Advances in
MICROBIAL PHYSIOLOGY

VOLUME 1

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Preface

The explosion in biological research which has taken place over the past two decades has inevitably induced a considerable degree of specialization among those engaged in this research. At the same time, there has been a move towards integration among the biological sciences, largely as a result of the realization that there exist many basic similarities among living organisms, particularly at the physiological level. These two trends have placed an even greater premium on the availability of review articles with which biologists, who are researching in one area, can become acquainted with progress that is being made in areas outside their immediate sphere of interest. The physiology of micro-organisms is now being studied by individuals representing widely diverse specializations in the chemical as well as the biological sciences. It was with the belief that work being carried out under these many specializations could profitably be brought together in the form of review articles that we agreed to edit the present series.

In *Advances in Microbial Physiology*, we aim to include articles covering as wide a range as possible of the specialized interests that constitute microbial physiology. Clearly there must be limits to our field of interest, and we have confined it to unicellular micro-organisms. Except in so far as they pertain to the physiology of the host micro-organism, the articles will not deal primarily with viruses and virus multiplication. Moreover, while we aim to include articles that deal with all of the major groups of unicellular micro-organisms, the concentration of research on bacteria—and indeed on just a very few species of bacteria—makes it difficult and probably undesirable to strike a true balance. Nevertheless, we hope that these reviews will be of value to all biologists who are interested in the physiological activities of micro-organisms.

A. H. ROSE
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January, 1967

ABBREVIATIONS AND SYMBOLS

Certain abbreviations in this book are used without definition. These abbreviations were recommended by the IUPAC-IUB Combined Commission on Biochemical Nomenclature, and have been reproduced in the *Biochemical Journal* **102**, 15 (1967), *Biochemistry* **5**, 1445 (1966), *Biochimica et Biophysica Acta* **108**, 1 (1965) and the *Journal of Biological Chemistry* **241**, 527 (1966). Enzymes are referred to by the trivial names recommended in the Report of the Commission of Enzymes of the IUB (Pergamon Press, Oxford; 1961). All temperatures recorded in this volume are in degrees Centigrade.

Contents

Viability Measurements and the Survival of Microbes Under Minimum Stress

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I. Introduction

The title of this review is obliquely phrased, because it is concerned with the "spontaneous" death of vegetative microbes. A moment's reflection will show that microbes, being uni- or non-cellular, have no simple analogue of the "natural" senescence and death undergone by multicellular organisms (though in molecular terms it is possible that processes analogous to the senescence of multicellular organisms could be demonstrated in individual microbes). Most studies on microbial survival have been concerned with the viability of populations after they have been subjected to some kind of stress: a population is examined after it has been heated, frozen, dried; exposed to adverse pH, pressure, salinity; to toxic chemicals or lethal radiation; or to biological antagonists (anti-sera, phage, phagocytes). Experiments on the viability of microbes after such treatments in fact constitute a great part of the literature of experimental microbiology, particularly that concerned with sterilization and disinfection or with the preservation of stock cultures, but such experiments require a control in which the viability of

a comparable population not subjected to the overt stress is assessed. Such "stress-free" controls have a "spontaneous" death-rate which may or may not act additively with respect to the stress imposed, and it is largely with the death of such "un-stressed" populations that this review is concerned.

In practice, stress-free conditions are difficult to realize, and the investigator must usually adopt a form of treatment in which as mild and simple stress as possible is imposed on the population. Three conditions are generally available for such studies: (1) Exposure of microbial populations to non-nutrient environments in conditions in which growth is in principle possible, but is prevented by withholding one or more essential nutrients. (2) Allowing a culture to age until multiplication has ceased. (3) Growing a continuous culture at so slow a rate that the spontaneous death-rate of the population makes a contribution to the dynamics of the steady state.

In contrast to the voluminous literature available on spore survival, remarkably little work has been published relevant to the survival of populations of vegetative organisms in such conditions, though experiments of this kind have obvious relevance to hygiene, water bacteriology, and microbial ecology. So far, published work has largely illustrated the methodological difficulties of the study of microbial death. Therefore, before reviewing such published data as exist, a critical consideration of the techniques used to assess microbial survival is desirable.

II. Methodology

A. Definitions and Principles

Viability, as used in this review, has the sense of the ratio of the number of viable units in a microbial population to the total number of microbes present. Thus a yeast with a bud is a single unit, viable or non-viable as the case may be, and the question whether it represents one or two individuals will not be discussed. Some authorities have also used the term viability in a clonal sense; a certain clone is less viable than another if the generations include a higher proportion of non-viable progeny. This sense of the term, though legitimate, can lead to ambiguity in the present context and will not be used.

