On the fiftieth anniversary of the Schaechter, Maaløe, Kjeldgaard experiments: implications for cell-cycle and cell-growth control

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Summary

The Schaechter–Maaløe–Kjeldgaard papers, which have their 50th anniversary this year, have major implications for understanding the cell cycle, control of cell growth, control of cell size, metabolic control, the basic bacterial growth curve, and myriad other bacterial and eukaryotic growth phenomena. These ideas have broad applications that should be considered in current studies of the cell cycle. In particular, the emphasis on steady-state growth conditions, and clear and sharp changes in growth conditions were fundamental to their experiments and have been codified in the principles of the Copenhagen School of Microbiology. ***BioEssays*** **30:1019–1024, 2008.**

Introduction

In graduate school in the laboratory of Norton Zinder, I studied the growth of f2, the RNA bacteriophage. As I began to think about a postdoctoral stint after graduation, I distinctly remember reading the Schaechter–Maaløe–Kjeldgaard papers (hereafter referred to as SMK). I believed that I knew how to grow bacterial viruses, as I had done it for four years. When I read the SMK papers, I thought that these people really knew how to grow bacteria. Therefore I would go to Copenhagen, and the Maaløe laboratory and study how to grow bacteria. Or perhaps I should say, grow bacteria really well. That was the simple idea behind my going to the laboratory of Ole Maaløe in 1963.

For a long time I didn't understand any of the deeper meaning of those papers. Yet, in a sense, I believe my career in science, with some small detours, has been related to understanding, thinking about, and applying the ideas embodied in SMK.

This year is the Fiftieth Anniversary of the SMK papers. There are more famous papers with a 50th anniversary this year—notably the Meselson–Stahl Experiment. But for subtlety, depth and broad applicability, the SMK papers deserve a special recognition.

I will describe the work of SMK, and then describe why this work is relevant to much of science today. They may not be as well known as other papers, but this work deserves reading in order to apply these ideas to current research.

Before the work of SMK, the dominant idea was that cells had some sort of obligatory life cycle. They were born small (as observed in overgrown cultures) and then they grew larger and eventually got small again as the culture became overgrown. This idea was overthrown by the use of steady-state growth in the work of SMK. As discussed below, the idea of a life of a cell culture is still with us, and this result is put in context by the discussion below.

What did SMK do? There were two related papers. The first[1] studied the growth of cells (classical gram-negative bacteria) at different growth rates, which were varied solely by changes in the richness or poverty of the media. Each different growth rate, no matter how determined by medium composition, provided cells of a particular physiological state, defined by the cell size and cell composition. Faster growing cells were larger, had more cytoplasm, more DNA and more nuclei per cell than slower growing cells. And it did not matter how the growth rate was achieved. Minimal media with 10 amino acids or minimal media with five vitamins and some nucleosides that achieved the same growth rate produced cells of the same cell size and composition (Fig. 1).

The second paper[2] studied the transition of cells between growth rates—a shift-up from slow to fast growth and a shift-down from fast to slow growth rate. In the transition studies, the question was how cell size changed when growth rate changed. In both the shift-up and the shift-down, cells were abruptly transferred to another medium supporting a different growth rate. In the shift-up experiments, cells at a slower growth rate were suddenly supplemented with medium constituents to produce a faster growth rate (Fig. 2). How did the smaller cells, produced at the lower growth rate, produce larger cells at the faster growth rate? The converse experiment, a shift-down, was accomplished by one of the first uses of membrane filters where cells were filtered out of rich

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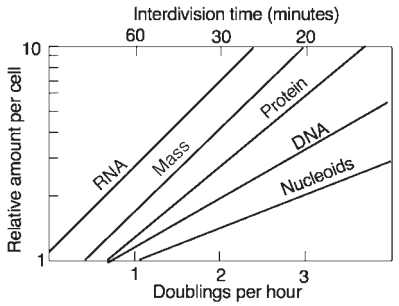

Figure 1. The SMK Experiment. Cells grown at different growth rates have different quantities of cell components. In general, there is more material per cell as cells grow faster, and the results fit a straight line on semi-logarithmic coordinates against the growth rate.

medium and resuspended in poorer medium. The question, in the shift-down, was how did cells get smaller?

