

Control of mammalian cell size and cell size maintenance:

**Analysis of the model of Conlon and Raff,
exponential and linear growth, checkpoints,
and control of the mammalian cell cycle**

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Running Title:

Regulation of mammalian cell size

Abstract

Mammalian cells growing in cell culture retain a relatively narrow size distribution and do not grow either larger or smaller during extended growth. Conlon and Raff (J. Biol. 2003; 2:7) propose that mammalian cells grow linearly during the division cycle. Linear growth means that cell growth is independent of cell size. According to the model of Conlon and Raff, this implies that such linearly growing cells do not need a size checkpoint to maintain a constant distribution of cell sizes. If there is no cell size control system, as Conlon and Raff propose, then exponential growth is not allowed as exponential growth would require a “cell size control” system. A reexamination of the model and experiments of Conlon and Raff indicates that exponential growth is fully compatible with size maintenance, that mammalian cells have a system to regulate and maintain cell size that is related to the process of S phase initiation, and that mammalian cell size control and its relationship to growth rate—faster growing cells are larger than slower growing cells—is explained by the initiation of S phase occurring at a relatively constant cell size coupled with relatively invariant S- and G2-phase times as interdivision time varies. This analysis of the mammalian cell cycle explains the mass growth pattern during the division cycle, size maintenance, size determination, and the results of a shift-up from slow growth to more rapid growth.

The Journal of Biology has published three articles devoted to a proposed new model of mammalian cell size regulation. The key article, by Conlon and Raff [1], is the experimental paper. An accompanying paper by the scientist/science writer Jonathan Weitzman [2], comments on and extols the Conlon/Raff experiments. Weitzman also interviews others regarding their responses to the new model proposed by Conlon and Raff. The third paper, a short commentary by Grewel and Edgar [3] on the general question of cell size control and its relationship to progression through the cell cycle, also praises and supports the Conlon/Raff model.

According to Weitzman [2], Conlon and Raff have described a series of experiments that question a basic assumption about the way mammalian cell size is maintained during proliferation. As described by Weitzman, the proposal of Conlon and Raff “demolish” the currently and widely accepted doctrine regarding control of mammalian cell size.

What have Conlon and Raff so critiqued and demolished with their experiments? The question revolves around how cells maintain constant cell size during cell growth. In a cell culture growing over many generations, the cell size distribution does not vary and get broader over time. Cells do not get progressively larger nor do they get progressively smaller. One formulation of this simple result is to say that cell mass increase is regulated during the cell cycle so that there is no disparity in the rate of cell mass increase and the rate of cell number increase. Total cell number and total cell mass increase in parallel during unlimited exponential growth. If there were a disparity or disproportion in the rate

of mass and cell number increase, cells would get either larger or smaller during extended growth.

According to Weitzman [2], the problem of cell size coordination has been a neglected field. Weitzman writes: “Raff was perplexed that such a fundamental issue has attracted relatively little attention over the last couple of decades...as the impressive advances in understanding the mechanisms of cell cycle progression have vastly overshadowed any insights gained into how cells coordinate their growth with their size.” Raff is quoted as saying “It’s quite inexplicable why this question has been so neglected.” Additional support for this idea comes from a quote (to Weitzman) from yeast cell biologist Doug Kellog (Santa Cruz, CA) who shares Raff’s amazement and says: “We really don’t know very much about this fundamental issue...It’s one of the last big unsolved problems in cell biology.”

What is the problem under analysis? In Weitzman’s article it is most succinctly summed up by Robert Brooks (Kings College, London) who says, “If [cell] growth is exponential, then cells must have a size control over division, since otherwise random differences in size at division would increase continuously from generation to generation. This does not happen. Conversely, if growth is not exponential, then such a size control is not necessary.” This quote from Brooks may be thought of in this way. Consider two newborn cells of slightly different size. Exponential growth means that cell mass would be made in proportion to the extant cell mass. The larger cell, having a head start, would increase its mass at a more rapid rate than the smaller cell. When the cells divide some time later, the dividing cell produced by the initially larger cell would be even larger compared to the dividing cell produced by the initially smaller cell. Given an

approximate equipartition of cell mass at division, the new daughter cells would have an even more disparate size difference. Exponential growth in the next cycle would lead again to larger differences than in the previous cycle. According to this reasoning, the cell size distribution would grow increasingly broader. Since this is not observed, it is suggested that either a cell must grow “linearly” or if a cell grows exponentially the cell must have a cell size control system. A similar reasoning process implies that if there is no cell size control system, then cells must grow linearly.

The experiments of Conlon and Raff [1] are presented as supporting linear cell growth. Linear cell growth means that there is a constant mass increase during each interval of the cell cycle. Furthermore, comparing their results on mammalian cells to what is referred to as the “yeast” model of cell size control, Conlon and Raff [1] conclude that mammalian cells have a different mechanism for cell size control. As Conlon and Raff summarize their experimental conclusion:

We show that proliferating rat Schwann cells do not require a cell-size checkpoint to maintain a constant cell size distribution, as, unlike yeasts, large and small Schwann cells grow at the same rate, which depends on the concentration of extracellular growth factors.”

Here I present a different view of cell size control and cell size maintenance. Not only has the size maintenance problem not been neglected, but the way in which cells maintain a constant cell size is very much understood. There is no problem with either linear or exponential mass increase for size maintenance. Size maintenance does not depend on which pattern of cell mass increase occurs within a cell cycle. As discussed

below, the preferred—and experimentally and theoretically supported—pattern for mass increase during the division cycle is exponential growth or exponential mass increase. But the exponential pattern poses no problem for size maintenance. Constancy of cell size is fully compatible with an exponential pattern of mass increase as well as the hypothetical linear pattern of mass increase. No major difference between yeast, mammalian, or bacterial cells need be postulated to account for the constancy of cell size during the growth of cell cultures. And, in contrast to the absence of a cell size control system in mammalian cells, it is shown that mammalian cells do have a very simple size control system. The formal elements of this system are similar to that found in the control of the bacterial cell cycle.

According to Conlon and Raff [1], it has been generally assumed that mammalian cells grow according to the tenets of the yeast model. The yeast model is described as a “checkpoint” system for cell size control. Cells with a checkpoint system have some mechanism to check the size of the cell in order to ensure that cells do not grow too large or too small. The main conclusion of the Conlon/Raff experiments is that the yeast model does not apply to animal or mammalian cells. That is, there is no cell size control system in mammalian cells.

Here it is shown that the problems raised by Conlon and Raff have been answered before, and that the solution to size maintenance has been available since 1968. It is merely that the solution to the size-constancy problem is not well known. It is this piece of missing information that accounts for the praise and endorsement of the Raff/Conlon experiments in the article by Weitzman [2].

Outline of the analysis

The analysis is composed of four parts. First it will be explained how exponential growth is compatible with the maintenance of a constant cell size. This idea is also extended to show that the same principles allow linear cell growth (although it is proposed that cells do not grow linearly). The second part of the analysis will deal with a rigorous discussion of what cell mass increase means, how it can be measured, and problems with the experiments of Conlon and Raff. The third part will explain the bacterial model of cell size determination. It is this successful model of size determination that will be applied to mammalian cells. The fourth part of the analysis applies the bacterial model to mammalian cell growth in order to extend the analysis to a mechanistic explanation.

Cell size maintenance with exponential and linear mass increase

Can cells grow exponentially during the division cycle and maintain a constant cell size? Consider two possible cases of exponential growth. For the first case (Fig. 1a), three cells of the same newborn size have slightly different rates of mass increase. If all three of the cells in Fig. 1a were to have the same interdivision time, the dividing cells would have disparate sizes. But if the interdivision times vary so that cells divide at the same cell size, then cell size is maintained even with exponential growth during the division cycle. A newborn cell that makes mass at a rate slightly faster than average will divide earlier than cells with an average or below average rate of mass increase (Fig. 1a; arrows indicate division times). Conversely, a newborn cell that makes mass at a rate slightly slower than average will divide later than cells with an average or above average rate of mass increase (Fig. 1a). Variation in interdivision times allows maintenance of

constant average cell size even with exponential mass increase. A second case (Fig. 1b) starts with different sized newborn cells that synthesize mass at the same rate. As in Fig. 1a, the earlier a cell reaches the division size, the earlier the cell will divide and the cell will have a shorter interdivision time. Size constancy is maintained even though mass increases at a constant rate for all three cells with initial different sized newborn cells (Fig. 1b). Mixtures of initial size variation and variation of rates of mass synthesis can be analyzed in the same manner; the analysis is strongly supported by experimental data on the variation of mammalian cell interdivision times determined by time-lapse cinematography [4].

Linear cell growth during the division cycle (Figs. 1c and 1d) can also produce size maintenance. Whether cells reach the division size earlier due to a larger initial cell size or more rapid mass increase, the cell size at division can be the same for all cells. Thus size maintenance is also consistent with linear growth

The patterns shown in Figs 1a,b,c,d show that there is no impediment to size maintenance as long as we do not consider interdivision times invariant. In all four panels in Fig. 1 the interdivision time varies depending on the time required for a newborn cell to reach a particular cell size.

To be rigorous and precise, it is not proposed that cells always divide at “exactly” the same size. There is a statistical variation in mass increase and interdivision times that can lead to variations in cell size at division [4]. The important point is that when cells deviate from the mean size there is a return to the mean size through compensating interdivision times during the next cell cycle. Large cells will have a relatively shorter

interdivision time, not adding as much cell mass relative to smaller cells, leading to a return to the average cell size.

Of course, variation in the rate of mass increase can also affect size homeostasis. A larger newborn cell that happens to have a slightly lower rate of mass increase during the division cycle may return to the mean size without having a shorter interdivision time. In this case, the interdivision time may or may not vary as the variation in mass increase may compensate for the different newborn cell sizes. The particular result depends on the relationship of the variation in mass increase and the variation in newborn cell size [4].

It is not proposed that a large, newborn cell “controls” its mass increase to have a slower rate of mass increase thus compensating for the initial larger cell size. Nor do small cells increase their rate of mass increase to compensate for their initial mass deficit. Mass increase variation is postulated to have some inherent statistical variation [4]. This means that there is a variation in cell mass increase for all cells and the precise rate of mass increase in a cell is independent of cell size. A large newborn cell could have a faster than average rate of mass increase. In this case, the interdivision time would be even shorter to compensate for both the larger initial newborn cell size and the greater than average rate of mass increase.

The overall pattern of size maintenance is illustrated in a different manner in Fig. 2, where the production of large and small cells can arise either by differences in interdivision time; newborn or baby cells (b) have a somewhat shorter interdivision time to produce small (s) cells or a slightly longer interdivision time to produce large (l) cells. Large and small cells may also occur by some deviation from equipartition of cell mass at

division so that an average-sized dividing cell produces one large (l) and one small (s) cell. The return of small and large cells to the average cell size occurs in the next generation by variation in interdivision times so that small cells (s) have a longer (on average) interdivision time than larger (l) cells (on average).

It may appear that this simple analysis is merely begging the question by not stating how the cell “knows” to divide at a particular size. This question will be answered below. But first, more preparation must be made before explaining how mammalian cells regulate their cell size and maintain a constant cell size even with exponential mass increase. Two issues should be dealt with. First, a discussion will clarify the relationship of cell mass increase to cell size. And second, problems with the proposal of linear growth during the division cycle will be described.

What is meant by the proposal that large cells grow faster than small cells?

What is meant by the Conlon and Raff proposal that, in yeast culture, large cells grow faster than small cells? And similarly, that in mammalian cells large and small cells grow at the same rate? There are four different meanings that can be given the notion of the rate of mass increase and its relationship to cell size. These different meanings lead to some verbal confusion that should be clarified.

One meaning of the proposal that large cells make mass faster than small cells is that given two cells of disparate sizes, the absolute rate of increase in cell mass is greater in the larger cells. A cell of size 2.0 might add, in some time interval, 0.2 units of cell mass, while a cell of size 1.0, in that same time interval might add only 0.05 units of cell

mass. This pattern illustrates a clear and unambiguous difference in the rate of mass increase that is related to cell size.

