Title
Effects of hyperthermia on Sphase [i.e. S phase] CHO cells

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Publication Date
1988

Peer reviewed|Thesis/dissertation

EFFECTS OF HYPERTHERMIA ON SPHASE CHO CELLS

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOPHYSICS

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

PREFACE

In general, it is futile to seek the elements of all existing things without distinguishing the various senses of 'be' and particularly when one investigates the nature of the elements of which things are composed. For it is surely impossible to discover the elements of activity or passivity or straightness; that can be done (if at all) only in the case of substances. It is therefore a mistake to look for, or to imagine that one has found, the elements of everything.

Aristotle, *Metaphysics*, Book A, Chapter IX, 20th Objection to Plato's Ideal Theory

Being is becoming.

Ilya Prigogine, *From Being to Becoming*

Most of this thesis is written in the styles required for the journals in which the individual chapters are either in press or have been submitted for publication. In particular, Chapter 2 is in press in *Radiation Research*; Chapter 3 has been submitted to *International Journal of Hyperthermia*; and Chapter 4 has been submitted to *Cancer Research*. Each of these chapters are self-contained journal articles, with separate Abstract, Materials and Methods, Results, etc. The Abstract of Dissertation, however, reflects the body of work on the whole.

I would like to thank all my friends, family, and associates who have supported me during these years spent in graduate school, without whom I would probably have never made it. I am especially grateful to my friends Douglas Spitz and Susan Davies for their friendship and support during this period. I am also deeply indebted to my advisor, Professor William C. Dewey, for all his time and effort spent in teaching and training me for a career as a scientist. And, last of all, I would like to thank Sandra for showing me the way.

ABSTRACT OF DISSERTATION

Title of Dissertation: Effects of Hyperthermia on S Phase CHO Cells

Cells in S phase of the cell cycle are more sensitive to the cytotoxic effects of heat than cells in G1 phase. We used aphidicolin, a specific inhibitor of DNA α polymerase to resynchronize cells near the G1/S border. Upon release from the drug-induced block in cell cycle progression, highly synchronous S phase cultures were obtained, allowing determination of the heat survival response over a range of temperatures (e.g. 41.5° to 45.5°C). The temperature dependence of the rate of cell killing of G1 and S phase cells was found to be very similar in this range, except that the S phase curves were offset from the G1 curves by about 1.5°C over the range of isosurvival levels studied.

While performing the G1/S comparison, a biphasic heat response was observed when S phase cells were heated at 41.5°C. This response was similar to the phenomenon of chronic thermotolerance, yet may have been due to progression of the cells during the heat treatment into a more resistant phase of the cell cycle. Further experimentation confirmed that such progression was occurring in the heated S phase cells, and that exponential killing occurred as long as the cells were in S phase. These results demonstrated that chronic thermotolerance is not expressed during S phase.

In the course of studying the progression of S phase cells heated at 41.5°C, a change in nuclear morphology was observed in the heated cells which developed upon recovery at 37°C after a 12 hr heat treatment. Further cytological examination of the heated cells revealed in about 50% of the population the development of a fragmented nuclear morphology which commenced after the time of the first division following the start of heating. We extended our observations in time and discovered that about 50% of the cells at the second division following the start of the heating showed premature chromosome condensation (PCC). These

results suggested that the nuclear fragmentation effect was causally related to this induction of PCC. Dramatic heat-induced ploidy changes were also observed, as increases in both aneuploid and tetraploid fractions of the cell population were observed. These cytogenetic changes appeared to be different from gross chromosomal aberrations induced by heating S phase cells.

Michael Austin Mackey

December 31, 1987

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CHAPTER 1
Introduction and Literature Review

I. Why Study Hyperthermia?

Ambient temperature variations have been demonstrated to produce biological effects upon species ranging from bacteria to man. Since environmental temperature changes may perturb the delicate metabolic balance within an organism, biophysical models of this phenomenon could provide an opportunity for study of the dynamic interplay between chemical and physical systems necessary for continued stability of the organism.

Living systems can be described quantitatively as complex systems wherein metabolic networks, through the agency of allosteric interactions and other nonlinear feedback controls, are able to maintain a nonequilibrium steady state (Prigogine and Nicolis, 1977; Schrödinger, 1944). Using thermodynamic arguments, it can be shown that the chemical affinity of each metabolic reaction is temperature-dependent (Katchalsky and Curran, 1965). However, an increase in temperature may act to either inhibit or stimulate specific reactions, and this effect may be highly nonlinear, thus precluding an analytical approach to prediction of temperature effects upon specific reactions. Any uncompensated changes in the reaction affinity could lead to destabilization of the steady state (Prigogine and Nicolis, 1977). Therefore, by disrupting the delicate homeostatic balance, environmental temperature changes are capable of generating thermodynamic instability in biological systems.

Since cellular control mechanisms are absent in isolated biochemical systems, kinetic investigations at this level cannot be extrapolated directly to the cellular state. In order to determine unequivocably the global effect of temperature change upon a biological system, a complete description of cellular metabolism, unattainable at the present state of knowledge,

would be necessary. Thus, an alternative, more phenomenological approach becomes necessary.

The influence of temperature upon biological stability is evident in the case of biological temperature optima (Johnson et al., 1974). This phenomenon is found in species ranging from microorganisms to vertebrates, is more pronounced in poikilotherms, and can be generalized as follows: At a given level of complexity (i.e. at the species level), variation of growth temperature leads to a change in a particular characteristic, such as body size of the adult, reproductive capacity, etc. Therefore, when such growth parameters are expressed as a function of temperature, extrema are observed which generally correspond to the same optimal temperature for multiple effects within a species. Furthermore, global characteristics of the particular systems often show a similar functional dependence, such as the ability to withstand high (Brock, 1985) or low (Rome et al., 1985) temperatures. Thus it would appear that responses to environmental temperature changes can lead to either an increase or a decrease in the evolutionary competence of organisms. Most of the hyperthermic effects to be described later have been determined in cell culture systems using modern techniques of cell biology, yet the study of heat shock in whole animals may lead to a better understanding of the relationship between this stress response and the generation of genetic diversity.

Regulation of gene expression in eukaryotes is a complex process. It has been estimated that about 99% of the genomic DNA in mammalian cells is transcriptionally inactive (Alberts et al., 1983). Therefore, investigation of transcriptional regulation of specific genes is currently of great interest. Heat shock-induced changes in eukaryotic gene expression have provided an excellent model system for the study of this regulation (for a review, see Lindquist, 1986). Furthermore, the study of generalized stress reponses in both prokaryotes and eukaryotes might lead to a better understanding of the interactions between biological systems and their environment.

Perhaps the main reason for studying the effects of elevated temperatures on cells lies in the application of hyperthermia in the cancer treatment clinic (Dewey and Freeman, 1980).

The therapeutic gain observed when heat is used with radiation is due to the fact that heat sensitizes cells to killing by ionizing radiation (Dewey et al., 1977). Heat alone and in combination with other modalities, such as radiation or chemotherapy, has been used in various clinical settings (Pettigrew et al., 1974; Dunlop et al., 1986). Although the greatest success has been observed following heat plus radiation therapy (Perez et al., 1983; Manning et al., 1982), heat in combination with chemotherapy has not been explored completely and may possibly yield the greatest benefit. This is because heat exposure can both increase activation (Riviere et al., 1986) and decrease clearance (Reinhold et al., 1986) of drug in the heated volume, thus minimizing normal tissue damage and enhancing tumor cure. It is also possible that hyperthermia reduces the radioresistant hypoxic fraction in tumors (Willett et al., 1987). The effectiveness of this approach depends upon the technology used in tumor heat delivery (Strohbehn and Douple, 1984), since the efficacy of the heat treatment has been shown to correlate with the minimum tumor temperature attained in the heat treatment (Dewhirst et al., 1983). In order to realize the potential of hyperthermia in the clinic, more investigations into the cell biology of heat shock are needed. An understanding of the basic modes of heat-induced cell death should assist in the optimization of its use as a cancer cure.

II. Historical Background of Interest in the Biology of Hyperthermia

The earliest scientific inquiry into the effect of elevated temperatures on biological systems was made in 1864 by Sachs who described the heat shock response in plants as a molecular change occurring without changing the appearance of the cells (Nover, 1984). A quarter of a century later, in 1888, Arrhenius began investigation into the temperature dependence of the catalytic effect of enzymes, then an area of considerable scientific interest. Arrhenius showed that isolated enzyme systems exhibited a log-linear relationship between initial reaction rate and reciprocal absolute temperature (for a review, see Johnson et al., 1974). In 1935 Bêlehràdek used the slope of the Arrhenius plot to calculate the temperature coefficient of thermal death in microorganisms. However, this author interpreted his results

as evidence supporting a currently popular philosophy, Vitalism, by postulating that death was caused by the loss of "vital energy" which occurred in a temperature-dependent fashion in the heated cells (Bêlehràdek, 1935).

With the advent of modern cell culture techniques in the late 1950's, a resurgence of interest in experimental heat shock studies occurred. In 1957, Selawry and co-workers selected HeLa cell sublines which were resistant to subsequent heat exposure. The method of selection used intermittent 42°C exposures, with intervening periods at 36°C (Selawry et al., 1957). In 1967 Harris, using a pig kidney cell line, employed clonal isolation techniques to verify that selection of a heat-resistant subpopulation alone did not account for the resistance observed by Selawry. In addition to characterizing certain cell biological parameters of the heat resistant variants, Harris applied Arrhenius analysis to the exponential rate of cell killing and discovered a log-linear functional dependence of the rate of heat inactivation of clonogenicity upon reciprocal absolute temperature, as Arrhenius had found in isolated enzyme systems (Harris, 1967).

Concomitantly with the development of the field of molecular genetics, heat shock became a laboratory tool in investigations of the genetic control of stress response. In 1962 Ritossa described heat-induced alterations in chromatin structure in *Drosophila*, a phenomenon known as chromosome puffing (Ritossa, 1962). These alterations in morphology have been shown to correlate with changes in gene transcription which are induced by stress. Similar morphological changes are also observed in the ontogeny of lower organisms, such as *Xenopus* oocytes and insects, which are associated with developmental changes (Alberts et al., 1983). This important discovery led to increased interest in the molecular aspects of the heat shock response, in particular the genetic control of the response.

In 1971 Westra and Dewey published a report (Westra and Dewey, 1971) which described the cell cycle dependence of heat-induced cytotoxicity. The results showed that cells heated in G1 phase were more resistant to heat killing than cells in S phase. These authors also reported a substantial increase in the fraction of tetraploid cells in the heated populations

at the first mitosis following the heat treatment administered to mitotic cells. Furthermore, the authors applied a theoretical interpretation to the Arrhenius inverse temperature dependence of the exponential rate of cell killing. This approach used Eyring's transition state theory (Johnson et al., 1974) to derive information pertinent to the mechanism of cell killing by heat. Specifically, it was asserted that the particular value of activation energy of heat-induced cytotoxicity (i.e. 141 kcal/mole), as derived from the slope of the Arrhenius plot, appeared to be within the same range of values obtained from thermal denaturation of isolated enzyme systems (see Johnson et al., 1974). Accordingly, it was hypothesized that the critical event in heat-induced cytotoxicity was thermal denaturation of proteins.

In 1974, Tissières et al. demonstrated that a specific class of proteins were preferentially synthesized following an acute heat shock in *Drosophila* (Tissières et al., 1974). These so-called heat shock proteins (HSP's) have since been found to be a large component of a wider class of stress proteins, and are implicated in a protective cellular response to stress. In the years that followed this discovery, HSP synthesis was seen in virtually every organism investigated and are induced by a variety of stresses, including heat and various kinds of oxidative stress (for reviews, see Ashburner and Bonner, 1979; Nover, 1984). Certain heat shock proteins have highly conserved DNA sequences showing homology in organisms ranging from bacteria to man. This sequence homology strengthens the proposition that the heat shock response has been important throughout evolution. Furthermore, since a mammalian HSP with an approximate molecular weight in the 70 kilodalton range is homologous to DNA K protein, a peptide involved in DNA replication in *E. coli*, a possible role for heat-shock proteins in the regulation of DNA synthesis has been postulated (Milarski and Morimoto, 1986). To date, however, no specific functional role for the protein products of heat shock genes has been demonstrated unequivocably.

III. Basic Phenomenology of Hyperthermia

A. Heat Survival Responses in General

When surviving fraction is expressed as a function of heating time in most *in vitro* mammalian systems, a sigmoidal response is observed. When the data are plotted on a semilog plot, the resulting survival curve is similar in shape to that observed for radiation survival: At short exposures a curving shoulder is seen, giving way to an essentially exponential region after higher exposure times. The presence of a shoulder region of the curve is generally interpreted to represent the ability of cells to accumulate sublethal damage (Harris, 1967; Dewey and Westra, 1971; Elkind and Sutton, 1960). Different phases of the cell cycle exhibit differing amounts of this shoulder, as S phase cells have much less shoulder than G1 cells (Dewey and Westra, 1971; Gerweck et al., 1975). In accordance with the interpretation of the shoulder as indicative of the ability to accumulate sublethal damage, S phase cells are also more sensitive to heat than G1 cells.

Heat exposure changes the morphology of cells, resulting in blebbing of the plasma membrane and collapse of the cytoskeleton (Borrelli et al., 1984; Borrelli et al., 1986b). In addition, heat induces a change in nucleolar morphology, resulting in an increase in the fibrillar component and a decrease in the granular component (Simard and Bernhard, 1967; Welch and Suhan, 1985). Also observed is the appearance in the nucleus of organized bundles of actin filaments (Welch and Suhan, 1985). When cells attempt to divide during chronic 41.5°C hyperthermia, multipolar division figures are observed, as well as early reformation of the nuclear membrane around chromosomes prior to anaphase (Coss et al., 1979).

B. Cell Cycle Dependence of Cell Killing by Heat

Heat sensitivity is cell cycle phase specific. Mitotic cells are the most sensitive to heat; G1 cells are the most resistant (Westra and Dewey, 1971; Palzer and Heidelberger, 1973). Mitotic cells probably die by disruption of the mitotic apparatus (Westra and Dewey, 1971),

since hyperthermia disassembles microtubules in cells and cell-free extracts (Coss et al., 1982), and also leads to the disruption of centrioles (Barrau et al., 1978), as well as the premature reformation of the nuclear membrane around mitotic chromosomes (Coss et al., 1979).

The sensitivity of S phase cells has been attributed to the induction of chromosome aberrations. Heated G1 cells exhibit only low frequencies of chromosomal deletions which are generally seen at the secondary constriction of the X chromosome and which show no dose dependence, while heated S phase cells yield aberrations in a dose-dependent fashion (Dewey et al., 1971). One study (Coss and Dewey, 1983) that compared cell morphology changes at the light microscope level to clonogenic survival, found that the percentage of cells heated in G1 which failed to reach mitosis following the heat shock was equivalent to the fraction of nonclonogenic cells, while the percentage of cells heated in S phase which reached mitosis with chromosome aberrations matched the percentage of cells killed. These authors suggested that G1 cells died in interphase, while S phase cells died a mitotic-linked cell death due to the induction of chromosome aberrations.

The pattern of cell cycle sensitivity to heat (Westra and Dewey, 1971) is complementary to that observed for X-irradiation, where S cells are the most resistant to X-rays and show a greater amount of shoulder than the sensitive G1 cells (Sinclair and Morton, 1965). This complimentarity was one of the main reasons for investigation of the clinical benefits of heat plus X-irradiation (Dewey et al., 1977), since the heat treatment would be highly toxic to the radioresistant S phase fraction in the tumor.

C. Effects on Cell Cycle Progression

Analysis of cell cycle progression of G1 and asynchronous cells during chronic heat exposure demonstrated delays of 5-7 hr and 40-60 hr at 41.5° and 42°C, respectively (Sapareto et al., 1978; Read et al., 1983). At higher temperatures (i.e. 45° - 45.5°C), a dose-dependent effect on cell cycle progression delay in all phases has been observed using the techniques of flow cytometry (Rice et al., 1984a; Rice et al., 1984b; and Read et al., 1984).

These studies demonstrated the longest delays in cells heated in G1 and the shortest delays in cells heated in S or G2/M phases. For example, a 20 min 45.5°C treatment of CHO cells induced a 7-fold decrease in the rate of progression through G1, while an equal treatment caused only a 2-fold decrease in progression of S phase cells through the DNA synthetic period. These findings are in contrast to the greater heat sensitivity of S phase cells, as compared to G1 cells.

D. Thermotolerance

The intrinsic heat sensitivity of biological systems depends on its past thermal history. Previous exposure of cells to subnormal growth temperatures (e.g. 35°C) sensitizes cells to the lethal effects of hyperthermia (Li and Hahn, 1980; Anderson et al., 1981), while exposure to 39°C before heating results in the protection of heat toxicity (Li and Hahn, 1980).

When G1 and asynchronous cells in culture are exposed to a single, nonlethal 45°C heat treatment and allowed to recover for several hours at 37°C, an induced resistance to further hyperthermic cell killing termed thermotolerance is observed (Gerner and Schneider, 1975; Henle and Leeper, 1976). Thermotolerance results in modification of both the shoulder and the final slope of the heat survival curve. Post treatment incubation at 0°C inhibits the development of this tolerance, suggesting that cellular metabolism is necessary for this effect (Gerner and Schneider, 1975). Furthermore, the kinetics of the development of acute thermotolerance, as expressed in an increase in cell survival, correlate with the increase in the relative rate of synthesis of HSP70 after the heat shock (Li and Werb, 1982). This correlation has led many investigators to suggest a causal role for HSP synthesis in the development of thermotolerance (Li et al., 1982). Cells grown in medium containing amino acid analogues are unable to develop acute thermotolerance even though analogue-containing HSP 70 is synthesized, presumably due to the lack of functional HSP synthesis (Laszlo and Li, 1983). When S phase cells are given a slightly toxic heat dose and allowed to recover at 37°C for several hours, an induced resistance similar to acute thermotolerance is observed (Rice et al., 1984b; Read et al., 1984). However, since cell cycle progression is not completely blocked

by this heat treatment as in G1 cells (Read et al., 1984), it is possible that this enhanced heat resistance is due to redistribution of cells into a more resistant compartment of the cell cycle.

When G1 and asynchronous cells are chronically exposed to temperatures of about 42.5°C and below, an induced resistance is observed which develops during the heat treatment (Read et al., 1983; Sapareto et al., 1978). In order to avoid confusion with acute thermotolerance, this phenomenon has been called chronic thermotolerance. Chronic thermotolerance can persist for up to several days during exposure to the elevated temperatures, yet eventually decays, resulting in the resumption of exponential cell killing (Read et al., 1983; Freeman, 1982; Dewey and Esch, 1982). One study comparing S and G1 phase CHO cells heated at 42°C found no development of chronic thermotolerance in S phase (Read et al., 1983). Furthermore, this study demonstrated a correlation between the eventual decay of chronic tolerance induced in G1 cells with the entry of cells into S phase. These results suggest that S phase is not permissive to the development of chronic tolerance, at least in the sense of that observed in G1 and asynchronous systems. Since S phase is more sensitive to the cytotoxic effects of heat shock, the response observed in asynchronous systems is probably dominated by the more resistant G1 cells (Dewey and Cole, 1962).

Other agents besides heat induce thermotolerance. Exposure of cells to low concentrations of sodium arsenite, zinc chloride, ethanol, and cadmium chloride induces HSP synthesis and thermotolerance (Li and Werb, 1982; Anderson et al., 1983). Treatment before and during the heat treatment with the inhibitors of protein synthesis cycloheximide or puromycin protect cells from heat killing at 43°C (Lee and Dewey, 1986), yet it is not clear that this phenomenon is truly thermotolerance, since post-heat incubation with these inhibitors can reduce thermotolerance (Henle and Leeper, 1982; Lee and Dewey, 1987). Heat-resistant variants of HA-1 cells show elevated levels of HSP70, suggesting that HSP levels are connected with intrinsic heat resistance (Laszlo and Li, 1985). However, pretreatment exposure to hydrogen peroxide appears to induce HSP70 without the development of thermotolerance, yet with the development of peroxide resistance. Nevertheless, pretreatment

of cells with heat induced resistance to a subsequent hydrogen peroxide challenge (Spitz et al., 1987). Thus, it is possible that in mammalian cells genetic control of the heat shock response overlaps with other stress responses, such as oxidative stress, as has been observed in bacteria (Christman et al., 1985), and it is also possible that HSP synthesis is associated with, but not necessary for thermotolerance induction.

E. pH Effect on Heat Sensitivity

Intrinsic heat sensitivity is also modulated by extracellular hydrogen ion content both at the time of treatment and in the interval following treatment (Freeman, 1978). This pH dependence is part of the rationale for the clinical use of hyperthermia, as the microenvironment of the radioresistant hypoxic cell fraction of tumors is at low pH (Dewey et al., 1980). Heat treatment at low pH (6.6-6.8) sensitizes cells as much as 5-fold and also inhibits the development of chronic thermotolerance (Freeman, 1978), while post-treatment incubation at low pH potentiates heat-induced cytotoxicity and inhibits the development of acute thermotolerance (Holahan et al., 1986).

F. Thermal Dose

In all the biological systems so far investigated, cells exhibit thermal clonogenic inactivation rates that are determined by the time-temperature combination of the heat treatment (Dewey et al., 1977; Field and Morris, 1983). Since the temperature dependence of the terminal rate of inactivation as a function of temperature has been determined for asynchronous and G1 CHO cells in culture (Dewey and Westra, 1971; Sapareto et al., 1978; Henle and Dethlefsen, 1980), this information can be used to predict an equivalent heating time at a reference temperature to reach a given isoeffect, thus providing a convenient concept of thermal dose for use in clinical applications of hyperthermia (Sapareto and Dewey, 1984). Specifically, this approach established that, for exposure temperatures in excess of about 42.5°C, an increase in temperature of 1°C requires that the time of exposure be reduced to 1/2 of that at the lower temperature in order to achieve the same survival level. Below

42.5°C, an increase of 0.5°C requires a reduction to 1/2 of the exposure time to achieve isoeffect. Although the development of thermotolerance during chronic treatment at temperatures of 42.5°C and lower, as well as tolerance development after an acute treatment, causes deviations from this idealized law, the functional dependence of the rate of cell killing upon temperature exposure can be extended to these situations by modifying the model accordingly (Sapareto, 1987).

G. Effects of Heat on Cell Membranes

Cells exposed to elevated temperatures demonstrate an alteration both in the structure and function of membraneous subcellular organelles. Effects upon plasma membrane structure (Anderson and Hahn, 1985; Borrelli et al.1986a) and mitochondria (Borrelli, 1984), are some of the most striking morphological changes observed after heat shock. The hypothesis has been presented that cellular membranes are the primary target for cell killing by heat (Yatvin, 1977; Hahn, 1982). One study (Borrelli et al., 1986b) demonstrated a clear connection between changes in membrane morphology and heat-induced cell killing, yet this correlation was measurable only at 45.5°C in G1 cells, and therefore may not be generally associated with heat-induced cell killing in other experimental systems. Membrane active agents such as procaine sensitize cells to hyperthermia (Coss and Dewey, 1983), suggesting a role for membrane damage in cell killing by heat. However, no membrane lipid phase transitions occur in the hyperthermic range of temperatures (Lepock et al., 1983), and membrane potential is unaffected during and immediately after a heat treatment (Borrelli et. al., 1986a). Although the heat-induced increase in total cell calcium content is reduced after heat treatment when intracellular calcium is lowered by lowering extracellular calcium, no change in heat sensitivity is observed (Vidair and Dewey, 1986). Furthermore, Na^+, K^+, Cl^-, (Vidair and Dewey, 1986; Borrelli et al., 1986a) and H^+ (Chu and Dewey, 1987) gradients across the cell membrane are unaffected by heat. Therefore, we conclude that the plasma membrane is probably not the primary target of cell killing by heat.

