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Structure of Parental Deoxyribonucleic Acid of Synchronized HeLa Cells†

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ABSTRACT: We have investigated the structure of parental DNA as a function of the cell cycle phase of HeLa cells. DNA was isolated from synchronized HeLa cells 0, 5, 8, and 12 h after release from a second exposure to 2 mM thymidine. These DNA preparations were characterized by $Cs_2SO_4/AgClO_4$ buoyant density, sensitivity to a single-strand specific nuclease, sedimentation in neutral and alkaline sucrose gradients, and sedimentation in neutral sucrose gradients after digestion with S_1 nuclease. The cultures were staged according to cell cycle phase by measurements of DNA content per cell by flow microfluorometry. The cell cycle phases were G_1/S (0-h culture), S (5-h culture), G_2 (8-h culture), and G_1 (12-h culture). There are no nuclease-sensitive sites in G_2. As the cells enter G_1, the number increases, with a maximum being reached in the S phase. The number of breaks in DNA with respect to cell cycle phase follows the same pattern. The amount of single strandedness, measured by buoyant density and nuclease sensitivity, is also minimal in G_2, increases in G_1, with a maximum achieved in the S phase. It appears that there is a chromosomal cycle, reflected as continuous structural changes in the DNA molecule, as cells traverse the cell cycle.

During the life cycle of a cell distinct periods are passed through which can be differentiated on the basis of DNA synthesis, mitosis, and DNA content. Howard and Pelc (1963) defined these periods as M (mitosis), G_1 (period after mitosis and preceding S), S (period of DNA synthesis), and G_2 (period after S and preceding M). The DNA content of a diploid G_1 cell would be 2N, that of a G_2 or mitotic cell would be 4N, and S phase would contain increments between 2N and 4N.

The nuclear DNA attaches to the nuclear membrane during the latter part of the G_1 period (Infante et al., 1976). Other G_1 events that may be preparatory for DNA synthesis are activation of chromatin for RNA synthesis (Teng and Hamilton, 1969), phosphorylation of histone f1 (Gurley et al., 1973), synthesis of non-histone chromosomal proteins (Gerner and Humphrey, 1973), deoxyribonucleotide triphosphate pool sizes (Walters et al., 1973), and activation of DNA (Collins, 1974a).

Biochemical studies of the cell cycle have been severely hampered by the lack of quick reliable means of determining the specific proportions of cells in various phases of the cell cycle. The recent advent of instruments capable of measuring the individual DNA content per cell of large numbers of cells has alleviated this problem (Van Dilla et al., 1969). By measuring the DNA content, cells can be staged according to the cell cycle. Such instruments can be used to monitor the cell-cycle stages of synchronized cells in ongoing experiments by sampling small aliquots of a culture (Crissman and Tobey, 1974). These instruments can also be employed to optimize the scheduling of synchronization protocols, thus maximizing the number of cells in a given period of the cell cycle (Collins, unpublished observations).

We have previously reported that when resting (G_0) cells

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of the iris of the adult newt are stimulated to enter the cell cycle, extensive changes occur in the structure of their DNA (Collins, 1974b). These observations have been confirmed and quantitated with resting human diploid fibroblasts stimulated to proliferate (Collins, 1977). This paper reports observations on the characterization of long-term labeled DNA extracted from synchronized HeLa cells accurately staged according to the G_1, S, G_2 and G_1/S boundary periods of the cell cycle by flow microfluorometry. These data lead us to suggest that in G_2 cells DNA exists as an intact structure with very few breaks and no single-stranded regions. However, as cells enter the G_1 period, a few breaks appear. As cells reach the G_1/S boundary, more breaks, some of which are single-stranded gaps, appear. S-phase DNA contains the greatest number of breaks and single-stranded regions.

Experimental Procedure

Cell Cultures. HeLa S-3 cells (kindly supplied by Dr. Thoru Pederson) 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 Fungizone at a concentration of 2–6 × 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.

Reagents. All tissue culture media and supplies were purchased from Flow Laboratories. [^{14}C]Thymidine (54.3 mCi/mmol) and [^{3}H]thymidine (20 Ci/mmol) were purchased from New England Nuclear. Pancreatic ribonuclease, Pronase, and S_1 nuclease were supplied by Sigma. Ultrapure Cs_2SO_4 was purchased from Accurate Chemical and Supply Corp., and anhydrous silver perchlorate was obtained from ICN Pharmaceuticals. R6K [^{3}H]DNA (a bacterial plasmid DNA) was the kind gift of Dr. Francis Maccina of the Medical College of Virginia, Virginia Commonwealth University.