To understand the origin of these experiments, one must look at the thinking of Ole Maaløe who had visited the laboratory of Max Delbruck who was then studying bacteriophage growth. Ole was impressed with the simple methodologies that Delbruck used to "synchronize" phage infection—for example, infection at high densities, and dilution down to lower densities to keep the phage infection of bacteria confined to a short time period. Ole began thinking about studying the bacterial cell cycle and using synchronized cells. The question arose, how does one synchronize cells?

Perhaps one could synchronize cells by simply putting cells at one growth rate into a different medium supporting a different growth rate. Would that synchronize cells? I will return to this question after commenting on some of the experimental details of the first paper.

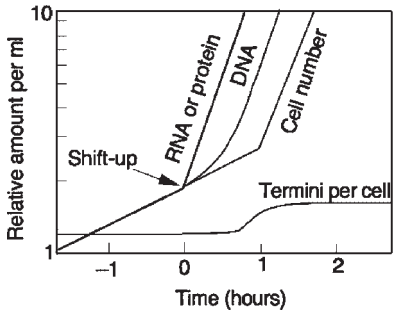

Figure 2. The shift-up. Cells growing in poor medium are then placed in richer medium (supporting a faster growth rate). Note that cell division continues for an extended period of time although mass and DNA replication increase earlier. This result, of necessity, leads to larger cells with more DNA per cell at faster growth rates.

Steady-state growth

One important contribution of SMK was to emphasize the notion of steady-state growth. Rather than study cells emerging from stationary phase and having the confusion of changing physiological conditions, the group studied cells growing for extensive periods in exponential or log growth. During unperturbed exponential growth, the cell composition was invariant, demonstrating that the cells were in the steady-state of growth.

As one reads the details of the experiments, one should be impressed with the rigor of the experimental work used to confirm steady-state growth. For example, the use of a spectrophotometer to determine cell mass at different cell densities was checked and confirmed by measuring the dry weight of cells at different absorbencies to show that absorbance at different cell densities truly measured cell mass.

The key result of SMK was that there was a relationship between growth rate (considered as the inverse of the doubling time) and cell composition. They found that the log of various cell constituents—mass per cell, nuclei per cell, DNA per cell—was proportional to growth rate. The faster that the cell grew the larger the cell, the more DNA per cell, and the more nuclei per cell. And the results fit an exponential or log function (Fig. 1).

When the measurements of various cell elements were determined, they found that the amount of each component followed a straight line on semi-logarithmic coordinates. It is worth noting the importance of this graph. Because there was scatter, even on rectangular coordinates the data would have been impressive and quite publishable. But the plotting of the data on logarithmic coordinates had an important implication with regard to the determination of cell size.

Determination of cell size

How was cell size and cell composition determined by the growth rate? The history of this is clear. Ten years after the SMK results, the pattern of DNA replication during the cell cycle was determined.[3,4] The two main results were a constant period for DNA replication and a constant period between termination of replication and division. These are the constant C and D periods for *E. coli*. Willie Donachie[5] was then able to combine these two results, the constant C and D periods, and the log graph of Schaechter, Maaløe, Kjeldgaard, to conclude that there was a constant "initiation mass" at which DNA replication was initiated. The "initiation mass", which is the mass per DNA origin at the moment of initiation of replication, was constant over a wide range of growth rates. One can wonder whether the constant initiation mass idea would have been so easily observed if the Schaechter, Maaløe, Kjeldgaard results had been plotted on rectangular coordinates, and Donachie would have had to convert the data to a logarithmic function.

Therefore one of the main results to stem from the Schaechter, Maaløe, Kjeldgaard work was the idea of a constant initiation mass. The idea of a constant initiation mass has been criticized,[6] but a reanalysis has shown that, in fact, the data support a constant initiation mass.[7] And it is the initiation of DNA replication when the initiation mass per origin is achieved during cell-cycle growth that leads to the determination of cell size at a particular growth rate. We do not yet completely understand the mechanism of the initiation mass, but this concept is certainly an important contribution of the Schaechter, Maaløe, Kjeldgaard results for understanding cell size determination. And the initiation mass model implies that it is the accumulation of material at a steady rate during the cell cycle rather than the cycle-specific synthesis of an initiator that starts DNA replication.