A second meaning of cell size affecting the rate of mass increase considers that a cell of size 2.0 adds 0.2 unit of mass and a cell of size 1.0 adds 0.1 unit of cell mass over the same time interval. Of course, this case could arguably be said to be a constant rate of mass increase, as the rate of mass increase is proportional to the amount of extant mass. This second proposal is equivalent to mass increasing exponentially. This is because as extant mass changes during the cell cycle the absolute rate of mass increase also changes to reflect the newly added cell mass. After the cell of size 1.0 grows to size 1.1, in the next time interval rather than 0.1 units of mass being added, there are 0.11 units of mass added to the cell mass. Just as interest is compounded in a bank account, and the funds grow exponentially, so mass in this second example increases exponentially.

A third meaning of the variation in mass increase with cell size is that the rate of mass increase is determined at birth and continues throughout the cell cycle, unaffected by continued cell size increase. A relatively small newborn cell could have a rate of addition of “X” units per time interval, and this rate would remain constant even as the cell increases its cell mass. The larger cell would add more than “X” units each time interval and not change this rate during the cell cycle. This pattern of increase would be called linear synthesis during the cell cycle.

It is interesting to think about these different meaning when considering the theoretical graph drawn by Conlon and Raff [1] to illustrate the return of cells of disparate sizes to the same cell size. As shown in Fig. 3 (redrawn from Fig. 1 of Conlon

and Raff [1]), consider two cells, one of size 1.0 and one of size 10.0. During one generation of growth 5.5 units of mass are added by the smaller cell to produce a dividing cell of size 6.5, and 5.5 units of mass are also added to the larger cell to produce a dividing cell of size 15.5. As discussed by Conlon and Raff, upon cell division the daughter cells produced by this pattern of growth would be sizes 3.25 and 7.75. Repeating this each generation (5.5 units added to each cell independent of the extant newborn cell mass) leads, according to Conlon and Raff, to a convergence of cell size as shown in Fig. 3.

But no indication of the length of the division cycles is given in Fig. 3. If the interdivision times are the same for the large and small cells, which is implicit in, and not excluded by, the analysis in Fig. 3 (Conlon and Raff's Fig. 1), the relative rate of mass increase for the larger cell is $5.5/10.0$ or 0.55 and the relative rate of mass increase for the smaller cell is $5.5/1.0$ or 5.5. From this point of view, the ratio of the rates of mass increase is a factor of 10, with the smaller cell making mass from its mass at 10 times the rate (relative to extant mass) compared to the larger cell.

But if the absolute rates of mass increase were the same, then the smaller cell would have a much longer interdivision time than the larger cell. If, over a unit time, 1.0 unit of cell mass were added to the larger newborn cell, and that cell divided at size 11.0, then the interdivision time would be that unit time. The smaller cell, however, would require 10 time units for its interdivision time, because that is the time required to reach size 11.0 as 1.0 unit of material is added to each small cell during each unit of time. The smaller cell grows for a longer time before division. After this first division the new

daughter cells produced by each of the initial cells would be the same size. By allowing interdivision time variation, cell size uniformity is restored in one generation.

A similar analysis can be made for exponential mass increase (i.e., mass added proportional to extant mass). If the cell of size 10.0 added 1.0 unit of mass in a unit of time, then the small cell would add 0.1 unit of mass in that same time interval. In this case, there would be even more time required for the small cell to reach the division size of 11.0. In any case, exponential growth coupled with interdivision time variation can allow size maintenance because both the large and the small newborn cells will divide at the same size.

Of course, the example given by Conlon and Raff (Fig. 3) as discussed here is unrealistic. Cell sizes do not vary over a factor of 10 in exponential culture. But this re-analysis of Fig. 3 illustrates the power of considering different interdivision times as a factor in maintaining constant cell size.

Robert Brooks (personal communication) notes that in some of his experiments cell size is observed as very variable. He states that in experiments with Shields they found that size varied over a range of at least 6-fold. In response it can be pointed out that recent careful measurements of the size variation during the division cycle of cells grown under ideal conditions indicates that size variation is not broad [5-8]. Helmstetter (personal communication) points out that when cells are not grown under optimal conditions there are always some cells of odd or abnormal size. But these cells are cells that are dying or in some way impaired. These abnormally sized cells should not be considered as typical of a well-maintained, exponentially growing cell culture.

The fourth part of our verbal analysis of mass increase as a function of cell size relates to bacterial cells. As will be seen below, one of the most important results in bacterial physiology is that as growth rate speeds up, cells get larger. As the growth rate of a cell is continuously varied by increasing the richness of the medium, there is a continuous variation in bacterial cell size with the faster growing cells being larger than slower growing cells. Bacterial cells with an interdivision time of 20 minutes are larger than cells with an interdivision time of 60 minutes. Although it could be said that larger cells make mass faster leading to the shorter interdivision time for larger cells, it is equally possible, and in fact preferred, to reformulate or verbalize this result by saying that faster growth produces larger cells. For a given medium the rate of mass increase is determined for the bacterial cells, and the cell size *results* from the growth rate. This idea, the fourth way of looking at the relationship of cell size and mass increase, will be illustrated below in the analysis of bacterial patterns of DNA replication and cell size maintenance.

As we shall see, in bacterial cells a constant period for DNA replication and a constant time between termination of replication and cell division explains the variation in bacterial cell size as a function of growth rate. This same explanation also applies to mammalian cells: the rate of growth determined by external conditions determines cell size. Rather than taking the results of Conlon and Raff and concluding that larger cells when placed in medium with more serum now grow faster, it is better, as with bacteria, to say that when cells are placed in a condition that provides faster growth (i.e., a shorter interdivision time), the cells grow larger. While this may appear, at first sight, to be a trivial and semantic difference, in fact this distinction lies at the heart of the problem and

is the key to the solution of size determination and size maintenance. Rather than thinking that cell size produces cells with a particular growth rate (e.g., large cells grow fast), it is preferable to think that a particular growth rate produces cells of a particular size (e.g, fast growing cells are made larger than slower growing cells).

What is wrong with linear growth?

There are problems inherent and unavoidable in any proposal of linear cell growth during the division cycle. Linear growth means that during the division cycle, as a cell proceeds from size 1.0 to size 2.0, cell mass is added at a constant amount per unit time. If a cell grows linearly, over tenths of a cell cycle time, a cell increases its size from size 1.0, to 1.1, to 1.2, and so forth.

One problem with linear growth is that as the cell gets larger, the cytoplasm becomes inefficient. More cytoplasm is present as the cell grows, but the extra cytoplasm does not increase the absolute rate of cell mass synthesis. There is a decrease in the relative rate of mass increase. One mechanistic model explaining this absence of a change in the absolute rate of new mass increase is to propose that the new mass does not enter into active participation in mass synthesis until a cell division. There is a constant rate of mass increase based on the original mass. As the cell grows larger, the efficiency of the cytoplasm decreases per unit of extant mass. As a cell approaches division, the efficiency of mass making new mass is half that of the efficiency of the initial, newborn cell mass. An alternative mechanistic proposal to explain linear growth is that during a cell cycle the amount of material able to be taken up by a cell is constant, and only upon cell division is there an activation of the new cell surface so that there is an increase in the ability of the cell to take in material.

Even more important and troublesome is the idea that if a cell grows linearly, at the instant of cell division there must be a sudden saltation or jump in the activity of the total cytoplasm. Toward the end of the cell cycle, 1.9 units of cell mass make 0.1 unit of cell mass to achieve a cell mass of 2.0. At the instant of division the 2.0 units of cell mass, now apportioned into two daughter cells, must now make, during the next time interval, 0.2 units of cell mass or twice as much as in the previous time interval. When the cell of size 2.0 divides, linear growth implies that the two new daughter cells now immediately activate the “quiescent” cytoplasmic material (or activate the previously inert cell surface uptake capabilities). Irrespective of mechanism, considering the two daughter cells together, linear growth during the division cycle inevitably implies that at division there is a sudden doubling in the rate of mass increase.

There is no known biochemical mechanism for these proposals to produce linear cell growth, or the sudden jump in the rate of mass increase. As currently understood, the new cytoplasm joins right in to make new mass. And there is no mechanism known to allow new cell surface to remain inert until a cell division. While the absence of any identification of these mechanisms does not mean that these mechanisms do not exist, there is no need to propose the existence of these mechanisms of cells grow exponentially. Occam’s Razor may be appropriately applied to deciding between linear and exponential growth, with exponential growth being the preferred choice.

The experimental evidence favors exponential mass increase during the cell cycle. In bacteria the evidence for exponential growth is extremely strong [9, 10]. Analysis of data on eukaryotic cell size increase also supports exponential growth during the division cycle [11].

What of the experiments presented by Conlon and Raff [1] that cell mass increases linearly. For their analysis Conlon and Raff studied cells cultured in 1% fetal calf serum, forskolin, and aphidicolin. Aphidicolin is an inhibitor of DNA synthesis. While mass increases, there is no concomitant increase in DNA content. DNA as a source of mRNA and other informational needs may become limiting. Conlon and Raff measured cell growth without any change in DNA content. The cells were incubated for 216 hours (9 days). The cell volume was measured using a Coulter Counter, although in one experiment total protein content was measured.

Conlon and Raff realized that it is extremely difficult to distinguish linear from exponential growth over one doubling time. Therefore they measured mass increase over a longer period of time (possibly 3 or more normal interdivision times). The problem with this experiment is that the inhibited cells do not allow exponential increase in cell number as DNA synthesis is inhibited. This experiment is subject to the critique that aphidicolin inhibition produced the observed results. Thus the results do not reflect the situation in normal, uninhibited, unperturbed cells. For example, there could have been exponential growth during the first "virtual cell cycle". Then the limitations of DNA content would lead to the observed linearity of growth as measured over the extended period of analysis. But this linearity should not be taken as an indication that during the normal cell cycle cell the cell mass increases linearly.

Even if cells grow linearly during the division cycle, if the rate of mass increase is measured over a number of cell cycles with uninhibited cells, there should be evidence of an approach to exponential mass increase. If the rate of mass increase during the first cycle is 1.0, during the second cycle it should be 2.0, during the third cycle 4.0, and so

on. Thus, even on its own terms, with linear mass increase during the division cycle, the experiments of Conlon and Raff [1] on the pattern of mass increase are flawed by the presence of an inhibitor of DNA synthesis. An analysis of this idea is presented schematically in Fig. 4.

Raff (personal communication) disputes this interpretation of the aphidicolin experiments, proposing that “while the aphidicolin-arrest strategy is certainly artificial, it is not unrealistic...as many cells, including Schwann cells, grow a great deal after they have stopped dividing. Moreover...hepatocytes grow linearly, independent of their size, if a mouse is re-fed after it has been starved for a couple of days.” As noted in Fig. 4, without inhibition, growing cells that grow and divide must, *a priori*, approach an exponential pattern (i.e., rate of 1, to 2, to 4, to 8 as cells multiply), and therefore the only meaningful discussion of the linear vs. exponential growth pattern relates to growth within the cell cycle. Regarding application of liver growth following starvation and refeeding, this complex situation seems particularly inapplicable to discussions of cell growth in cell culture as there are so many complicating factors. A detailed analysis of the proper systems for cell-cycle analysis has been presented [8].

The experiments of Conlon and Raff also show some internal inconsistencies that weaken the actual data. Comparison of cell volume increase and protein per cell increase in the same cells over a 96 hour period (Fig. 3 of Conlon and Raff) shows that the volume increase was 4.75-fold ($\sim 2,000 \mu\text{m}^3/\text{cell}$ increasing to $\sim 9,500 \mu\text{m}^3/\text{cell}$) but the protein increase was only 2.93-fold ($\sim 0.16 \text{ ng}/\text{cell}$ increasing to $\sim 0.47 \text{ ng}/\text{cell}$). Until these differences are resolved, it is difficult to accept these experiments as supporting linear

cell growth—or any pattern of cell growth—during the normal division cycle. The discrepancies pointed out here suggest that the quantitative measures of cell size by Coulter Counter may not be able to distinguish different growth patterns.

