The Bacteria

Edited by

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Volume IV: THE PHYSIOLOGY OF GROWTH

The Bacteria

A TREATISE ON STRUCTURE AND FUNCTION

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VOLUME IV: THE PHYSIOLOGY OF GROWTH

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PREFACE

The contributions contained in the present volume deal with a variety of subjects, often referred to collectively as "bacterial physiology." Perhaps this area can best be defined by saying that it is concerned with biochemical phenomena that are expressed at the level of the intact microbial cell and often of the growing cell.

The more restricted subtitle "Physiology of Growth" was chosen to exclude those reactions involved in energy release, precursor formation, and biosynthesis which, not infrequently, find their way into textbooks and lecture syllabi on "microbial physiology." For our purposes Volumes II and III subtitled "Metabolism" and "Biosynthesis" consider these topics, while Volume V, "Heredity," considers microbic replication and variation at both genetic and chemical (metabolic) levels.

The classic and often quoted view from the Preface to Rahn's book*

> While practically all the discussion in the book refers to bacteria, the principles developed reach beyond the domain of bacteriology, and apply to biology generally. More than that, I believe that some of the principles of biology can be found and studied *only* with the simplest forms of life, and that general physiology has much to learn from the physiology of bacteria. . .

predated much of the chemical knowledge upon which the chapters of this volume are based, yet his early text emphasized the requisites for growth, regulation, and maintenance. In contrast, recent synthesis and summary by Lwoff† is far forward in present knowledge and terminology:

> . . . The organism has to cope with the variation of the environment. In order to survive, it must adjust its enzymatic equipment according to the nature of its food and to the nature of its needs. And also, the machine must be regulated in such a way that each one of the twenty amino acids is manufactured in just the right amount and proportion. . . .
> . . . Obviously, in a microorganism, no single molecule or group of molecules can be held responsible for the harmonious dynamic cellular balance. The functional order is the result of the interplay of the hereditary material, of the enzymes, of the metabolism, and of the dual feedback mechanism. The metabolism itself is the result of the interaction of enzymes and food, as provided by the environment. . . .

The terms of the definition have changed, the depth of understanding expanded, but the "physiology of growth" still remains a sharp tool—a

*"Physiology of Bacteria," Blakistan, 1932. [Cf., van Niel, C. B., *Bacteriol. Rev.* 8, 225 (1944), footnote 2.]

†See *Biological Order* (MIT Press, 1962), particularly conclusion of Chapter IV, Functional Order, p. 60.

method—providing clues and insight. Still persists also the naiveté afforded to each generation to consider that its branch of science may have accumulated enough of the total knowledge to put the pieces in proper relationship. To those so inclined, these chapters are offered as an extrapolation from structure, metabolism, and biosynthesis to the whole cell; each reader may assemble the aspects in accordance with his choice.

The editors wish to thank the contributors for their consideration and cooperation in supplying this current and clear set of discussions of problems on bacterial growth. They regret that a mathematical description of cultures in static and continuous growth is omitted from the volume; this became necessary because of timing beyond our control.

As in the case of earlier volumes, the publishers and the members of their staff have furnished much expert help and encouragement for which we are deeply grateful.

I. C. GUNSALUS
R. Y. STANIER

December 1962

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CHAPTER 1

Synchronous Growth

O. MAALØE

I. Introduction

For more than a century, bacteriologists have added to our knowledge about growth requirements, metabolic pathways, individual enzymes, and a variety of other functions and components of the bacterial cell; and in recent years, several aspects of the genetic organization of bacteria have been successfully analyzed. In contrast, little is known about the physiology of cellular and nuclear division in bacteria and about the sequence in which these and other elementary acts of replication are linked together to form the normal division cycle. This is not surprising: a culture of growing bacteria is a mixture of cells representing all phases of the division cycle. Physiological and chemical studies on such a culture therefore yield average values only, and few relevant observations can be made on individual, living cells.