Rate maintenance

The study of the transition between different growth rates, particularly the shift-up, revealed the ''rate maintenance'' phenomenon. When slow-growing cells are supplemented with richer media, the cell number rate does not abruptly increase in rate although the rate of mass increase changes almost immediately. The combination of a continued slow increase in cell number with a more rapid increase in cell mass in the shift-up culture means that the mass per cell increases.

We now understand the origin of rate maintenance. Simply put, when a shift-up occurs, there may be a rapid insertion of new initiations of DNA replication, but none of these new initiations can pass through the C and D periods faster than the sum of C and D. Thus, an increase in the rate of cell division is delayed for approximately 60 minutes. This is the time for new initiations of DNA replication to be associated with new cell divisions.

On whole-culture methods to synchronize cells

The initial impetus for studying cell growth in different media was to try to use a shift between growth rates to synchronize cells but in their attempt to synchronize cells using a shift-up, the Schaechter, Maaløe, Kjeldgaard group appeared to fail spectacularly as there was no synchronization of cell divisions following a medium shift. However, following a shift from slow growth to rapid growth, there was a simple pattern. Shortly after a shift-up, the cell mass (measured by absorbance) increased immediately, but cell division continued unaltered for an extended period of time—the rate maintenance period—about an hour. The combination of increased mass without a concomitant change in cell division led to cells growing in size.

In retrospect, this result is a clear demonstration that one cannot synchronize cells by any use of whole-culture methods. Consider cells growing in steady state growth. At some particular time ''zero'', there are cells of different ages. One can consider the increase in cell number during exponential growth as the sequential division of cells from the oldest down to the youngest and this pattern repeating again and again. There is what I have called a ''conservation of cell age order''.[8]

The deeper reason that cells conserve age order is that there is no change in the cell size distribution when cells are subjected to a shift-up or a shift-down. If the size distribution in exponential growth varies over approximately a factor of two (the dividing cells are twice as large as newborn cells) then any change in the entire culture, where all cells are treated equally, does not narrow this size distribution. Since initiation of DNA replication is related to the cell achieving a certain cell size, this means that cells of different sizes initiate DNA replication at different times and thus are not synchronized by a shift of media. This is the fundamental reason that cells are not synchronized by whole-culture methods.

Bacterial application to the mammalian cell cycle

It is of interest to apply the Schaechter, Maaløe, Kjeldgaard results to the analysis of the eukaryotic or mammalian cell cycle. Briefly, the study of the eukaryotic cell cycle is a hot field. It has been proposed that a large number of genes are expressed at different times during the cell cycle. But essentially all of this work was done using whole-culture methods (along with a few selective synchronization methods). For example, cells have been allowed to overgrow and stop growing—the so-called G_0 phase—and then these cells were diluted into fresh growth conditions. Alternatively cells have been inhibited by various chemicals—thymidine, mimosine, hydroxyurea, nocodazole—or starved of serum or various amino acids. All of these methods are whole-culture methods where all of the cells in a growing culture are treated equally. All of these ''whole-culture'' methods do not work. I have written elsewhere of the detailed proof of why whole-culture methods do not work[9] but, perhaps, it is of interest to just touch on a few experimental proofs of this proposition.

For example, when cells are inhibited by thymidine, there is no narrowing of cell size.[10] Nocodazole-inhibited cells similarly do not show any narrowing of size distribution.[11] With Nocodazole the DNA distribution does narrow, with all cells achieving a G_2-phase amount of DNA (as they are arrested at mitosis), but these cells are not representative of cells at a particular cell age during the normal, unperturbed cell cycle. To summarize the basic result, in all of these whole-culture synchronization methods there is no observed synchronization of cell divisions.

The major implication of this critique of whole-culture synchronization, and hence a major implication of the shift-up results of Schaechter, Maaløe, Kjeldgaard is that one must be skeptical of the vast amount of data on cell-cycle patterns of gene expression in eukaryotic cells because the vast majority of the methods used do not synchronize cells.[12–14] This critique is in addition to the well-accepted problem that

whole-culture methods may introduce artifacts that are not present in unperturbed cells.

On selective methods and the baby machine

Does this mean one cannot synchronize cells? No, it only means that whole-culture methods, where all cells are treated identically, cannot synchronize cells. One can, however, synchronize cells by using selective methods. In contrast to a whole-culture method—where all cells are retained after a common treatment of all cells—selective methods remove a subset of cells from a culture, so the cells removed from the culture share a narrow age distribution, and thus a narrow size distribution, and these cells produce a synchronized culture.

