Rates of DNA Synthesis during the S-Phase of HeLa Cells*

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The rates of DNA synthesis were determined for each of two consecutive journeys through S-phase by highly synchronized HeLa cells. Cells at various times after release from the metabolic block were pulsed with [3H]thymidine. The amount of radioactive in whole cells, purified DNA, and Okazaki fragments provided indexes of the rates of DNA synthesis. Measurements of the average DNA content per cell by the diphenylamine method and the individual DNA content per cell by DNA:propidium fluorescence provided better estimations of the actual rates of DNA synthesis, independent of thymidine metabolism. Unsynchronized cells that had been pulsed with [3H]thymidine were sorted into early, middle, and late S-phase preparations for estimations of the amount of radioactivity per cell. There were differences in the rates predicted by each of the various methods. Rates estimated by fluorescence measurements of DNA content per cell, or by diphenylamine measurements of average DNA content per cell exhibited a pattern of an initial burst, followed by a decreased rate then a final burst. Similar patterns were obtained for the amount of radioactivity in Okazaki fragments, and in early, middle, and late S-phase cells separated from a log-phase culture by electronic cell sorting. Rates estimated by measurements of the amount of radioactivity in whole cells, and the specific activity of purified DNA exhibited a different pattern of an initial slow rate, followed by a maximal rate then a slow rate.

In mammalian cells, the synthesis of DNA is confined to a discrete period of the cell cycle called the S-phase (Howard and Pelc, 1963). The rates of DNA synthesis of a population of cells in tissues, or in culture, depend on the number of cells in S-phase and the rate of polymerization of nucleotides in each S-phase cell. The use of radioactive nucleosides to measure "rates of DNA synthesis" adds the complications of uptake, pool sizes, separate endogenous and salvage pools, activities of nucleotide-synthesizing enzymes, and turnover.

To obtain an accurate picture, the use of synchronized cells is necessary, but adds the further complications of the induction of metabolic imbalances, especially if metabolic inhibitors are used, of accurately staging the cells according to phases of the cell cycle, and of determining the actual numbers of cells in S-phase. The recent availability of machines capable of measuring the DNA content of individual cells, at rates approaching 3000 cells/s (Horan and Wheelless, 1977) has made it easy to follow the progression of a synchronized culture through the cell cycle (Collins et al., 1977).

The steps whereby mammalian DNA is synthesized are still unclear (Weissbach, 1977). Replication apparently occurs in discrete steps resulting in discontinuous units tandemly arranged on the chromosom (Huberman and Horwitz, 1973), analogous to "Okazaki fragments" in bacterial chromosomal replication (Okazaki et al., 1968). These short 4 to 4.5 S DNA intermediates, about 200 nucleotides in length (Tseng and Goulian, 1975), are eventually ligated together to form chromosomal sized DNA of about 400 S, or 1.5 x 10^7 nucleotides (Rawles and Collins, 1977).

It was discovered by Taylor (1969) and confirmed by Huberman and Riggs (1968) that specific segments of chromosomes are replicated in a particular and temporal order simultaneously at several sites on the chromosom. The segment of DNA replicated from a single initiation site was called a replicon (Plaut et al., 1966), after Jacob and Brenner (1963).

The electron micrographs of Kriegstein and Hognes (1974) clearly show the bubble-like replicating segments formed by the two diverging forks of each replicon in chromosomal DNA. Painter et al. (1966) have measured the rate of fork movement in HeLa cells, about 1.2 μm/min (60 nucleotides/s), and the number of replicons active at any one time (on the order of 10^5). That the average rate of fork movement is similar in all mammalian cells, yet S-phases range from 5 to 24 h in length (Painter and Schaefer, 1969), is explained by different numbers of replicons operating at any one time. Thus, fluctuations in the rate of DNA synthesis during S-phase would also be attributed to different numbers of active replicons (Terasima and Tolmach, 1963; Remington and Klevecz, 1973).