^{14}C-Labeled Parental DNA and Cell Synchronization. Cells were cultured on media containing 10 Ci/mL of [^{14}C]thymidine for 5 h. The cells were harvested by centrifugation under sterile conditions and resuspended in media without [^{14}C]-thymidine but which was 2 mM unlabeled 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 mM thymidine for 14 h. This procedure yields cells synchronized at the G_1/S boundary, which contain ^{14}C parental DNA. The synchronized cells are released from the metabolic block by resuspension in normal media and allowed to progress through the cell cycle.

Incorporation of [^{3}H]Thymidine. Samples (1 mL) were removed from the cultures and incubated with 10 Ci/mL of [^{3}H]thymidine for 30 min at 37 °C. Cold media were added and the cells collected by centrifugation at 200*g* for 3 min. The cells were resuspended in 3 mL of cold 5% trichloroacetic acid and collected by filtration onto a Whatman 4S 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 filters was determined in a toluene–fluors cocktail 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.

Purification of DNA. DNA was purified essentially according to the procedure of Mamur (1961) as previously described by one of us (Collins, 1974b).

Determination of Radioactivity. DNA which had been applied to filters was counted in toluene/fluors (2,5-diphenyloxazole/1,4-bis[2-(5-phenyloxazolyl)benzene]) in a Beckman liquid scintillation counter (Model LS-335).

Determination of Molecular Weight of DNA. The approximate sedimentation coefficient, *s*, of DNAs in the sucrose gradients was determined by the method of Martin and Ames (1961) using R6K [^{3}H]DNA, which contains 51S and 39S components (Kontoichalov et al., 1970), as a marker. Molecular weights were calculated from the *s* values using the equations of Studier (1965).

Sucrose Gradient Centrifugation. DNA was sedimented through neutral and alkaline 5-mL sucrose gradients as previously described (Collins, 1974b). Recoveries were greater than 95%. The tubes were punctured and 25-drop fractions collected from the bottom onto Whatman no. 3MM filter disks. Carbon-14 was determined by counting the disks.

Isopycnic Centrifugation of DNA in Cs_2SO_4/$AgClO_4$. Purified DNA was dialyzed, mixed with Cs_2SO_4 in the presence of $AgClO_4$, and centrifuged according to the procedure of Jensen and Davidson (1966) as previously described (Hoffman and Collins, 1976). Under these conditions, single-stranded DNA bands have a density of 1.67 g/mL, while intact DNA bands have a density of 1.50 g/mL. [^{3}H]DNA from WI-38 cells in G_1 was included as a marker in each gradient. This DNA has a density of 1.50 g/mL (Collins, 1977).

S_1 Nuclease Digestion of DNA. DNA was treated with S_1 nuclease (Ando, 1966; Godson, 1973; Wiegand et al., 1975) as previously described (Collins, 1977), To ensure maximum activity of the enzyme preparation used, heat-denatured [^{14}C]DNA was digested. The percent digestion of aliquots taken at various times from the reaction mixture was calculated by subtracting the radioactivity of a zero-time sample from the radioactivity of each time point. After 20 min, 88% of the denatured DNA was rendered acid soluble. Further incubation for an additional 40 min did not lead to more digestion. When intact [^{14}C]DNA from WI-38 cells in the G_0 phase of the cell cycle was digested, 3% of the radioactivity was rendered acid soluble, in good agreement with the value reported previously (Collins, 1977). The nuclease was found to be free of double-strand hydrolytic activity, and incapable of hydrolyzing nicks (where hydrolysis of single phosphoester linkages has occurred) as described previously (Collins, 1977).

Cell Cycle Distribution. Approximately 15 mL of the cell suspension (about 4 × 10^6 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 Crissman and Steinkamp (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 instrument utilizes an argon laser rated at 35 mW at the principal band of 488 nm. Cells in suspension flow in coaxial 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 histogram of the counts in each channel (the number of cells) vs. 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 3000 cells/s. Analysis of a log phase culture (data not shown) revealed that the G_1 channel was 33 and the G_2 + M channel was 66. The percent of cells in G_1, S, and G_2 + M was calculated with a

