# SYMPOSIUM ON INITIATION OF BACTERIAL GROWTH[1]

## III. Physiological Aspects of Growth Initiation[2]

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The science of Bacteriology depends in large measure on the ability to obtain pure cultures of microorganisms on artificial media. It is not surprising, therefore, to find that the problem of bacterial growth has occupied the serious attention of microbiologists for several decades. Much is known of the factors influencing microbial growth and considerable attention has been devoted to the kinetics of the bacterial growth cycle, the several phases of which are well recognized even by the beginning student. Yet there is still some confusion concerning the theoretical and operational definition of growth, and the criteria for bacterial death are controversial.

Information relating to bacterial growth is based usually on measurements of the increment in viable plating units rather than the synthesis of protoplasm. However, it is well known that the bacterial cell does grow quite apart from the process of reproduction. Thus, growth should be limited to increases in cell mass rather than cell numbers. Since under optimal conditions each bacterial cell divides regularly into two daughter cells, cell number and cell mass may be directly correlated. The rate of cell division is dependent on the physical and chemical environment and on the genetic constitution of the particular species. It should be emphasized that changes in mass and number may not always be proportional. For example, during the lag phase increases in cell mass are detectable almost immediately, whereas cell division may be delayed substantially. Thus, during the initial growth phase optical density measurements of culture turbidity are better indices of growth than are viable counts. It should be noted, however, that turbidity is affected by changes in the refractive index of the cells, as well as by changes in the shape of the cells, and that dead cells contribute to the total turbidity. It is therefore clear that methodology plays an important role in determining the early period of the growth cycle, namely the lag phase. The work of Hershey (1) supports the thesis that the lag phase is in reality a delay in reproduction rather than in protoplasmic synthesis. The fact that growth and reproduction are indeed distinct and separate entities has been demonstrated elegantly by the studies of Webb (2) and of Nickerson and Sherman (3). The technique utilizing magnesium-deficient media introduced by Webb makes it possible to obtain nondividing bacilli with cell lengths of 20 to 50 $\mu$. Using Webb's technique, Nickerson and Sherman obtained cells of *Bacillus cereus* in the form of long filaments. They established that increased mass of protoplasm was the same for normal dividing cultures and comparable filamentous cultures. It was thereby confirmed that cell division is not an obligatory requirement for cellular growth.

At the other end of the growth cycle we encounter some difficulty in formulating criteria for cell death. A reasonably satisfactory definition is based on the inability of the individual bacterium to reproduce. However, it has long been recognized that cells taken from adverse environments can initiate growth in one medium but not in another. More recent findings on chemical- (4) and photoreactivation (5) of irradiated cells have added to the difficulty of drawing the boundary between viability and death.

The present account is intended to review briefly the physiological factors controlling the primary stage of bacterial growth, namely the initiation of bacterial growth. Since it is not designed to be inclusive, only selected areas will be covered.

When a bacterial cell is seeded into a new

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medium, the factors that will determine whether or not growth will initiate, as well as the rate and extent of growth are (a) the suitability of the physical and chemical environment, (b) the availability of the medium components to the cell, namely, permeability, and (c) the adaptability of the cell, namely, the ability of its biochemical machinery to handle the new situation via synthesis of essential nutrients, vitamins, coenzymes, and enzymes. Although for convenience we shall discuss these three areas separately, it will become manifest that overlapping does occur. For example, environment plays a marked role in determining the nutritional requirements of organisms as well as affecting the permeability of the cell and its capacity to induce enzymes.

Let us therefore first consider the environment. It can be stated that the more suitable the environment the shorter will be the lag period or phase of adjustment and the more rapidly will growth commence. Of the many environmental factors operative we will discuss temperature, pH, and oxidation-reduction potential. These have been demonstrated to affect quite profoundly the nutritional requirements of microorganisms as well as the initiation of growth.