Another problem arises in Conlon and Raff's [1] analysis of the pulse-chase experiments where cells starved for different times are pulsed and chased to measure protein turnover. They concluded: "...the rate of decrease in radiolabeled protein increased as the cells increased in size." That is, there was a greater release of labeled amino acids from cells that were inhibited with aphidicolin for longer periods of time and which were therefore larger [1]. But the release data were plotted on rectangular coordinates. This led to the observation that the slope between the 0 hour and 2 hour points in their Fig. 4b is steeper for the cells arrested for 72 hours compared to the cells arrested for 48 hours. The 72 hour cells were larger than the 48 hour cells. But looking at the actual values, reading the results from the published graph, the counts for the 72 hour arrested cells went from ~179 to ~121 in two hours, or a ratio of 0.67 for the two hour chase, while the 48 hour arrested cells went from ~138 to ~94 for a ratio of 0.68. Thus, in contrast to the conclusion of Conlon and Raff [1] there is no apparent difference in the turnover of proteins as a function of cell size.

Robert Brooks (personal communication) has argued against this analysis, noting that the cells starved for 24 hours appeared to show "no turnover" as the line for this graph (Conlon and Raff's Figure 4b) was flat. But in the text in the legend to their Fig. 4(b) Conlon and Raff state "The shallowness of the curve for the 24-hour-arrested cells is likely to be the result of the lower than expected value at 0 hours." This

explanation comes from the initial counts in Fig. 4(a) where it can be seen that there is some apparent error in the zero time value for the 24 hour starved cells in their Fig. 4(b).

But an even more egregious, significant, important, and fundamental error in analysis precedes even these technical problems. The cells studied by Conlon and Raff were not synchronized. The cells were not aligned and were in all phases of the cell cycle. It is a theoretical fact that it is impossible to analyze the pattern of synthesis during the cell cycle on cells that are not synchronized. (For complete details see reference [12].) This is because of the age distribution of cells in a growing culture. The age distribution for growing cells in culture is given by 2^{1-X} , where X is the cell age during the cell cycle; X varies between 0.0 and 1.0 (newborn cells are age 0.0 and dividing cells are age 1.0). At age 0.0 the relative number of newborn cells is 2.0 ($2^{1-0} = 2^1 = 2$) while at age 1.0 the relative cell number of dividing cells is 1.0 ($2^{1-1} = 2^0 = 1$). This distribution of cell ages means that any incorporation measurement on asynchronous cells must, and will, yield an exponential pattern of uptake. This is illustrated in Fig. 5 for an extreme idealized *Gedanken* case where we can imagine cells making all of their cytoplasm only at age 0.5. Because of the age distribution an exponential pattern of incorporation is observed when the entire culture is analyzed (Fig 5a,b). The details of the analysis are presented in the legend to Fig. 5. If the cells had been synchronized then one would have measured a peaked pattern as illustrated in Fig 5c.

Robert Brooks (personal communication) argues that this critique is incorrect because “they [Conlon and Raff] started with quiescent (G0/G1) cells.” It can only be pointed out that the arguments that quiescent cells with a G1-phase amount of DNA are

not synchronized and cannot be synchronized has been described in both general [13, 14] and rigorous [15] presentations. The reader is referred to these papers for a detailed analysis. Despite the widespread belief and acceptance of the idea that cells can be synchronized by growth arrest (i.e., by whole-culture synchronization methods), this idea is incorrect. Cells can only be synchronized by selective methods [15].

How can one determine whether mass increases exponentially or linearly during a normal, unperturbed, division cycle? To illustrate one approach to determining the pattern of mass increase during the division cycle, consider the following experiment. Grow cells for many generations in a radioactive amino acid (e.g., C-14 labeled amino acid) so that cell protein is totally labeled. Then add a pulse of a counter-labeled amino acid (e.g., H-3 labeled). As shown in Table 1, if cells grow linearly, the ratio of tritium (H-3) to C-14 should decrease as the cells become larger. With exponential growth the ratio of tritium to C-14 should be constant over the cell cycle. If one now one took such double-labeled cells, fixed them, and spread the cells out on a gradient such that the larger cells were preferentially at the bottom and the smaller cells at the top, if cells grew linearly there would be a decrease in the H-3/C-14 ratio as the larger and larger cells were assayed. If cells grew exponentially there would be a constant radioactivity ratio over the entire set of cell size fractions. The idealized results from Table 1 are illustrated in Fig. 6.

To summarize this critique of the aphidicolin-inhibition experiments, it is proposed that the experiments of Conlon and Raff do not measure the mass increase during the cell cycle. The experiments using inhibition of DNA replication merely measure the pattern of mass increase in an abnormal, unrealistic, artificial, and perturbed experimental situation. This experiment is not supportive of any particular pattern of

mass increase during the normal division cycle. And even more important, as shown in Fig. 5, without synchronization of cells, it is impossible to determine the pattern of mass increase during the division cycle.

The bacterial cell cycle: Rules, patterns, and regulation

In 1968 the rules for the replication of DNA in a simple bacterium (*Escherichia coli*) as well as the relationship of cell size to control of DNA replication were worked out [16-20]. The pattern of DNA replication and cell size are determined by three rules:

1. A round of DNA replication is invariant (40 minutes) over a wide range of growth rates [18, 19].
2. The time between termination of replication and cell division is invariant (20 minutes) over a wide range of growth rates [18, 19]
3. At the time of initiation of replication, the cell mass per origin is a constant [16, 17, 21].

These rules are illustrated in Figs. 7 and 8. These three rules predict, as shown in panel (c) of Fig. 8, that cell size should be a logarithmic function of growth rate. Cell size plotted on semi-logarithmic coordinates against the reciprocal of the interdivision time gives a straight line [22]. Faster growing cells are larger than slower growing cells. Ten years earlier, in 1958, before the rules predicting the size-growth rate relationship were determined, this experimental result [22] was clearly obtained in what has been called “the Fundamental Experiment of Bacterial Physiology” [12]. An analysis of the history, origins, and meaning of this experiment has been published [23].

The important consequence of Figs. 7 and 8 is that we understand how cell size is controlled in bacteria. Cells initiate DNA replication at a certain cell size. This cell size (sometimes referred to as the “initiation mass”) is a constant size within experimental limits [21]. The cell size at initiation is constant per origin present in the cell, and so a cell with two origins being initiated is twice as large as a cell with only one origin. The number of origins present at initiation and the cell age during the division cycle at which initiation occurs determines the average cell size of a cells growing in culture.

Analysis of size maintenance in animal cells

The ideas of the bacterial cycle can be directly applied to animal cells. Cells of different growth rates are shown in Fig. 9(a). The different lines, a-g, identify cells of different sizes because they pass through size 1.0 at different times during a relative cell cycle span. Cell “g” is a faster growing cell than cell “a” with the others of intermediates growth rates. The earlier a cell reaches size 1.0, the larger the cells will be. Thus, in Fig. 9(a), the cell “g” is larger than the cell “a” because the “g” cells reaches size 1.0 earlier than the “a” cell. As drawn in Fig. 9(a), the newborn “g” cell is size 1.0. The mother or dividing cell is size 2.0. We can imagine that the mean size of cells growing at this rate is approximately 1.5. In contrast, the “a” cell varies between newborn size of approximately 0.6 and dividing size of 1.2. The average size of the “a” cells is smaller than the “g” cell, approximately size 0.9. (The precise calculation of the average cell size requires consideration of the age distribution and the actual pattern of mass increase during the division cycle; for purposes of this analysis, these complications are omitted.) Other cells (b-f) may be similarly analyzed to see that faster growing cells are larger than

slower growing cells. And as will now be seen, this variation in size is related to, and determined by, the growth rate.

It is proposed that mammalian cells initiate DNA replication, or the S phase, as in bacteria, at some relatively constant cell size. As the time for S and G2 phases are relatively constant or invariant as the interdivision time varies [24], the time of initiation occurs earlier and earlier as the growth rate increases (or as the interdivision time decreases). This is shown in Fig. 9(b), where the interdivision time is varied but S- and G2-phase lengths are constant. In Fig. 9(c) the cell cycle patterns in Fig. 9(b) are normalized to a constant length. In Fig 9(c) it is clear that the faster cells initiate S phase earlier in the cell cycle. This is because faster cells have a relatively shorter G1 phase. These faster growing cells achieve the initiation mass earlier in the cell cycle and thus these cells will be larger. As in bacteria, faster growth leads to larger average cell sizes. (For a discussion of the case of cells growing so fast as to not have a G1-phase as in cell “g” in Fig. 9(b), see [24].)

The rate of cell growth is determined by the content of the medium. For example, as more and more nutrients are added to a minimal medium, bacterial cells grow at faster and faster rates. The interdivision time shortens as the medium becomes richer. For bacteria the mechanism for growth rate variation with medium composition is, in outline, well understood [25]. The addition of nutrients to a medium represses the synthesis of unneeded enzymes. This leads to a shift in synthetic capacity of the cell to the protein synthesizing system—RNA polymerase, ribosomes, related materials, etc.—as these functions are not repressible by external components [25]. This leads to a more rapid rate of mass increase as expressed in a shorter mass doubling time [24, 26]. Although the

details may vary, it is proposed here (and in fact supported by the experiments of Conlon and Raff) that the richer a medium is (e.g., more serum rather than less serum), the faster the cells will grow. And the faster a cell grows the larger it will be (Fig. 9). The variation of G1-phase length with interdivision time variation has been analyzed in detail [24, 26].

Conlon and Raff [1] supply evidence for the relationship of cell size and growth rate in their Fig. 7, where cells that have become overcrowded by not being diluted back (their Fig. 7b) decrease their volume (their Fig. 7a).

This analysis explains the variation of cell sizes as function of growth rate as observed by Conlon and Raff (slower growing cells are smaller than faster growing cells). Furthermore, the analysis can also explain the maintenance of cell size, even with exponential mass increase during the division cycle, as shown in Fig. 9. Larger than average cells will divide sooner as they reach the initiation mass earlier and smaller than average cells will delay initiation until the initiation mass is achieved. Cell division will follow after relatively constant S- and G2/M-phases. This is the underlying and fundamental explanation for the patterns described in Fig. 1.

Thus, in answer to the question “how does the cell ‘know’ when to divide so that size homeostasis is maintained?”, the answer is that the initiation of S phase is determined by the cell mass. A cell with a larger size initiates S phase earlier than a smaller cell, and this earlier initiation is played out in an earlier cell division after a period equal to the S and G2/M phases. While the analysis in Fig. 1 at the start of this paper discussed the size maintenance problem in terms of the cell dividing earlier if a newborn cell was larger and later if a newborn cell was smaller (or if the rate of mass

increase was high or low), the analysis presented here proposes that the division decision is determined somewhat earlier than the moment of cell division. The initiation of S phase is determined by cell size and the faster a cell reaches the initiation sizes the earlier it will initiate S phase and then the earlier it will divide. This is because, in some way not yet understood, there is a relationship between initiation and cell division such that once S phase is initiated the cell will ineluctably proceed to division.

We therefore see the answer to the problem of size at division. Cell size at division is merely a surrogate indication of the size at initiation or the time of initiation of S phase. A cell that initiates S phase earlier in the cell cycle will have less time to increase its mass prior to division. Thus, the larger cell, having initiated S phase relatively early compared to its sister and cousin cells, will divide earlier as described in Fig. 1 above. Conversely, smaller cells will delay initiation of S phase, and that delay will allow more mass increase before the actual cell division. In this way, the cell size distribution is maintained.

