DNA REPLICATION: DIRECTION AND RATE OF
CHAIN GROWTH IN MAMMALIAN CELLS

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ABSTRACT

Using pulse labeling techniques with \(^{3}H\)thymidine or \(^{3}H\)cytidine, combined with DNA fiber autoradiography, we have investigated the direction and rate of DNA chain growth in mammalian cells. In general, chain elongation proceeds bidirectionally from the common origin of pairs of adjacent replication sections. This type of replication is noted whether the DNA is labeled first with \(^{3}H\)thymidine of high specific activity, followed by \(^{3}H\)thymidine of low specific activity or the sequence is reversed. Approximately one-fifth of the growing points have unique origins and in these replication units, chain growth proceeds in one direction only. Fluorodeoxyuridine and hydroxyurea both inhibit DNA chain propagation. Fluorodeoxyuridine exerts its effect on chain growth within 15-23 min, while the effect of hydroxyurea is evident within 15 min under conditions where the endogenous thymidine pool has been depleted by prior treatment with fluorodeoxyuridine. Puromycin has no effect on chain growth until 60 min after addition of the compound, even though thymidine incorporation is more than 50% reduced within 15 min. After 2 h of treatment with puromycin, the rate of chain growth is reduced by 50%, whereas thymidine incorporation is reduced by 75%. Cycloheximide reduces the rates of DNA chain growth and thymidine incorporation 50% within 15 min, and, on prolonged treatment, the decrease in rate of chain growth generally parallels the reduction in thymidine incorporation.

INTRODUCTION

DNA chain growth in prokaryotic cells proceeds bidirectionally from a single initiation point (Masters and Broda, 1971; Prescott and Keumpel, 1972; Bird et al., 1972) and is independent of protein synthesis (Maaløe and Hanawalt, 1961; Lark, 1969). The situation in eukaryotic cells is not as clear-cut. Huberman and Riggs (1968) first observed that replication proceeds in opposite directions from the common origin of adjacent replication sections in mammalian cell DNA. Lark et al. (1971) however, reported experiments showing that chain propagation need not be exclusively of this type, but that replication can proceed via a single replication fork in each replication unit. Both these groups of investigators used DNA fiber autoradiography to determine the direction of chain growth. Weintraub (1972) later concluded that replication is bidirectional from data derived from rate zonal centrifugation of DNA pulse labeled with bromodeoxyuridine and sheared by exposure to ultraviolet light. As to the requirement for protein synthesis during chain growth, we (Hand and Tamm, 1972) have shown that rate of chain growth is reduced by inhibition of protein synthesis for 2 h with puromycin, and Weintraub and Holtzer (1972) have shown that cycloheximide also decreases rate of chain growth. However, data obtained from rate-zonal gradient centrifugation analyses have been interpreted to show that
chain growth is unaffected by inhibition of protein synthesis (Fujiiwara, 1972). We have therefore examined both the rate and direction of chain growth in mammalian cells in an effort to resolve some of these differences. We have examined the direction of chain growth using some of the techniques of both Lark et al. (1971) and Huberman and Riggs (1968). We have investigated the kinetics of inhibition of rate of chain growth using two different inhibitors of protein synthesis, puromycin and cycloheximide, and examined chain growth during inhibition of DNA precursor synthesis by fluorodeoxyuridine or hydroxyurea.

MATERIALS AND METHODS

Radioisotopes and Chemicals

For our initial experiments, [3H]thymidine ([3H]-dThd) was obtained from Amersham-Searle Corp., Arlington Heights, Ill. at 22-25 Ci/mmol. For later experiments, [3H]dThd at 42-55 Ci/mmol was obtained from Schwarz-Mann Div. Becton, Dickinson & Co., Orangeburg, N.Y. [3H]cytidine ([3H]Cyd), 23-25 Ci/mmol, was obtained from Amersham-Searle Corp. Fluorodeoxyuridine (FdUrd) was a gift from Dr. W. E. Scott of Hoffman-La Roche, Inc., Nutley, N.J. Cycloheximide was obtained either from Calbiochem, San Diego, Calif., or Polysciences, Inc., Warrington, Pa. Lots from either company gave comparable results. Puromycin was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and hydroxyurea from Calbiochem.

