*ONSET OF DNA SYNTHESIS DURING THE CELL CYCLE IN CHEMOSTAT CULTURES**

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Chemostat cultures have been used for several extensive studies of mutation, but little has been known about the regulation of synthesis of DNA in these cultures. A knowledge of the timing of DNA synthesis during the cell cycle would be especially useful, for example, in distinguishing between mutagenesis initiated in the absence of DNA synthesis and mutagenesis requiring synthesis of the locus. In this paper we describe a method generally applicable to the study of intracellular events throughout the growth and division cycle, although its present usage has been confined to the determination of the timing of initiation of DNA synthesis in continuous bacterial cultures.

The method described involves the separation of cells into different size classes by velocity sedimentation in a sucrose gradient so that appropriate intradivision measurements can be made as a function of cell volume. In order to interpret these measurements, it is necessary to establish a scale for cell age as a function of volume; in particular, it is necessary to determine mean cell volumes at the beginning and at the termination of the cycle by fission. Although precise relationships of these kinds would require knowledge of the age distributions, which are unknown for these bacterial populations, a suitable approximation of the mean cell volume at birth $\bar{V}_b$ and the mean cell volume just before division $\bar{V}_d$ is obtained by assuming that cell size increases linearly with time from birth to fission and that there is no variability in cell volume at birth or at fission. In this case, the average cell volume $\bar{V}$ for the culture is related to the mean volume at birth and at division by

$$\bar{V}_b = \bar{V} \ln 2 = \bar{V}_d/2. \quad (1)$$

This relationship establishes a comparative scale for the cell populations in exponential growth. Although this scale is not precise, it is sufficiently accurate for our purposes. For example, if cell volumes were assumed to increase exponentially rather than linearly during the division cycle, then the mean cell volume would be decreased by about 4 per cent.

Materials and Methods.—Escherichia coli 15 THU, requiring thymine, histidine, and uracil (kindly supplied by S. S. Cohen, University of Pennsylvania), was grown in chemostat cultures at 37°C in a minimal medium containing M9 salts,[1] 2 μg/ml thymidine, 50 μg/ml histidine, and 10 μg/ml uracil. Growth was limited with 100 μg/ml glucose, and generation times ranged from about 80 minutes to 12 hours by varying the rate of addition of the nutrient medium to the culture. For those experiments requiring generation (doubling) times less than 100 minutes, cultures were grown freely in well-aerated tubes in which the glucose concentration was increased to 1 mg/ml.

Chemostat cultures, about 25 ml of 1–2 × 10^8 cells/ml, were pulse-labeled with

0.2 μg/ml of methyl-H^3-thymidine, 12.5 c/mM (Schwarz Laboratories). Freely growing cultures in tubes were pulsed with the same concentration of tritiated thymidine during exponential growth when cell concentrations reached about 10^8 cells/ml. After 0.1 generation, unlabeled thymidine was added to give 20 μg/ml, and the cells were immediately fixed with formaldehyde at a final concentration of 0.4 per cent.

Sucrose gradients, ranging in concentration from 5 to 15 per cent, were established in 13-ml volumes in 12 $\times$ 100 mm cellulose nitrate centrifuge tubes by the method of Britten and Roberts.[2] These gradients contained 1 per cent KCl as an electrolyte. Cells were layered upon these gradients, but within a confining smaller cylinder of polycarbonate to reduce impingement of cells upon the walls of the tube during centrifugation. The polycarbonate cylinders had internal diameters of 12 mm and extended into the gradients for a distance of about 2 mm. Labeled cultures were centrifuged to obtain a pellet that was then resuspended in 0.1 ml of a 2.5 per cent sucrose solution. About half of this suspension was layered upon the gradient with a hypodermic needle, bent to provide an inverted point, then spun at 3000 rpm (approx. 2500 $\times$ g) in a swinging-bucket centrifuge (Sorvall centrifuge, RC2-B; rotor, HB-4). The period of centrifugation, 6 minutes for freely growing cultures and up to 11 minutes for chemostat cultures, was chosen to give a turbid band extending from a depth of about 2 cm below the meniscus to a depth of about 4 cm. The samples (0.1 ml) were removed from the gradients with a bent hypodermic needle, at intervals of about 2.5 mm along the tube, and diluted into 1 ml of 0.1 N HCl.