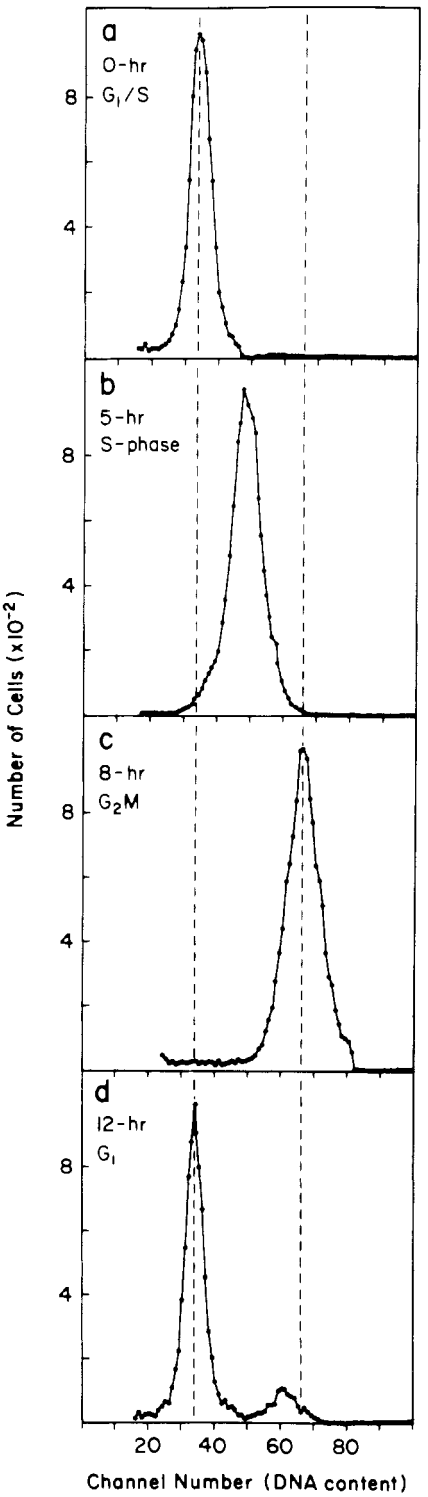

FIGURE 1: Staging of synchronized HeLa cells. Cells at various times after release from a double thymidine block were analyzed for DNA content. The vertical dashed lines indicate the peak channel modes of G_1 cells (channel 33) and G_2 + M cells (channel 66). Hours after release: (a) 0, (b) 5, (c) 8, and (d) 12 h.

computer program kindly donated by Dr. Jerrold Fried (Fried et al., 1976).

Results

Cells were synchronized and released from the second thymidine block. For the experiments reported herein, cells in a single 500-mL spinner flask were used. Aliquots were taken at various times for the determination of [^{3}H]thymidine incorporation and mitotic index. The incorporation of [^{3}H]-thymidine in a 30-min period reached a maximum 2 h after release and was relatively constant up to 5 h after release and declined thereafter (Table I). The number of mitotic cells monitored at 30 min intervals was negligible until 9 h after release, with a peak of 39% mitotic cells occurring at 10 h after release (Table I). These data argue for extremely well-synchronized cells.

TABLE I: Incorporation of [^{3}H]Thymidine and Mitotic Index after Release from Second Thymidine Block.[a]

Hours[b]	cpm × 10^3 [c]	Mitotic index[d]
0	0	0
1	4.1	0
2	12.8	0
3	11.2	0
4	10.8	0
5	12.1	0
6	8.4	0
7	4.2	0.01
8	2.1	0.03
9	0.9	0.11
9.5		0.28
10	0.8	0.39
10.5		0.26
11	0.6	0.16
11.5		0.03
12		0.02

[a] At various times after release, separate aliquots of cells were pulsed with [^{3}H]thymidine for 30 min and stained with acetylcarmine prior to determination of incorporation and mitotic index. [b] Hours after release from block. [c] Incorporation of [^{3}H]thymidine into acid-insoluble DNA of released cells. [d] Number of mitotic cells per nonmitotic cells.

At various times after release, samples were also taken for DNA purification and cell-cycle analysis. The DNa distribution of cells 0, 5, 8 and 12 h after release is presented in Figure 1. The dashed line at channel 33 corresponds to the G_1 DNA content (1 unit), while the dashed line at channel 66 corresponds to the G_2 + M DNA content (2 units). Cells with a DNA content corresponding to the S phase will be distributed between channels 33 and 66. As can be seen, the majority of the blocked cells (0 h) have the DNA content of G_1 cells and hence should be considered to be at, or near, the G_1/S boundary (Figure 1a). Five hours after release, the cells have entered the S phase (Figure 1b). Eight hours after release most cells have the DNA content of G_2 + M cells, but as the percent of cells in mitosis was 3% (Table I) most of these cells are G_2 cells (Figure 1c). Twelve hours after release the cells have passed through mitosis and are back in the G_1 period of the cell cycle (Figure 1d). The ability of these cells to divide argues for good viability.

