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Correlation Between Size and Age at Different Events in the Cell Division Cycle of *Escherichia coli*

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The variability of (i) the B period between birth and initiation of chromosome replication, (ii) the U period between initiation of chromosome replication and initiation of cell constriction, and (iii) the interdivision period (τ) have been estimated for slowly growing *Escherichia coli* B/r F. Cultures synchronized by the membrane elution technique were pulse-labeled with [^{3}H]thymidine or continuously labeled with [^{3}H]thymine. After fixation, the pattern of deoxyribonucleic acid replication was analyzed by electron microscopic radioautography. Cell length was found to increase exponentially with age at two different slow growth rates. The coefficient of variation of the B period was estimated to be 60%, that of the U period was 29%, and that of the interdivision period was 12%. From these values and the coefficient of variation of length at different cell cycle events we calculated a negative correlation between the B and U period ($r = -0.9$) and a positive correlation between length at birth and cell separation ($r = 0.6$). Initiation of chromosome replication and cell constriction were strictly correlated both with respect to age ($r = 0.7$) and length ($r = 0.8$). On the other hand, length at initiation of chromosome replication was distantly correlated with age ($r = 0.1$) or length at birth ($r = 0.3$). This low correlation excludes a model in which chromosome initiation is controlled by a random event in the B period. It favors a model in which chromosome initiation occurs at a particular distributed size independent of cell division.

An early approach to the study of the cell cycle was to look at individual cells in a light microscope and to record the sizes or ages at which events occur. From these studies and from later interpretations (17) it is apparent that there is considerable variation in the age and size at which individual cells initiate chromosome replication and at which they divide (17, 18). These studies also demonstrate that there are correlations between the interdivision times of related individuals in a family tree up to the third generation (9, 22, 28, 30). Powell concluded that "the generation time of an individual is determined partly by molecular accidents, partly by heredity" (28). Thus the control of cell cycle events cannot conclusively be described by constants within the life span of an individual cell. A model for the control of chromosome replication and cell division should explain variability and dependence on ancestral events.

Koch and Schaechter put forward a hypothesis to explain the variability of cell division and the correlation between the interdivision times of related cells in a steady-state culture (18). Based on the observation of a twofold-higher coefficient of variation (CV) of age at division as compared to that of size, they postulated that cells divide when they attain a critical size, rather than age, independent of their size at previous divisions. Because of a higher CV of size at chromosome initiation as compared to that at cell division, Koch later suggested that both events could be independently triggered by "some aspects of cell growth" (17). In contrast, Newman and Kubitschek reported a lower CV for the interinitiation time of chromosome replication as compared to the interdivision time of cells, and they implied that cell division may be driven primarily by chromosome replication (26).

In a preceding paper we calculated a high coefficient of correlation (r) between length at initiation of chromosome replication and length at initiation of cell constriction in slowly growing steady-state cultures of *Escherichia coli* B/r K. We explained that such a correlation favors a model in which an event before or at chromosome initiation triggers cell division (19).

In this paper we investigate the variability of length and age at successive cell cycle events in slowly growing cultures of *E. coli* B/r F synchronized by the membrane elution technique (13). In our previous work with *E. coli* B/r, K cells were classified primarily by length, whereas

in the present work with *E. coli* B/r, F cells are classified primarily by age, taking advantage of the better synchronizability of the latter strain by membrane elution. At slow growth rates, both strains show a period devoid of DNA synthesis in the beginning of the cell division cycle before initiation of chromosome replication (B period), which is more pronounced than that of the closely related strain *E. coli* B/r A (21). We have again determined the kinetics of length extension. From the data we were able to calculate the coefficient of correlation between lengths at successive events. We found a high correlation between chromosome initiation and cell constriction, but a low one between birth and chromosome initiation.

MATERIALS AND METHODS

Culture and medium. *E. coli* B/r F *thy his* was obtained from C. E. Helmstetter. Cells were grown in a minimal medium (12) supplemented with 0.05 mg of thymine and 0.05 mg of L-histidine per ml, with either 0.04% L-alanine plus 0.04% L-proline (doubling time, T_D = 82 min) or 0.2% sodium acetate (T_D = 150 and 165 min) as carbon sources. For each experiment 100 ml of minimal medium was inoculated with bacteria and incubated under aeration by shaking in a water bath at 37°C. Growth was measured turbidimetrically at 450 nm in a Zeiss PMQ II spectrophotometer. Cell number was determined in samples fixed with 0.4% formaldehyde by using a Coulter Counter (model Z_B) equipped with a 30-μm orifice.

Synchronization. Synchronization was carried out by the membrane elution technique as has been previously described (24). The experiments in which T_D was 82 min and 165 min have been published before (24); in the present study they were analyzed in more detail. In some experiments (T_D = 150 min), preelution was at a reduced rate of 7 ml/min for 1 h, after which newborn cells were collected at the same rate in an ice bath for 5 to 10 min.

Pulse-labeling with [^{3}H]thymidine. Every 10 min, a 3-ml sample of synchronized cells was incubated with [^{3}H]thymidine (17 μCi/ml, 27 Ci/mmol; Radiochemical Centre, Amersham, England) at 37°C for 5 min. The incorporation was stopped by adding 3 ml of ice-cold minimal salts medium supplemented with 1 mg of thymidine per ml and, subsequently, 1 ml of ice-cold 1% OsO_4. After fixation for at least 30 min on ice, the cells were sedimented by centrifugation at 6,000 × *g* for 1 h at 4°C, washed in 5 ml of minimal salts medium supplemented with 0.01 M NaN_3, and suspended in 0.05 ml of minimal salts medium with 1% bacitracin.

Agar filtration and radioautography. These techniques were carried out as previously described (references 40 and 20, respectively).

Analysis of radioautograms. Cell length was measured and grains were counted from electron micrographs projected at a final magnification between 10,000 and 40,000 onto a transparent tablet digitizer (Summagraphics, Fairfield, Conn.), which was connected to a calculator (HP 9825A).

The average number of grains above radioactive cells was at least 4; the average number of background grains above nonradioactive cells in controls was about 0.2. Therefore, cells were considered to be labeled when they were associated with at least two grains. More than 100 cells of a particular age class were screened. Alternatively, the fraction of labeled cells was estimated by fitting a Poisson distribution to the observed frequencies of grains per cell [see Appendix (i)]. Both methods gave about the same result.