Cell Line

The continuous line of mouse fibroblasts, L-929, was used throughout this study. The cell line was maintained in monolayer cultures using Eagle's minimal essential medium (MEM) with 5% fetal calf serum (FCS).

Pulse-Labeling Techniques

All experiments were performed in a 37°C warm room. Cells were maintained in a moist CO2 atmosphere and were removed from this only for changes of medium or additions of radioisotopes or other chemicals. Cells were seeded in 60-mm Petri dishes at a concentration of 250,000 cells/dish 24 h before the beginning of an experiment; they were in logarithmic growth at the start of the experiment. At the beginning of a labeling period, fresh MEM with 5% FCS containing the appropriate amounts of isotope was added to the cultures. All other chemicals were added when needed as 100 times concentrated solutions. Experiments were terminated by removing the radioactive medium and washing the cells twice with cold phosphate-buffered saline (PBS).

DNA Fiber Autoradiography

Cells were detached from Petri dishes by trypsinization. They were then collected by centrifugation, washed, and suspended in PBS without calcium or magnesium (PBS-def) at a concentration of 40,000-60,000 cells/ml. One drop of this suspension containing 2000-3000 cells was placed on a subbed glass slide near a drop of PBS-def containing 1% sodium dodecyl sulfate and 0.01 M ethylenediaminetetraacetate. A clean glass rod was then touched simultaneously to both drops, and the rod was drawn gently over the surface of the slide. The material from the two drops mixed during this maneuver and the DNA fibers released from the lysing cells were spread over the slide. Slides prepared in this manner were allowed to dry in air, washed four times in 5% trichloroacetic acid and dehydrated in graded alcohols. They were then dipped in Kodak NTB-2 emulsion and exposed at 4°C for 3-6 mo. This procedure for DNA fiber autoradiography is essentially that of Lark et al. (1971), and differs markedly from that used by us previously (Hand et al., 1971). The newer procedure offers the advantages of simplicity and speed, and it also is free of the technical artifacts, such as high background and grain clumping, that may be introduced by the use of membrane filters for support of the DNA strands. Autoradiographs were developed as described previously (Hand et al., 1971).

Quantitation of DNA Fiber Autoradiographs

DIRECTION OF CHAIN PROPAGATION The direction of replication was inferred from the changes in grain density along DNA fibers in autoradiograms produced by pulse-labeling the DNA sequentially with [3H]dThd of different specific activities (Huberman and Riggs, 1968; Lark et al., 1971). This was done by labeling the DNA first with [3H]dThd of high specific activity (50 Ci/mmol, 250 µCi/ml) and then with [3H]dThd of low specific activity (5 Ci/mmol, 250 µCi/ml). This sequence is referred to as high-low, the reverse as low-high. Before the beginning of the pulse the cells were treated with 2 X 10⁻⁷ M FdUrd for 1 h to exhaust the endogenous dThd pool. The FdUrd was kept in the medium during the pulse period to increase incorporation of [3H]dThd into DNA. In a high-low experiment, if chain growth proceeded bidirectionally from the common origin of adjacent replication sections, the autoradiograms were of two types, depending whether replication began on individual replication sections before or during the high pulse. In those sections where replication began before the high pulse, a
small clear area (indicating DNA replicated before the pulse) was flanked by linear arrays of high grain density proceeding to low. In autoradiograms resulting from replication that began during the high pulse, there was a linear array of high grain density adjoined at either end by an array of low grain density. In low-high experiments, the patterns of grain density were reversed. If replication, initiated at an origin, proceeded in a single direction only, then, regardless of the order of the pulses or whether replication began before or during the pulses, the patterns were alternating stretches of high and low grain density. In all experiments, the labeling periods were sufficiently short so that unlabeled regions existed at the termination points in a significant number of replication sections.