The computer analysis of these DNA distribution histograms is presented in Table II. The 0-h culture contains 91% cells at or very near the G_1/S boundary. The 5-h culture contains 89% cells in the S phase. The 8-h culture contains 78% cells with a DNA content of G_2 + M, but only 3% mitotic cells; hence, 75% of the cells are in G_2. The 12-h culture contains 88% G_1 cells.

Single-Stranded Regions in DNA from Various Phases of the Cell Cycle. Isopycnic banding of DNA in Cs_2SO_4/$AgClO_4$ causes DNA with single-stranded regions to band at a greater buoyant density than DNA with no single-stranded character (Jensen and Davidson, 1966; Hoffman and Collins, 1976). DNA was purified from 0-, 5-, 8-, and 12-h cultures and

TABLE II: Staging of Synchronized HeLa Cells.[a]

Hour[b]	G_1	S	$G_2 + M$
0	0.91	0.07	0.02
5	0.07	0.89	0.04
8	0.08	0.14	0.78
12	0.88	0.03	0.09

[a] Phases of the cell cycle (G_1, S, and $G_2 + M$) were calculated from the data of Figure 1. All methods are described under Experimental Procedure. [b] Hours after release from the double thymidine block.

TABLE III: Single-Stranded Character of DNA from Various Phases of the Cell Cycle.[a]

Hour[b]	Phase[c]	P^d	S_1 nuclease sensitivity[e] (%)
0	G_1/S	1.535	9
5	S	1.580	16
8	G_2	1.510	4
12	G_1	1.520	6

[a] The amount of DNA hydrolyzed by S_1 nuclease was determined as described under Experimental Procedure. For purposes of comparison, the buoyant densities from Figure 2 are also given. [b] Hours after release from block. [c] Phase of cell cycle (from Figure 1). [d] Buoyant density from $CsSO_4/Ag^+$ (Figure 2). [e] Percent radioactivity removed by S_1 nuclease.

subjected to isopycnic centrifugation (Figure 2). As the cells enter the S phase, the DNA bands at a greater density (compare Figure 2c with 2b). As the cells enter the G_2 period, the density decreases (Figure 2c). After mitosis, when the cells are back in the G_1 period, the density increases again (Figure 2d). In separate experiments, when the ^{14}C-labeled HeLa cells were mixed with ^3H-labeled WI-38 cells in G_0 (density = 1.50 g/mL), and the DNA was extracted and centrifuged in $Cs_2SO_4/AgClO_4$, the resulting isopycnic patterns were those expected of a mixture of DNAs of different densities (data not shown). These mixing experiments are important to rule out such artifacts as differential accumulation of nucleases or lysosomes, or slightly different purification and centrifugation conditions as an explanation of these data.

Sensitivity to Single-Strand Specific Nuclease. The same DNA preparations used for isopycnic centrifugation were subjected to digestion by S_1 nuclease from *Aspergillus oryzae* (Ando, 1966). The results are presented in Table III which, for comparison, also lists the phases of the cell cycle (based on Table II) and the buoyant densities (obtained from Figure 2). There is an excellent correlation between the buoyant densities and the S_1 nuclease sensitivities (Table III). When one of these parameters is graphed relative to the other, a straight line is obtained (data not shown). Subsequent linear-regression analysis yields a correlation coefficient, r, of 0.99 (when $r = 1$ the correlation is said to be exact). These experiments were duplicated with the same results.

Presence of Breaks in DNA. Neutral and alkaline sedimentation studies were performed on the same DNA preparations used above to quantitate the average number ef breaks in the DNA chains. Under the ionic conditions employed in these studies, DNA single chains in alkali should sediment faster than corresponding duplex molecules in neutral gradients [e.g., an intact duplex molecule with a molecular weight of 4.44×10^7 sediments with an s value of 39, whereas the denatured single chain with molecular weight of 2.22×10^7 sediments with an s value of 46 (Studier, 1965)]. Thus, if s