Symbols. Symbols used for the different parameters of the cell cycle are listed in Table 1.

RESULTS

Degree of synchronization. In the present experiments we synchronized *E. coli* B/r F in two different growth media by the membrane elution technique. The cells eluted from the membrane were collected in an ice bath for about 1/10 of the doubling time (T_D). Then they were incubated at 37°C to initiate synchronized growth (t = 0). Because the further analysis depends heavily on the success of the synchronization procedure, we first calculated a synchronization index F: the fraction of the population at t = 0 that really has age zero.

Figure 1 (histograms) shows length distributions of *E. coli* B/r F at t = 0. The steady-state cultures had T_D values of 82 min (Fig. 1A) and 165 min (Fig. 1B). The constricted cells (hatched areas) indicate contaminating older cells in the sample. On the assumption that these cells have been randomly eluted from the membrane (and, therefore, have the length and age distribution characteristic of the steady-state culture), F can be estimated: $F = 1 - g(0)/g_e$, in which $g(0)$ is the fraction of constricted cells in the sample at t = 0 and g_e is this fraction in the exponentially growing population. F cannot be calculated very precisely due to the low number of constricted

TABLE 1. *Symbols used for the parameters of the cell cycle*[a]

Event	Length at event	Age at event	Period between events
Birth	L_0	$a_0 = 0$	B
Initiation of DNA replication	L_i	a_i = B	C
Termination of DNA replication	L_f	a_f = B + C	U
Initiation of cell constriction	L_c	a_c = B + U	D
Separation of daughters	L_s	$a_s = \tau$	T

[a] Note that the interdivision time τ = B + C + D = B + U + T.

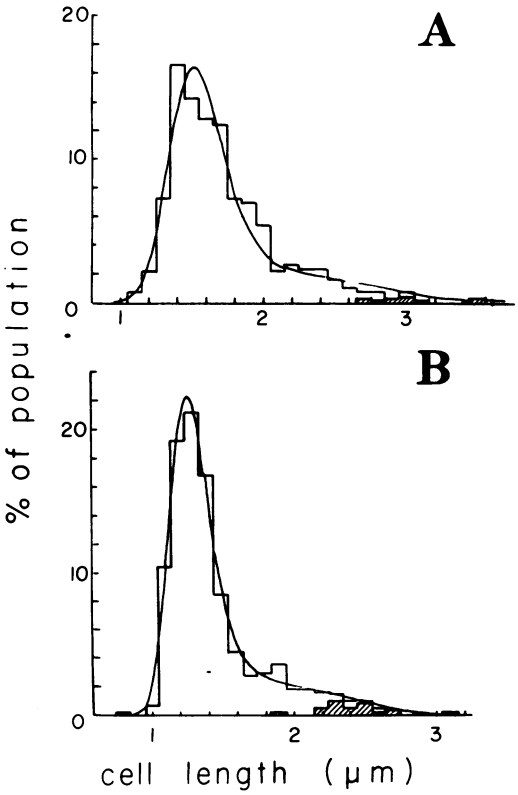

FIG. 1. *Length distributions of E. coli B/r F obtained by membrane elution (sample from t = 0). (A) T_D = 82 min; N = 698; $\bar{L}$ = 1.71 μm; CV = 22.3%. (B) T_D = 165 min; N = 616; $\bar{L}$ = 1.44 μm; CV = 23.6%. Constricted cells are indicated by the hatched areas. The curves were calculated from the length distributions at birth and cell separation in the steady-state culture assuming that cells elongate exponentially and that a part (F) is newborn and the rest (1 − F) has been randomly eluted from the membrane. The parameters of the curves are: (A) F = 0.75 ± 0.03 (±standard deviation); $\bar{L}_0$ = 1.57 ± 0.01 μm; D krit = 4.6%; D max = 3.4%. (B) F = 0.68 ± 0.03; $\bar{L}_0$ = 1.28 ± 0.01 μm; D krit = 4.9%; D max = 2.5%.*

cells: $F = 0.77 \pm 0.09$ (±standard error) for T_D = 82 min and $F = 0.68 \pm 0.08$ for T_D = 165 min. Therefore, we also estimated F by fitting a model to the length distribution of all cells at $t = 0$. The assumptions of the model are as follows. (i) Random elution of the asynchronous fraction from the membrane. (ii) Log normally distributed lengths of newborn (L_0) and separating cells (L_s). The CV of L_0 equals that of the distances between cell pole and site of constriction (i.e., the lengths of the prospective siblings of the constricted cells in the steady-state population); the CV of L_s equals that of the lengths of the constricted cells in the steady-state population (19). (iii) Exponential length extension of individual cells (see below); the steady-state length distribution can then be calculated by means of the Collins and Richmond equation (3). The parameters of the model are F and $\bar{L}_0$ (mean length at birth). The best fits were calculated by the method of maximum likelihood (33) and are shown as the curves in Fig. 1. They could not be rejected by the Kolmogorov-Smirnov test for goodness of fit at a level of significance $\alpha = 0.10$. In addition, the values of F ($F = 0.75 \pm 0.03$ for T_D = 82 min and $F = 0.68 \pm 0.03$ for T_D = 165 min) did not differ from that estimated from the constricted cells alone (see above). The value of $\bar{L}_0$ (1.57 ± 0.01 μm) was equal to that estimated from the constricted cells in the steady state (1.58 ± 0.03 μm) for T_D = 82 min. For T_D = 165 min, the former (1.28 ± 0.01 μm) was somewhat larger than the latter (1.22 ± 0.01 μm). These findings indicate that the assumptions of the model are reasonable.

