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THE INHIBITORY EFFECT OF HEAT AND RADIATION ON THE INITIATION OF DNA SYNTHESIS AND THE MEDIATION OF THE HEAT EFFECTS THROUGH DNA SUPERCOILING.

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THE INHIBITORY EFFECT OF HEAT AND RADIATION ON THE INITIATION OF DNA SYNTHESIS AND THE MEDIATION OF THE HEAT EFFECTS THROUGH DNA SUPERCOILING

by

René Cathleen Davis

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For the Degree of

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In the Graduate College

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ABSTRACT

The pH step alkaline elution and alkaline sucrose gradient techniques were utilized to evaluate alterations in DNA replication. Initiation and elongation were radioresistant at the G_1/S boundary (four hours after mitosis), while in mid S phase (nine hours after mitosis), initiation was sensitive to X-irradiation. Initiation and elongation of DNA synthesis which were radiation resistant at the G_1/S boundary could be inhibited by a hyperthermia treatment (43 C for one hour, four hours after mitosis). The impairment of initiation was maintained through late S phase while that of elongation was reversible as judged by full recovery at 15 hours after mitosis. Neutral sucrose gradients showed that the impairment of the initiation by heat treatment may be mediated by unwinding of the DNA supercoiled configuration. Whole cells and isolated nuclei from asynchronous cultures were used in nucleoid sedimentation and alkaline elution to show that heat (40 C-45 C) serves to unwind the DNA superhelix.

CHAPTER 1

INTRODUCTION

Hyperthermia (temperatures above 41 C) has been used as an agent for cancer therapy since the early 1900's. Although some encouraging results were obtained, there was not enough consistency in the findings for sustained efforts in using hyperthermia alone or combined with radiation. Within the last five years, cell culture, animal studies, and a few select preliminary clinical trials have obtained encouraging results indicating the potential of hyperthermia or hyperthermia and radiation in cancer therapy. Better control over heating temperatures has contributed to recent consistency and success in the hyperthermia trials.

Clinically, heating of tumor tissue is obtained by regional perfusion of extremities, water bath immersion, ultrasound, and non-ionizing electromagnetic radiation such as radiofrequency (13.56 MHz) and microwave (434 - 2450 MHz). The following findings make hyperthermia relevant to clinical therapy: 1) cancer cells usually are more sensitive to heat than normal cells; 2) hypoxic cancer cells appear to be more sensitive than oxic cells to heat; 3) cells in late S phase which are radioresistant are heat sensitive; 4) combination of heat and radiation kill more tumor cells in vivo and in vitro than either treatment alone; and 5) the microenvironment of tumor tissue (low pH, chronic hypoxia, and nutrient deprivation) favors preferentially the lethal effects of heat, and tumor vasculature may favor increased heat retention over that of normal tissue. Kim, Hahn, and Benjamin (1) used radiofrequency to heat and 6,000 rads of X-irradiation in six weeks to treat malignant melanoma and medullary carcinoma of the thyroid and found a large synergistic interaction with the regression of tumor nodules. In studies with 72 patients with advanced cancers, Horback, Shupe et al. (2) evaluated the synergism possible with heat before or after irradiation. When heat was given before radiation, 53% of the patients showed a complete remission of symptoms and, if heat followed radiation, 92% showed a complete remission. Stewart and Denekamp (3) determined the thermal enhancement ratios of normal skin and fibrosarcoma tissue in mice. It was observed that with a single dose, there was a therapeutic advantage with heat three hours after X-ray, but none when heat is applied immediately after radiation. With two to five daily fractions, heat immediately following irradiation gave less of a gain, and by three hours after, there was no therapeutic gain.

In vitro results have supported the rationale for heat and radiation in therapy. Sapareto (4) found temperatures above 41 C to kill mammalian cells and sensitize them to X-irradiation, however, with great variability with a 0.5 C change in temperature. Hyperthermia has also been shown to reduce recovery from sublethal (5) and potentially lethal (6) radiation damage and may, therefore, selectively kill tumor cells existing in the G_0 compartment. Other synergistic mechanisms include the production and repair of DNA strand breaks. Lunec, Hesslewood, et al. (7) compared the hyperthermic enhancement of radiation cell killing in HeLa S3 cells and found that pre-irradiation heat treatment did not alter the initial levels of radiation-induced DNA strand breaks. However, the rate and extent of break repair were significantly reduced by pre-irradiation heat treatments. Survival studies by Dewey et al. (8) showed heat can cause the slope of the X-ray survival curves to be increased and the shoulder to be reduced.

Many target theories are being evaluated to explain the observed synergistic interaction between hyperthermia and X-irradiation. For example, heat radiosensitization during both G_1 and S phase appears to be related to an increase in chromosomal aberrations (9) or also to the observed delay in repair of X-ray-induced strand breaks in DNA (10). The delay in rejoining these breaks might enhance the frequency of exchanges between DNA molecules that would result in chromosomal aberrations. Secondary effects from membrane changes are also possible in that changes in intracellular ionic concentrations may in turn modify protein structure or function and affect the normal events in the nucleus (11). In addition to these mechanisms, many workers have reported the increase in the protein to DNA ratio following heat treatments (12-15). This heat-induced increase in the protein to DNA ratio of chromatin is likely to play a major role in the inhibition of DNA-level enzymatic processes observed on the cellular level.

The purpose of this study was to examine DNA replication as a target for heat and radiation effects. It was important to evaluate the steps of initiation and elongation in DNA replication, and the stepwise alkaline elution assay is useful in this analysis. It separates nascent DNA intermediates according to size and function, and the elution patterns obtained show effects on these two processes based on relative proportions of specific replication intermediates eluted at each pH. Initiation can be assayed by monitoring the percentage of intermediates eluting at the lower pHs, while pulse-labeling studies and intermediates eluting at the higher pHs (larger molecular weight joined replicons) can be used to study elongation (16). Both processes of initiation and elongation occur simultaneously during DNA replication and heat or radiation effects could be mediated via separate mechanisms. Synchronized CHO cell cultures were used to distinguish between subsequent DNA replication effects resulting from heat or radiation prior to or after the G_1/S boundary. This study found initiation and elongation to be radioresistant at the G_1/S boundary, but during S phase only initiation is radiosensitive. In contrast, heat given before or at the G_1/S boundary caused a persistent inhibition of DNA initiation and a transient inhibition of DNA elongation. In an attempt to explain the persistent heat effect on DNA initiation, I investigated heat effects on DNA supercoiling. Heat caused a persistent decrease in DNA supercoiling that corresponded to the persistent effect on DNA initiation. Heat and radiation effects on DNA initiation and elongation will be discussed in the context of a model for DNA replication which stresses the importance of DNA supercoiling. Each chromosome in mammalian cells has multiple origins of replication, separated from each other by 60,000 to 180,000 base pairs (17). Replication proceeds bidirectionally from these origins and ends at some defined termination sequence or where the two replication forks happen to meet. The progeny molecules (replicons) synthesized from adjacent origins are then joined. Clusters of adjacent replicons tend to initiate in synchrony as if under coordinate control. Alterations of the normal supercoiled configuration could affect normal initiation patterns. Cook and Brazell (18) have suggested a model for the structure of the chromosome in which topological constraints acting over great lengths of DNA are obtained by the organization of the linear duplex into loops. Specific pairing of base sequences along different regions of one duplex could give rise to loop formation, and supercoils could be the basis of any subunit structure of the chromosome for RNA or protein interactions with DNA. Since the supercoiled configuration is necessary for normal DNA replication, it is also probable that a gyrase-like topoisomerase is responsible for maintaining the negative supercoils found in mammalian DNA, though no such gyrase has been isolated to date. Gellert et al. (19) discovered the gyrase enzyme in E. coli in 1976, and it has served as a prototype for other prokaryotic and eukaryotic topoisomerases that interconvert complex forms of DNA by transient double-strand breaks. Nucleoid sedimentation on neutral sucrose gradients has been shown to be useful for evaluating the superhelical structures of DNA (20). In particular, titration curves with various concentrations of ethidium bromide are useful for determining the degree of superhelicity of the DNA molecule (21).

In this study, nucleoid sedimentation has revealed the possibility for an uncoiling of the DNA helix after heat treatments to be responsible for the impairment of initiation observed with the stepwise alkaline elution.

Although the synchronized cell experiments were necessary to best evaluate DNA replication effects, asynchronous cells were used to assay general heat effects on DNA supercoiling at various temperatures. Temperatures of 40 C to 45 C were assayed by nucleoid sedimentation in neutral sucrose gradients for the extent of supercoiling, and by alkaline elution for the presence of strand breaks as a possible cause of observed unwinding of the DNA supercoils.

