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DNA Chain Growth during Replication of Asynchronous L1210 Cells. Alkaline Sedimentation Studies†

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ABSTRACT: The growth of replicating DNA chains was studied in log-phase L1210 cells by an alkaline sucrose gradient sedimentation method which avoids shearing or degradation of the DNA. Three distinct phases of chain growth were identified: (1) rapid joining of short (<4 S) segments to form intermediate size (approximately 25 S) segments; (2) continuous growth from the 25S stage to 50S–70S pieces which may represent completed replicons; and (3) the discontinuous joining of completed replicons to form larger strands that sediment in an aggregate. The first phase was completed within 2–3 min, the second extended over about 15 min, and the third required about 2 hr.

The sequence of chain growth in the replication of nuclear DNA in eukaryotic cells appears to have two points of discontinuity. The first, revealed by alkaline sucrose gradient sedimentation after short pulse labeling (Schandl and Taylor, 1969; 1971; Sato et al., 1970; Nuzzo et al., 1970; Goldstein and Rutman, 1973), may be analogous to the joining of "Okazaki fragments" in bacteria and viruses (Okazaki et al., 1968). This step may involve the replication of all or only part of the DNA in eukaryotic cells. The second, revealed by autoradiography, occurs much later in the course of replication and involves the joining of completed replicons (Huberman and Riggs, 1968; Amaldi et al., 1972; Huberman and Tsai, 1973).

In order to examine the effects of physiological perturbations and drugs on replication (Hori and Lark, 1973; Gautschi et al., 1973), it is desirable to be able to observe the kinetics of chain growth in a quantitative way. With this in mind, we have used alkaline sedimentation to study the sequence of chain growth events from the joining of short strands to the joining of replicons. The procedure used in this study has avoided shear or degradation of the DNA and provides a clearer view of the chain growth sequence than has previously been reported.

Materials and Methods

Cells. L1210 cells (Moore et al., 1966) were grown in suspension culture in RPMI 1630 medium with 20% heated fetal calf serum, plus penicillin and streptomycin. Stock cultures were maintained in static bottles without antibiotics and were used to initiate suspension cultures at weekly intervals. Cultures were periodically tested for PPLO and were free of contamination. Suspension cultures proliferated logarithmically up to $1.5–2.5 \times 10^6$/ml with a doubling time of 12–14 hr; cells harvested for the experiments were at a density of approximately 1.0×10^6/ml.

Alkaline Sedimentation. Cells labeled with [2-^{14}C]thymidine or [*methyl*-^{3}H]thymidine were diluted with 20 volumes of ice-cold PBS (phosphate-buffered saline), centrifuged at 1000 rpm for 5 min at 4°, and resuspended in 0.5–1 ml of cold PBS. Between 0.5 and 1.0×10^6 cells were slowly pipetted onto a 1.0–1.5 ml layer of 0.45 *M* NaOH, 0.55 *M* NaCl, and 0.01 *M* EDTA overlying 30–33 ml of a 5–20% sucrose gradient in 0.1 *M* NaOH, 0.9 *M* NaCl, and

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0.01 *M* Na_2EDTA. In some experiments, Sarkosyl was present in the lysis layer and in the gradient, as indicated in the figure legends. A 5-ml cushion of 60% sucrose was present underneath the gradient. The entire procedure of cell layering, lysis, and centrifugation (unless otherwise stated) was carried out at 2–4° in the dark. After layering the cells onto the precooled solutions, the tubes were allowed to stand for 4–5 hr before centrifugation. Centrifugation was usually for 18 hr at 4° in a Beckman SW27 rotor. The speed of the centrifuge is indicated in each experiment and was selected to produce the desired degree of sedimentation in 18 hr. Gradients were calibrated with ^{14}C-labeled T7 DNA and R1-cleaved linear SV40 DNA (gift of Dr. A. G. Kasselberg). The assumed sedimentation constants were 37 S for T7 DNA and 16 S for SV40 DNA single strands. The apparent sedimentation constant scales shown in the figures are only approximate values. Gradients were collected from the top in 1.2-ml fractions. In short labeling experiments, the fractions were precipitated with 12% trichloroacetic acid in 50% ethanol and filtered on glass fiber filters (Gelman Type A). DNA on the filters was solubilized by heating with 0.4 ml of 1 *N* HCl at 70° for 1 hr, followed by 2.5 ml of 0.4 *N* NaOH at room temperature for 1 hr. Radioactivity was counted after addition of 10 ml of Aquasol (New England Nuclear).