I. Changes in Nuclear Non-Histone Protein Content Induced by Heat

Heat shock causes a change in the non-histone protein content of the nucleus. Following a short, acute heat treatment, the protein content of the nucleus is reversibly increased (Tomasovic et al., 1978). The initial rate of entry of excess protein is temperature dependent, and the kinetics of the exit of excess nuclear protein correlate with the development of acute thermotolerance (Roti Roti et al., 1979; Roti Roti et al., 1986). Furthermore, agents which are believed to act at the nuclear membrane, such as straight-chain alcohols, are synergistic with respect to both heat-induced nuclear protein increases and cell killing (Roti Roti et al.,1984). This influx of protein into the nucleus associated with hyperthermic treatment has also been postulated to account for the phenomenon of heat radiosensitization (Roti Roti, 1982), as it presumably interferes with the accessibility of repair enzymes to radiation-damaged DNA.

J. Effects of Heat upon Macromolecular Synthesis

Heat shock is a potent inhibitor of macromolecular synthesis in the cell. A heat treatment of 15 min at 45.5°C rapidly inhibits 80% of protein synthesis in an asynchronous population of CHO cells (Lee and Dewey, 1987). Heat also disrupts polyribosomes and this effect is probably responsible for the rapid inhibition of protein synthesis by heat (McCormick and Penman, 1969), as both initiation and elongation of nascent peptides are blocked by heat exposure (Oleinick, 1979). RNA synthesis is also inhibited by heat shock (Levine and Robbins, 1970). Furthermore, heat inhibits the processing of ribosomal RNA precursors, resulting in a change in nucleolar morphology (Amalric et al., 1969; Simard and Bernhard, 1967). DNA synthesis is rapidly inhibited by acute heat shock (Wong and Dewey, 1982; Warters and Stone, 1983). Following a 15 min heat shock at 45.5°C, replication is inhibited about 80-90% (Wong and Dewey, 1982). Heat-induced blockage of replicon initiation occurs immediately and lasts for at least 4 hrs, while replicon elongation ceases for about 20 min following the heat treatment, and then continues at near the control rate. The long delay in initiation has been postulated to lead to the induction of chromosome aberrations and may account for the observed sensitivity of S phase cells. This hypothesis is supported by the

observation that when bromodeoxyuridine (BrdUrd) is added prior to heat shock of S phase cells, an additive effect on survival is observed, suggesting that both heat and BrdUrd are affecting the same subcellular components (Dewey et al., 1971).

Hyperthermia also decreases the activity of DNA α and β polymerases (Spiro et al., 1982). The rate of loss of these activities show time-temperature relationships similar to that observed for cell killing (Spiro, et al., 1982; Dewey and Esch, 1982). The restoration of activity of α polymerase occurred later than that of polymerase β, and the kinetics of the recovery of polymerase β activity correlated with the loss of heat radiosensitization following the heat treatment (Mivechi and Dewey, 1985). Thus, heat inactivation of DNA polymerase β may account for heat radiosensitization.

IV. Studies Performed in this Thesis

This thesis is an investigation into some of the effects of heat shock on synchronized populations of S phase CHO cells. Whereas earlier reports have described some effects of heat on asynchronous and G1 cells, S phase responses to heat have been more difficult to obtain. This is due to the challenge of obtaining highly synchronous S phase cultures. A population which was 95-99% mitotic, as obtained by the mitotic shakeoff technique, will demonstrate only about 90% purity by the time these cells have entered S phase. Since we were interested in studying survival responses, this degree of synchrony would render the system unusable, as only one decade of a logarithmic survival scale would be available for analysis. Therefore, we resynchronized initially mitotic cells at the G1/S border using aphidicolin, a specific inhibitor of DNA α polymerase. Upon evaluation of this experimental system, we discovered 99% of the cells were in early S phase, which then allowed a quantitative comparison of heat killing of G1 and S phase cells to be performed over a range of hyperthermic temperatures.

While performing this G1-S comparison, a biphasic response of S phase cells to 41.5°C exposure was observed. Further investigation demonstrated that S phase cells heated at 41.5°

- 42°C were able to progress out of S phase into a more resistant phase during the heat treatment. While studying the morphology of those cells progressing during the heat treatment, we observed several changes occurring in the population. Eventually, we were able to describe a quantitative relationship between heat-induced nuclear fragmentation, induced by attempted division during the 41.5°C exposure, and the appearance, at the next mitosis, of prematurely condensed chromosomes (PCC) in metaphase chromosome preparations. The frequency of heat-induced nuclear fragmentation was equivalent to the incidence of metaphase-associated PCC among the mitotic cells, yet nuclear fragmentation preceded the PCC effect. This study provided evidence suggestive of a causal relationship between nuclear fragmentation and metaphase-associated PCC. Furthermore, changes in ploidy occurred immediately following the heat exposure, presumably demonstrating the heat-induced disruption of mitotic division caused by the heat exposure.

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CHAPTER 2

Time-Temperature Analyses of Cell Killing of Synchronous G1 and S Phase Chinese Hamster Cells in vitro

ABSTRACT

Time-temperature analyses of durations of heating required to achieve isosurvival were used to compare hyperthermic cell killing of synchronous Chinese hamster ovary (CHO) cells heated in G1 or S at temperatures of 42° to 45.5°C. G1 populations were obtained by incubation of mitotic cells for 90 min at 37°C. S phase populations were obtained by incubation of mitotic cells for 12 hrs at 37°C in medium supplemented with 2 μg/ml aphidicolin, a reversible inhibitor of DNA α polymerase; S phase survival was also determined in an aphidicolin-free system by using high specific activity ^{3}H-thymidine. In both systems, the thermosensitivity was similar and decreased as the cells progressed from early S phase, in agreement with earlier studies (Read, RA, Fox, MH, and Bedford, JS, Rad Res 98:491-505 (1984)).

A comparison of Arrhenius plots of the inverse of durations of heating required to achieve isosurvival for cells heated in G1 or S phase showed similar temperature dependence above 43.5°C; yet the plots for heat-sensitive S phase cells were offset from those for heat-resistant G1 cells by about 1.5°C, i.e. S phase cells respond to 43°C with a rate similar to that observed in G1 cells heated at 44.5°C. Using least-squares regression of the semilog plots, the curves were analyzed either as continually bending curves or as two straight lines with a break at 43.5°C. When the data were analyzed using two straight lines, no significant differences in the slopes of the time-temperature plots of G1 or S phase cells was observed. A quantitative comparison between the two methods of data analysis demonstrated that in both phases the data were better fit with a continuously curving line, rather than two straight lines.

INTRODUCTION

Previous studies have demonstrated that the mechanism of hyperthermic cell killing of S phase cells probably differs from that involved in the killing of G1 cells. S phase cells are much more sensitive to heat than asynchronous or G1 populations (1,2,3), and hyperthermia-induced chromosomal aberrations correlate with the decrease in survival of S phase cells, but not that of G1 cells (4). Therefore, the effects of heat on DNA replication may be a critical event in S phase (5,6,7), especially since heating of CHO (8) or HeLa (9) cells has been shown to inhibit both the replicon initiation and elongation processes of DNA synthesis. The hypothesis was presented (8) that the long delays in replicon elongation and increases in the amount of single-stranded DNA are responsible for the chromosomal aberrations induced when cells are heated in S phase.

The event responsible for the killing of cells heated in G1 has eluded investigators. A popular hypothesis (3) is based upon the application of a particular thermodynamic model, Eyring's Transition State Theory (10), to results obtained from the analysis of survival data. The theoretical basis for this model has been described in detail elsewhere (11,10,3); essentially, it is assumed that some rate-limiting reaction(s) dominate the temperature dependence of the rate of hyperthermic cell killing. Eyring's theory then predicts that these reaction(s) are characterized by a particular value of activation enthalpy($\Delta H^{\pm}$), as obtained from the slope of an Arrhenius plot. This analysis has also been applied to a variety of *in vivo* and *in vitro* data (12,13,14,15) and may be summarized as follows: the temperature dependence of the rate of cell killing was found to be biphasic, with a discontinuity at approximately 43°C. Above this "break" temperature, the calculated activation enthalpy, $\Delta H^{\pm}$, was found to be about 140 kcal/mole for a great variety of cell types and tissue endpoints; this value is in the range observed for protein denaturation in cell-free systems. Below 43°C, the corresponding $\Delta H^{\pm}$ value is usually at least twice the magnitude of that of the higher temperature interval, suggesting that different mechanisms occur in the two

temperature ranges. According to this hypothesis, then, protein denaturation is the rate-limiting step for cell killing in the upper hyperthermic range (i.e. above the break).

The applicability of the Eyring approach to the analysis of time-temperature relationships in hyperthermic cell killing has not been established unequivocably (11). Eyring's equilibrium thermodynamic model requires linearity between the rate of cell killing and inverse absolute temperature (10), and the variability inherent in survival assay systems makes the demonstration of such linearity a difficult task to pursue. Furthermore, this type of analysis has only been possible for killing of G1 cells, since G1 cells are much more heat resistant than S phase cells, therefore dominating the asynchronous heat survival response.

To date no studies of hyperthermic cell killing of S phase cells over a wide range of temperatures have been reported. One possible reason for this is that highly synchronous S phase cultures are difficult to obtain. Typically, a population which is better than 95% synchronous at mitosis retains only approximately 90% synchrony after having progressed into S phase. Survival curves derived from such populations are characterized by a biphasic response, as the survival of heat resistant non-S phase cells dominates below the 10% survival level. Thus, in these systems, survival curves for S phase cells cannot be evaluated for survival levels below about 10%.

In this study we present G1 and S phase survival data obtained using highly synchronous cultures exposed to a range of temperatures (i.e. 41.5 - 45.5°C). S phase synchrony was accomplished by combining mitotic selection (1) with the use of aphidicolin, a reversible inhibitor of DNA polymerase α (16,17), to resynchronize cells near the G1/S border. The recent development in our laboratory of a technique used to freeze mitotic cells (18) allowed experiments to be performed using the same synchronous cell populations. Then, cell killing of S phase cells was compared to that of G1 cells using time-temperature analyses of curves obtained from the durations of heating required to achieve various iso-survival levels.

MATERIALS AND METHODS

Culture Conditions and Synchrony Methods

Chinese Hamster Ovary (CHO) cells were grown in McCoy's 5a modified medium (Gibco) containing 10% heat inactivated fetal calf serum (J.R. Scientific Co.), and the antibiotics neomycin sulfate (0.1 g/l), potassium penicillin G (0.07 g/l) and streptomycin sulfate (0.1 g/l) (Sigma). Exponential cultures were grown at 37°C in a humidified atmosphere and maintained at pH 7.4 with 5% CO^2. Mitotic cells were obtained from these stocks by the mitotic selection technique, as described previously (1); mitotic indices ranged from 98-99%. For the survival curves shown in Figures 3 and 4, the mitotic cells used were frozen in a solution which has been shown to protect against adverse effects of freezing on plating efficiency, heat survival, and cell cycle progression (18). Plating efficiencies ranged from 50 to 70%.

For S phase synchrony experiments, mitotic cells were incubated in suspension culture for 12 hours at 37°C in McCoy's 5a medium supplemented with 2 μg/ml aphidicolin (Sigma). At the end of the incubation period, the cell suspension was centrifuged (900 rpm - 10 min @ 4°C), and the pellet was resuspended in fresh drug-free medium. Then, the cells were plated into flasks containing medium at room temperature and equilibrated at pH 7.4. The flasks were then placed into an incubator at 37°C to allow the cells to attach before they were treated. After incubation for 1 to 8 hours, some of the cultures were placed at 20°C for up to an hour to prevent cell cycle progression during the treatment periods; this treatment had no effect upon subsequent cell progression or survival (data not shown). Furthermore, the exposure to aphidicolin did not effect the plating efficiency, as compared to control cultures held for 12 hours in suspension, i.e. 58 $\pm$ 3% (plating efficiency $\pm$SEM) for both the drug-treated cells and untreated controls.

For the full survival curves shown in Figure 4, S phase heat exposure began 2 hrs after the cells were plated. G1 survival experiments used frozen mitotic cells obtained on the same day of synchrony as those used in the S phase studies and were plated as described above for S phase cells, with the exception that G1 cells were not treated with aphidicolin in suspension. Heating of G1 cells commenced 90 min after plating.

Survival Assay

Surviving fraction was estimated by plating cells into T25 flasks (Falcon) to which lethally irradiated (25 Gy.) feeder cells were added 24 hrs prior to the experiment to control cell density at 10^5 cells/flask while heating (19). To help minimize systematic variations in survival, only one lot of powdered medium and serum was used in all survival curves shown in Figures 3 and 4. Immediately prior to the heat exposure, flasks were sealed with paraffin and placed in racks. Survival was normalized to the apparent plating efficiency (55-65%) obtained by extrapolation of the survival curves to zero dose, by assuming a zero initial rate of cell killing. Although the multiplicity was 1.6 for G1 cells and 1.0 for S cells, no multiplicity correction was applied because the viable multiplicity for G1 cells was estimated to be close to 1.0, using the method outlined in Appendix I of this thesis (data not shown). Following incubation for colony formation (7-11 days @37°C), the flasks were fixed and stained with crystal violet, dried, and colonies of 50 or more cells were scored. All determinations were performed using at least 5 replicates, and the standard errors of the means of the survival values are indicated when larger than the size of the data points in figures.

The curves shown in Figs. 3a and 4a were obtained on the same day; 43.5°, 44.5°, and the lower 45.5°C curves in Figs. 3b and 4b were performed on the same day one week later. The 43° and upper 45.5°C curves shown in Figs. 3b and 4b were obtained on a day 3 weeks later. All the data shown in Figs. 3 and 4 were derived from the same population of mitotic cells. In each case, G1 curves were obtained on the same day as the S curves.

Tritiated Thymidine Suicide

Forty minutes before the initiation of hyperthermic exposure, the medium was removed from flasks containing feeders and viable cells, and was replaced with 2 ml of pH- and temperature-equilibrated bactopeptone-free McCoy's 5a medium supplemented with 10% dialyzed fetal calf serum containing either high specific activity (h.s.a.) tritiated thymidine (75 Ci/mM-Amersham Corp.) at a concentration of 4 μCi/ml, or an equivalent amount of stable thymidine (Sigma) for sham controls. 5-10 replicate flasks were used in each survival determination. After a 30-minute pulse at 37°C, the cultures were rinsed twice with 5 ml room temperature, pH-equilibrated Hank's BSS; then, 5 ml of regular McCoy's medium supplemented with 10 μg/ml stable thymidine was added. Previous experiments have demonstrated that these conditions result in ^{3}H incorporation of about 8 disintegrations per labelled cell per minute which issufficient to kill all the S phase cells in the population (data not shown). Sham plating efficiencies were 40-45%. For the experiment in which differences between survival of heat+sham and heat+^{3}H-TdR groups were calculated (Fig. 2), an estimate of the SEM of the difference was obtained by pooling the variances of the plating efficiencies of the two groups for each time point.

Labeling Index

The labeling index was determined by plating 5 X 10^{4} cells into T25 flasks; 20 min pulses using low specific activity tritiated thymidine (25 Ci/mM - Amersham Corp.) at a concentration of 4 μCi/ml in the regular culture medium were followed by fixation with fresh Carnoy's fixative (3:1 methanol:glacial acetic acid). The cultures were then rinsed 3 times with Carnoy's and allowed to dry overnight, after which the flasks were cut open, coated with Kodak NTB Nuclear Track emulsion and exposed for 9 days at 4°C. Following development, the flasks were stained using Wright's stain and scored for percent labeled cells, counting at least 200 cells for each determination.

Hyperthermic Treatment

All hyperthermia exposures were performed in Precision GCA waterbaths (GCA Corporation) which adhere to a precision of ± 0.03°C. Thermometers were calibrated by reference to a Brooklyn thermometer which was factory calibrated against reference standards conforming to National Bureau of Standards specifications (20). In order to standardize warm-up and cool-down transients, prior to and immediately following hyperthermic exposures the flasks were maintained in a 37°C waterbath (± 0.1°C) for at least 10 minutes. Under these conditions, exponential warm-up and cool-down half-times at 45.5° were 24 sec, as determined from thermistor temperature measurements of 4.5 ml of medium in the flasks.

Time-Temperature Analysis

In order to quantitatively compare the temperature dependence of the rate of cell killing in the two cell cycle phases, time-temperature plots were constructed. Traditional analysis using the slopes of the exponential portion of the survival curves was not used since the curves did not manifest a truly exponential portion. Furthermore, G1 and S survival curves differ primarily in the amount of shoulder on the curve; such nonlinear contributions to an average rate of hyperthermic cell killing are not included in the analysis of slopes. Accordingly, an isoeffect approach was chosen for the analysis (13,21). The rationale for the use of this endpoint for determining time-temperature relationships has been explained in detail elsewhere (21). The intersection of survival curves with the appropriate isosurvival level was extrapolated directly to the time axis; then, the logarithm of the reciprocal of this isosurvival exposure time was expressed as a function of temperature.

To perform least-squares regression upon these data, normal equations were constructed, after logarithmic transformation of the reciprocal time values, using a basis which was quadratic in temperature. Solution of the resulting matrix yielded unique values for the coefficients $\{a_i\}$

(1) $\log(1/\text{time}) = a_0 + a_1 T + a_2 T^2$.

where T is temperature in °C (see Appendix II of this thesis for details of the method used in the analysis). This polynomial produced the best fit to the data when compared to polynomials of higher or lower degree.

As a measure of the temperature dependence of the rate of cell killing, the relative decrease in rate over a temperature change of 1°C, R, is often used (21). R is defined as:

(2) R = 1/time for T°C / 1/time for T+1°C

for a given isoeffect.

To account for temperature transients, the isosurvival time values were corrected for the biologically equivalent time at each exposure temperature, using warm-up and cool- down data from thermistor measurements of the temperature of the medium in flasks obtained under conditions identical to those used experimentally. Equivalent time was calculated using a modification of a computer program described previously (22). The program was modified to allow specification of the R value by the user, and this single R value was used in the numerical integration to calculate the equivalent time at the specified temperature. After an initial regression analysis of the data, the R value obtained from Eq. (2) was used to compute the appropriate equivalent time at the particular temperature, and this time was used to calculate a correction to the isosurvival time values. Following this correction, the data was again analyzed using regression analysis. For example, for flasks containing G1 cells heated at 45.5°C to give 50% isosurvival, the difference between the observed warm-uptime (4.5 min) and the equivalent time at 45.5°C during this warm-up (2.50 min) was used to calculate a correction to the overall heating time at the elevated temperature (i.e. 2.50 - 4.50 = -2.00 min). Similar computations showed that a total of 0.07 min of equivalent time at 45.5°C was

experienced by the system during cooling, so the total correction was determined to be -2.00 + 0.07 = - 1.93 min. When an identical analysis was performed upon 44.5°C warm-up and cool-down transients at this same isosurvival level, a correction value of -1.24 min was calculated. Subsequent iterations of the regression-correction procedure described above failed to produce results significantly different from the initial correction.

RESULTS

Thermal Sensitivity of S Phase Cells

Hyperthermic sensitivity of cells decreased as the cells progressed from early S through the DNA synthetic period into G2, M, and probably G1 (Figure 1 and our unpublished data showing cell division between 5 and 8 hrs after release from the G1/S border). In this experiment, more than 99% of the cells were labeled at 2 hours after release. These results confirm observations by other investigators using S phase CHO (8) and HeLa (9) cells synchronized with either hydroxyurea or aphidicolin. These previous studies attributed much of the decrease in sensitivity in CHO cells to progression through S phase, but in HeLa cells, to progression into G2.

To determine if a decrease in thermosensitivity could be observed as cells progressed through S phase without using any drug treatment to achieve synchrony, a synchronous population of mitotic cells was pulse labeled with h.s.a. tritiated thymidine at various times after mitosis to selectively kill the S phase cells. Immediately after labeling, the cells were heated for 10 min at 45.5°C (Figure 2). The difference between the surviving fraction obtained from heat alone and from heat + ^{3}H-suicide can be attributed to the survival of the S phase cells in the population (dashed line in Figure 2). Survival comparable to that seen in the aphidicolin system was observed in this experiment, and increased 10-fold as the cells progressed through S phase.[1]

Survival of Cells Heated in G1 or Early S Phase

Since heat survival increased as cells progressed from early S phase (Figures 1 and 2), cells were heated, in all cases 2 hrs after release from the aphidicolin block near the G1/S border (Figure 4). These curves, when compared to curves from cells heated in G1 (Figure 3), show that S phase cells were much more sensitive to hyperthermic exposure than G1 cells. For example, after 60 minutes at 43°C, survival for G1 cells was about 0.60, compared to about 0.03 for S phase cells. In addition, the S phase curves exhibited much less of a shoulder than the G1 curves, as has been described previously (1). The biphasic survival curves observed for survival values less than 0.01 - 0.05 for cells heated in S is due to the lower heat sensitivity of the non-S phase population (mostly G1), as, below these survival levels, the survival of the resistant population predominates (24). This phenomenon is illustrated in Figure 4b for temperatures of 43.5°, 44.5° and 45.5°C. Because of this complication, only S phase data for survival values above 0.01 - 0.05 can be analyzed.

S phase cells exposed to 41.5°C for more than 3 hours exhibited some resistance to heat (Figure 4a) which was similar to the phenomenon termed chronic thermotolerance observed in G1 ((15) and Figure 3a) and asynchronous systems (15). This thermoresistance was not attributed to any non-S contamination in the population, as confirmed by ^{3}H suicide experiments (data not shown). However, this resistance could be related, at least in part, to cell cycle progression, as heat sensitivity decreased as the cells progressed from early S into late S and G2 (Figures 1 and 2) and resynchronized systems (24,25).

Experiments in our laboratory have shown that at 42°C cells undergoing continuous heating which commenced in early S are capable of progressing through S at a reduced rate during the heat treatment (manuscript in progress, also see (23)). At 42°C, cells progressed through S at about 1/5 the control rate; at 41.5°C, the rate was about 1/2 of control. Therefore, we suggest that the biphasic response observed in S phase cultures heated at 41.5° and 42°C (see Figure 4a) is due to progression to a more resistant phase of the cell cycle. Note that the survival curves for S phase cells heated at 41.5° and 42°C do not exhibit a

plateau as observed for cells heated in G1 (Fig. 3a). This suggests that cells heated in S phase do not develop chronic thermotolerance. At 42.5°C, however, the flattening of the survival curve observed at 10^{-3} survival in S phase experiments (Figure 4a) must be due to non-S phase cells contaminating the population, as h.s.a. ^{3}H-TdR pulses administered before heating failed to produce any significant difference in survival when compared to sham treated controls (see Table I).

Figure 5 shows a semilogarithmic plot of the reciprocal of the time required to achieve various survival levels in the populations heated either in G1 or S phase as a function of temperature. A combined plot of both the G1 and S data from Figure 5 (shown in Figure 6) demonstrates that the curves were roughly parallel from 43.5° to 45.5°C and that the S cells were more sensitive to heat than the G1 cells. For a given isosurvival level, the S phase curve was displaced towards a shorter time to reach iso-effect, or, stated differently, the S phase curve was shifted relative to the G1 curve about 1.5°C lower on the temperature scale. These results confirmed two previous experiments performed using different populations of mitotic cells in the synchrony protocols (data not shown).