Size variation during a shift from slow to fast growth

Immediately following the discovery of bacterial cell size variation with growth rate [22], shift-up experiments of cells from slow growth (relatively small size) to faster growth (relatively large size) were performed [27]. The phenomenon of “rate maintenance” was discovered in this shift-up experiment. Rate maintenance is the continuation of the rate of cell division for a constant period after the shift-up. The rate of mass increase changes immediately to the new rate at the instant of shift-up, while the rate of cell division continues for a period of time before abruptly changing to the new rate. This phenomenon occurs over a wide range of shift-ups [20]. The continuation of

cell increase at the original, slow rate of cell increase, combined with an immediate transition to the new rate of mass increase, led to an increase in cell size over the period of rate maintenance (Fig. 10a). Rate maintenance is now understood to result from the constant S and G2 periods (C and D periods in bacteria) that do not allow new divisions to occur until the newly inserted replication forks pass through the S (i.e., C) period and the G2 (i.e., D) period. Without going into details here (see [12] for a complete analysis and explanation), suffice it to say that the rate maintenance phenomenon leads to the observed variation in bacterial cell sizes as the rate of cell growth varies over a wide range.

Conlon and Raff [1] have studied a shift-up from slow growth to rapid growth and small to large cell size in mammalian cells. Upon shifting slow cells to faster medium (i.e., shifting cells from low serum to high serum) there is a concomitant increase in cell size (Fig. 6e of Conlon and Raff [1]). One major difference from the bacterial shift-up result is that with animal cells the time for cell size to increase took a much longer time, between 6 and 9 days. To explain the difference between the bacterial shift-up result (Fig. 10a) and the mammalian cell shift-up result (Fig. 10b) one can postulate that for reasons unrelated to the cell cycle but merely related to cellular metabolism occurring continuously throughout the cell cycle, the change in external conditions does not immediately lead to the new rate of mass increase (Fig. 10b). It is predicted that the rate of mass increase will change relatively slowly as mammalian cells are shifted from serum-free (slow growth) medium to serum-containing (fast growth) medium. Of course this is the result reported by Conlon and Raff (their Fig. 6e).

This view of the change in cell size following a shift from slow growth to rapid growth is quite different from the description Conlon and Raff present for the case of yeast cells switched from a nutrient-poor to a nutrient-rich medium. They write [1], “When switched from a nutrient poor medium to a nutrient-rich medium, the cell cycle arrests and resumes only when the cells have reached the appropriate size for the new condition, which occurs within one cell cycle... Thus, the cells can adjust their size threshold rapidly in response to changing external conditions.” The bacterial model of the shift-up allows a rapid change in cell size within one cell cycle without postulating any “arrest” of passage through the cell cycle.

Rather than postulate a mechanism that slows or actively shuts down the cell cycle, it is simpler to propose that no change in cell division occurs until the increased initiations of S phases pass through the S and G2/M phases, as in the bacterial model. No special additional mechanism needs to be proposed to “stop” some event of the cell cycle until cell size has increased.

What is a checkpoint?, and are checkpoints required for cell cycle and cell size control?

The analysis presented here did not invoke “checkpoints”, a common and ubiquitous explanation for many aspects of cell cycle control. The idea of a “checkpoint” probably comes from the metaphor of “checkpoint Charlie”, the famous (or infamous) gate between East and West Berlin during the Cold War. This military checkpoint existed and acted outside of the traveler, checking the papers and ensuring that the traveler was allowed to proceed through the “checkpoint”. In the same way, the idea of a cell-cycle “checkpoint” is that a cell mechanism “checks” that required functions have been

performed or the required material has been synthesized before the cell is allowed to proceed through to the next function such as initiation of S phase or cell division.

But there is an alternative view of controls on cell cycle passage that should be considered, where the “checking” is not external to the system (or traveler). The “checking” function may be inherent in the system itself.

A simple metaphor or analogy will explain the difference between these two ways of looking at checkpoints. Let us say we are allowing a young child to take the family car out for the first time. We wish to prevent the child from traveling more than 100 kilometers. One way to accomplish this is to insert into the car a “checking” apparatus that will keep checking how far the car has gone and that will shut off the engine when 100 kilometers is reached. This checkpoint is “external” to the motor and the car. If it were not for the checking shut-off control, the car would be able to go more than 100 kilometers.

An alternative approach to controlling the distance driven would simply be to put into the car enough gas to go precisely 100 kilometers (with a locked gas cap). In this way the car will not be able to go further than 100 kilometers because the engine will run out of gas. The limitation on driving is inherent in the fuel volume. There is no need for an external checking device. In this case we would say that the checking was not external to the car, but inherent in the car or the fuel.

Applying this to the cell cycle, one view of a classical “checkpoint”—for example, for the initiation of S phase—is that there is some system of molecules that checks to see that all of the required processes proposed to occur before initiation of S

phase have actually occurred. If the required processes have occurred, then the checkpoint allows the initiation of S phase.

The alternative, non-checkpoint approach to ensure that everything required has happened before initiation is to make the control inherent in the system. In the description of cell-cycle control presented here, it is merely the accumulation of material, continuously throughout the cell cycle, and independent of cell-cycle phases, that controls the initiation of S phase as well as the final mitosis and cytokinesis. As described here, there is no need to have an external “checking” mechanism looking at the cell and continuously checking to see whether the proper mass has been achieved. The mass accumulation itself is the checking mechanism [28-30].

The age-size distribution summarizes size control

One way to consider a growing culture is to see that every cell in a growing culture has an age and a size. What is the relationship of age and size for these cells? The precise answer is not known, and the result may be very complicated, but it is possible to think about this by reference to a graphic representation of the relationship of size and age for a growing culture (Fig. 11). The age-size structure of a population is a representation of each of the cells in the culture and its age and size. In panel (a) of Fig. 11 a purely deterministic age-size structure is illustrated. The projections of the dots on the exponential line in this panel to the age axis (bottom) and the left axis (size) indicate that when cells are growing exponentially, there are a greater number of smaller cells relative to the population than there are younger cells. This reflects the age distribution discussed above.

When variation in interdivision times and sizes at birth and division are introduced, the age-size distribution is as illustrated in panel (b) of Fig. 11. The shaded area indicates a cloud of points preferentially collected around the middle of the shaded area, with fewer cells at the outer edges. If this were a three-dimensional graph, there would be a peaked “ridge” up the center of the shaded area indicating that more cells reside with a particular age size distribution than those at the edges of the age-size distribution.

It is possible to indicate the cells at particular times during the division cycle such as birth, division and initiation of DNA synthesis, and this is indicated in panel (c) of Fig. 11. There is no distribution in age at birth, since by definition age at birth is 0.0. The graphs at the upper and right sizes of this panel are representations of the spread of the various distributions. There is some variability in the age at initiation of DNA replication and the age at division is also variable. The size at birth is not constant. It is expected, however, that the size at division will be slightly less variable than the size at birth. This is attributable to the probability that unequal division of cells leads to a broadening of the size distribution in newborn cells. It is clear from panel 11(c) that larger newborn cells will reach the size at initiation earlier than smaller newborn cells.

An alternative way of looking at the age-size distribution is to replot the ages using the age at initiation of DNA synthesis as a starting point (Fig 11(d)). By defining age at initiation as 0.0 one gets negative ages for cells before initiation and positive ages for cells after initiation. This panel shows that there is no distribution in the age at initiation (since by definition the age at initiation is 0.0 for all cells) but there is a variation in the age of newborn cells. The “bottleneck” at initiation of DNA synthesis

enables cells born of different sizes to retain size homeostasis. Since all cells to the left must pass through the bottleneck of initiation on the way to division, all cells, of any newborn size, are realigned and assigned a new age and a new size as they pass through the act of initiation.

Understanding mammalian cell size control

The analysis presented here explains how cell size is maintained through a combination of interdivision time variation and cell growth rate variation. Exponential growth is certainly possible and allowed during the division cycle, in contrast to the proposal of Conlon and Raff [1]. The ideas presented here are a fresh way to look at the cell cycle and cell growth, even though the ideas have been around for 25-35 years [24, 31]. The model of the cell cycle presented here explains many experimental results without postulating checkpoints, G1-phase events, restriction points, or similar phenomena. Experimental support for these ideas [32, 33], and the application of these ideas to other problems of cell growth and differentiation [4, 13-15, 26, 34-39] have been published. These ideas have also been reviewed [28-30].

It may be best to summarize these two contrasting views of size maintenance by looking at cell growth in a simple manner, and asking how the rate of mass increase is related to the passage of the cell through the cell cycle. The model of Conlon and Raff looks at the events of passage through the cell cycle as occurring independently of mass increase. The problem then remains as to how mass increase fits into the pattern or timing of passage through the cell cycle. It is as though the cell moves through the cell cycle without considering the mass problem, and then the mass of the cell looks at the cell cycle and says “I must grow at some rate so that I do not get too big or too small.” In the

Conlon/Raff model, there must exist a control that coordinates mass increase with the rate of cell division.

The model presented here situates mass as the driving force of the cell cycle. The mass increases at some rate determined by external conditions (medium, growth factors, pH, etc). As the mass increases, the mass starts or regulates passage through the cell cycle. This model proposes that a cell cannot grow to abnormal size because at a certain cell size or cell mass the S phase is initiated and this event starts a sequence of events leading to mitosis and cytokinesis. A cell cannot get too small because if mass grows slowly then the later events of the cell cycle (S-, G₂-, and M-phases) are delayed until mass increases sufficiently to start S phase. A cell cannot get too large because at a certain size the cell initiates S phase leading to the relatively early cell division. Thus, there is no problem relating mass increase and the cell cycle. Cell mass growth and cell cycle passage cannot be dissociated because one (mass increase) is the determinant of the other (S-phase initiation). For this reason one needs neither checkpoints nor control elements outside of mass increase itself.

The time for mass to double in a particular situation determines the doubling time of a culture. This is because initiations of S phase occur every mass doubling time, and cell divisions similarly occur every mass doubling time. Thus total mass increases at the same rate as total cell number.

The model presented here explains size determination, size maintenance, and the relationship of mass increase and cell number increase in a growing, exponential, unperturbed, mammalian cell culture.

The relationship of models and experimental data

In the analysis presented here, a number of experimental results have been presented as supporting a particular view of cell size control, cell size maintenance determination, and the control of the cell cycle. Some of the proposals may not fit all of the experimental data, particularly since there is so much experimental data available. What can we make of these “exceptions?” I can only suggest that we read the words of Francis Crick in his autobiography (What Mad Pursuit, Basic Books, 1988) where he writes:

The failure on the part of my colleagues to discover the alpha-helix made a deep impression on Jim Watson and me. Because of it I argued that it was important not to place too much reliance on any single piece of experimental evidence. It might turn out to be misleading, as the 5.1 Å reflection undoubtedly was. Jim [Watson] was a little more brash, stating that no *good* model ever accounted for *all* the facts, since some data was bound to be misleading if not plain wrong. A theory that *did* fit all the data would have been “carpentered” to do this and would thus be open to suspicion.

The analysis presented here should be looked at as a way of critically examining the experimental evidence available. There is evidence on both sides of the issues discussed here. Thus one must examine the evidence, and see whether one or the other way of looking at the growth of cells fits the experimental evidence better.

Thus, Robert Brooks (personal communication) points out that experiments performed many years ago with Shields indicated that “...mammalian cells don’t initiate

S phase at the same size....” In line with the ideas proposed by Crick in the quote above,, my analysis of the classical data indicates that the evidence criticizing the proposals made is so weak as to not invalidate the discussion of cell size control presented here. Of course more experiments and evidence is needed. But these experiments should be performed on exponentially growing, well-behaved mammalian cells. A discussion of the criteria for a good experimental system has been presented [8].

Acknowledgements

This paper is dedicated to Moselio Schaechter, who has supported and encouraged me over the years, who was a participant in what has been termed “the Fundamental Experiment of Bacterial Physiology”, the experiment that is the basis for the eukaryotic ideas presented here, and who has been a model scientist, person, and mentor.