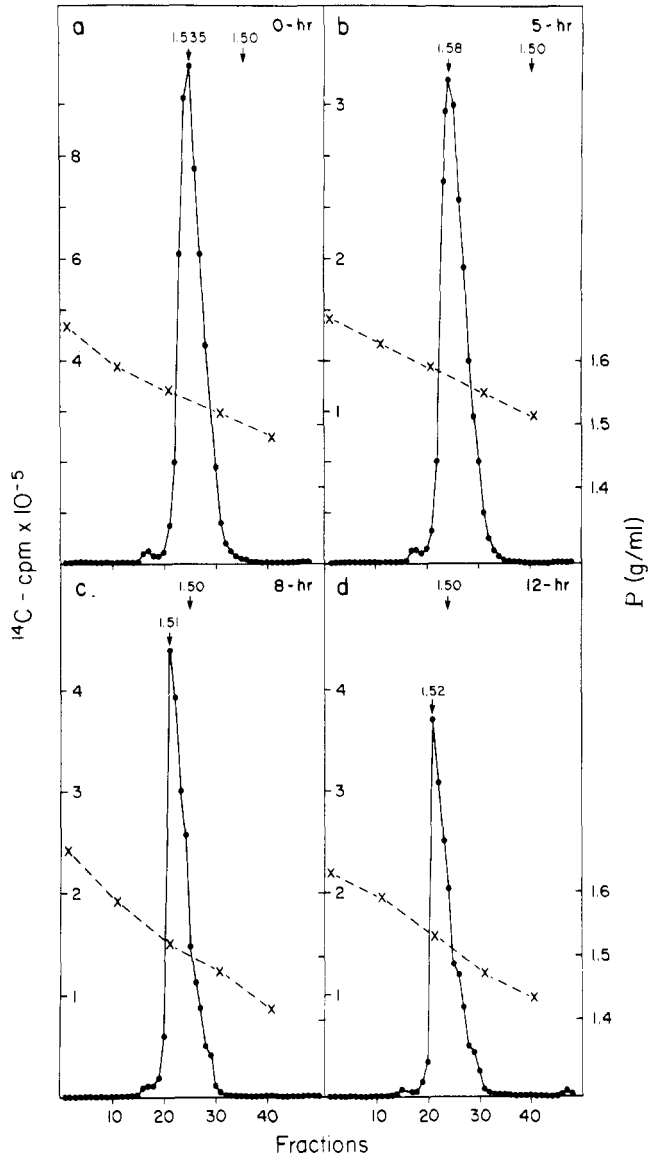

FIGURE 2: $CsSO_4/AgClO_4$ centrifugation of DNA isolated at various times after release. The final volume was 8 mL. The tubes were filled with paraffin oil and centrifuged at 35 000 rpm at 25 °C for 70 h in a 65 rotor with a Spinco Model L3-50. The tubes were punctured and fractions of approximately 0.15 mL were collected onto filter disks. Radioactivity was determined as described under Experimental Procedures. The density of selected fractions was determined from the refractive index measured with a Bausch and Lomb refractometer. The specific activities of the DNA preparations ranged from 14 000 to 40 000 cpm/μg. The arrow at the top of each figure indicates the buoyant density of the peak fraction and of G_0 DNA from WI-38 cells (1.50 g/mL).

values in alkali correspond to molecular weights less than one-half of those in neutral solutions, the presence of breaks is indicated (Collins, 1977). The sedimentation patterns obtained with the same DNA preparations used above are presented in Figure 3. DNA from cells at or near the G_1/S boundary sediments as DNA containing breaks (Figure 3a). S-phase DNA exhibits a profile consistent with even more breaks (Figure 3b). In contrast, G_2 DNA contains very few breaks (Figure 3c). G_1 DNA exhibits more breaks (Figure 3d) than G_2 DNA (Figure 3c).

For ease of presentation, the data are tabulated in Table IV. The DNA preparation from cells at or near the G_1/S boundary contains molecules with an average of 14 breaks per 10^5 bases. This is in good agreement with previous data from WI-38 cells, which contained 15 breaks per 10^5 bases for late G_1 cells pre-

TABLE IV: Breaks in DNA during the Cell Cycle.[a]

DNA		Neutral gradient		Alkaline gradient		Breaks per
Hours[c]	Phase	s	Mr × 10^{-7}	s	Mr × 10^{-7}	10^5 bases[b]
0	G_1/S	21	0.74	21	0.32 (29%)[d]	1.4 (29%)
				15	0.14 (71%)	14.3 (71%)
5	S	30	2.07	30	0.77 (45%)	1.1 (45%)
				12	0.08 (55%)	39.3 (55%)
8	G_2	33	2.73	36	1.21	0.31
12	G_1	39	4.42 (37%)			14.5 (37%)
		24	1.09 (63%)	18	0.21	2.4 (63%)

[a] DNA isolated from cells at various times after release from the double thymidine block was sedimented in neutral and alkaline sucrose gradients to determine the number of breaks as described under Experimental Procedure. The data are taken from Figure 3. [b] Calculated as (number of breaks per strand/number of bases per strand) × 10^5. The number of breaks per strand was calculated as (Mr neutral ÷ 2 ÷ Mr alkaline) − 1. (Mr is the average molecular weight). [c] Hours after release from block. [d] Where two peaks were obtained, the percentage represented by each is given in parentheses.