Viable microbes, for microbiologists, are microbes capable of dividing to form one or more live daughter cells when provided with a favourable environment. What constitutes a favourable environment can rarely be stated precisely, and must therefore be derived from a consensus of informed opinions. The usage of the term thus differs from that current among scientists concerned with higher organisms, where a creature may be viable without being capable of multiplication. It is possible,

in special conditions, to obtain microbes having many of the characters of normal, living organisms but unable to divide and form a colony. Such microbes are committed, as it were, to death and will be termed "moribund". Because of the need to use colony formation as a test, the viability of an individual microbe can only be rigidly determined retrospectively: as soon as its viability is established, it ceases to be the same individual. Since bacteria, for example, do not necessarily multiply the moment they are given the opportunity to do so, an element of ambiguity arises in deciding whether a given cell is dead or in a state of division lag. Undoubtedly, in fact, organisms from a dying population may continue to die during a lag phase, so that an assessment of the viability of such a population may actually measure the resultant of two theoretical viabilities: the viability at the time of sampling supplemented by the mortality of those organisms that died during the lag phase. These elements of uncertainty in the assessment of viability are often trivial, but they can assume importance on occasions. Death during the lag phase takes place among the survivors of frozen populations (Postgate and Hunter, 1963b), and has been proposed as a partial explanation of "metabolic injury", a phenomenon in which the recovery of viable bacteria from frozen populations may be greater the "richer" the recovery medium used. The possibility that comparable metabolic injury may precede death by starvation must be borne in mind, though Postgate and Hunter (1962) did not observe it in their experiments. Bacteria subject to stress become hypersensitive to secondary stresses, such as toxicity of certain medium components (see Jacobs and Harris, 1960, 1961), and the possibility of comparable hypersensitivity among starved populations must also be considered. Starved *Escherichia coli* K–12, for example, show increased sensitivity to infection by λ bacteriophage (Arber, 1963). Sykes and Tempest (1965) studied a slow-growing continuous culture of magnesium-limited *Pseudomonas* which showed nearly 100% viability when the sample for assay was diluted in a cold saline solution, but gave considerable evidence of lysis when diluted in distilled water; normal populations were indifferent to the diluent used. Postgate and Hunter (1962) routinely centrifuged their populations for starvation studies and observed that the survival patterns of the populations were influenced by whether distilled water or a saline solution was used to resuspend the bacteria.

Viability is normally assessed by growth tests. Because such tests are slow and can give ambiguous results, many indirect procedures have been proposed for determining viability. Though these can have value for the study of populations subject to major stresses, they are uniformly unsuitable for the examination of starved populations. A critical summary of the major indirect procedures follows.

B. Indirect Assessment of Viability

1. *Staining and Dye-Uptake Tests*

Vital staining, either with fluorescent dyes (Strugger, 1948) or ordinary dyes such as methylene blue (see Gilliland, 1959), depends on the assumption that microbes become permeable to dye as they die. Thus they fail to score moribund cells as dead. This point is almost certainly unimportant when the stress applied is heating or disinfection with, for example, a quaternary bactericide; it is far from trivial when a mild stress such as chilling or starvation is applied. Postgate and Hunter (1962) showed that the permeability barriers of starved *Aerobacter aerogenes* persisted for many hours after loss of viability; Postgate *et al.* (1961) observed that methylene blue, as a vital stain, under-estimated the viability of old baker's yeast by a considerable margin, doubtless because it administered the *coup de grâce* to the moribund members of the population (see, for example, Ketterer, 1956). Gilliland (1959), on the other hand, found that brewer's yeast was tougher, and that vital staining with methylene blue over-estimated the viability of old yeast populations. Razumovskaya and Osipova (1958) reported a lack of correlation between the ability of *Acetobacter* cells to multiply and their permeability to a fluorescent vital stain. Differential staining (e.g. White, 1947) likewise depends on assumed differences in the inherent stainability of dead and live cells, which are expected to persist after the surviving organisms have been killed during the staining procedure.

Permeable microbes take up more dye than impermeable ones, and the assumption that permeability and death are coincident has led to proposals for the determination of the gross viability of populations by dye uptake tests (Borzani and Vairo, 1958, 1960). Like vital staining, these procedures are excluded for use with unstressed or mildly stressed populations because stainability and viability do not necessarily correlate. Fluorescence when stained by dyes of the anilino-naphthalene-sulphonic acid class has, in fact, been used to demonstrate persistence of osmotic integrity in certain classes of moribund cells (Postgate and Hunter, 1962; Mathews and Sistrom, 1960; Strange and Postgate, 1964).

