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Rhythmic Response of *Serratia marcescens* to Elevated Temperature

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ABSTRACT

DIMMICK, ROBERT L. (University of California, Berkeley). Rhythmic response of *Serratia marcescens* to elevated temperature. J. Bacteriol. **89**:791–798. 1965.—Populations of *Serratia marcescens* of varied ages and pretreatments, which had been grown in a chemically defined medium, were subjected to thermal stress at 50 to 56 C. The numbers of survivors were plotted vs. time to form survivor curves, and the curves were assembled to form three-dimensional models. The manner in which survivors varied as a function of age and time of heating was variable and often rhythmic. Different three-dimensional patterns were found when different inoculum for the test culture was used. Apparently some "dead" cells again produced colonies after extended heating periods (recuperation); this tendency varied with the age of the culture. Diminutive colony forms, which produced normal colonies upon transfer, appeared and disappeared during heating; this tendency fluctuated with age. It is suggested that survivor curves represent a distribution of resistant forms within the population, and that this distribution varies in a manner best described in terms of servomechanistic response within each cell and within a given culture. Difficulties of attempting to relate changes in specific molecular species to subsequent whole-cell responses are discussed.

Hansen and Riemann (1963) recently published an excellent review of heat resistance of bacteria, and other workers (Sykes, 1963; Postgate and Hunter, 1963; King and Hurst, 1963) have presented data concerned with the general field of bacterial survival. But the variety of response patterns of microorganisms to elevated temperatures noted in these and many other papers leads one to believe that our knowledge of the subject is far from complete.

Although it is well established that "young," actively growing cells are more sensitive to almost any trauma than "mature" cells, no one has examined the manner in which this change occurs in context with the implied change in the single-hit theory (either young cells have fewer sensitive sites or the sites are more easily inactivated), especially in regard to the genetic apparatus. The question is important, because the survival of bacteria is employed as an index of disinfectant efficacy, or, in terms of decay rates, survival is used to measure implied molecular changes in the internal structure of the cell (Webb, 1959).

In an attempt to create a model system, I have examined survivor curves of *Serratia marcescens* as a function, primarily, of test temperature and age of culture. Although the study is not complete, a sufficient body of data has been accumulated to indicate that the reaction of this species to thermal stress is extremely complex, and is certainly not explainable by the assumption of logarithmic death kinetics (single-hit hypothesis) or on the basis of simple thermodynamic principles.

MATERIALS AND METHODS

Organism. A strain of *Serratia marcescens*, labeled 8 UK, was obtained from the U.S. Department of Agriculture, Western Regional Research Laboratory. Normal cultures (i.e., those between 4 and 24 hr old, in growth medium, and held at constant growth temperature) consistently produced light-red colonies about 2 mm in diameter on peptone or Blood Agar Base (BAB, Difco) after 24 hr of incubation at 31 C, and bright-red colonies on a solid chemically defined medium (CDM) containing (per liter): dibasic ammonium citrate, 2.5 g; glycerol, 5 ml; K_2HPO_4, 7.8 g; $MgSO_4$, 0.25 g; NaCl, 0.13 g (all anhydrous); and agar, 20 g. The medium was minimal; reducing the concentration of any component reduced the total cell yield of 10^{10} to 2×10^{10} viable cells per milliliter in shake-flask cultures of the liquid menstruum at 31 C after 24 hr of incubation.

Assay of viability. Ten-fold serial dilutions of a culture sample were made in the same medium in which cells were grown or tested. A pipette having a stainless-steel tip with an orifice calibrated to deliver 0.02 ml per drop was used to plant five drops on each of three plates of nutrient agar; the plates,

rotated sufficiently to spread the drops slightly, were incubated at 31 C for 24 hr unless noted. The number (mean of three plates) of colonies was obtained with the aid of an electronic colony counter (Leif and Wolochow, 1958). This technique has been shown to produce replicate assays of normal cultures having a 95% confidence interval of ±8%. All colonies were counted regardless of size.

Cultures and suspensions. Unless noted, a loopful of the organism was transferred from a stock slant into 100 ml of CDM contained in a 500-ml flask. The flask was incubated on a rotary shaker at 31 C for 24 hr. A 1-ml amount of the culture was transferred to a second flask and incubated for either 10 or 24 hr (see below); 1 ml from the second flask was planted in a third flask. Immediately, and at the noted intervals thereafter, 0.5-ml samples were removed for testing. In some instances, Heart Infusion Broth (Difco) was used instead of CDM; in others, the third flask was inoculated with 1 ml of a 10-fold, or higher, dilution of the suspension in the second flask rather than with 1 ml directly.

