LINEAR CELL GROWTH IN *ESCHERICHIA COLI*

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ABSTRACT Growth was studied in synchronous cultures of *Escherichia coli*, using three strains and several rates of cell division. Synchrony was obtained by the Mitchison-Vincent technique. Controls gave no discernible perturbation in growth or rate of cell division. In all cases, mean cell volumes increased *linearly* (rather than exponentially) during the cycle except possibly for a small period near the end of the cycle. Linear volume growth occurred in synchronous cultures established from cells of different sizes, and also for the first volume doubling of cells prevented from division by a shift up to a more rapid growth rate. As a model for linear kinetics, it is suggested that linear growth represents constant uptake of all major nutrient factors during the cycle, and that constant uptake in turn is established by the presence of a constant number of functional binding or accumulation sites for each growth factor during linear growth of the cell.

INTRODUCTION

Although the major events occurring during the cell growth and division cycle can be outlined broadly, we have little knowledge at present of the explicit nature of growth processes and their controls. Optical studies of large single cells have given markedly different growth kinetics for different organisms (see Prescott (1)), implying that several different modes of control of growth may be involved. Our understanding of bacterial growth processes has been in an even more primitive state, since optical measurements are more difficult for these. Measurements of bacterial cell length (2) and mass (3) have been made, but the data are not precise enought to distinguish between the two simplest alternatives of linear or exponential increase (which differ by less than 6% during one doubling), let alone more complex patterns. More recently, the limitations of direct observation were avoided by a method of analysis of bacterial volume distributions obtained with an electronic counter (4). However, this method at present is only approximate because assumptions must be made about the size distributions of cells at birth and at division, and because, as shown by Koch (5), this procedure requires extremely accurate determinations of cell size distributions, to a degree as yet perhaps unattainable.

Nevertheless, there has been some concurrence that bacteria grow in an essentially exponential manner during the divison cycle. This viewpoint seems to stem primarily from observations with synchronous cultures that proteins accumulate essentially exponentially during the cycle (6, 7) as well as from the rationale that the rate of protein synthesis should increase exponentially if ribosomes are made continuously and if protein synthesis is proportional to ribosomal content (see Koch (5), for example). This viewpoint, however, assumes that cell growth results only from macromolecular synthesis, and thereby neglects the contribution made by the accumulation of precursor pools required for macromolecular synthesis.

The technique developed by Mitchison and Vincent (8) for obtaining synchronous cultures suggested an effective method for determining cell growth kinetics. For these cultures, cell sizes can be measured far more accurately with a Coulter counter analyzer system than is possible optically. Such measurements, reported in this paper, reveal that mean cell volume increases linearly, rather than exponentially, during most of the growth-duplication cycle.

MATERIALS AND METHODS

Bacterial Strains

Escherichia coli, strains B_{s-1}, WP2-HCR$^-$ (requiring tryptophan), and 15 THU (requiring thymine, histidine, and uracil) were used.

Media

Three different media were used to establish three different growth rates for parental cultures at 37°C: nutrient broth (Difco Laboratories, Inc., Detroit, Mich.), M9 salts (9) with glucose (0.1%), and M9 salts with sodium acetate (0.25%). When required for growth, the following compounds were added: thymidine, 2 μg/ml; uracil, 10 μg/ml; histidine monohydrochloride, 50 μg/ml; and tryptophan, 5 μg/ml. Sucrose (2%, by weight), a nonutilizable sugar, was added to some cultures to increase osmotic pressure. The corresponding cell number doubling times in the three media approached 20, 60, and 140 min for each of the three strains.

Synchronous Cultures

Parent cultures were maintained at 37°C in the exponential growth phase for at least 8 doublings in order to ensure balanced growth before selecting a synchronous subpopulation, by the method of Mitchison and Vincent (8). A parent culture (50–100 ml, 2–6 × 10^7 cells per ml) was concentrated by centrifugation (Servall SS-1, Ivan Sorvall, Inc., Norwalk, Conn.) to form a pellet or by filtration through a membrane filter (Bact-T-Flex, Schleicher & Schuell Co., Keene, N.H.), and was resuspended in 0.1–0.2 ml of growth medium. It was then layered upon a linear sucrose density gradient (5–15% sucrose in M9 salt solution, in 8 ml, at 37°C) and centrifuged for 1.5–5 min, depending upon cell size, in a swinging bucket centrifuge (Servall HB-4 rotor, RC2-B, 1500 × *g*). For most experiments a sample of the smallest cells (0.1–0.3 ml, about 3–10% of the culture) was removed from the upper region of the visible band of cells and was inoculated into growth medium (usually 20–30 ml) to give a cell con-

centration of about 1–3 $\times$ 10^6 per ml. Further details of these procedures may be found in an earlier paper (10).