Many problems concerning cell multiplication, which cannot be studied on conventional cultures or by direct observation, might be solved by analyzing samples drawn from a culture in which the cells were induced to divide more or less simultaneously. General considerations of this kind have led investigators to look for possible means of producing the desired "phasing" or "synchronization" of cell division in bacterial cultures. Stimulated by the work of Scherbaum and Zeuthen[1] and of Hotchkiss[2] several attempts have been made during the last few years.

A successful experiment of this kind may be very exciting to the experimenter, but it should be realized that the fact that stepwise cell division has been obtained in various ways poses at least as many questions as it

answers. To clarify the situation at this early stage of development, it may therefore be useful to sort out some of these questions.

If nuclear or cellular division can be induced to occur at nearly the same time in the majority of the cells of a culture, it may be possible to demonstrate that certain synthetic or metabolic activities are associated with division. It is, however, at least as important and perhaps more rewarding to analyze the mechanisms underlying these particular induction phenomena. In general, we may look upon induction and inhibition of nuclear or cellular division as complementary experimental approaches.

To study the physiology of the normal division cycle is a different proposition. There are serious objections to accepting artificial synchrony as a means of studying normal growth at the cellular level.[3, 4] Ideally, a system is not acceptable for this purpose unless it consists of normal cells undergoing repeated synchronous divisions. In practice, however, the best we can do to evaluate the usefulness of a given system is to analyze it carefully, and if we find that *unphysiological* states, e.g., abnormal chemical composition or aberrant nuclear morphology, are being produced the system must be disregarded as a source of information about the normal division cycle. An additional, but in itself insufficient, criterion of acceptability is that the sequence of events observed in one cycle is repeated in the next.

II. Technical and Theoretical Problems

A. Procedures and Definitions

With few exceptions the basic criterion of synchrony has been cell division and the colony count *technique* therefore plays an important role. It is not always remembered that individual samples must yield 400 or more colonies if, on an average, 19 out of 20 counts are to fall within 10% of the mean value; if plates with only about 100 colonies are counted 1 count in 3 must be expected to fall outside this interval. These theoretical limits cover the sampling error only; the dilution errors can, with care, be reduced to the point where they may be neglected.[5] It is perhaps pedantic to stress these simple statistical facts; but, in synchronization experiments, short segments of the growth curve must be analyzed in detail, and this simply cannot be done unless many samples are taken and all counts are sufficiently high. Sometimes this basic requirement is neglected.

Another seemingly trivial concern, which does not always receive proper attention, is the culture density. In dilute liquid cultures the cell number increases exponentially with time and the average chemical composition of the cells remains constant. This mode of growth may be described as *balanced*, and it can be maintained almost indefinitely by appropriate

dilution. We shall assume that, under these ideal conditions, *all* cell components double in quantity between successive divisions, and the *normal division cycle* can thus be defined as the sequence of events by which this complete replication is achieved.

When a culture approaches saturation the cells become progressively smaller; this implies that at least some components do not double between successive divisions, and the cycle therefore cannot be called normal in our sense. The critical density, above which growth is no longer balanced, is different in different systems. In aerated broth cultures of *Escherichia coli* or *Salmonella typhimurium* it is usually between 1 and 2×10^8 cells/ml.[6, 7] It is desirable to work near this limit because chemical analysis can then be made on samples of reasonable size, but it is also dangerous because the limit cannot be precisely defined.

Our definition of the normal division cycle implies that changes in the physical or chemical properties of the medium may upset the cycle. We must therefore be prepared to find that many of the treatments which have been used to induce synchrony also modify the division cycle to some extent.

In practice, it is very difficult to decide how profound the modification is in a given system. Some treatments which produce synchrony at the same time produce rather obvious artifacts. In such cases it is clearly impossible to interpret the observations in terms of the normal division cycle, and attention can therefore be focused directly on the effects on cellular or nuclear division which have been produced (see Section III,B, 2 and 3). In other cases artifacts are not demonstrable and a definite pattern is found to repeat itself in successive growth cycles. It is tempting to interpret the observations made on such a system in terms of the normal division cycle; we shall see later that it is not always safe to do so (see Section III,B,3).