A number of different selective methods have been used over the years—elutriation to select cells of a particular cell size is one notable example. But I suggest that the baby machine, developed by Helmstetter,[15,16] is a superb example of how a selective method works. Cells are bound to a membrane so that on the membrane there are cells of all ages. As cells grow on the membrane, newborn cells are released by division. And these released cells collected from the membrane are all cells of a common age (newborn) and thus they produce a synchronized culture. Thus from the cell culture growing on the membrane, with cells of all ages present, one selects out the newborn cells and discards the rest.

Recently Helmstetter has extended the method to eukaryotic cells[17,18] with results that may be even more impressive than the bacterial results. We have studied the mammalian cell cycle using cells produced by the baby machine.[19] Cells at time zero are smaller and have a narrower size distribution than the cells in the total culture and, with time, cell size increases until cells divide synchronously. Further, the pattern of DNA content is just what one would expect from synchronized growth, where cells go from G_1-phase amount of DNA to S-phase to G_2-phase amount of DNA before cell division occurs.

As an aside to this issue, it is important to consider a small but exciting experiment by Schaechter, Bentzon and Maaløe[20] on the pattern of DNA replication in *Salmonella*. A few years before, Karl Lark and Ole Maaløe had used a whole-culture method to synchronize cells. They used repeated temperature shifts to "synchronize" cells.[21,22] They found in those cells that DNA replication occurred in the middle of the cell cycle, with the total cell-cycle pattern reminiscent of G_1, S, and G_2 phases of animal cells. What Schaechter, Bentzon, and Maaløe found was that if one just took exponentially growing cells and pulse labeled them with thymidine, all of the cells were labeled. This clearly indicated that the results from the temperature-shift experiments were artifacts of the treatments and that, in unperturbed exponentially growing normal cells, there was a continuous synthesis of DNA throughout the cell cycle. This experiment is a clear proof that one must be wary of whole-culture methods of synchronization.[23]

On the classical bacterial growth curve

The SMK experiments, and the reaction of cells to shift-ups and shift-downs, have meaning for what is perhaps one of the central ideas in basic microbiology as taught in classes all over the world: the classical bacterial growth curve. There is a classical pattern of growth following inoculation of an overgrown culture into fresh medium. First there is the lag phase, where cell number remains the same for a period of time. Then cell number begins to increase and, for a period of time, there is log or exponential growth for all cell components, and when the cell concentration gets too high cell number increase ceases and one enters a stationary phase. This may be followed, if one uses viable counts to measure cell number, a death phase where cells not only cease dividing but also cease to be able to grow and produce a colony.

The SMK experiments indicate that the bacterial growth curve as studied and taught is merely a special case of a shift-up and a shift-down (Fig. 3). Consider an overgrown culture where cells have ceased growing. These cells have a zero growth rate. Now shift these cells to a fresh medium supporting a more rapid growth rate. The rate maintenance phenomenon means that there will be a zero growth rate in cell numbers, while mass increase starts immediately. The lag in the increase in cell number—rate maintenance at zero growth rate—and the increasing mass during lag phase means cells get larger.

Conversely, as cells overgrow the medium, mass first ceases to increase, while cell division continues at the original rate—rate maintenance. Thus, increasing cell numbers without a concomitant increase in cell mass leads to smaller cells as cells enter stationary phase.

Thus the classical bacterial growth curve is really a laboratory artifact of using overgrown cultures taken from the

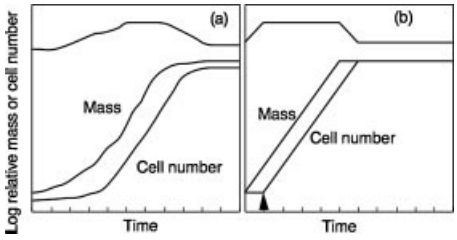

Figure 3. Reinterpretation of the classical growth curve of bacteria. **a:** The classical result of starting an overgrown culture in fresh medium illustrates the early increase in mass with a later increase in cell number. **b:** This result is shown in idealized terms as a shift-up from slow growth (zero growth rate with rate-maintenance at zero growth rate for cell number) to faster a growth rate. The cell size changes are illustrated at the top.

previous day to start up a growing culture. I suggest that the results of SMK indicate that one should teach the shift-up and shift-down results in classes, and then consider the classical growth curve as a special case of shift-ups and shift-downs.