Portions of each diluted sample were used to determine tritium incorporation into DNA, as well as cell counts and size distributions. To assay for tritium in cold acid-insoluble material, 0.5 ml of each sample was added to 4 ml of ice-cold 0.1 N perchloric acid. After 30 minutes the suspension was filtered (Millipore membrane, HA, 0.45-μ pore size), and washed three times with 5-ml portions of ice-cold perchloric acid. The membrane, containing acid-insoluble tritium, was then dissolved in XDC scintillation fluid,[3] and the activity of the sample was measured with a liquid scintillation spectrometer (Tri-Carb, Packard Instrument Co.). The remaining portion of each sample was used for cell counts and size distributions, measured with a Coulter counter having a sensing aperture about 12 μ in diameter (preamplifier, amplifier, and pulse height analyzer were obtained from Radiation Instrument Development Laboratory).

Results.—The resolution achieved in separating cells according to their volumes is shown in Figure 1 for a representative experiment. In all experiments, resolution was better for samples taken near the top of the gradient than for samples near the bottom because larger cells are more rod-shaped. Also, the deeper samples may have included some cells that had impinged upon the centrifuge tube wall, had aggregated with other cells, and were later released into suspension.

Measurements of DNA synthesis in samples from chemostat cultures indicated that little or no synthesis occurred in the samples containing the smaller cells. The results of two experiments are shown in Figure 2, which displays cell activities (*open circles*) as a function of mean cell size in each sample. In all of the experiments, activities rose from an initial background level (*dashed lines*) to a final value some three to ten times larger. Since the H^3-thymidine was not rechro-

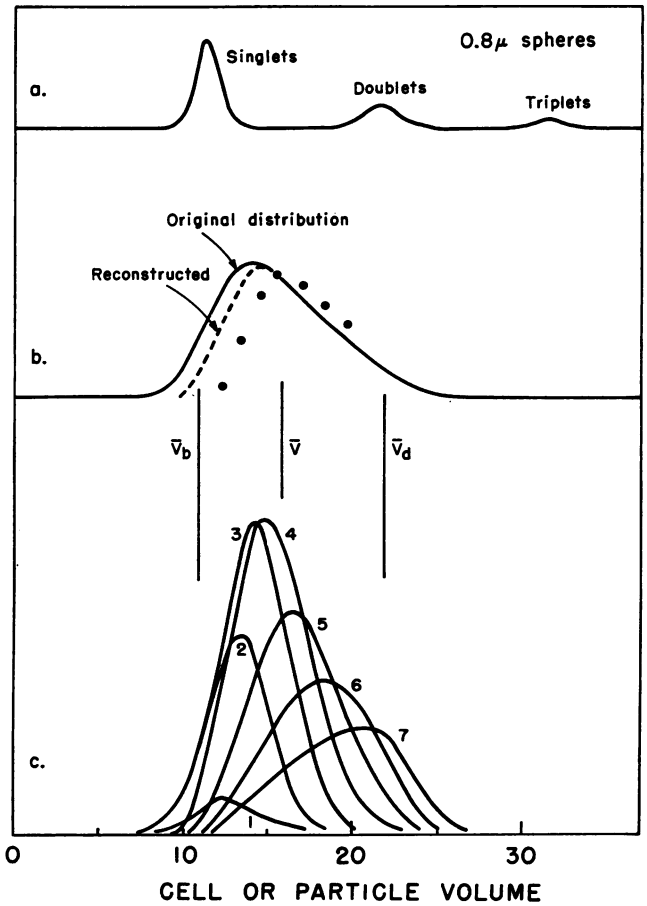

FIG. 1.—Cell size distributions in samples from a gradient. Particle concentrations are shown as a function of volume. (*a*) Calibration with 0.8-micron polystyrene latex spheres. Doublets and triplets refer to the volumes measured when two and three particles pass through the counting aperture simultaneously. (*b*) Size distributions for bacteria grown in a chemostat culture at a generation time of about 8 hr. Measured distribution, —. Distributions reconstructed from samples shown below in *c*: ●, number of cells *vs.* mean cell volume for each sample; —, sum of distributions of individual samples. (*c*) Cell size distributions for seven successive samples taken from a sucrose gradient.