B. General Effects of Temperature and Medium Changes

In a number of cases synchrony has been obtained by single or repeated changes of temperature or of the composition of the medium. For a better understanding of the possible mechanisms of synchronization, which will be discussed in Section III of this chapter, it may be helpful first to consider more generally how the *average* properties of growing cells are influenced by temperature or medium changes. Systematic studies recently carried out in the author's laboratory may serve as illustration.

It has been found that prolonged, balanced growth leads to the establishment of one out of a great number of possible physiological states, each of which can be identified by the average size and chemical composition of the cells.[7] The state that is finally established depends on the

medium, and not to any appreciable extent on temperature. *At a given temperature,* the quantities that characterize the physiological state—i.e., the *per cell* values for dry weight, RNA, DNA, and average number of nuclei—all vary exponentially with the growth rate afforded by the medium (see Table I). The so-called "resting state" finds a natural place in this system; thus, in an outgrown broth culture, or under nitrogen starvation, the cell size is reduced to approximately the value predicted for zero growth rate. The data further indicate that the stained bodies referred to as the "bacterial nuclei" contain a constant amount of DNA as would be expected if these bodies represent nuclei in the genetic sense.

TABLE I

EFFECT OF GROWTH MEDIUM ON CHEMICAL COMPOSITION[a]

Medium	Generation time at 37°C., (minutes)	Dry weight/cell	Nuclei/cell	RNA/nucleus	Particles[b]/nucleus	Protein[c] synthesized/minute	
						Per nucleus	Per particle
Lysine-salts	96	240[d]	1.1	22[d]	3,900	1.3[d]	3.3
Glucose-salts	50	360	1.5	31	6,600	3.1	4.6
Broth	25	840	2.4	64	16,000	8.8	5.3
Heart infusion broth	21	1090	2.9	84	24,000	10.6	4.6

[a] Adapted from Schaechter *et al.*[7]

[b] For simplicity, 80% of the total RNA of the cell is assumed to be in particles with molecular weight 2.6×10^6 and containing 63% RNA (the 70 *S* ribosomes; see Tissières *et al.*[8]).

[c] Based on an estimate of total protein; the assumptions involved are discussed by Schaechter *et al.*[7] HCf. Addendum on p. 32.

[d] All weights in grams $\times 10^{15}$ (except in the last column where the unit is 10^{-19} g.).

From the figures of Table I it can be seen that the number of nuclei/cell as well as the mass/nucleus increase with the growth rate. Per nucleus, the mass increment is largely accounted for by an increase in the number of ribosomes. It can be estimated that, *per unit RNA,* the amount of protein synthesized *per minute* is nearly independent of the growth rate.

The transition between two physiological states has been shown to follow a simple pattern (see Fig. 1).[9, 10] If the cells are shifted to a medium affording a *higher* growth rate it is regularly found that the low preshift rates of DNA synthesis and of cell division are maintained for well-defined periods, whereas the rate of mass increase and, in particular, that of RNA synthesis immediately go up. Conversely, if the shift involves a decrease in growth rate, both DNA synthesis and cell division continue

for some time in the absence of net increase in mass and RNA. Clearly, the transition between two physiological states involves a transient period of unbalanced growth.