In an ongoing study of the relationships of the different cell cycle phases to the replication of DNA, we have examined the structure of parental DNA in resting and proliferating cells (Collins, 1974a) and, in various cell cycle phases (Collins, 1977; Collins et al., 1977), the ability of DNA from various phases to serve as a template for heterologous DNA polymerase (Collins, 1974b), the formation of Okazaki-like fragments (Collins, 1975), and the assembly of 4.5 S DNA intermediates into chromosomal sized DNA (Rawles and Collins, 1977). In the present study we have accurately estimated the rate of increase in cellular DNA (i.e. rate of DNA synthesis) by two means not dependent on [3H]thymidine metabolism, that is, the intensity of DNA:propidium fluorescence, and the absorbance of the diphenylamine reaction, of synchronized cells progressing through S-phase. These rates are compared to relative rates estimated by means dependent on [3H]thymidine metabolism, that is, the amount of incorporation into: whole cells, purified DNA, 4.5 S DNA, and cells electronically sorted according to their DNA content.

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Rates of DNA Synthesis

EXPERIMENTAL PROCEDURES

Cell Cultures

HeLa S-3 cells (kindly supplied by Dr. Thoru Pederson, Worcester Foundation for Experimental Biology, Shrewsbury, MA 01545) were maintained in spinner culture by daily dilutions with Joklik's modified Eagle's minimal essential medium containing 3.5% calf serum and 1.25 μg/ml of Fungizone at a concentration of 2 to 6 x 10^5 cells/ml. The cultures were routinely checked for mycoplasma (agar plating technique) by the Clinical Microbiology Laboratory of the Medical College of Virginia, to whom we are grateful for this service.

Cell Synchronization

Cells were adapted to recover from a 2 μM thymidine block by a procedure described by Thilly et al. (1977). The cells underwent 12-hour cycles of exposure to 2 μM thymidine media, followed by exposure to “normal” media, for a 3-week period. Following the last exposure to normal media, the cells were harvested by centrifugation under sterile conditions and resuspended in media which was 2 μM thymidine for 14 h. The cells were then harvested and resuspended in normal media for 9 h. The cells were again placed in media containing 2 μM thymidine for 6 h. This procedure yields cells synchronized at the G1/S boundary. The synchronized cells are released from the metabolic block by resuspension in normal medium and allowed to progress through the cell cycle.

Reagents

All tissue culture media and supplies were purchased from Flow Laboratories, [3H]thymidine (20 Ci/mmol) was purchased from New England Nuclear, and found to contain >99% of the [3H] in thymidine when examined by paper chromatography (Fink and Adams, 1966) prior to use in these experiments. Pancreatic ribonuclease and pronase were supplied by Sigma. Propidium iodide was supplied by Calbiochem and recrystallized before use.

Incorporation of [3H]Thymidine into Whole Cells

Samples (1 ml) were removed from the cultures and incubated with 10 μCi/ml of [3H]thymidine for 30 min at 37°C. Cold media were added and the cells were centrifuged at 2000 x g for 3 min. The cells were resuspended in 2 ml of cold 5% trichloroacetic acid and collected by filtration onto a Whatman 41S glass-fiber filter. The filter was washed two times with 3 ml of cold 5% trichloroacetic acid, once with 10 ml of cold 70% ethanol, and allowed to dry. Radioactivity on the filter was determined in a toluene/fluors mixture with a Beckman LS 355 scintillation counter.

Incorporation of [3H]Thymidine into DNA

Samples (2 to 10 ml) were incubated with 10 μCi/ml of [3H]thymidine for 30 min at 37°C. Cold media were added and the cells were centrifuged at 2000 x g for 30 min. DNA was purified as previously described (Collins et al., 1977). Aliquots of DNA were applied to filters with a Beckman LS 355 scintillation counter.

Mitotic Index

One volume of cells in media was combined with 1 volume of acetic acid/carmine solution (2 g of carmine in 100 ml of 45% acetic acid) for 30 min, according to the method of Japa (1942). A minimum of 300 cells were examined for each determination. The cell number of each aliquot was determined with a hemacytometer.