For estimation of the size of DNA chains from the short pulse label experiments (30 sec at 26°), 5–25% constant-velocity alkaline sucrose gradients were utilized (Noll, 1967; Mego, 1970). These gradients were centrifuged in a Beckman SW40 Ti rotor, for 16.5 hr, 35,500 rpm, 20°. The gradients were fractionated from the top into 0.5-ml fractions and precipitated with 10% Cl_3CCOOH, and the DNA was collected on Millipore HAWP filters. Filters were dried at 70° and counted with 5-ml Econofluor (New England Nuclear).

For isopycnic centrifugation of pulse-labeled DNA, the top six fractions from an experiment such as that shown in Figure 3a were collected. The material was dialyzed against 0.1 *M* $NaHCO_3$, 0.05 *M* EDTA, and 0.5 *M* sodium trichloroacetate (pH 10.0); 6 g of solution was then mixed with 7.25 g of CsCl (Harshaw Chemical Co.), and ^{14}C-labeled heat-denatured L1210 DNA was added as a single-strand DNA density marker. After centrifugation in a Beckman 65 rotor for 48 hr at 40,000 rpm, 25°, the gradients were fractionated into 5-drop fractions which were precipitated and counted as described above.

Results

Because of the complexity of the results that have been reported for various methods of sedimenting DNA from mammalian cells lysed on alkaline sucrose gradients, the method selected for the current work was examined in some detail. A major concern is the interpretation of the rapidly sedimenting peak of intact, fully labeled DNA. This peak has been reported to contain lipid and is thought to be an aggregate or "complex" (Elkind and Chang-Liu, 1972). The DNA in this peak is extremely sensitive to X-irradiation, indicating that very few single-strand breaks are required to release DNA from the aggregate; the released DNA sediments as expected for free single strands and has a molecular weight of the order of $2–4 \times 10^8$ (Ormerod and Stevens, 1971; Lehmann and Ormerod, 1972). These observations suggested that only essentially intact DNA strands sediment with the aggregate, and that strands that have not completed replication would probably not sediment with the