Distribution of interdivision times. The cells in a synchronized population will divide at different times due to the distribution of interdivision times $f(\tau)$. Figure 2 shows the increase in relative cell number $N(t)$ as a function of time after incubating the membrane-eluted cells at 37°C. $N(t)$ was calculated from the fraction of constricted cells $g(t)$ at successive times t according to (see appendix of reference 38):

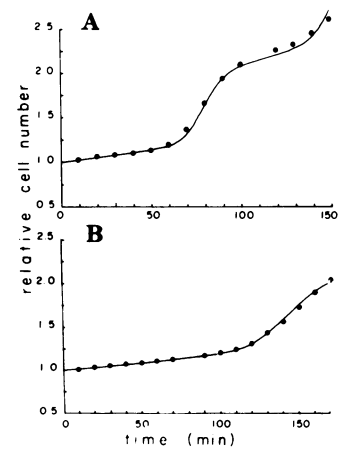

FIG. 2. *Increase of relative cell number during synchronized growth. Relative cell number calculated from the fraction of constricted cells in successive samples on the assumption of a constant T period. (A) T_D = 82 min; T = 15 min. (B) T_D = 165 min; T = 25 min. The curves were calculated using the renewal equation of Harris (8) for the synchronous fraction (F) and the exponential growth equation for the asynchronous fraction (1 − F). In the calculation it was assumed that interdivision times are log normally distributed with parameters: (A) $\bar{\tau}$ = 81 min; CV = 11.4%. (B) $\bar{\tau}$ = 146 min; CV = 13.4%.*

$$N(t) = \exp\left(\frac{1}{T} \cdot \int_0^t g(u) \cdot du\right)$$

in which T is the constant average duration of the constriction process. T was estimated from g_e (40): $T = 15$ min for $T_D = 82$ min and $T = 25$ min for $T_D = 165$ min. The same result within errors was obtained when cell number was estimated with a Coulter Counter for $T_D = 82$ min. To estimate the mean and CV of $f(\tau)$, we calculated theoretical relative cell numbers $N(t)$ assuming the following. (i) The synchronous fraction F of the synchronized population at $t = 0$ increases according to the renewal equation (ref. 8):

$$N(t) = 1 + \int_0^t [2N(t-u) - 1]f(u) \cdot du$$

This equation assumes independence of successive interdivision times. In fact, a negative correlation has been reported (9). However, we think that this correlation can be disregarded in view of experimental errors. (ii) Interdivision times are log normally distributed with parameters $\bar{\tau}$ and CV_τ. We chose the log normal distribution for computational convenience. Other distributions that are truncated at zero and positively skewed (for instance, reciprocal normal and gamma distributions) would probably satisfy equally well. (iii) The asynchronous fraction $1 - F$ at $t = 0$ increases exponentially according to:

$$N(t) = \exp(\ln 2 \cdot t/\bar{\tau})$$

The small difference between T_D and $\bar{\tau}$ (27) is ignored in this equation. This model was fitted to the observed $N(t)$ by the method of least squares (curves in Fig. 2; for parameters see the legend). The mean interdivision time $\bar{\tau}$ of the slow-growing cells is shorter than T_D in the steady state. The CV_τ is about 12.4%. The CV_τ in an independent experiment was 14.3% (T_D = 150 min). The CV_τ of the closely related A strain was 19.3 ± 0.4% averaged over four experiments ($T_D = 67 \pm 7$ min).

Distribution of the B period. The B period is defined as the time interval between birth and initiation of DNA replication. It is the analog of the eucaryotic G1 period. Because G1 is the most variable period of the mitotic cycle (31), we were interested to see whether this also holds for B in *E. coli*.

Part of the membrane-eluted cells ($T_D = 165$ min) were incubated at $t = 0$ in the presence of [^{3}H]thymine at 37°C. At successive times samples were fixed, and the fraction of radioactive cells (i.e., cells that had initiated DNA replication) was estimated by electron microscopic radioautography. In Fig. 3, 95% confidence intervals for the labeled fractions are plotted as a function of time after start of synchronized growth. Such a figure represents the kinetics of entrance of cells in the C period (period of DNA synthesis) as a function of their age. It can be seen that from the beginning more than 10% of the cells are already labeled, probably due to the contaminating asynchronous cells (see above). The curve is a best fit calculated on the assumption of a log normally distributed B period. In the calculation, a different kinetics of labeling of the asynchronous fraction were taken into account [see Appendix (ii)]. The fit is rather poor, as indicated by the chi-square test ($P < 0.005$), and was not much improved by assuming an exponentially distributed B period (36).

Our data are not accurate enough to establish the precise kinetics of initiation of chromosome replication. Nevertheless, they do indicate a large spread in the age at which this event occurs: $CV_B = 60\%$.

Distribution of the U period. The U period is defined as the time interval between initiation of DNA replication and initiation of visible cell constriction. We have estimated the variability of this period by application of the "fraction of unlabeled constrictions" technique to *E. coli* as we described earlier (19).

Strain B/r F ($T_D = 150$ min) was synchronized by the membrane elution technique. The degree of synchronization was limited ($F = 0.52$), but

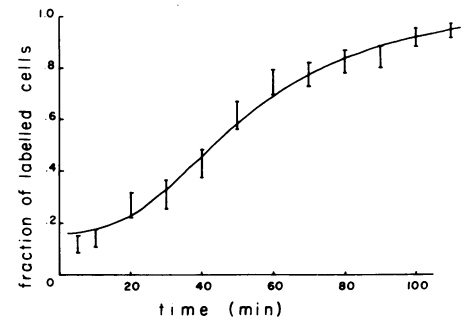

FIG. 3. *Fraction of labeled cells as a function of time after start of synchronized growth. Membrane-eluted cells ($T_D = 165$ min) were incubated at $t = 0$ at 37°C in the presence of [^{3}H]thymine. At successive times afterwards, samples were fixed and the fraction of labeled cells was estimated by radioautography. The 95% confidence intervals for the labeled fractions are given by the bars. The curve was calculated assuming a log normal distribution of the B period with parameters: $\bar{B} = 61 \pm 2$ min; $CV = 60 \pm 4\%$; $\chi^2 = 26.7$; $df = 10$. The kinetics of labeling of the asynchronous fraction (0.32) were assumed to be that of a steady-state culture and are outlined in Appendix (ii).*

this is no drawback because the fraction of unlabeled constrictions technique is rather insensitive to the age distribution in the population (38). The culture was pulse-labeled with [^{3}H]-thymidine for 10 min at 30 min after the start of synchronized growth (note that the B period is about 60 min under these conditions; see Fig. 3) and subsequently chased with an excess of unlabeled thymidine at 40 min after the start of synchronization.

