indicate the possibility of such a cycle. Just how many dead cells are needed to supply the energy for the growth of one living cell could only be determined by more accurate analytical methods. It is no doubt true that a 1 per cent suspension of bacteria autoclaved at their height of development is not comparable with the cells of very old cultures. Nevertheless, the results secured in growing *S. marcescens* in a saline suspension of killed cells of the same species would seem to have particular significance in this respect. This medium, in which the only source of food was the dead bacterial cells, supported growth to a degree almost as great as nutrient broth. For the want of a better word this relationship has been referred to as "cannibalism," a term used by Kollath (1924) in reference to a similar phenomenon. This phase of the work is being further investigated.

SUMMARY

Periodic plate counts made of aging broth cultures of *Sarcina lutea* and *Serratia marcescens* showed the numbers of cultivable bacteria remaining in the cultures to be remarkably high even after two years incubation. Similar findings have been obtained with a number of other organisms after one year of incubation. Readjusting the reaction of the cultures of *S. marcescens* from pH 9.0 to 7.0 gave a definite increase in the number of viable organisms.

Microscopic examination of these aging cultures did not show much cellular variation, although on plating colonial variation was evident in some instances, especially with *S. marcescens*. The species retained their physiologic and gram stain characteristics. Rough determinations indicated that the aging cells possessed a marked resistance to adverse environmental conditions.

When the counts were plotted logarithmically, the resulting curve, after the initial drop following the maximum stationary phase, tended to level off and maintain an almost imperceptible decline. Small rises and falls in the curve were still apparent

during this period. The term "senescent phase" is suggested for this period of the curve.

Varying degrees of growth were obtained with 21 strains of organisms on media in which the only source of nutriment was a large quantity of washed and autoclaved bacterial cells. In general, the proteolytic organisms grew more luxuriantly on such media than did the non-proteolytic organisms.

When *S. marcescens* was inoculated into a liquid medium consisting of a suspension of killed cells of *S. marcescens*, the counts obtained were almost as great as those obtained in the nutrient broth controls.

The term "cannibalism" is used to designate the growth obtained in cultures where the dead cells are serving as a source of food.

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REFERENCES

CHICK, H. 1908 An investigation into the laws of disinfection. J. Hyg., **8**, 92–158.

GAY, F. P. 1936 Agents of disease and host resistance, p. 201. Charles Thomas, Baltimore.

KENDALL, A. I., FRIEDEMANN, T. E., AND ISHIKAWA, M. 1930 Methods for the study of "resting" bacteria. J. Infectious Diseases, **47**, 186–193.

KOLLATH, W. 1924 Vitaminähnliche Substanzen in ihrer Wirkung auf das Wachstum der Influenzabazillen. Zentr. Bakt. Parasitenk., Abt. I., Orig., **93**, 506–519

QUASTEL, J. H., AND WHETHAM, M. D. 1924 The equilibria existing between succinic, fumaric, and malic acids in the presence of resting bacteria. Biochem. J., **18**, 519–534.

SHERMAN, J. M., AND ALBUS, W. R. 1923 Physiological youth in bacteria. J. Bact., **8**, 127–139.

TOPLEY, W. W. C., AND WILSON, G. S. 1936 The principles of bacteriology and immunity, p. 82. William Wood & Co., Baltimore.