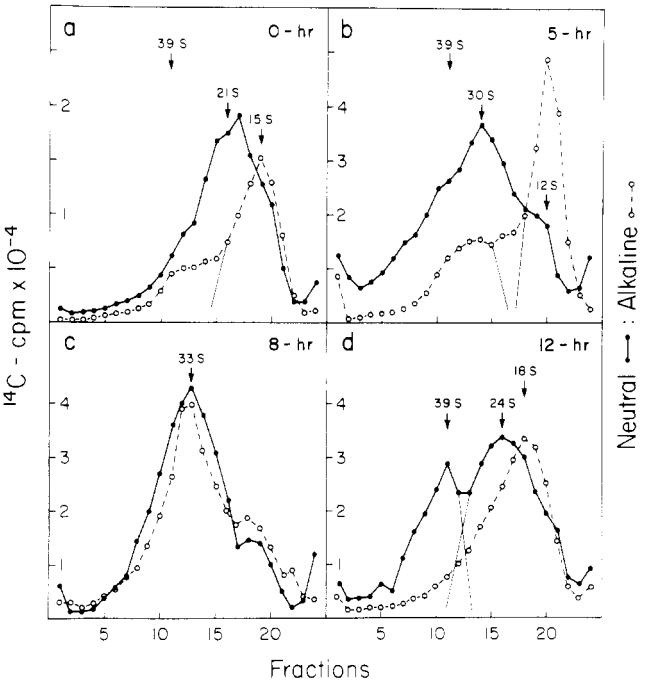

FIGURE 3: Sedimentation of DNA in neutral and alkaline sucrose gradients. Approximately 0.5 mL of [^{14}C]DNA purified from cells harvested at various times after release of the double thymidine block was layered onto 5 to 20% sucrose gradients (5 mL). In the alkaline gradients, the DNA was denatured by the addition of 1 N NaOH before layering. The tubes were centrifuged at 40 000 rpm at 4 °C for 2 h in an SW 50.1 rotor. The gradients were punctured and the radioactivity of the subsequent fractions was determined as described under Experimental Procedure. Neutral gradients (●) and alkaline gradients (○). Approximately 20 μg of DNA was used for each experiment.

paring to enter the S phase (Collins, 1977). Onec the cells have entered the S phase, the number of breaks of half of the molecules has increased to 39 per 10^5 bases (Table IV). Upon entering the G_2 period the number of breaks decreases to a minimum value of 0.3 per 10^5 bases, and after division and entry into the G_1 period the number of breaks increases again (Table IV). These experiments were repeated with essentially the same results. For example, the number of breaks in 8-h (G_2) DNA varied from 0.11 to 0.43 per 10^5 bases, but the pattern of breaks in DNA from 0-, 5-, 8-, and 12-h DNA was always the same as those in Table IV.

It can be seen that the molecular weights of the purified DNAs vary from 0.74 × 10^7 to 4.42 × 10^7 (Table IV). We attribute this to random variations in shear forces during the purification procedures. For example, when 0-h cells were mixed with 12-h cells, all of the isolated DNA had the same molecular weight of 2.73 × 10^7 (data not shown). It should be mentioned that when patterns such as that shown in Figure 3d are obtained, whereby DNA in neutral sucrose exhibits two bands and DNA in alkali exhibits a "single" band, there is no problem in interpretation, as the denatured DNA band must have been contributed by each of the native DNA bands. However, had two bands been exhibited in alkali, there would be no way of knowing which denatured band corresponded to which native band. When this situation occurs, we shear the DNA preparation lightly with a syringe to obtain a single band in native gradients, and sediment the preparation again. In fact, this is why the DNA isolated from 0-h cells has a low molecular weight (Table IV). Before shearing, two bands with molecular weights of 2.73 × 10^7 and 4.42 × 10^7 were obtained with DNA from 0 h cells (data not shown).

S_1 Nuclease Sedimentation Studies. A break in a DNA chain could arise from a nick (single phosphodiester linkage hydrolyzed), a gap (run of missing nucleotides), or a mixture of both. An attempt to distinguish between these two possibilities can be made by digestion of the DNA preparation with S_1 nuclease prior to sedimentation in neutral sucrose gradients (Collins, 1977). The nuclease should hydrolyze only single-stranded regions and not nicks (Collins, 1977).