These data were analyzed using least-squares regression analysis, as described in Materials and Methods and Appendix II of this thesis, and the results are presented in Table II. Previous studies (13,21) have employed the Eyring formalism in the analysis of time-temperature data. Using this model,the logarithm of the rate of cell killing is expressed as a function of inverse absolute temperature, 1/T(K), and the data are fit by a biphasic linear function with a discontinuity in the mid-temperature range. Since our data were curvilinear over the temperature range studied, we decided to investigate whether these data were better fit by a quadratic function of absolute temperature, as compared to the 1/T fit. A biphasic fit to the data in Figures 5 and 6 was optimal when a break was assumed at 43.5°C, accordingly, 43.5°C was used for the "break" temperature. When residuals were calculated based upon the two models, a quadratic function of temperature produced the best fit to the experimental data (see Table III). For comparison with other data in the literature, however, a biphasic

linear plot was constructed and used to calculate R values. Since there was very little data below 43.5°C at 0.1% isosurvival, these data were excluded from the analysis. As shown in Table IV, there were no significant differences observed between the slopes of the isosurvival time plots for both G1 and S phase cells. R values ranged from 0.23 (S phase - 10% isosurvival - 42-43.5°C) to 0.49 (G1 phase - 10% isosurvival - 43.5-45.5°C). These R values are very similar to those obtained previously (13,21) in a variety of experimental systems, and correspond to calculated values for $\Delta H^{\pm}$ of about 140 and 300 kcal/mole, respectively for G1 (43.5-45.5°C) and S (42-43.5°C) populations. Although these results are obtained from what is essentially one experiment, an earlier experiment using a different population of synchronous cells yielded similar values: i.e. R values of 0.36 and 0.35 in the range 42-43.5°C and 0.53 and 0.47 in the range 43.5-45.5°C for 50% isosurvival in G1 and S phase, respectively.

DISCUSSION

While cells synchronized in early S phase were much more heat sensitive than G1 cells (Figures 3 and 4), the temperature dependence of the rate of cell killing, as determined from the slopes of the isosurvival plots in Figures 5 and 6, appeared to be roughly the same in the two phases in the temperature range studied. It is interesting to note that the S phase curves in Figure 6 below 43°C bend more than those for G1 cells. The break in the usual Arrhenius curve of heat survival data at about 43°C has been attributed to the development of chronic thermotolerance for asynchronous cells and for cells heated in G1 (12,15,27). In our experiments, however, cells heated in G1 were analyzed through periods during which very little chronic thermotolerance had developed, i.e. from zero to less than 3 hours of heating, which may account for the lack of an obvious break in these analyses. As mentioned in Results, the curvature observed in S phase curves is complicated by cell cycle progression and is probably not due to the development of chronic thermotolerance. This would seem to

indicate that, at least for S phase curves analyzed using an isoeffect endpoint, this interpretation of the break is not appropriate.

Analysis of the slopes of Arrhenius curves is often used to deduce the mechanism of thermal effects on biological systems (10,3). However, the similarity between the slopes of the G1 and S curves in Figure 6 at temperatures above 43.5°C appears to be in contrast to the observation that heated S phase cells divide and die by a mechanism related to the appearance of chromosomal aberrations, while cells heated in G1 die without completing cytokinesis (5,28). This would suggest that the use of Arrhenius analysis, as applied to cellular endpoints, may be inappropriate for determining information concerning the mechanism of cell killing by heat. Since the isosurvival analyses presented in this study demonstrate that the data are fit better by a quadratic function of temperature, as compared to the usual biphasic linear Arrhenius plot, inference as to the mechanism of heat killing derived from values of the inactivation enthalpy $\Delta H^{\pm}$ would also seem to be questionable, especially since absolute linearity is required in the Eyring hypothesis (10). It is interesting to note that when the *in vitro* and *in vivo* survival data in the literature are reviewed (13,14,21), at temperatures in the vicinity of which thermotolerance is developing, the uncertainty in the data would permit fitting a quadratic function of temperature to the heat inactivation rates, as well as the usual biphasic 1/T dependence, with a break at about 43°C. The advantage of the quadratic fit lies in its ability to characterize the hyperthermic response using a single function of temperature, without a discontinuity in the temperature dependence of the rate of cell killing by heat. Regardless of the theoretical interpretation of this temperature dependence, a single-function empirical fit to the data would simplify the concept of thermal dose, providing a more accurate prediction of thermal response in the clinic.

ACKNOWLEDGEMENTS

This research was supported by NCI Grant CA 31813 awarded by DHHS. The authors also would like to thank Ms. Louise Thompson and Mr. Todd Adair for their excellent technical assistance.

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1. Normalized survival values with and without ^{3}H-TdR, respectively, were .083 $\pm$.002 and .087 $\pm$.002 for 8hrs.; .045 $\pm$ .001 and .056 $\pm$.002 for 10 hrs.; .020 $\pm$.001 and .040 $\pm$.002 for 12 hrs.; .063 $\pm$.003 and .104 $\pm$.005 for 14 hrs. (all values $\pm$1 SEM).

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TABLE I - SURVIVAL OF S PHASE POPULATIONS EXPOSED TO HIGH SPECIFIC ACTIVITY ^{3}H-TDR AND HEATED AT 42.5°C

Experimental Group	Surviving Fraction
Sham Treated	0.45 ± .12
Sham + 6 hrs. - 42.5°C	0.0017 ± .0003
Sham + 8 hrs. - 42.5°C	0.0019 ± .0002
^{3}H-TdR + 6 hrs. - 42.5°C	0.0020 ± .0001
^{3}H-TdR + 8 hrs. - 42.5°C	0.0017 ± .0001
^{3}H-TdR Treatment Only	0.013 ± .001

All groups used aphidicolin-resynchronized cultures; labeling index was 95% and mean ^{3}H incorporation was 19.1 ± 9.1 dpm/labelled cell. Background was 0.02 dpm/cell, and counting efficiency was 38.1%. Heated and ^{3}H-treated survival values were corrected for sham plating efficiency. All values shown are ± 95% confidence intervals.

TABLE II - RESULTS OF REGRESSION ANALYSIS OF DATA IN FIG. 6

G1 Phase			
50% Isosurvival	$a_0 = -55.92$	$a_1 = 2.121$	$a_2 = -0.0201$
10% Isosurvival	$a_0 = -54.75$	$a_1 = 2.078$	$a_2 = -0.0198$
0.1% Isosurvival	$a_0 = -63.83$	$a_1 = 2.450$	$a_2 = -0.0243$
S Phase			
50% Isosurvival	$a_0 = -103.80$	$a_1 = 4.311$	$a_2 = -0.0448$
10% Isosurvival	$a_0 = -156.16$	$a_1 = 6.614$	$a_2 = -0.0702$

The values a_i represent the least-squares coefficients in the following expression relating the time required at a given temperature to achieve a specified isosurvival:

$$\log(1/\text{time}) = a_0 + a_1 T + a_2 T^2,$$

where T is temperature in degrees Celsius.

TABLE III - A COMPARISON OF TWO METHODS OF ANALYSIS OF ISOSURVIVAL PLOTS IN FIGS. 5 & 6

	RESIDUALS			
	G1 Phase		S Phase	
Isosurvival	1/T	T^2	1/T	T^2
50%	0.1000	0.0418	0.0215	0.00533
10%	0.00408	0.00406	0.0603	0.0335

Residuals were calculated from:

$$\text{Residual} = \Sigma(Y_i - Y_i')^2$$

where Y_i is the logarithm of the observed reciprocal isosurvival time and Y_i' is the value calculated from the respective fit, and the summation is performed over the entire range of the dataset.

Note that although the regression coefficients shown in Table II were obtained using temperature expressed in degrees Celsius, this comparison was performed using temperature in degrees Kelvin.

TABLE IV - TEMPERATURE DEPENDENCE OF THE RATE OF CELL KILLING OF G1 AND S PHASE CELLS

Isosurvival Level	G1 Phase				S Phase			
	Below 43.5°C		Above 43.5°C		Below 43.5°C		Above 43.5°C	
	Slope	R	Slope	R	Slope	R	Slope	R
50%	.41±.26	.39	.34±.18	.46	.48±.16	.33	.32±.04	.48
10%	.41±.07	.39	.31±.05	.49	.64±.28	.23	.36±.05	.44

All slopes shown were calculated using a log-linear fit of the data over the temperature intervals designated and are ± 95% confidence.

R values were calculated from the linear regression coefficients using the following relations:

$$\log(1/\text{time}) = a_0 + a_1 T$$

$$R = 1/\text{time for } T°C \;/\; 1/\text{time for } T+1°C$$

$$= 10^{a_0 + a_1 T} \;/\; 10^{a_0 + a_1 (T+1)}$$

$$= 10^{-a_1}$$

FIGURE LEGENDS

Figure 1

Variation in heat sensitivity as cells progress from early S phase. Mitotic CHO cells were incubated in suspension for 12 hours at 37°C in medium supplemented with 2 μg/ml aphidicolin, then the cells were resuspended in fresh drug-free medium, plated into T25 flasks, and allowed to progress at 37°C through the DNA synthetic period. At intervals they were either heated for 10 min at 45.5°C and incubated at 37°C for colony formation (left ordinate, solid symbols) or pulsed for 20 min with 4 μCi/ml ^{3}H-TdR, fixed, and processed for autoradiography (right ordinate, open symbols).

Figure 2

Variation in hyperthermic sensitivity through S phase in CHO cells, as determined by tritiated thymidine suicide experiments in an aphidicolin-free system. At various times after mitosis, cultures were either sham-treated or pulsed with high specific activity (h.s.a.) ^{3}H-TdR (30 min.- 4 μCi/ml), and heated (10 min-45.5°C) 10 minutes later. Abscissa shows the time of the start of heating, following the sham- or ^{3}H-pulse. Open triangles represent survival (G1+S+G2) after heat exposure following sham treatment; solid squares represent heat survival (G1+G2) following the tritiated thymidine pulse. All the data were corrected for sham treatment plating efficiencies; error bars represent $\pm$1 sem, unless smaller than symbol size. To determine the fraction of clonogenic cells actively synthesizing DNA during each pulse period, some groups were treated with h.s.a. ^{3}H-TdR only, and allowed to form colonies. The fraction of clonogenic cells in S phase (open circles - dotted line, right ordinate) was calculated from:

$$\text{Fraction in S} = \frac{1 - \text{P.E.}(^{3}\text{H only})}{\text{Sham P.E.}} .$$

S phase heat survival, as a function of time after mitosis, (solid circles, dashed line) was calculated from:

$$\text{S phase Survival} = \frac{\text{P.E.(heat only)} - \text{P.E.(heat+}^{3}\text{H)}}{\text{(Sham P.E.) x (Fraction in S)}},$$

where P.E. = no. colonies/no. cells plated. Since there was no significant difference between heat alone and heat + tritium suicide at 8 hrs. after mitosis, the maximum S phase survival possible is indicated; the error bar represents the upper limit on the SEM of the difference.

Figure 3

G1 phase hyperthermic survival of CHO cells. Mitotic cells were plated into flasks and incubated for 90 min to allow progression into G1 before heating. Error bars indicate ± 1 SEM, unless smaller than symbols. Control plating efficiencies were 63-68%. Panel A: 41.5 - 42.5°C. Panel B: 43.0 - 45.5°C.

Figure 4

S phase hyperthermic survival of CHO cells. Mitotic cells were resynchronized near the G1/S border by incubation in suspension for 12 hrs in 2 μg/ml aphidicolin and allowed to attach at 37°C for 2 hrs before the start of heating. Labeling indices were 99% except for the upper 45.5°C and the 43°C curves in panel B, for which 97% of the cells were labeled at the start of the heating. Control plating efficiencies were 55-67%. Error bars indicate ± 1 SEM, unless smaller than symbols. Panel A: 41.5 - 42.5°C. Panel B: 43.0 - 45.5°C.

Figure 5

Time-temperature analysis of G1 and S phase CHO cells. The reciprocals of the durations of heating required to achieve an arbitrary isosurvival level are expressed as a function of temperature on a semilogarithmic scale. Data were obtained from the survival curves in Figures 3 and 4. Solid lines and solid symbols indicate the raw data; open symbols and dashed lines represent the data corrected for temperature transients, as described in Materials and Methods. All lines shown are the least-squares estimator obtained from regression analysis of the data, as described in Materials and Methods. Panel A: G1 phase, circles represent 50% isosurvival, squares - 10% isosurvival, and triangles represent 0.1%

isosurvival. Panel B: S phase cells, circles represent 50% isosurvival, and squares - 10% isosurvival.

Figure 6

Comparison of S phase and G1 isosurvival plots for synchronous G1 and S phase cells. Data were corrected for temperature transients, as explained in Materials and Methods, and correspond to the corrected curves in Figure 5. Data points are omitted for clarity. Dashed curves refer to S phase data; solid curves refer to G1 data.

FIGURE 1

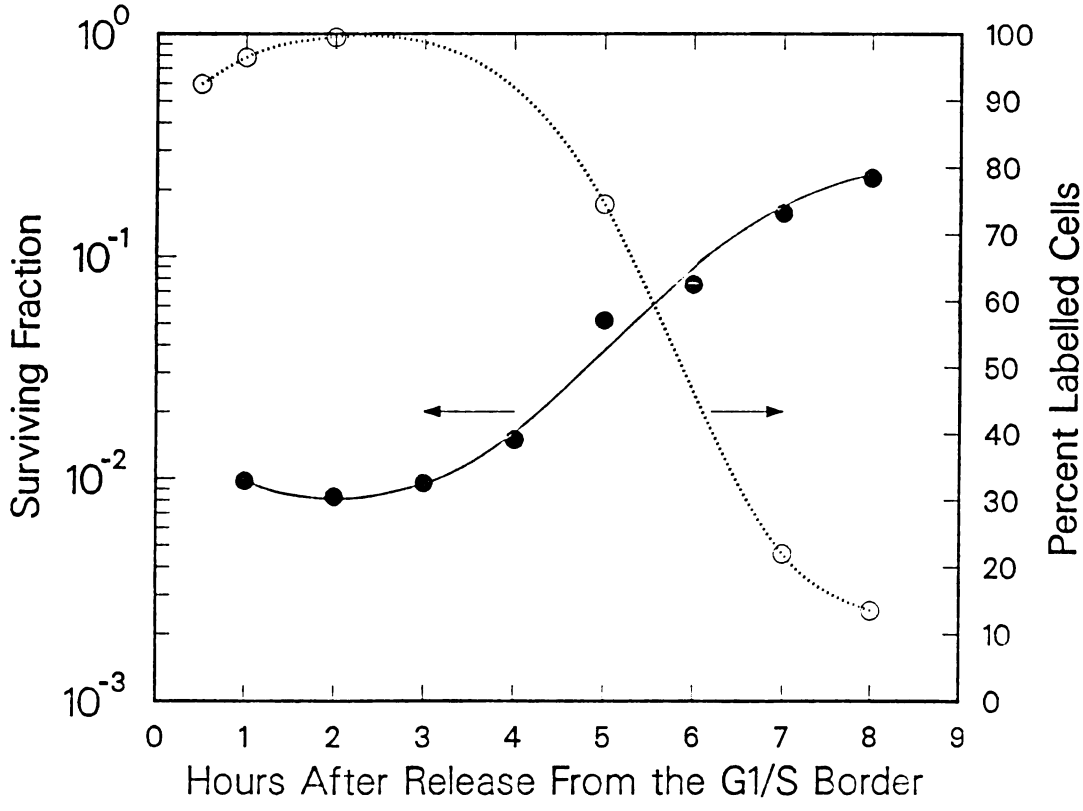

Surviving Fraction
10^0
10^{-1}
10^{-2}
10^{-3}
Percent Labelled Cells
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9
Hours After Release From the G1/S Border

FIGURE 2

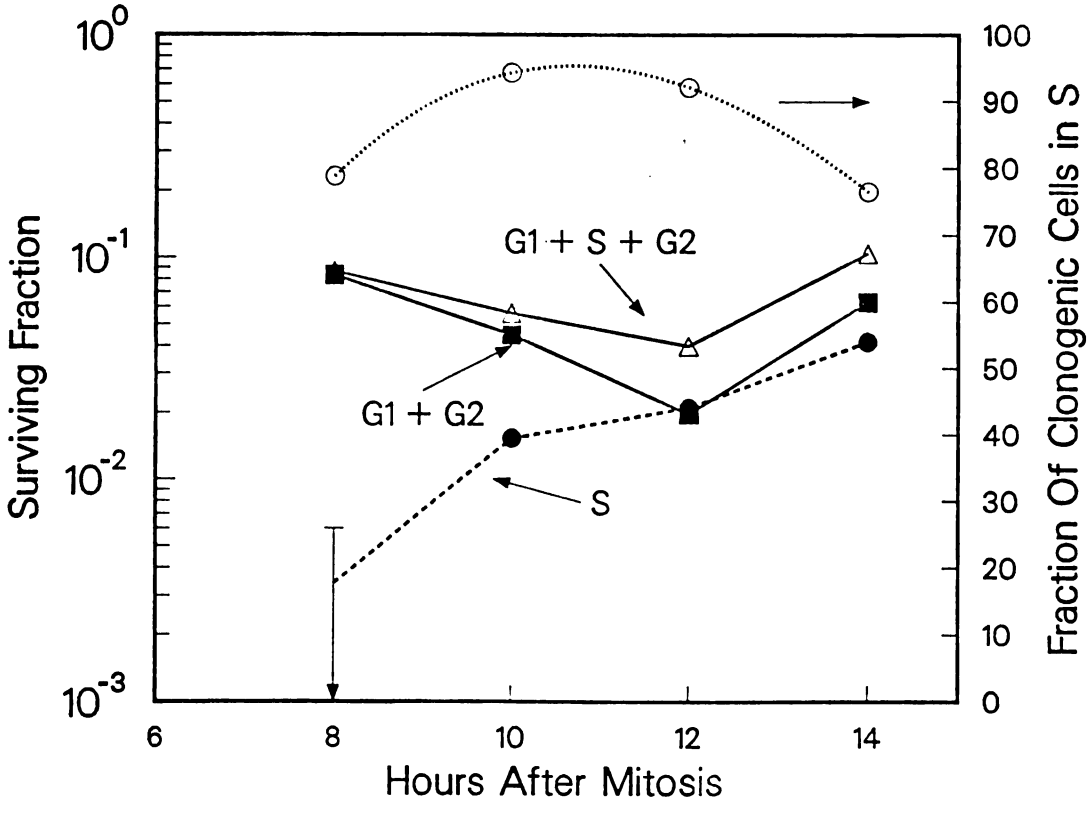

Surviving Fraction
10^0
10^{-1}
10^{-2}
10^{-3}
G1 + S + G2
G1 + G2
S
6
8
10
12
14
Hours After Mitosis
100
90
80
70
60
50
40
30
20
10
0
Fraction Of Clonogenic Cells in S

FIGURE 3A

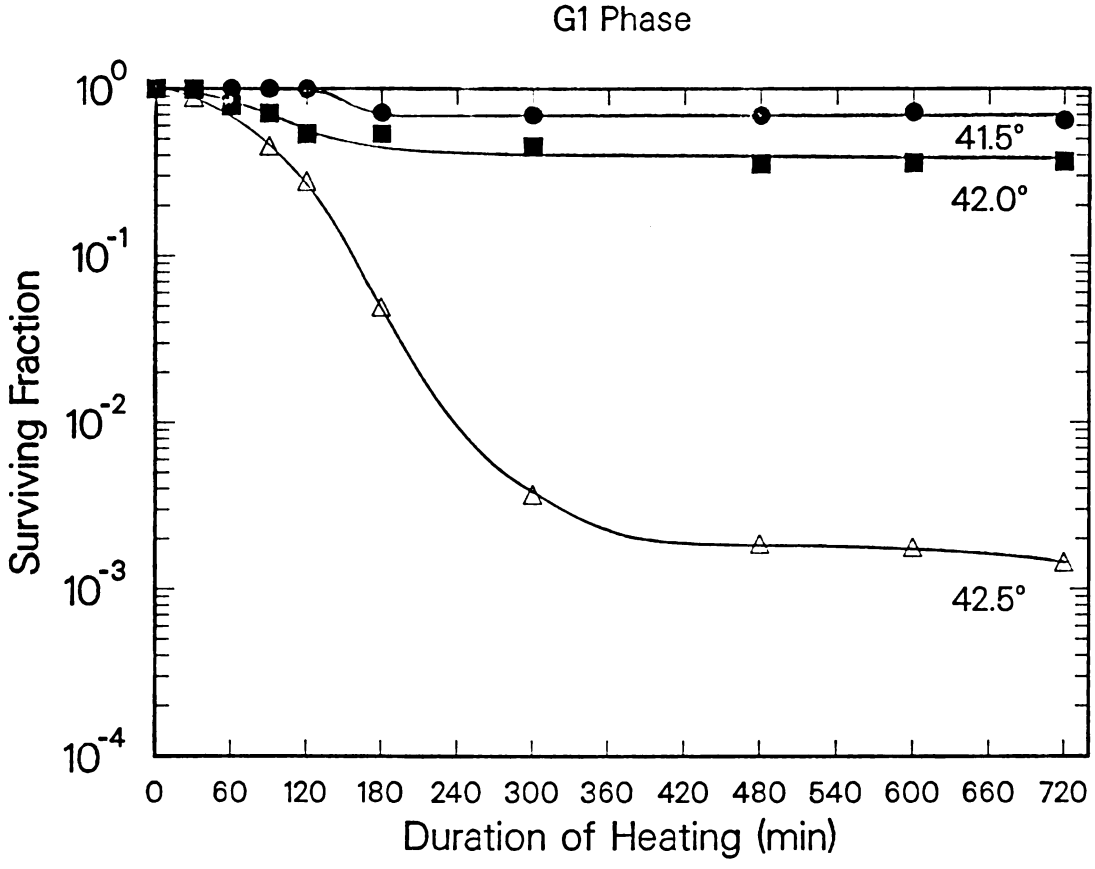

G1 Phase
Surviving Fraction
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
41.5°
42.0°
42.5°
0
60
120
180
240
300
360
420
480
540
600
660
720
Duration of Heating (min)

FIGURE 3B

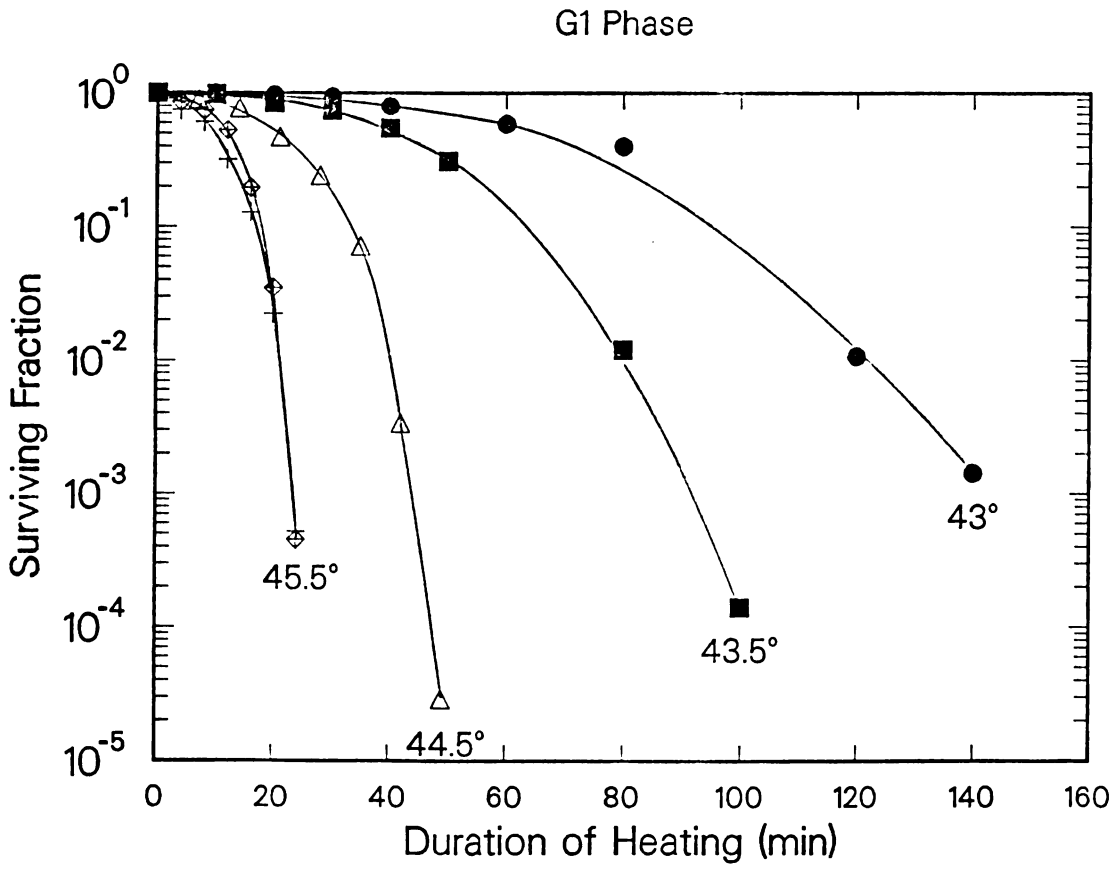

G1 Phase
Surviving Fraction
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
45.5°
44.5°
43.5°
43°
0
20
40
60
80
100
120
140
160
Duration of Heating (min)