Amount of DNA in Cell Suspensions

The amount of DNA in 1-ml aliquots was determined by the diphenylamine procedure of Burton (1968), using calf thymus DNA as a standard.

Amount of DNA per Cell by Fluorescence Measurement

Approximately 10 ml of the cell suspension (about 4 x 10^5 cells) was removed and washed three times with calcium/magnesium-free phosphate-buffered saline, pH 7.0. The cells were fixed in 70% ethanol and stained for DNA fluorescence analysis by the procedure of Cridman and Steenkamp (1973), using propidium iodide. Cells stained with propidium were then analyzed with a Coulter Electronics TPS-1 at a laser setting of 488 nm. This machine utilizes an argon laser rated at 35 milliwatts at the principal band of 488 nm. Cells in suspension flow in co-axial fashion past the intersection point of the laser beam. The resultant fluorescent pulses are focused onto a photomultiplier tube. The resultant electrical signals are then stored in the memory of a pulse-height analyzer containing 128 channels. The data are recovered either as counts in each channel or as a distribution of the counts in each channel (the number of cells) versus the channel number (the DNA content). For DNA distribution analysis, the high voltage detector was set at 750 and an amplification of 10 was used. Cells were analyzed at a rate of about 500 cells/s. Analysis of a log phase culture revealed that the G1 channel was 30 and the G2 + M channel was 90. To use, the machine was calibrated with fluorescent beads, as previously described (Collins, 1978). This assures that all experiments are relative to the same standard, from day to day. The computer analysis of Fried (1975, 1977) was used to compute the centroids of the individual Gaussian distributions that underlie each overall fluorescent distribution, for each aliquot of cells from a synchronized culture, as a function of time after release from the metabolic block. As the fluorescence intensity of the centroids of each Gaussian, measured in channel numbers, is proportional to the DNA content (Horan and Wheelless, 1971; Collins et al., 1977), the DNA content of a population of cells progressing through S-phase can be reliably estimated.

Cell Cycle Distribution by Computer Analysis

The overall DNA distributions of each aliquot of cells analyzed by flow microfluorometry (described above) were analyzed for the proportions of cells in the G1, S, and G2 + M phases of the cell cycle according to two models, one described by Fried (1975, 1977) and one developed at our institution, similar to the trial and error evaluation method described by Gray (1976), but modified so that the coefficients of the normal Gaussian distributions are not constant, but are allowed to vary (Brunsting et al., 1978). The use of these modifications in our model will be described in a separate publication. For the experiments described herein, there were no significant differences in the number of cells in each cell cycle phase predicted by each of the two programs (data not shown). There are several computer models for estimation of numbers of cells in each phase of the cell cycle, each based on different assumptions of which assume that the rate of DNA synthesis is linear (Watson and Taylor, 1977; Dean and Jett, 1974), in spite of numerous reports to the contrary (for example: Terasima and Tolmach, 1963; Denly and Cleaner, 1964; Painter and Schaefer, 1971; Remington and Klevecz, 1973). It should be stressed that the two computer models used in this study make no such assumptions.

Incorporation of [3H]Thymidine into Okazaki Fragments

Aliquots (1 ml) of cells were pulsed with 100 μCi/ml of [3H]- thymidine for 60 s at 37°C. As a control for nonspecific absorption of radiolabeled, a chilled aliquot was pulsed at 0°C. Incorporation was terminated by adding cold (0°C) media containing 2 mM thymidine and rapid chilling on ice. Cells were washed with cold thymidine-containing media, fixed in 70% ethanol and centrifuged at 0°C, and centrifuged as previously described (Rawles and Collins, 1977). The gradients were punctured, and fractions collected from the bottom were applied to filter discs for the determination of radioactive activity as previously described (Rawles and Collins, 1977). The resulting data were analyzed by a general nonlinear least squares computer program to quantitate the amount of radioactivity in the 4.5 S region of the gradients, assuming the shape of the radioactive distribution to be Gaussian (see Fig. 7). The fitting function used is of the form $g(x) = A_0 \exp(x^2) - c_1 \frac{x}{c_2} \frac{x}{c_3} \frac{x}{c_4}$, where $A_0$ is the amplitude of the exponential background under the center of the first Gaussian peak and $c$ is the slope. The $A_1, A_2, A_3, c_1, c_2,$ and $c_3$ are the amplitudes, center, and standard deviation of each respective Gaussian. The number of Gaussians to be fitted is $N$.