Determination of survivor curves and patterns. A water bath was maintained at the required temperature (50 to 56 C) by means of a Thermistemp (Yellow Springs Instrument Co., Yellow Springs, Ohio) control unit. Test tubes (2.5 by 20 cm) containing 9.5 ml of the same medium in which the test organism was suspended were immersed in the water bath to within 2.5 cm of their tops. After the medium equilibrated for 30 min, 0.5 ml of the test culture was carefully added, the tube was shaken for 5 sec, and a 0.5-ml sample was removed to a dilution blank and assayed. Thereafter, at 10, 20, 30, 40, 60, 90, and 120 min, unless noted, 0.5-ml samples were removed from the heated sample and assayed. Every 10-fold or every 100-fold dilution (0 to 7) was plated, as above, for each assay. Culture samples to be heated in this manner were removed from the third flask at hourly intervals for 8 hr, and then at 10, 16, 22, and 24 hr. Minor variations from the latter time sequence were sometimes made for practical purposes.

Each assay datum was plotted on semilog paper above an appropriate time scale, and a line was drawn through the points to create a survivor curve. For model construction, the curve was traced on plastic sheet, the sheet was cut to shape, and sheets were mounted serially in direct relationship to the culture age. Spaces between the sheets were filled in with clay to produce a three-dimensional model of best fit. The models were photographed, and tracings of the photographs are presented.

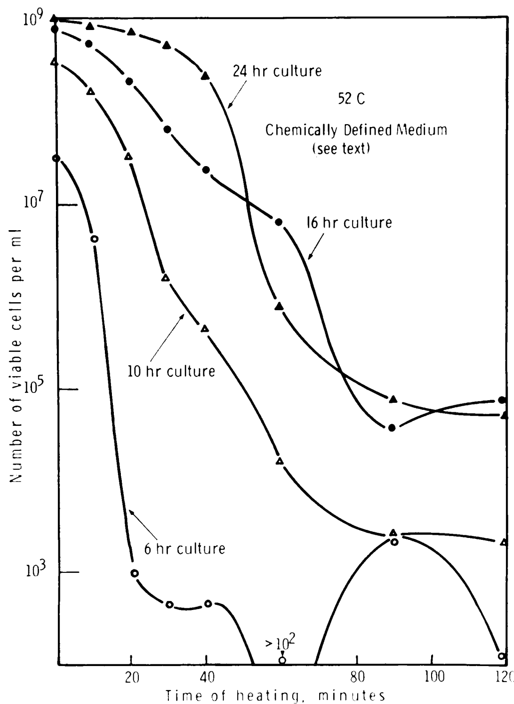

FIG. 1. *Effect of age of culture on survivor curves of Serratia marcescens during thermal stress at 52 C. In all instances, points listed as zero heating time were taken immediately after sample was added to heated media, and represent at least 10 sec of heating.*

RESULTS

Figure 1 shows typical survivor curves from a culture during growth. Repeated experiments showed that enhanced sensitivity usually occurred with younger cultures, together with a tendency for the number of viable cells to increase upon prolonged heating (recuperation), and that sigmoid curves without recuperation always occurred with older cultures. It is important to note that these recuperation periods never exceeded 30 min; i.e., they were less than one generation time (47 min in CDM at 31 C).

To test the possibility that clumping caused the results to appear nonlogarithmic, cultures of several ages were filtered through membrane filters (1.5-μ pore size), and some were treated with nonlethal ultrasonic energy. I found essentially the same results as above, including evidence of "tailing" and periods of recuperation.

Figure 2 shows the results of assembling data in the form of three-dimensional models. When cultures were started from 10-hr-old inocula, the most pronounced periods of recuperation (labeled R) occurred at about 6 hr after inoculation, regardless of the stress temperature (A, B, C, and D in Fig. 2). When a culture started from a 24-hr inoculum (F) was tested, no marked recuperation period was noted, and the generally sigmoid shape of the curves was reduced. Repeated tests confirmed the general "hill and valley" appearance of the models.

A culture that had been stored 5 weeks at 4 C in CDM was used directly as an inoculum. The

survival pattern from the resulting culture is shown in E (Fig. 2). Slight recuperation periods were noted, though the curves were more linear in shape than those resulting from the use of a 10-hr inoculum.