CHAPTER 2

MATERIALS AND METHODS

Cell Culturing and Synchronization

Chinese hamster ovary cells (CHO) were grown in monolayer culture with McCoy's modified medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), penicillin (100 U/ml), and streptomycin (100 ug/ml). Cultures were checked periodically for mycoplasma contamination (22) and found to be negative.

Synchronized cells were obtained by incubating pre-confluent roller bottles with colcemid (0.6 ug/ml) for two hours. The mitotic index was 90-95% in all experiments. Cells were removed by mechanically shaking of bottles, washed twice (kept at 4 C), and plated in 60 mm dishes at a density of 1.5-2.0 x 10^6 cells before subsequent heat treatments during G_1 and S phases. For asynchronous experiments, 60 mm dishes were seeded with 1.0 x 10^6 cells 24 hours prior to use. Under these conditions, the cell doubling time was 12-14 hours with G_1, four to five hours; S, six to seven hours; G_2, one to two hours; and M, one-half an hour (data not shown).

For neutral sucrose gradient experiments, the synchronized and asynchronous cells were prelabeled with ^{14}C-thymidine (0.02 uCi/ml, 57 mCi/mmol, Research Products International, Mount Prospect, IL) for 18-24 hours, and chased subsequently for four to ten hours with unlabeled McCoy's medium.

Hyperthermia and X-Irradiation

Cells growing attached to 60 mm dishes were wrapped twice in parafilm strips to seal edges and submerged in a 43 C water bath for one hour. The water bath temperatures were uniform and constant to $\pm$ 0.1 C. The plates were then returned to a 37 C incubator to await subsequent pulse-labeling and harvesting. X-ray doses of 500 rads were delivered at a dose rate of 400 rads/minute to the cells by a four MeV linear accelerator (Varian Associates, Palo Alto, CA). Cells were kept on ice during irradiation, after which the medium was changed and the plates were post-incubated at 37 C before pulse-labeling and harvesting.

Pulse-Labeling and Harvesting

At appropriate times following heat and X-ray treatments, the cells were pulse-labeled for alkaline elution with ^{3}H-thymidine (50 uCi/ml, 80.1 Ci/mmol, New England Nuclear, Boston, MA) for five, 15, and 25 minutes. For alkaline sucrose experiments, the cells were pulsed for 15 minutes and chased with thymidine, deoxycytidine (2 ug/ml each) in McCoy's medium for various time intervals. Pulse-labeling was terminated by submerging the plates in cold PBS. The cells were harvested by scraping and resuspended in 5 ml HBSS + 0.02% EDTA until used in the alkaline elution or sucrose gradient assays.

pH Step Alkaline Elution and Alkaline Sucrose Gradient

Replication intermediates of DNA synthesis were studied using a pH stepwise alkaline elution technique as described by Erickson and Kohn (16). Nascent cellular DNA from 1.0 x 10^{6} cells was eluted with tetrapropyl ammonium hydroxide solutions of pH 11.0, 11.3, 11.5, and 12.1 at a flow rate of 0.1 ml/minute. The amount of total radioactivity being eluted at the various pHs for the heated cells was always decreased compared to control cells. Therefore, the data presented in all the figures are normalized to the total amount of radioactivity eluting in the controls. For example, in Figure 1, for pH = 11.0 and a five minute pulse, the normalized data point is determined by the following equation:

$$\text{normalized percent of radioactivity eluting} = \frac{\text{dpm eluting at pH=11.0}}{\text{total dpm*}} \cdot \frac{\text{total dpm in heated}}{\text{total dpm in control}} \cdot 100$$

(*total dpm from pH 11.0, 11.3, 11.5, and 12.1 and the filter). Elongation was also studied using pulse-chase experiments with linear, continuous 5-25% alkaline sucrose gradients (0.3 M NaOH, 0.7 M NaCl, 0.001 M EDTA Na_2, 0.1 M Tris-base). Cells (2×10^5) suspended in PBS + 0.01 M EDTA were loaded onto the gradients, centrifuged 17.5×10^3 rpm for 12 hours as described by Bowden et al. (23). The data gathered from the alkaline sucrose gradients was also normalized to controls as described for the alkaline elution data.

Neutral Sucrose Gradients and Nuclei Extraction

The sedimentation properties of nucleoids from heated synchronous and asynchronous cells and also of heated nuclei from asynchronous cells were assayed by a modified procedure from Mattern and Painter (24). Cells (2.0×10^6) were suspended in 0.5 ml of 1.5 M NaCl/0.15 M sodium citrate buffer and layered in a lysis solution (1.95 M NaCl, 0.01 M EDTA, 0.5% Triton X-100, 2 mM Tris-HCl, pH 8.0)

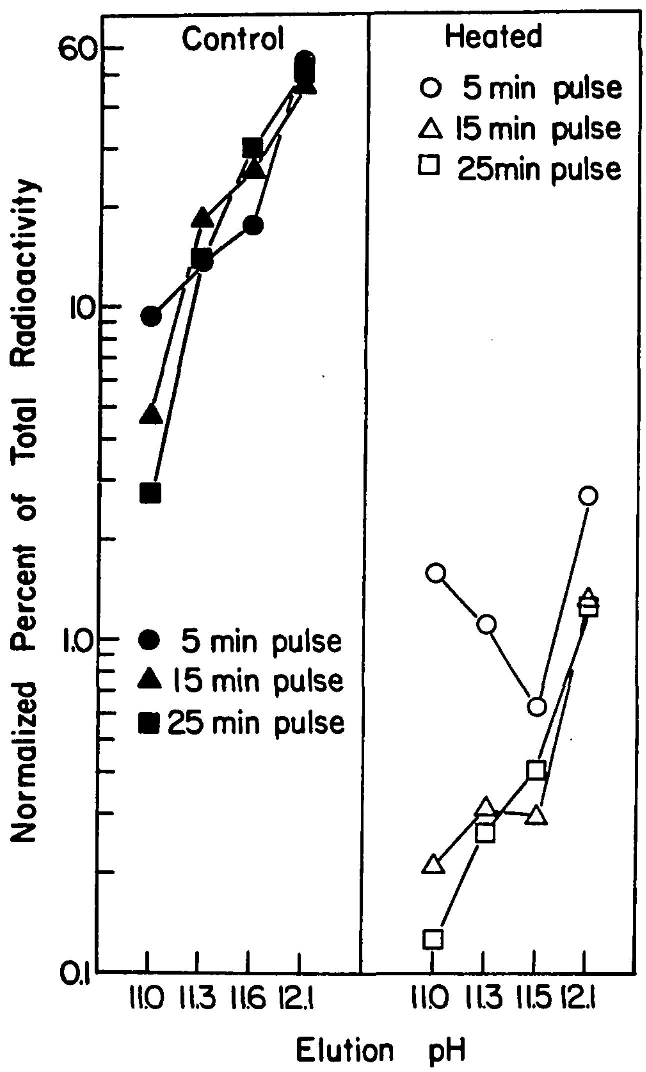

Figure 1. Heat Four Hours Post-Mitosis, pH Stepwise Elution Seven Hours Post-Mitosis

Heat 43 C for one hour, pulse-labeled five, 15, and 25 minutes. Results shown are the average of at least three experiments, and the standard error ranged from 10-20% of these averaged values.

on top of 15-30% neutral sucrose gradients (1.9 M NaCl, 0.01 M EDTA, 0.01 M Tris-HCl, pH 8.0, 36 ml total), before centrifuging at 11.1 x 10^3 rpm for 95 minutes. Some experiments utilized permeablized cells pre-incubated with ethidium bromide, while others contained intact nucleoids sedimenting through ethidium bromide containing sucrose gradients to establish the necessary titration curves.

Nuclei were obtained by a modification of the extraction procedure described by Roti Roti and Winward (25). Cells were washed four times in 20 ml spinner salts solution, five times in 20 ml of a 1% Triton X-100, 20 mM EDTA, 80 mM NaCl solution, and five times in 20 ml of 0.15 M NaCl. After heat treatment of the nuclei in sealed glass centrifuge tubes, they were layered on top of neutral sucrose gradients as described above.