For the determination of the percentage of autoradiograms that showed bidirectional replication from a common origin, we adhered to the following guidelines: autoradiograms were scored only in areas of the slides where the DNA fibers were well separated. Only autoradiograms that were part of a discrete DNA chain (internal autoradiograms) were scored. (This minimized the inclusion of broken DNA strands in the data. These would have been interpreted as showing unidirectional replication, regardless of whether the autoradiograms resulted from unidirectional or bidirectional replication.) Those replication sections where DNA replication began prior to the onset of the radioactive pulse were scored as examples of bidirectional chain propagation if the grain density gradients were in opposite directions and appropriate for labeling protocol followed (either high-low or low-high). Autoradiograms that showed uniform grain density were not scored. These resulted from sections where replication was completed entirely within the period of time of either a high or low specific activity pulse. Along some stretches of DNA fibers, replication had terminated in multiple adjacent sections, which were fused and appeared as long alternating regions of high and low grain density without intervening clear areas. These longer stretches of labeled DNA were not scored since they could be interpreted as representing either unidirectional or bidirectional chain propagation. The autoradiograms of uniform grain density and the fused long segments were infrequent, representing less than one-third of the internal autoradiograms in areas where the DNA fibers were well separated.

**Rate of DNA Chain Growth** We have described previously the method used for quantitation of rate of DNA chain growth (Hand and Tamm, 1972). This was modified in the present experiments only by the use of larger doses of radioactivity. In brief, the cells were treated for 1 h with FdUrd (2 \( \times \) 10\(^{-6}\) M) and then, still in the presence of FdUrd, labeled with 5 \( \times \) 10\(^{-6}\) M [\(^3\)H]dThd of high specific activity (50 Ci/mmol, 250 µCi/ml) for 30 min. This was followed by a pulse with 5.5 \( \times \) 10\(^{-6}\) M [\(^3\)H]dThd of low specific activity (3 Ci/mmol, 250 µCi/ml) for 30 min. The autoradiograms from replication sections where replication began before the onset of the high specific activity pulse and continued into the low specific activity pulse were measured and the rate of DNA chain growth calculated by dividing the length of the autoradiograms by the time of the high specific activity pulse. The beginning of the pulse was marked by the appearance of grains in the autoradiograms, and the end by the transition from heavy to light grain density. We determined that the distance between initiation points in L cells is 62.6 ± 1.78 µm (\( \bar{x} \) ± SE). Thus the high specific activity pulse was of sufficiently short duration to allow measurement of rate of chain growth in individual replication sections. Fused adjacent replication sections (with twice the modal length of measured segments) were seen infrequently under the conditions of these experiments.

In this method of measurement of rate of DNA chain growth the pool-dependent transit time of the tritium from dThd to thymidine triphosphate is not taken into account. This transit time is extremely short, however, since equilibrium concentrations of [\(^3\)H]dThd during pulse labeling in L cells are reached within 5 min in the absence of FdUrd, and current theories of pool kinetics suggest that the pool size would be markedly reduced in the presence of FdUrd (Cleaver, 1967). Thus, our method of determining rate of DNA chain growth may give only a slight underestimate of the true rate, and variations in pool size under control and experimental conditions probably minimally alter the observed rate of chain growth.

**RESULTS**

**Direction of Chain Propagation**

Cells were pulsed sequentially with [\(^3\)H]dThd of different specific activities, and the percentage of growing points involved in bidirectional replication from shared origins of adjacent replication sections was determined.

In high-low experiments, 83.0% of the growing points showed bidirectional replication. When a low-high protocol was followed, 77.5% of growing points showed bidirectional replication. Our results suggest that in L cells, DNA chain propagation occurs predominantly in a manner where adjacent growing points proceed in opposite directions from a common origin.