Sorting of [3H]Thymidine-labeled Cells

Cells were incubated with 1 μCi/ml of [3H]thymidine for 30 min, stained with propidium iodide as described earlier in this section, and sorted according to fluorescence as previously described (Collins, 1978). At least 20,000 cells were sorted directly into scintillation vials, or in some cases, into 5-ml plastic centrifuge tubes, prior to determination of radioactive activity, as previously described (Collins, 1978).

Calculation of Rates of DNA Synthesis

DNA-Propidium Fluorescence—The rate of increase in channel number as a function of time from $t_1$ to $t_2$ was calculated as: 

$\frac{\text{channel number at } t_2 - \text{channel number at } t_1}{t_2 - t_1}$

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no. at \(t_2 - \text{channel no. at } t_3\) + \(t_2 - t_1\), where channel no. refers to the centroid of the major Gaussian at \(t_3\), and at \(t_2\). For these experiments, the machine was calibrated such that channel 30 represents 16.1 pg of DNA and channel 60 represents 32.2 pg of DNA. For example, a rate of increase of 5 channels/h would equal a rate of synthesis of 2.6 pg of DNA/h.

Diphenylamine—The rate of DNA synthesis was calculated as:

\[
\frac{1}{\text{picograms of DNA + number of cells \times fraction of S-phase cells, at } t_3} - \frac{1}{\text{picograms of DNA + number of cells \times fraction of S-phase cells, at } t_1} + t_2 - t_1.
\]

Okazaki Fragments—The relative rate of DNA synthesis per cell for a 1-min period at \(t_1, t_2, \ldots, t_n\) was calculated as:

\[
\text{amount of } ^{3}H\text{ in 4.5 S DNA + number of cells \times fraction of S-phase cells, at } t_3, t_2, \ldots, t_1.
\]

Amount of \(^{3}H\) in Whole Cells—The relative rate of DNA synthesis per cell for a 30-min period at \(t_1, t_2, \ldots, t_n\) was calculated as:

\[
\text{amount of } ^{3}H + \text{number of cells \times fraction of S-phase cells, at } t_1, t_2, \ldots, t_n.
\]

Specific Activity—The relative rate of DNA synthesis per cell for a 30-min period was calculated as:

\[
\text{specific activity + number of cells \times fraction of cells in S-phase, at } t_1, t_2, \ldots, t_n.
\]

Sorting of Labeled Log Phase Cells—The relative rate of DNA synthesis for a 30-min period, during various parts of S-phase, was calculated as:

\[
\text{amount of } ^{3}H\text{ in } \log + \text{number of cells in sort \times fraction of S-phase cells in sort}.
\]

RESULTS

In initial experiments, we tested various synchronization protocols where the times of inhibition and release were varied, by using flow microfluorometry to follow the progression of cells through the cell cycle. We found that the particular schedule used in these experiments was optimal for obtaining highly synchronous cells located at the G\(_1\)/S boundary (data not shown).

Cells that had been adapted to thymidine for a 3-week period were synchronized and released from the second thymidine block. All of the experiments reported herein using synchronized cells were performed with a single 500-ml culture in order to minimize variations due to slight differences in synchronization protocols, growth conditions, or the inherent instability of HeLa cells (Thilly et al., 1977). All quotes were taken for the determination of DNA:propidium fluorescence, \([^{3}H]\)thymidine incorporation into whole cells, purified DNA and Okazaki fragments (4.5 S DNA), mitotic index, and amount of DNA by the diphenylamine procedure. Log-phase cultures were used for the sorting of \(^{3}H\)-labeled cells.