At successive times after the pulse, samples were fixed and analyzed by electron microscopic radioautography. The percentage of labeled cells remained relatively constant at 39 ± 5%. The percentage of labeled cells in controls that had been chased for 20 and 190 min immediately after the start of the pulse was 3%. These findings indicate that the chase was effective. Figure 4 shows 95% confidence intervals for the fraction of unlabeled constricted cells as a function of time after the end of the pulse. This plot represents the appearance of unlabeled cells (i.e., cells that were in the B period at the end of the pulse) in the constriction "window" (or T period) of the cell cycle. From the slope the variability of the U period can be estimated. The curve in Fig. 4 is a best fit calculated on the assumption of a log normally distributed U period and a constant T period of 25 min (see reference 19). The fit cannot be rejected by the chi-square test ($P > 0.5$). The parameters of the best fit are $\bar{U} = 98 \pm 3$ min and $CV_U = 29 \pm 2\%$. The U period

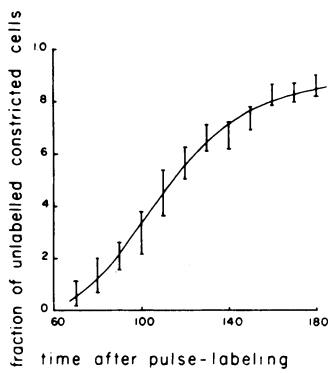

FIG. 4. *Fraction of unlabeled constrictions as a function of time after pulse-labeling. Membrane-eluted cells (T_D = 150 min) were pulse-labeled with [^{3}H]thymidine for 10 min at 30 min after the start of synchronized growth and chased with an excess of unlabeled thymidine at 40 min. At successive times after the pulse, samples were fixed and the fraction of unlabeled constricted cells was estimated by radioautography. The 95% confidence interval for this fraction is given by the bars. The curve was calculated assuming a log normal distribution of the U period with parameters: $\bar{U}$ = 93 ± 3 min; CV = 29 ± 2%; T = 25 min; χ^2 = 8.5; df = 10.*

appears to be less variable that the B period (CV_B = 60%), but more variable than the interdivision period (CV_τ = 11 to 14%).

Relationship between length and age. In Fig. 5 the natural logarithm of total cell length (5) has been plotted as a function of time after the start of synchronized growth. The same data of reference 24 were analyzed again in more detail for strain B/r F growing with T_D = 82 min and with T_D = 165 min. Total cell length represents the relative cell number (Fig. 2) times the average cell length at the given time. The straight lines were calculated by linear regression (r = 0.99). The doubling times of total cell length, as derived from the respective slopes of the regression lines, are 84 and 138 min, not very different from $\bar{\tau}$ estimated from the relative cell numbers alone (81 and 146 min, respectively; see the legend to Fig. 2). If total cell length increases linearly with a doubling of the rate at some time (5), Fig. 5 would show long runs of residuals of the same sign around the fitted lines. No evidence for such runs was indicated by a runs test [see Appendix (iii)], i.e., the scatter of the points along the fitted lines appears to be random (α = 0.05), compatible with exponential but not with linear elongation of individual cells during the cycle.

Correlation between length and age at initiation of DNA replication. The large variability of the B period could be explained if cells initiate rounds of chromosome replication when they reach a particular size rather than a particular age. To investigate this possibility, we estimated the average length $\bar{L}_i$ and CV_i at initiation of DNA replication in successive samples during synchronized growth by the same procedure as described elsewhere (19).

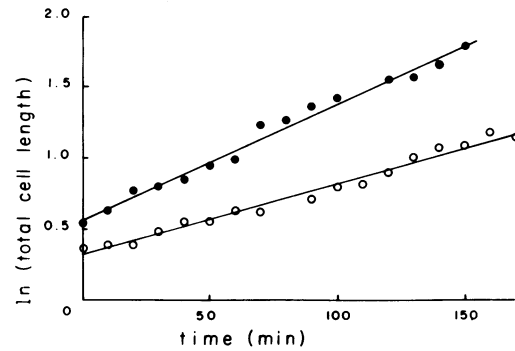

FIG. 5. *Total cell length as a function of time after the start of synchronized growth. Note the use of a logarithmic length scale. The straight lines were calculated by linear regression. The parameters are: (●) $\bar{L}_0$ = 1.75 μm; doubling time of total length (estimated from the slope) is 84 min for T_D = 82 min. (○) $\bar{L}_0$ = 1.37 μm; doubling time of total length is 138 min for T_D = 165 min.*

Figure 6 shows $\bar{L}_i \pm$ standard error as a function of time (≈age). The closed circles refer to the culture (T_D = 165 min) incubated at $t = 0$ in the presence of [^{3}H]thymine. The large standard error is probably due to variable amounts of radioactivity incorporated by the cells. The cells in the sample at $t = 5$ (not shown) and that drawn at $t = 10$ and 30 min were covered with only few grains, resulting in an overestimate of $\bar{L}_i$. Therefore, the values at these times are shown within parentheses and were disregarded. The open circles relate to a culture (T_D = 150 min; $F = 0.43 \pm 0.05$) in which DNA synthesis was followed by pulse-labeling samples at successive times with [^{3}H]thymidine for 5 min. The latter method is more reliable than the method of continuous labeling. This is reflected in a smaller standard error for $\bar{L}_i$. The straight line was calculated by linear regression. The coefficient of correlation ($r = 0.11$) did not differ significantly from zero. The $\bar{L}_i$ and CV_i, averaged over all times, were, respectively, $\bar{L}_i = 1.27 \pm 0.06$ μm and $CV_i = 22.9 \pm 5.2\%$.

Cells of different ages appear to initiate DNA replication, on the average, at the same length (size). This finding implies that cells that are born small have to accrue more and that they are older at initiation of DNA replication than cells that are born large.