Several precautions were taken to maintain steady-state growth in synchronized cultures. First, all operations were carried out at or near 37°C. Second, for cultures in minimal medium, sucrose was frequently added to decrease the osmotic excursion later during banding. Sucrose also aided pellet formation during centrifugation of acetate cultures; without sucrose, a large fraction of the cells was dispersed over the bottom and the walls of the centrifuge tube. Such cells failed to give good synchrony. Third, the subculture selected for synchrony was reinoculated into the parent growth medium, which had been filtered to remove residual cells.

Asynchronous Controls

Asynchronous control cultures were obtained by removing all cells in the band, mixing, and inoculating a small fraction of these into parent growth medium. When only 3–5% of the parent culture was removed for the synchronous culture, the asynchronous control sometimes was composed of a suitable fraction taken from a mixture of the remaining cells.

Cell Counts and Volumes

Cell counts and volume distributions were obtained with a Coulter counter-analyzer system (amplifier and multichannel analyzer from Nuclear-Chicago Corp., Des Plaines, Ill.). Culture samples (0.5 ml) were diluted about 20-fold into 0.1 N HCl for counting and sizing. Cell size distributions were the same in HCl and saline. Volume scales were calibrated with standard latex microspheres (Dow Chemical Company, Midland, Mich.).

RESULTS AND DISCUSSION

Linear Growth in Synchronous Bacterial Cultures

The degree of synchrony and its rate of decay are illustrated in Figs. 1 and 2 for a synchronous acetate culture of *E. coli* WP2 HCR^-. Both cell counts (Fig. 1) and cell volume distributions (Fig. 2) show that partial synchrony was retained for at least two successive divisions. During the first two cycles there were two doublings of the initial cell count to plateau values, and cell size distributions were narrower than those in randomly dividing cultures. The decay of synchrony in successive cycles is evident from the increase in the fraction of the cycle during which dividing cells may be found, and from the broadening of the cell size distributions.

Although the distributions in Fig. 2 were unimodal at first, they became bimodal as cell numbers increased. The emergence of the second peak corresponds to the formation of small cells by fission of cells in the original peak. This interpretation is supported by the agreement between values for the divided and undivided cell fractions calculated from the increase in cell numbers and the corresponding values calculated from the areas of the two peaks in the bimodal distributions.

Fig. 2 also shows that the mean cell volume of the undivided fraction apparently increased linearly, at least for the first seven samples. In view of the supposed exponential increase in size of bacteria, the values for the mean volumes of these first seven distributions were tested by the variance ratio (F) test. The data were fitted significantly better by a linear increase than by an exponential increase both

for the first five points before any observable cell division as well as for the first seven points. The corresponding F values (ratio of the variance for exponential increase to the variance for a linear increase) were 12.3 and 19.0, respectively, and the corresponding probabilities of these or larger ratios occurring by chance are less than 0.05 and 0.01.

To ascertain whether or not linear growth in synchronous cultures was established only with the smallest cells in the parent population, the following experiment was carried out. Cell samples were taken from three regions of the band so that small, medium, and large cells were included, and synchronous cultures were established from each of these. The initial cell volume distributions of the three cultures are

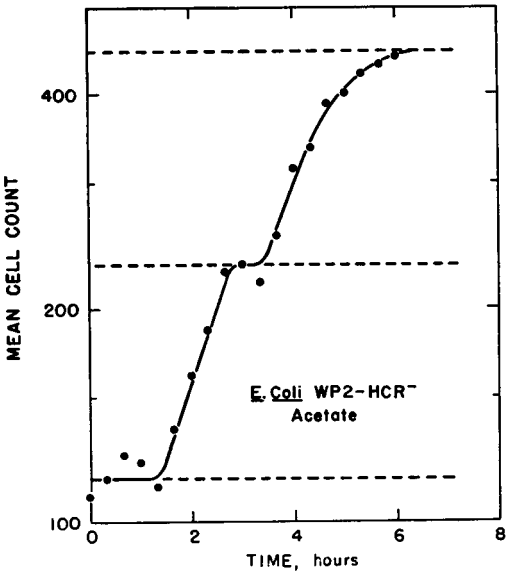

FIGURE 1 Average cell counts during growth of a synchronous culture of *E. coli* WP2-HCR$^-$ in acetate medium. For concentrations, per ml, multiply values by 10^4.