With regard to temperature of incubation, several reports can be cited demonstrating that an increase in temperature results in a gain of nutritional requirement. For example, Maas and Davis (6) obtained a mutant strain of *Escherichia coli* which requires added pantothenate for growth initiation only when the temperature of incubation exceeds 30 C. On the basis of further detailed study they concluded that the mutation resulted in the production of an altered excessively heat-labile enzyme concerned with the synthesis of pantothenate. We have recently encountered a similar temperature effect with *Saccharomyces cerevisiae* (7). Several strains were unable to initiate growth or grew very poorly at 38 C in a chemically defined medium adequate for optimal growth at 30 C. In sharp contrast, these organisms proliferated equally well at both temperatures after inoculation into a complex medium. The addition of yeast extract to the defined medium permitted growth of the organisms at 38 C. Subsequent investigation revealed that the active component of yeast extract was replaceable by calcium pantothenate. Addition of this vitamin to the defined medium supported growth of the yeasts at 38 C to the extent of about 60 per cent of that obtained at 30 C in the same medium devoid of pantothenic acid. It seems likely, in agreement with the studies on *E. coli*, that one or more of the enzymes concerned with pantothenate synthesis in the temperature-sensitive yeast strains are heat labile.

Higher incubation temperatures do not always result in increased fastidiousness. Campbell and Williams (8), for example, found three types of response to temperature change exhibited by 13 strains of thermophiles. Some revealed the same nutritional requirements regardless of the incubation temperature, some required additional nutrients as the temperature increased, whereas others required additional nutrients when the temperature was decreased. They observed that *Bacillus coagulans* and *Bacillus stearothermophilus* required several amino acids and nicotinic acid at 36 C whereas growth took place at 45 to 55 C without an exogenous supply of these compounds.

It is pertinent that the temperature differential need not be very great before nutritional problems arise. Borek and Waelsch (9), for example, found that a temperature differential of 2 C determines the essentiality of phenylalanine for *Lactobacillus arabinosus*. Optimal growth occurred at 35 C but not at 37 C unless the medium was supplemented with phenylalanine.

Turning now to hydrogen ion concentration, this has been demonstrated to play an important role in controlling growth initiation of microorganisms. Recent studies suggest that pH may exert some of its effect by controlling the permeability of the bacterial cell to some substance essential for growth initiation. During studies on the growth promoting effect of autoclaved carbohydrate media on propionibacteria we observed (10) that several 4- and 5-carbon dicarboxylic acids, such as succinate, malate, and $\alpha$-ketoglutarate, were effective replacing agents under certain conditions. The most important condition found was pH. At the usual initial growth pH of 6.8 these acids substituted only partially for the autoclaved glucose effect. However, at a pH of 6.4 their activity was enhanced markedly, whereas at pH 6 they were able to replace completely the growth promoting effect of the heated carbohydrate. Such results are in keeping with the interpretation that the penetration of dicarboxylic acids into the bacterial cell is improved by reduction of the pH.

With regard to oxidation-reduction (O/R) potential, this has been studied most extensively with respect to growth initiation of anaerobic bacteria. We recognize that a reduced potential must be developed before such microorganisms can commence growth. Comparatively little has been done regarding the influence of O/R potential on the growth of aerobic organisms. That the rate of growth of strict aerobes in stationary culture can be a function of the rate at which oxygen diffuses into the medium has been demonstrated by several groups. In such cases forced aeration or mechanical agitation of the culture will improve both the initiation and the subsequent rate of growth (11). Finally, like other environmental factors, O/R potential may affect the nutritional requirements of a microorganism. For example, Koditschek *et al.* (12) found that the requirement for vitamin $B_{12}$ by *Lactobacillus lactis* and *Lactobacillus leichmannii* could be eliminated by growth under anaerobic conditions. Also, Shockman (13) reported that although *Streptococcus faecalis* exhibits an absolute requirement for acetate or lipoic acid and thiamin for aerobic growth, these substances are not required under anaerobic conditions of cultivation.

It is clear therefore that factors such as temperature, pH, and O/R potential influence profoundly the capacity of microorganisms to initiate growth. It is equally manifest that these agents may exert their influence by altering the synthesis of essential materials as evidenced by increased nutritional demands, or by affecting the selective permeability of the cell. It is pertinent to reemphasize that relatively minor changes in the environment may affect markedly the ability of microorganisms to initiate growth. Thus, more careful attention should be paid to the environment when considering problems of growth.