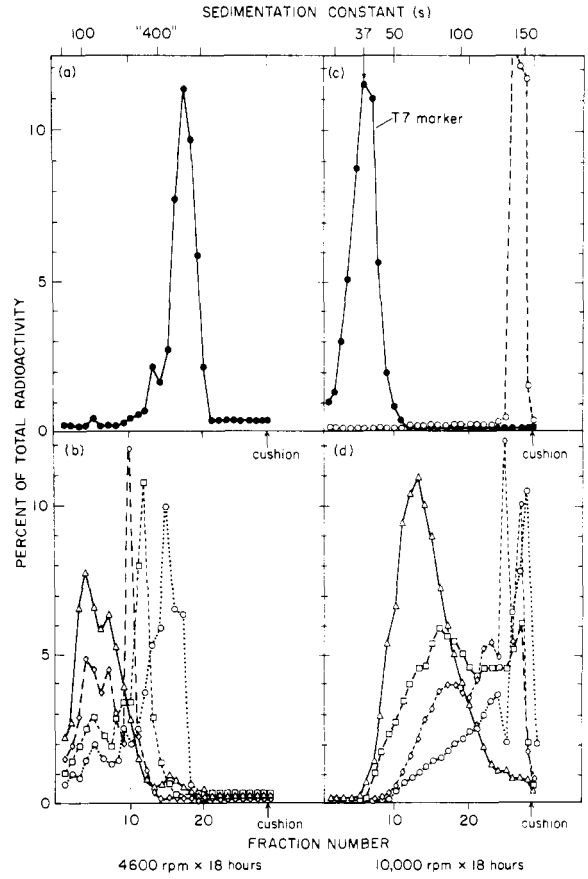

FIGURE 1: Sedimentation of fully labeled DNA from cells lysed on alkaline gradients, showing the effects of room-temperature incubation of the alkaline lysate (b) and of exposure of the lysate to light (d). Cells were labeled for 20 hr with [2-^{14}C]thymidine (0.01 μCi/ml, 10 Ci/mol). Centrifugation in SW27 rotor at 4° for 18 hr at 4600 rpm (a and b) or 10,000 rpm (c and d). (a and c) Cells lysed at 2° in the dark. (Sedimentation of a T7 DNA marker in a separate gradient is included in (c).) Total radioactivity (cpm): a, 4889; c, 7079. (b) Alkaline lysate incubated at room temperature (23°) for (○ . . . ○) 30 min (total radioactivity 5219 cpm); (□- - -□) 1 hr (4918 cpm); (◇—◇) 2.5 hr (3561 cpm); or (△—△) 5 hr (2685 cpm). (d) Alkaline lysate exposed to room fluorescent light at 2° for (○ . . . ○) 45 min (8261 cpm); (◇- - -◇) 2.4 hr (11,991 cpm) (□—□) 4.5 hr (13,488 cpm); or (△—△) 24 hr (14,800 cpm).

aggregate. Therefore, sedimentation conditions for the current work were selected so as to retain the aggregate, and to avoid procedures such as shear (Goldstein and Rutman, 1973), alkaline lysis at room temperature (Elkind, 1971; Lett et al., 1970; Gautschi and Kern, 1973; Gautschi et al., 1973), or ionizing radiation (Lehmann and Ormerod, 1972) which have been used to free the DNA.

The sedimentation of the aggregate peak from cells labeled with [^{14}C]thymidine for 20 hr is shown in Figure 1a. The peak appears to sediment at about 450 S and is relatively sharp. Although this peak was unchanged when cell lysates were let stand for 24 hr at 2°, exposure of the alkaline lysates to room temperature released what appears to be freely sedimenting DNA (Elkind, 1971) and reduced the sedimentation of the sharp peak (Figure 1b). DNA was also released from the aggregate by exposure of the alkaline lysates to visible light at 2° (Figure 1d). These findings agree with the previously reported properties of the aggregate DNA peak (Elkind, 1971).

We selected a long sedimentation time in our experiments so as to reduce the likelihood of trapping of uncompleted DNA strands in the aggregate material. The possi-

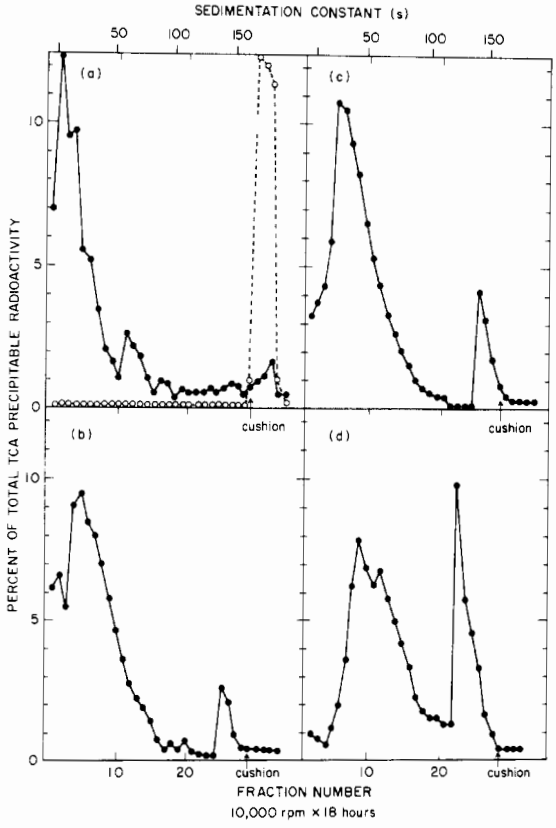

FIGURE 2: Alkaline sedimentation of DNA labeled for (a) 0.5 min, (b) 1 min, (c) 3 min, or (d) 20 min. Cells were labeled at 37° with 5 μCi/ml (a and b) or 2 μCi/ml (c and d) of [^{3}H]thymidine (20 Ci/mmol), and lysed on alkaline sucrose gradients at 2°. Total ^{3}H cpm in each gradient: a, 1370; b, 1770; c, 1995; d, 16,100. Centrifugation at 10,000 rpm, 18 hr, 4°. Fractions were subjected to Cl_3CCOOH precipitation.