It was logistically unfeasible to obtain complete survivor curves of a single growing culture during the entire 24-hr period at intervals of less than 1 hr. Instead, survival was assayed every 20 min during growth by sampling cells after 90 min of heating. The results (Fig. 3) are equivalent to a cross-section of the survival patterns shown in Fig. 2. In Fig. 3, a distinction is made between samples that had countable numbers of colonies as the result of a direct transfer to agar surfaces and those that were countable only in a diluted sample, to show that the rapid rise and fall of surviving numbers included more than one order of magnitude. Evidently, the survival patterns contained "fine structure" not apparent in Fig. 1 and 2.

This "fine structure" was also demonstrated by sampling a heated culture every minute for a 30-min interval, with samples placed directly on agar surfaces and the order of single droplets (ca. one per 10 sec) on each plate recorded. A curve, from data obtained by a second technician testing the same culture, based on samples at less frequent intervals, followed the general trend of the rapid samples (Fig. 4). The periodicity of the fluctuations appeared to lengthen as a function of time of heating. The first 12 plates obtained in this manner contained mixtures of white diminutive (see below), red diminutive, and red normal colonies; the next 8 plates contained red diminutive and red normal colonies; and the last 10 contained red normal colonies only. The number of colonies per drop on a single plate varied as much as 1:50 and sequentially, in a manner suggesting a trend rather than a random change.

To ascertain whether a cold shock before heating would cause temporal changes, 10 ml of a 16-hr culture were placed in a tube cooled to 16 C in a water bath. At intervals, 0.5-ml samples were removed and tested for survival at 52 C. The results (Fig. 5) show that survival fluctuated as a function of time of cooling in a manner best described as a damped wave.

Identical inocula were placed in CDM and in Heart Infusion Broth (Difco), and samples of different ages were tested with both CDM and BAB agar as assay media. The shapes of survivor curves of the two cultures were sometimes different, and the two sets of curves, based on colony numbers found on CDM or BAB plates, varied (Fig. 6). The number of colonies formed on BAB agar, divided by the number found on CDM,

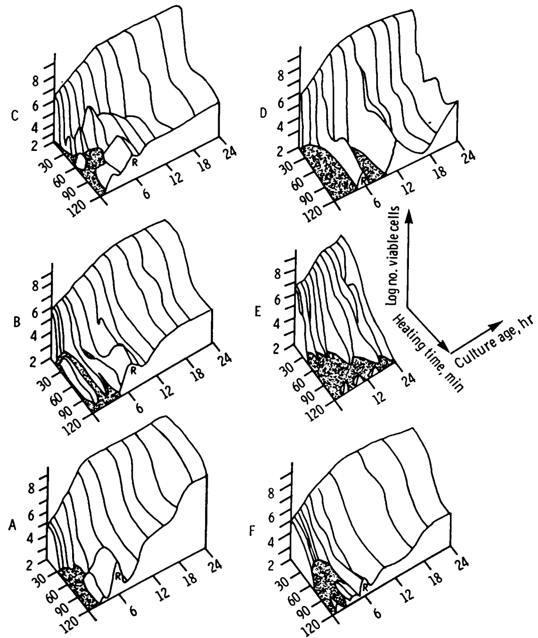

FIG. 2. *Three-dimensional survival patterns of Serratia marcescens during thermal stress: (A) 50 C, 10-hr inoculum, third pass; (B) 52 C, 10-hr inoculum, third pass; (C) 54 C, 10-hr inoculum, third pass; (D) 56 C, 10-hr inoculum, third pass; (E) 52 C, inoculum stored 5 weeks at 4 C, CDM medium, direct transfer; (F) 52 C, 24-hr inoculum, third pass; (R) position of most evident recuperation period.*