2. *Optical Tests*

Mager *et al.* (1956) described the "optical effect", whereby the optical density of a suspension of live bacteria is greater in a dilute salt solution than in distilled water by a factor of 30–100% (the percent change is now known to depend on the geometry of the apparatus used to measure extinction; see Powell, 1963). Heat- or phenol-killed bacteria showed no optical effect. Though proposed by its discoverers as a rapid method of

assessing viability, the method is in fact useless for this purpose unless the populations have been violently damaged. On the other hand, the optical effect provides an invaluable test of the integrity of the osmotic barrier in cell suspensions (Postgate and Hunter, 1962; Strange, 1964) and is very useful in permeability studies.

Immersion refractometry is based on the principle that breakdown of the osmotic barrier leads to a decline in refractive index and consequent loss of contrast under the phase-contrast microscope. Barer *et al.* (1953), Fikhman (1959a, b) and Fikhman and Pryadkina (1961) adopted this phenomenon using gelatin solutions of various refractive indices to abolish light-scattering by one class of cells ("dead") while leaving others visible ("live"). The method suffers from the same formal objections as others based on permeability changes.

3. *Leakage of Pool Materials*

Koch (1959) estimated death during growth of a culture by measuring leakage of purines from labelled bacteria. Since bacteria appear to undergo quite dramatic changes in permeability during active growth, without loss of viability (see, for example, Strange and Postgate, 1964), this test is of doubtful validity.

4. *Enzyme Activity*

Loss of viability in practice is often paralleled by a decline in dehydrogenase enzyme activity, and the ability to reduce a dye such as tetrazolium or methylene blue has been proposed as the measure of the viability of microbial populations (e.g. Delpy *et al.*, 1956). In specialized circumstances, such procedures may work satisfactorily, even with unstressed populations, but their lack of sound theoretical basis makes them unsuitable as a tool for studying the physiology of microbial survival.

C. Direct Assessment of Viability

1. *Plate and Total Counting*

It is probably desirable here to repeat certain textbook truisms concerning the accuracy of plate and total counts. Plate counts record the numbers of organisms in a known volume able to form a macrocolony on the medium provided and, since their distribution is Poissonian, the standard deviation is the square root of the number counted. Thus it is necessary to count about 300 colonies, but little gain in accuracy arises from counting more. Many errors arise in the serial dilution process, and it is usually preferable to perform a single 1 in 10^4 dilution (e.g.

rinsing a 10 μl. capillary of culture into 100 ml. of diluent) than to do four 1 in 10 dilutions. The necessity to use a non-toxic and osmotically suitable diluent is widely realized, but the fact that cold diluents can cause errors due to cold shock in sensitive populations (e.g. Gorrill and McNiel, 1960; Strange and Dark, 1962) seems less well known. Replicate plate counts on a population of discrete bacteria should always fall within the fiducial limits of a Poissonian distribution and, if they do not, some aspect of the technique should be suspect. For completeness, a dutiful reviewer should cite de Silva's (1953) claim to be able to influence plate counts of typhoid bacilli by mental concentration.

On the other hand, microscopic total counts are normally subject to a systematic error that exaggerates the count. The depth of the chamber varies considerably according to the manner in which it is set up, and for complete accuracy the depth of the chamber should be checked interferometrically each time it is used (Topley and Wilson, 1955; Norris and Powell, 1961). If this precaution is not taken, cultures that are 100% viable often give ratios of total to viable bacteria indicating viabilities of 50–80%, and historically this technical error led to a widespread belief that normal bacterial cultures contained many dead organisms (e.g. Wilson, 1922, 1926). This view is no longer tenable, but the existence of the technical error should alert one to view with suspicion statements of the form: "plate and microscopic counts were equal".

Weibull (1960) reported an ingenious technique for total counts: the suspension under test is set in agar between an ordinary slide and coverslip and whole microscopic fields are counted throughout their depth. The areas of such fields are known from the optical characteristics of the objective, and their depths may be found using the micrometer scale of the fine focusing adjustment, after due allowance for the refractive index of the agar environment. In practice, the accuracy of the method is limited by the extent to which sloping coverslips can be avoided and the exactness with which the micrometer scale can be used. Collins and Kipling (1957) evaporated a known volume of stained aqueous suspension to near-dryness in the presence of about 5 μl. of glycerol; the bacteria concentrated in the tiny glycerol droplet in which, provided clumping did not occur, every cell could be counted. Gabe (1957) counted small volumes of suspensions in flat-walled capillaries; special techniques are needed to manufacture these capillaries. The problems of total counts have been discussed further by Meynell and Meynell (1965). Provided certain conditions regarding orifice size and interference by dust are observed, electronic particle counters such as the Coulter counter (Kubitschek, 1960) can be used to obtain very exact and reproducible total counts of large (>1 μ^3) bacteria. This instrument, however, measures a conductivity signal that is determined by the cell volume as

it flows through a tunnel, and the user should remember that live and dead microbes of the same nominal volume may not have the same effective volume: the writer has observed an apparent net shrinkage of *Aerobacter aerogenes* on adding formaldehyde to a live population. With yeasts or erythrocytes, such alterations are usually readily allowed for in adjusting the instrument's setting; with bacteria, however, the particles are so small that the counter is, perforce, operating near to the electronic noise level and such counts need far more care than those with larger microbes.