Analysis of DNA Single-Strand Breaks by Alkaline Elution

The alkaline elution technique followed was essentially that of Kohn et al. (26). Cell suspensions (1.0 x 10^6 cells) were diluted with cold phosphate-buffered saline and filtered under mild suction onto 25 mm diameter, 2 um pore size poly (vinyl chloride) filters (Millipore Corporation, Bedford, MA). The filters were then washed with cold phosphate-buffered saline and the cells were lysed with 5 ml of 0.2% sodium lauroylsarcosine (Sarkosyl, Ciba-Geigy Corporation, Ardsley, NY), 2 M NaCl, 0.04 M EDTA, pH 10. The lysis solution was allowed to flow through the filter without suction, and the filters were then washed twice with 3 ml of 0.02 M Na_2 EDTA, pH 10, and the DNA eluted with a solution consisting of 0.02 M H_4 EDTA plus tetrapropylammonium hydroxide (Eastman Kodak Company, Rochester, NY, 25% in water) added to give a pH of 12.2. A modified filter funnel was used with the output passing through a peristaltic pump at a rate of 0.04 ml/minute to a fraction collection. At 64-minute intervals, fractions were collected and mixed with a scintillation fluid (Westchem Products, San Diego, CA) containing 0.7% acetic acid. Radioactivity remaining on the filters was recovered by treating with 0.4 ml of 1 N HCl at 60 C for one hour, followed by 2 ml of 0.4 N NaOH at room temperature for 60 minutes and ACS. The funnels were washed with 10 ml of 0.4 N NaOH and a 2 ml aliquot of this was counted.

CHAPTER 3

RESULTS

Effect of Heating (43 C, One Hour) in Late G_1 Phase on the Initiation and Elongation Processes of DNA Replication

Synchronous CHO cells heated at four hours after release from a mitotic block with colcemid were returned to 37 C for two hours post-heat incubation followed by ^{3}H-thymidine pulse-labeling. pH stepwise alkaline elution assay performed at seven hours after mitosis (early S phase) shows initiation and elongation to be decreased at this time. In Figure 1 is shown a comparison of the normalized percent of total radioactivity being eluted at the various pHs for the respective increasing pulse times in control and heated cells (see Materials and Methods).

The pieces of nascent DNA eluting at pH 11.0 and 11.3 have been suggested to correspond to Okazaki and subreplicon size fragments, respectively. The larger DNA sizes eluting at pH 11.5 and 12.1 correspond to elongated fragments. Large decreases in the relative percent of the small DNA fragments being eluted with short five-minute pulses of ^{3}H-thymidine would suggest a decrease in the amount of DNA initiation (16).

In Figure 1 is illustrated that the percent of radioactivity eluting at pH = 11.0 and 11.3 with a five-minute pulse is decreased in the heated cells when compared to controls. These data indicate that initiation is depressed in heated cells. The effects on elongation are seen when the normalized percent of total radioactivity is plotted versus the elution pH for the various pulse times. For control cells, a precursor product relationship is apparent. For example, as the percentage of radioactivity eluting decreases in the pH = 11.0 fraction, with increasing pulse time, it increases in the pH = 11.3, 11.5, and 12.1 fractions. The precursor product relationship exists in synchronized CHO cells (27). This, however, is not the case with the heated cells. A decrease in the lower pH fractions is not followed by a subsequent increase in the higher pH fraction after a longer pulse time with ^{3}H-thymidine. This suggests that there is also an inhibitory effect of heat on DNA elongation.

Since elongation and initiation are decreased by heating, it was important to determine if the defects were reversible. Therefore, cells were treated and elongation and initiation were evaluated at post-heat treatment times of up to 15 hours after mitosis. In Figure 2 is shown the state of initiation at 15 hours after mitosis after heating the cells at the G_1/S boundary. Initiation is still depressed as judged by a decrease in the amount of radioactivity eluting at pH 11.0 and 11.3 for a five-minute pulse. A comparison of the data in Figure 2 with the data in Figure 1 shows that the magnitude of the inhibition is essentially the same at ten hours after heating as at the G_1/S boundary. A post-incubation of cells at 37 C following heating does not result in a recovery of DNA initiation. In addition, in Figure 2 is shown that there is an increase in the incorporation of

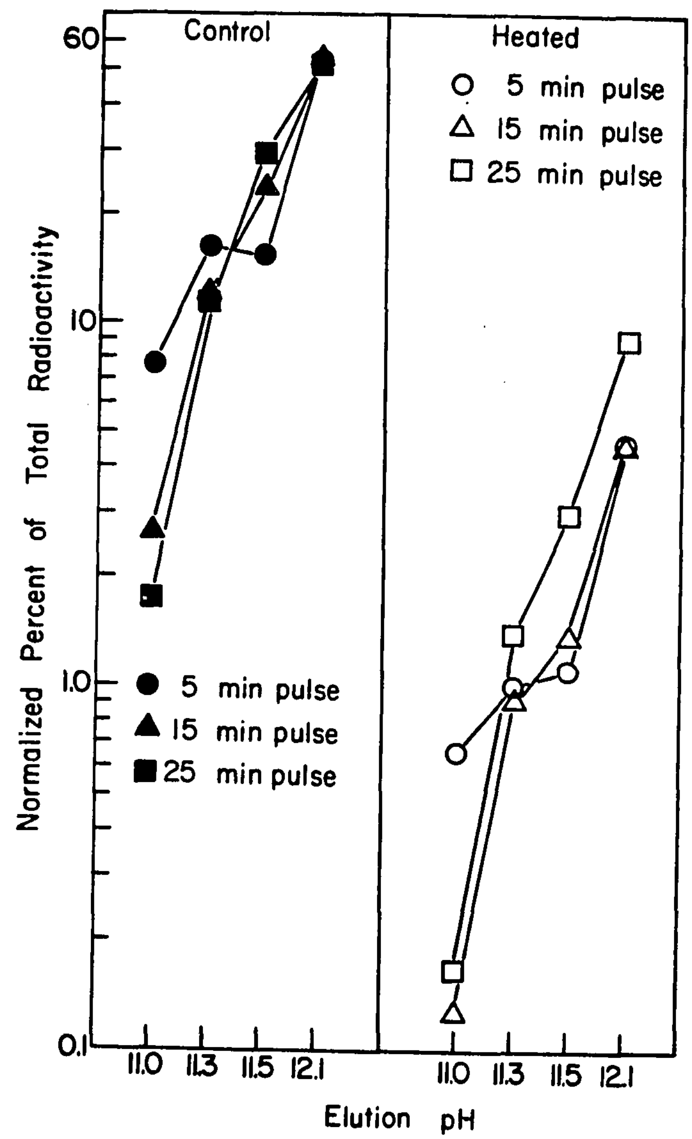

Figure 2. Heat Four Hours Post-Mitosis, pH Stepwise Elution 15 Hours Post-Mitosis

Heat 43 C for one hour, pulse-labeled five, 15, and 25 minutes. Points plotted represent the average of at least three experiments. Deviations were not more than 10-20% of average values.

radioactivity in the pH = 11.5 and 12.1 fractions in the heated cells with increasing pulse time. This is in contrast to Figure 1, which showed an impairment of elongation in the heated cells and indicates that elongation recovers during the ten-hour incubation at 37 C.

Effect of Heating (43 C, One Hour) at Mid S Phase on the Initiation and Elongation Processes of DNA Replication

Since initiation and elongation are both affected at the G_1/S boundary by heating, I investigated mid S phase for these defects in DNA synthesis. Synchronized cells were heated at nine hours post-mitosis for one hour at 43 C, post-incubated at 37 C for two hours, and analyzed by pH stepwise alkaline elution. In Figure 3 is shown an inhibition of initiation and elongation. Figure 3 when compared to Figure 1 shows that the inhibition of initiation during S phase is similar to the inhibition seen at the G_1/S boundary. For example, there is a comparable depression of radioactivity in the small DNA fragments with a short pulse time (pH = 11.0 and 11.3; five-minute pulse). An inspection of the pH = 11.5 and 12.1 fragments in both figures shows that elongation may be depressed in S phase as well as at the G_1/S boundary after heating. The data in both figures indicate a defect in elongation, since an increase in the pulse time does not result in an increase in radioactivity in the larger DNA fragments. Pulse-chase experiments with alkaline sucrose gradients were utilized to confirm both the results seen with the alkaline elution and my interpretation of the effect of heat on elongation. During mid S phase, untreated cells will elongate DNA fragments which can be monitored by