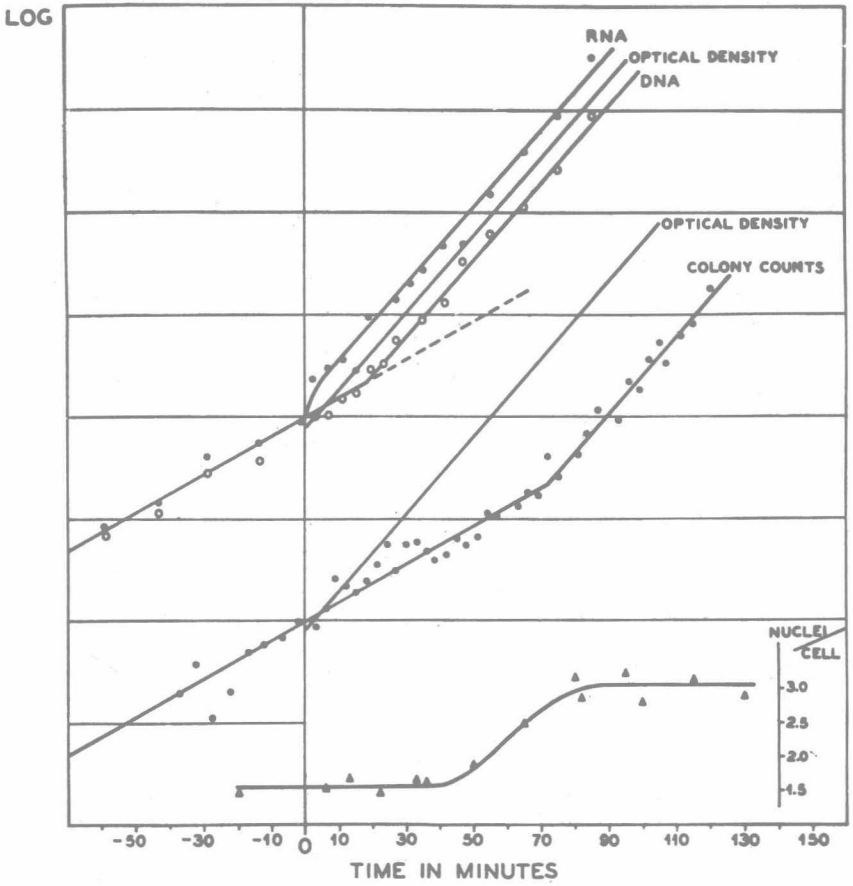

FIG. 1. *Salmonella typhimurium* culture at 37°C., shifted from glucose-salts medium to broth at time zero. Two separate sets of curves are presented: Top: optical density, RNA, and DNA; Below: optical density and viable counts. In all cases the logarithms of the measured values are plotted against time and all values are transposed so as to make the curves representing balanced growth in minimal medium coincide. The distance between horizontal lines corresponds to one doubling. In lower right corner the average number of nuclei per cell obtained from direct counts on stained preparations are plotted against time. (From Kjeldgaard *et al.*[9])

The maintenance of the established rates of cell division and of DNA synthesis for well-defined periods of time after shifting to a new medium suggests the existence of *distinct rate-controlling mechanisms*. In the in-

tact cell, DNA synthesis presumably takes place in the "nuclear regions," and it is natural to imagine that the system responsible for the synthesis of wall material forms part of the cell wall itself or of the cell membrane. The two functions therefore seem to be separable physiologically as well as anatomically.

The control mechanisms invoked to account for the rate maintenance effects observed after a medium shift are not yet fully understood. Recent studies, however, indicate that the most prominent feature of a shift to a richer medium, i.e., the immediate increase in the rate of synthesis of ribosomal and soluble RNA, may be explained in terms of a specific repression mechanism. Genetic experiments by Stent and Brenner[11] and physiological studies by Kurland and Maaløe[11a] independently have led to the conclusion that the soluble RNA itself (or a class of compounds with very similar properties) effects repression of the synthesis of ribosomal and soluble RNA. *Derepression*, i.e. stimulation of RNA synthesis, is observed under conditions which increase the degree of saturation of soluble RNA with amino acids; in other words, a soluble RNA molecule ceases to function as a repressor when it combines with an amino acid.

It has further been shown by Kjeldgaard[10] that, during the transition from slow to fast growth following a medium shift, the rate of protein synthesis increases in strict proportion to the increase in number of ribosomes per nucleus. Thus the number of protein molecules synthesized per minute per ribosome remains more or less constant even during the transition period (cf. the last column of Table I). We shall return to these control mechanisms in Section III,C, of this chapter when discussing a system for synchronization of the DNA replication cycle.