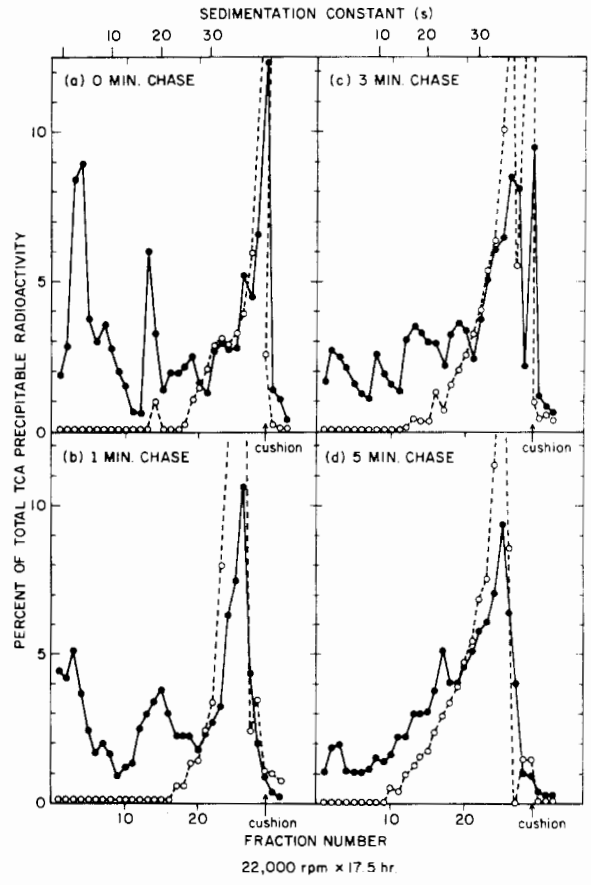

FIGURE 3: Early stages of replication at 26°. Cells were labeled with [^{3}H]thymidine (10 μCi/ml, 55 Ci/mmol) for 0.5 min and then chased with 10^{-5} *M* unlabeled thymidine for 1, 3, or 5 min. (●—●) ^{3}H label; (○- - -○) ^{14}C from cells prelabeled for 20 hr as in Figure 1. Cells were lysed in a test tube by mixing with alkaline lysis solution plus 1% Sarkosyl at 23° in the dark. The lysates were then layered on alkaline sucrose gradients containing 0.015% Sarkosyl. Centrifugation at 22,000 rpm, 18 hr, 4°. Total radioactivity (cpm): (a) ^{3}H, 1082, ^{14}C, 11,121; (b) ^{3}H, 1738, ^{14}C, 7073; (c) 2585, ^{14}C, 10,461; (d) ^{3}H, 2984, ^{14}C, 8791.

bility of artefactual trapping of DNA in the aggregate was tested by examining the effects of a large excess of unlabeled cells on DNA sedimentation. It was found that 10^6 cells did not influence the sedimentation of closed circular SV40 DNA (53 S) nor of linear R1-cleaved SV40 DNA (16 S), and there was no significant trapping of newly synthesized DNA in the bottom peak when the labeling time was sufficiently short (Figure 2a). In a further test, it was found that the sedimentation distribution of the 50S peak in an experiment similar to that of Figure 7a (except using only 10^5 labeled cells) was not changed by mixing the labeled cells with 10^6 unlabeled cells. These findings argue against trapping as a significant factor in the results to be described.

The sedimentation distributions of labeled DNA from cells labeled for 0.5–20 min with [^{3}H]thymidine are shown in Figure 2. The cells were lysed on alkaline sucrose gradients at 2° in the dark, as described under Materials and Methods. Under these conditions of lysis and centrifugation, all of the "old" DNA (20 hr prelabel with [2-^{14}C]thymidine) sediments to the bottom (Figure 2a, open circles). DNA labeled for 0.5 min with [^{3}H]thymidine (Figure 2a, closed circles) sediments in a distribution near the top of the gradient, and very little ^{3}H radioactivity is found with the peak of old DNA at the bottom or in DNA sedimenting faster than about 40 S. With increasing time of labeling with [^{3}H]thymidine (Figure 2b–d: 1, 3, and 20-min labeling), the sedimentation peak gradually increased from about 25 S to about 50 S, and the amount of labeled DNA found at the bottom of the gradient increased progressively.

The sequence of events from the shortest practicable labeling times to the formation of DNA sedimenting entirely to the bottom with the aggregate peak was examined in a series of pulse-chase experiments. The earliest events of chain growth are shown in Figure 3. In this experiment the labeling of cells was conducted at 26° instead of 37° to slow the replication events, and the speed of centrifugation was increased to 22,000 rpm in order to resolve the slow-sedimenting components.