matographed, the initial background level was most likely due to nonspecific incorporation of minor radioactive components present with H^3-thymidine, such as that found by Forro.[4] The results are consistent with the absence of DNA synthesis until a critical mean cell size (or possibly age) is reached, after which DNA synthesis takes place at a constant rate for most of the remainder of the cycle. Rapidly growing cultures showed no such large change in sample activities; DNA synthesis occurred to about the same extent in all samples.

It is possible to determine the mean cell volume at which synthesis of DNA begins if we assume as a first approximation that the rate of synthesis, after initiation, is constant until the end of the cycle. A visual estimate of the location of this initiation point was made on the following basis: such a point would divide all active samples into two classes, one of smaller cells not yet synthesizing DNA, and

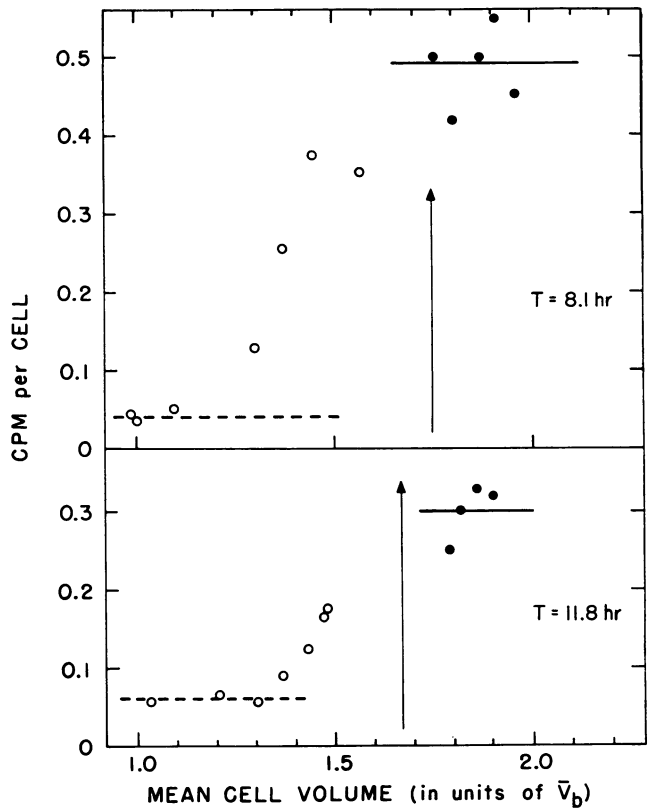

FIG. 2.—Counting rates as a function of mean cell volume for each sample. Vertical arrow, estimated mean cell volume at which DNA synthesis begins; O, observed counting rates; ●, calculated counting rates per cell in those cells with volumes equal to or greater than that shown by the vertical arrow (synthesis in smaller cells).

the other of active larger cells synthesizing at the maximum rate. Proper location of the initiation point, then, should give approximately the same calculated activity per cell for the active cell fractions, as is illustrated for two experiments in Figure 2. In this figure the calculated activities (*filled circles*) are plotted at their correspondingly increased mean cell volumes. They were calculated only for each sample in which the initial activities (*open circles*) were in excess of the background level (*dashed line*).

The mean value of the activity per cell in the active fraction was used in a second approximation to provide an improved estimate of the initiation point for each experiment. Using this mean as a reference level, the location of the initiation point was redetermined for each sample as that point for which the active fraction would have the same mean count per cell. Then these values of the initiation point were averaged in turn. The mean values for the cell volumes at onset of DNA synthesis, calculated in this way, are shown in Figure 2 by vertical arrows.