Fig. 1 shows several examples of bidirectional replication and one example of unidirectional replication.
replication where adjacent growing points proceeded in the same direction from separate origins. These were taken from an experiment where a high-low protocol was followed. Fig. 2 shows several examples of bidirectional replication where a low-high protocol was followed.

**DNA Chain Growth during Inhibition of Synthesis of DNA Precursors**

**FdUrd** Cells were exposed to $3.3 \times 10^{-8} \text{ M}$ $[^3\text{H}]\text{Cyd}$ (25 Ci/mmol, 80 µCi/ml), and after 1, 7, or 15 min, FdUrd, $2 \times 10^{-8} \text{ M}$, was added. The experiment was terminated after 30 min. Controls received no FdUrd. If FdUrd blocked chain growth, then the mean length of simple pulse autoradiograms of DNA labeled with $[^3\text{H}]\text{Cyd}$ would be reduced. Table I shows that control autoradiograms produced by labeling with $[^3\text{H}]\text{Cyd}$ had a mean length similar to that observed after simple pulse labeling with $[^3\text{H}]\text{dThd}$, (Hand et al., 1971). FdUrd, present in the cultures for 23 or 29 of the 30 min of the $[^3\text{H}]\text{Cyd}$ pulse, reduced the length of DNA segments. FdUrd present for 15 of the 30 min had little effect. The results are best explained by postulating that FdUrd inhibits chain elongation, but requires 15–23 min in order to exert this effect in L cells.

This may represent the time required to exhaust the cellular pool of dThd.

**Hydroxyurea** Cells treated with hydroxyurea can be labeled with $[^3\text{H}]\text{dThd}$ without reversing the inhibitory effect of the hydroxyurea.

The 30 min high specific activity $[^3\text{H}]\text{dThd}$ pulse was begun at various times after the addition of hydroxyurea ($2 \times 10^{-3} \text{ M}$). The time points were taken as the midpoints of the 30 min pulse. All cells had been exposed to FdUrd for 1 h before the start of the $[^3\text{H}]\text{dThd}$ pulse, and FdUrd remained in the medium during the pulse. Thus the cells were exposed to both hydroxyurea and FdUrd during the time that the rate of DNA chain growth was measured. However, we assumed that the high concentration of $[^3\text{H}]\text{dThd}$ ($5 \times 10^{-8} \text{ M}$) reversed the effect of the FdUrd. The pattern of DNA replication was markedly altered by treatment with hydroxyurea for 15 min. The autoradiograms did not show the characteristic long segments seen in untreated controls. Instead, the grains were arranged in multiple short segments below the minimal length permitting accurate measurement. Linearity was preserved suggesting that the autoradiograms represent small chains of replicating DNA on intact template strands and that the most evident effect of hydroxyurea is a marked slowing of
FIGURE 2 Chain propagation in an experiment where a pulse with $[^3H]$dThd of low specific activity was followed by a pulse with $[^3H]$dThd of high specific activity. Figs. 2a, b, c, and d are examples of bidirectional replication where the low grain density is in the center and the high grain density segments are on either side of the autoradiogram. X 720.

TABLE I Effect of Fluorodeoxyuridine on DNA Chain Growth

| Time of addition of FdUrd after beginning of [3H]Cyd pulse | Length of FdUrd present during [3H]Cyd pulse | Length of DNA segments in μm |
|-----------------------------------------------------------|---------------------------------------------|-------------------------------|
| min | min | μm ± SE |
| -- | 0 | 20.9 ± 0.84 |
| 15 | 15 | 18.9 ± 0.80 |
| 7 | 23 | 13.1 ± 0.72 |
| 1 | 29 | 12.1 ± 0.58 |

rate of chain growth. (Fig. 3, compare a, untreated cells, with c, hydroxyurea-treated cells). On each slide there were a few autoradiograms that showed the longer tandem arrays characteristic of the controls not treated with hydroxyurea. They tended to be clustered together, suggesting that they were derived from the same cell. In these few DNA strands, the rate of chain growth approached that in controls.