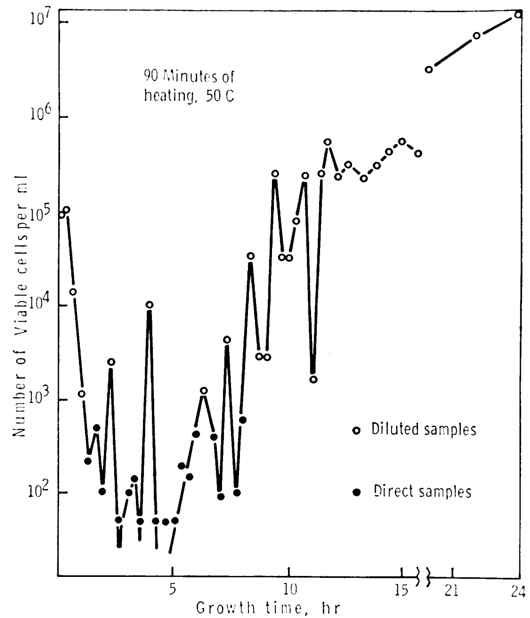

FIG. 3. *Survival of Serratia marcescens after 90 min at 50 C as a function of age of culture.*

from the same dilution (plating ratio) of normal cultures was 1.05 with a standard error of 0.05; no deviation greater than 1.1 or less than 0.8 was noted. Plating ratios as high as 700 and as low as 0.1 were often found with injured cells, the number tending to increase, then decrease, as a function of time of heating.

The influence of the level of inoculum (10^4 to 10^7 bacteria per milliliter) on the "resistance" of fully grown cultures was tested by first determining the incubation time required for each culture to progress 2 hr into the stationary phase. By use of this information, duplicate cultures 2 hr into the stationary phase were tested at 52 C for

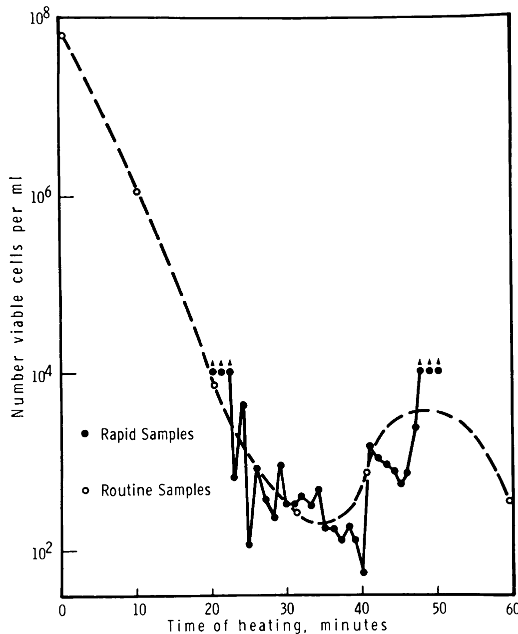

FIG. 4. *Comparison of rapid sequential sampling to routine sampling procedure during thermal stress of Serratia marcescens.*

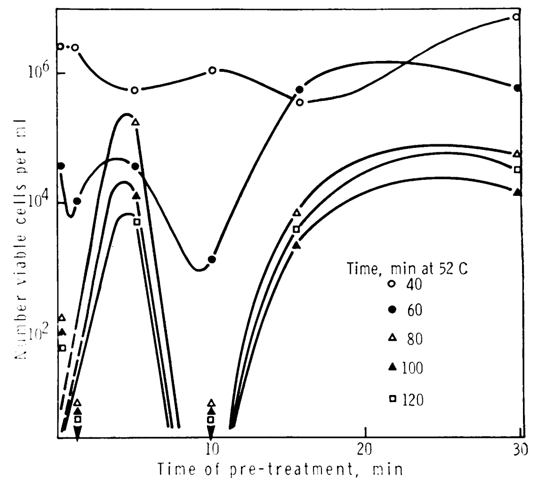

FIG. 5. *Influence of time of pretreatment at 16 C on the number of surviving cells of Serratia marcescens stressed at 52 C.*