FIGURE 4A

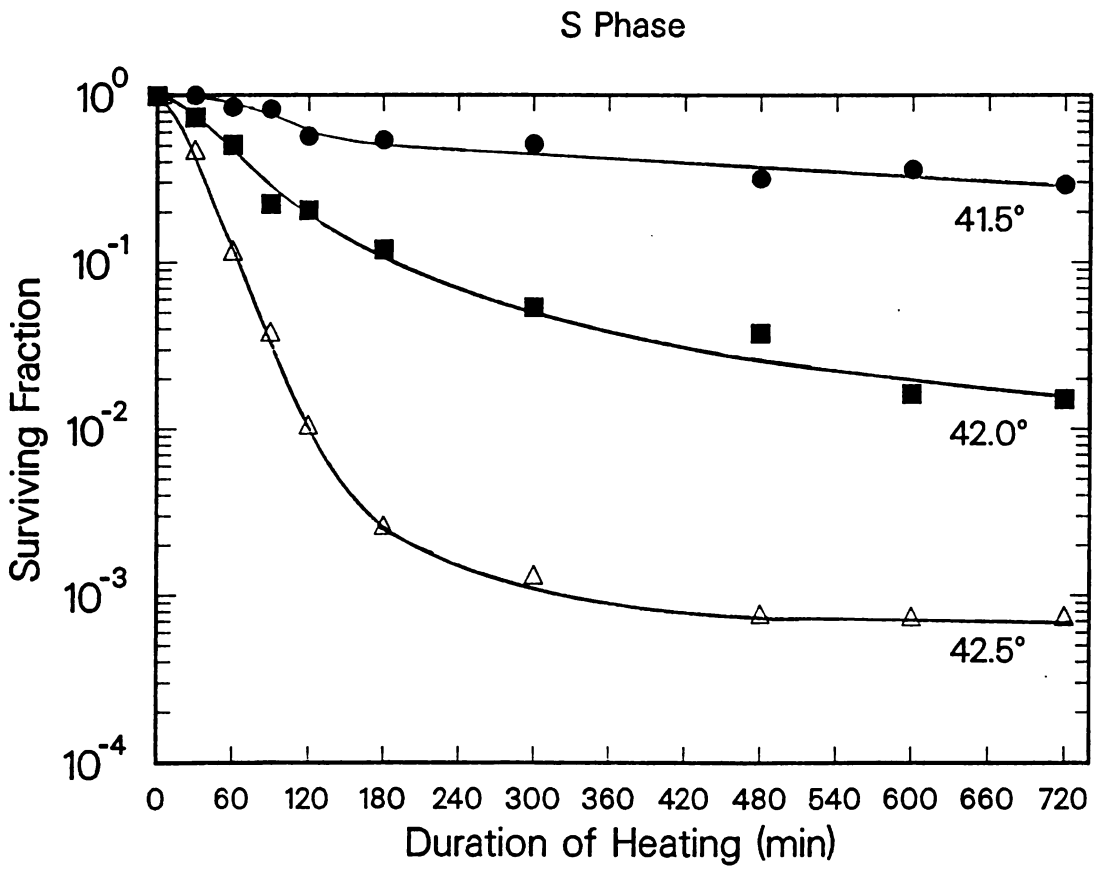

S Phase
Surviving Fraction
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
41.5°
42.0°
42.5°
0 60 120 180 240 300 360 420 480 540 600 660 720
Duration of Heating (min)

FIGURE 4B

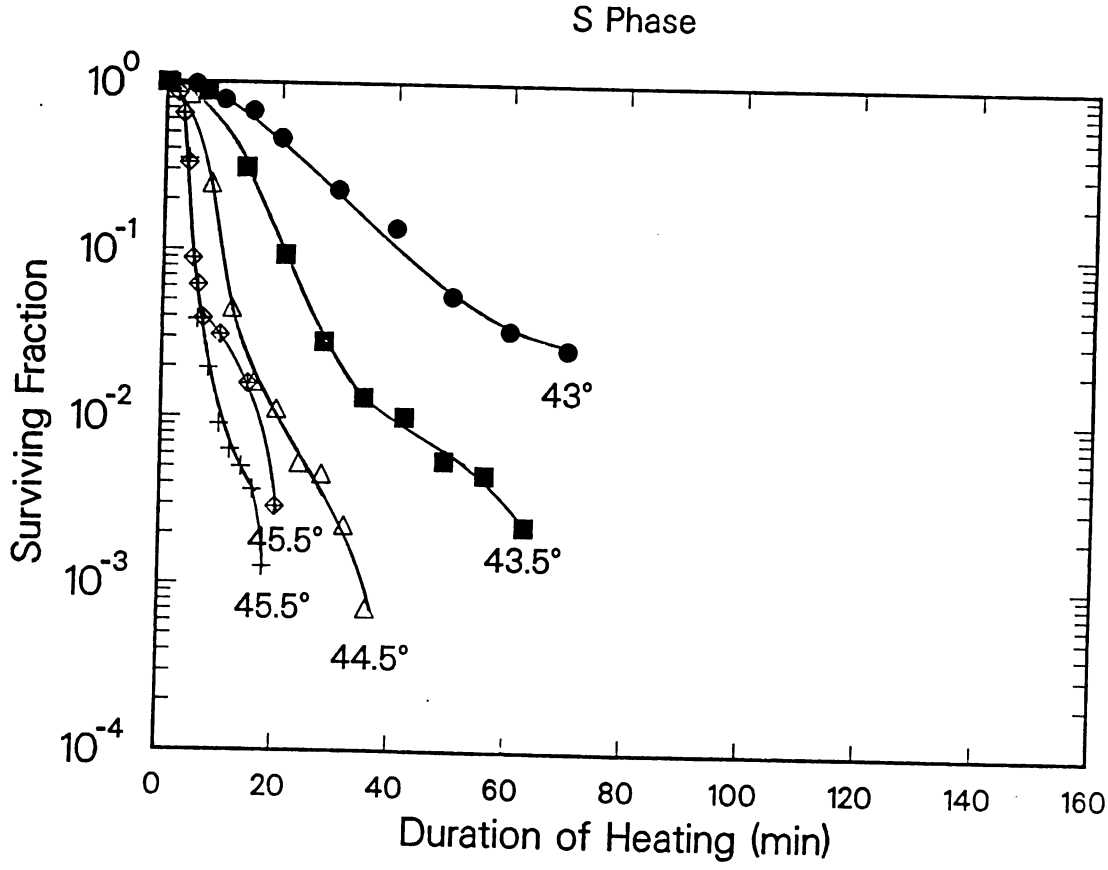

S Phase
Surviving Fraction
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
0
20
40
60
80
100
120
140
160
Duration of Heating (min)
43°
43.5°
45.5°
45.5°
44.5°

FIGURE 5A

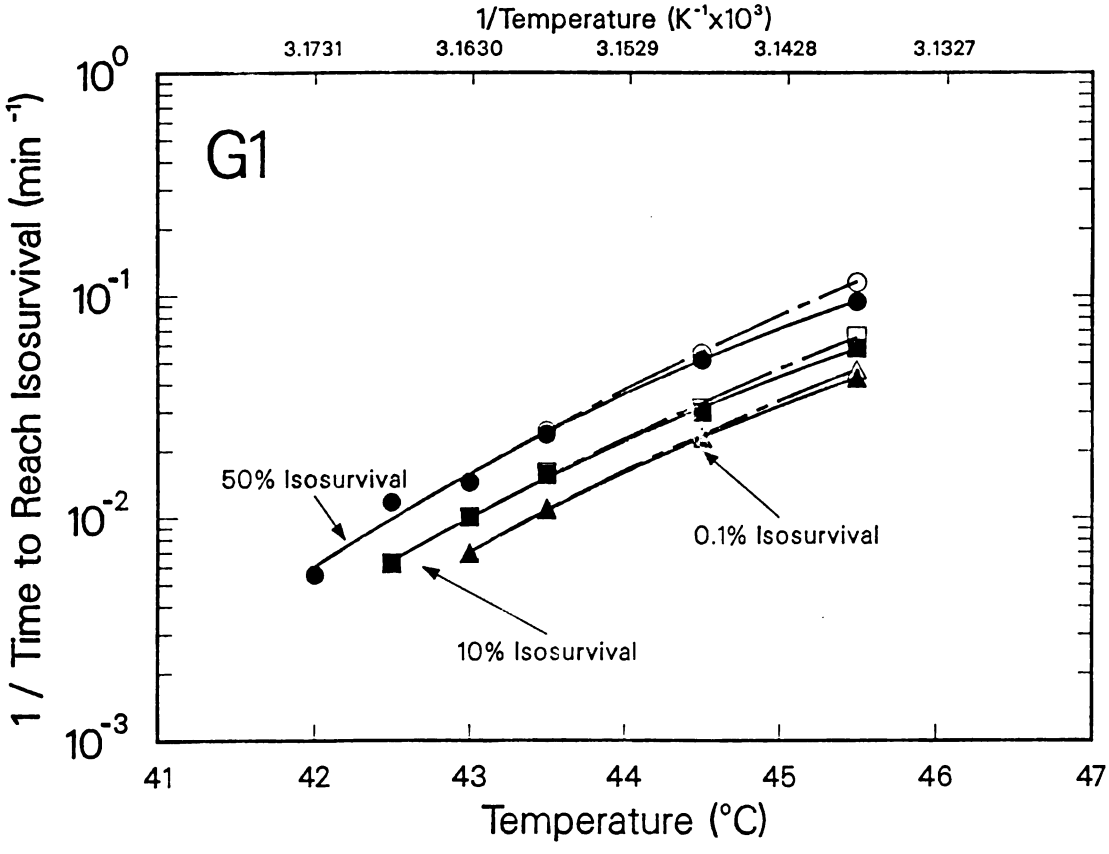

1/Temperature ($K^{-1}x10^3$)
3.1731
3.1630
3.1529
3.1428
3.1327
G1
10^0
10^{-1}
10^{-2}
10^{-3}
1 / Time to Reach Isosurvival (min^{-1})
50% Isosurvival
0.1% Isosurvival
10% Isosurvival
41
42
43
44
45
46
47
Temperature (°C)

FIGURE 5B

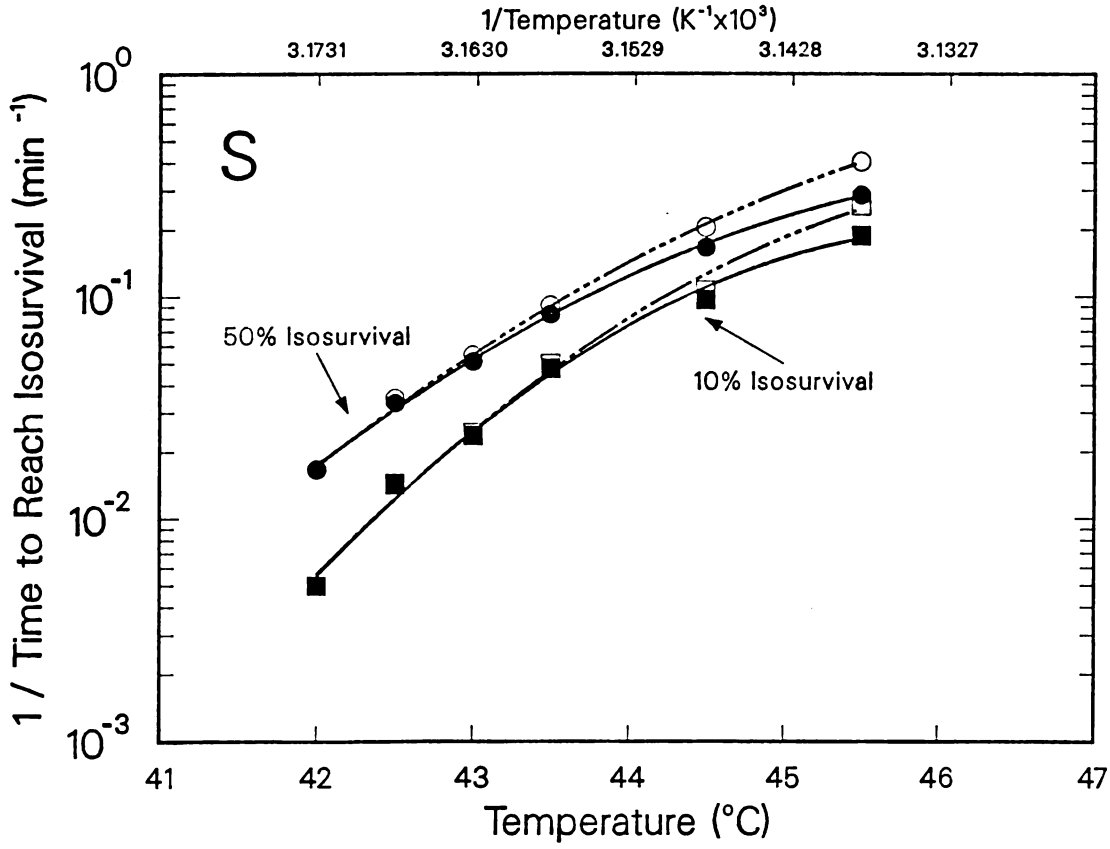
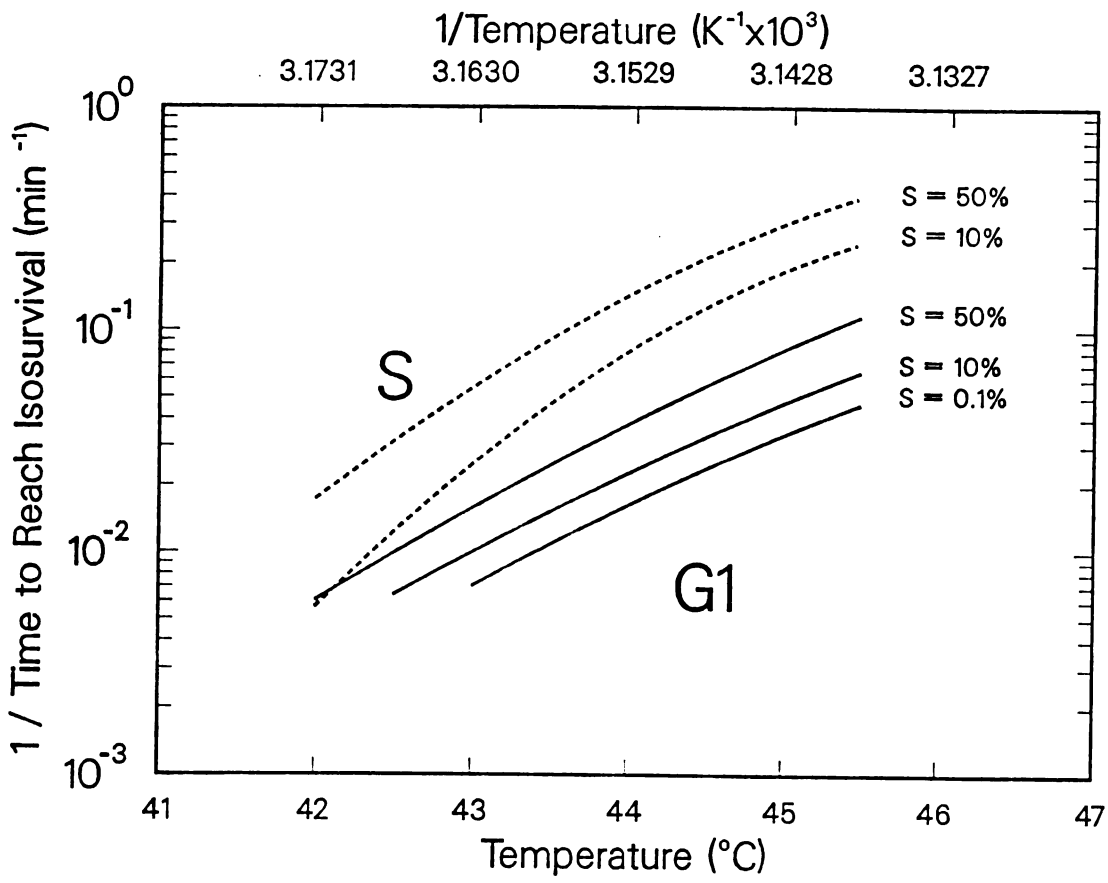

1/Temperature ($K^{-1}x10^3$)
3.1731
3.1630
3.1529
3.1428
3.1327
S
50% Isosurvival
10% Isosurvival
1 / Time to Reach Isosurvival (min^{-1})
10^0
10^{-1}
10^{-2}
10^{-3}
41
42
43
44
45
46
47
Temperature (°C)

FIGURE 6


1/Temperature ($K^{-1}x10^3$)
3.1731
3.1630
3.1529
3.1428
3.1327
10^0
10^{-1}
10^{-2}
10^{-3}
1 / Time to Reach Isosurvival (min^{-1})
S = 50%
S = 10%
S = 50%
S = 10%
S = 0.1%
S
G1
41
42
43
44
45
46
47
Temperature (°C)

CHAPTER 3

Cell Cycle Progression During Chronic Hyperthermia in S Phase CHO Cells

ABSTRACT

S phase CHO cells chronically exposed to 41.5°C exhibit an apparent resistance to further cell killing after 4 - 6 hrs. During the treatment interval, cells were observed to progress out of S phase and into G2, mitosis, and the next cell cycle. Progression through S was delayed by about 2 hrs and an equivalent mitotic delay was observed. After entry into mitosis, heated cultures showed an altered nuclear morphology, presumably as the result of abnormal division occurring during the treatment. Tritium suicide experiments at this temperature showed that clonogenic, as well as non-clonogenic cells progressed during this period. When S phase cultures were chronically heated at 42°C, however, the delay in transit through S phase was 8 - 10 hrs, and an accumulation in G2 phase was observed. Survival during this treatment decreased continuously. Therefore, our results show that in S phase cultures heated at 42°C, exponential cell killing continues while cells progress through S, suggesting that chronic thermotolerance cannot be expressed in S phase. Furthermore, at 41.5°C, cells which progress out of S during the treatment express resistance to heat killing in subsequent cell cycle phases. In summary, our results indicate that, although chronic tolerance is not expressed during heating in S phase, it is expressed after the cells progress out of S phase.

INTRODUCTION

When G1 or asynchronous CHO cultures are chronically heated at temperatures below about 42 - 42.5°C, a period of cell killing occurs for about 2 - 3 hrs, followed by a cessation of further killing (Sapareto et al., 1978). Studies have shown that this form of thermotolerance, termed chronic thermotolerance, can persist for up to 60 hrs, at which time tolerance decays, resulting in the resumption of heat-induced cytotoxicity (Freeman, 1982; Dewey and Esch, 1982; Read et al., 1983). Furthermore, G1 cells heated at these lower temperatures are blocked in cell cycle progression in a temperature-dependent fashion (Sapareto et al, 1978). Other studies have demonstrated a correlation between the decay of chronic thermotolerance and the transition from G1 into S phase which occurred during the 42°C treatment (Read et al., 1983). G1 cells, being more heat resistant than S phase cells, dominate the asynchronous heat survival response (Mackey and Dewey, 1987). Therefore, these observations of synchronized G1 cells probably account for chronic thermotolerance as observed in asynchronous systems. In spite of the increased thermal sensitivity of S phase cells when compared to G1 cells, we found that both populations shared the same temperature dependence of the rate of cell killing (Mackey and Dewey, 1987). In the course of performing this comparison of the two cell cycle phases, we observed resistance in S phase cells heated at 41.5°C which developed after an initial period of cytotoxicity. This phenomenon, though similar to chronic thermotolerance, may be due to cell cycle progression, since heat sensitivity decreases as cells progress out of S phase (Read et al., 1984; Mackey and Dewey, 1987). Accordingly, we decided to determine if cell cycle progression out of S was responsible for this decreased heat resistance.

MATERIALS AND METHODS

Culture Conditions, Synchrony Methods, and Heat Treatment

Chinese Hamster Ovary (CHO) cells were grown in McCoy's 5a modified medium (Gibco) containing 10% heat-inactivated fetal calf serum (J.R. Scientific Co.), and the antibiotics neomycin sulfate (0.1 g/l), potassium penicillin G (0.07 g/l) and streptomycin sulfate (0.1 g/l) (Sigma). Exponentially growing cells were cultured at 37°C in a humidified atmosphere and maintained at pH 7.4 with 5% CO^2. Mitotic cells were obtained from these stocks by the mitotic selection technique, as described previously (Gerweck et al., 1975); mitotic indices ranged from 98-99%. For some experiments, mitotic cells were frozen in a solution which has been shown to protect against adverse effects of freezing on plating efficiency, heat survival, and cell cycle progression (Borrelli et al., 1987). Plating efficiencies ranged from 50 to 70%.

Synchronous S phase populations were obtained by incubating mitotic cells in suspension culture for 12 hours at 37°C in McCoy's 5a medium supplemented with 2 μg/ml aphidicolin (Sigma), as described in detail elsewhere (Mackey and Dewey, 1987). At the end of this incubation period, the cell suspension was centrifuged (120 x g - 10 min - 4°C), the pellet was resuspended in fresh drug-free medium, and cells were plated into 25 cm^2 flasks at a density of 2.0×10^4 cells/cm^2. After two hours incubation in a 37°C incubator, flasks were sealed with paraffin and then placed in Precision waterbaths (GCA) at either 37°C or the appropriate hyperthermic temperature. For the tritium suicide experiments shown in Figure 5, flasks were heated at either 37° or 41.5°C in water-jacketed incubators (Wedco).

Mitotic and Labeling Indices

Both labeling index and mitotic index were determined from the same slide preparations. Cultures were pulse labeled for 20 minutes with low specific activity tritiated thymidine (25 Ci/mM - Amersham Corp.) at a concentration of 4 μCi/ml in the regular culture medium. All labeling was performed at 37°C; for the heated groups, an interval of 10 min at 37°C immediately preceded the tritium pulse. After the pulse label, cultures were trypsinized on ice using a Hepes-buffered trypsin solution (Wong and Dewey, 1982); all solutions were saved, including the medium in the flasks and the trypsin rinses, and this suspension was centrifuged (120 x g - 10 min - 4°C). After discarding the supernatant, the pellet was resuspended in 5 ml Hanks BSS and centrifuged as before, and the pellet was fixed with 0.5 ml fresh Carnoy's fixative (3:1 methanol:glacial acetic acid) for 5 min, followed by gentle resuspension with a small-bore pipette and the addition of 9.5 ml Carnoy's. After an additional centrifugation, the resuspended pellet was diluted with an appropriate amount of fixative to yield a good density of cells on the slide (usually 0.2 - 0.5 ml), and slide preparations were made by dropping 2-4 drops of the suspension onto clean slides wetted with ice-cold distilled water. After drying overnight, the slides were either stained with 2% aceto-orcein and permanently mounted for mitotic index sampling, or the slides were coated with photographic emulsion (Ilford L4 - Polysciences) and exposed for 1-5 weeks, before developing according to the manufacturer's recommendations, and staining using Wright's stain. Both labeling and mitotic indices were determined by counting at least 200 cells for each determination.