displayed in Fig. 3, along with mean cell volumes calculated as a function of time. Since some of the large cells divided during the course of manipulations, the mean cell volume of only the undivided fraction was determined for the third sample. As shown in this figure not only were straight lines obtained, but there were no significant differences in the rate of increase of mean cell volume in the three cultures: growth rate was essentially independent of initial cell volume, strongly supporting the interpretation of linear growth.

Linear increases in mean cell volume during the first cell cycle were also observed for synchronous cultures of *E. coli* THU in glucose-salts media (Fig. 4). This figure also shows the effect of growth in fresh medium: although the rate of cell volume increase was diminished, a linear increase was observed. Again, growth in broth was linear (Fig. 5). Furthermore, in all of these cultures (Figs. 2, 4, and 5) the asymptotic volume growth rate during the second cycle was also linear and essentially the same as that during the first cycle.

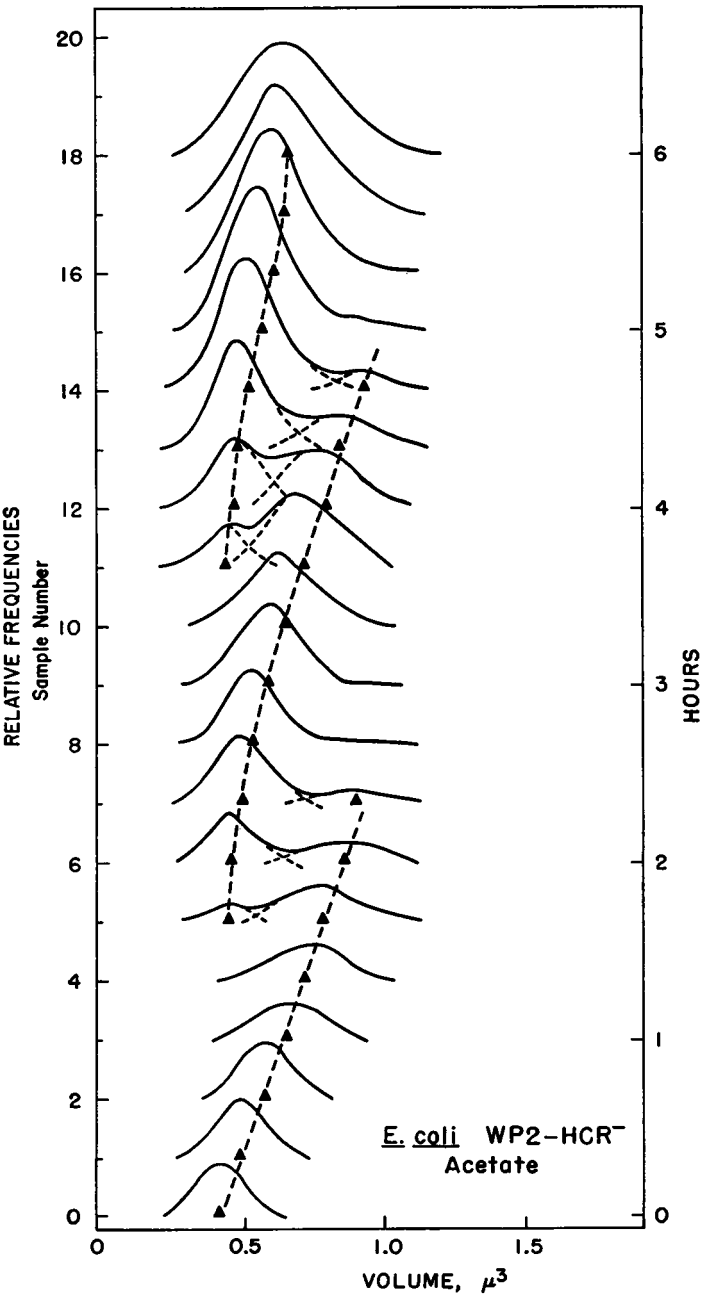

FIGURE 2 Cell size distributions during growth of the same culture as shown in Fig. 1. Relative frequencies vs. cell volume are shown for consecutive samples. Numbers and times at which samples were taken are shown on the ordinates. Triangles indicate mean cell volume. The dashed lines show the time-dependence of mean cell volume.