Correlation between length and age at cell constriction. In Fig. 7 the average length $\bar{L}_c \pm$ standard error of slightly constricted cells and that of deeply constricted cells are plotted as a function of time after the start of synchronized growth (T_D = 165 min). The straight lines were calculated by linear regression. The coefficients of correlation ($r = -0.23$ and -0.38 for slightly and deeply constricted cells, respectively) did not differ significantly from zero ($P > 0.10$). $\bar{L}_c$ averaged over all times was: $\bar{L}_c = 2.33$ μm, $CV_c = 8.7\%$ for slightly constricted cells and $\bar{L}_c = 2.58$ μm, $CV_c = 9.1\%$ for deeply constricted cells. The same result was obtained in two other experiments (T_D = 82 min, $r = -0.26$; and T_D = 150 min, $r = 0.28$) and for the related A strain (T_D = 60 min, $r = 0.07$). In one experiment a significant positive correlation between $\bar{L}_c$ and time was found (T_D = 150 min; $r = 0.79$; $P < 0.01$). However, this culture had been chased with an excess of unlabeled thymidine (see above), which could have inhibited DNA synthesis and thus cell division.

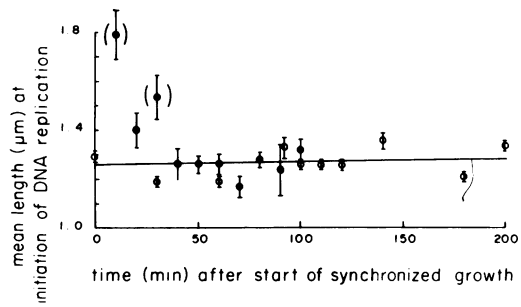

FIG. 6. *Average length at initiation of chromosome replication as a function of time.* (●) $\bar{L}_i \pm$ *standard deviation in a synchronized culture* (T_D = *165 min) continuously labeled at* $t = 0$ *with* [^{3}*H]-thymine.* (○) $\bar{L}_i \pm$ *SD in a synchronized culture* (T_D = *150 min) pulse-labeled at successive times with* [^{3}*H]thymidine.* $\bar{L}_i$ *was estimated by radioautography from the length distribution of labeled cells as previously described (9). The straight line (r = 0.11) was calculated by linear regression disregarding the values in parentheses.*

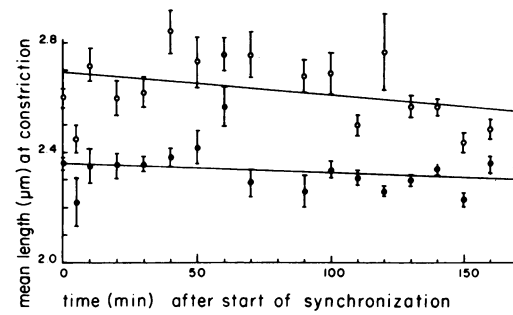

FIG. 7. *Average length at cell constriction as a function of time. A culture* (T_D = *165 min) was synchronized by membrane elution. At successive times samples were fixed and analyzed by electron microscopy.* (●) $\bar{L}_c \pm$ *standard deviation of slightly constricted cells;* $\bar{L}_c$ = *2.33 μm; CV = 8.7%.* (○) $\bar{L}_s \pm$ *standard deviation of deeply constricted cells;* $\bar{L}_s$ = *2.58 μm; CV = 9.1%. The straight lines (r = −0.23 for* $\bar{L}_c$ *and r = −0.38 for* $\bar{L}_s$*) were calculated by linear regression.*

Cells of different ages appear to divide, on the average, at the same length. This implies a negative correlation between size at birth and interdivision time.

Correlation between successive cell cycle events. The most direct way to obtain correlations between cell cycle events is to observe individual cells growing in a culture chamber with a light microscope. This method is rather laborious because a sufficient number of cells have to be watched and steady-state conditions have to be maintained. Another way is to deduce correlations from the CV of sizes at successive events and from the CV of the period between them as estimated for cells growing in suspension [Appendix (v)].

In Table 2 the average lengths and CVs at different events are summarized for slowly growing synchronized cells (T_D = 150 min). For comparison, the corresponding values for a steady-

state culture growing with T_D = 240 min (Table 3 of reference 21) are shown in parentheses. In Table 3 the average duration and CV of the B, U, and interdivision (τ) periods are summarized. From the average duration and CV of B, U, and τ (assuming that $CV_{B+U} = CV_\tau$), it follows that there is positive correlation between the age at which a cell initiates chromosome replication and the age at which it starts cell constriction: r(B, B+U) = 0.66. There is a negative correlation between successive B and U periods: r (B, U) = −0.85.

Table 3 also gives values of the CV of B, U, T, and τ calculated from the length distributions at inception and termination of the period (parameters in Table 2) on the assumption of independent events [Appendix (iv)]. It can be seen that the calculated CVs exceed the observed ones to different degrees, indicating that the assumption of independence is not valid for our material. From the CV we next calculated the coefficient of correlation r between lengths at inception and termination of the period [Table 3, values in parentheses; see Appendix (v)]. The net coefficient of correlation (i.e., $r_{obs} - r_{cal}$) of length at birth and length at initiation of DNA replication is low: $r(L_0, L_i) = 0.3$. On the other hand, the net coefficient of correlation between length at initiation of DNA replication and length at initiation of cell constriction is high: $r(L_i, L_c) = 0.8$. The coefficient of correlation between length at birth and length at cell separation is intermediate: $r(L_0, L_s) = 0.55$.

In summary, initiation of chromosome replication is more positively correlated with cell division than with cell birth. In other words, initiation of chromosome replication (or something associated with it) appears to affect cell division and not vice versa.

TABLE 2. *Average lengths and CVs at different events during the cell cycle*

Event	Symbol	$\bar{L}$ (μm)	CV (%)
Cell birth[a]	L_0	1.2 (1.1)[b]	10 (12)
Initiation of DNA replication[c]	L_i	1.5 (1.4)	23 (17)
Initiation of cell constriction[c]	L_c	2.2 (2.0)	8 (12)
Cell separation[a]	L_s	2.4 (2.2)	9 (11)

[a] $\bar{L}_0$ and $\bar{L}_s$ were estimated from the far-constricted cells in the steady-state culture (T_D = 150 min).

[b] Values within parentheses refer to a steady-state culture (T_D = 240 min; see reference 21).

[c] $\bar{L}_i$ and $\bar{L}_c$ were calculated from the relative duration of the periods (see Table 3) on the assumption of exponential elongation.

TABLE 3. *Average durations and CVs of different periods in the cell cycle*

Period	Relative duration (%)	CV (%)	
		Observed	Calculated[a]
B	33	60 (0.94)[b]	77 (0.64)
U	53	29 (1.00)	55 (0.18)
T	14	ND[c]	73 (0.66)
τ	100	12 (0.55)	18 (0.00)

[a] The CV was calculated from the relative duration and length at inception and termination of the period (Table 2) on the assumption that the latter are independent of each other [Appendix (iv)].