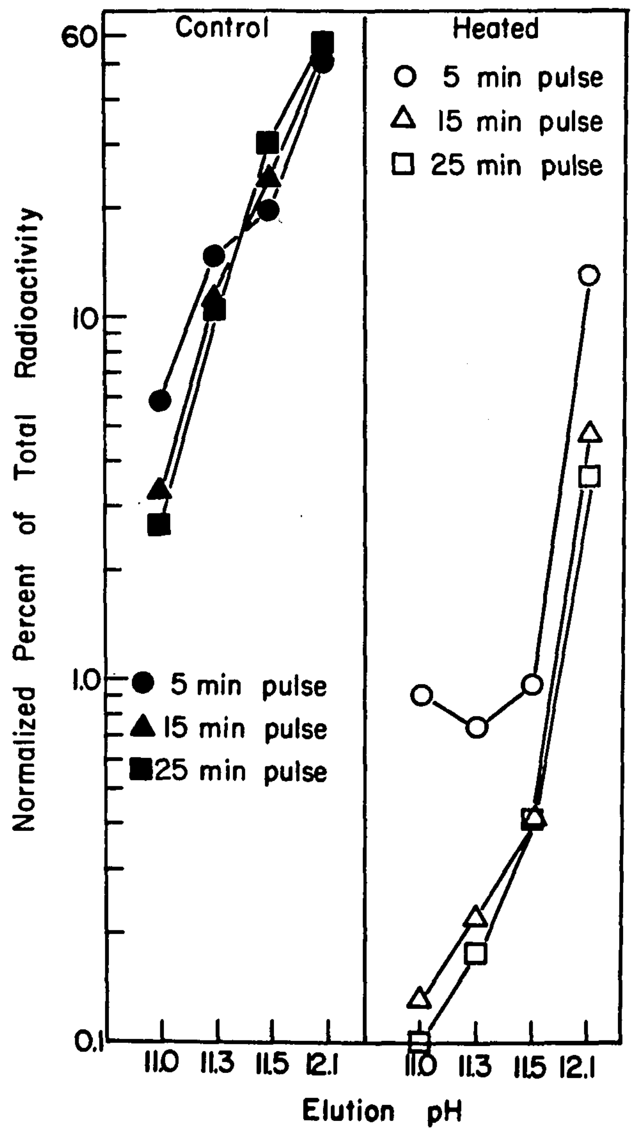

Figure 3. Heat Nine Hours Post-Mitosis, pH Stepwise Elution 12 Hours Post-Mitosis

Heat 43 C for one hour. Standard deviations were 10-20% of the averaged values of at least three experiments plotted here.

an increasing size of labeled DNA fragments as the chase time increases (Figure 4). The increase in DNA size is illustrated by a shift of the sedimentation pattern to the right (higher molecular weights). A comparison of a zero-minute chase to a 15-minute chase in the control cells results in a shift of the sedimentation pattern to the right of the figure, which is not seen in the heated cells. Interestingly, the heated cells do demonstrate an apparent increase in DNA size from a 15 to 30-minute chase.

Effect of X-Ray (500 Rads During G_1 and S Phase on Replication Intermediates)

Synergistic cell killing obtained with heat and ionizing radiation may involve a defect of DNA initiation and elongation. Since heat alone causes an inhibition of initiation and elongation, I determined the effect of X-irradiation alone on DNA replication. In Table I is illustrated the differences seen when irradiating cells in G_1 or in S phase. At four-hour post-mitosis (G_1), little effect is seen on initiation or elongation at 20-minute post-irradiation, with no effect seen after three hours post-irradiation. By three hours after X-ray, the elution patterns from irradiated cells are essentially the same as those of control cells since the percent of radioactivity in each elution pH does not vary considerably from the control. Irradiation in S phase at nine hours after mitosis causes the largest decrease in the relative amounts of DNA eluting to be at the lower pH fractions, suggesting a decrease in the amount of initiating fragments. For example, pH = 11.0 and 11.3 drop to approximately 55% of the control values for

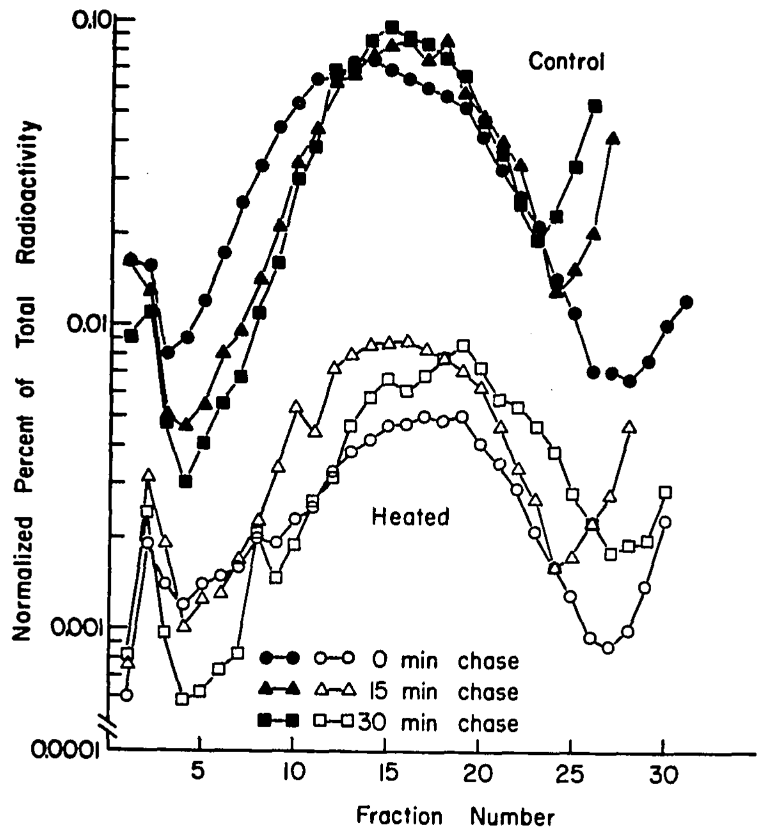

Figure 4. Alkaline Sucrose Gradients; Cells Heated Nine Hours Post-Mitosis

Heat 43 C for one hour, pulse with ^{3}HTdR and chased for zero, 15, and 30 minutes. The direction of sedimentation is from left to right. Standard errors were 5-10% of those values plotted here, which represent the average of three to four experiments.

Table I. Synchronous CHO Cells (X-Ray 500 Rads)

Pulse	Time After X-Ray	Percent of Control at Elution pH 11.0	11.3	11.5	12.1 + Filter
S-Phase (Nine Hours Post-Mitosis)[a]:					
25 min. pulse	20 min.	55.87	55.37	81.59	64.94
	1 hr.	53.70	51.14	58.79	84.04
	3 hr.	65.88	77.18	68.08	101.0
G_1-Phase (Four Hours Post-Mitosis):					
25 min. pulse	20 min.	72.82	92.71	72.27	109.6
	1 hr.	122.0	129.0	73.65	98.07
	3 hr.	103.0	107.3	113.0	97.97

[a]Cells irradiated on ice, media then changed for 20 minutes, one hour, and three hour post-irradiation 37 C incubations.

cells assayed 20 minutes after irradiation, and this depression at pH 11.0 and 11.3 continues through three hours after irradiation, though to a lesser degree. pH step fractions of pH = 11.5 and 12.1 contain a higher percentage of the control total radioactivity, indicating that elongation is not as affected as initiation.

Comparison of Heat and X-Ray on G_1 and S Phase

In Table II is illustrated the comparison of heat (43 C for one hour) and X-irradiation (500 rads) on synchronized CHO cells. The cells were treated at four and nine hours post-mitosis and assayed by the stepwise alkaline elution at seven and 12 hours post-mitosis, respectively. Treatment in S phase (nine hours post-mitosis) and assaying at 12 hours post-mitosis causes an impairment of initiation after both X-irradiation and heat. This is illustrated by only 65.88% and 4.74% of control eluting at pH 11.0, and 77.18% and 7.47% eluting at pH 11.3. X-irradiation gives little effect on elongation at this time, while heat causes a marked reduction in normal elongation levels (6.55% and 3.60% for pH 11.5 and 12.1 plus filter, respectively). Heat responses were generally more dramatic than those seen with X-irradiation. A comparison of the treatments given at four hours post-mitosis and assayed at seven hours post-mitosis shows a marked difference between heat and X-irradiation. A dose of 500 rads caused no effect on initiation or elongation, while heat (43 C for one hour) caused a marked impairment on initiation and elongation (2-4% of control eluting at pH 11.0-12.1, plus filter).

Table II. Comparison of Heat and X-Ray (Percent of Control Values)

pH	7 Hours Post-Mitosis		12 Hours Post-Mitosis	
	X-Ray	Heat	X-Ray	Heat
11.0	103.0[a]	2.8	65.88	4.74
11.3	107.3	4.15	77.18	7.47
11.5	113.0	2.07	68.08	6.55
12.1 + filter	97.97	2.33	101.0	3.60

[a]Percent eluted values were normalized to that of the control.