These experiments at different growth rates, and others, including some with strain B_{s-1}, provide prima facie evidence for a linear increase in cell volume during most of the growth-duplication cycle of these bacteria.

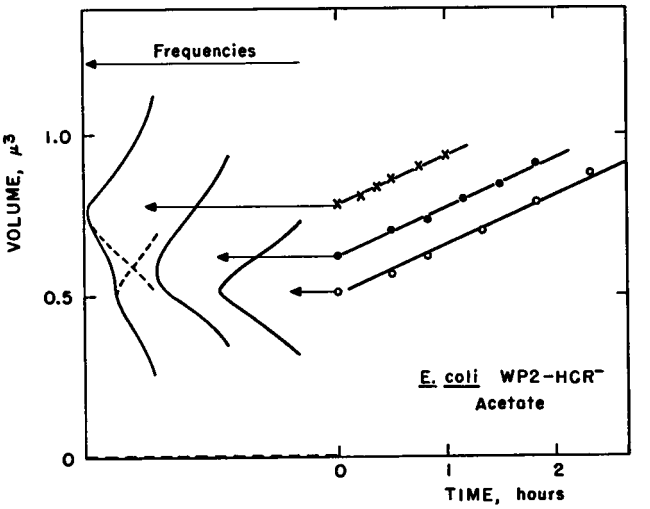

FIGURE 3 Independence of growth rate and initial cell size. An acetate culture of *E. coli* WP2-HCR$^-$ was banded in a sucrose density gradient. Three samples of different mean cell volume were removed and used as inocula for synchronous cultures. The initial cell size distributions are shown to the left of the volume growth curves. The mean values, X, for the sample with the largest cells were calculated only for the undivided cell fraction (the larger of the two peaks).

Evidence for the Absence of Perturbation of Steady-State Growth

During the second cycle, mean cell volumes first increased slowly, reaching the first cycle rate only asymptotically (Figs. 2, 4, and 5). This curvature might suggest that technical procedures altered cell growth so that the cultures were not in steady-state growth during the first cycle or the second, or perhaps either cycle. However, none of the evidence supports an interpretation of this kind. Instead, the following evidence supports the absence of any significant growth perturbation.

(*a*) There is no perceptible initial lag in growth or change in growth rate during the first division cycle in any of the experiments (Figs. 2, 4, and 5).

(*b*) In all of these experiments, the asymptotic growth rate during the second cycle was indistinguishable from that during the first cycle.

(*c*) Asynchronous control cultures, carried through the same procedures, showed no detectable lag or change in rate of cell division.

(*d*) When the total volume of cells in the culture was calculated, it was found to be linear during both the first and second cycles. This is shown in Fig. 6 for the

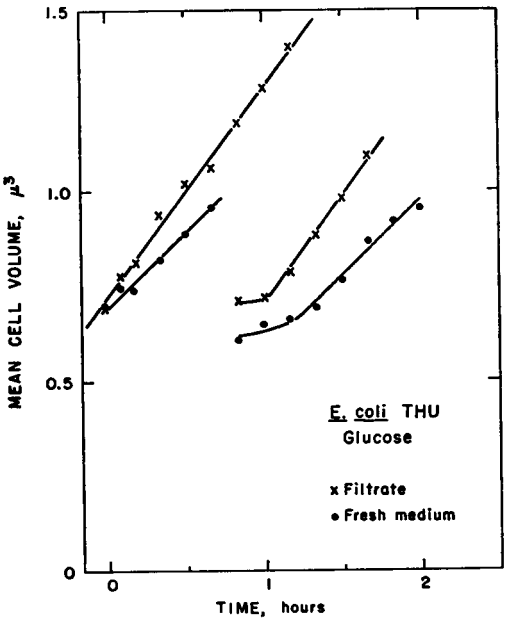

FIGURE 4 Mean cell volumes for synchronous glucose cultures of *E. coli* THU. This figure shows the effect of inoculating the culture into fresh growth medium, as well as that for inoculating into filtered parent growth medium. Data are shown for the first two cycles.