[b] Values in parentheses given the coefficient of correlation (r) between length at inception and termination of the period [Appendix (v)].

[c] Not determined.

DISCUSSION

In the present experiments we synchronized *E. coli* B/r F by the membrane elution technique and analyzed samples drawn at successive times, first, by radioautography to follow DNA replication, and second, by scoring constricted cells to follow cell division. Our purpose was to estimate the variabilities of the B, U, and interdivision periods and to calculate from them correlations between cell cycle events. Such an approach requires knowledge about the degree of synchronization obtained by membrane elution and about the relationship between length and age. We will first consider these points, then discuss the τ, B, and U periods and make inferences about the control of initiation of chromosome replication and the coordination with cell division.

Degree of synchronization. The index proposed by Blumenthal and Zahler (2) can be used within limits for the measurement of synchronization of cells obtained by membrane elution. Because of the distribution of interdivision time, the index can never be 1. To assess the extent of synchronization, the calculated index should, therefore, be compared with the maximal index attainable given a certain distribution of interdivision times. An alternative index proposed by us equals the fraction of the membrane-eluted cells that can be considered to have age zero and was estimated in two ways: (i) by comparing the fraction of constricted cells with that in the steady-state population (this method is rapid but not so precise); and (ii) by comparing the shape of the length distribution with that of the newborn cells in the steady-state population. This method is precise but needs more arithmetic. The agreement between the outcome of both methods indicates that our index is useful.

Rate of length extension. A relationship between length and age is needed for the com-

putation of correlations and the design of models. We found an exponential rate of length extension during synchronized growth by classifying cells by age and averaging their lengths (Fig. 5). Exponential elongation was also found during steady-state growth by the use of the Collins-Richmond principle (classification of cells by length and averaging their ages) for strains B/r F (unpublished data), B/r A and K (Fig. 4 of reference 21), and a K-12 strain derivative (Fig. 1 of reference 20).

Previously (24) we observed an alteration of length growth about midway in the division cycle of synchronized strain B/r F (T_D = 82 min and T_D = 165 min). Since synchronized B/r A strain showed a different cell elongation pattern, it was then concluded that the pattern is substrain dependent and more complex than simply linear with a doubling in rate or exponential. We have also mentioned that, on the basis of a Collins-Richmond analysis, an exponential mode of cell elongation could not be excluded. In this paper we have reutilized the data on strain B/r F and made some more detailed calculations (Fig. 5). Though the data points may indicate alterations in the rate of length growth (Fig. 5), we now find that the scatter of points along the fitted lines appears random (see Results). For the time being, we therefore prefer to use the simpler exponential model (see also concluding remarks of reference 34).

Interdivision time. The average interdivision time ($\bar{\tau}$ = 146 min) estimated from the time course of cell division in the synchronized culture (Fig. 2B) was 12% shorter than T_D (165 min). In addition, the cells eluted from the membrane were bigger (1.28 ± 0.01 μm) than newborn cells in the steady-state population (1.22 ± 0.01 μm). These observations can be explained in several ways: (i) cells are eluted from the membrane on the average some time after division; (ii) cell division is asymmetrical with respect to cell wall (R. W. H. Verwer and N. Nanninga, submitted for publication), cell size, and growth rate (6); (iii) cells on the membrane and, after elution, in suspension grow faster. The doubling time of total cell length (138 min; Fig. 5) and the average length at cell separation (2.58 μm; Fig. 7), which is twice that of newborn cells eluted from the membrane (see above), hint at a faster growth of the synchronized population. In addition, it has been reported previously that cells growing on a membrane have a 10% shorter interdivision time as compared to T_D of the steady-state culture (12). Therefore we choose possibility (iii) to account for the short average interdivision time of the synchronized population. A small shift up by the ample supply of fresh medium is likely to be responsible for this phenomenon.

The CV_τ of interdivision times (12 to 19%) agrees with the range of values (13 to 22%) reported by others for gram-negative bacteria (34). For the F strain at least, it is less than twice the coefficient of variation of length at cell separation (CV_τ = 12% and CV_s = 9%), which implies that there is positive correlation between length at birth and length at cell separation: $r(L_0, L_s) = 0.55$ (see Table 3). Such a correlation has also been observed by direct microscopic observation of individual cells (r = 0.17 to 0.63; 6) and is in contrast with the major postulate of the Koch and Schaechter hypothesis that a cell divides on the attainment of a critical size, independent of size at birth (see Introduction). As noted by Errington et al. (6), positive correlation between size at birth and size at cell separation accounts for the hereditary element in the interdivision time beyond the first generation (i.e., up to the second and third generation; 22).

Membrane elution is usually applied to strains of *E. coli* B/r. Different strains arranged in order of increasing CV of age at initiation of chromosome replication and age at cell division (as is apparent from a decreasing quality of synchronized growth after membrane elution) are: F < A < K (14). Our observation of a higher CV_τ for the A strain (19%) as compared to the F strain (12%) agrees with this arrangement.

B period. Our data (Fig. 3) indicate that the B period is the most variable part of the cell division cycle (CV = 60%). The B period can be compared to the G1 period of the eucaryotic cell cycle. It is well known that growth conditions affect the length of G1 rather than the other periods (S, G2, and M) of the cell cycle. At one particular growth condition, it is the variation in G1 that would primarily be responsible for the distribution of interdivision times (31). We found that the variation of age at initiation of chromosome replication is considerably larger than the variation of size. (This suggests a size control over chromosome replication rather than an age control.) Two hypotheses have been put forward to explain this variation, as follows. (i) According to Smith and Martin (36), cells enter an indeterminate state in G1 from which they leave at random with a constant probability per unit of time. The variable age at initiation of chromosome replication is the consequence of the variable time spent in the hypothetical indeterminate state. Therefore, this hypothesis predicts positive correlation between size and age at initiation of chromosome replication. (ii) According to Donachie (4) and Pritchard (R. H. Pritchard, Heredity **23**:472, 1968), cells initiate chromo-

some replication when they achieve a particular size, independent of size at birth. The variable age at initiation is the consequence of the independent sizes at birth and initiation of chromosome replication. The hypothesis predicts no correlation between size and age at initiation of chromosome replication. We found no correlation between mean length and age at chromosome initiation (Fig. 6). This finding is not consistent with the model of Smith and Martin in which initiation is primarily controlled by time. (However, the introduction of a size element in the model [L. Martin, personal communication] would reduce the expected correlation between size and age.) We favor the view that initiation occurs at a particular size independent of birth. The low net correlation $r(L_0, L_i) = 0.3$ (Table 3) is consistent with this notion. A similar size control over initiation of chromosome replication has been described for fission yeast (25), budding yeast (39), and animal cells (16). Size as such is a vague concept, and it is not clear how it should be understood in terms of, for instance, the critical concentration of a chemical compound or a characteristic cytological feature.

