Regulation of cell growth is a major biological mystery. Even the kinetics of cell growth, although studied for many years, remain elusive. This state of affairs reflects, in large part, the fact that cell growth represents the interaction of a large set of complex and interrelated biochemical processes that allow the cell to survive and respond to a tremendous variety of challenges and stimuli. Thus, it might seem at first that studies of cell growth during the cellular division cycle may be of little help. Nevertheless, interest in cell growth patterns has continued to increase because such studies reveal the presence of major processes that contribute to the regulation of cell growth and may also provide clues to their biochemical identification.

Methods and criteria

Cell growth has been studied far more extensively in *Escherichia coli* than in any other prokaryotic or eukaryotic cell type. Several experimental approaches have been used, including studies of growth in individual cells, synchronized cultures, or exponential-phase populations. Because each of these approaches has produced a variety of results and conclusions for the kinetics of cell growth during the cell cycle in this micro-organism, no common consensus on the pattern of cell growth has developed, much less an understanding of its regulation. The slow progress in this area reflects both the complexity of growth and the technological difficulties in its study, as well as the involvement of semantic differences. For example, in these different studies, 'growth' has variously been defined and measured as increase in cell length, surface area, volume, mass, or content of cellular RNA and protein.

The accumulated evidence now makes it possible to resolve some of the earlier conflicting results, especially as we have become more cognizant of the kinds of difficulties associated with these experiments. In addition to reproducibility, there are three major requirements for experiments on cell growth. (1) Resolution must be sufficient to distinguish among the three major growth models that have been proposed – exponential, linear, or bilinear growth. For example, the experimental resolution must be far better than the largest difference, about 6%, between exponential and linear growth (Fig. 1). (2) Growth characteristics of cells in synchronized cultures must remain unchanged from their values in steady-state or exponential phase cultures; that is, growth should not be perturbed by procedures used to synchronize cells. (3) Data analysis must concern an appropriate growth parameter. In particular, as discussed later, averages over measured values of divided and undivided cells in synchronous cultures are inappropriate because the two groups represent different generations.

The earliest method for measuring cell growth was to observe individual cells by light microscopy as they progressed through the division cycle. Results were not very meaningful until the late 1950s, however, when the concept of maintaining balanced growth conditions was established (Campbell, 1957). Nevertheless, even when these conditions were adopted, very different patterns of length increase were found for the single strain *E. coli* B/r, including exponential (Schaechter et al., 1962), bilinear (Cullum & Vicente, 1978) and interrupted (Hoffman & Frank, 1965). These disparate patterns reflected insufficient optical resolution, approximately 0.4–0.5 μm, in the measurement of cells 1–2 μm in length.

This lack of sufficient optical resolution led N. Nanninga, C. L. Woldringh and their colleagues at the University of Amsterdam to study cell growth and division by a technique capable of much higher resolution: electron micrography. However, with this technique, they could not follow the growth of individual cells; consequently, cell growth kinetics were obtained with a method based upon analysis of steady-state population distributions of cell size, as pioneered by Collins & Richmond (1962). This method provided greater consistency for the measurement of the kinetics.

† Shortly after completing work on this paper, Dr Herbert E. Kubitschek died on 28 November 1989, at the age of 69.
of cell length extension, which was exponential during
the division cycle (Koppes et al., 1978a, b; Kubitschek
& Woldringh, 1983). Furthermore, if cell width were
constant, these results implied that cell volumes should
increase essentially exponentially. However, Trueba
& Woldringh (1980) found that cell diameters did not
remain constant but diminished by as much as 8% during
part of the cycle, corresponding to a change in cross-
sectional area of about 15%. In this case, cell volume
should increase less rapidly than exponentially. In
support of this view, Kubitschek (1981) found that when
both lengths and diameters were measured for individual
cells, then cell volume appeared to increase bilinearly,
i.e. with a step-up in growth rate during the cycle.