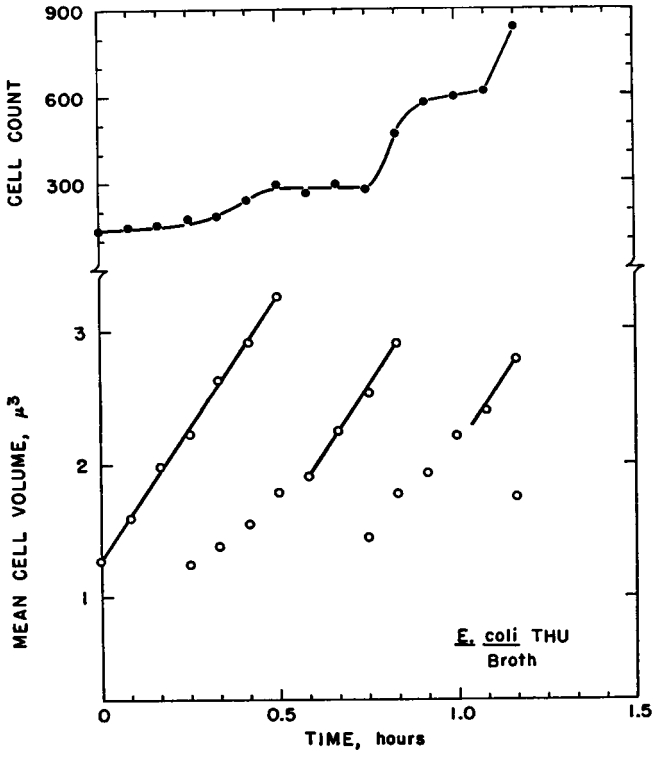

FIGURE 5 Average cell counts and mean cell volumes for a synchronous broth culture of *E. coli* THU. Growth (mean cell volume) is shown for the first, second, and third cell cycles.

acetate culture already discussed. Total volume of cells per ml was computed from the values in Figs. 1 and 2: for each sample, the mean cell count given by the line in Fig. 1 was used to determine the numbers of divided and undivided cells in the culture, and these numbers were multiplied by the corresponding mean cell volumes shown in Fig. 2. These data show that total cell volume per ml increased linearly (a significantly better fit than exponential increase) during the second cycle as well as during the first, and that the rate of increase was essentially doubled.

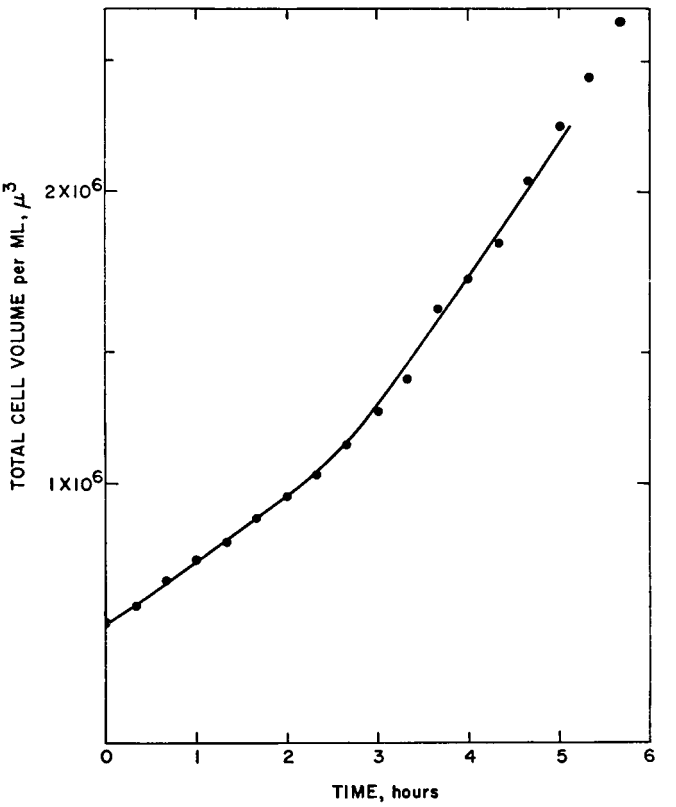

FIGURE 6 The increase of total cell volume per ml, as obtained from the data in Figs. 1 and 2 (see text).