U period. The CV_U (29%) is similar to that estimated before for the K strain (25%) in the steady state (19). The value of this parameter indicates, as deduced before (19), that there is positive correlation between age at initiation of chromosome replication and age at initiation of cell constriction ($r = 0.7$) and also between the respective lengths at these events ($r = 0.8$). As we suggested earlier (19), this high correlation means that some event before or at chromosome initiation triggers the onset of cell division presumably long before a constriction is visible. A further aspect is that segregation of sibling chromosomes, which of course is coupled to initiation of the replication of the parent chromosome, is a prerequisite for the completion of normal division. In this connection it would be interesting to know the correlation between termination of chromosome replication and initiation of cell constriction in individual cells of slowly growing *E. coli* B/r. This question is experimentally accessible by radioautography.

From the independence of mean length and age at initiation of chromosome replication (Fig. 6) and the high correlation between length at initiation of chromosome replication and length at initiation of cell constriction, it follows that also the mean length and age at cell constriction are largely independent (Fig. 7). This finding agrees with the model of Koch and Schaechter (18), in which cells divide when they achieve a critical size rather than age.

The variabilities of lengths and ages at different events (Table 2, 3) indicate that there is negative correlation between successive periods within the cell division cycle (r[B, U] = −0.9) and between length at inception of a period and the duration of that period: $r(L_0, B)$, $r(L_0, \tau)$, $r(L_i, C)$, $r(L_i, U) < 0$. Such negative correlations have also been reported for eucaryotic cells (7, 15, 35, 41) and could imply a feedback mechanism by which time-invariant length and age distributions are maintained in the steady state.

Initiation of chromosome replication. It has been proposed that initiation of chromosome replication is controlled by the previous initiation (1, 10, 11, 23). The I period is the time interval between successive initiations of chromosome replication during which an initiator accumulates (37), a replisome matures (1), or an inhibitor is diluted (32). It has the same average duration as the interdivision time. In this model the B period is merely the difference between the I period and the C+D period of the preceding cycle (Fig. 8). The variance of the B period is a function of the variances and covariance of I and C+D. The B period, therefore, is not a suitable measure of age as far as chromosome replication is concerned. Age should be defined as time since the last initiation rather than time since the last division, as is usually done (see reference 26). Then both size and age (time) could be equally important to describe the control of chromosome initiation. From Fig. 8 it is also apparent that cells cannot cycle with respect to chromosome replication (I) and cell division (τ) at the same time.

In conclusion, our results suggest that cells initiate chromosome replication at a particular

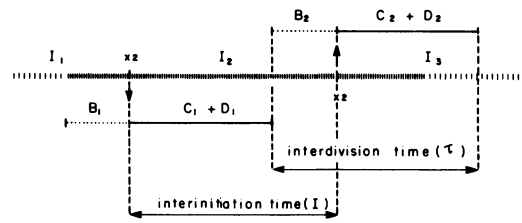

FIG. 8. *Diagram to illustrate the relationship between the chromosome replication cycle (I) and the cell division cycle ($\tau = B + C + D$). At the end of the I period, chromosome replication is initiated, resulting in the doubling of the number of chromosome origins. The factor x2 indicates that two new I periods are initiated. The arrow indicates that chromosome initiation is usually followed by cell division after C + D min. From the diagram it can be seen that the B period is the difference between the I period and the C+D period of the preceding division cycle: $B_2 = I_2 - (C+D)_1$. The duration of I, B, C+D, and τ should be conceived not as constant, as suggested by the diagram, but as distributed.*

distributed size which is independent of the size at birth. After initiation, two processes are started which proceed at a rate increasing with size: (i) preparation for the next initiation of chromosome replication (I period), and (ii) preparation for cell division (C+D period). In such a model (Fig. 8), chromosome replication is cyclic and the pacemaker for cell division. Further work is concerned with the development of a stochastic model for the control of size and age at initiation of chromosome replication and the subsequent cell division.

APPENDIX

(i) Calculation of the fraction of cells engaged in DNA replication. During a pulse-label with [^{3}H]thymidine, cells that are synthesizing DNA (fraction S) will incorporate a relatively high amount of radioactivity; on the other hand, cells that are not engaged in DNA replication (fraction $1 - S$) will incorporate a low amount. After radioautography the former cells will, on the average, be covered with C grains, and the latter will be covered with G background grains. If grain numbers in both subpopulations follow the Poisson law, then the probability $P(n)$ of observing a cell with n grains is given by:

$$P(n) = (1 - S)\cdot e^{-G}\cdot G^n/n! + S\cdot e^{-C}\cdot C^n/n! \quad (1)$$

This equation was fitted to the observed distribution of grains per cell in each length or age class by the method of maximum likelihood (33). If the best fit could not be rejected by the chi-square test ($\alpha = 0.10$), the parameters S, G, and C were accepted.