Since the cells were pretreated with FdUrd before the start of the $[^3H]$dThd pulse, these experiments are not strictly comparable to the experiment using FdUrd as the inhibitor and $[^3H]$Cyd as the label. It seems clear that normal chain growth could not proceed in the presence of hydroxyurea when the FdUrd block was reversed by the addition of $[^3H]$dThd, however, we cannot rule out an additive effect of the two inhibitors on rate of chain propagation.

In cells treated for 30 min and 1 h with hydroxyurea, the long tandem arrays were rarely seen, and after treatment for 2 h, none were seen. The reason for the relative resistance to hydroxyurea of these few strands is unknown, however it might be related to differences in the deoxynucleoside pools in certain subpopulations of cells.

DNA Chain Growth during Inhibition of Protein Synthesis

We have reported previously that inhibition of protein synthesis for 2 h by puromycin reduces the rate of DNA chain elongation by half (Hand and Tamm, 1972). However, we observed also that the degree of inhibition of $[^3H]$dThd incorporation exceeded the degree of chain growth inhibition suggesting that some other aspect of DNA replication in addition to chain propagation might be affected by this compound. Cycloheximide is an inhibitor of protein synthesis that acts differently from puromycin. It was therefore important to determine the kinetics of inhibition...
FIGURES DNA chain growth during inhibition of DNA synthesis by cycloheximide or hydroxyurea.

Cells treated with either of the two compounds were labeled with [3H]dThd as indicated in the text. Fig. 3 a, control; Fig. 3 b, cycloheximide, 1.8 \times 10^{-4} \text{M} for 15 min; Fig. 3 c, hydroxyurea, 2 \times 10^{-3} \text{M} for 15 min. The labeled DNA segments are shorter in the cycloheximide-treated cells than in the control. In the hydroxyurea-treated cells, the DNA segments are so short as to be unmeasurable. "X "275.

of chain growth during puromycin and cycloheximide treatment.

**Puromycin** Cells were treated with puromycin (4.2 \times 10^{-4} \text{M}) and rate of DNA chain growth determined as in experiments with hydroxyurea. Samples, taken at the midpoint of the 30 min high specific activity pulse were collected at 15, 30, 60, and 120 min after addition of puromycin. Rate of DNA chain growth was unaffected for 30 min, but was inhibited more than 50% by 120 min. The data are shown in the upper graph in Fig. 4. Control cells showed a rate of chain elongation of 0.6-0.7 \mu\text{m/min} (3600-4200 bases/replication fork/min), and they maintained this rate throughout the 2 h of the experiment. As shown in Table II, the reduction in rate of chain growth was always less than the degree of inhibition of [3H]dThd uptake as determined by pulse labeling in replicate control and puromycin-treated cultures that had not been treated with FdUrd or large amounts of [3H]dThd for purposes of chain growth measurement.

**Cycloheximide** Similar experiments were performed using cycloheximide (1.8 \times 10^{-4} \text{M}) to inhibit protein synthesis. In contrast to the results with puromycin, cycloheximide produced an immediate reduction in the rate of DNA chain growth (Fig. 3 b; Fig. 4, lower graph) which paralleled the inhibition of incorporation of [3H]-dThd into replicate cultures (Table II). Again, control cells maintained a rate of chain growth of 0.6-0.7 \mu\text{m/min} for the duration of the experiment.

**Discussion**

Our data indicate that DNA chain propagation in mammalian cells proceeds predominantly in a bidirectional manner from the common origin of paired replication sections. In addition, we have shown that inhibitors of DNA precursor synthesis and protein synthesis slow the rate of DNA chain growth and that the sensitivity of this process to different inhibitors of protein synthesis varies.

**Bidirectional Chain Propagation**

We have shown that under conditions of either high-low or low-high specific activity [3H]dThd uptake as determined by pulse labeling in replicate control and puromycin-treated cultures that had not been treated with FdUrd or large amounts of [3H]dThd for purposes of chain growth measurement.
Figure 4  Rate of DNA chain growth during inhibition of DNA synthesis with puromycin (upper panel) or cycloheximide (lower panel); •—•, control; △—△, drug treated. The standard error for each determination is indicated. The abscissa indicates the time in minutes after addition of the inhibitory compound. The calculation of rate of chain growth is explained in the text.