Desynchronization

The curvature during the second generation might arise from transient departures from linear growth or from desynchronization, even though the growth pattern during the cycle was identical with that for the parent culture. There are at least three possibilities:

(*a*) Growth rates might change just before or after division. Thus, curvature would be observed if growth rates of newly-divided cells were reduced, or if growth

rates of cells near the end of the cycle were increased. Of course, if growth of the culture was not perturbed, then a growth lag present at the beginning of each cycle should have been observed during the first generation unless the lag was very short or the reduction in growth rate was small, so a pronounced change in growth rate at the beginning of the cycle seems unlikely since it is not seen during the first cycle. At present, rates of growth cannot be determined for cells remaining in the undivided fraction at the very end of the cycle; here growth rates may vary undetectably.

(*b*) Desynchronization could result in part from an orderly division of cells as they reach a critical volume. If the coefficient of variation of cell size decreased, for example, because only the largest cells divided, then the mean cell volume of the residual undivided fraction would be decreased. This possibility, however, also fails to fit the data because the coefficient of variation for the size distribution of the undivided cell fraction did not vary appreciably for the first seven undivided cell fractions. (The average value was 0.17 for the first seven samples, and 0.15 for the eighth.) This relatively constant value for the coefficient of variation also indicates that the growth rate of the undivided fraction represents the growth rate of the entire culture with fair accuracy.

(*c*) The most likely explanation for desynchronization is that it must arise, even if growth is completely linear, because of the variability of cell generation times.[1] This is most easily seen by considering a model population of cells initially all of the same size, all growing linearly at the same rate at all times, and all of which split precisely in two at division. The growth kinetics of this model culture are shown in Fig. 7 by the heavy lines. The dashed line represents the "birth line," that is, cell volumes at birth for cells born at any time; this line has half the slope and half the amplitude of that for growth during the first cycle. More strictly, the birth line is a segment extending from the shortest generation time, at *A*, to the longest, at *D*. Since we have assumed that newly-born cells immediately increase in volume at the same rate as their progenitors, individual cells born at the times *A*, *B*, *C*, and *D* will increase in volume according to the individual trajectories shown by the arrows. Consider the first cells to divide, at *A*. Since their trajectory has a slope that is twice as great as the slope of the birth line, these cells must always be larger during their second cycle than all progeny born later. In the same manner, cells born at times *B* and *C* must also be larger than cells born later. The result is that the mean cell volume during the second cycle cannot immediately increase at the rate for individual cells, but will continue to be weighted toward lower growth rates by the addition of newborn cells until all cells have divided. Thus the finite range of generation times leads to partial desynchronization during the second cycle, with an apparent lag in growth.

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[1] Sinclair, W. K. 1967. Personal communication.