Cells were labeled for 0.5 min at 26° with [^{3}H]TdR (Figure 3a) and chased at 26° with nonradioactive TdR for 1 min (b), 3 min (c), and 5 min (d). Immediately after the 0.5-min pulse (Figure 3a), labeled DNA sediments in three major components: slow sedimenting (<4 S), fast sedimenting (>42 S), and a sharp peak at about 15 S. This pattern, was consistently reproducible at 26°, except for the 15S peak which varied in size, shape, and position. Radioactivity in the top peak and in the sharp 15S peak declined within 3 min of chase either at 26 or 37° and was replaced by increasing radioactivity in the 30S–45S region of the gradient.

In similar experiments at 37°, 0.5-min labeling produced patterns resembling Figure 3b. The top peak was visible but not as large as that produced by 0.5-min labeling at 26°,

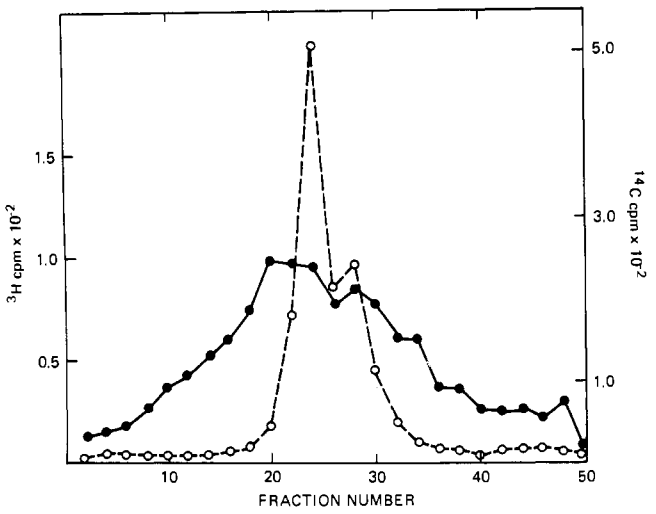

FIGURE 4: Isopycnic centrifugation of small pulse-labeled DNA. Cells were labeled for 0.5 min at 26° with 100 μCi/ml of[^{3}H]thymidine. The cells were then lysed and centrifuged through an alkaline sucrose gradient as in Figure 3. The top six fractions of the gradient were combined, dialyzed, and centrifuged in a CsCl solution as described under Materials and Methods. (●—●) ^{3}H; (O- - -O) ^{14}C-labeled heat-denatured L1210 DNA added as density marker. Centrifugation was at 40,000 rpm for 48 hr in a Beckman 65 rotor at 25°.

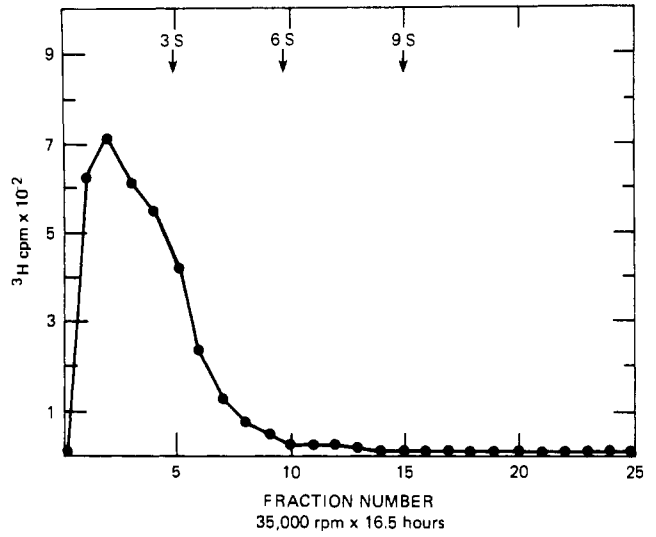

FIGURE 5: High-speed sedimentation of small pulse-labeled DNA. A portion of the material collected from the top of an alkaline sucrose gradient and used for CsCl centrifugation in Figure 4 was centrifuged in a "constant velocity" alkaline gradient as described under Materials and Methods. Centrifugation was at 35,500 rpm for 16.5 hr ($\omega^2 t$ = 78872 × 10^{7} rad^{2}/sec) in a Beckman SW40 Ti rotor at 20°.

and most of the radioactivity by this time already sedimented to near the bottom of the gradient. The central part of the gradient was occupied by a broad sedimentation distribution which became skewed toward the rapid sedimenting side within a few minutes of chase. The top peak disappeared within 1 min of chase. The sharp 15S peak (Figure 3a) was never seen in experiments at 37°, and it is not clear whether this peak is a characteristic of short labeling times or of low temperature.