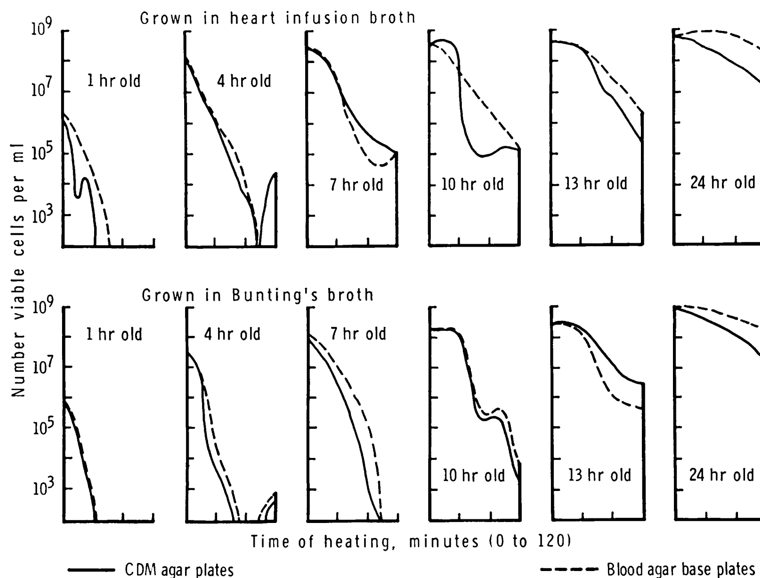

FIG. 6. *Influence of age of culture, growth medium, and sampling medium on thermal death curves of Serratia marcescens.*

survival (Fig. 7). Cultures inoculated with the least number of cells (hence, older cultures at time of test) were the most resistant, although the number of cells tested was the same. All were more sensitive than cultures of the same ages started from the usual 10^8 bacteria (see Fig. 6).

Samples from cultures less than 16 hr old that had been heated more than 10 min but less than about 90 min usually produced numerous diminutive colony forms on both types of media. The population of colonies varied from those just visible to those of normal size. The smaller colonies were usually white after 24 hr of incubation; most became red after 48 hr, and some increased in size. Numerous transfers of these colonies to fresh agar yielded no evidence of mutations; i.e., all new colonies appeared normal (pseudoauxotrophy; Zamenhof, 1960); and when survivors were recultivated in liquid medium and tested, their progeny evinced no enhanced resistance. Growth curves of subcultures of heated samples often showed extended lag times and reduced growth rates compared with normal cultures, although some finally attained a normal rate of growth. But after cultures had been heated approximately 100 min, the surviving cells produced colonies that were uniform in size and color when the plates were incubated for 24 hr. Reversion to "normal" colony production was especially evident during the recuperation period: the number of cells that produced normal colonies after 120 min of heating and 24 hr of incubation was sometimes 1,000 times the number that produced normal colonies after 30 min of heating and 48 hr of incubation.

In addition, instances of a gross lack of agreement between the expected numbers of colonies arising from sequential dilutions were often observed during the 15- to 100-min period, a phenomenon I prefer to term "incoherence," because the true extent of viability was no longer determinable. Usually these instances involved a lack of agreement between the direct-transfer sample (zero dilution) and the first 10-fold dilution. Sometimes the plates containing a zero dilution produced no colonies, whereas plates containing the next higher dilution contained too many colonies to count; sometimes the reverse was observed. No instances of this kind were observed in thousands of assays of cultures that had not been treated in some way generally considered to be detrimental to the life of the cell. When heat-killed cells were added to living cells before heating, no differences in either the time of occurrence or the extent of incoherence was observed.

Twenty duplicate platings from several dilutions of a single sample of a 6-hr-old culture

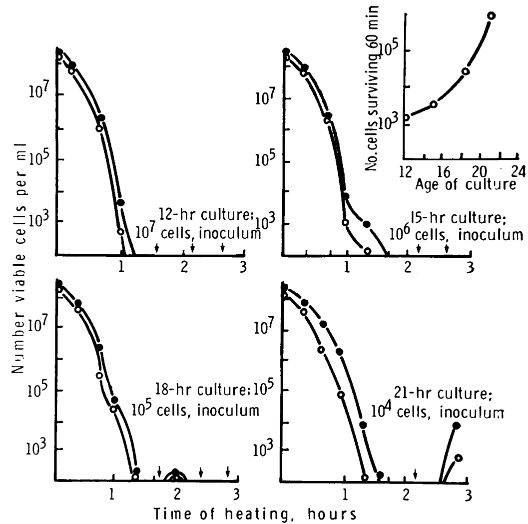

FIG. 7. *Influence of level of inoculum on thermal death curves of Serratia marcescens in the early stationary phase. Duplicate survivor curves were made for each test.*