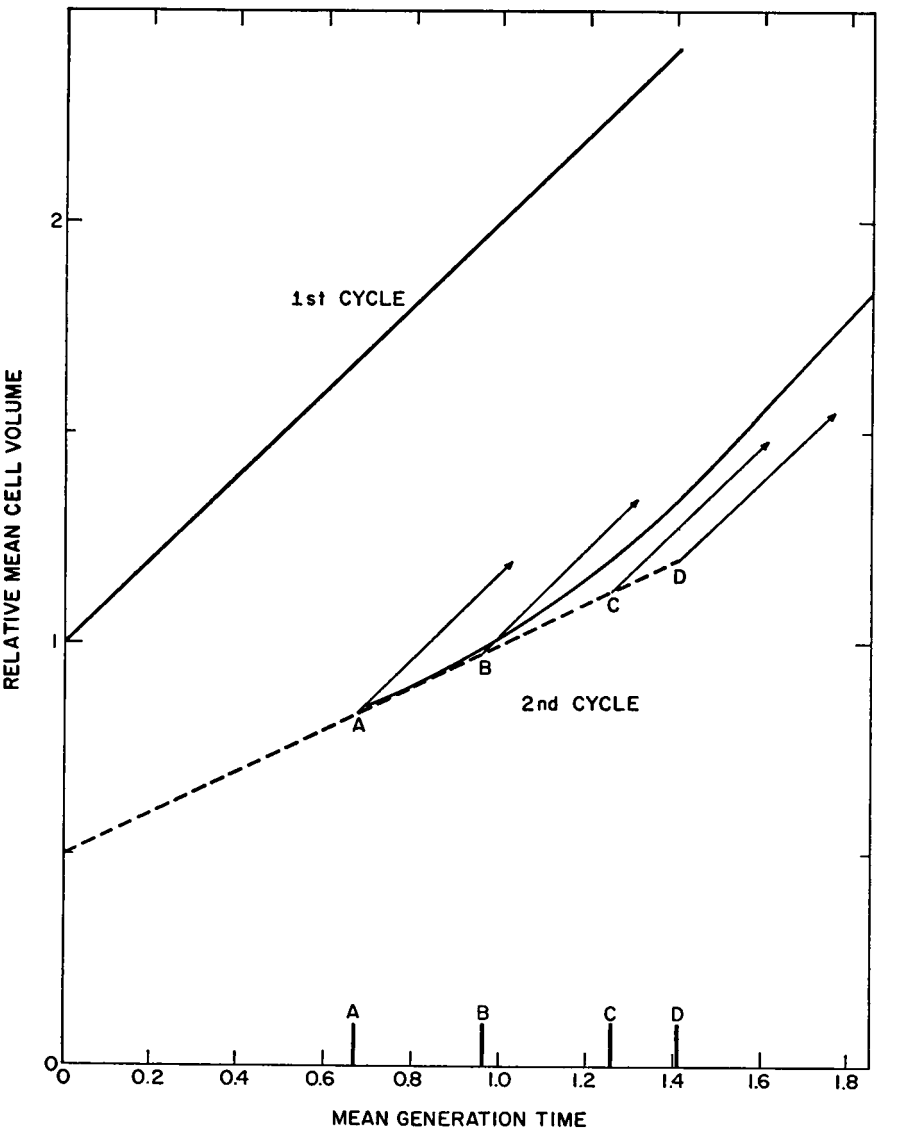

FIGURE 7 Growth kinetics expected for a synchronous model culture of cells having a finite range of generation times. All cells grow linearly and at the same rate; at division, two daughters of equal volume are formed; initially, all cells have the same volume. Generation times range from *A* to *D*. Mean cell volumes during the first and second cycles are represented by the heavy lines. The dashed line segment between times *A* and *D* is the "birth line," representing the volumes at birth of cells born by fission during this period. The arrows represent the growth kinetics of only those cells born at times *A*, *B*, *C*, or *D*.

Linear Growth After a Metabolic Shift

To test further the possibility that linear growth might be an artifact, a synchronous culture of strain WP2-HCR⁻ in acetate medium was metabolically "shifted up" by adding glucose (0.1%) and nutrient broth (5%). Although cell volumes increased 4-fold, cell numbers did not change. Mean cell volumes again increased linearly during the first volume doubling (Fig. 8), and coefficients of variation remained constant within experimental error.

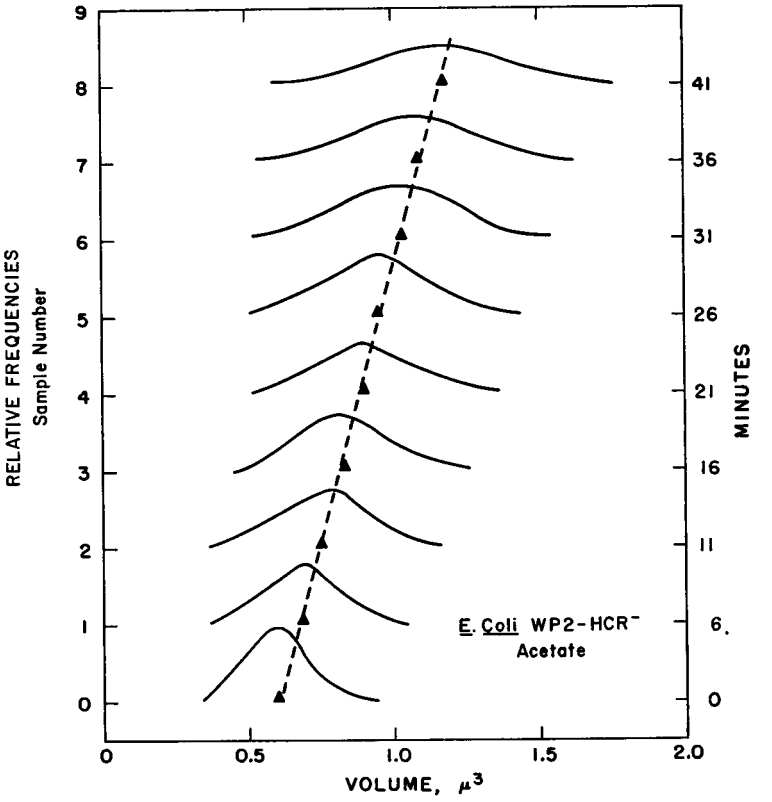

FIGURE 8 Cell size distributions (frequency vs. volume) for a synchronous acetate culture of *E. coli* WP2-HCR⁻ during the first volume doubling after a "shift up" by adding glucose and nutrient broth.