Cell Cycle Progression—Measurements of the DNA:propidium fluorescence of single cells with the flow microfluorometer are presented in Fig. 1. The panel designated “log” represents a log-phase culture, included for comparison. The G\(_1\) distribution of the log-phase cells corresponds to DNA content of 16.1 pg/cell, determined by calibrating the centroid of the fluorescence intensity distribution relative to WI-38 and 2RA cells for which the DNA content is known (Lin et al., 1974). This corresponds to DNA of molecular weight 9.7 \(\times\) \(10^{6}\), in excellent agreement with that reported by Lee and Puck (1960) for this cell line. The log-phase distribution (Fig. 1) corresponds to a cell cycle distribution of 68% G\(_1\), 23% S-phase, and 9% G\(_2\) + M cells, respectively, as determined with each of the cell cycle analysis computer programs employed.

As can be seen, cells in the released culture do not begin to enter the S-phase until about 2 to 2.5 h (Fig. 1). From 3 to 7.5 h, the cells continue to travel through mitosis and divide as evidenced by the increasing numbers of cells back in G\(_1\). From 13 to 24 h, the cells remain in G\(_1\) (data not shown), then journey through S-phase again (24 to 31 h), then divide once more (31 to 36 h). The overall distributions for the second journey through S can be seen to be much broader than for the first journey through S, due to individual cell to cell variations in cell cycle progression. Nevertheless, the culture still exhibits good synchrony. The ability of the cells to divide argues for excellent viability.

Cell Cycle Distribution—The data of Fig. 1 can be used to compute the precise numbers of cells in each of the cell cycle phases, based on cellular DNA content (DNA:propidium fluorescence), using computer programs (“Experimental Procedures”). The results obtained with the Fried model are shown in Fig. 2. Initially, 92% of the released cells have a DNA content of 16.1 pg/cell.
content corresponding to G1 cells, hence are at, or very near, the G1/S boundary. From here, they proceed in a highly synchronous fashion through S, and G2 + M (1 to 12 h). They remain in G2 from 12 to 24 h, whereupon they again proceed through S, and G2 + M (24 to 36h). At 40 h, 91% of the cells were back in G1 (data not shown).

Mitotic Index and [3H]Thymidine Incorporation— Measurements of the amount of [3H]thymidine incorporated into whole cells and purified DNA, following a 30-min pulse, and the number of mitoses are presented in Fig. 3. The incorporation of [3H]thymidine into whole cells and into DNA exhibited a similar pattern throughout both of the S-phases. The first peak of mitosis (42%) occurs at 10 h, and the second peak (26%) occurs at 35 h, consistent with a total cycle time of about 25 h. Similarly, a Tc = 25 h is indicated by the distance (in hours) between the centroids of the two waves of [3H]thymidine incorporation, determined by analysis with a nonlinear least squares computer program (see “Experimental Procedures”). The width of the second wave of [3H]thymidine incorporation and the second wave of mitosis is increased by about 1 h, and the number of mitoses of the second peak (35 h) is decreased by 40%, again consistent with an increase in the variation of cycle progression of individual cells with time. Still, as mentioned previously, the culture retains excellent synchrony 35 h after release from the metabolic block.

It should be apparent from a comparison of Fig. 3 with Fig. 2 that, whereas incorporation of [3H]thymidine can predict the onset and end of S-phase, it cannot predict the precise numbers of cells in any of the cell cycle phases. Similarly, the mitotic index, while a useful indication of the degree of synchrony, does not predict the number of cells in G1 or S-phase. A pattern similar to that of Fig. 3 for the incorporation of [3H]thymidine into synchronized cells has been reported by numerous laboratories, for example, Terasima and Tolmach (1963), Dendy and Cleaver (1964), and Painter and Schaefer (1971). This has been interpreted to indicate a pattern where “the rate of DNA synthesis” is maximal in the middle of S-phase and minimal in the early and late stages of S.