The results of S_1-nuclease treatment on the sedimentation of the same DNA preparations used above are seen in Figure 4. There are a few S_1 sites in DNA from cells at the G_1/S boundary (Figure 4a). The number of sites increases as the cells enter the S phase (Figure 4b). In contrast, G_2 cells contain DNA with no S_1 sites (Figure 4c). After division, G_1 cells contain DNA with S_1 sites again. For ease of presentation, the data from Figure 4 are tabulated in Table V. DNA from 0-, 5-, 8-, and 12-h cultures contain 6.2, 32.4, 0 and 2.8 S_1 sites per 10^5 base pairs, respectively (Table V). These experiments were repeated with essentially the same results. While the calculated number of S_1 sites varied by 6%, the pattern shown was the same. However, G_2 DNA (8-h DNA) always contained no S_1 sites. It might be noted that two bands are present in the neutral gradient of Figure 2d, whereas only one band (of lower molecular weight) is present in the neutral gradient of Figure 4d, for the same (12-h) DNA. We attribute this to the result of shear forces during the lengthy dialysis procedures used prior to S_1-nuclease treatment (Collins, 1977).

Number of Nicks in DNA Chains. If one assumes that the average number of nicks is equal to the average number of breaks minus the number of S_1 sites, it can be calculated that 0- and 12-h DNAs contain an average of 7.5 and 5.5 nicks per 10^5 bases, respectively, with 8-h DNA containing 0.31 nicks per 10^5 bases. The calculated value for 5-h DNA is 8.3; how-

TABLE V: S_1 Nuclease Sensitive Sites in DNA during the Cell Cycle.[a]

DNA		Control		Digested		Sites per 10^5
Hours[c]	Phase	s	Mr $\times 10^{-7}$	s	Mr $\times 10^{-7}$	base pairs[b]
0	G_1/S	26	1.37	20	0.60	6.2
5	S	33	2.73	13	0.19	32.4
8	G_2	33	2.73	33	2.73	0
12	G_1	30	2.07	24	1.13	2.8

[a] DNA isolated from various phases of the cell cycle was digested with S_1 nuclease and sedimented in neutral sucrose gradients as described under Experimental Procedure. The data are taken from Figure 4. [b] Calculated as (number of S_1 sites per duplex/number of bases per duplex) $\times 10^5$. The number of S_1 sites was calculated as (Mr control/Mr digested) $-$ 1. Mr is the average molecular weight). [c] Hours after release from block.

ever, the presence of single-stranded regions in replication bubbles (Blumenthal et al., 1973) probably indicates that this is a meaningless value regarding S-phase DNA.

Discussion

The inhibitory effect of excess thymidine on DNA synthesis was first reported by Xeros (1962). Numerous others have utilized this technique to synchronize cells (for reviews, see Bootsma et al., 1964; Galavazi et al., 1966; Pedersen and Anderson, 1964). It can be argued that inhibited cells should not be used for biochemical studies. However, we know of no other method which will afford the large numbers of cells needed for biochemical analysis. Mitotic detachment (Terasima and Tolmach, 1963) is a gentle procedure, but it can be calculated that over 900 75-cm^2 culture flasks would be needed in order to perform monolayer culture to obtain the numbers of synchronized cells used in this study. Regarding the effect of excess thymidine on HeLa cells, several investigators have reported that the cells appear to penetrate into the S phase (Rueckert and Mueller, 1960; Galavazi and Bootsma, 1966; Studzinski and Lambert, 1969). This does not appear to be the case under our conditions, as the blocked cells clearly have a G_1 DNA content (Figure 1a) and the uptake of [^{3}H]thymidine was low over the first 1-h period (Table I). However, some penetration into the S phase cannot be ruled out. Cells damaged by a metabolic block at early S phase will not enter S phase in large numbers (Tobey and Crissman, 1975). Figure 1 clearly shows that over 93% of the HeLa cells traversed the cell cycle when the block was removed. It has been reported that cells enter mitosis sooner than expected when excess thymidine is removed (Bootsma, et al., 1964). In our experiments, the peak of mitosis occurred at 10 h, exactly as expected (T_S = 6 h, T_{G_2} = 2 h, and T_M = 1 h) of cells near the G_1/S boundary. Furthermore, the mitotic index of 0.39 for 0.5-h intervals reflects a remarkable degree of retention of synchrony 10 h following release of the metabolic block.

In our previous study, whereby resting WI-38 cells were stimulated to proliferate, the actual number of cells entering the S phase (for example) was not known (Collins, 1977). It should be stressed that the recent availability of machines for the rapid analysis of the individual fluorescence of large numbers of cells now permits a precision of cell-cycle analysis not heretofore possible. Thus, as reported herein, small samples of a culture can be used to monitor ongoing experiments and quickly and reliably stage the cell cycle. This adds a powerful new dimension to the study of the biochemistry of the cell cycle.