Survival Assay

Surviving fraction for Figure 5 was obtained as described in the next section. For the survival curves shown in Figures 1 and 3, aphidicolin-resynchronized cells were plated into 25 cm^2 flasks at a density of 2.0 x 10^4 cells/cm^2 and allowed to attach and progress into early S phase for 2 hrs in a 37°C incubator. Immediately prior to the heat exposure, the flasks were

sealed with paraffin and placed in racks. After the appropriate heating interval, flasks were trypsinized on ice using Hepes-buffered trypsin and an appropriate number of cells to yield 100 - 200 colonies per flask were plated into 25 cm^2 flasks (5 replicates) to which lethally irradiated (25 Gy) feeder cells were added 24 hrs prior to the experiment to control cell density at 10^5 cells/flask (Highfield et al., 1984). Following incubation for colony formation (7-11 days - 37°C), the flasks were fixed and stained with crystal violet, and colonies of 50 or more cells were scored. Figures 1 and 3 show the mean survival of the replicate flasks; no errors are given as each point is the result of a single observation in a given experiment.

Tritiated Thymidine Suicide

Aphidicolin-resynchronized cells were plated into 25 cm^2 flasks containing lethally-irradiated feeders at the density necessary to yield 100 - 200 colonies per 25 cm^2 flask after the heat treatment, and were allowed to attach and progress into S phase for 2 hrs in a 37°C incubator. Heat exposure and tritiated thymidine labeling were performed in 37°C or 41.5°C incubators. Thirty minutes before the end of the heat exposure, the medium was removed from flasks and was replaced with 2 ml bactopeptone-free McCoy's 5a medium supplemented with 10% dialyzed fetal calf serum containing either high specific activity (h.s.a.) tritiated thymidine (75 Ci/mM - Amersham Corp.) at a concentration of 4 μCi/ml, or an equivalent amount of stable thymidine (Sigma) for sham controls. All solutions used in this procedure were pH- and temperature-equilibrated overnight in the incubator used for the treatment; 5 replicate flasks were used in each survival determination. After a 30 min pulse the cultures were rinsed twice with 5 ml Hanks BSS; then, 5 ml of regular McCoy's medium supplemented with 10 μg/ml stable thymidine was added. Under these conditions, this treatment results in ^3H incorporation in excess of 8 disintegrations per labeled cell per minute which is sufficient to kill all the S phase cells in the population (data not shown).

Flow Cytometric Analyses

At various times after the start of the experiment, cultures were pulse labeled for 30 min at 37°C with 10μM bromodeoxyuridine (BrdUrd); heated groups were transferred to 37°C for 10 min prior to labeling. Following the labeling period, the cultures were trypsinized as for labeling index, saving all medium and rinses. For each experimental point, four 75 cm^2 flasks plated at a density of 2.0 x 10^4 cells/cm^2 were pooled for the analysis. After centrifugation of the pooled cell suspensions (10 min - 120 x g - 4°C), the supernatants were decanted, and the resuspended pellets were fixed in 5 ml of ice-cold 50% ethanol, and stored for up to 2 weeks at 4°C in sealed centrifuge tubes. On the day of analysis, samples were centrifuged as before, and the pellets were resuspended in 1 ml of Ca^{++}- and Mg^{++}-free phosphate-buffered saline (PBS), and transferred to 1.5 ml microfuge tubes. The samples were then centrifuged using a Beckman Microfuge B for 45 sec at room temperature, the supernatants were discarded, 1 ml of ribonuclease A (Sigma) solution (1 mg/ml in Ca^{++}- and Mg^{++}-free PBS) was added to the resuspended pellets, and the samples were incubated for 30 min at 37°C. Following the ribonuclease treatment, the samples were centrifuged again as before, the supernatant was discarded, 1 ml of ice-cold 0.1N HCl was added to the resuspended pellet, and the samples were incubated for 10 min at 0°C. The samples were microfuged again, the supernatant was discarded, and 1 ml of double distilled water was added to the resuspended pellet in preparation for the heat denaturation step; the samples were then heat-treated at 95.5°C for 10 min in a waterbath, followed by immediate transfer to an ice-water bath. After centrifugation as before, the supernatants were discarded, and 1 ml of 0.5% Tween-20 - 0.5% bovine serum albumin (both available from Sigma) in PBS was added to each tube. Following an additional centrifugation, 0.5 ml of a 1:5000 dilution of the primary antibody, mouse monoclonal anti-BrdUrd (a gift of Dr. Frank Dolbeare of Lawrence Livermore Laboratory), was added to each tube and allowed to stain for 30 min at 37°C. The samples were then centrifuged again and 0.5 ml of a 1:100 dilution of secondary antibody, fluorescein isothiocyanate-conjugated sheep anti-mouse IgG, was added to each tube, which

was then incubated for 30 min at 37°C. Following centrifugation as before, the pellet was resuspended in 0.5 ml of 10 μg/ml propidium iodide in PBS, and allowed to stain at room temperature for 20 min. Before analysis of the samples on the flow cytometer, the samples were pipetted through a 37 micron nylon mesh into tubes used for FCM sampling to remove cell clumps and debris.

Cell samples were analyzed using a Becton-Dickinson FACS II flow cytometer, located at the Laboratory for Cell Analysis, University of California, San Francisco. The flow cytometer was equipped with an argon ion laser emitting 488 nm light at 400 milliwatts, and was also equipped with a 560 nm dichroic mirror. For the anti-BrdUrd fluorescence measurements, a 530/30 band pass filter was used; propidium iodide fluorescence was detected using a 580 nm long pass filter. Data from 10,000 cells per sample were collected in list mode. Data analysis and contour plot construction were performed using Electric Desk FCM Analysis software (Becton-Dickinson), running on a VAX 11/750 (Digital Equipment Corporation), also located at the Laboratory for Cell Analysis. To determine the percentage of cells in the various phases of the cell cycle, compartments were assigned as shown in Figure 4, and the data were integrated numerically.

RESULTS

When early S phase cells were chronically heated at 41.5°C, survival decreased for about 4-6 hrs; after this period of cell killing, no further decrease in survival was observed (see Figure 1A). In this experiment, transit through S phase was delayed by about 2 hrs (as determined by the time required to achieve 50% labeling index), and a similar 2 hr mitotic delay was also observed (see Figure 1B-C). This phenomenon was observed in two other experiments; a representative experiment is shown in Figure 1. Cell number increased by about 15% in the heated population, as determined by an electronic particle counter, and this increase was not observed until 8 hrs after the start of the heat treatment, in agreement with

the mitotic and labeling index data. At this time, a bimodal volume distribution was observed in these populations, confirming that some cell division had occurred (data not shown).

Upon inspection of the cell preparations used for mitotic and labeling indices, changes in nuclear morphology were observed to develop after about 8 hrs of heating (see Figure 2), presumably as the result of abnormal division induced by the heat exposure. Cell killing also correlated well with the frequency of chromosomal aberrations (about 1 aberration for 40% survival). A more complete description of these morphological effects is included in another report (Mackey et al., 1987).

In order to determine if similar cell cycle progression occurred at a higher temperature, an identical experiment was performed at 42°C (see Figure 3). Three replicate experiments at this temperature showed a delay of 8-10 hrs in traversal of the S period, as well as entry into mitosis after 12 hrs of heating. Since 42°C inhibits ^{3}H-thymidine incorporation (Wong and Dewey, 1982), we used bromodeoxyuridine - propidium iodide (BrdUrd - PI) flow cytometric analyses to confirm that the decrease in labeling index shown in Figure 3B was not complicated by a heat-induced decrease in tritium incorporation (see Figure 4). The results of compartmental analyses of these data (see Table I) confirmed that at this temperature progression occurred at a reduced rate through S phase. This method of FCM data analysis produced minor discrepancies when compared to the labeling index data (shown in Figure 3B), presumably due to complications associated with doublets and cell clumping induced by the harsh treatment of these cell samples. We also observed the accumulation of cells in G2 phase after 8 - 12 hrs of heating, in agreement with an earlier report (Read et al., 1983). At 42°C, exponential cell killing continued throughout the treatment interval (see Figure 3A), which, when coupled with our observations at 41.5°C, showed that cell killing correlated with the temperature-dependent delay in transit through S phase.

The use of aphidicolin in the S phase synchrony technique did not induce the delays in cell cycle progression we observed, as experiments performed using S phase cultures obtained from mitotic cells not treated with the drug showed similar results (data not shown). These

experiments without aphidicolin confirmed progression out of S during heating at 41.5° and 42°C, but were not as easily interpreted because of increased heterogeneity associated with poorer synchrony.

Since we observed decreases in cell killing concomitantly with progression out of S phase during the heat treatment, we used tritium suicide to verify that clonogenic cells were progressing out of S phase (see Figure 5). In this experiment, a tritium pulse was delivered to the cultures during the last 30 min of the heat treatment, under conditions which were cytotoxic to S phase cells. We observed that the survival of the heat plus tritium suicide group increased as the cells progressed through S; indicating that the clonogenic survivors were indeed progressing out of S during the treatment interval.

DISCUSSION

Progression through S phase during a chronic 41.5°C treatment was delayed by about 2 hrs, and cell killing continued until the cells had progressed out of S (see Figures 1 and 2). By the time cells had progressed out of S phase, no further cell killing was observed. Tritium suicide experiments at 41.5°C confirmed that clonogenic cells were progressing out of S phase during the treatment interval (see Figure 5). When progression was measured at 42°C, however, a much greater S phase delay of 8 - 10 hrs was observed (see Figure 3), and cell survival decreased exponentially throughout the experiment. These results show that at lower hyperthermic exposure temperatures progression out of S phase can occur, and this progression is followed by the development of resistance to further cell killing which resembles chronic thermotolerance. Further studies are needed to determine if those cells which progress during the heat treatment are tolerant to further heat exposure at other exposure temperatures, as pretreatment of asynchronous CHO cells at 40°C increased resistance to a subsequent exposure at 45°C (Henle et al. 1978).

Earlier reports have shown that the induced resistance observed in heated G1 cultures is accompanied by a delay of progression into S phase (Read et al. 1983, Sapareto et al. 1978). These findings, coupled with the correlation between the decay of chronic thermotolerance and entry into S phase of initially G1 cultures (Read et al. 1983), suggests that the expression of chronic tolerance is restricted to non-S populations. Support for this conjecture is found in our results which showed a cessation in cell killing at 41.5°C only after cells had progressed out of S phase (see Figure 1), however, we do not know whether the viable cells have divided successfully, thus making it impossible to distinguish between G1 and G2 populations. S phase cells heated at 42°C failed to completely leave S during our experiments, and no induced resistance was observed, even though 42°C induces chronic tolerance in G1 CHO cells (Read et al. 1983, Sapareto et al. 1978). Therefore, it is possible that those cells exiting from S phase at 41.5°C may have already induced tolerance to heat killing in subsequent cell cycle phases, because survival did not decrease as the cells were heated from 8 to 12 hrs. 42°C treatment, however, induced a much greater lengthening of S phase, and virtually all the S phase cells were killed before progression out of S could occur. We conclude that cells heated in S phase may be able to induce chronic thermotolerance, yet are unable to express it until progression out of S phase occurs.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude to Dr. Rosemary S. L. Wong, Ms. Louise Thompson, and Mr. Todd Adair for their expert technical assistance in conducting these experiments, and to Mr. Bill Hyun of the Laboratory of Cell Analysis, UCSF, for assistance in flow cytometric analyses.

This research was supported by NCI grant CA 31813.

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TABLE I - CELL CYCLE ANALYSIS OF FCM PROFILES SHOWN IN FIGURE 4

Group	% G1	% S	% G2/M	% Doublets
Asynchronous	41.1	49.1	9.8	32.0
37 - 0 hrs	12.3	85.6	2.2	36.5
37 - 4 hrs	50.4	21.2	28.5	23.9
37 - 8 hrs	40.3	50.3	9.5	19.3
37 - 12 hrs	41.1	45.5	13.4	28.8
42 - 4 hrs	8.7	89.3	2.0	34.7
42 - 8 hrs	12.1	74.1	13.9	34.6
42 - 12 hrs	13.1	55.3	31.2	35.1

Cell cycle phase percentages were obtained by numerical integration of flow cytometric data (Stanford University-developed Electric Desk FCM software). Compartments of the contour plots were assigned to each cell cycle phase, as shown in the asynchronous plot in Figure 4. These data are from the same experiment shown in Figure 3.

FIGURE LEGENDS

Figure 1.

Cell cycle progression during a chronic 41.5°C hyperthermia treatment which began in early S phase. Mitotic CHO cells were incubated for 12 hours in medium containing 2 μg/ml aphidicolin in suspension culture, during which time cells accumulated near the G1/S border. After centrifugation and resuspension in drug-free medium, the cells were plated into 25 cm2 flasks and allowed to attach for 2 hrs at 37°C, after which the flasks were sealed with paraffin and heated in waterbaths set to either 37°C or 41.5°C. A: surviving fraction; B: labeling index; C: mitotic index. The abscissa shows elapsed time after the start of heating, which began 2 hrs after release from the aphidicolin block.

Figure 2.

Representative examples of the change in nuclear morphology observed in heated populations when the heat treatment was begun in early S phase. A: Control unheated cells; B: Cells heated for 12 hrs at 41.5°C exhibiting an altered nuclear morphology. 500 K magnification, phase contrast.

Figure 3.

Cell cycle progression of S phase cells during chronic 42°C hyperthermia. The experimental design was identical to that shown in Figure 1, except that cultures were heated at 42°C, instead of 41.5°C. A: surviving fraction; B: labeling index; C: mitotic index. The abscissa shows elapsed time after the start of heating, which began 2 hrs after release from the aphidicolin block.

Figure 4.

Bromodeoxyuridine (BrdUrd) - propidium iodide (PI) bivariate flow cytometry contour plots showing cell cycle progression during 42°C hyperthermia. These data were obtained from the same experiment as that shown in Figure 3, and the results of the cell cycle analysis is shown in Table I. Cultures were pulse labeled with 10μM BrdUrd for 30 min at 37°C 0,

4, 8, and 12 hrs after the start of the experiment, and the cells were trypsinized immediately afterwards. Cultures which were heated at 42°C were moved to 37°C 10 min prior to labeling. Samples were then stained with PI and a fluorescein isothiocyanate (FITC) conjugated monoclonal antibody to chromatin substituted with BrdUrd and were analyzed using a Becton-Dickinson FACS II flow cytometer. An asynchronous control sample was used as an internal standard in order to aid in the determination of the location of the various cell cycle compartments. For each plot, the abscissa shows PI fluorescence (a measure of relative DNA content) and the ordinate shows FITC fluorescence, which is proportional to anti-BrdUrd binding.

Figure 5.

The rate of cell killing decreases as cells progress out of S phase during a chronic 41.5°C heat treatment. S phase cultures were heated at 41.5°C for up to 8 hrs, followed by either sham treatment or tritium suicide. Abscissa is the duration of the heat treatment in hrs, the ordinate is surviving fraction. Solid circles - surviving fraction of cells sham-labeled during the last 30 min of the heat exposure; open triangles - surviving fraction of cells labeled with h.s.a. ^{3}H-thymidine during the final 30 min of the heat treatment. All curves were corrected for sham plating efficiencies.

FIGURE 1A

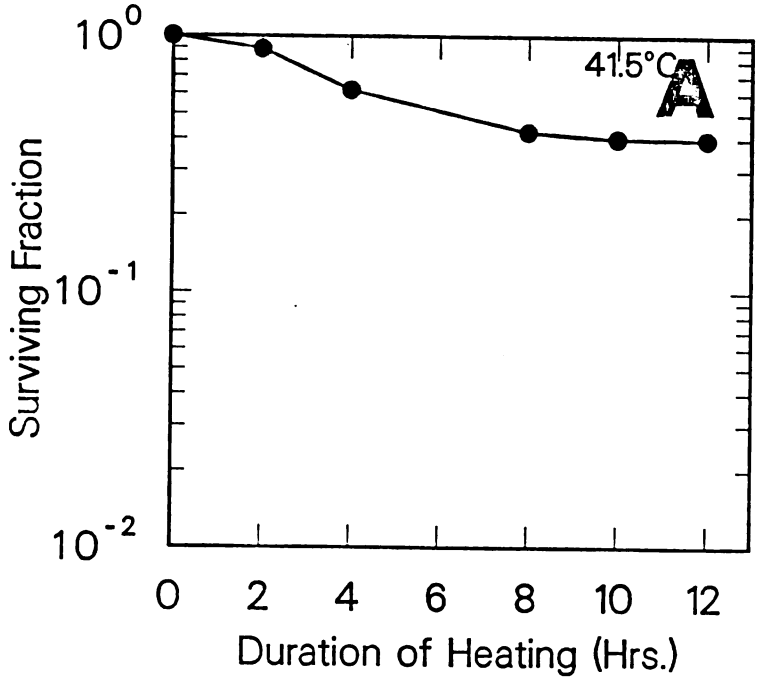

10^0
10^{-1}
10^{-2}
Surviving Fraction
41.5°C
A
0
2
4
6
8
10
12
Duration of Heating (Hrs.)

FIGURE 1B,C

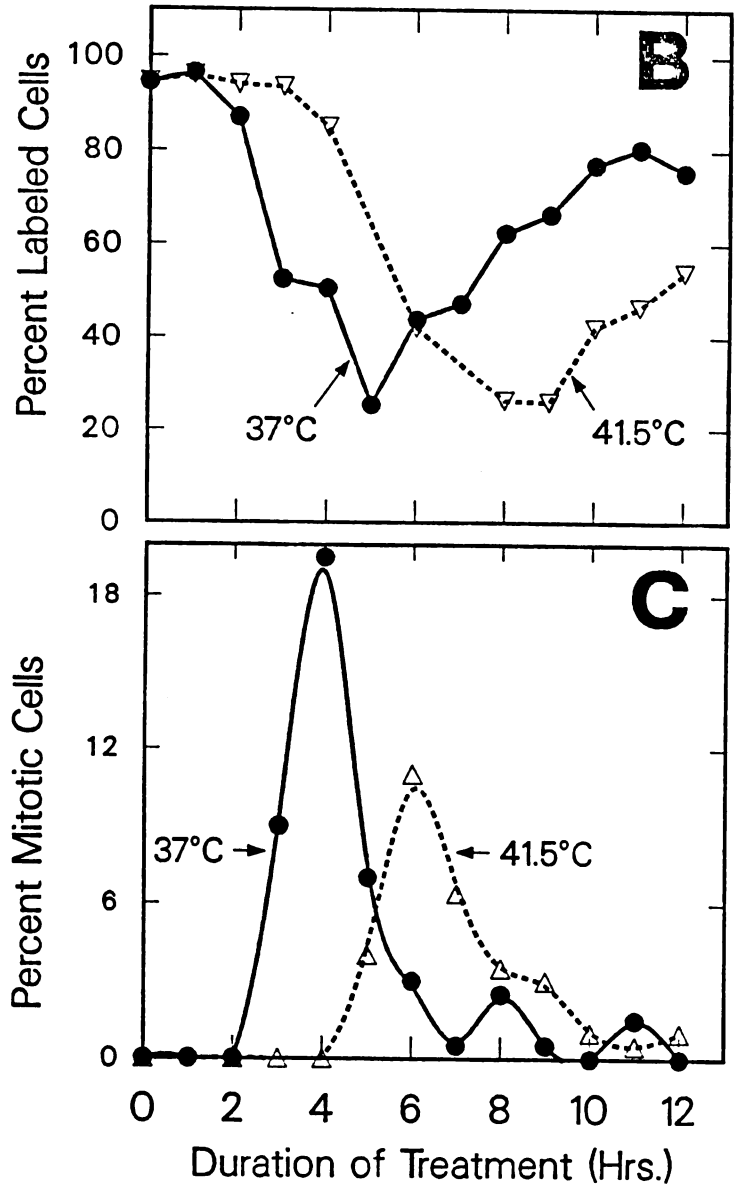

B
Percent Labeled Cells
100
80
60
40
20
0
37°C
41.5°C
C
Percent Mitotic Cells
18
12
6
0
37°C
41.5°C
0
2
4
6
8
10
12
Duration of Treatment (Hrs.)

FIGURE 2

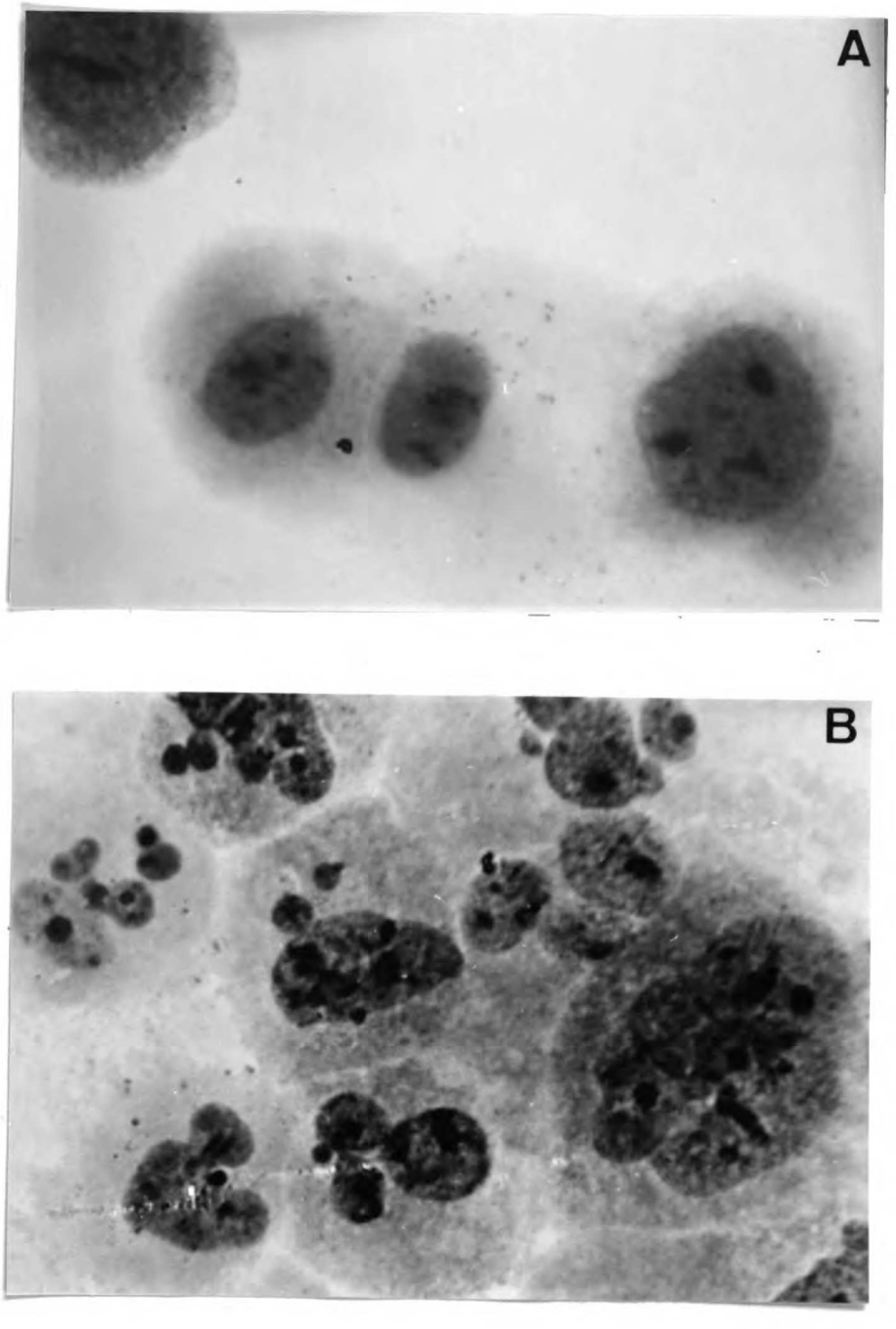


FIGURE 3A

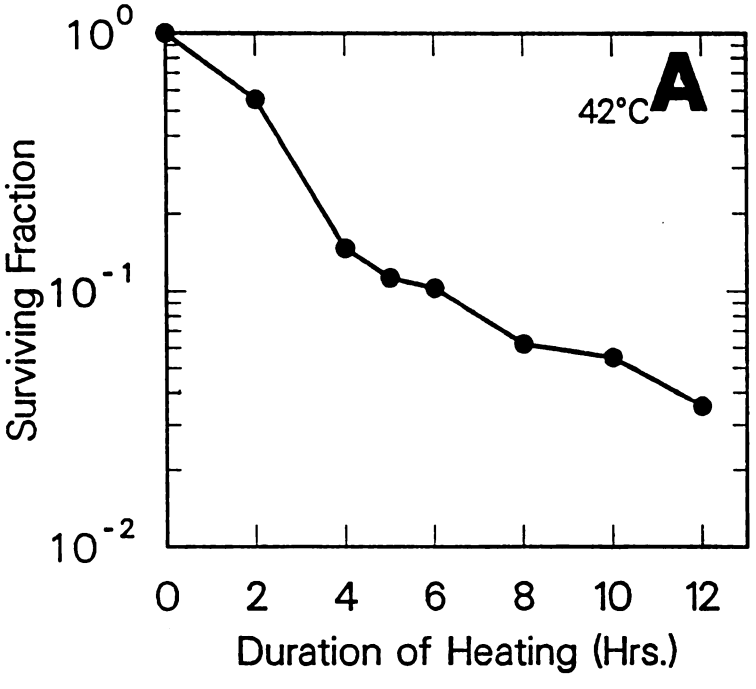

10^0
10^{-1}
10^{-2}
Surviving Fraction
42°C
A
0
2
4
6
8
10
12
Duration of Heating (Hrs.)