On ribosomes and protein synthesis

The central role of ribosomes in protein synthesis is now well understood. It is interesting to note that the measurements of RNA and protein content in the growing cells were important support of the idea that ribosomes are the protein-synthesizing machines. More important, the data from different growth rates implied that the ribosomes are working at constant efficiency. That is, at different growth rates the ribosomes are fully employed in protein synthesis—no ribosomes are idle—and that the rate of protein synthesis per ribosome is constant and independent of growth rate.

On abrupt changes

The SMK experiments studied abrupt changes in growth conditions. During a shift-up, they suddenly added nutrients to a poorer medium. During a shift-down, they filtered the cells away from the richer medium and resuspended the cells immediately in poorer medium. Thus, rather than seeing the slow change in mass synthesis rate that occurs during the classical bacterial growth cycle, they were able to time alterations in growth and synthesis from the moment of the abrupt change.

The alternative experimental analysis is perhaps best seen during the approach of cells to stationary phase. As cells achieve a greater cell density the medium becomes depleted of some nutrients, and various deleterious changes occur in the medium. Perhaps the pH gets lower or perhaps oxygenation is reduced. In any case the cells stop growing as mass accumulation stops. But the physiological changes are the result of a complex sequential series of adaptations of the cells to the slowly changing medium. The implication of this idea is simple. If one wanted to study the physiology of cells in stationary phase, it would be better to take medium from an overgrown culture, filter out the cells, and resuspend cells growing in steady-state exponential growth into this ''overgrown'' medium at high density. One would thus see the response to immediately changing conditions rather than the consequence of slow historical changes as the medium deteriorated for growth.

Perhaps the best current application of this idea is in the study of sporulation. When sporulation is studied, cells are inoculated into a growth medium that will eventually yield spores in all cells. But the cells, as they grow to higher densities, are changing the medium slowly to the final sporulation medium conditions. The SMK experiments suggests that one could get better control of the sequence of events during sporulation if cells are taken during exponential growth, and resuspended at high density in conditioned sporulation medium prepared by removing spores from an overgrown medium. All cells would then respond in a more responsive manner to the medium rather than to accumulated changes over time.

Conservation of cell age order

Microbiology is an overwhelmingly experimental science. Overriding generalizations or laws are rare, and it appears to be the accumulation of large amounts of data that explains the nature of microorganisms. Therefore one may be suspicious of an idea that is extremely important, and that has general theoretical applicability as a critique of all whole-culture methods of synchronization. This is the law of ''Conservation of cell age order''. I suggest that, if there is a proposal of an experimental proof that the Law of Conservation of Cell Age Order is violated—for example, by synchronizing cells with a whole-culture method, the right response is to state that this is impossible. This law is a direct result of the SMK experiments.

The Copenhagen school

The summation of the ideas discussed above may be considered within a simple set of ideas embodied in the concept of the Copenhagen School. The Copenhagen School of Microbiology is not an actual bricks and mortar school, but rather a school of ideas descending from the Ole Maaløe and his colleagues.

The central tenet of the Copenhagen School is to not perturb or disturb cells. Cell should be treated gently, or at least precisely, and under very well-controlled conditions. Without going into the details of the history of these ideas, it is important to recognize that the best results regarding cell physiology and the cell cycle came from experiments that did not perturb cells.

The Copenhagen School ideas should be studied in classes and incorporated into the current work. Most important, the implications of the shift-up to the problem of whole-culture synchronization should be the main take-home lesson. There may be cyclical patterns of gene expression as shown by various microarray experiments, but the Law of Conservation of Cell Age Order would attribute many of these changes to the perturbations of the experimental approach rather than to the natural cell cycle. To summarize, results may be related to the perturbing methodology, but they are not related to the normal, unperturbed cell cycle.

Although many of the ideas from the Copenhagen school are clear and obvious, and are easily incorporated into the work in laboratories today, many ideas are quite subtle. I suggest that it is time to explicitly incorporate the ideas of Schaechter, Maaløe, Kjeldgaard into current work, and to recognize the importance of these ideas.

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