Additional material on the ideas presented here may be viewed at

www.umich.edu/~cooper.

I thank Charles Helmstetter, Robert Brooks, Arthur Koch, Martin Raff, Ian Conlon, and Sanjav Grewal for their comments and suggestions. Further comments may be sent to

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FIGURE LEGENDS:

Fig. 1. Exponential and linear growth patterns are both compatible with cell size maintenance. In panel (a) newborn cells of identical size increase mass at slightly different rates with an exponential pattern of mass increase. If cells divide at a constant cell size, here size 2.0, size will be maintained even though the rate of mass increase varies. This occurs as the cells divide at different times (division indicated by the downward arrows) as they reach the same size. In panel (b), exponential growth at identical rates from initial cells of different cell sizes also gives size maintenance as cells divide at the same size because there are different interdivision times for each cell; the larger initial cells have a shorter interdivision time and the smaller initial cells have a longer interdivision time. As shown in panels (c) and (d), linear cell increase (note the different ordinate scale compared to panels (a) and (b)) can also lead to cell size maintenance as cells divide at the same size, 2.0.

Fig. 2. Interdivision time variation allows a return of slightly deviant sizes to a constant cell size. In panel (a) newborn “baby” cells (b) grow for slightly different times, producing either large (l) or small (s) newborn cells from large or small dividing cells. Deviation from equipartition for an average sized dividing cell can also produce large and small cells. The resolution of size differences is illustrated in (b) and (c) where the larger cell (l) has a shorter interdivision time dividing at average (a) cell size and the smaller cell (s) has a longer interdivision time also dividing at average cell size. The dividing average sized cells (a) produce newborn baby cells (b) of the original newborn size.

Fig. 3. Hypothetical model of Conlon and Raff where constant size increase independent of cell size allows return of deviant cell sizes to a constant cell size over time (Adapted from Conlon and Raff [1]). If large and small cells grow and progress through the cell cycle with the same mass added each cell cycle, then cell size converges on one particular cell size. As drawn here, cells of initial sizes 10 and 1 both increase size by 5.5 units and then divide to produce cells of sizes 3.25 and 7.75. This continues each generation and cells progress to the same size.

Fig. 4. Approach of cell mass to exponential even if cells had linear synthesis within cell cycle. Panel (a) illustrates cells dividing to produce two, four or eight times the original number of cells. The mass, thin line, increases linearly. It is clear that the cell size will not be maintained. In panel (b), even with linear mass growth within the cell cycle (thin line), as cells divide the rate of mass synthesis doubles and then quadruples as cell numbers increase. There is no change in the cell mass over a number of cell cycles. Note that it is not proposed that mass increases linearly, but merely that even linear synthesis should show up, in an uninhibited situation, as appearing close to exponential mass growth.

Fig. 5. Unsynchronized cells cannot be used to determine cell-cycle pattern of synthesis. Panel (a) shows a series of age distributions starting with the initial age distribution reflecting the pattern $\text{Age Distribution} = 2^{1-X}$, where X is the cell age going from 0.0 to 1.0. In this *Gedanken* analysis, it is assumed that cells of age 0.5 (i.e., cells in mid-cycle) are the only cells incorporating amino acid (cross-hatched bars). The asterisk (*) on a bar in each pattern indicates the newborn

cells. One reads the cell ages by going from the asterisked bar to the right and then back to the left to finish off the age distribution. The number to the right of each pattern is the relative number of cells incorporating amino acid. Thus, in the uppermost pattern in Panel (a) the relative number is 1.46. After one-tenth of a generation we see that the oldest cells in the first pattern have divided to give double the number of cells and these cells are now the youngest cells in the culture. All of the other cells move up one-tenth of an age so that the cells that were age 0.4 are now age 0.5 (cross-hatched bar) and the rate of synthesis increases to 1.57. This is because there are more cells in the original culture of age 0.4 than there were of age 0.5. Continuing down the patterns in Panel (a) we see that as cells move to age 0.5 there is a continuous, and exponential, increase in the radioactivity. The cells above age 0.5 (in the original topmost diagram) divide and produce two cells each tenth of a cell cycle, so that over one total cell cycle there is an exponential increase in the rate of amino acid incorporation (a measure of cytoplasm increase). The total pattern of incorporation is plotted in panel (b) where the exponential incorporation during one cell cycle is indicated. Panels (a) and (b) thus show that even with a non-exponential pattern of incorporation, if a total culture is studied, the measured incorporation pattern will be exponential. If, however, cells are truly synchronized, as illustrated in Panel (c), a peaked incorporation pattern is observed, accurately reflecting the mid-cycle incorporation of amino acids into the cells at a particular cell-cycle age. Starting with newborn cells at age 0.0 and moving through the cell cycle at one-tenth of an

age each pattern in Panel (c) the incorporation (noted by the numbers to the right of the diagrams in Panel (c)) shows a peaked pattern.

Fig. 6. Comparison of the ratio of pulse label to total label for exponential and linear patterns of mass increase as described in Table 1.

Fig 7. Diagram of patterns of DNA replication during the division cycle in bacteria. The different patterns go from an infinite interdivision time (i.e., essentially no or extremely slow growth) to cells with 90, 60, 50, 40, 35, 30, 25, and 20 minute interdivision times. In all cases, the rate of fork movements is 40 minutes for a round of replication or one-quarter of the genome every 10 minutes. All rounds of replication end 20 minutes before the end of the cell cycle. This is most clearly seen in the 60-minute cells where a newborn cell has one genome, which replicates for 40 minutes ending replication 20 minutes before cell division. The same rules are drawn here for a 90-minute and a very slow growing cell (infinite interdivision time). The large numbers in each pattern at the left indicate the number of origins to be initiated at each time of initiation of replication. Thus, in the 60-minute cells there is one origin in the newborn cell. Consider that the cell mass is given a unit value for each origin to be initiated. Thus, the newborn cell in the 60-minute case is given a size of 1.0 unit of mass. This means that the dividing cell in the 60-minute cells is size 2.0. Mass increases, in the 60-minute case, from 1 to 2. In the 90-minute cells the cell of size 1 is one third of the way through the cell cycle. Since mass increases continuously during the division cycle it is clear that the newborn cell in the 90-minute culture is less than 1.0 in size. Let us say it was something like size 0.7. In this case the newborn cell in the

90-minute cells would be size 0.7 and the dividing cell would be size 1.4. It is clear that the 90-minute cells are, on average, smaller than the 60 minute cells. Similarly, if we consider the very slow cells, the cell of size 1.0 is very near the end of the cell cycle, and the newborn cell is slightly above size 0.5. Since the very slow growing cells (top panel) go from sizes 0.5 to 1.0 and the 60 minutes cells go from size 1.0 to 2.0, the 60 minute cells are twice as large as the very slow growing cells. The 30-minute cells have two origins in the newborn cell and thus the newborn cells can be considered size 2.0 with the dividing cells 4.0. The 20-minute cells have a newborn cell of size 4.0 (four origins in the newborn cell) and a dividing size of 8.0. As one goes from extremely long interdivision time, to 60, to 30 to 20, the relative sizes go from 0.5, to 1, to 2 to 4, with the growth rates expressed as doublings per hour, or 0 (infinite interdivision time), 1 (60 minute interdivision time), 2 (30 minute interdivision time), and 3 (20 minute interdivision time). Cells that initiate DNA replication in the middle of the cycle may be considered as follows. The 40-minute cell has two origins in the middle of the cell cycle so the mid-aged cell is size 2.0. The newborn cell might be some size like 1.5 and the dividing cell something like 3.0. Thus, the 40-minute cell has an average size intermediate between the 60 and the 30-minute cell. Similarly, the 25-minute cell also initiates mid-cycle, but there are 4 origins at the time of initiation. Thus, the mid-aged cell in this case is size 4.0 and the newborn cell may be considered something like size 3.0. The cell sizes go from 3.0 to 6.0, and these cells are larger than the 30-minute cells and smaller than the 20 minute cells.

Fig. 8 . Size determination in bacteria. In panel (a) the rates of growth of cells from infinitely slow (very long interdivision time) minutes to 20 minutes (as illustrated in Fig. 6) are plotted with the relative sizes shown. Thus, the 60 minute cell goes from size 1.0 to 2.0 over 60 minutes. The 30-minute cell (third angled line from top) goes from size 2 to 4 over 30 minutes. And the 20-minute cell (top angled line) goes from size 4 to 8 over 20 minutes. Other rates of growth for 25, 35, 40, 50, 90 and “infinite” interdivision times are also shown. In panel (b) the same results are plotted over relative cell ages from age 0 (newborn) to 1.0 (dividing cell). The open circles indicate when initiation occurs, and corresponds to the numbers in the individual panels. Thus, in Fig. 6 the cells with a 60, 30, and 20 minute interdivision time initiation DNA replication in the newborn cell (age 0.0) at sizes 1, 2 and 4. Besides the cell age at initiation, the open circle also indicates the relative size of the cell at initiation (see numbers in Fig. 6). The cell sizes at age 0.0 for all cells is a measure of the average cell size in the culture. (Given an identical pattern of cell growth during the division cycle the relative cell size of the cells in a culture is precisely proportional to the newborn cell size). These size values are then plotted against the rate of cell growth (the inverse of the interdivision time or doublings per hour) as shown in panel (c). The log of the cell sizes are a straight line when plotted as a function of the rate of cell growth (the inverse of the interdivision time).

Fig. 9. Mammalian cell size variation. Panel (a) shows a given mammalian cell growing at different rates and with different sizes. The lines are parallel because the interdivision times are normalized to a relative cell age as cells are born at age 0.0

and divide at age 1.0. Thus, all lines are exponentially increasing cell sizes from smallest to largest. Where they cross the thick horizontal line indicates a cell of size 1.0. Since the fastest cell (cell g) has a size 1.0 at the start of the cell cycle these cells must go from a newborn sizes of 1.0 to a size at division of 2.0. The slowest cell (cell a) has size 1.0 toward the end of the cell cycle, so the newborn cell is slightly larger than size 0.5 at age 0.0. Thus we see that the size ranges of these cells goes over a factor of 2. In Panel (b) the size patterns are re-interpreted in terms of initiation at a particular time during the cell cycle. Given that S and G2 (the thick, short line on each pattern is the S phase and the line to the right is the G2 phase; the line to the left is the G1 phase).are relatively constant in length then the slower cells (e.g., cell “a”) have a longer G1 phase than the faster growing cells (e.g., cell “g”, which has no measurable G1 phase). This is because the interdivision time is the sum of S+G2+G1, and if S and G2 are relatively constant as the interdivision time decreases (i.e., as cells grow at faster growth rates) the G1 phase gets smaller. When the interdivision time equals the sum of S and G2 as in cell g, there is no G1 phase. Such a situation has been analyzed before [24]. It is clear from Panel (b) that as cells grow faster, the time during the division cycle at which initiation of S phase starts is earlier and earlier. This is illustrated even more directly in panel (c) where the phases are normalized to a unit length. The slowest cell (cell a) has the shortest fraction of cells with an S or G2 phase and the fastest growing cell (cell g) has the entire division cycle occupied by S and G2 phases. The topmost line in panel (c) is the fastest cell and it starts S phase early in the cell cycle. Thus we see that the faster a cell grows the

earlier in the cell cycle the cell achieves a size of 1.0. This accounts for the result that the slower cell has a smaller cell size than the faster growing cell.)

Fig. 10. Comparison of shift-up of bacterial cells and mammalian cells. In the panel (a), after a shift of bacterial cells from slow-growth medium to fast growth medium there is an immediate change in the rate of mass synthesis to the new rate while the rate of cell division continues at the old rate for a fixed period of time (rate maintenance). At the end of this “rate maintenance” period, there is a sudden shift in the rate of cell number increase to the new rate. The thick line in panel (a) shows the change in cell size following the shift-up. In contrast, in panel (b) a slower and more gradual change in the rate of mass synthesis, concomitant with the cell number pattern also changing slowly over a period of time, will give a longer period of change in cell size. Conlon and Raff observed this slow pattern of mammalian cell size change.