2. *Viability by Micro-culture*

Powell (1956, 1958) and Quesnel (1963) used micro-culture to study the multiplication of individual bacteria, and incidentally, to record viability. Valentine and Bradfield (1954) adapted slide culture for the rapid assessment of viability by adding urea to the population and assuming that elongated forms appearing after brief incubation were derived from originally viable cells; Gilliland (1959) used slide culture in a haemocytometer for assessing the viability of yeast populations; Postgate *et al.* (1961) described a simple method of slide culture on agar for the assessment of viability and reported on its statistical accuracy in practice; Bretz (1962) described a similar procedure using blotting paper in place of agar; Jebb and Tomlinson (1960) cultured mycobacteria in agar films suspended in wire loops in order to determine clonal multiplication rates, a procedure which could obviously be adapted for viability determinations. Kogut *et al.* (1965) used micro-culture to follow growth of bacterial clones.

Slide culture is accurate within the range 5–100% viability, and since that is the operational range of most workers concerned with the physiology of death, it is often the method of choice. It is relatively rapid and, though it does not give either a total or a viable count, it gives the ratio of these with Poissonian precision. Results obtained with it differ slightly from conventional plate counts because pairs are normally scored as representing one live parent, though both may be dead; the error so introduced is normally trivial. It is unsuitable for use with organisms that lyse or that form filamentous networks; these and other limitations were discussed by Postgate *et al.* (1961).

3. *An Oblique "Direct" Method*

Wade and Morgan (1954), using the principle that bacteria accumulate "fluctuating RNA" when about to divide, devised a rational vital stain whereby populations were inoculated for a brief period in defined conditions and those that had amassed RNA were distinguished by a

staining procedure from those that had not. Though successful in model mixtures of dead and live organisms, this procedure was unsatisfactory with starved populations of *Aerobacter aerogenes* when tested jointly by H. E. Wade and myself: too many organisms of ambiguous staining reaction were observed.

D. Cryptic Growth

When members of populations of starved microbes die, the survivors are no longer starved. Leakage and lysis products from the dead organisms may support growth of the survivors. This phenomenon, known as cryptic growth (see Ryan, 1959), can interfere with studies on the physiology of starvation. Postgate and Hunter (1962) recorded that death of fifty members of a starved population of *Aerobacter aerogenes*, derived from a chemostat, allowed the doubling of one survivor, a figure closer to four or five deaths per multiplication probably applied to organisms from batch cultures. Cryptic growth can be prevented by adding a non-toxic inhibitor of multiplication, such as penicillin or chloramphenicol; it is particularly important to allow for cryptic growth in studies in which a carbon substrate is added to a starved population (e.g. those concerned with maintenance energy or substrate-accelerated death), because then situations can arise in which death of one cell supports the growth of one and sometimes, in the writer's experience, more than one survivor. Slide culture can give qualitative evidence of cryptic growth because the distribution of division lags in the populations become highly irregular: cryptically grown newcomers among starved populations have typically a short lag phase and show large micro-colonies. Systematic differences in colony sizes after plate-counting also suggest cryptic growth.

Cryptic growth interfered with attempts by Postgate and Hunter (1964) to demonstrate substrate-accelerated death with *Bacillis subtilis*, *Pseudomonas ovalis* and *Candida utilis*. Jannasch (1965) found it interfered with determinations of the lowest substrate concentration accepted by *Spirillum serpens*, and he had recourse to continuous culture for that measurement. Its importance in the design of experiments is not appreciated sufficiently widely. For example, Theil and Zamenhof (1963), in a discussion of DNA turnover in a mutant of *Escherichia coli*, dismiss cryptic growth on the grounds that the species shows no tendency to lysis. In fact, all studies on cryptic growth have used non-lysing bacteria for the simple reason that slide culture would be inapplicable if lysis of the dying population were extensive. Many experiments on "maintenance energy" could be interpreted as yielding the resultant of cryptic growth and substrate-accelerated death; this point is discussed further below.