Increase in DNA Content during S-Phase—As DNA:propidium fluorescence is proportional to the DNA content per cell (Horan and Wheeless, 1977; Collins et al., 1977), the increase in DNA content (e.g. DNA synthesis) as a function of time can be estimated from the increase in the fluorescence intensity of the major distributions or “peaks,” as the cells progress through the S-phase (see Fig. 1, 2 to 8 h and 25 to 32 h). However, this approach is compromised by the fact that at certain times the major distribution is composed of both S-phase and G1 or G2 cells (e.g. 2, 8, 9, 24, 25, 30, 31, and 32 h refer to Figs. 1 and 2). This approach can be vastly improved by the use of computer analysis (Fried, 1977) to produce separate G1, S and G2 - M distributions, with corresponding intensity values, for each experiment depicted in Fig. 1. Selected distributions were thus obtained for 2, 3, 4, 5, 6, 7, 8, 26, 27, 28, 29, 30, 31, and 32 h (data not shown). These distributions thus represented the major cohort of DNA-synthesizing cells progressing through S-phase. The intensity values for the center of each distribution are presented in Fig. 4b. The triphasic pattern is similar to that reported by Remington and Klevecz (1973), who utilized a fluorescence method to measure the average DNA content of a large number of synchronized cells. For comparison with Fig. 4b, the average DNA content of large numbers of cells analyzed by the diphenylamine method (Burton, 1968), which has been adjusted to represent the number of S-phase cells, using the data of Fig. 2, are presented in Fig. 4a. The data of Fig. 4a are remarkably similar to an experiment reported by Thilly et al. (1977) although they interpreted their data as indicative of a linear rate of increase in DNA. As can be seen, there is good correlation between the two methods (Fig. 4, a
and b). For each S-phase, there is a rapid initial increase in DNA during the 1st h, followed by a slower increase, then a rapid increase over the last hour. The dashed lines in Fig. 4 represent the expected data if the increase in DNA were linear. When the data of Fig. 4a are uncorrected to represent the proportions of S-phase cells, and when the fluorescence data of Fig. 1 are used without subsequent computer analysis, the same pattern, more rapid increases during the first and last hour of S-phase, is obtained (data not shown).

Rates of DNA Synthesis—The rates of DNA synthesis at different times of S-phase, calculated by taking the first derivative of the data of Fig. 4, are presented in Fig. 5. For each S-phase, the rate is initially rapid for the 1st h, declines to almost linearity over the next 4 h, then is even greater over the last hour. The calculated rates of DNA synthesis are: diphenylamine method, 3.6 pg/h for the 1st h, 1.7 pg/h for the next 4 h, and 5.3 pg/h for the last hour; DNA:propidium fluorescence, 3.6 pg/h for the 1st h, 1.7 pg/h for the next 4 h, and 5.7 pg/h for the last hour. It should be stressed that the latter calculation would result in an exact doubling of DNA over the 6 h of S-phase. A rate of 5.7 pg/h would correspond to $2.9 \times 10^8$ nucleotides/s.

Rate of Synthesis of Okazaki Fragments—The amount of radioactivity in 4.5 S DNA (Okazaki fragments) of two selected times are presented in Fig. 6. There is relatively little

![Fig. 5. Rates of DNA synthesis. The data of Fig. 4 were used to calculate rates which are plotted as per cent of duration of S-phase. a, diphenylamine method; , time after release of culture, 2 to 8 h; , time after release of culture, 26 to 32 h. b, intensity of DNA:propidium fluorescence; , times after release of culture, 2 to 8 h; , times after release of culture of 26 to 32 h.](http://www.jbc.org/)

![Fig. 6. Synthesis of Okazaki fragments. Cells at 2 h (a) and 3 h (b) after release were pulsed with $[^3H]$thymidine for 60 s and lysed on alkaline sucrose gradients, and the radioactivity was determined, as described under "Experimental Procedures." The temperature during the pulse was: , 37°C; , 0°C.](http://www.jbc.org/)
incorporation of [³H]thymidine into 4.5 S DNA at 2 h (Fig. 6a), but a dramatic incorporation at 3 h (Fig. 6b), reflecting the relatively sudden progression of the culture into S-phase. In each case, there is very little incorporation at 0°C, indicating that our procedure is measuring the production of Okazaki fragments and not nonspecific binding of thymidine.