The Collins–Richmond analysis, however, requires
data of much greater precision than do more direct
measurements of cell growth because the shape of the
population distribution depends not only upon cell
growth during the cycle, but also upon the distributions
of cell size at birth and division. Analysis requires
estimations of these contributions from birth and
division, which must be either assumed or derived from
distributions of deeply constricted cells. Because these
different techniques are so strongly influenced by
statistical variation, experimental errors can only be
reduced by the labour-intensive approach of collecting
far more data than is required by other methods (Koch,
1966; Kubitschek & Woldringh, 1983).

The third method of determining cell growth kinetics
uses synchronous cultures. Ideally, a synchronously
growing cohort of cells of a given age or size is selected
from a steady-state or exponential-phase culture, and the
growth of this cohort is then used to represent the growth
pattern of the average individual cell. The use of
synchronized cultures, however, has led to observations
of a variety of growth patterns for E. coli. For length
extension alone, these patterns include, in addition to
those discussed above, linear (Donachie et al., 1976;
Pierucci, 1978), approximately exponential (Marr et al.,
1969), or even a more complex response (Meyer et al.,
1979).

When cell volume increase was examined in synchron-
ized cultures, the growth patterns were mainly of two
kinds and appeared to depend upon the synchroniza-
tion technique. Cell volume increase (already concluded to be
approximately exponential from length measurements
alone) was also approximately exponential when cells
were synchronized by membrane elution or elutriation
centrifugation and their sizes were measured microgra-
raphically (Olijhoek et al., 1982; Olijhoek, 1983). On the
other hand, cell volume increase was found to be linear
when cells were synchronized by sucrose gradient
centrifugation and their sizes determined with a Coulter
electronic counter analyser system (Kubitschek, 1968a,
1986, 1990). This apparent dependence upon methodology
highlighted a requirement for consistency of experi-
mental results with those from cultures in steady-
state or exponential growth for adequate comparisons to
be made.

The second criterion, the absence of perturbation of
cell growth, is commonly recognized, and tests for
perturbation are usually performed. However, these tests
are customarily based only upon the timing of increase in
cell numbers (Nanninga & Woldringh, 1985; Kubits-
chek, 1987), and as such are incomplete because cell
volumes may also be perturbed in synchronized cultures.

Several observations support the existence of cell
growth perturbation in cultures synchronized by mem-
brane elution (Kubitschek, 1987, 1990). First, when cells
of E. coli B/r were filtered on membranes, synthesis of
protein D and other altered outer-membrane proteins
was induced, changing the chemical composition of the
outer membranes of these cells (Boyd & Holland, 1977).
Second, buoyant densities of E. coli cells synchronized
by membrane elution were significantly higher than those of
cells in their unsynchronized parent culture (Kubitschek
et al., 1983, 1984). Third, rates of increase of cell length
and volume changed periodically during the cell cycle
after membrane elution (Olijhoek & Nanninga, cited by
Nanninga et al., 1982). Fourth, in slowly growing
membrane-eluted cultures of E. coli B/rA, DNA replica-
tion was consistently initiated at or near the beginning of
the cell cycle, with a large gap in synthesis for most of the
final quarter or third of the cycle (Helmstetter &
Pierucci, 1976; Koppes et al., 1978b). In exponential-
phase cultures, however, the gap in DNA synthesis
occurred, instead, during the first part of the division.
cycle as measured by DNA:DNA hybridization (Chandler et al., 1975), decay of incorporated $^{125}$I (Kubitschek & Newman, 1978), and flow cytometry (Skarsted et al., 1985). These comparisons provide very strong evidence for perturbation of cell growth by the membrane elution technique. Thus, although this technique has yielded fundamental information on DNA replication, one cannot conclude that it provides synchronized cultures with cell characteristics representative of exponential-phase growth, as stated recently (Cooper, 1988a).