Fig. 11. Age-size structure of a growing culture. Panel (a) is the age-size structure for a perfectly deterministic population growing exponentially in mass during the division cycle. The dots on the exponentially increasing line are placed at equal age intervals shown by their representation at the bottom of the panel. The representation of the dots at the left of panel (a) indicates that there is a greater concentration of smaller cells than younger cells. In panel (b) the age-size structure for a population with variation in size and interdivision times is illustrated. The cloud of points (indicated by a few points as representative of the population) is one possible age-size structure. In panel (c) the newborn cells are indicated by the filled circles, the dividing cells by open circles, and the cells in

the act of initiation of DNA synthesis by + signs. It can be seen that the larger cells at birth will, on average, reach the size required for initiation of DNA replication more quickly than smaller cells. This is because the larger cells are closer to the initiation size (represented by I on the right side of panel (c)). The B and D distributions at the right of panel (c) indicate the size distributions of newborn (B) cells and dividing (D) cells. The B, D, and I distributions at the top of panel (c) illustrate the age distributions for newborn, dividing, and initiating cells. Note that the size distribution of initiating cells is drawn with a narrower distribution. Variations in mass increase during the period after initiation lead to the widening of the size distribution at division. Panel (d) is a replotting of the pattern in panel (c) with the bottom time scale defined by the time of initiation of DNA synthesis. Cells before initiation have a “negative age value”, and cells after initiation have a “positive age value.” Initiation takes place, by definition in this panel, at age 0.0. There is some variation in the size of cells at initiation, but it is proposed that this variation is less than the variation at other events of the cell cycle. The narrowing of the age-size structure at the time of initiation is a graphic representation of the size-homeostasis mechanism. No matter what size cells are present at birth or division, these cells are returned to their proper age-size relationship at the instant of initiation of DNA synthesis. Larger cells at division produce larger newborn cells which then reach initiation size earlier than smaller cells which were produced by the division of smaller dividing cells.^f At the top and right panels of (c) and (d) are representation of the presumed variation of the sizes and ages of cells at particular events. The size at birth is always a little more

widely distributed than the size at division due to a slight inequality of partition of mass at division. The size at initiation of DNA replication is drawn with a relatively small variability.

Table 1

LINEAR GROWTH			Cell age	EXPONENTIAL GROWTH		
Cell size	Size increase	Inc/size		Cell size	Size increase	Inc/size
1	0.1	0.100	0	1.00	0.07	0.072
1.1	0.1	0.091	0.1	1.07	0.08	0.072
1.2	0.1	0.083	0.2	1.15	0.08	0.072
1.3	0.1	0.077	0.3	1.23	0.09	0.072
1.4	0.1	0.071	0.4	1.32	0.09	0.072
1.5	0.1	0.067	0.5	1.41	0.10	0.072
1.6	0.1	0.063	0.6	1.52	0.11	0.072
1.7	0.1	0.059	0.7	1.62	0.12	0.072
1.8	0.1	0.056	0.8	1.74	0.12	0.072
1.9	0.1	0.053	0.9	1.87	0.13	0.072
2			1	2.00		

The center column lists the cell ages from 0 to 1.0. At the left the linear increase of mass is related to the absolute increase in mass per interval (0.1 each interval for linear increase in mass during division cycle), and the ratio of incorporation per extant cell mass is given in the third column (0.1 to 0.053). Similar results for exponential growth except the mass increase per interval goes from 0.07 at the start of the division cycle to 0.13 at the end. The ratio of incorporation per extant mass in the right-most column is thus constant.