C. The Distribution of Generation Times

As pointed out in the introduction, it is a serious handicap that so few quantitative observations can be made on individual, living cells. Those that can be made are therefore most valuable, and for the analysis of synchronization experiments observations on the distribution of generation times are of particular importance. The classical studies of Kelly and Rahn[12] have been greatly extended by Powell,[13-15] whose observations have almost certainly been made under conditions of balanced growth. In all instances bacterial generation times have been found to fluctuate very considerably. Powell[13] estimated the coefficients of variation (the standard deviation as fraction of the mean) in strains of *E. coli, Klebsiella, Proteus,* and *Streptococcus*. Most experiments gave estimates between 0.25 and 0.31, with exceptional values as low as 0.07. Powell also described the markedly skew distributions from which these values were obtained.

Imagine that all the cells of a culture were brought to divide simultane-

ously once. If the large variation in generation times observed during normal, unrestricted growth applied to this cell population, a fair degree of synchrony would be preserved in the first but very little in the second of the succeeding divisions.[4, 16] On theoretical grounds the chances therefore are that division synchrony induced by a single stimulus will die out rapidly.

Powell[15] has demonstrated a definite positive correlation between the generation times of sister cells and weak, perhaps also positive correlation between mother and daughter cells. He points out that this difference may be due to *a relatively long and variable time* elapsing between complete *subdivision* of a cell into two, physiologically independent units and cell division proper. Powell concludes that cell division must be considered "a phenomenon of secondary importance in growth," and that it "certainly ought not to be too readily assumed to be linked directly with fundamental rate-controlling processes."

We discussed above the indications that a strict rate control is involved in cell division (see Section III,D,1). Following Powell's reasoning it seems natural to assume that this control applies to the fundamental and rather precisely timed process of subdivision (Powell's p-fission); cell division (Powell's o-fission) may then be assumed to follow after a detachment period of considerable variability, which is not under the influence of the control mechanism.

Recent studies by Kubitschek[16a] and by Schaechter *et al.*[16b] and Koch and Schaechter[16c] have confirmed Powell's estimates of the coefficients of variation normally observed during balanced growth on solid surfaces. In the study by Schaechter *et al.* not only the time between cell divisions but also that between *nuclear* division was recorded. The finding that the coefficient of variation is as high for the nuclear as for the cellular interdivision times argues against Powell's conclusion that division is less rigorously controlled than physiological separation. Unfortunately this conflict of evidence is difficult to resolve since the observations involved are made at the limit of the resolution of the light microscope and therefore subject to considerable uncertainty. The importance of the measurements of generation times to synchronization experiments will be discussed in Section III,D.

III. Synchronization Experiments: Methods, Applications, and Interpretations

A. Natural versus Artificial Synchrony

In bacterial physiology whole cultures usually take the place of the individuals studied by animal or plant physiologists. The substitution of a large number of virtually identical cells for a whole animal or a plant

may be a great advantage; in particular if the process to be studied can be precisely induced. The importance of timing is borne out by the rapid advances made in the studies of bacteriophage infection and of induced enzyme synthesis; in both fields, some of the most critical experiments have been possible only because the desired process, infection or induction, can be initiated in a whole population of cells at a precisely defined time. By analogy, we may expect to obtain useful information from the study of synchronized cellular or nuclear division.