FIGURE 3B, C

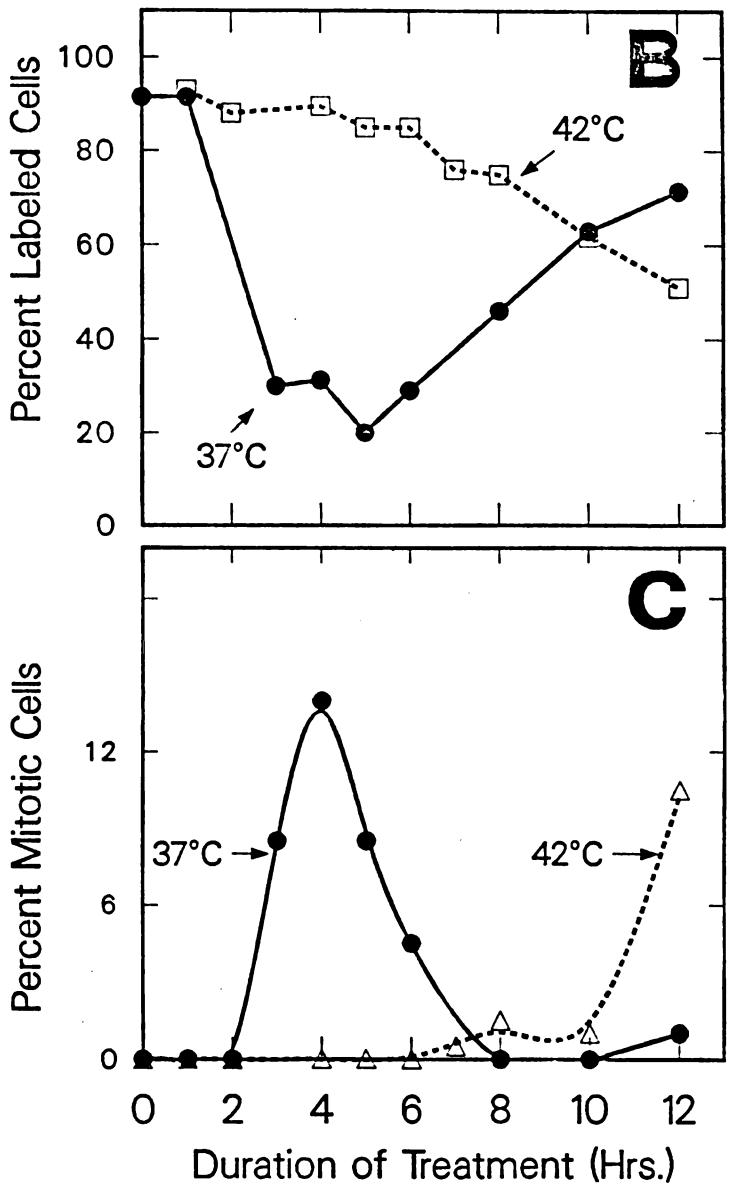

B
Percent Labeled Cells
100
80
60
40
20
0
42°C
37°C
C
Percent Mitotic Cells
12
6
0
37°C
42°C
0
2
4
6
8
10
12
Duration of Treatment (Hrs.)

FIGURE 4

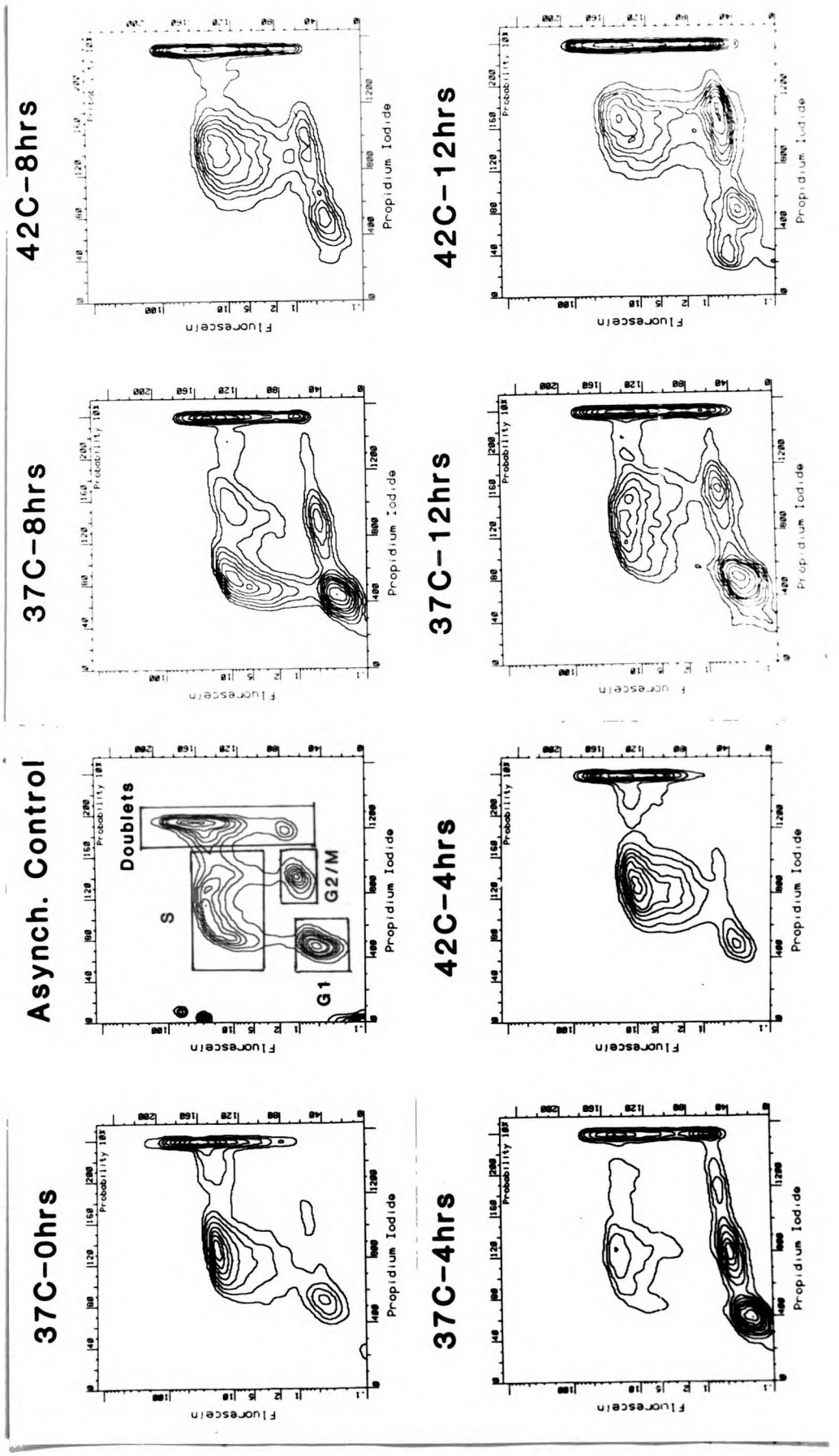
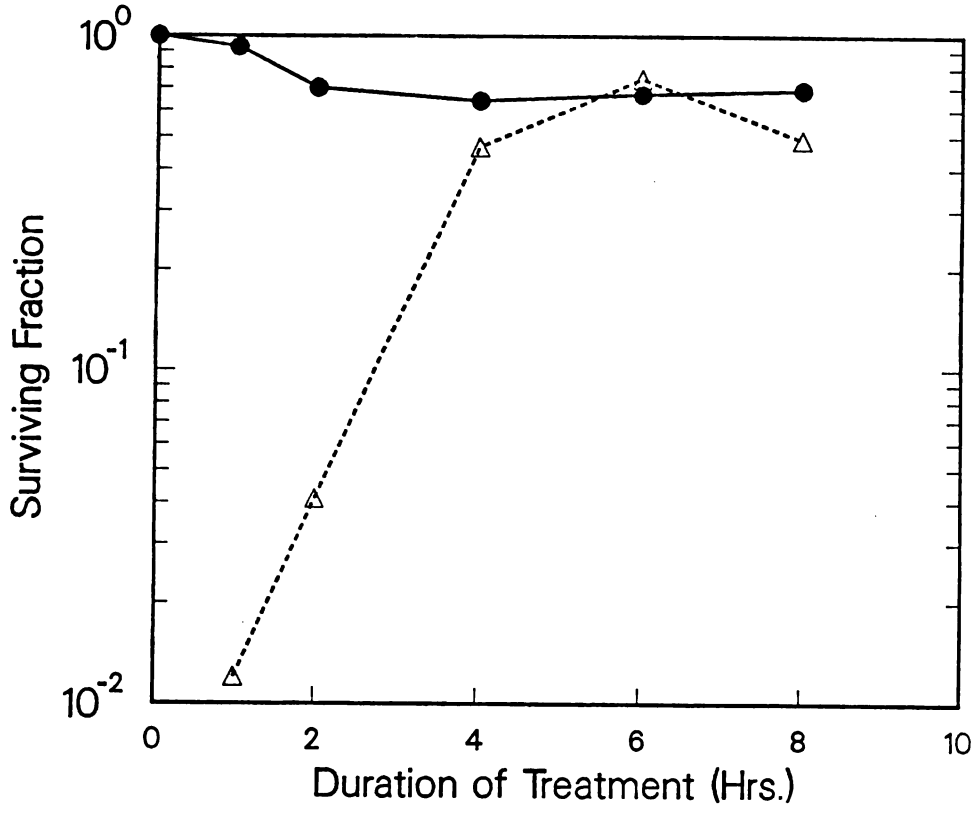

37C-0hrs
Asynch. Control
37C-8hrs
42C-8hrs
37C-4hrs
42C-4hrs
37C-12hrs
42C-12hrs
Doublets
S
G2/M
G1
Propidium Iodide
Fluorescein
Probability

FIGURE 5


10^0
10^{-1}
10^{-2}
Surviving Fraction
0
2
4
6
8
10
Duration of Treatment (Hrs.)

CHAPTER 4

Nuclear Fragmentation and Premature Chromosome Condensation Induced by Heat Shock in S Phase CHO Cells

ABSTRACT

Beginning in early S phase, synchronized CHO cells were heated for 12 hours at 41.5°C, during which time the cells progressed through S, G2, and into mitosis. Upon return to 37°C, some of the interphase cells developed a fragmented nuclear morphology, and the fraction of cells exhibiting this effect reached 40% of the population by late S or G2 of the next cell cycle. About 50% of the mitotic cells accumulated with Colcemid at the second mitosis exhibited premature chromosome condensation (metaphase-associated PCC) in an amount equal to approximately 1 - 2 chromosomes per diploid cell. There was also a low frequency of spontaneous S and G2 PCC. Heated cells in the first mitosis exhibited a chromosome break frequency of about 0.9 breaks per cell. This value agrees well with the observed surviving fraction of 40%, if we assume that 1 break per cell is a lethal event, and that chromosome damage has a Poisson frequency distribution in the cell population. Between the first and second mitoses, an increase in hyperthermia-induced aneuploidy was observed. This was apparently due to unequal division, since mitotic cells containing PCC were excluded from the chromosome number frequency analyses. Although this induction of aneuploidy was observed independently of PCC, we conclude that abnormal division is necessary for both effects, and both PCC and unequal division result in an increase in aneuploidy. We suggest that the heat-induced PCC reported here is caused by the heat-induced inhibition of DNA

synthesis during the first S phase (in S and G2 PCC), and by the inhibition of DNA synthesis in nuclear fragments during the second S phase (in metaphase-associated PCC).

INTRODUCTION

Mitotic division in mammalian cells is closely regulated by events occurring in G2 phase which require *de novo* protein synthesis (1). Although the biochemical nature of these events has not been elucidated completely, it is clear that soluble factors synthesized in G2 induce chromosome condensation in mitosis (2). Whe a mitotic cell is fused with an interphase cell using either Sendai virus or polyethylene glycol, chromatin is condensed, allowing visualization of the interphase organization of chromatin (3,4). The morphology of this premature chromosome condensation (PCC) depends on the location of the interphase cell in the cell cycle (5,6). Protein synthesis is required in the fused heterokaryon for the expression of fusion-induced PCC, as the addition of either cycloheximide or puromycin inhibits this change in nuclear morphology (7).

When inhibitors of DNA synthesis are used to delay cells in S phase, a disruption of normal mitotic events occurs, resulting in spontaneous PCC (8,9). Spontaneous PCC occurs without any cell fusion, requires *de novo* protein synthesis, does not require RNA synthesis, and has only been observed with the morphology characteristic of cells in the S or G2 phases (9). This type of PCC generally affects all of the chromatin in a cell to about the same extent, and can occur before the completion of DNA synthesis. The morphology observed in the affected cells ranges from the completely diffuse, pulverized chromosomes of a PCC in early S to that of a G2 PCC, which exhibits thin, extended double chromatids.

Another type of PCC which has been observed occurs in only a fraction of the chromosome complement and appears only in metaphase. This metaphase-associated PCC has been observed in cells possessing micronuclei induced by X irradiation (10), and appears to be caused by incomplete DNA synthesis in micronuclei (11,6).

Interest in the use of hyperthermia for cancer therapy, either alone or in conjunction with other therapeutic agents, has provided an impetus for understanding the various mechanisms of heat-induced cytotoxicity. Knowledge of specific modes of cell death should allow the design of more effective treatment regimens. Previously we found that when S phase cells were chronically heated at 41.5°C, cell cycle progression continued at a reduced rate, although reproductive cell death occurred in about 60% of the population (see Chapter 3 of this thesis). Based upon these earlier data, experiments were conducted to examine the effects of chronic 41.5°C hyperthermia upon nuclear and chromosome morphology when the heat exposure was confined to the period of time in the cell cycle from early S through G2 and into mitosis. In this report we present evidence suggesting that the development of a fragmented morphology in the nucleus of interphase cells, following entry into mitosis during a chronic heat stress, is later expressed in the appearance of metaphase-associated PCC at the next mitosis.

MATERIALS AND METHODS

Culture Conditions, Synchrony Methods, and Heat Treatment

Chinese Hamster Ovary (CHO) cells were grown in McCoy's 5a modified medium (Gibco) containing 10% heat-inactivated fetal calf serum (J.R. Scientific Co.), and the antibiotics neomycin sulfate (0.1 g/l), potassium penicillin G (0.07 g/l) and streptomycin sulfate (0.1 g/l) (Sigma). Exponentially growing cells were cultured at 37°C in a humidified atmosphere and the medium was maintained at pH 7.4 with 5% CO_2. Mitotic cells were obtained from these stocks by the mitotic selection technique, as described previously (12); mitotic indices ranged from 98-99%. For some experiments, mitotic cells were frozen in a solution which has been shown to protect against adverse effects of freezing on plating

efficiency, heat survival, and cell cycle progression (13). Plating efficiencies ranged from 50 to 70%.

Synchronous S phase populations were obtained by incubating mitotic cells in suspension culture for 12 hours at 37°C in McCoy's 5a medium supplemented with 2 μg/ml aphidicolin (Sigma), as described in detail elsewhere (14). At the end of this incubation period, the cell suspension was centrifuged (120 x g - 10 min - 4°C), the pellet was resuspended in fresh drug-free medium, and cells were plated into flasks at a density of 2 X 10^4 cells/cm^2. After two hours incubation in a 37°C incubator, flasks were sealed with paraffin and then placed in Precision waterbaths (GCA) set at either 37° or 41.5°C.

Mitotic and Labeling Indices

Both labeling index and mitotic index were determined from the same slide preparations. Cultures were pulsed for 20 minutes with low specific activity tritiated thymidine (25 Ci/mM - Amersham Corp.) at a concentration of 4 μCi/ml in the regular culture medium. All labeling was performed at 37°C; for the heated groups, an interval of 10 min at 37°C immediately preceded the tritium pulse. After the pulse label, cultures were trypsinized on ice using a Hepes-buffered trypsin solution (15); all solutions were saved, including the medium in the flasks and the trypsin rinses, and this suspension was centrifuged (120 x g - 10 min - 4°C). After discarding the supernatant, the pellet was resuspended in 5 ml Hanks BSS and centrifuged as before, and was fixed in the pellet with 0.5 ml fresh Carnoy's fixative (3:1 methanol:glacial acetic acid) for 5 min, followed by gentle resuspension with a small-bore pipette and the addition of 9.5 ml Carnoy's. Following an additional centrifugation, the resuspended pellet was diluted with an appropriate amount of fixative to yield a good density of cells on the slide (usually 0.2 - 0.5 ml), and cell preparations were made by dropping 2-4 drops of the suspension onto clean slides wetted with ice-cold distilled water. After drying overnight, the slides were either stained with 2% aceto-orcein and permanently mounted for mitotic index sampling, or the slides were coated with photographic emulsion (Ilford L4 -

Polysciences) and exposed for 1-4 weeks, before developing according to the manufacturer's recommendations and staining using Wright's stain. Both labeling and mitotic indices were determined by counting at least 200 cells for each determination.

Preparation of Metaphase Spreads

To obtain metaphase chromosome preparations, Colcemid (Ciba) was added at a final concentration of 0.2μM, and mitotic cells were accumulated for 2 hours at 37°C, followed by trypsinization as described above for mitotic and labeling indices. After centrifugation (120 x g - 10 min - 4°C), the pellet was resuspended in 10 ml 0.075M KCl and incubated at room temperature for 6 min. The cell suspension was centrifuged again as before, and the pellet was fixed for 10 min using 10 ml fresh Carnoy's fixative. After recentrifugation and resuspension of the pellet in fresh fixative, cell preparations were made by dropping the cell suspension onto wetted slides, followed by drying overnight and staining with 2% aceto-orcein. Slides prepared in this manner allowed visualization of the cytoplasm, which ensures accurate determinations of chromosome number per cell.

Determination of Surviving Fraction

Synchronized S phase cells were plated into 25 cm^2 flasks containing 10^5 lethally irradiated feeders (25 Gy - 250 kVp X rays), at a density yielding about 100 colonies per flask. These flasks were then heated as described above. After 12 hours exposure to 41.5°C, the flasks were removed from the waterbaths, dried, and placed in a 37°C incubator for colony formation for 7-9 days. Following staining with crystal violet, colonies of 50 or more cells were scored as survivors.

RESULTS

Figure 1 shows the kinetics of cell cycle progression of cells chronically exposed to 41.5°C for 12 hrs beginning in early S phase. Surviving fraction after the 12 hr heat treatment was found to be 40%. This value only pertains to the fraction of cells initially in early S which survived the chronic treatment. Even though these effects were seen in three separate experiments, all of the data shown in this report are from a single representative experiment; therefore, Figure 1 also serves as a guide to the cell cycle dependence of the phenomena to be described.

Exposure of S phase cells to chronic 41.5°C hyperthermia resulted in a lengthening of S phase by 4 hrs, or by about 75% of the control transit time (Figure 1A). This extension of the S period was also evident in an apparent 4 hr mitotic delay (Figure 1B); it should be noted that the entry of cells into mitosis occurring during the heat exposure was essentially complete by the end of the treatment interval (e.g. 14 hrs after release), at which time some of the cultures were returned to 37°C to continue monitoring the effects of the heat shock.

Several interesting cytological observations were made upon examination of the cells after the heat exposure. One striking observation was that a fraction of the population possessed fragmented nuclei (see Figure 2B). We have chosen to avoid classifying these cells as multinucleated, as this term usually refers to syncytia containing multiple numbers of individual nuclei (16). Likewise, the term micronucleated is usually associated with cells which possess a large macronucleus, surrounded by 1 or 2 smaller micronuclei (17). Accordingly, we use the term fragmented to refer to any abnormal nuclear structure, from a cell with a macronucleus and micronuclei, to the more extreme damage shown in Figure 2B. In control unheated populations few cells showed fragmented nuclei (i.e. 5-25%). Of those with fragmented nuclei, the average number of fragments per cell was 2 ± 1 (mean ± 1 SD), and 96% of these cells had one or two micronuclei per cell. In the heated population, however, the fragmentation was much more dramatic: the fragments were generally much

larger than micronuclei and the average number of fragments per cell was 4 ± 3; this mean number of nuclear fragments was essentially constant in those heated cells exhibiting nuclear fragmentation through the course of the experiment. To differentiate the micronucleation seen in controls from the larger, more numerous fragments observed in heated cells, we scored a cell as fragmented only if it possessed more than 2 micronuclei or had larger nuclear fragments. A rapid increase in the fraction of fragmented cells appeared following the first mitosis and remained at about 30-40% up to the second division (see Figure 3).

We also observed PCC in metaphase cells accumulated at the second division, i.e. the first division following the division that occurred during the chronic heat exposure (see Figure 2C,D). Incompletely condensed chromosomes, as well as amorphous chromatin patches, were observed to increase in frequency by the time of the second mitosis (see Figure 4).

Chromosomal damage was scored on cells at the first division and the results are shown in Table 1. Cells from the first two accumulation periods showed mainly gaps and deletions, while the final collection showed an increase in exchanges over the earlier periods. This may be related to the fact that these were those cells delayed the longest in S phase during the heat treatment, but might be explained by the longer duration of heating (e.g., this later group was heated 2-4 hrs longer than the earlier collections).

There was also a low level (e.g. 2-6%) of apparent S and G2 PCC (see Figure 5A). The PCC figures showed morphology similar to the prematurely condensed chromatin resulting from fusion of a mitotic cell with an interphase cell (18), except that there was no corresponding mitotic cell needed to induce chromosome condensation.