The third criterion is that the analysis of cell growth must concern the proper cohort in a synchronous culture. Cell growth during the division cycle has sometimes been interpreted as the increase in mean, modal or total cell volume in the synchronized culture (Scott et al., 1980; Olijhoek et al., 1982). Although each of these parameters represents growth of the average individual cell early in the cycle, each ceases to represent the average as soon as any cells divide, because the newly divided cells advance to the following generation and are no longer representative of the kinetics of cell growth of the first generation (Kubitschek, 1986). Because ages and volumes of newly divided cells are smaller than those of the remaining undivided cells, if both groups are averaged together, then average cell size and age will decrease as more and more cells divide near the end of the cycle. Furthermore, the mean cell volume cannot then double during the cycle, as occurs for undivided cells during steady-state or exponential phase growth. Thus, average individual cell growth is correctly represented only by the growth of the undivided cells alone, for which both age and volume continually increase in the same manner as they do in steady-state or exponential-phase cultures. The misuse of mean, modal or total cell volume to describe cell growth is readily apparent from graphs of cell growth: these incorrect calculations yield a single, sinuous curve connecting the points of successive division cycles. In contrast, proper analysis of the growth of undivided cells provides distinct curves of volume increase for each generation.

Even when the three criteria (resolution, absence of perturbation, and appropriate cohort) appear to be satisfied, experimental results may still differ for different techniques. To ascertain which set of results is correct, the guiding principle is again to compare the experimental results with those obtained in steady-state or exponential-phase cultures. If a direct comparison cannot be made, perturbation may be revealed by examining some alternative measurable characteristic. The timing of DNA replication of *E. coli* B/rA in slowly growing exponential-phase cultures is an example. As observed by autoradiography of individual cells from an exponential-phase culture, initiation of DNA replication starts very near the beginning of the division cycle and is associated with a long D period (Koppes et al., 1978b). However, when measurements were made upon exponential-phase cultures by methods of DNA:DNA hybridization, by survival of cells exposed to decay of incorporated $^{125}$I, or by flow cytometry, initiation was found to occur much later in the cycle, following a large gap in DNA synthesis and associated with much shorter D periods. Again, the concurrence obtained with the latter three widely different techniques strongly supports a large initial gap in DNA replication during the division cycle of this strain of bacterium.

The source of the alternative kinetics observed with electron micrography is not yet identified. Possibly, the different kinetics concern the consequences of fixation. Although electron micrography provides excellent dimensional resolution, the cells are flattened and deformed in cross-section during specimen preparation (Woldringh et al., 1977). Also, although the fixation technique was carefully designed to match optical dimensions of cell length and width, the unusual pattern produced for initiation of DNA synthesis in *E. coli* B/rA continues to raise questions about the consequences of the fixation technique. These questions also apply to more recent electron micrographic results obtained for cells in steady-state cultures exposed to shifts in growth rate. In particular, in an experimental and analytical tour de force, Zaritsky et al. (1982) examined a number of models of cell growth for their agreement with the experimental results during a shift in growth rate. Their results supported a model of linear increase in cell surface area and a gradual increase in growth rate during the growth shift. As they pointed out, however, assumptions of constancy of cell mass at initiation and constant periods of chromosome replication might invalidate their conclusions.

The three major criteria for *E. coli* cell-growth studies, as discussed above, appear to have been achieved for synchronous cultures by measurements of cell volume with a Coulter counter analyser system in cultures synchronized by selection of cells from velocity gradients. Results of tests for perturbation of growth were negative, with the same doubling periods for cell numbers and cell volumes in synchronous cultures and parent cultures, and the same sizes of cells at birth and at division (Kubitschek, 1986, 1987, 1990). The accuracy of the Coulter counter analyser system also was examined by comparing cell volumes observed with the counter to those obtained with a common biophysical technique, the measurement of excluded volume in packed cells. The results supported the same cell volumes within experimental errors (Kubitschek & Friske, 1986) and therefore establish that the counter analyser measured the cell volume within the outer membrane, as expected (rather than, say, that within the inner cell membrane).
Growth models: comparison and implications

In the cultures just described, cell volumes increased linearly in *E. coli* B/rA at doubling times of 25 and 40 min, in *E. coli* 15THU at doubling times of 25 and about 105 min, and in *E. coli* 12 WP2s at a doubling time of about 105 min (Kubitschek, 1986, 1990). In two ways, these results effectively ruled out an exponential increase in cell volume during the cycle. Regressions fitted to the data had slopes and intercepts that were in significant disagreement with those predicted by the exponential growth model. In addition, the nonrandom distribution of residuals (i.e. differences between observed and theoretical values) invalidated the model of exponential growth. Bilinear growth was not completely ruled out, but in one case at least it was shown that if growth is bilinear, then growth rate doubling must occur very near cell division, within 0-03 generations, and the pattern would appear almost indistinguishable from linear growth (Kubitschek, 1986).