grown in CDM and heated for 10 min at 52 C were made on two types of media. All plates were immediately incubated. At hourly intervals on Trypticase Soy Agar plates, and at 2-hr intervals on CDM plates, duplicate sets of each media type were treated by spreading the surface moisture with sterile dally rods. The plates were reincubated and examined 24 and 48 hr after they had been dallied. Colony numbers, both total and diminutive, increased as a function of time after plating (Fig. 8). Although there was no increase of numbers of colonies on undallied CDM plates as a result of additional incubation, the number increased on plates dallied during the first 10 hr. The number of diminutive colonies approximately doubled, and then approached zero when rapid growth started, indicating, I believe, that the diminutive characteristic was passed to at least one generation. Since the total number of colonies also increased and the colony sizes were heterogeneous, there must have been a heterogeneity of initial generation times greater than in "normal" populations, indicating either a variety of injured sites or a variety of responses to a similar injury in all cells. In this instance, approximately 10 times the number of colonies were formed on CDM plates compared with the number on Trypticase Soy Agar plates (plating ratio ca. 0.1). There were too few colonies, including diminutive colonies, on Trypticase Soy Agar plates in the lowest dilution tested to be significant for the purpose of this experiment, although I have shown the total number in Fig.

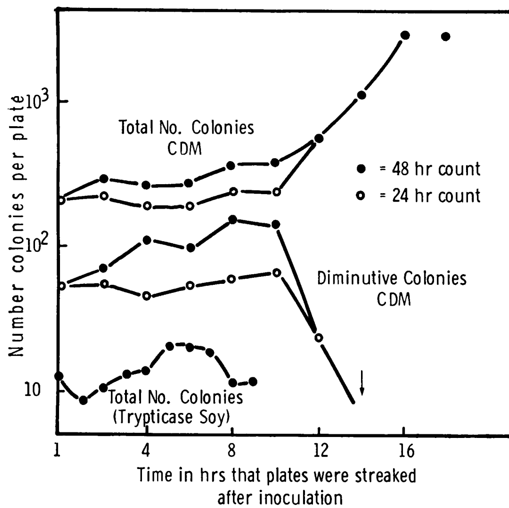

FIG. 8. *Change in colony number as a result of streaking plates at intervals after they had been inoculated from the same dilution sampled from a 6-hr culture of Serratia marcescens heated to 52 C for 10 min. Two types of media were used: Blood Agar Base and chemically defined medium (CDM).*

8 for comparison. Colonies that arose on Trypticase Soy Agar were almost all normal in size after 48 hr of incubation.

Discussion

The object of these exploratory experiments was to obtain a notion of factors that might influence the general form (shape) of survivor curves without initial reference to "rate" theories or to any specific molecular species in the cell. In other words, if one discards preconceived concepts and assumes that a suspension of bacteria is a "black box" system (Rescigno and Segre, 1961) about which little is actually known, but a system that nonetheless has measurable "output" characteristics (colony number, size, growth rate, temperature response, etc.), one may adjust the "input" (environment), measure the output, and hope to gain knowledge of internal mechanisms by deductive means. This concept is in contrast to that embodied in many current studies of cellular physiology that prefer to take the box apart and hope that structural properties retain those characteristics present in the intact assembly; both avenues obviously yield useful information.

On the basis of these data, several general statements can be made about suspensions of *S. marcescens* cells. First, the apparently irregular action of mild heat on *S. marcescens* is in general agreement with the findings presented by White (1953) and Lemcke and White (1959) for *Streptococcus faecalis* and for *Escherichia coli*. Second, cell populations did not die logarithmically. In fact, and despite the weighty arguments of Wood (1956), the most reasonable conclusion is that of Jordan, Jacobs, and Davies (1947) and Vas and Proszt (1957); i.e., a survival curve represents a kind of cumulative distribution of the "resistance" of the individual cells in the population. Third, the distribution varied, sometimes rhythmically, as a function of culture age or of pretreatment. The inoculum for a given culture influenced the extent of rhythm. Responses such as these were predicted by Hinshelwood (1951). Fourth, the capacity of some cells to form colonies after varied periods of stress was influenced by the act of sampling, age of culture, and a temporal function of a change in environment prior to test. Fifth, ability to recuperate from such lethal conditions, again depending on age, was most often enhanced by enriched plating medium, but sometimes enriched medium discouraged the growth of many cells. Harris (1963) reviewed this phenomenon in relationship to several other species. Sixth, the duration of all these responses varied from seconds to hours, often in a manner resembling a damped wave phenomenon or a self-regulating system seeking a point of stability.