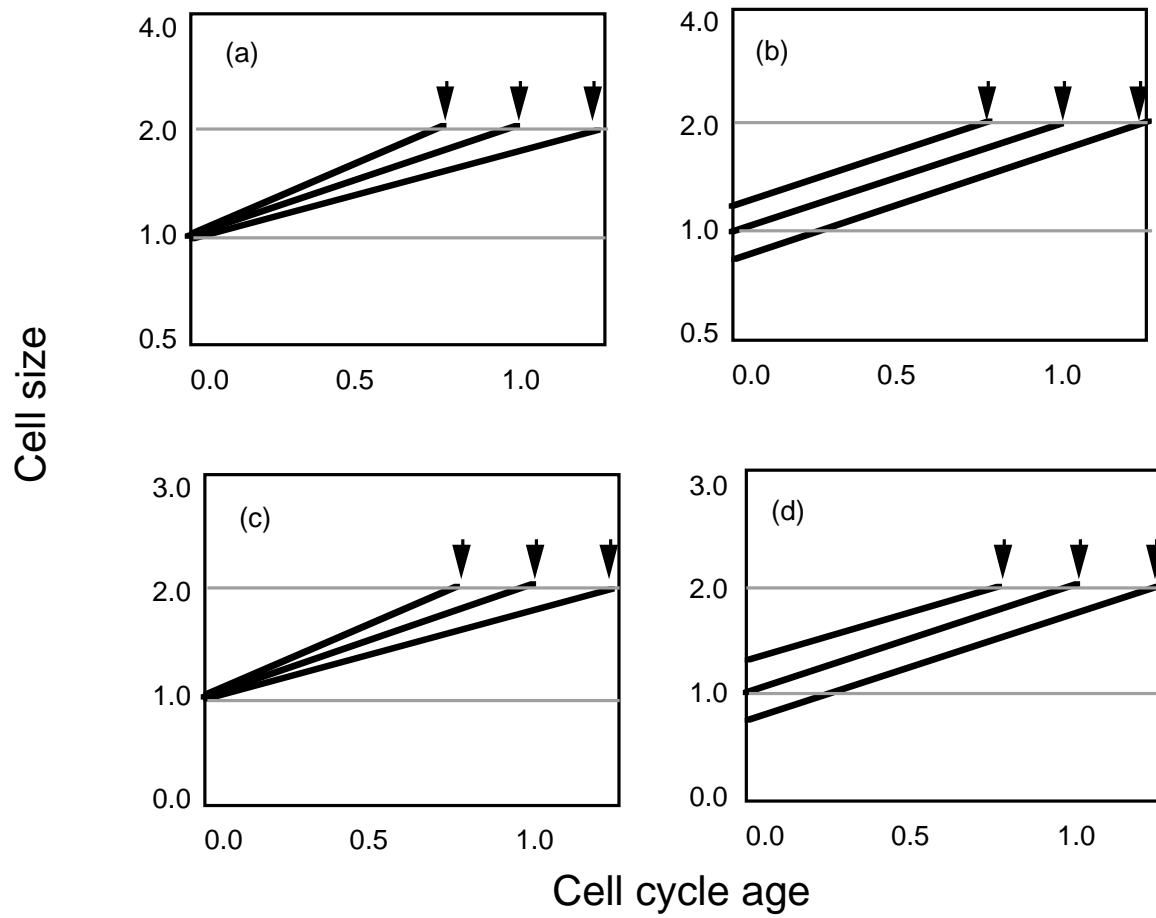


FIGURE 1

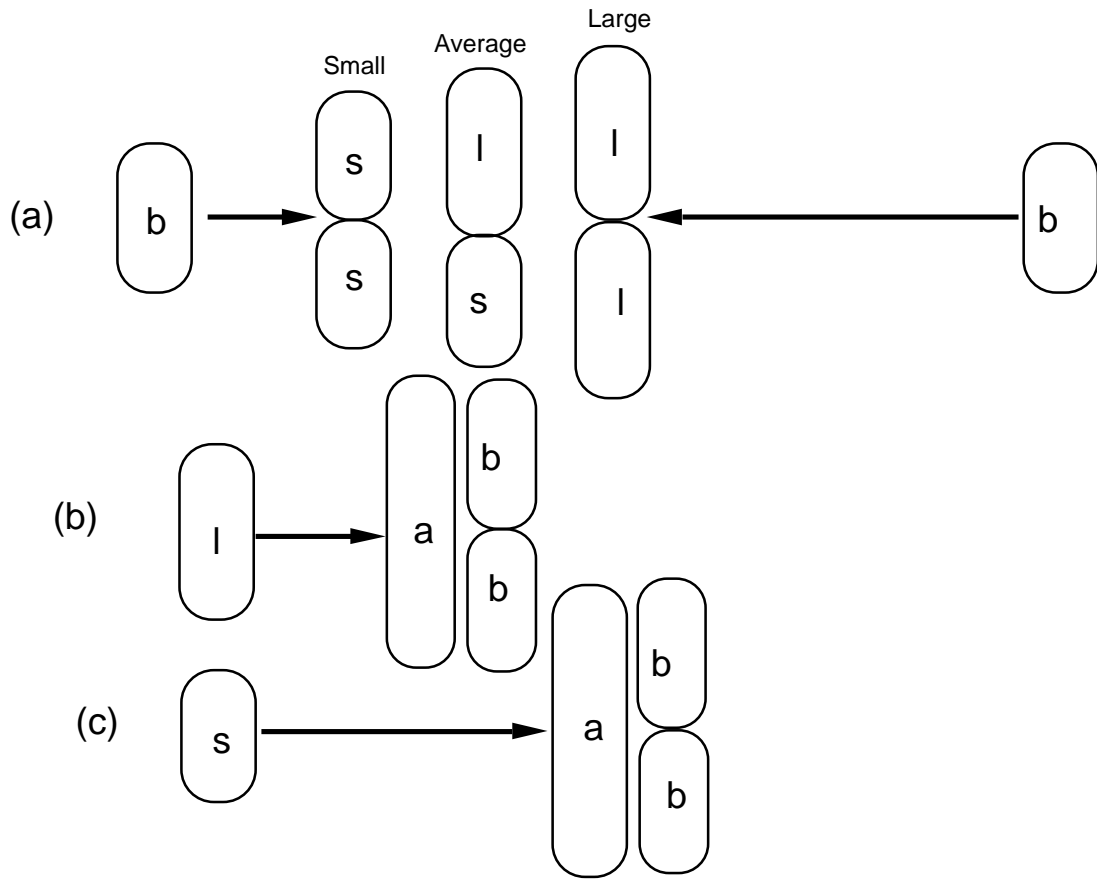


FIGURE 2

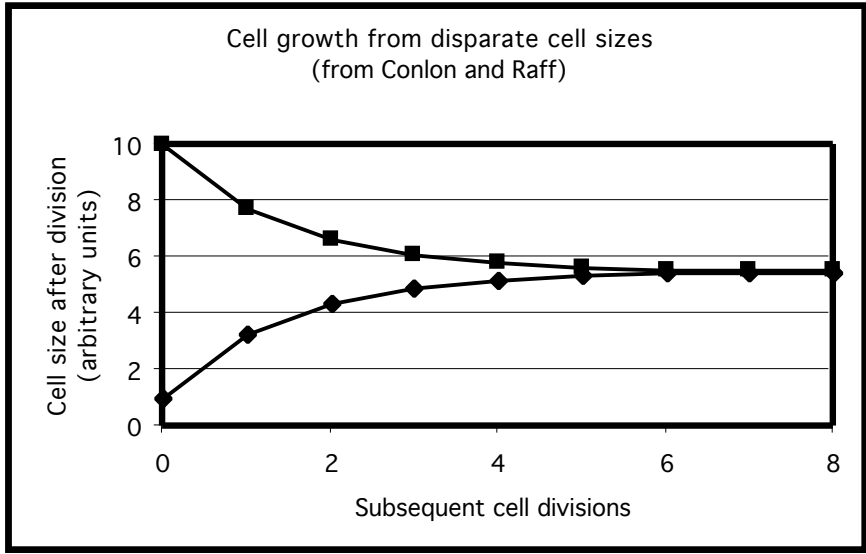


FIGURE 3

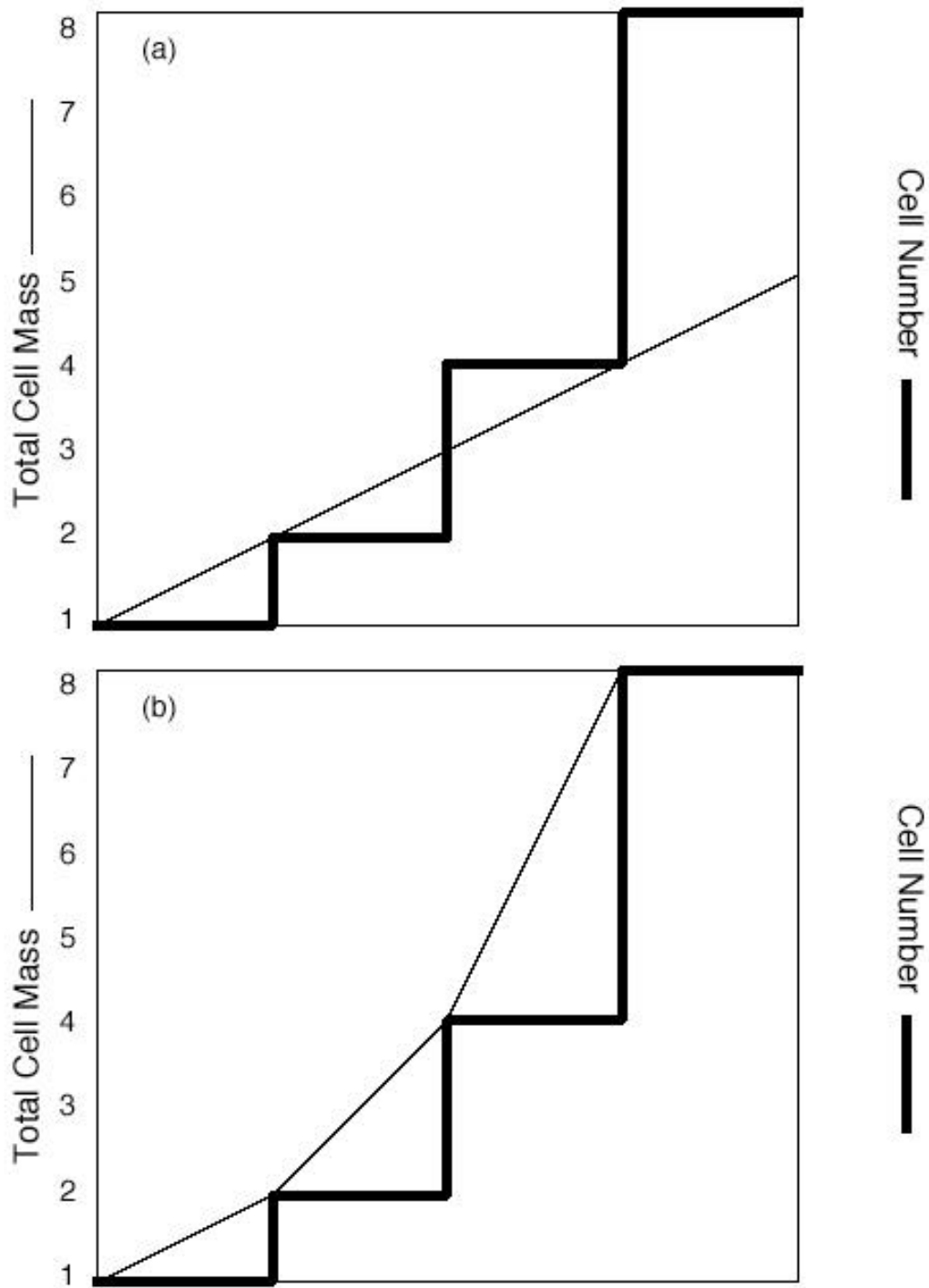


Fig. 4.