These results also effectively ruled out an exponential increase in cell mass, the fundamental growth parameter. It has been shown that cell buoyant density in *E. coli* is essentially independent of age (Kubitschek et al., 1983); and thus cell mass, which is the product of cell volume times density, must remain proportional to volume. Studies of buoyant cell density and the criteria that must be satisfied for experimental determination thereof were reviewed earlier (Kubitschek, 1987).

Despite this evidence against exponential growth, Cooper (1988a) argued that cell growth in *E. coli* must be exponential because protein and RNA are known to accumulate essentially exponentially in cells during the division cycle. That argument, however, neglects the contribution of soluble material in the cell pools to the total cell mass. In the eukaryote *Schizosaccharomyces pombe*, for example, this ratio is approximately 15% during midcycle, as observed long ago by Mitchison (1957). Although the fraction of pool mass is only a few percent of the total in *E. coli*, the kinetics of cell growth depend strongly upon pool size variation during the cycle. Assuming that macromolecular synthesis increases exponentially during the division cycle, linear growth predicts that soluble pools will increase to midcycle maxima and then decrease to their initial values again at the end of the cell cycle, whereas exponential cell growth predicts the absence of such a midcycle maximum. The linear growth model was supported by the behaviour of cell pools for uracil, histidine and methionine in *E. coli* THU (Kubitschek & Pai, 1988).

What is the implication of linear cell mass increase during the cycle? Clearly, constant rates of mass increase require constant rates of accumulation of compounds from the surrounding medium. To test that possibility for individual compounds, Kubitschek (1968b) examined rates of accumulation of glucose, acetate, phosphate, sulphate, leucine, glycine and thymidine in synchronous cultures of *E. coli*. In every case, the relative constancy of the net uptake rates both supported the linear growth model and provided evidence against the increasing rates of uptake during the cycle predicted by the exponential model. Uptake of potassium in synchronous cultures also supported the linear growth model (Kubitschek et al., 1971).

Accumulation rate experiments inherently have much greater resolution than measurements of cell mass or volume and consequently are more capable of discriminating between alternative cell growth models. Despite this advantage, I am aware of only one other attempt to measure uptake rate, rather than incorporation, of a protein or RNA precursor in *E. coli*, an experiment by Cooper (1988a). He treated cells with a pulse of radioactive leucine, then washed away the free radioactivity with warm medium before inverting the membrane and sampling at intervals. However, these samples could not have represented leucine uptake rates during the cell cycle as stated because unincorporated leucine would have almost immediately been dissipated by diffusion from the cells or have become incorporated into protein before the cells were eluted from the membrane. Thus, those results cannot reveal the sizes of the leucine pools later in cells of different ages or the fraction of the pool leucine that becomes incorporated into the cell at different ages.

Models have been proposed for both exponential and linear growth. In his passive continuum model, Cooper (1979, 1982) proposed that cell mass increase is essentially exponential because the increase in cell mass is the sum of the different macromolecular and micromolecular components of the cell and the rates of synthesis of these components are assumed to be essentially proportional to their masses. This proportionality has been found to hold, to good approximation, for RNA and protein synthesis. Cooper’s (1984) model also postulates that, at most, a very few ‘events’ occur during the cell cycle, and therefore that ‘cell division is the end of a process and the beginning of none’. That is, cell growth between divisions is predicted to be simple and continuous (Cooper, 1988b). The model thus predicts that sudden transitions and new modes of regulation at the whole-cell level do not occur.