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Pulses, replication is predominantly bidirectional with replication forks in adjacent replication sections sharing a common origin. Our results agree with the original observations in eukaryotic cells by Huberman and Riggs (1968) and those made subsequently by us (Hand and Tamm, 1972) and by Callan (1972), using the technique of fiber autoradiography, and with those made by Weintraub (1972), using ultraviolet light-induced specific strand breakage and rate-zonal centrifugation of DNA. Lark et al. (1971) observed DNA replication that proceeded via unpaired replication forks with a single replication fork per replication unit. This type of replication was observed only during sequential low-high [3H]dThd labeling. When Lark et al. used a high-low pulse sequence they obtained results similar to ours. Even in their low-high experiments, close to 40% of their autoradiograms showed grain density gradients pointing in opposite directions. As Lark et al. suggested, these may well represent bidirectional replication from a common origin. Huberman and Tsai (1973) have performed experiments similar to those of Lark et al. and those reported here. They found approximately 90% of replication bidirectional, and this percentage was independent of the presence of FdUrd. They have emphasized several technical points that may explain in part Lark et al. findings. These are the small difference in specific activity between the high and low pulses and their scoring of autoradiograms that may have been from broken DNA strands.

Table II

Comparison of the Degree of Inhibition of [3H]dThd Incorporation and Rate of DNA Chain Growth Caused by Cycloheximide or Puromycin

| Drug              | Time after addition of inhibitor | [3H]dThd incorporation* | Rate of DNA chain growth | % of control | % of control |
|-------------------|---------------------------------|-------------------------|--------------------------|--------------|--------------|
| Puromycin, 4.2 × 10⁻⁴ M | 15 min                         | 46.2                    | 83.9                     |              |              |
|                   | 30 min                         | 33.6                    | 87.0                     |              |              |
|                   | 60 min                         | 25.9                    | 63.7                     |              |              |
|                   | 120 min                        | 23.9                    | 40.3                     |              |              |
| Cycloheximide, 1.8 × 10⁻⁴ M | 15 min                        | 49.2                    | 47.6                     |              |              |
|                   | 30 min                         | 43.6                    | 54.2                     |              |              |
|                   | 60 min                         | 40.9                    | 51.6                     |              |              |
|                   | 120 min                        | 44.8                    | 41.4                     |              |              |

* Control or inhibitor treated cells were pulse labeled with [3H]dThd (1.0 μCi/ml) for 15 min. The incorporation of radioactivity into trichloroacetic acid-insoluble material was determined by liquid scintillation counting.
It seems clear, however, that autoradiographic patterns compatible with unidirectional replication do occur. Whether these represent true unidirectional replication occurring as a physiologic alternative to the more common bidirectional mode or result from experimentally induced artifacts, such as unrecognized strand breakage, remains to be determined.

**Rate of DNA Chain Growth**

The very high concentrations of radioisotope used in the present experiments seem to have had little effect on the rate of chain elongation over the short term period of the experiment. The rate in the various controls (0.6-0.7 µm/min), using the present high doses, was closely similar to the control rate using the more moderate doses employed in our earlier experiments (Hand and Tamm, 1972).