Analysis of frequency distributions of chromosome number per cell showed the induction of aneuploidy in the heated population. Cells accumulated in mitosis at the first division were in the diploid range, with about 2-15% of the cells not containing 20-22 chromosomes. At the second division, however, an increase in aneuploidy was observed in the heated cells (see Figure 6 and Table 2), as chromosome distributions of heated groups exhibited 42-76% aneuploid character, (i.e. not exactly diploid or tetraploid chromosome number). In controls,

however, even though an increase from 6-9% to 14% tetraploid was observed in the second division, the incidence of aneuploidy was much lower, (i.e. 17-22%). The aphidicolin synchrony technique itself induced abnormal mitoses at the first division, as control distributions obtained from cells synchronized only at mitosis showed a much lower incidence of aneuploidy (see footnote to Table 2).

DISCUSSION

Spontaneous PCC has been attributed to the uncoupling of mitotic events from the completion of DNA synthesis (9). The qualifier "spontaneous" is usually used to avoid confusion with a popular technique, also known as PCC, involving the Sendai virus- or polyethylene glycol-mediated fusion of mitotic cells with interphase cells (3,4) whereby interphase chromatin structure is studied. In this report, PCC refers to any cytologically-observable aberrant condensation of chromosomes in metaphase or interphase cells. This occurred in the absence of any chemical- or virus-induced fusion. The PCC effects discussed here belong to two classes of phenomena. One type of spontaneous PCC, which we term metaphase-associated PCC, appears in metaphase cells amidst otherwise normal mitotic chromosomes (see Figure 2C,D). This type of PCC was usually observed in metaphase spreads in an amount consisting of approximately 5-10% of the mitotic chromosome complement (i.e. 1-2 chromosomes per diploid cell).

The other distinct type of PCC which we observed is distinguished by the fact that all the chromatin in the cell is affected to about the same extent. These PCC-containing cells appear with various morphologies which are characterized by their location in the cell cycle (6), and usually are observed by applying the PCC fusion technique. In our experiments we observed a low frequency of PCC in cells from early S through G2 (see Figure 5). However, due to their low frequency (2-6%) and sometimes indistinct morphology, no quantitative account of these results is presented here. Of the 15,000 cells scored for this study, only one was found

with morphology characteristic of an interphase cell fused with a mitotic cell (see Figure 5B), and so we conclude that spontaneous fusion is not responsible for the other PCC effects we observed.

Other investigators have shown that inhibition of DNA synthesis in synchronized S phase CHO cells, using either hydroxyurea, or hydroxyurea plus caffeine, results in spontaneous S and G2 PCC. Hydroxyurea produced PCC in cells in late S or G2 (8). When hydroxyurea was used with caffeine, however, cells exhibited an early S phase PCC morphology (9). In this study we found that heat shock caused late S and G2 PCC. Since heat has been shown to inhibit DNA replication (15), it would appear that the inhibition of DNA synthesis, either by drugs or heat, can lead to PCC.

Early S phase cells exposed to chronic 41.5°C hyperthermia for 12 hrs progressed through S more slowly than controls and entered mitosis with an aberration frequency of about 0.9 breaks per cell. If we assume that a chromosome break is a lethal event, this break frequency corresponds (19) to a surviving fraction of about 40%, which was the survival observed in our experiments. Attempted cell division during the chronic heat exposure preceded the development of a fragmented nuclear morphology in a substantial fraction of the population, and this proportion reached a maximum towards the end of the second S phase. As the fraction of cells exhibiting nuclear fragmentation began to decrease, there was a dramatic increase in the fraction of second division mitotic cells that contained metaphase-associated PCC. The fact that nuclear fragmentation preceded metaphase-associated PCC suggests that nuclear fragmentation caused this PCC effect. Nuclear fragmentation appears not to be related to gross chromosomal damage because the frequency of this damage (i.e. about 1 break per cell) is much lower than the observed fragmentation (about 4 fragments per cell). Electron microscopy of initially asynchronous cultures dividing during chronic 41.5°C hyperthermia revealed the precocious reformation of the nuclear envelope around adjacent chromosomes while still in metaphase (20). This early reformation of the nuclear envelope may be responsible for the appearance of fragmented nuclei in the subsequent G1 phase.

Presumably, these membrane-encased chromosomes are unable to complete karyokinesis and cytokinesis. It is also possible that some of these nuclear fragments, upon entry of the cell into the second S phase, are deficient in some component necessary for the timely completion of DNA replication, as has been found in micronuclei (21). X-irradiated S phase CHO cells require at least one additional mitotic cycle before manifesting metaphase-associated PCC (22). Since ionizing radiation-induced micronuclei appear within a few hours of irradiation (23), we suggest that, as in our hyperthermia experiments, micronucleus formation precedes x-ray-induced metaphase-associated PCC. X-irradiation of bean root tip cells (*V. faba*) produces some micronuclei that do not contain nucleoli and these micronuclei did not synthesize DNA at the same time as their neighboring macronucleus during S phase (21). Furthermore, studies using binucleate human cells indicated that the presence of ^{3}H-thymidine labeled metaphase-associated PCC were the result of interference with the completion of DNA replication in micronuclei (11). All these earlier studies suggest that when DNA replication is incomplete in micronuclei, chromosome condensation cannot occur properly. Accordingly, we present the following hypothesis on the mechanism of induction of metaphase-associated PCC caused by chronic heat shock. Cells arriving at mitosis during the heat treatment are delayed in division and experience a premature reformation of the nuclear envelope before anaphase, leading to the inhibition of karyokinesis and, ultimately, cytokinesis. This results in a fragmented interphase nuclear morphology. When these cells enter the next S phase some of the fragments are unable to complete DNA synthesis in synchrony with the rest of the nuclear material and are then prematurely induced to begin chromosome condensation in preparation for mitosis, resulting in PCC formation. We also observed changes in the ploidy of the heated cell population over the course of a single cell cycle (see Table 2). The chromosome number frequency distributions show that an increase in both aneuploid and tetraploid cell fractions was observed after the heat treatment (see Figure 5). These changes in ploidy are attributable to abnormal division at the first mitosis, and are distinct from ploidy changes related to induction of metaphase-associated PCC, because metaphase spreads

containing PCC were not scored for chromosome number. If we assume that metaphase-associated PCC is followed by the irreversible loss of genetic material, PCC induction then would cause a change in ploidy. Therefore, we conclude that cell division during chronic 41.5°C hyperthermia causes an increase in aneuploidy because of unequal division and because of the induction of metaphase-associated PCC after the development of nuclear fragmentation.

ACKNOWLEDGEMENTS

This research was funded by NCI grant CA 31813 (W.C.D. and M.A.M.) and the Office of Health and Environmental Research of the U.S. Dept. of Energy, Contract No. DE-AC03-76-SF012 (W.F.M.). The authors are grateful to Dr. Robert B. Painter for critical review of the manuscript.

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TABLE 1 - HYPERTHERMIA-INDUCED CHROMOSOMAL DAMAGE

Hours After Release	Deletions[a]	Exchanges[b]	Gaps	Other[c]	Number of Cells Scored	Breaks per Cell[d]
37°C						
5	1	0	0	0	50	0.02
7	0	0	0	0	.50	0.00
Total	1	0	0	0	100	0.01
41.5°C						
7	12	3	56	0	50	0.36
9	29	7	42	1	50	0.90
11	24	22	38	0	50	1.36
Total	65	32	136	1	150	0.86

Note: All values for hours after release are the midpoints of the 2 hour Colcemid accumulation period.

[a]Chromatid and isochromatid deletions were included in this category.

[b]Includes chromatid inter- and intra-changes, as well as isochromatid exchanges.

[c]Chromosome aberrations (e.g. dicentrics, rings, etc.) are included in this category.

[d]Breaks per cell was calculated by assuming that deletions resulted from one break, while exchanges resulted from two breaks; gaps were not included in these calculations.

TABLE 2 - EFFECTS OF HYPERTHERMIA UPON PLOIDY[a,b]

Hours[c] After Release	Number of Cells Scored	Percent Mitotic	Percent Hypo-diploid	Percent Diploid	Percent Hyper-diploid	Percent Hypo-tetra-ploid	Percent Tetra-ploid	Percent Hyper-tetra-ploid
37°C								
5	51	33	16	71	0	8	6	0
7	67	9	13	76	0	2	9	0
19	71	11	6	76	4	6	7	1
21	70	5	7	69	4	3	14	3
23	50	3	10	64	2	10	14	0
41.5°C								
7	63	4	8	90	0	0	2	0
9	66	20	6	88	3	0	3	0
11	74	16	14	76	1	3	7	0
21	62	5	23	50	10	10	8	0
23	75	20	21	33	16	9	19	0
25	73	8	48	19	14	14	5	0

[a]Classifications used were the following:
Hypodiploid: less than 21 chromosomes
Diploid: 21 chromosomes
Hyperdiploid: 22-30 chromosomes
Hypotetraploid: 31-41 chromosomes
Tetraploid: 42 chromosomes
Hypertetraploid: more than 42 chromosomes

[b]Analysis of the chromosome frequency distribution of cells which were not resynchronized using aphidicolin, (i.e. mitotic cells allowed to progress through a single cycle without perturbation), yielded 88% diploid, 7% hypodiploid, and 5% tetraploid.

[c]Mitotic cells were accumulated for 2 hrs at 37°C using Colcemid; time after release refers to the midpoint of the two hour collection period.

FIGURE LEGENDS

Figure 1.

Cell cycle progression kinetics of S phase cells at 37°C (solid circles), or heated for 12 hrs at 41.5°C beginning at 2 hrs after the start of the experiment (triangles) followed by treatment at 37°C for the duration of the experiment. A: 3H-thymidine pulse labeling indices; B: mitotic indices. Abscissa shows the elapsed time after release from the G1/S border.

Figure 2.

Representative examples of fragmented nuclei and metaphase-associated PCC induced by 12 hrs heating at 41.5°C which began in early S phase. A: Morphology of control cells at 37°C; B: heated cells presenting a fragmented nuclear morphology; C and D show PCC-containing metaphases derived from the heated population which were accumulated at the second mitosis following the start of the experiment (arrows indicate PCC patches). All photos 800X.

Figure 3.

Kinetics of the induction of fragmented nuclei in cells exposed to 41.5°C hyperthermia beginning in early S phase. The abscissa represents the elapsed time from release from the G1/S border; the ordinate shows the frequency of fragmented nuclei (see Figures 2A, 2B) in the total cell population. Solid circles: 37°C; triangles: cultures exposed to 41.5°C from 2 to 14 hrs after release (up to 12 hours total treatment time).

Figure 4.

Induction of metaphase-associated PCC in S phase cells heated for 12 hrs at 41.5°C, followed by recovery at 37°C. The experimental protocol used was identical to that shown in Figure 1, except that 2 hr Colcemid treatments were used to accumulate mitotic cells at 37°C. The abscissa shows the time after release from the G1/S border (points plotted at the midpoint of the 2-hr collection period); the ordinate is the percentage of mitotic cells exhibiting PCC. Triangles: cultures heated for up to 12 hrs at 41.5°C; circles: 37°C control cultures.

Figure 5.

Examples of spontaneous PCC observed in cells heated at 41.5°C for 12 hrs beginning in early S phase. A: spontaneous S phase PCC; B: spontaneous fusion-induced PCC. Note that PCC derived from spontaneous fusion was discovered in only one cell out of the 15,000 cells scored for this report, and is therefore not responsible for the other spontaneous PCC effects which we observed. All photos 800X.

Figure 6.

Induction of aneuploidy in cells heated for 12 hrs at 41.5°C starting in early S phase. These data are the frequency distributions of chromosome number per cell in the same Colcemid-accumulated samples as shown in Figures 3 and 4 at the first division (e.g. 5 hr - 37°C and 9 hr - 41.5°C points) and the second division (19 hr - 37°C and 25 hr - 41.5°C points), and were obtained by scoring at least 75 cells without metaphase-associated PCC. Panel A: first division - 37°C; panel B: second division - 37°C; panel C: first division - heated cells; panel D: second division - heated cells.

FIGURE 1

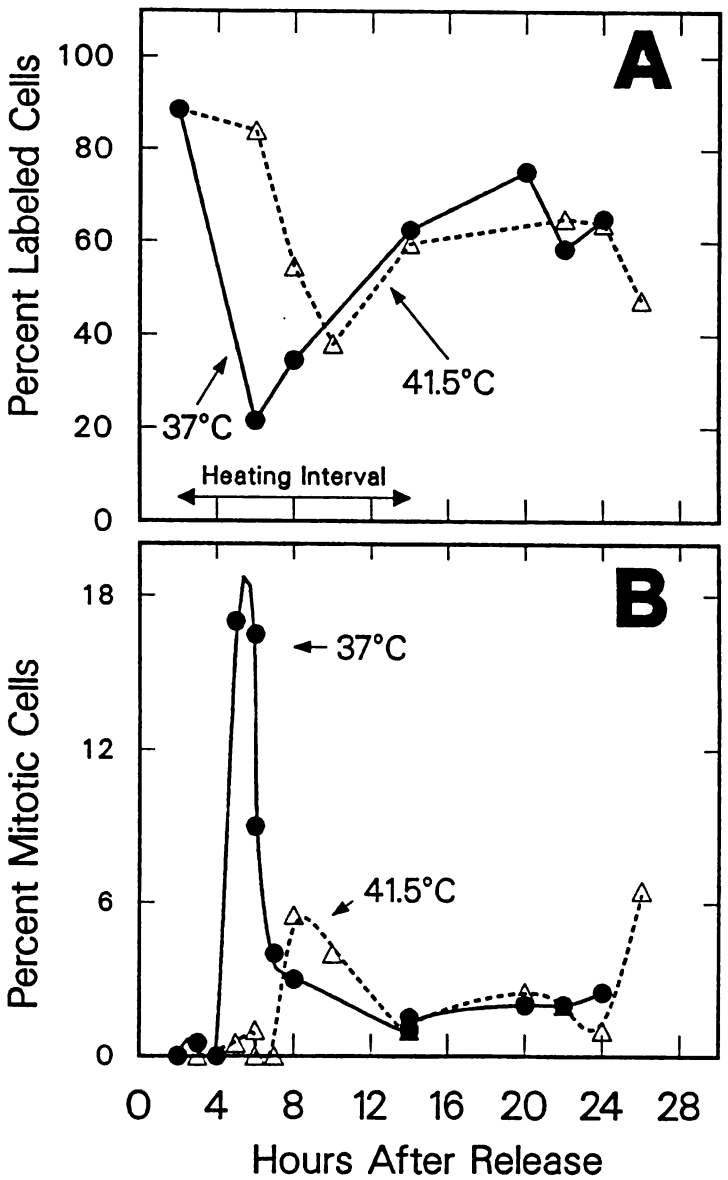

A
B
Percent Labeled Cells
Percent Mitotic Cells
100
80
60
40
20
0
18
12
6
0
37°C
41.5°C
Heating Interval
0 4 8 12 16 20 24 28
Hours After Release

FIGURE 2

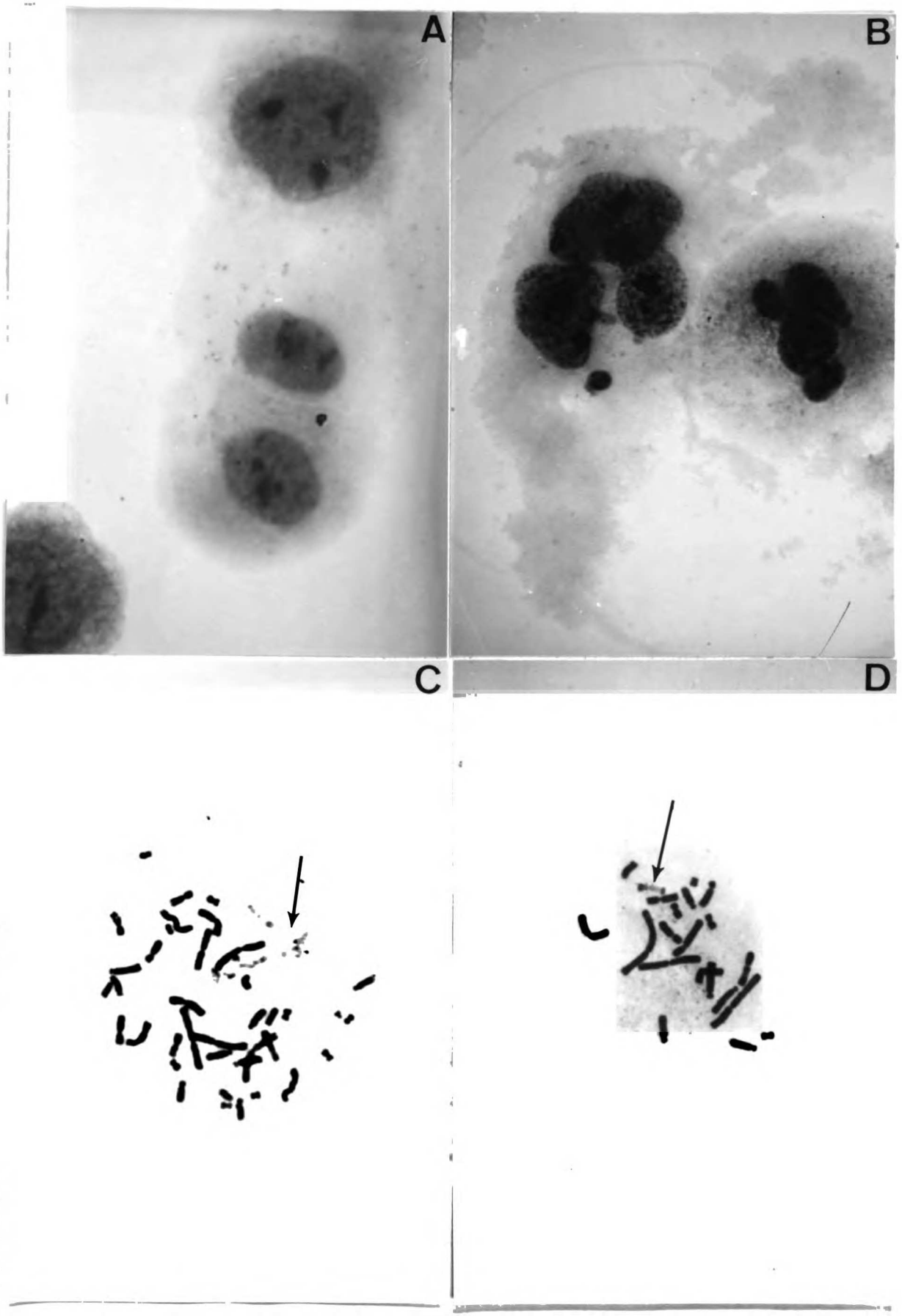


FIGURE 3

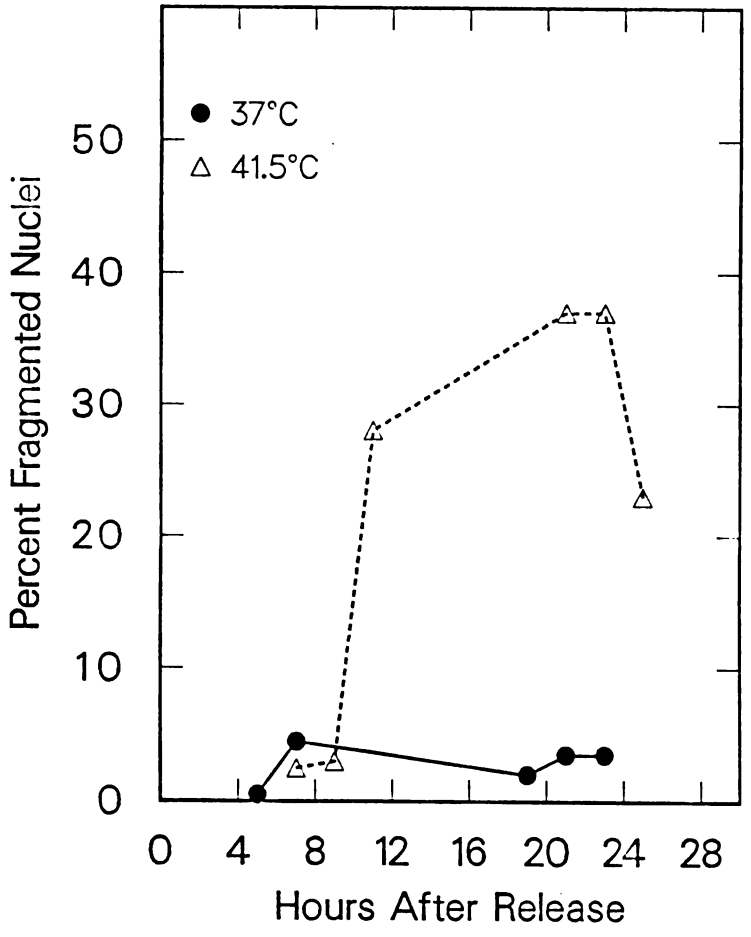

37°C
41.5°C
Percent Fragmented Nuclei
0
10
20
30
40
50
0
4
8
12
16
20
24
28
Hours After Release

FIGURE 4

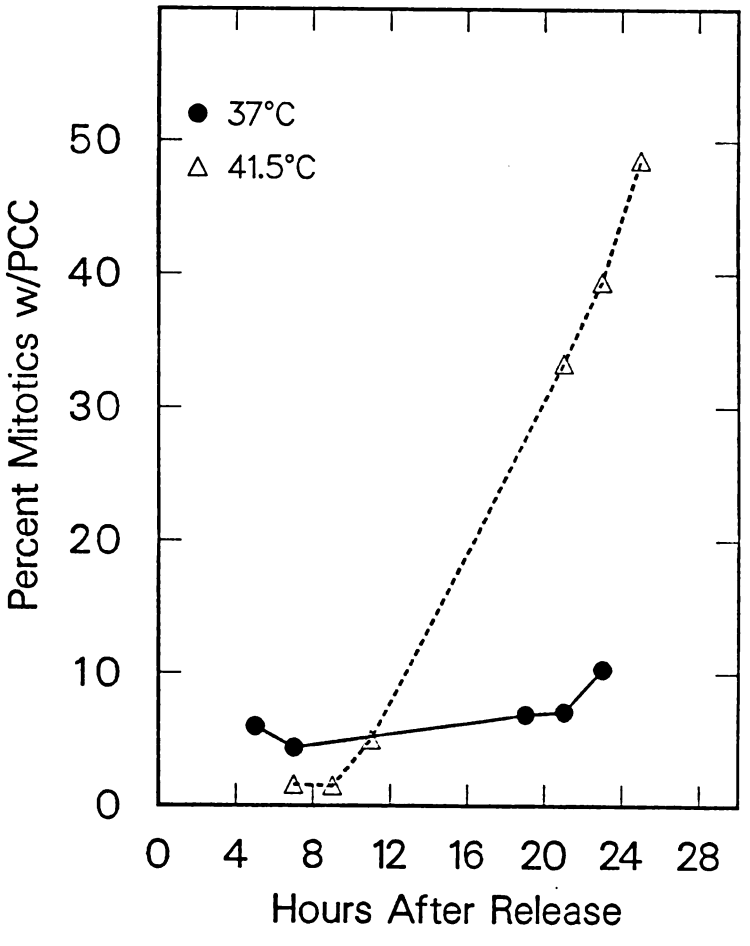
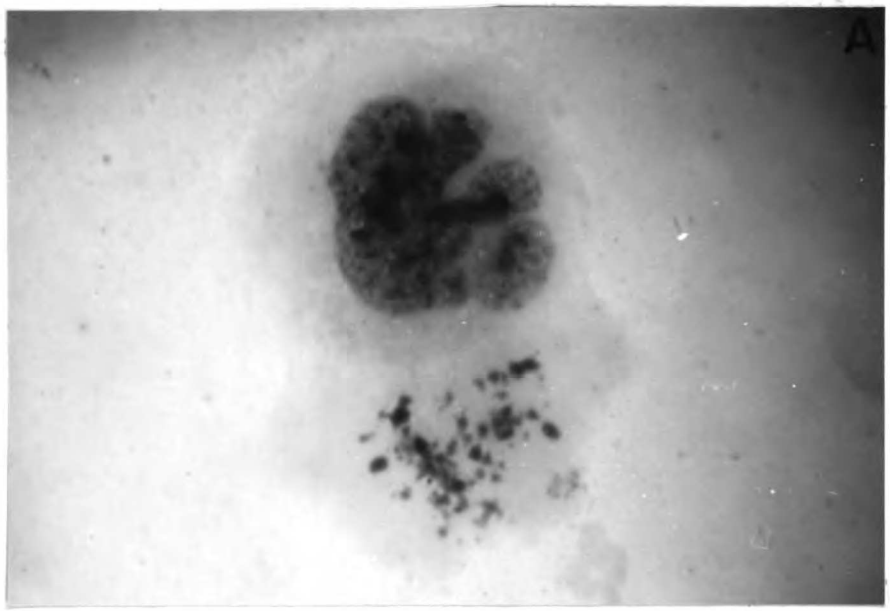