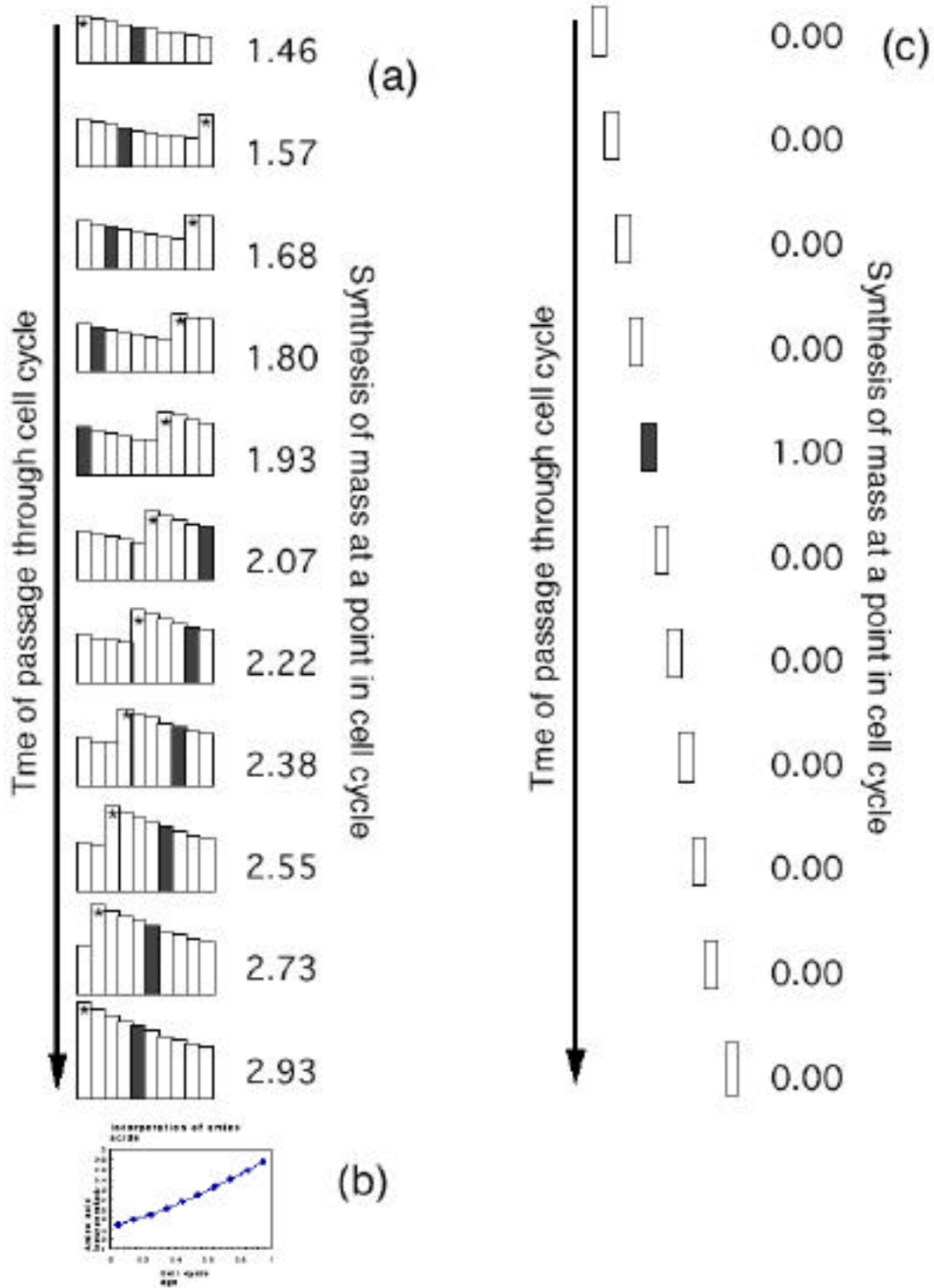


FIGURE 5

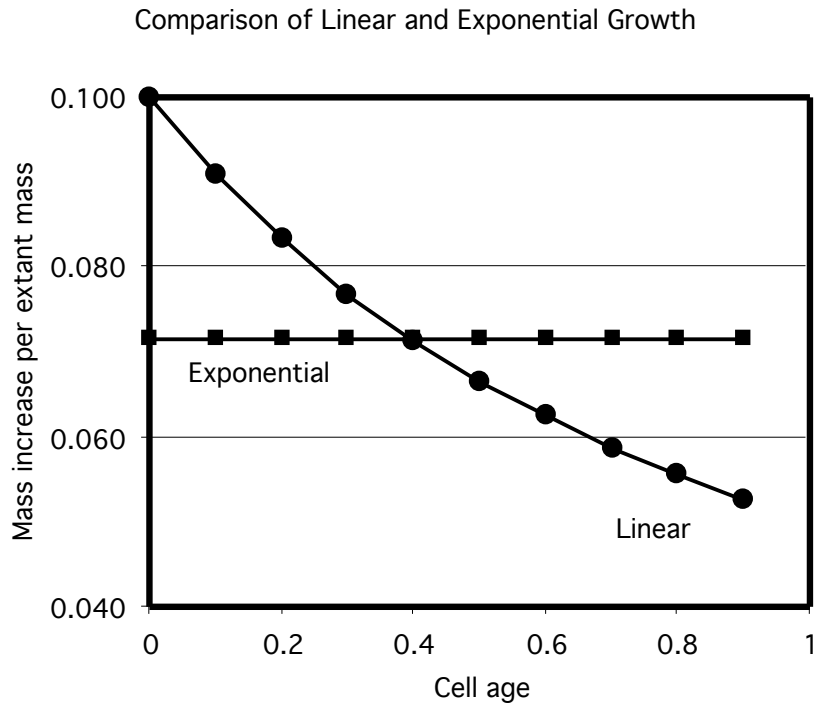


FIGURE 6

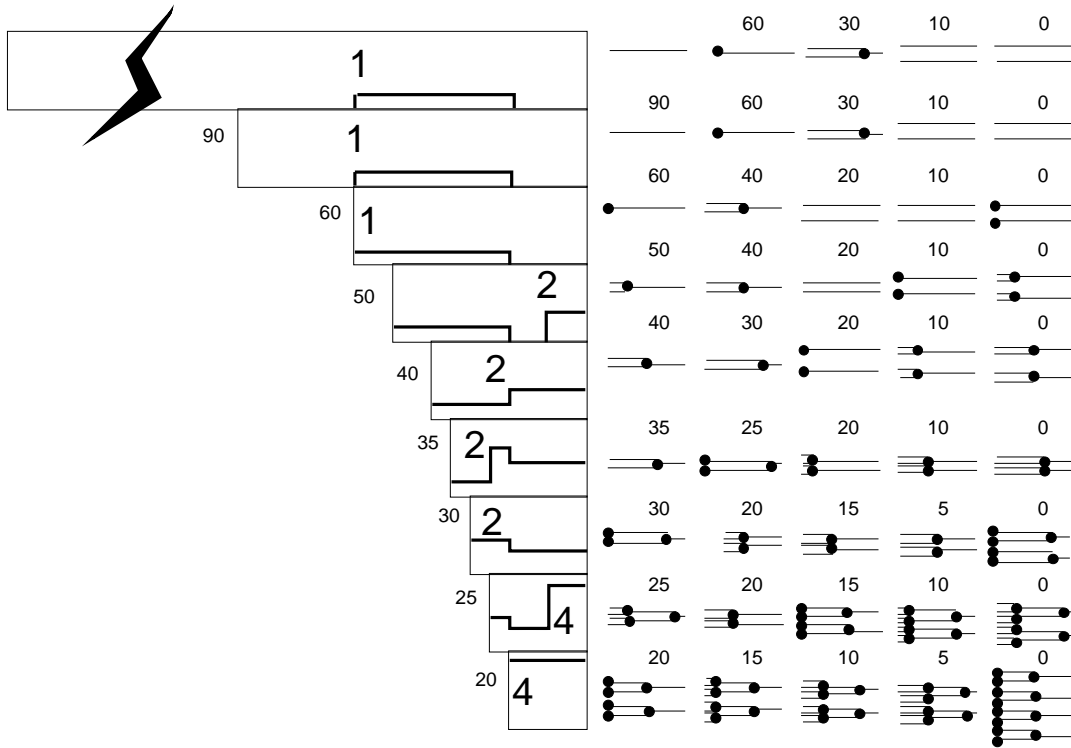


FIGURE 7

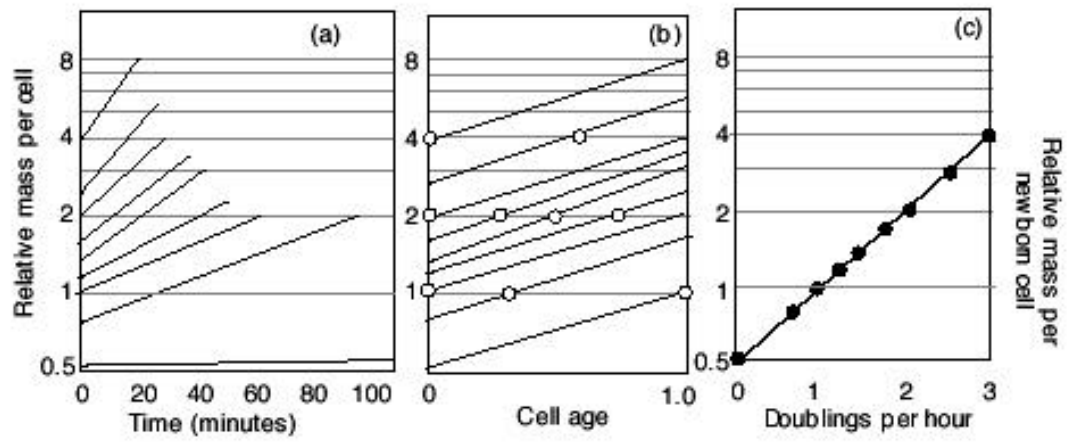


Figure 8.

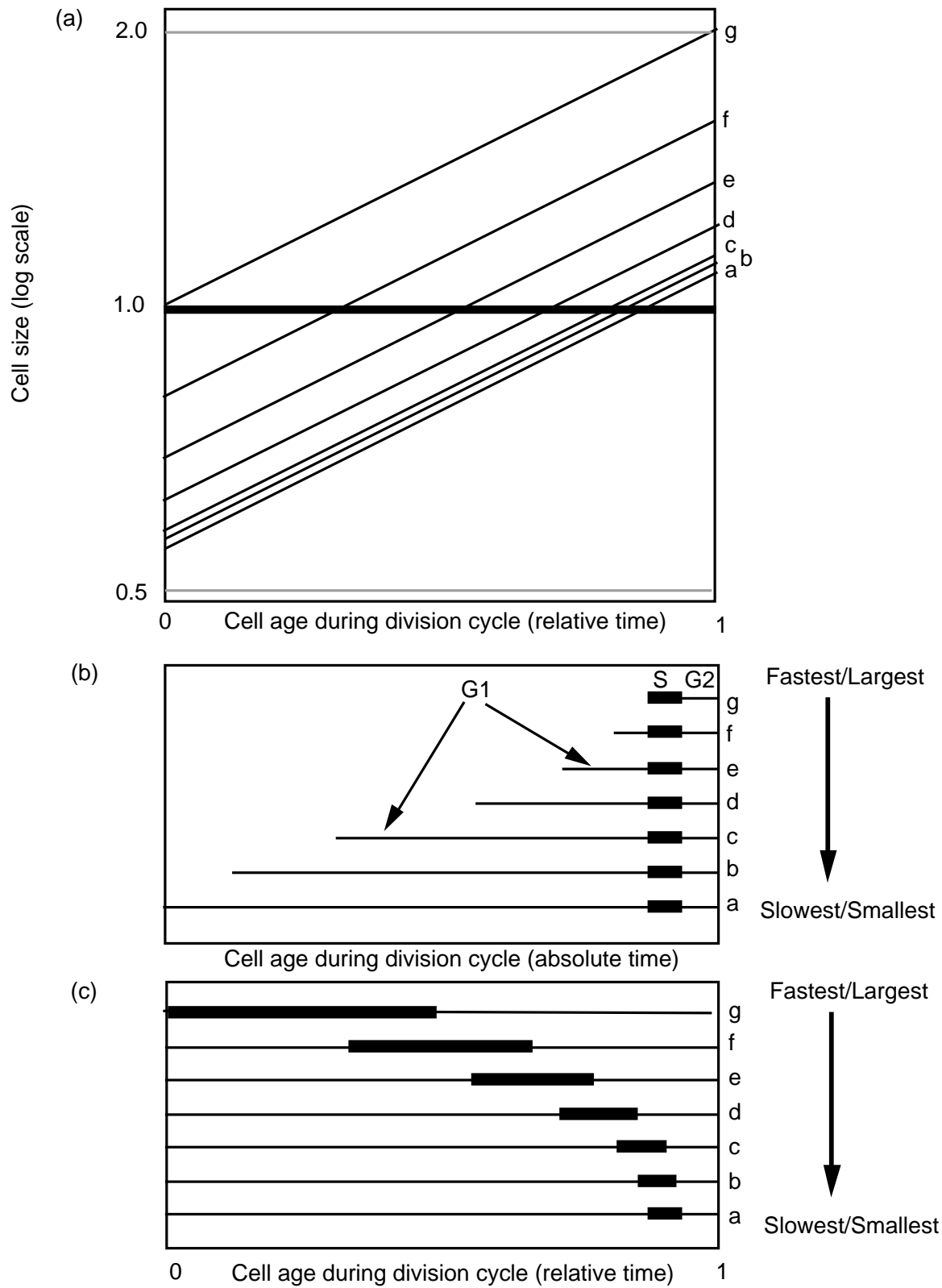


FIGURE 9

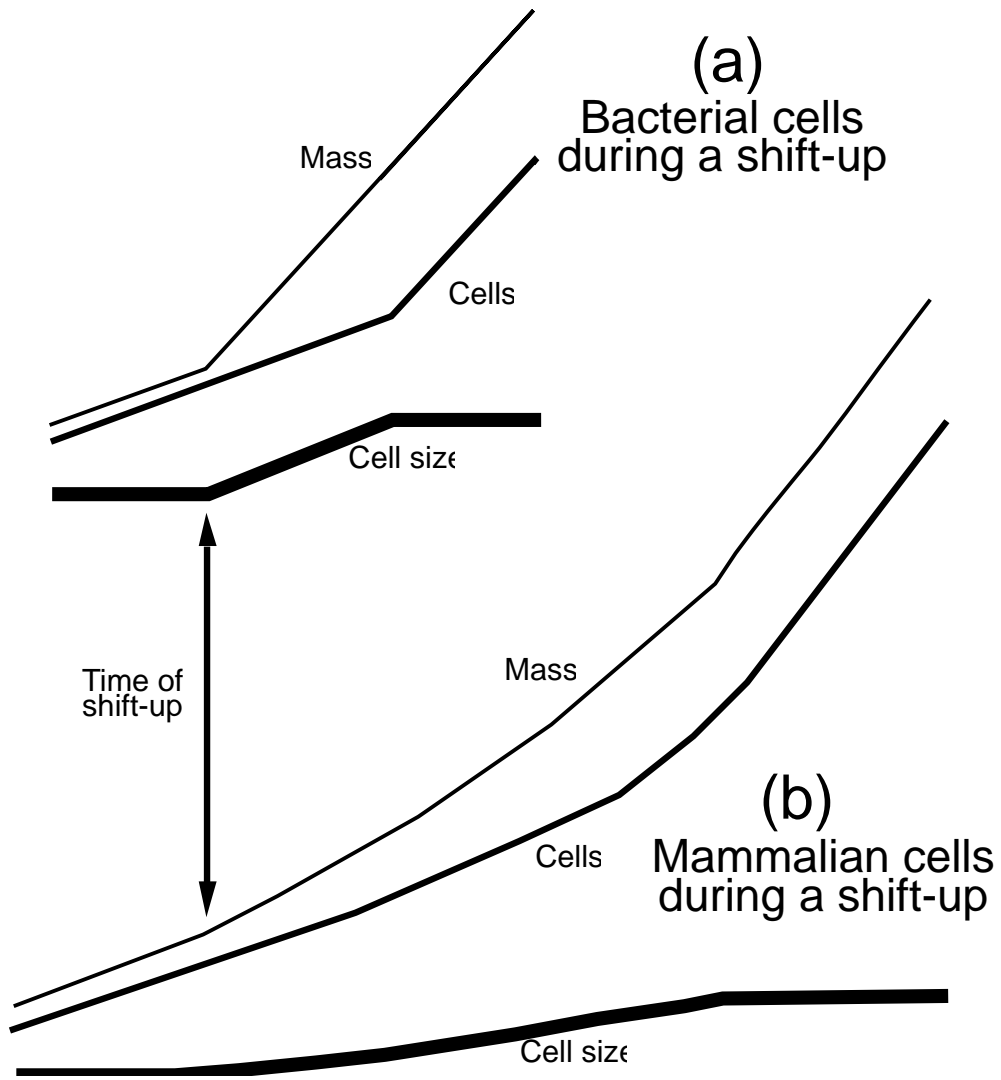


FIGURE 10

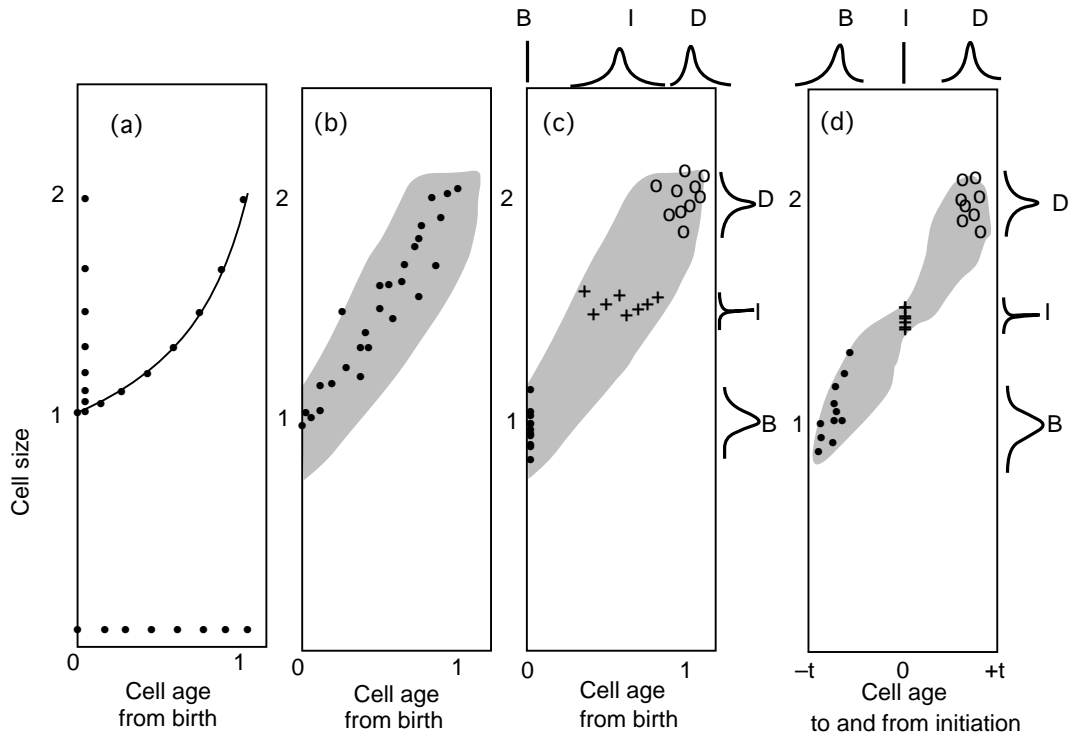


Figure 11.