Mean cell volumes at initiation of DNA synthesis were plotted as a function of cell generation time in Figure 3. At the most rapid growth rate (doubling time of 57 min) most, but not all, newly born cells appeared to be synthesizing DNA; if all active cells were synthesizing at the same rate, the number of cells not synthesiz-

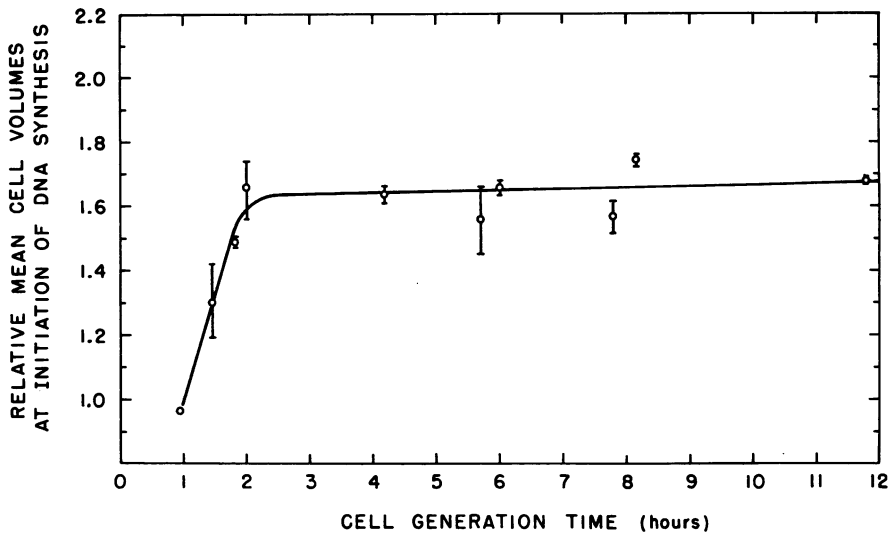

FIG. 3.—Mean cell volumes at onset of DNA synthesis as a function of cell generation time. The mean cell volume at onset of DNA synthesis is given in units of the mean volume at birth. The vertical bars above and below points represent the standard errors arising from fluctuations of values within each experiment.

ing DNA was 4.1 per cent (SE, 0.8%). The nonsynthesizing cells were among the smallest newly born cells since only those samples containing cells that were smaller than the mean cell volume at birth showed a decreased activity per cell. As generation times were increased, the onset of synthesis of DNA was increasingly delayed, and for generation times greater than two hours onset approached a maximum value of about two thirds of the interdivision time.

Discussion.—Our results for the onset of DNA synthesis (Fig. 3) are in qualitative agreement with earlier findings. Rapidly growing bacteria, with mean generation times less than one hour, synthesize DNA almost continuously.[5-7] Observations on the cell fraction of *E. coli* 15T$^-$ that was immune to thymineless death led to the conclusion[8,9] that there was an undetected interval during which no DNA synthesis occurred. Lark and Bird[10] were able to estimate the fraction of cells in this interval from their autoradiographic studies of relatively rapidly growing cells, and C. Lark[11] found that this interval was lengthened when growth rates were reduced. At generation times of three hours or more, she found that only about 60 per cent of the cells were synthesizing DNA. She also concluded that synthesis of DNA occurred primarily during the latter half of the cell cycle.

In our most rapidly growing cultures, almost all cells were synthesizing DNA. Our estimate that 4.1 per cent of the cells were not synthesizing agrees well with extrapolated values from the data in the tables by Lark and Bird,[10] and by Lark.[12] Both values must be underestimates, however, since the cultures were exposed to label for a period of 0.1 generation rather than to an indefinitely short pulse.

For chemostat cultures with generation times longer than two hours we found that DNA synthesis does not begin until cells have passed through about two thirds of the cell cycle (Fig. 3). Again, the values shown in this figure are underestimates because the labeling period was 0.1 generation.