In order to characterize the top-peak material, the top six fractions of a sedimentation run similar to that shown in Figure 3a were pooled, dialyzed, and centrifuged as described in Materials and Methods. On isopycnic centrifugation in CsCl (Figure 4), the material distributed in a broad

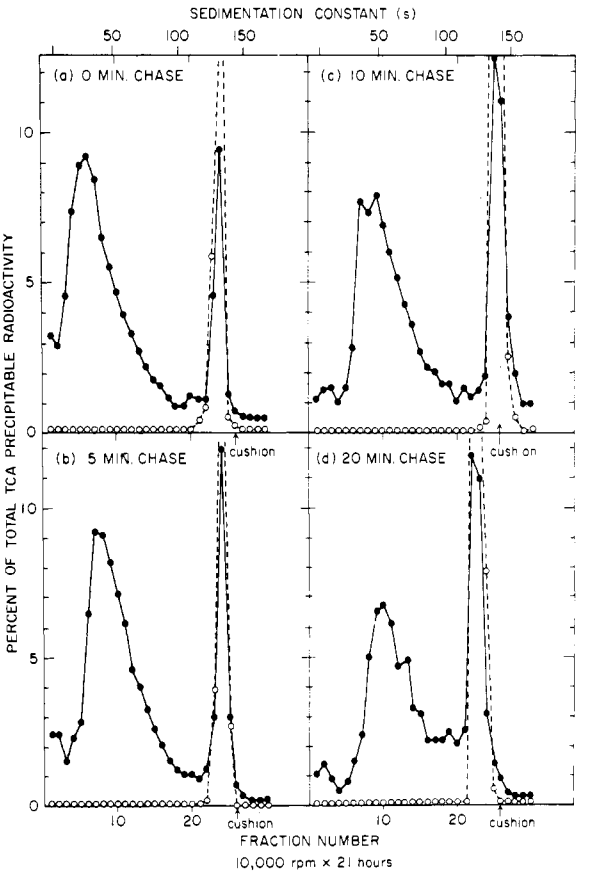

FIGURE 6: Intermediate stages of replication at 37°. Cells were labeled for 2 min at 37° with [^{3}H]thymidine (1 μCi/ml, 27 Ci/mmol) and then chased for the indicated times by addition of 10^{-5} *M* thymidine. The cells were also prelabeled with [2-^{14}C]thymidine (0.01 μCi/ml, 10 mCi/mmol) for 20 hr. (●- - -●) ^{3}H; (O- - -O) ^{14}C. Cells were lysed on the gradients at 2°. Centrifugation at 10,000 rpm, 21 hr, 4°. Total radioactivity (cpm): (a) ^{3}H, 2634, ^{14}C, 2973; (b) ^{3}H, 5883, ^{14}C, 3187; (c) ^{3}H, 5580, ^{14}C, 2804; (d) ^{3}H, 5375, ^{14}C, 1934.

band which centered at the buoyant density of the single-strand DNA marker. The position and broadness of the band indicates that the top-peak material consists of very short DNA single strands. In velocity sedimentation (Figure 5), most of the material sedimented at less than 3 S, and there was essentially none sedimenting faster than 4 S.

The intermediate sequence of events is shown in a pulse-chase experiment at 37° in Figure 6. Cells were labeled for 2 min with [^{3}H]TdR (Figure 6a) and then chased for 5 min (Figure 6b), 10 min (Figure 6c), and 20 min (Figure 6d) with nonradioactive thymidine. The sedimentation of DNA immediately after the 2-min pulse was a broad distribution with a peak at 25 S. After 20 min of chase, the peak was 50 S. After intermediate chase times, the position of the sedimentation peak was at intermediate *s* values. The growth of DNA strands from the 25S to the 50S modal size distributions appeared to be gradual, and there was no evidence of a discontinuous step in this part of the replication process.