(ii) Kinetics of labeling of a steady-state culture. We define age α for a cell in the D or B period as the time after termination of DNA replication. Suppose that cell separation and initiation of DNA replication occur independently of each other after termination. (In fact, initiation can occur before termination of the previous round of DNA replication, but at slow growth rates such events are probably rare and can be disregarded.) Suppose further that a steady-state culture has been growing in the presence of [^{3}H]thymine during t min. Then only those cells will be unlabeled that have been in the D or B period all the time after addition of [^{3}H]thymine to the culture, i.e., the cells in D or B that have ages $a > t$. The fraction of labeled cells $I_L(t)$ at time t after addition of the label will therefore be given by:

$$I_L(t) = 1 - \int_t^\infty \nu(a)\cdot da \quad (2)$$

in which ν (a) is the time-invariant age distribution of cells that are either in the D or B period and is given by an expression analogous to that for the whole culture (see equation 8 of reference 27):

$$\nu(a) = A\cdot\left[1 + \int_0^a p(x)\cdot dx\right] \cdot \mu e^{-\mu a}\cdot \int_a^\infty q(x)\cdot dx \quad (3)$$

in which μ is the specific growth rate; $p(x)$ is the probability density of the D period; $g(x)$ is the probability density of the D+B period; and A is the relative rate of termination of DNA replication as compared to cell separation at each time:

$$1 \leqslant A = g + 1 \leqslant 2 \quad (4)$$

in which g is the fraction of cells in the D period.

The factor $1 + \int_0^a p(x)\cdot dx$ in equation (3) arises because each separation results in two new cells. In the estimation of $\bar{B}$ and CV_B (Fig. 3) we made a number of assumptions to facilitate the calculations: (i) the average duration of the D period was equalized to that of the T period, $\bar{D} = \bar{T}$; (ii) the D, B, and D+B periods were all assumed to be log normally distributed with the same CV; (iii) the factor A was approximated by $\exp(\mu\bar{T})$. These assumptions concern the labeling kinetics of the fraction of steady-state cells in the synchronized population (T_D = 165 min). This fraction was estimated to be about 0.32. We do not think that the assumptions invalidate our conclusion about the B period.

(iii) χ^2 test for runs of residuals of the same sign. In the present study the equation for a straight line containing two parameters ($m = 2$) has been fitted to n measurements of the logarithm of total cell length. Suppose that the probability of a change of sign between successive residuals is $z = \frac{1}{2}$. Then the probability $P(s)$ of finding actually s changes will approximately be given by the binomial term:

$$P(s) = \binom{n-3}{s-2}\cdot\left(\frac{1}{2}\right)n - 3 \quad (5)$$

and the χ^2 for the fitted line will be given by:

$$\chi^2 = \frac{(2s - n - 1)^2}{n - 3} \quad (6)$$

with 1 degree of freedom (6).

(iv) Calculation of the distribution of a period Q. Two postulates of the hypothesis of Koch and Schaechter (18) are used in the derivation of the distribution $f(Q)$ of the period Q between two events that occur sequentially in

the cell division cycle: (i) exponential elongation of individual cells, so that the relationship between length at inception L_b and length at termination L_t of period Q is given by: $L_t = L_b \cdot \exp(\mu \cdot Q)$, in which $L_b \leq L_t$ and $Q \geq 0$; and (ii) the probability that a cell with extant length L will terminate in the length interval $(L_t, L_t + \Delta L_t)$ is independent of L_b and is given by:

$$\frac{h(L_t) \cdot \Delta L_t}{\int_L^\infty h(x) \cdot dx} \tag{7}$$

in which $h(x)$ is a hypothetical intrinsic distribution. The $f(Q)$ distribution is then obtained by integrating all possible combinations of L_b and L_t:

$$f(Q) = \mu \cdot \exp(\mu \cdot Q) \cdot \int_0^\infty \frac{L \cdot \mathcal{L}(L) \cdot h[L \cdot \exp(\mu \cdot Q)] \cdot dl}{\int_L^\infty h(x) \cdot dx} \quad Q \geq 0 \tag{8}$$

(cf. equation 2 of reference 29), in which:

$$h(L) = \frac{L \cdot \mathcal{R}(L) \cdot [1 - \int_0^L h(x) \cdot dx]}{\int_0^L x \cdot [A \cdot \mathcal{L}(x) - \mathcal{R}(x)] \cdot dx} \tag{9}$$

(cf. equation 27 of reference 29), in which $\mathcal{L}(L)$ is the length distribution at inception; $\mathcal{R}(L)$ is the length distribution at termination; and A is the relative rate of entrance of cells in period Q (see above): $A \approx \exp(\mu \cdot \bar{Q})$. These rather complex equations are simplified if $\mathcal{L}(L)$ and $\mathcal{R}(L)$ do not overlap (29).

(v) Coefficient of correlation r between lengths at inception and termination of a period. Length at inception L_b and at termination L_t of period Q are stochastic variables, which belong to the same cell and are related by the exponential growth law:

$$\mu \cdot Q = \ln L_t - \ln L_b \tag{10}$$

$$\operatorname{var}(\mu \cdot Q) = \operatorname{var}(\ln L_t) + \operatorname{var}(\ln L_b) - 2 \operatorname{cov}(\ln L_b, \ln L_t) \tag{11}$$

Assuming that μ and Q are independent, and approximating the variance of the logarithm of length by the square CV of length, equation (11) can be written as:

$$(\bar{\mu} \cdot \bar{Q})^2 \cdot [\mathrm{CV}^2(\mu) + \mathrm{CV}^2(Q)] = \mathrm{CV}^2(L_t) + \mathrm{CV}^2(L_b) - 2\mathrm{CV}(L_b) \cdot \mathrm{CV}(L_t) \cdot r \tag{12}$$

Since $\mathrm{CV}^2(\mu) \ll \mathrm{CV}^2(Q)$ and $(\bar{\mu} \cdot \bar{Q})^2 \approx \frac{1}{2} \cdot w^2$, in which $w = \bar{Q}/T_D$ is the relative average duration of the period as fraction of the doubling time, equation (12) simplifies to:

$$\frac{w^2}{2} \cdot \mathrm{CV}^2(Q) = \mathrm{CV}^2(L_b) + \mathrm{CV}^2(L_t) - 2\,\mathrm{CV}(L_b) \cdot \mathrm{CV}(L_t) \cdot r \tag{13}$$

which on rearrangement gives:

$$r = \frac{\mathrm{CV}^2(L_b) + \mathrm{CV}^2(L_t) - w^2 \cdot \mathrm{CV}^2(Q)/2}{2\,\mathrm{CV}(L_b) \cdot \mathrm{CV}(L_t)} \tag{14}$$

In practice, we estimated the CV in different cell samples. The small error thus introduced in r is probably within sampling error and was therefore disregarded.

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