Response of Asynchronous Cells to X-Rays (500 Rads)

Asynchronous cultures were irradiated in exponential phase with 500 rads of X-rays. Elution patterns suggested a decrease in initiation because of a decrease in the amount of radioactivity eluting at pH = 11.0 and 11.3 as compared to controls. There is little or no effect on elongation when assayed within ten minutes after irradiation since the amount of radioactivity eluting at pH 11.5 and 12.1 did not change (Table III). Longer time intervals before assaying caused slight deviations from these patterns. These results are consistent with that found by other techniques in which initiation proved to be more sensitive than elongation to low dose irradiation (28-30).

Effect of Heat on the Supercoiling of DNA

Alterations of supercoiling of DNA is a possible mechanism responsible for the inhibition of initiation observed at the G_1/S boundary and in mid S phase. Neutral sucrose gradients with various concentrations of ethidium bromide were used to evaluate the extent of supercoiling in the heated cells. Titration curves with various concentrations of ethidium bromide have been shown to be useful for determining the degree of superhelicity of DNA in that as the amount of ethidium bromide is increased, the amount intercalating into the DNA increases, and the strands are unwound. An equivalence point is reached beyond which further increases in ethidium bromide cause a subsequent rewinding of the DNA. This equivalence point varies depending upon the coiled state of the DNA duplex (21).

Table III. Asynchronous CHO Cells (X-Ray 500 Rads)

Time After X-Ray	pH	Normalized Percent of Total: Control	Normalized Percent of Total: X-Ray	Percent of Control
10 min.	11.0	5.52	2.56[a]	46.38
	11.3	16.82	6.28	37.34
	11.5	25.27	15.14	59.90
	12.1 + filter	47.54	43.28	91.04
20 min.	11.0	5.22	1.88	36.00
	11.3	16.42	6.03	36.70
	11.5	27.39	13.35	48.74
	12.1 + filter	46.31	33.89	73.18
30 min.	11.0	4.73	1.90	40.17
	11.3	16.91	4.12	24.36
	11.5	27.10	9.67	35.68
	12.1 + filter	43.84	23.94	54.61

[a]Results shown represent the average of three experiments, and standard errors never reached more than 10-20% of these values. X-ray values were normalized to control totals.

As can be seen by the three panels in Figure 5, there were large differences in the equivalence points obtained for untreated cells in different phases of the cell cycle and in comparing DNA from heated and control cells. Panel A, Figure 5 shows that the DNA from heated and control cells have essentially the same equivalence point (approximately 50 ug/ml of ethidium bromide) or the same degree of supercoiling immediately following the 43 C heat treatment. At seven hours after mitosis (Panel B) following a two-hour post-heat incubation, the configuration of the DNA from heated cells has changed dramatically as compared to that shown in Panel A. DNA from control cells (Panels A and B) still has approximately the same equivalence point, but in the heated cells the DNA now rewinds positively with ethidium bromide at a concentration 50 times less than before (1.0 ug/ml ethidium bromide), suggesting that DNA from heated cells is in a more uncoiled state than in control cells. As illustrated by Panel C, the uncoiling of the DNA must occur during the two-hour post-heat incubation and continues until at least 15 hours after mitosis. The equivalence point for DNA from heated cells is about 1.0 ug/ml ethidium bromide, while that of DNA from control cells is 50 ug/ml, indicating the same uncoiled state as seen in Panel B. Figure 6 illustrates the migration patterns with no ethidium bromide for the various time points. Panels B and C emphasize that the DNA unwinding pattern observed in the heated cells results in a peak shift to the left of that of the control at seven and 15 hours after mitosis, indicating that the DNA becomes uncoiled. Also apparent from these graphs is the

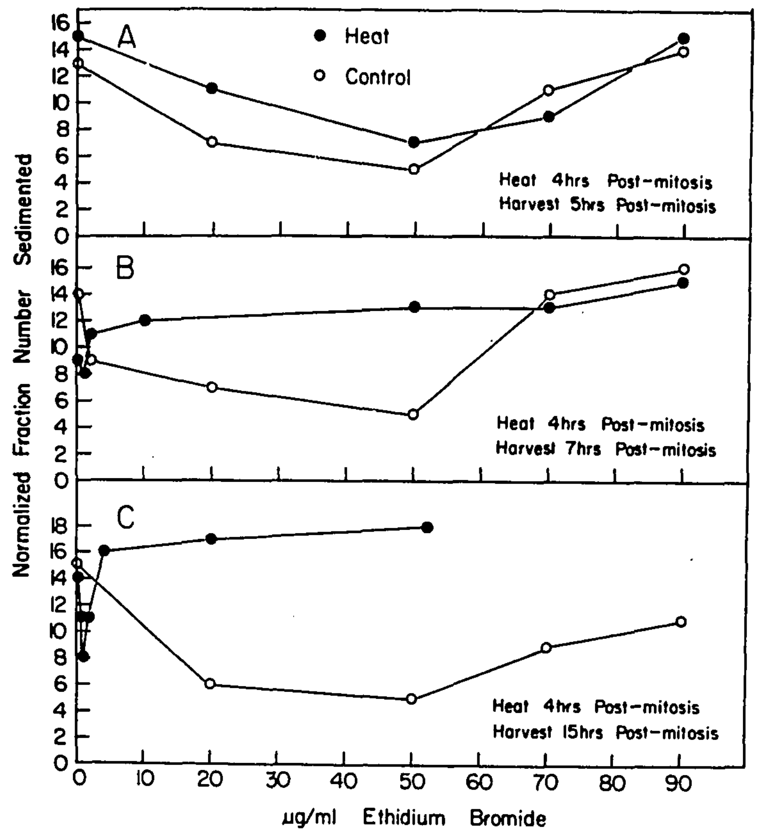

Figure 5. Ethidium Bromide Titration-Nucleoid Sedimentation

Results represent the average of at least three experiments with standard errors of no more than 10% of average values. Migration patterns were always as shown, though exact fraction number migrated were normalized to 30 fractions where gradients contained more or less than 30 fractions.

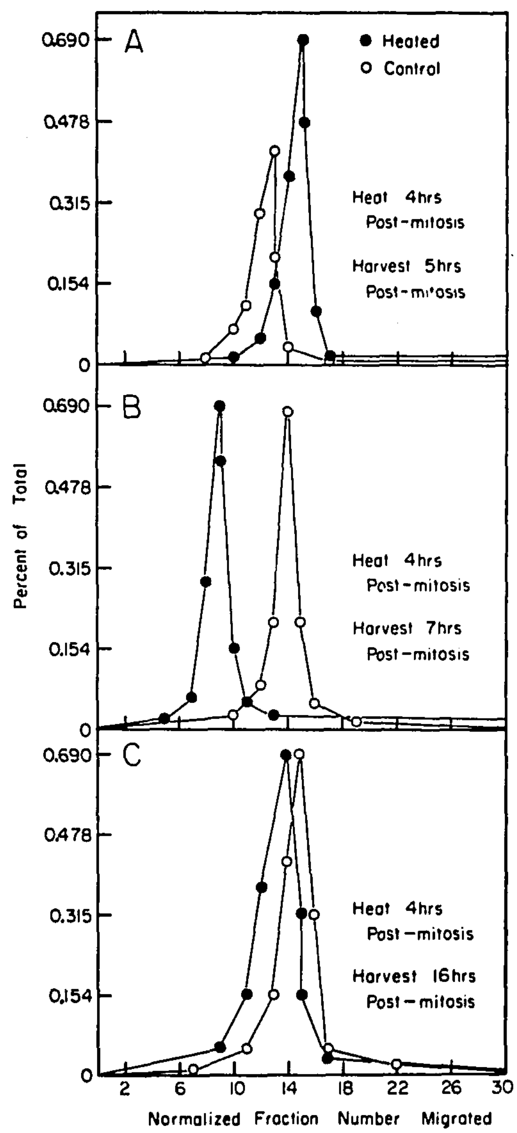

Figure 6. Nucleoid Sedimentation

Peaks represent the results of at least three experiments, and standard errors were near 10% of those plotted. Values were again normalized to 30 fractions when gradients contained more or less than 30.

peak shift to the right of DNA from control cells from G_1 to S phase. As DNA replication begins, the DNA duplex becomes more condensed and supercoiled, consistent with Painter's cluster theory for initiation (15).