In contrast, the model for linear cell growth is a transport model of growth regulation (Kubitschek, 1968a, b). It postulates that increase in cell mass during steady-state growth is limited by and proportional to the net rate of transport of materials into the cell, which in turn is proportional to the number of functional transport
sites on the cell surface. Cell growth rate constancy requires that the (functional) number of these sites remain constant during the cell cycle and that the number be doubled at or near cell division, thus maintaining the same average numbers of sites on daughter cells.

Two predictions of this latter model have now been supported. The first is that some cell proteins associated with the surface should double abruptly; abrupt doubling has been observed for a number of envelope proteins of *E. coli* (Ohki, 1979; Churchward & Holland, 1976; Boyd & Holland, 1979) and for binding proteins for lactose and maltose (Shen & Boos, 1973; Dietzel et al., 1978), as discussed below. Second, assuming that cellular macromolecular synthesis increases essentially exponentially during the division cycle, linear growth predicts that soluble pools of RNA and protein precursors should increase to midcycle maxima, then decrease again to their initial values by the end of the division cycle. Measured values of pool sizes for uracil, histidine and methionine in *E. coli* THU supported these predicted pool kinetics (Kubitschek & Pai, 1988).

**Abrupt doubling of transport proteins**

Perhaps the most distinctive difference between the exponential and the linear growth models concerns the latter’s prediction of a set of transport proteins that double abruptly during the cell cycle. This difference will almost certainly distinguish between the two models and has already begun to do so, as described below. First, however, in opposition to that interpretation, it can be argued that although a number of candidates have been found, the evidence for the sharpness of the doubling is insufficient because these doublings occurred at many different times during the cell cycle and the relationships of some of these proteins to transport was not established. Furthermore, stepwise increases contrast with the results of Lutkenhaus et al. (1979), who observed by means of two-dimensional electrophoresis essentially continuous synthesis of 750 individual proteins of *E. coli*. For an even more precise estimate of synthesis rates, they also measured the radioactivity of some 30 of these proteins synthesized during the cell cycle and found that the variability of each was 15% or less. These results would negate the linear growth model unless the proteins measured are structural or ‘housekeeping’ proteins not involved in the regulation of cell growth. The transport sites model predicts that only a small fraction of the cell proteins should double abruptly. In agreement, most or all proteins observed to double abruptly are envelope or periplasmic proteins.

Another objection to the evidence presented below, for abrupt doubling of specific proteins during the cell cycle, might be that this evidence is obtained from experiments that have not been shown to satisfy the critical tests for absence of growth perturbation. Indeed, in some cases, data were obtained from cultures synchronized by membrane elution and, consequently, are probably perturbed. However, although the precise kinetics of abrupt doubling of specific proteins may not be established, there is no question about the occurrence of the phenomenon. In each study, the time interval required for doubling of envelope/periplasmic protein was much shorter than the cell generation time.

The first membrane proteins found to increase abruptly in *E. coli* K12 were cytochrome b₁ and L-α-glycerolphosphate dehydrogenase (Ohki, 1972). The two proteins appeared to double simultaneously during the cell cycle, but the cell age at doubling depended upon the method used for synchronization and occurred at ages of either 0.6 or 0.2 generations. This dependence of time of synthesis upon the synchronization method appears to be a common feature in measurement of abruptly doubling proteins and suggests that different methods of synchronization perturb the timing of doubling to different degrees. The following year, using nutritionally synchronized cultures, Shen & Boos (1973) found that the periplasmic galactose-binding protein was synthesized only during or near cell division. Within a few years, several other proteins were found to be produced in a cycle-dependent manner. These included envelope proteins [the α-receptor protein (Ryter et al., 1975) and protein D (Gudas et al., 1976; Boyd & Holland, 1977)], inner-membrane proteins [L-α-glycerolphosphate dehydrogenase (Ohki & Mitsui, 1974) and lactose permease (Ohki & Sato, 1975)], and another cytoplasmic protein [the maltose-binding protein (Dietzel et al., 1978)].

Churchward & Holland (1976) studied the synthesis of 29 envelope proteins in cultures synchronized by the membrane elution technique. They found that rates of synthesis of the great majority of these proteins increased stepwise and that these steps occurred early in the cycle. (However, one envelope protein, the D protein of *M. bactera* 76000, was synthesized periodically near the time of division.) To be a prediction of the linear growth model, it would seem that abrupt doublings in rates of synthesis of these surface proteins would be limited by the numbers of functional transport sites.