Perhaps the most important environmental agent is the chemical composition of the medium. The completeness of the medium with respect to the nutritional demands of the organism will control both the initiation and the subsequent rate of growth. If the medium is complete, growth initiation will depend primarily on the rate of penetration of the essential components into the cell and the enzymatic constitution of that cell. There are instances, however, where an exogenous supply of a growth factor is not mandatory for growth initiation yet the lag may be reduced significantly if the material is present in the medium. These so-called stimulatory materials are compounds the cell can synthesize but the rate of synthesis, especially from small inocula, may be too slow to meet the demands of the cells for initiation of growth. For example, Morrison and Hinshelwood (14) have studied the lag period of *E. coli* in a synthetic medium containing ammonium salts as the sole source of nitrogen. Incorporation of glutamate or $\alpha$-ketoglutarate into the medium almost abolished the lag. Thus, the lag period may have been principally a reflection of the time required to synthesize $\alpha$-ketoglutarate from glucose, and its subsequent amination to form glutamate.

Another interesting aspect along these lines has developed from the reports of beneficial effects of autoclaved carbohydrate media on the growth of fastidious microorganisms such as lactobacilli, streptococci, and propionibacteria. Information has come from many laboratories with no uniformity in results (15). In our own studies, already mentioned (10), we found that the growth of propionibacteria is not initiated at all or only after a prolonged lag of several days when small inocula are introduced into media containing glucose which was added aseptically after autoclaving of the other medium constituents. However, prompt growth resulted when the carbohydrate was autoclaved with the medium. Of the compounds reported to have some effect in other organisms, only glucosylglycine was partially effective in replacing the growth enhancement produced by autoclaving glucose with the medium, and then only under certain defined conditions. We have already mentioned that certain dicarboxylic acids are effective in this respect, especially when the initial growth pH is reduced to 6. Subsequent work revealed that incubation in an atmosphere of carbon dioxide completely replaces the requirement for autoclaved carbohydrate. Whether the dicarboxylic acids act as sources of metabolic carbon dioxide or as end products of $CO_2$ fixation is not clear at the present time. It is pertinent that the use of large inocula makes the addition of autoclaved glucose unnecessary. Since $CO_2$ replaces the carbohydrate effect, it is possible that larger inocula carry over sufficient metabolic $CO_2$ or $CO_2$-fixation products to initiate growth. Conversely, the larger number of cells may produce $CO_2$ or $CO_2$-fixation products more rapidly than the smaller inocula thus

piling up concentrations sufficient to initiate growth.

One of the more interesting and vexing problems associated with nutrition is that the requirement for a given metabolite may not remain constant, but rather may vary considerably depending on the kind and amount of other components in the medium. This relationship is demonstrated by the peptide requirements induced by amino acid imbalances. The recent report by Demain and Hendlin (16) on growth stimulation of a mutant strain of *Bacillus subtilis* by glycine peptides has been very revealing. A tripeptide of glycine was found to decrease markedly the length of the lag phase and to increase the subsequent exponential rate of growth. Of interest was the observation that the peptide was not required when the amino acids of the medium were deleted. Further work revealed that the amino acid inhibitory to growth was histidine. Thus, the peptide was required only under conditions of amino acid imbalance. One may anticipate peptide requirements to be found more frequently among the fastidious microorganisms whose complex requirements necessitate the use of amino acid-containing media.

In this brief survey of the role of nutrition in growth initiation, we have considered the difference between essential and stimulatory nutrients. It was emphasized that the rate of penetration into the cell plays an important role in the essential nutrients, whereas the rate of synthesis by the cell is an important consideration in the case of stimulatory materials.