Effect of Heat on Asynchronous CHO Cells

In attempts to evaluate the hypothesis that generally heat serves to uncoil the DNA superhelix, possibly by altering a heat sensitive structure, asynchronous CHO cells were heated for extended times at temperatures ranging from 40 C to 45 C. In Panel A of Figure 7 is illustrated the apparent uncoiling of the helix at temperatures below 43 C, and an apparent positive supercoiling when heated at temperatures of 43 C and above. An uncoiling of the DNA is represented by a shift to a lower relative sedimentation value (less migration in neutral sucrose gradients) and a positive supercoiling is represented by an increase in relative sedimentation values (increased migration in neutral sucrose gradients). By six hours of heating, cells at 40 C to 42 C contain very unwound DNA, while after 43 C and 45 C, the DNA sediments in a pellet at the bottom of the polyallomer tubes, with an apparent tightly-coiled configuration possibly due to protein association with the DNA. In Panel B, however, is illustrated the effect of post-heat incubation at 37 C on cells heated at 45 C for 30 minutes. Immediately after heating, the DNA has an increased sedimentation over the control (see Panel A), but with time at 37 C, the DNA exhibits a decreased sedimentation over the control cell DNA. Alkaline elution was utilized to assay for the presence of strand breaks following these heat

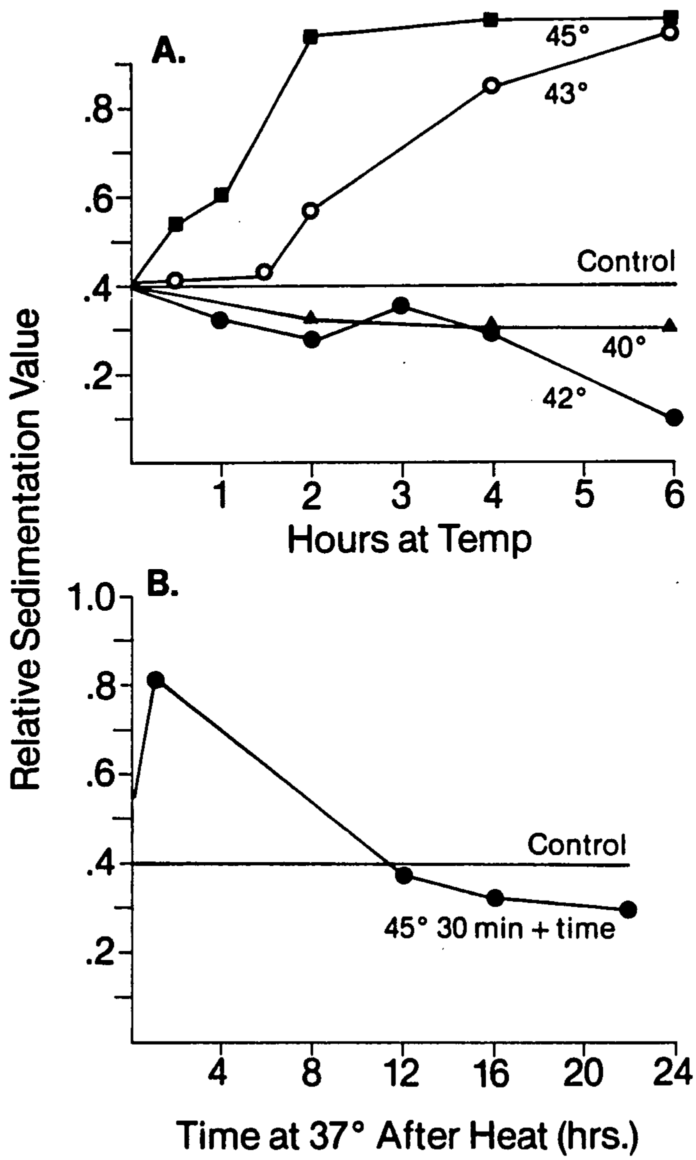

Figure 7. Nucleoid Sedimentation of Heated Asynchronous Cells

Results represent the average of at least three experiments, and deviations were less than 10% of those plotted. Relative sedimentation values were determined because the number of fractions collected varied.

treatments. In Figure 8 is illustrated the results of this experiment. Control cell DNA showed a normal elution pattern with most of the DNA remaining on the filter after 16 hours of elution with a pH 12.2 solution. DNA from cells heated four hours at 42 C showed no strand breakage as it also elutes at a rate similar to control. When these findings are compared to the decrease in sedimentation in neutral sucrose gradients (see Figure 7, Panel A), this decrease in sedimentation is probably because of an unwinding of the intact supercoiled helix and not because of strand breakage. Heating at 45 C for four hours, however, causes severe strand breakage and degradation, as illustrated by a rapid elution rate as compared to control (only 8% of the DNA remained on the filter by 16 hours). When comparing this with Panel A of Figure 7, which shows a rapid sedimentation, indicative of a tightly-coiled configuration, this data suggests there to be an additional restriction, most likely protein, on the DNA helix causing an apparent supercoiled structure in neutral sucrose gradients when DNA is actually fragmented. If nuclei are isolated and heated at 45 C for 30 minutes, the neutral sucrose gradient sedimentation shows the DNA to be in an unwound configuration. This is illustrated in Figure 9 as the heated nuclei sediment slower (peak to the left of control). These results, in addition to those seen with the alkaline elution (Figure 8), suggest the DNA helix to be in a more unwound configuration than the control, as was seen with temperatures below 43 C, though this unwinding is masked by protein effects as exhibited in Panel A, Figure 7.

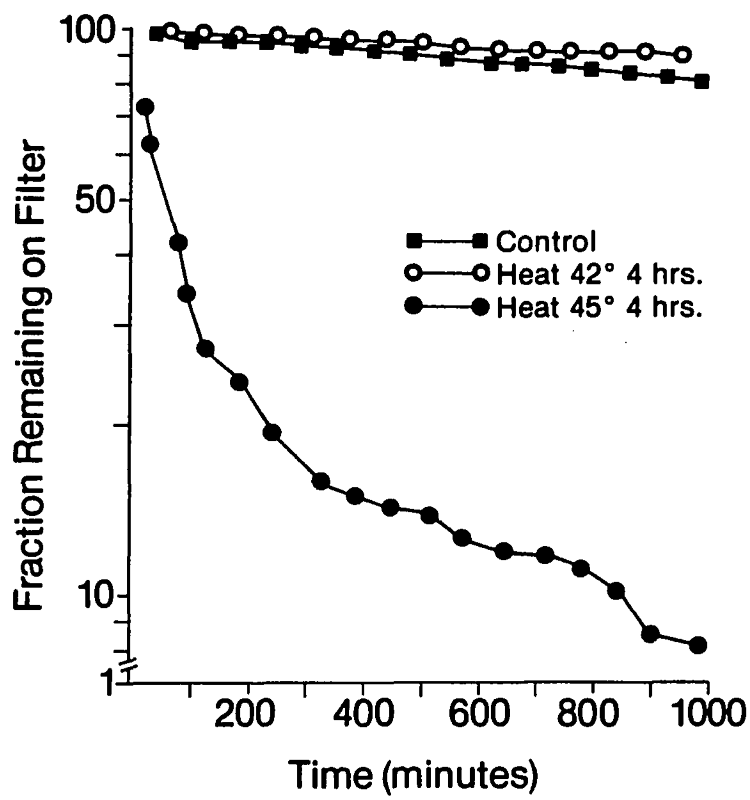

Figure 8. Alkaline Elution of Heated Asynchronous Cells

Results represent the average of duplicate samples, and deviations were less than 15% of those plotted.