Ohki (1979) identified 11 proteins, each of which doubled 10–20 min before cell division, and also described their cellular locations; these proteins were primarily inner-membrane proteins, but a few were outer-membrane or cytoplasmic. Ohki assigned these proteins to cell-cycle control A. He assigned the maltose- and galactose-binding proteins and other periplasmic proteins to cell-cycle control B because these proteins
were synthesized 11 min later. His studies clearly demonstrated that there may be more than one cell-cycle control mechanism that produces abrupt protein doubling; they suggest, however, no more than two.

Boyd & Holland (1979) also found that the rate of synthesis of outer-membrane proteins, and in particular of a 36-5 kDa porin, doubled during the final part of the cell cycle. Doubling of phospholipid synthesis, observed by Pierucci (1979) and Pierucci et al. (1981), was confirmed by Joseleau-Petit et al. (1984), and again later by the same group (Joseleau-Petit et al., 1987), using the relatively new technique of automatic synchronization (Kepes & Kepes, 1980). The confirmation also provides support for this synchrony technique, which uses a forcing method rather than selection to obtain synchronization. The abrupt synthesis of the λ-receptor protein was confirmed by Ohki & Nikaido (cited in Ohki, 1979), and later, by a very different method, by Vos-Scheperkepter et al. (1984), who showed that the lamB protein also doubled before division.

There is little or no evidence that abrupt doubling is related to DNA replication per se. When DNA synthesis was blocked, rates of synthesis of total and outer-membrane and of cytoplasmic proteins continued to increase in parallel (Boyd & Holland, 1979). Nor is there evidence that the control for abrupt doubling operates at the level of gene expression: while total RNA and trp mRNA synthesis continued at almost normal rates at a nonpermissive temperature in the cell-cycle control mutant tsC42, the mutant failed to synthesize lac mRNA at the nonpermissive temperature (Ohki, 1979). However, Benner et al. (1985) provided evidence that the control of the maltose transport system is at the transcriptional level. Furthermore, results for doubling of the rate of phospholipid synthesis are compatible with models in which some event is linked to replication termination or nucleoid segregation (Joseleau-Petit et al., 1987). Thus, the mechanism(s) for cell-cycle regulation of abrupt doubling of selected proteins have not yet been definitively identified.

The isolation of a cell-cycle control mutant by Ohki & Mitsui (1974) is an important step in the elucidation of this mystery. The defect of the tsC42 mutant (divE42) is phase specific. The divE42 gene functions only about 20 min before cell division and is essential for cell growth and membrane synthesis (Ohki & Sato, 1975). When shifted to 42°C, the mutant increased in protein by a factor of about 1-8 before ceasing growth, and at this stage the cells were large and homogeneous in size. When returned to a permissive temperature, cells divided synchronously after an immediate burst of synthesis of particular enzymes, such as succinate dehydrogenase. In synchronous cultures, the cell cycle stage at which mutant cells were arrested coincided with the time of synthesis of the particular proteins whose synthesis was controlled by divE42, leading Ohki (1979) to propose that a defect in the divE42 gene is involved in both cell cycle regulation of specific protein synthesis and of cell division. The defect produced by this mutation was later identified as a single alteration of the D stem of the isoacceptor serine tRNA^Ser^ (Tamura et al., 1983).

Finally, these results for the divE42 gene suggest that the abrupt doubling of specific proteins in the absence of gene doubling may also have a role in cell division. Other evidence has recently been obtained by A. Robin & D. Joseleau-Petit (personal communication), who found that the ftsZ gene was transcribed bilinearly, but with a doubling in rate around the time of initiation of DNA synthesis rather than near the end of the cell cycle; they used an ftsZ::lacZ fusion carried on a λ prophage in cultures under automatic synchronization. Thus, the phenomenon of abrupt doubling of proteins near the end of the cell cycle may offer crucial information on the development of cell division, as well as on the regulation of cell growth.

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