The observations reported herein reflect the structure of parental DNA chains, since the DNA was labeled and subjected to a 37-h chase in the absence of radioactive thymidine before the cells were released from the synchronization block. The various mixing experiments tend to rule out the

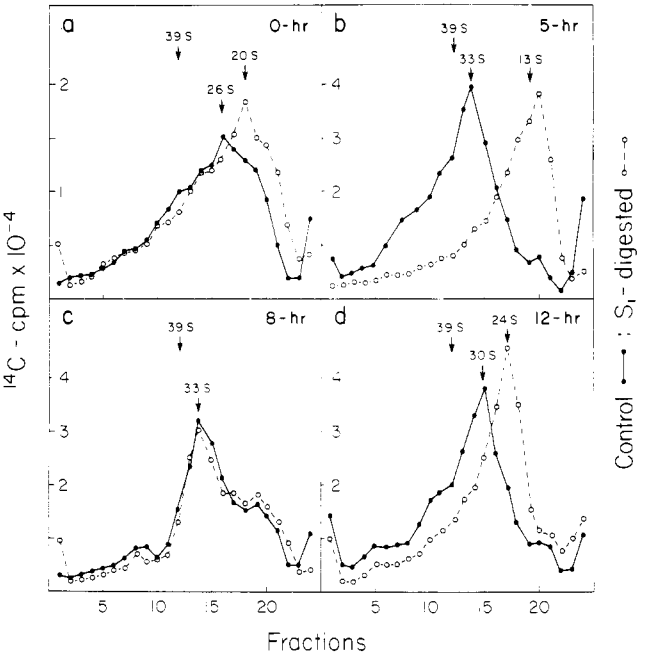

FIGURE 4: Sedimentation of DNA after S_1 digestion. All procedures were as described in the legend of Figure 3 for neutral gradients, except that the DNA preparations were first digested with S_1 nuclease as described under Experimental Procedure. Control undigested DNA (●) and S_1 nuclease digested DNA (○).

differential action of endogenous nucleases or ligases as the cells traverse the cell cycle. We have previously reported the characterization of DNA from resting WI-38 cells stimulated to enter the cell cycle by addition of serum (Collins, 1977). Although that study suffered from lack of a means to accurately determine the exact numbers of cells in each period of the cell cycle, certain comparisons with the present study can be made. In each case, early G_1 cells contain a minimum of breaks and S_1 sites. These increase in number as the cells enter the S phase, and decline as the cells enter G_2. The present study extends our earlier observations (Collins, 1977) in that relatively pure populations of G_2 cells were not available with the WI-38 system. The amount of single strandedness measured by isopycnic centrifugation in $Cs_2SO_4/AgClO_4$ and digestion with S_1 nuclease increases as HeLa cells leave G_2, divide, and traverse through G_1 and S (Table II), in good agreement with that reported for stimulated WI-38 cells (Collins, 1977).

Upon first reflection, the presence of nicks and single-stranded gaps in G_1 might seem puzzling. However, it should be recalled that all of the current models of DNA synthesis (Oishi, 1968; Klein and Bonhoeffer, 1972; Kornberg, 1969; Mitra and Kornberg, 1966; Okazaki et al., 1968; Erhan, 1968; Edenberg and Huberman, 1975) seem to require that such

structural alterations occur. In particular, Kornberg (1969) has reported that the POL 1 enzyme, while not the "true" polymerase, will not attach to DNA unless single-stranded regions are present. We have reported that DNA isolated from late G_1 cells, which contains a large number of gaps, was a better template for rat liver DNA polymerase than even S-phase DNA (Collins, 1974).

It was first suggested by Mazia (1963) and later elaborated by Pederson (1972) that continuous changes in the structure of chromatin occur throughout the cell cycle. One would expect that these changes are aimed at the goal of DNA replication, although an involvement in RNA synthesis cannot be ruled out (Collins, 1977). Our model suggests that this chromosomal cycle is reflected at the level of the DNA molecule itself. During the G_2 period, as the cells prepare for mitosis, the DNA molecule exists as an intact, duplex structure. After division, in early G_1, the DNA rapidly acquires nicks and single-stranded gaps, which increase in number during the S phase. As the replication machinery shuts down and the cells enter G_2, the number of breaks and single-stranded gaps declines to zero.

Acknowledgments

We thank Annie K. Chu for her excellent technical assistance and Dr. W. L. Banks, Jr., for his continued encouragement and support.

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