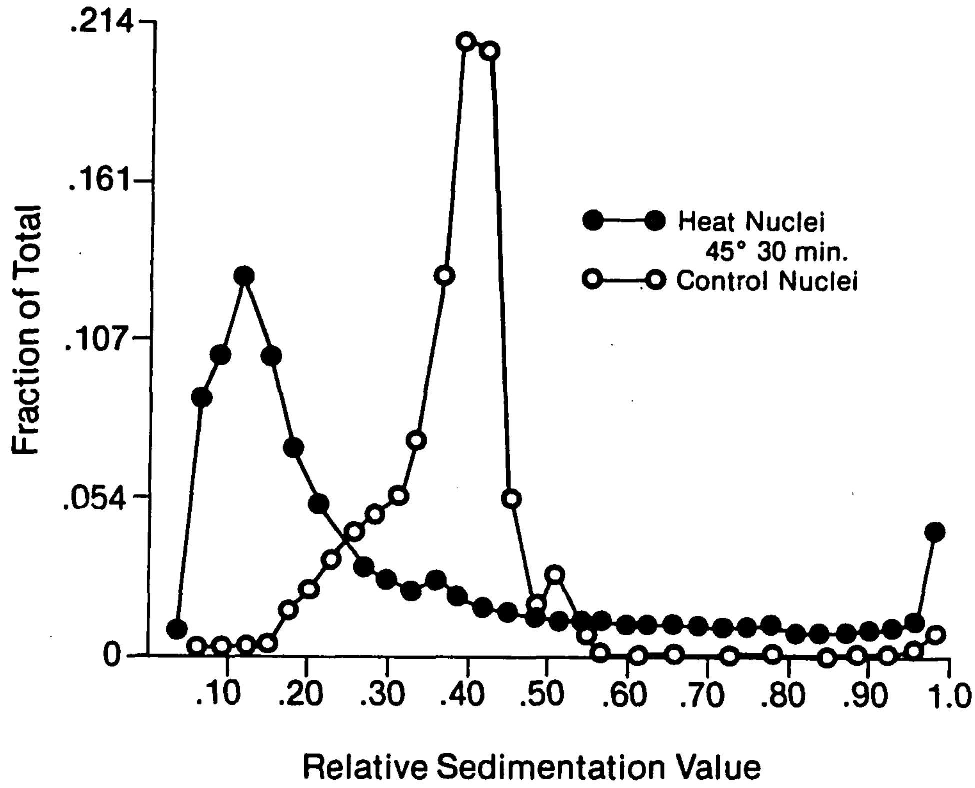

Figure 9. Nucleoid Sedimentation of Isolated Nuclei

Results shown represent the average of three experiments, and standard errors did not exceed 10% of those plotted. Relative sedimentation values were determined as fraction number varied among gradients.

CHAPTER 4

DISCUSSION

Through the use of synchronized CHO cells, the DNA initiation and elongation processes can be monitored at various times throughout the cell cycle. Both processes occur simultaneously during DNA replication, and it can be shown that despite an inhibition of a normal initiation rate, elongation can continue with nascent strands which were initiated before or during the firing of replicons. Cells which are irradiated with 500 rads at nine hours post-mitosis and assayed 20 minutes, one hour, and three hours later have initiation affected while elongation appears to be normal for those replicons already initiated (Table I). These findings confirm those found by Laughlin and Taylor (30) with alkaline, sodium iodide self-generating gradients. Initiation was not found to be significantly affected at four hours post-mitosis in what appears to be a radioresistant portion of the cell cycle. This radioresistance at four hours post-mitosis has also been suggested by Gerner (31) using an integrative DNA replication assay. In Table I, I showed no significant difference between eluted fractions if the cells were irradiated in G_1 and pulsed three hours later, but a large decrease in smaller replication intermediates was apparent when cells were irradiated in S phase. It would be of interest to investigate this difference in the replication processes and the vulnerability or resistance to various agents when in G_1 as compared to S phase. Lower percentages of nascent DNA eluting at pH 11.5 and 12.1 can be explained by the precursor-product relationship by which a significant decrease in the small molecular weight fragments would cause an additional decrease in the remaining pH fractions.

In contrast to radiation, heat treatment either in G_1 or S phase caused a significant inhibition of the initiation of DNA synthesis. This difference suggests possible reasons for the synergism found with combined modalities of heat and irradiation. This synergism may involve a defect of either the initiation or elongation processes and a complementation of the effects of heat and irradiation. The radioresistance at the G_1/S boundary may be overcome by replication-impairment heat treatments. DNA elongation is inhibited immediately after heating (Figures 1 and 3) and returns to normal rates of elongation at 15 hours after mitosis (Figure 2). Initiation is a process which seems to be permanently impaired, although not totally inhibited, at least within the time interval investigated here, while elongation can return to normal. Analyses of eluting patterns were also obtained for 12 hours after mitosis and showed elongation to be inhibited at this time. Since elongation is inhibited at 12 hours after mitosis but not at 15, the DNA elongation process recovers sometime during this time interval. A return to normal elongation rates at 15 hours post-mitosis was observed by Gerner (32) in his studies of DNA replication using CsCl hybrid density assays on cells heated three to four hours after mitosis. This observation may, in part, be due to a restoration of normal elongation rates.

The different effects heat appears to have on initiation versus elongation may be partially explained by changes in the supercoiled state of the DNA. Supercoiling analysis by neutral sucrose gradients was performed to address this question. It has been suggested by Painter (33) that initiation occurs via a supercoiled configuration in which clusters of replicons are fired at one single moment. Any perturbation of this coiled configuration could interrupt normal initiation patterns.

My results show that the heated cells reach the equivalence point at a concentration 50 fold less than control cells, suggesting that the heated cells are in a more relaxed state initially and less ethidium bromide is therefore required for complete relaxation. It is apparent from Figure 5 that the heated cells never fully unwind as compared to control cells. The difference in sedimentation with no ethidium bromide and that at the equivalence point is greater for the control cells than for the heated cells. This suggests that the amount of DNA available for unwinding is less in heated cells, possibly as a result of increased protein associated with the DNA. Nonetheless, the large differences in equivalence points for seven and 15 hours after mitosis suggest this unwinding of the DNA to be partially responsible for the observed inhibition of initiation. Figure 5, Panel A shows no difference in equivalence points immediately after heating. However, by seven hours post-mitosis, at the start of DNA replication, the unwound state is obvious. These two observations suggest that heat at the G_1/S border does not directly affect the supercoiling of DNA, but instead affects possibly a hypothetical gyrase-like enzyme responsible for the unwinding and supercoiling of the DNA. It should be noted that the 50 ug/ml equivalence point for the control cells may be abnormally high as asynchronous cells have a much smaller equivalence point when compared to populations synchronized with colcemid. In order to evaluate this discrepancy, non-colcemid treated cells also should be used in these experiments to determine if there exist any changes because of colcemid exposure.

Novobiocin, which is a competitive inhibitor of the ATPase subunit of the bacterial gyrase molecule, causes the same shift to a more unwound state with neutral sucrose gradients and no ethidium bromide (24). Many models have been proposed for the supercoiling reaction catalyzed by the E. coli DNA gyrase (34-36). Forterre (34) suggests a mechanism by which both strands of the duplex are cleaved inside the segment that is coiled around the enzyme subunits. The uncoiling is driven by an ATP dependent movement of the two subunits, and as the DNA ends are in front of each other again, the DNA duplex can be resealed. A possible mechanism by which the DNA becomes unwound includes the ATP driven uncoiling reaction being altered by heat damage to the thermal sensitive gyrase molecule. No detectable changes were seen in the levels of ATP at this time as assayed by a highly sensitive luciferase-ATP assay system (data not shown). Therefore, insufficient ATP levels are not likely the source of the heat effects. It is possible that the resealing step of the duplex strand breaks may be affected, leading to the persistence of these cleaved areas and a subsequent unwinding of the DNA molecule. It should be noted, however, no single-stranded breaks have been detected following short heat treatments of 44 C for ten minutes, for example (7). Instead, one could imagine the cleaved areas to be resealed as planned, but faulty movements by the two subunits could lead to rejoining the ends while the DNA is in an unwound, intermediate state. Sugino and Cozzarelli (37) have shown the ATP hydrolysis step to have a temperature optimum of 30 C, with the rate of hydrolysis at 37 C being only 75% of that at 30 C. This also suggests the thermal instability of the *E. coli* DNA gyrase molecule and, specifically, of the uncoiling, ATP-dependent step in the reaction.

Although heat served to unwind DNA of synchronized CHO cells during G_1 and S phases, it was necessary to assay asynchronous cells at various temperatures before a general conclusion that heat causes an unwinding of the DNA supercoiled helix could be made. Figure 7, Panel A suggests this unwinding to occur at temperatures less than 43 C. Figures 8 and 9 show a contradiction of the effect at 43 C and 45 C. These figures indicate the heat causes the DNA to become less supercoiled while the neutral sucrose gradients show the opposite to be true. It is suggested that a secondary effect from an increase in protein being bound to the DNA masks the true sedimentation in the nucleoid sedimentation assay. Roti Roti and Winward (12) have shown with HeLa cells an increase in the protein to DNA ratio after heating at

45 C for 30 minutes. It has also been shown that this ratio decreases with a subsequent increase in time at 37 C after heat, which corresponds to the data in Figure 7, Panel B. As the amount of bound protein is decreased at 37 C, there is a corresponding decrease in the sedimentation values over the control cell DNA. This probably represents the true configuration of the helix, which soon becomes devoid of these heat-induced proteins. Roti Roti and Winward (25) have shown that heating of isolated HeLa nuclei 45 C for 30 minutes causes no increase in the protein to DNA ratio, suggesting these proteins may be coming from the cytoplasm. The data in Figure 9 for the heat treatments of nuclei 45 C for 30 minutes show the absence of any protein effects and a decrease in the nucleoid sedimentation. This contrasts the sedimentation observed when intact cells are heated under the same conditions, indicating the latter case may involve secondary modifications because of protein accumulation.