Because of the broad range of cells sizes in those samples containing the larger cells, we cannot estimate the time during the cycle at which DNA synthesis ceases, and therefore we cannot determine the period of DNA synthesis from our present data. However, it is most unlikely that DNA synthesis, once initiated, continues until the end of the division cycle. If this were the case, label incorporated just before division would be found in newly formed daughter cells after fission, and a large specific activity would have been observed for the smallest cells. But our experiments gave no evidence for increased labeling of the smallest cells (see Fig. 2, for example). It can be argued that the carry-over of activity to small cells might be prevented if cells failed to separate after fission for a period of 0.1 generation or more, and furthermore, that the distribution of cell volumes would then be distorted, invalidating our sizing scale. Because of these possibilities, we routinely examined cultures under phase microscopy. Some "doubles," i.e., cell pairs still attached although constriction had begun, were found, but their frequency in chemostat cultures was less than 2 per cent. Since unconstricted cells of the same lengths as "doubles" were very rare, we assume that the value of 2 per cent is a reasonable estimate of the fraction of cells that accidentally fails to divide or separate. If so, the final 8 per cent or more of the cell cycle would be devoid of DNA synthesis, and DNA synthesis could take place only over about a quarter of the cycle. Because of the broad range of cell sizes in samples containing larger cells, these results do not have sufficient precision to test Maaløe and Kjeldgaard's suggestion[13] that the rate of DNA synthesis, once initiated, is independent of growth rate in all normal, balanced cultures. For example, if we assume that DNA synthesis is always completed in the first hour after onset in chemostat cultures, the calculated initiation points are not significantly changed, although calculated activities per cell are.

Our results from cultures of slowly growing bacteria show that the division cycle in these bacteria is partitioned in a manner similar to that found in higher organisms. That is, the stages in the division cycle of these bacteria are formally analogous to the G_1, S, and G_2 periods of more complex cells. Since slowly growing bacteria appear to contain a single replicating genome,[11,12] this similarity suggests that the controls upon DNA replication and cell division in such bacteria are similar to those of uninucleate cells in higher organisms.

Summary.—The onset of DNA synthesis during the growth and division cycle has been measured for continuous, glucose-limited cultures of *E. coli* THU, and for cultures in exponential growth in the same medium. The method used was the measurement of H^3-thymidine incorporation in cells partially separated by size in a sucrose gradient. Cell volumes were related to their mean value at birth by a comparative scale that assumes that mean cell volumes increase linearly from birth to division. For freely growing cultures, only the unusually small cells at birth fail to synthesize DNA. As growth rates are decreased in chemostat cultures, the initiation of DNA synthesis is increasingly delayed, requiring the passage of about two thirds of the interdivision cycle when generation times become longer than two hours. Synthesis of DNA ceases before the cycle is terminated by fission. Thus, for these cultures the cell cycle is partitioned into stages similar to the G_1, S, and G_2 periods of more complex cells.

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[1] Kubitschek, H. E., and H. E. Bendigkeit, *Mut. Research*, **1**, 113 (1964).
[2] Britten, R. J., and R. B. Roberts, *Science*, **131**, 32 (1960).
[3] Bruno, G. A., and J. E. Christian, *Anal. Chem.*, **33**, 1216 (1961).
[4] Forro, F., Jr., *Biophys. J.*, **5**, 629 (1965).
[5] McFall, E., and G. S. Stent, *Biochim. Biophys. Acta*, **34**, 580 (1959).
[6] Schaechter, E., M. W. Bentzon, and O. Maaløe, *Nature*, **183**, 1207 (1959).
[7] Young, I. E., and P. C. Fitz-James, *Nature*, **183**, 372 (1959).
[8] Maaløe, O., in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 26 (1961), p. 45.
[9] Maaløe, O., and P. C. Hanawalt, *J. Mol. Biol.*, **3**, 144 (1961).
[10] Lark, K. G., and R. E. Bird, these PROCEEDINGS, **54**, 1444 (1965).
[11] Lark, C., *Biochim. Biophys. Acta*, **119**, 517 (1966).
[12] Lark, K. G., *Bacteriol. Rev.*, **30**, 3 (1966).
[13] Maaløe, O., and N. O. Kjeldgaard, *Control of Macromolecular Synthesis* (New York: W. A. Benjamin, Inc., 1966), p. 163.