The concept of the cell as a complex, dynamic servomechanism has been presented, or at least implied, by Hinshelwood (1951), Heinmetz (1960), Elsasser (1960), and Chance (1961). Ideas presented by Mora (1963) and by Dean and Hinshelwood (1963) add emphasis to this concept. Apparently overlooked in these discussions is the fact that the "output" of one cell can be the "input" of another. Thus, in a culture of sufficient numbers, groups of cells may act together (a biophase; Perret, 1960), and the culture may possess some of the same servomechanistic properties as the cell, but with longer cycles. There is evidence that the culture as a whole might have acted autonomously during and after this type of stress, and it is evident that individual cells changed in some way during stress according to conditions imposed upon them by the culture as a whole before stress.

I suggest that the death of most cells resulted from a lack of over-all control (i.e., an imbalanced state), and that this occurred in a variety of ways. Of course, real logarithmic death rates can occur in a population—many lethal, quantitized phenomena could kill a cell if that cell is dependent on, and is "hit" in, a vital site; i.e., if a one-to-one relationship exists. But other, less lethal stresses can so damage a cell that it either does not grow, or does not grow immediately in the environment imposed for purposes of recultivation. Conversely, the finding of a linear decay on a semilog plot

need not imply that a monomolecular reaction is occurring, or that, having demonstrated an exponential relationship in one instance, it must apply to all instances. The problem is to distinguish between the two kinds of damage. Time-dependent repair mechanisms that interpose "noise" between the lethal event and the measurement of that event, as well as differences in individual cell structure and response coupled with cell-to-cell interaction, cause the interpretation of every survival curve to be suspect, especially if "decay constants" are to be estimated. The presence of a colony is proof of life; the absence of a colony is not proof that death had occurred.

Harrison and Lawrence (1963) and Hess and Shon (1962) attributed periods of enhanced viability, or of "tailing" of survivor curves as a result of stress, to the growth of selected mutants or variants. My data show instances of fluctuation of viable numbers too rapid to be considered a result of cell replication during stress, and fluctuations during growth too repeatable (qualitatively) to be entirely random in nature. I found no instances of enhanced resistance of populations grown from resistant cells. The progeny from small white colonies produced red colonies of normal size after a single transfer. Apparently, some type of nonlethal injury (e.g., lengthened generation time) can be passed to a limited number of generations within a colony.

Obviously, the majority of these data rest on the behavior of a small fraction of the total population tested, but this is the fraction upon which concepts of sterility or epidemiology are usually based. Whether these resistant cells are true "mutants" or not is of no consequence to the present argument, because survivors are undoubtedly members of the species and therefore represent overall species capabilities; it is the transient nature of their presence and absence that is important. Certainly, there was no selection pressure to encourage varieties resistant to heat during culture growth. Recuperation, or even incoherence in specific instances, might be explained on the basis of thermal renaturation of deoxyribonucleic acid (DNA; Marmur and Doty, 1961), or by assuming random repair of genetic injury, but this does not explain the rapid and consistent rise and decline of either resistance or recuperative capacity during growth of the culture.

Attempts to relate survival mechanisms to specific biochemical changes, observed from studies of enough cells to be analyzed by available techniques (for example, analysis of DNA content), may yield information pertinent to average behavior, but would not reveal individual cellular capabilities. Conversely, colony formation (as a phenotypic measurement) is a whole-cell response. Death of some cells could be attributed to metabolic deficiencies induced by the stress, but not in instances where more colonies were found on chemically defined medium than on enriched medium.

Again, differences in cellular resistance seemed to be more closely associated with time-dependent organization than with a given molecular complement or with genetic accidents, although these attributes are not easily separated and measured in large populations. There exists no proven technique for distinguishing live cells from dead ones other than ability to reproduce, and the argument is circular, because the definition of life, with regard to bacteria, implies reproduction. An explanation of the behavior of microbial cultures in terms of servomechanisms may also be circular, but the theory can be tested by available techniques, mathematical models can be constructed, and computer techniques can be applied (Sugita, 1961).

One such highly informative model was suggested by Goodwin (1963). In discussing the epigenetic system, he pointed out that probably no oscillation would be observed in bacteria because of the small numbers of a given species of messenger ribonucleic acid (RNA) per cell; he added, "There is one rather comforting observation which we can make at this point, however, and that is that no rhythmic or cyclic behaviour has ever been observed in bacteria analogous to the tidal, diurnal, lunar and other rhythms which are such an obtrusive feature of behaviour in higher organisms, from the protozoa up." I suggest that two types of rhythmic behavior, with age and with environmental shift, have now been demonstrated in at least one species of bacteria, and that we ought to be aware of this possibility in other species under other test conditions.

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