Inhibitors of synthesis of DNA precursors had a marked effect on the rate of chain propagation. Hydroxyurea, a compound that inhibits ribonucleotide reductase (Adams and Lindsay, 1967; Young et al., 1967), caused an immediate alteration in the pattern of DNA replication compatible with a marked slowing of chain growth. We could not use a direct method to measure rate of chain growth with FdUrd. The block induced by this inhibitor at the level of thymidylate synthetase is circumvented by exogenous dThd (reviewed by Heidelberger, 1965). [3H]Cyd was used as a label, and although it produces readable autoradiograms, the labeling density is lighter than with dThd and changes in grain density cannot be interpreted with confidence. The results of our experiments indicate that FdUrd slows the rate of chain growth with FdUrd. The block induced by this inhibitor at the level of thymidylate synthetase is circumvented by exogenous dThd (reviewed by Heidelberger, 1965). [3H]Cyd was used as a label, and although it produces readable autoradiograms, the labeling density is lighter than with dThd and changes in grain density cannot be interpreted with confidence. The results of our experiments indicate that FdUrd slows the rate of chain growth, but they do not permit quantitation of the effect. The inhibition of chain growth by FdUrd in this system occurs 15-23 min after addition of the compound. This may be taken as an estimate of the time necessary for the cells to clear their endogenous thymidine pools and it agrees well with estimates by alternative methods (Holford, 1965 as quoted in Cleaver, 1967).

Both puromycin and cycloheximide inhibit the rate of DNA chain elongation. The inhibitory effect of puromycin is not evident until 60 min and the degree of inhibition of dThd incorporation is always greater than the reduction in rate of chain growth (Hand and Tamm, 1972).

Cycloheximide causes a rapid decrease in rate of chain growth that parallels the reduction in dThd incorporation. Weintraub and Holtzer (1972) and Gauschi and Kern (1973) measured rate of DNA chain growth during cycloheximide treatment using methods based on the shift in density of nascent DNA strands labeled with bromodeoxyuridine. Their results are very similar to ours, with a 50% reduction of chain growth occurring almost immediately after introduction of the compound and good correspondence between the reduction in rate of chain growth and dThd incorporation.

Fujinawa (1972) has claimed that cycloheximide has no effect on chain growth. He has based this on size measurements of DNA in alkaline sucrose gradients after labeling for 3 h with [3H]dThd. However, since the size range of replication sections is 7-30 µm (Huberman and Riggs, 1968), most of the replication sections in cycloheximide-treated cells would be able to complete replication within 3 h even with a 50% reduction in rate of chain propagation. Alkaline sucrose sedimentation analysis of pulse-labeled DNA in mammalian cells cannot give quantitative information of rate of DNA chain elongation. By proper utilization of pulse-chase techniques it has been determined by such sedimentation analyses that both puromycin and cycloheximide allow nascent DNA chains to reach a size of 100-200 million daltons (Ensminger and Tamm, 1970). The size of labeled DNA in alkaline sucrose gradients reflects not only chain growth on individual replication sections, but also fusion of the newly replicated sections with adjacent sections that may have been replicated before the pulse or during the chase period. Quantitative determinations of rate of chain propagation require the use of autoradiographic techniques (Huberman and Riggs, 1968; Hand and Tamm, 1972) or isopycnic ultracentrifugation of density substituted DNA (Taylor, 1968; Painter and Schaeffer, 1969; Lehmann and Ormerod, 1970).

The kinetics of inhibition of DNA chain propagation by puromycin and cycloheximide clearly differ (cf. Fig. 4). The rapid onset of the cycloheximide effect might be due to direct action on DNA replication. This has been suggested (Gale et al., 1972), however there is also evidence that cycloheximide does not inhibit DNA synthesis except through its effect on protein synthesis (Weintraub and Holtzer, 1972). The modes of action of puromycin and cycloheximide on ribosomal protein synthesis differ and perhaps this

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results in a differential effect on the synthesis of the proteins required in DNA chain propagation.

These two compounds, at the concentrations used, also differ in their effects on \(^{3}H\)dThd incorporation into DNA (cf. Table II), and this difference cannot be explained solely on the basis of the more rapid inhibition of chain growth by cycloheximide. It has been proposed that they also inhibit DNA chain initiation (Ensminger and Tamm, 1970; Hand and Tamm, 1972; Fujiwara, 1972). Further work is needed to clarify the effects of puromycin and cycloheximide on DNA chain initiation in the replication of chromosomal DNA.

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