Growth kinetics were no longer linear, however, during the second volume doubling of this culture (Fig. 9). Instead, volumes increased in an essentially exponential fashion. Presumably, growth controls were modified in some way between the first and second volume doublings. Whatever the nature of the change, these results demonstrate that exponential growth kinetics can be distinguished from linear kinetics.

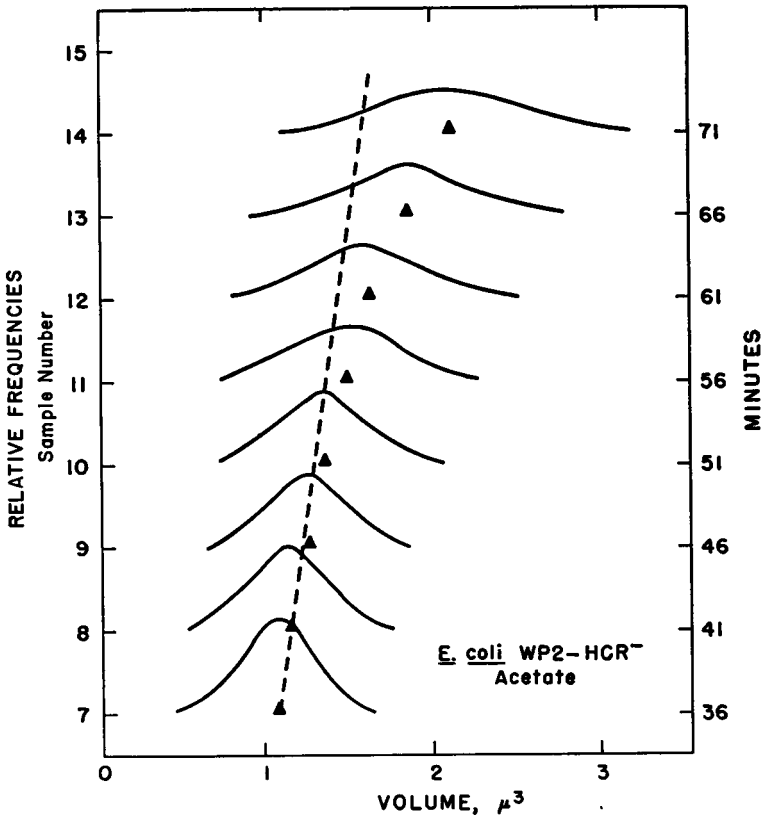

FIGURE 9 Cell size distributions for the same culture as shown in Fig. 7, during the second volume doubling. The average cell count remained constant during the consecutive volume doublings.

Model for Linear Cell Growth

Although growth rates in *E. coli* are shown to be constant during most of the cell cycle, the rates of synthesis of RNA and protein increase during the cell cycle (6, 7, 11). These findings for bacteria parallel the findings of Mitchison and his collaborators for yeast (12): cell mass increased linearly over most of the cycle, but macromolecular mass increased exponentially. As an explanation, Mitchison and Wilbur (13) postulated the existence of a pool of substances of low molecular weight that varied in a regular manner, first increasing, then decreasing during the cell cycle. Later, the existence of regularly varying pools in yeast was demonstrated more directly by interference microscope measurements of cell mass with and without extraction of soluble pools (13). In the same manner, our present knowledge of mass increase and of macromolecular synthesis in bacteria would be explained by the presence of similar pools that vary in the same manner during the bacterial cell cycle.

As Mitchison indicated, a linear mass increase requires a constant rate of accumulation of nutrients (12). The simplest interpretation of a constant accumulation would be that it is due to a constant rate of uptake of low molecular weight compounds from the growth medium. If so, we would expect cell uptake to be limited by a constant number of functional binding or accumulation sites involved in the uptake of exogenous growth factors during the major part of the cycle. Furthermore, this over-all constancy might in turn be due to the presence of constant numbers of each of the several (or possibly many) different kinds of binding or accumulation sites needed for each of the major components taken up by the cell, primarily amino acids, sugars, and inorganic ions.

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