The total amount of radioactivity in Okazaki fragments as a function of time after release of the culture is presented in Fig. 7. These data represent the number of S-phase cells (from the data of Fig. 2). Although this procedure does not measure the amounts of DNA synthesized, it should adequately reflect the relative rate of DNA synthesis. The pattern obtained (Fig. 7) very closely follows the pattern obtained with the diphenylamine procedure (Fig. 5a) and by DNA:propidium fluorescence (Fig. 5b). Even when the data of Fig. 7 are not adjusted to represent the number of S-phase cells, the same pattern of more rapid rates during the 1st and last hours of S-phase is exhibited (data not shown). When the data of Fig. 7 were expressed as per cent of total counts in each gradient, in order to minimize fluctuations in pool sizes, or the addition of a slightly different amount of [³H] (Rawles and Collins, 1977; Friedman et al., 1975), the same pattern resulted (data not shown).

Sorting of [³H]-pulsed Cells—Log-phase cells were pulsed with [³H]thymidine for 30 min, stained with propidium iodide, and analyzed for DNA:propidium fluorescence as described under “Experimental Procedures.” A typical DNA distribution is presented in Fig. 1 (log). Electronic sorting of cells corresponding to early, middle, and late S-phase DNA content was accomplished with sort windows set for early S (channels 36 to 42), mid-S (channels 42 to 50), and late S (channels 50 to 60). Stained cells were either sorted directly into scintillation vials for determination of radioactivity, or sorted into tubes and treated with trichloroacetic acid prior to determination of radioactivity. Direct sorting into scintillation vials without acid treatment yielded the greatest "incorporation" in tubes and treated with trichloroacetic acid prior to determination of radioactivity. Acid treatment of the sorted cells removed radioactivity such that the lowest amount of incorporation occurred in the middle of S-phase, with values of 3.3, 4.7, and 3.5 x 10⁻² cpm/cell for early, middle, and late S-phase. However, acid treatment of the sorted cells removed radioactivity such that the lowest amount of incorporation occurred in the middle of S-phase, with values of 3.2, 2.5, and 3.1 x 10⁻² cpm/cell for early, middle, and late S-phase. When the amount of incorporation into acid-treated sorted cells was adjusted to represent the number of S-phase cells, the differences in the amounts of incorporation (4.6, 2.5, and 6.1 x 10⁻² cpm/cell for early, middle, and late S-phase) are comparable to the differences in the rates of DNA synthesis obtained from the diphenylamine procedure and the fluorescence method (Fig. 5), and to the different relative rates from measurements of radioactivity in Okazaki fragments (Fig. 7). The data cited above were the averages of nine separate sorts of a single culture sorted with identical electronic windows yielding a range of variation of 4%. When four separate cultures were examined in the same manner, the range of variation within a culture never exceeded 4% but the range between cultures varied as much as 20%. We attribute this to culture variations, rather than to differences in the placement of the electronic sort windows, as the different cultures were sorted on the same day with identical windows.

Comparison of Rates of DNA Synthesis—The rates predicted by each of the various methods are summarized in Table I, for early, middle, and late portions of S-phase. It can be seen that there is excellent agreement of the proportional rates predicted by the first four methods, based on DNA:fluorescence/cell, average DNA content (DPA), amount of radioactivity in Okazaki fragments (4.5 S), and amount of radioactivity in sorted S-phase cells. All four methods reveal a triphasic pattern of a burst, a decline, and then a final burst. The last two methods, based on amount of radioactivity in whole cells and specific activity in purified DNA, even though adjusted to represent the number of S-phase cells, follow a quite different pattern. These experiments were duplicated, and essentially the same patterns were observed.