In a few instances synchronous division is observed in natural populations of protozoa (see Campbell[4]), and from time to time it has been claimed that significant degrees of synchrony could be demonstrated to occur spontaneously in bacterial cultures. Thus, Hegarty and Weeks[17] observed that the sensitivity of growing cells of *E. coli* to cold shock seemed to change cyclically, and they suggested that this might be due to some degree of synchrony persisting in their cultures. This notion derived some support from previous observations of "fission waves" in cultures of *Bacillus megaterium*.[18] None of these demonstrations is very convincing. By direct observation of the development of microcolonies from single, old cells of *E. coli*, Houtermans[19] found that the first two divisions showed signs of synchrony; the phenomenon was difficult to demonstrate and not very reproducible. Takebe and Yanagita[20] also claimed to have observed weak synchrony during the first divisions in cultures of *E. coli*; the published colony counts hardly support this conclusion. More conclusive is the demonstration that spores of *B. cereus* or *B. megaterium* can be induced to germinate more or less simultaneously. Fitz-James[21] and Young and Fitz-James[22] have used such material for cytological experiments; their results will be discussed later (Section III, B, 3).

The attempts to produce artificial synchrony, which will be described below, constitute a very heterogeneous group. They show that synchrony may be obtained in several ways, and it may be surmised that few of the possible methods have actually been tried. It is practical to distinguish between three techniques: "initial treatments," "repeated stimulus or entrainment methods," and "mechanical selection procedures." A variety of treatments involving changes of temperature or of medium have been used; some of these changes can be readily reversed and repeated stimuli can therefore be applied; in other cases reversion of the change is not practicable.

B. Experiments Involving One or More Temperature Shifts

1. Pretreatments at Reduced Temperature

First we shall discuss the synchronizing effect, originally observed by Hotchkiss,[2] of an initial, low temperature treatment (see Fig. 2). An ex-

ponentially growing culture of pneumococci which is taken from 37 to 25°C., held at the lower temperature for 15 minutes and then returned to 37°C., has been found to be "phased" in two respects: (*a*) during the 5 minutes following the 25°C. period about ⅔ of the cells divided, and for two generations thereafter cell division remained partially synchronized; and (*b*), after returning the cells to 37°C., their susceptibility to the transforming effect of pneumococcal DNA changed in a cyclic manner.[2]

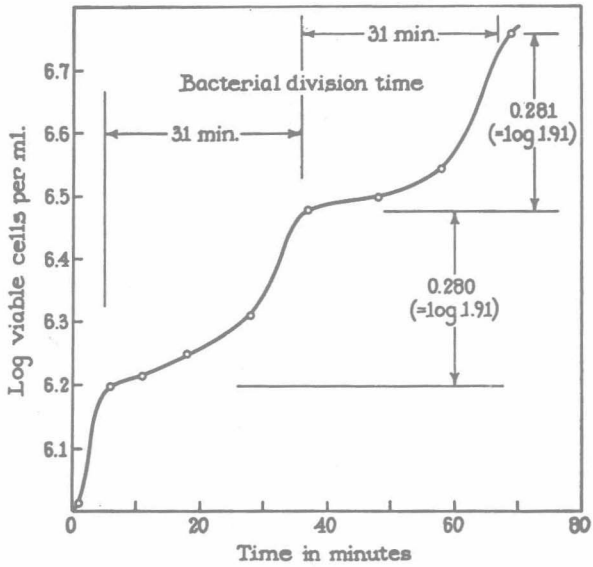

FIG. 2. Course of pneumococcal growth immediately following 15 minutes conditioning at 25°C. Time shown is time of reincubation at 37°C. Each point is the result of 4 to 6 determinations with estimated error of about ±10%. The indicated division time and logarithmic increments are formally derived from the drawn curve and only approximately determined by the experimental data. (From Hotchkiss.[2])

This experiment was actually designed to test the independently derived hypothesis that individual cells are susceptible to transformation only during a small part of the normal division cycle. As pointed out by Hotchkiss, this possibility is not only interesting from the point of view of the mechanism of interaction between cell and agent, but also because slight degrees of synchrony, which would be difficult to register by colony counts, might suffice to produce very marked changes in susceptibility. In his experiments the fraction of the cells transformed by DNA was always increased just after the 25°C. period and then dropped by as much as a factor of 10 to 20 during the next 20 minutes. One or two more cycles of rise and fall in susceptibility were usually observed, but these did not coin-