In conclusion, it is suggested the observed synergism of heat and radiation to be explained in part by the contrasting effects on initiation, which is radioresistant but heat sensitive at the G_1/S border, that heat generally causes an unwinding of the supercoiled DNA helix, and that alterations of this supercoiling may be a molecular explanation for the inhibition of DNA replication by heat.

REFERENCES

1. J.H. Kim, E.W. Hahn, and F.J. Benjamin, Treatment of superficial cancers by combination hyperthermia and radiation therapy. Clin. Bull. vol.9, no.1, 13-16 (1979).

2. N.B. Hornback, R. Shupe, et al., Radiation and microwave therapy in the treatment of advanced cancers. Radiology 130, 459-464 (1979).

3. F.A. Stewart and J. Denekamp, Fractionation studies with combined X-rays and hyperthermia in vivo. Br. J. Radiol. 53, 345-356 (1980).

4. S.A. Sapareto, L.E. Hopwood, and W.C. Dewey, Combined effects of X-irradiation and hyperthermia on CHO cells for various temperatures and orders of application. Radiat. Red. 73, 221-233 (1978).

5. E. Ben-Hur, M.M. Elkind, and B.V. Bronk, Thermally enhanced radioresponse of cultured Chinese hamster cells: Inhibition of repair of sublethal damage and enhancement of lethal damage. Radiat. Res. 58, 38-51 (1974).

6. G.C. Li, R.G. Evans, and G.M. Hahn, Modification and inhibition of repair of potentially lethal X-ray damage by hyperthermia. Radiat. Res. 67, 491-501 (1976).

7. J. Lunec, P. Hesslewood, P. Parker, and S. Leaper, Hyperthermic enhancement of radiation cell killing in HeLa S3 cells and its effect on the production and repair of DNA strand breaks. Radiat. Res. 85, 116-125 (1981).

8. W.C. Dewey, L.E. Hopwood, S.A. Sapareto, and L.E. Gerwick, Cellular responses to combinations of hyperthermia and radiation. Radiology 123, 463-474 (1977).

9. W.C. Dewey, S.A. Sapareto, and D.A. Betten, Hyperthermic radiosensitization of synchronous Chinese hamster cells: Relationship between lethality and chromosomal aberrations. Radiat. Res. 76, 48-59 (1978).

10. E.P. Clark, S. Robinson, J.T. Lett, and P. Corry, Inhibition of DNA strand break rejoining by hyperthermia, in Proceedings of the International Symposium on Cancer Therapy by Hyperthermia and Radiation, J.E. Robinson and M.E. Wizenberg, eds. American College of Radiology, Chevy Chase, Md., pp.27-33 (1976).

11. P.S. Lin, L. Kwock, K. Hefter, and D.F.H. Wallach, Modification of rat thymocyte membrane properties by hyperthermia and ionizing radiation. Int. J. Radiat. Biol. 33, 371-382 (1978).

12. J.L. Roti Roti, R.T. Winward, The effects of hyperthermia on the protein to DNA ratio of isolated HeLa cell chromatin. Radiat. Res. 74, 159-169 (1978).

13. G. Bouch, F. Amalric, M. Caizerques-Ferrer, and J.P. Zalta, Effects of heat shock on gene expression and subcellular protein distribution in Chinese hamster ovary cells. Nucl. Acid Res. 7, 1739-1747 (1979).

14. J.L. Roti Roti, K.J. Henle, and R.T. Winward, The kinetics of increase in chromatin protein content in heated cells: A possible role in cell killing. Radiat. Res. 78, 522-531 (1979).

15. R.L. Warters, J.L. Roti Roti, and R.T. Winward, Nucleosome structure in chromatin from heated cells. Radiat. Res. 84, 504-513 (1980).

16. L.C. Erickson, W.E. Ross, and K.W. Kohn, Isolation and purification of large quantities of DNA replication intermediates by pH step alkaline elution. Chromosoma 74, 125-139 (1979).

17. H.J. Edenberg and J.A. Huberman, Eukaryotic chromosome replication. Annual Review of Genetics 9, 245 (1975).

18. P.R. Cook and I.A. Brazell, Supercoils in human DNA. J. Cell Sci. 19, 261-279 (1975).

19. M. Gellert, K. Mizuuchi, M.H. O'Dea, and H.A. Nash, DNA gyrase: An enzyme that introduces superhelical turns into DNA. Proc. Natl. Acad. Sci. 73, 3872 (1976).

20. P.R. Cook, I.A. Brazell, and E. Jost, Characterization of nuclear structures containing superhelical DNA. J. Cell Sci. 22, 303-324 (1976).

21. M.R. Mattern and R.B. Painter, Dependence of mammalian DNA replication on DNA supercoiling. I. Effects of ethidium bromide on DNA synthesis in permeable Chinese hamster ovary cells. Biochim. Biophys. Acta 563, 293-305 (1979).

22. K.W.C. Peden, A rapid and simple method for the detection of mycoplasma and other intracellular contaminants. Experientia 31, 1111-1112 (1975).

23. G.T. Bowden, B. Giesselbach, and N.E. Fusenig, Post-replication repair of DNA in ultraviolet light irradiated normal and malignant transformed mouse cell cultures. Cancer Res. 38, 2709-2818 (1978).

24. M.R. Mattern and R.B. Painter, Dependence of mammalian DNA replication on DNA supercoiling. II. Effects of novobiocin on DNA synthesis in Chinese hamster ovary cells. Biochim. Biophys. Acta 563, 306-312 (1979).

25. J.L. Roti Roti and R.T. Winward, Factors affecting the heat-induced increase in protein content of chromatin. Radiat. Res. 81, 138-144 (1980).

26. K.W. Kohn, R.A.G. Ewig, L.C. Erickson, and L.A. Zwelling, Measurements of strand breaks and crosslinks in DNA by alkaline elution, in Handbook of DNA Repair Techniques, E. Friedberg and P. Hanawalt, eds. Marcel Dekker Inc., New York, pp.473-477 (1979).

27. A.E. Cress and G.T. Bowden, pH stepwise alkaline elution of DNA replication intermediates during S phase. Biochem. Biophys. Res. Commun. 102, 845-853 (1981).

28. R.B. Painter and B.R. Young, Formation of nascent DNA molecules during inhibition of replicon initiation in mammalian cells. Biochim. Biophys. Acta 418, 146-153 (1976).

29. R.A. Walters and C.E. Hildebrand, Evidence that X-irradiation inhibits DNA replicon initiation in Chinese hamster cells. Biochem. Biophys. Commun. 65, 265-271 (1975).

30. T.J. Laughlin and J.H. Taylor, The effects of X-rays on DNA synthesis in synchronized Chinese hamster ovary cells. Radiat. Res. 83, 205-209 (1980).

31. E.W. Gerner, DNA replication kinetics in X-irradiated Chinese hamster ovary cells. Radiat. Res. 71, 387-397 (1977).

32. E.W. Gerner and D.H. Russell, The relationship between polyamine accumulation and DNA replication in synchronized Chinese hamster ovary cells after heat shock. Cancer Res. 37, 482-489 (1977).

33. L.F. Povirk and R.B. Painter, The effect of 313 nanometer light on initiation of replicons in mammalian cell DNA containing bromodeoxyuridine. Biochim. Biophys. Acta 432, 267-272 (1976).

34. P. Forterre, Model for the supercoiling reaction catalyzed by DNA gyrase. J. Theoret. Biol. 82, 255-269 (1980).

35. K. Mizuuchi, L.M. Fisher, M.H. O'Dea, and M. Gellert, DNA gyrase action involves the introduction of transient double-strand breaks into DNA. Proc. Nat. Acad. Sci. USA 77, 1847-1851 (1980).

36. A. Morrison, N.P. Higgins, and N.R. Cozzarelli, Interaction between DNA gyrase and its cleavage site on DNA. J. Biol. Chem. 255, 2211-2219 (1980).

37. A. Sugino and N.R. Cozzarelli, The intrinsic ATPase of DNA gyrase. J. Biol. Chem. 255, 6299-6306 (1980).