**DISCUSSION**

The use of high concentrations of thymidine to achieve cell synchrony has been criticized, as such conditions can lead to a temporary imbalance of metabolism (Studzinski and Lambert, 1969); however, the abnormal cellular composition reverted to control levels quickly, at least by the time of the first mitosis (Studzinski and Lambert, 1969). The fact that essentially the same data were obtained for the second journey through S-phase, as for the first, in our experiments, argues that these thymidine-adapted cells were not grossly abnormal, at least for the processes we examined.

In mammalian cells, such as HeLa, [³H]thymidine en route...
to DNA does not enter the endogenous, intracellular pool of de novo synthesized nucleotides, but, rather, enters a separate pool via the salvage pathway (Kuebbing and Werner, 1975).

It should not be surprising that the estimates of relative rates of DNA synthesis based on \(^{1}H\)thymidine incorporation into either whole cells or DNA (Table 1) did not correlate with the rates estimated by more direct means (e.g. DNA:propiodum fluorescence or diphenylamine). The literature is fraught with examples of the pitfalls encountered in relying upon the metabolism of exogenous thymidine for estimations of rates of DNA synthesis. For example, in HeLa cells, there are variations during S-phase of: the size of the TTP pool (Bray and Brent, 1973), thymidine kinase activity (Brent et al., 1965) and transport of thymidine into the cells. A further complication is that radioactive nascent DNA can be degraded (Klievecz et al., 1974).

Numerous reports have utilized flow microfluorometry to examine the synchrony of cells (Kübler et al., 1974; Watson and Taylor, 1977; Barlogie et al., 1976; Krishan et al., 1978; Tobey and Greenman, 1972). However, the emphasis was on the numbers in G1, S, and G2 + M, not on precise estimations of the increase in DNA content of Gaussian distributions, hence we are unable to use the data of others for calculations of rates of DNA synthesis, for comparisons with our findings. Kübler et al. (1974) have demonstrated the use of flow microfluorometry to monitor the synchrony of HeLa S phase in suspension culture, as we have done. Their data reveal a 3- to 4-fold lower synchrony, and the presence of a large number of cells apparently unable to progress past the G1/S boundary (Kübler et al., 1974). We attribute this to the fact that in their experiments the cells were not adapted for thymidine synchronization, and the synchronization protocol was not optimized. Our 3-week adaption procedure probably selects for a population of cells which are able to quickly overcome the inhibitory effects of 2 mM thymidine (see Fig. 1 and Fig. 2), and our synchronization protocol assures that all of the cells are at (or very near) the G1/S boundary (see "Experimental Procedures").

Our use of DNA:propiodum fluorescence to measure the rates of DNA synthesis of a synchronized cell population yielded rates which predicted an exact doubling of the DNA content during the 6 h of S-phase; hence this approach appears to be adequate. The excellent correlation with rates predicted from use of the diphenylamine technique (Fig. 5) further argues for the accuracy of this approach. The correlation coefficient, \( r \), was 0.981 (when \( r = 1.0 \), the correlation is said to be exact). The triphasic pattern obtained by sorting labeled S-phase cells from a log-phase culture was similar to the pattern obtained with the fluorescence and diphenylamine methods, hence should be suitable for routine estimations of perturbation effects (e.g. drugs) on DNA synthesis. However, the possibility of perturbation effects on thymidine metabolism would still remain.

With all of the problems associated with the use of \(^{1}H\)thymidine to measure "rates" of DNA synthesis, it may seem surprising that the amount of radioactivity in Okazaki fragments (Fig. 7) closely followed the same triphasic pattern as the increase in the amount of DNA (Fig. 5), whereas the specific activity of isolated DNA (Fig. 2 and Table 1) did not.

However, it should be recalled that the synthesis of Okazaki fragments (polymerization of nucleotides to form nascent DNA) is not compromised by the action of terminal deoxynucleotidyltransferases or repair processes as might be the case with the specific activity of total DNA.

It has been reported that the rate of replicon movement in HeLa cells is twice as fast 5 h after release from a thymidine

1 J. M. Collins, unpublished data.
Rates of DNA synthesis during the S-phase of HeLa cells.

J M Collins


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