Later stages of replication are shown in Figure 7. Cells were labeled for 20 min at 37° with [^{14}C]TdR, spun down at 37°, and resuspended in conditioned medium without radioactivity or additional TdR. The sedimentation profiles are shown in Figure 7 for samples taken at chase times from 6 min to 5 hr. Shortly after the 20-min labeling period, most of the DNA sedimented in a distribution that peaked at about 50 S. With increasing time of chase, this material gradually moved into the bottom peak. In the intermediate

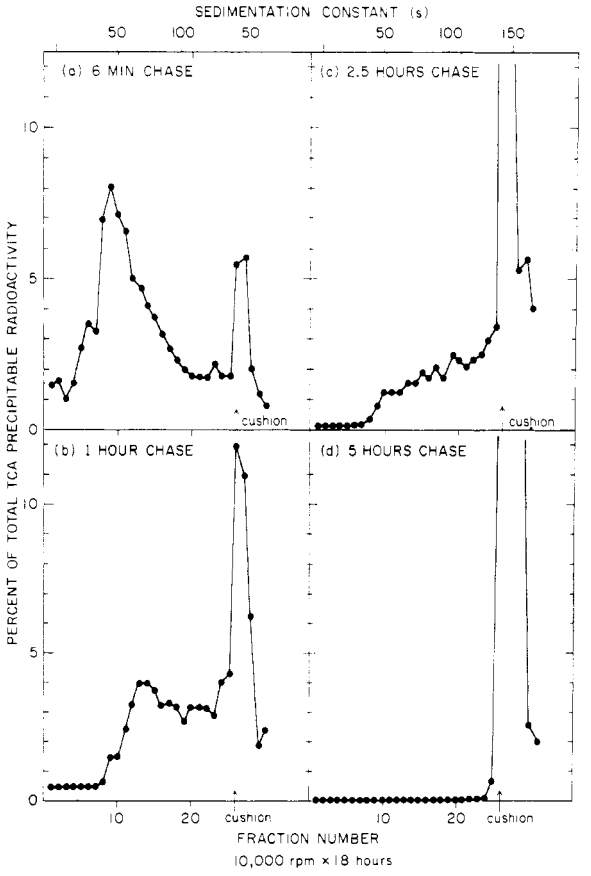

FIGURE 7: Late stages of replication at 37°. Cells were labeled for 20 min with [2-^{14}C]thymidine (0.1 μCi/ml, 10 mCi/mmol) and were then centrifuged and resuspended in conditioned medium without added thymidine. Chase time is indicated from the time of spinning down the cells. Cells were lysed on alkaline gradients at 2°. Centrifugation at 10,000 rpm, 18 hr, 4°. Total radioactivity (cpm): (a) 3314; (b) 2608; (c) 1901; (d) 2872.

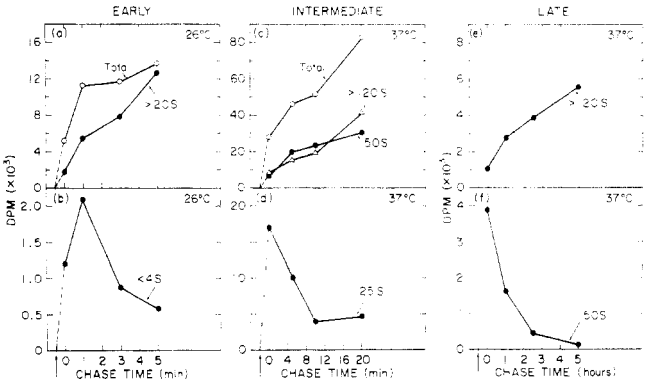

FIGURE 8: Kinetics of labeling of various DNA size classes in pulse-chase experiments. The data are from the experiments presented in Figures 3, 6, and 7.

stages of this process, the peak near 50 S declined as the bottom peak grew. Between the two peaks, there was only a broad distribution of material without defined peaks. The material near 50 S thus converts relatively quickly into bottom-peak material without passing through well-defined stages of intermediate sedimentation. A similar sequence was observed in HeLa cells.

The kinetics of appearance and disappearance of label in the various DNA size classes in pulse-chase experiments are summarized in Figure 8. During the early phase, label rapidly leaves the top peak and accumulates in the lower half of the gradient (>20 S). In the intermediate time scale, radioactivity declines in the 25S region and accumulates both in the 50S region and in the bottom peak (>120 S). At late times, radioactivity is transferred from the 50S region to the bottom peak.

Discussion

In the present study, we have examined the growth of DNA single strands during the normal replication of logarithmically growing L1210 cells. The replication sequence was not perturbed in this study by any synchronization procedure and the DNA was not subjected to shearing. In order to minimize DNA breakage, cells were lysed on alkaline sucrose gradients at 2° in the dark. The chain growth sequence was examined from the earliest observable size distribution to the formation of strands long enough to sediment with the DNA aggregate peak.