We turn now to the second major factor determining growth initiation, namely, permeability. The importance of permeation as a physiological control mechanism is well documented. Furthermore, it is manifest that we could not avoid this aspect when considering environment, since many environmental agents exert a demonstrable influence on the cell membrane. Indeed, permeability represents one of the most exciting areas of biological research today. The concepts of active transport and of stereospecific permeation systems have contributed richly to our understanding of the mechanisms operative in the penetration of organic molecules into the cell (17). However, we are not presently concerned with the process of permeation itself but rather with its role in controlling growth initiation of microbial cells. Initiation of growth will depend primarily on the ability and the rate of penetration of the nutrient through the permeability barrier surrounding that cell, assuming of course that once this is accomplished the cell has the available biochemical machinery to make proper use of the nutrient. Furthermore, since many of the enzymes appear to be organized in subcellular particles of varying sizes, future work will of necessity be directed toward internal permeation problems.

The inhibition of bacterial growth by selective interference with the passage of basic amino acids into the cell has been studied by Mandelstam (18). He established that the permeation of arginine and ornithine into coliform bacteria was competitively inhibited by aliphatic diamines. Then, he tested the possibility that such diamines might inhibit the growth of organisms requiring these amino acids as growth factors by restricting the entry of the amino acids into the cell. If the diamines inhibited growth in this manner and not by interfering with the metabolism of the amino acid after its entry into the cell, one would expect to find inhibition only in those strains of bacteria in which the amino acid concerned must be supplied in the external medium. Employing a mutant strain of *E. coli* requiring arginine or ornithine and the parent wild type which could synthesize these amino acids, he obtained precisely these results. Therefore when studying growth inhibition, interference with the penetration of a required nutrient into the cell must be considered as well as interference with its utilization.

In spite of the increased tempo of research in permeability, little is known of the conditions affecting the transport of B vitamins into bacterial cells. In the course of studies on the nutrition of lactobacilli we observed that the growth of *Lactobacillus arabinosus* from small inocula was characterized by a prolonged lag of about 24 hr (19). Incorporation of a surfactive agent, *i.e.*, Tween 40, into the medium or reduction of the initial pH from 6.8 to 6 resulted in marked reduction of the lag period. On the basis of these and other results we were led to the hypothesis that these organisms have a limited permeability to biotin and that the rate of entry of this vitamin into the cell controls the initiation of growth. While this problem has not yet been resolved, we have found that the penetration of biotin into these cells requires energy and is inhibited

by homobiotin, a homologue of the vitamin (20). Thus the system possesses the properties of active transport and stereospecificity. It is entirely possible that growth initiation may be prolonged in certain bacterial species because of limited permeability to a vitamin rather than, or as well as, to some substrate.

We turn now to the last category influencing growth initiation, namely, the adaptability of the bacterial cell itself. By this we mean the ability of its biochemical machinery to handle the new situation via synthesis of essential nutrients, vitamins, coenzymes, and enzymes. Greater or lesser adjustments in the cells' enzymatic apparatus may be necessary depending to some extent on the age of the cells inoculated and the suitability of the environment. For example, older cells may possess partially denatured enzymes or the labile prosthetic groups may be decomposed or dissociated from the apoenzyme. In such cases the cell may be obliged to resynthesize these apoenzymes or coenzymes before growth will initiate. Finally, if the bacterial cells are seeded into a medium containing a substance not present in the previous medium, and if this substance is essential for growth in the new environment, obviously growth will not commence until an enzyme system capable of handling this substance is developed by the cell. As an example of just such a situation one need only mention the well documented studies on the induced $\beta$-galactosidase system (21).

In summary, the major physiological factors determining growth initiation are the suitability of the chemical and physical environment, the availability of the medium components to the cell, and the adaptability of the bacterial cell itself. To put it quite simply the cell must be provided with the proper environment including nutrients, these nutrients must be able to permeate the cell, and once inside, the cell must have the biochemical machinery to make proper use of these materials. It is clear that none of these factors can be treated individually since each affects the other. Furthermore, although much is known, much work is still required before we can have a total understanding of the intricate relationships among these varied factors. However, the methodology is available and the newer techniques of synchronization and continuous culturing should provide further interesting information. Lest we should have any complacency, however, let us admit that almost all that is known has been accumulated from studies with pure cultures and even here only a relatively few genera have been investigated reasonably well. The growth patterns and growth problems of mixed cultures either purposely prepared or as found in nature are still foreign to us and provide a stimulating and challenging area for further study.

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