Most of the initially synthesized DNA was found in the form of short strands that sedimented at less than 4 S in alkaline sucrose gradients. This is in agreement with the findings of Nuzzo et et al. (1970), Huberman and Horwitz (1973), and Gautschi and Clarkson (1975). Others have found somewhat larger sedimentation values for this peak (Schandl and Taylor, 1969, 1971; Goldstein and Rutman, 1973).

The possibility that some of the synthesis occurs directly at the ends of much longer strands is difficult to exclude. Our minimum labeling conditions of 0.5 min at 26° in fact showed about 40% of the label already in DNA sedimenting faster than 30 S. This could be due either to synthesis directly at the ends of long strands or to a sufficiently rapid rate of joining the short strands.

The formation of rapidly sedimenting DNA after short pulse labels was also observed by Gautschi and Clarkson (1975). This material was no longer apparent after deproteinization and gel electrophoresis of single-stranded DNA, leading these authors to conclude that the rapidly sedimenting peak is artefactual and hence that nearly all of the synthesis occurs initially in short segments. An alternative, however, would be that the larger strands were lost during preparation of the DNA for electrophoresis.

These findings are all consistent with DNA replication models that envisage multiple initiations behind rapidly unwinding template strands. A physiological need for multiple initiations on both strands could arise from a limit in the rate of polymerization at chain ends. Multiple initiations of short strands would overcome this limit and could make the unwinding process the rate-limiting step.

The intermediate stages of chain growth in our experiments revealed sedimentation distributions with modal values ranging from 25 S after 2 min of labeling to 50 S after 20 min. These values agree with the findings in Ehrlich ascites cells by Goldstein and Rutman (1973), but we do not confirm their conclusion that the conversion from the smaller to the larger values is discontinuous. Instead, we find peaks at intermediate positions, indicating a gradual increase in size of these strands, in agreement with Huberman and Horwitz (1973).

The interpretation of sedimentation distributions of pulse labeled DNA after shearing must take into account end-labeling effects, as has been repeatedly warned by Lehmann and Ormerod (1969, 1970, 1974). Previous studies of the intermediate stages of chain growth have employed shear, alkali, or X-ray to break DNA sufficiently to eliminate the aggregate peak. In our experiments, however, no DNA

breaking procedure was used. That our methodology did not significantly break the DNA was shown by exhibiting normally sedimenting DNA distributions with modal values much greater than 50 S, thereby demonstrating that the methodology did not limit DNA size to 50 S. Such sedimentation peaks in excess of 50 S were observed after low extents of DNA breakage by visible light and alkali (Figure 1). These observations also show that DNA strands much larger than 50 S are readily released from the aggregate peak.

The possible selective trapping of long DNA strands in the aggregate material is an important question since this could artifactually distort the shapes of sedimentation distributions. Arguing against this possibility are our findings that purified DNA does not become trapped in the aggregate and that addition of a large excess of unlabeled cells does not distort the sedimentation of newly synthesized DNA in cells.

In the late states of chain growth (Figure 7), we find that the sedimentation distribution becomes low and diffuse as it enters the aggregate peak. This behavior is analogous to what was seen during the early states of replication, and may have a similar basis. Whereas during early replication, short segments were joining into intermediate lengths, during late replication completed replicons are joining to form larger units. The highest modal-sedimentation values achieved before the distributions become diffuse were 50S–70S. This corresponds to 30–60 μm single-strands lengths, and is in good agreement with replicon lengths observed by autoradiography (Huberman and Riggs, 1968).

The latest stage in DNA strand joining that we could observe by alkaline sedimentation was the transfer of replicon-length segments into the rapidly sedimenting aggregate material. This occurred with a half-time of about 0.8 hr. In a previous report, we showed that later stages of the joining sequence can be resolved by the method of alkaline elution (Kohn et al., 1974). By both methods, we found that the strands labeled in 20 min have a modal distribution of about 50 S. By alkaline elution, however, it was possible to examine the behavior of strands of a size of at least 100 S. The half-time for conversion to slow-eluting DNA was 1.4 hr, as compared to 0.8 hr for the half-time of conversion to the rapidly sedimenting aggregate. The formation of rapidly sedimenting aggregate, therefore, occurs at an earlier stage of strand or replicon joining than does the formation of slow-eluting DNA.

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