37°C
41.5°C
Percent Mitotics w/PCC
0
10
20
30
40
50
0
4
8
12
16
20
24
28
Hours After Release

FIGURE 5



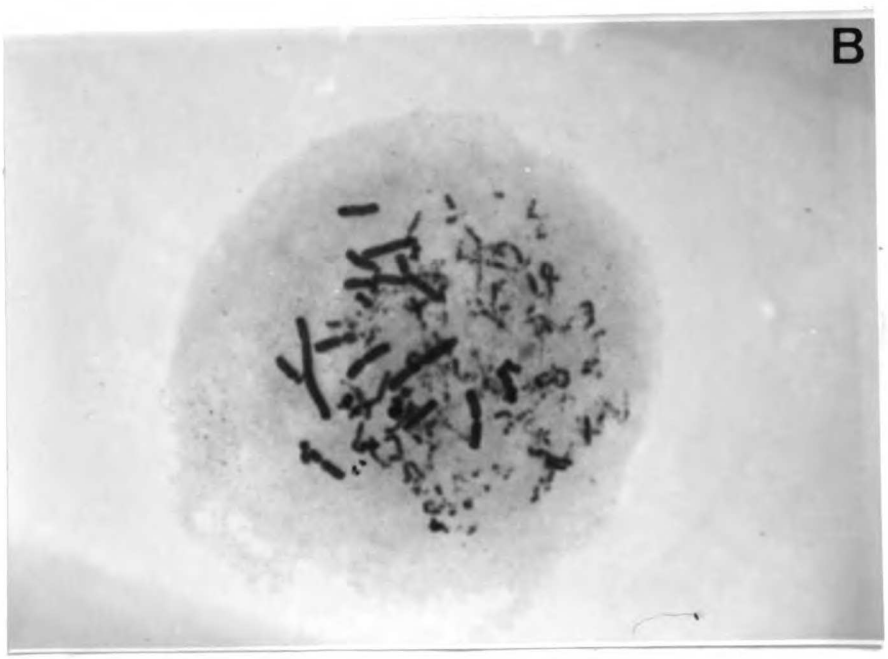


FIGURE 6A, B

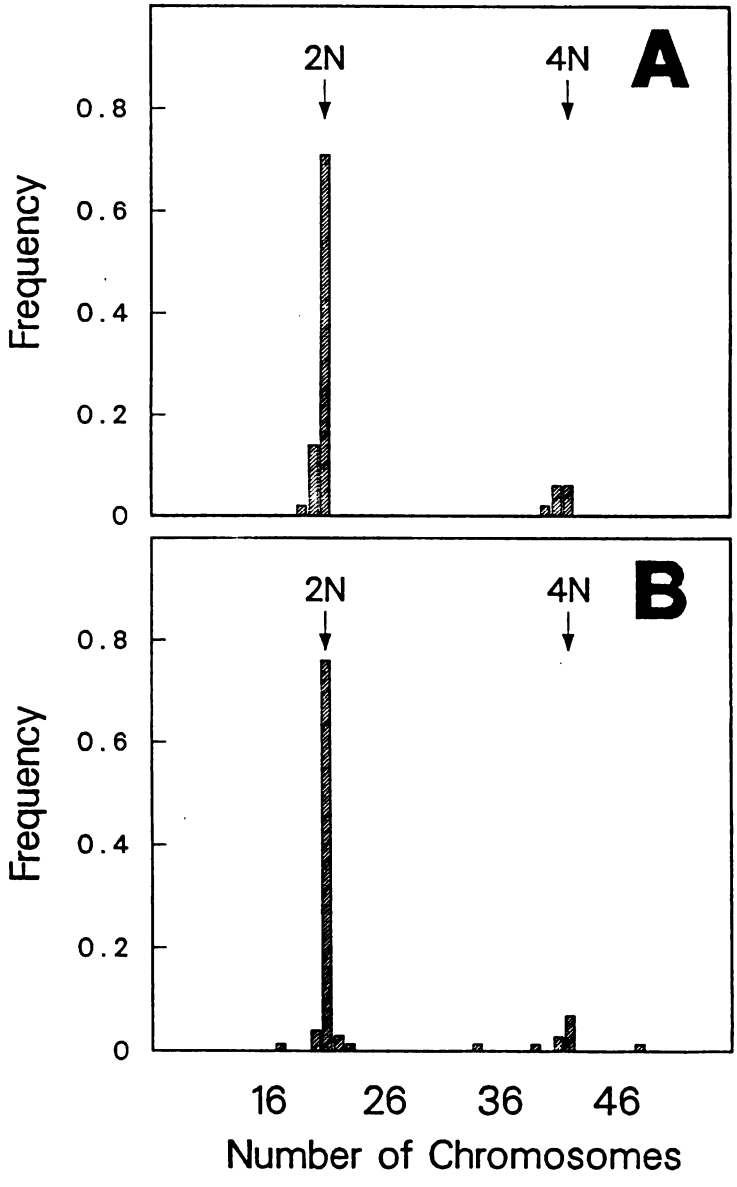
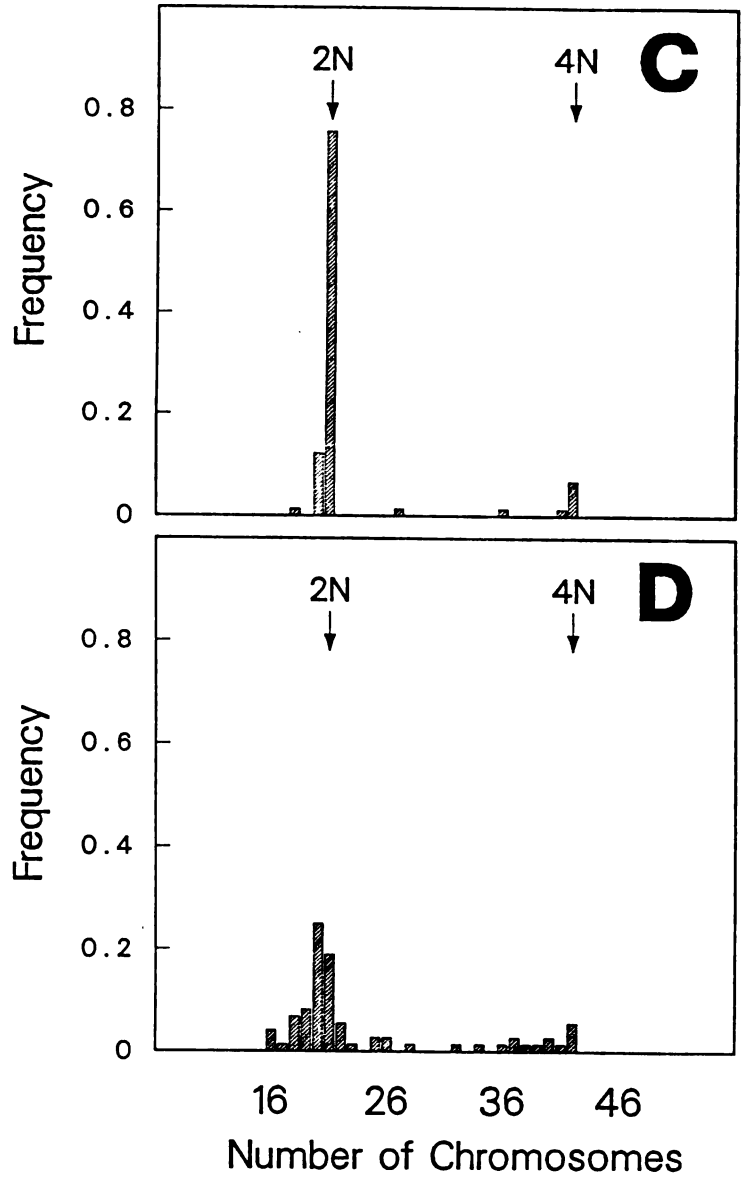

2N
4N
A
0.8
0.6
0.4
0.2
0
Frequency
2N
4N
B
0.8
0.6
0.4
0.2
0
Frequency
16
26
36
46
Number of Chromosomes

FIGURE 6C, D


2N
4N
C
0.8
0.6
0.4
0.2
0
Frequency
2N
4N
D
0.8
0.6
0.4
0.2
0
Frequency
16
26
36
46
Number of Chromosomes

CHAPTER 5
Conclusion

Several conclusions are apparent from the comparison of the heat survival responses of G1 and S phase cells presented in Chapter 2. Although heat-induced cytotoxicity is apparently manifest in different ways in G1 versus S phase cells, the temperature dependencies of the rates of decrease in survival in these two phases were essentially the same. S phase cells die by a mechanism apparently related to chromosome aberrations; such a relationship between heat killing of the resistant G1 cells and induction of chromosome damage is not found. These differences in the apparent mechanisms of cell death in the two populations, when coupled with similarities in the temperature dependencies of the rates of cell killing, suggest that the temperature-dependence of the rate of cell killing is independent of mechanism. This conclusion is contrary to the usual interpretation of the slope of the Arrhenius plot. If this slope is not related to the mechanism of heat killing, then there is no *a priori* reason to suspect that protein denaturation is involved with this mechanism of heat-induced cytotoxicity.

The notion that mechanistic information is not forthcoming from the slope of the Arrhenius plot is further supported by the observation that the temperature dependence of the rate of cell killing was better fit by a quadratic function of temperature in both cell cycle phases, rather than the usual biphasic linear fit. According to the thermodynamical hypotheses of Eyring in his original formulation of Transition State Theory, linearity of this plot is absolutely necessary to justify the proportionality between this slope and the activation energy of a rate-limiting step in a reaction sequence. This is not surprising when the thermodynamic state of the cell is taken into account. The cell is at a non-equilibrium steady-state, frequently termed homeostasis in the biological literature, which requires the dynamic flow of substances into and out of the open system in order to ensure thermodynamic stability. The implications of this consideration of state upon the aforementioned theoretical framework

of the Eyring hypothesis is highly nontrivial, as the basic assumption of the Eyring model is that the system is initially in a state of minimum free energy. In the non-equilibrium milieu, free energy is a poorly defined, ambiguous quantity. In such dynamic systems, a better criterion of thermodynamic stability is the rate of entropy production. It is possible that, given a proper theoretical framework, a new model may be constructed which could explain the temperature dependence of the rate of cell killing by heat. Such an ambitious undertaking is beyond the scope of this thesis.

The observation that during a chronic heat exposure S phase cells are capable of progressing out of S explains the change in the rate of cell killing at 41.5°C which was observed while performing the G1/S comparison, as these cultures were able to progress to a more resistant phase of the cell cycle during the treatment. Furthermore, once this complication was understood, it was determined that chronic thermotolerance is not expressed in S phase cells, as in G1 cells, thus providing another aspect of the difference between the heat response of the two phases. This fact of cell cycle progression during a chronic treatment allowed investigation of the cytogenetic effects of growth under these stress conditions.

Since heat affects the ability of cells to perform normal mitotic division, it is not surprising that changes in ploidy were observed after cells attempted division during the heat treatment. Although not unexpected, the ramifications of this phenomenon in the cancer treatment clinic have not been fully explored. In some cases, the effectiveness of treatment of human tumors has been found to be inversely proportional to the degree of karyotypic abnormality present histologically at the time of treatment. If treatment of the tumor with any cytostatic agent induces ploidy changes in the tumor cell population, this may result in some degree of refractive response to further treatment with the same or other cancer treatment modalities.

The observation that heat shock induces premature chromosome condensation (PCC) is a novel observation that may have important ramifications in the study of the mechanism and

effects of the induction of aneuploidy. Although certain combinations of drugs which interfere with DNA replication have been found to produce these effects, hyperthermic induction of PCC is significant in several ways. One possible significance of this phenomenon lies in the fact that the exposure temperature which causes this effect is low enough (i.e. 41.5°C) to occur environmentally. If such environmental temperature fluctuation-induced changes in ploidy can occur in the biosphere, its effects on speciation throughout evolution may be important, as it has been found that both the rate of new species appearance as well as climate have fluctuated considerably in the past. To date, it appears that no study of a possible temporal connection between these two parameters has been made.

Other reasons for interest in heat-induced PCC lies in deducing the role of PCC in the mechanism of induction of aneuploidy and its relationship to the cancer cell phenotype, as well as the study of the cellular control of chromosome condensation and mitotic division. The discovery of a physical agent which is capable of producing PCC in abundance may provide an experimental tool superior to the drug combinations currently in use, especially since these effects in heated cells are observable under conditions where there is much less cell killing than in drug-induced PCC systems.

Finally, the observation that heat causes PCC may provide an insight into the mechanism of cell killing by heat. Although no dose response of the yield of chromosome aberrations is observed in heated G1 cells, but is observed in S phase cells, PCC induction may resolve this contradiction and, furthermore, may explain the similarity of the temperature dependence of the rate of cell killing by heat. This is possible because cells heated in G1 may possess nuclear damage which prevents normal chromosome condensation in the ensuing mitosis, preventing the accumulation of damage cells in mitosis with Colcemid, thus preventing any assay of chromosome damage at that time. In support of this idea is the observation by others that some cells heated in G1 eventually exhibit a fragmented nuclear morphology, which has been shown in this thesis to result in metaphase-associated PCC. If this aspect of the nuclear effects of heat on S phase cells is observable in cells heated in G1, perhaps the spontaneous

induction of PCC prior to the first posttreatment mitosis can account for the lack of observable cytogenetic effects upon these cells, by preventing the timely chromosome condensation of damaged cells in mitosis. Alternatively, incomplete or aberrant chromosome condensation may be involved in the mechanism of the appearance of chromosome aberrations at mitosis. If such a temperature-induced effect upon chromosome condensation is responsible for the ultimate death of the cells, the temperature dependence of the rate of induction of damage causing this PCC may be similar in both G1 and S phases, therefore yielding similar temperature dependencies in the rates of cell killing by heat, as observed in the G1/S comparison presented in this thesis.

APPENDIX I - A Method for the Determination of Viable Multiplicity

In order to obtain single cell survival, a correction for cellular multiplicity must be performed (1). An estimate of viable multiplicity can be obtained by plating 10000 mitotic cells into a gridded T25 flask, determining which cells successfully divide and scoring the percentage of cells which completed the next division within three days. In our studies comparing G1 and S phase heat sensitivity starting with frozen mitotic cells this was especially important, since our synchrony techniques produced G1 microcolonies which were more than 80% doublets and S phase microcolonies which were more than 90% single cells at the start of our experiments. In order for a meaningful comparison to be made between these two systems, these data had to be corrected for viable multiplicity. The following is a brief description of the technique we used to determine that the viable multiplicity was essentially 1.0 in each system.

The locations of 25 microcolonies within a gridded flask were recorded and observed for 72 hrs at 8 - 12 hr intervals. 66% of the attached frozen mitotic cells formed doublets and the remainder formed singlets, however only in 44% of these doublets did both daughter cells successfully complete the next cytokinesis during the ensuing three days, although every doublet had at least one daughter which completed division. All the singlets were viable. This is equivalent to a viable multiplicity of 1.29 for the cells which had attached.

As an alternative method for the determination of viable multiplicity in flasks which were not viewed microscopically, and in order to employ a larger sample size, another technique was used to estimate viable multiplicity. Replicate flasks were plated at 200 cells per flask, allowed to attach, and gently trypsinized to disaggregate the newly-formed microcolonies. 5 flasks were treated with trypsin and an additional 5 flasks were sham-treated with Hanks BSS. From each group one additional flask, plated with 5000 cells was fixed in Carnoy's (3:1 methanol : glacial acetic acid) after treatment and 200 cells were scored for multiplicity, as

described previously, while the other flasks were incubated for 8 days at 37 }C for colony formation. Sham-treated groups survived with an efficiency which was 90 ~ 2% of untreated controls; survival in the trypsinized group was 95 ~ 2% of the untreated control population (values shown are mean ~ SEM). The total cellular multiplicities for the sham-treated and trypsinized groups were 1.60 and 1.22, respectively, indicating that about 64% of the doublets were successfully disaggregated by the trypsin procedure. Since the plating efficiencies in the two groups were not significantly different, while the total multiplicity controls demonstrated separation of more than half of the doublets, we conclude that the viable multiplicity in this system must be close to 1.0. Both the visual observations and the disaggregation results demonstrate that in this system the viable multiplicity is less than the observed total multiplicity. From these considerations, survival was determined based upon a viable multiplicity of 1.0 for both G1 and S phase survival: i.e., the data were not corrected for multiplicity.

REFERENCES

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APPENDIX II - General Formulation of the Polynomial Least-Squares Problem

Consider a set of m observations (y_i, x_i). In our application each $y_i = \log(1/t_i)$, where t_i is the isosurvival time value, and x_i is the corresponding temperature. A system of equations is then constructed of the m observations as a linear combination of terms of an n^{th} degree polynomial $(m > n + 1)$:

$$
\begin{aligned}
y_1 &= a_0 + a_1 x_1 + a_2 x_1^2 + \ldots + a_n x_1^n \\
y_2 &= a_0 + a_1 x_2 + a_2 x_2^2 + \ldots + a_n x_2^n \\
&\vdots \\
y_m &= a_0 + a_1 x_m + a_2 x_m^2 + \ldots + a_n x_m^n
\end{aligned}
\qquad \text{(A.1)}
$$

where the a_i $(i = 0, 1, \ldots, n)$ represent the least-squares coefficients. Eq. A.1 can also be written in vector notation:

$$
\mathbf{Ba} = \mathbf{y} \qquad \text{(A.2)}
$$

where

$$
\mathbf{y} = \begin{bmatrix} y_1 \\ y_2 \\ y_3 \\ \vdots \\ y_m \end{bmatrix} \qquad \mathbf{a} = \begin{bmatrix} a_0 \\ a_1 \\ a_2 \\ \vdots \\ a_n \end{bmatrix} \qquad \mathbf{B} = \begin{bmatrix} 1 & x_1 & x_1^2 & \ldots & x_1^n \\ 1 & x_2 & x_2^2 & \ldots & x_2^n \\ 1 & x_3 & x_3^2 & \ldots & x_3^n \\ \vdots & \vdots & \vdots & \vdots & \vdots \\ 1 & x_m & x_m^2 & \ldots & x_m^n \end{bmatrix}
$$

Each column in the $m \times n$ matrix $\mathbf{B}$ is actually a single function of the independent variable x_i, i.e. each column is a basis vector $\psi_i(x)$:

$$\mathbf{B} = \begin{bmatrix} \psi_0(x_1) & \psi_1(x_1) & \psi_2(x_1) & \cdots & \psi_n(x_1) \\ \psi_0(x_2) & \psi_1(x_2) & \psi_2(x_2) & \cdots & \psi_n(x_2) \\ \psi_0(x_3) & \psi_1(x_3) & \psi_2(x_3) & \cdots & \psi_n(x_3) \\ \vdots & \vdots & \vdots & \vdots & \vdots \\ \psi_0(x_m) & \psi_1(x_m) & \psi_2(x_m) & \cdots & \psi_n(x_m) \end{bmatrix} .$$

For a polynomial fit:

$$\psi_i(x) = x^i \quad , \quad i = 0, 1, \ldots, n. \tag{A.4}$$

Although the basis functions ψ_i can in principle be any function of the parameter x, it can be shown (1) that the polynomial basis shown in Eq. A.4 ensures that a solution to the overdetermined system A.2 always exists, provided that the points $x_1, x_2, x_3, \ldots, x_m$ are not all identical. From geometrical considerations (2), the formulations of the least-squares problem is to find the components of the vector $\mathbf{a}$, such that

$$(\mathbf{B}^T\mathbf{B})\mathbf{a} = \mathbf{B}^T\mathbf{y} , \tag{A.5}$$

where $\mathbf{B}^T$ is the transpose of the matrix $\mathbf{B}$. Eq. A.5 is equivalent to minimizing the error E:

$$E = \left|\left| \mathbf{Ba} - \mathbf{y} \right|\right| \tag{A.6}$$

Minimization of the vector norm in eq. A.6 requires that the error vector $\mathbf{Ba} - \mathbf{y}$ be normal (perpendicular) to the column space of $\mathbf{B}$ (i.e. the vector space spanned by the basis functions $\psi_i(x)$). Hence eqs. A.5 are referred to as "normal equations".

Numerically, eqs. A.5 are solved as follows: First, the described basis functions are calculated. For this application, functions of the form shown in eq. A.4 of the desired order were chosen, such as $\psi_0 = 1$, $\psi_1 = T$, $\psi_2 = T^2$. The matrix **B** is then constructed from these basis functions as in eq. A.3. Eq. A.5 can be rewritten as

$$\mathbf{Ma} = \mathbf{c} \tag{A.7}$$

where $\mathbf{M} = \mathbf{B}^T\mathbf{B}$ and $\mathbf{c} = \mathbf{B}^T\mathbf{y}$. Since the columns of **B** are linearly independent, the inverse of **M**, $\mathbf{M}^{-1}$, exists and a is found to be

$$\mathbf{a} = \mathbf{M}^{-1}\mathbf{c} \, . \tag{A.8}$$

Following inversion of the matrix **M** and vector multiplication by **c**, the desired least-squares coefficients are found in the components of the vector **a**. The actual algorithm used to construct the normal equations was modified from that found in (2). The matrix inversion and vector multiplication shown in eq. A.8 were accomplished using LINPACK routines (1). The program used for analysis of the data in the text was compiled in Microsoft C and Microsoft Fortran, and was run on an IBM PC-compatible equipped with an INTEL 8087 numeric coprocessor.

REFERENCES

1. Vandergraft, JS, *Introduction to Numerical Computations*, Second Edition, Academic Press, New York, 1983.

2. Strang, G., *Linear Algebra and its Applications*, Second Edition, Academic Press, New York, 1980.

APPENDIX III - Visualization of 5'-Bromodeoxyuridine (BrdUrd) Pulse Incorporation as Discrete Replication Bands in Chromosome Preparations of CHO cells

(Procedure modified from that found in Vogel, W., Boldin, S., Reisacher, A., and Speit, G. *Chromosoma* (Berl) **92**: 363-368, 1985)

MATERIALS:

(All labeling and culturing solutions are kept sterile)

1. General cell culturing and specimen preparation supplies.
    a. Medium - McCoy's 5a (modified) supplemented with 10% fetal calf serum.
    b. 25 cm^2 tissue culture flasks (T-25).
    c. Conical 15 ml centrifuge tubes.
    d. Permount (or equivalent) and coverslips for permanent specimen mounting.
    e. Rinsing dishes, slide racks, and a humidified chamber, as described in section 3, for the antibody staining steps.
2. Labeling reagents
    a. BrdUrd stock solution (0.5mM in Hanks BSS).
    b. Thymidine chase solution (10μg/ml in McCoy's 5a).
3. Chromosome preparation reagents
    a. Colcemid (10^{-5} M stock solution in water).
    b. Hypotonic medium - 0.075 M KCl (7.5 ml of 1 M KCl stock + 92.5 ml distilled water).
    c. Carnoy's fixative - 1:3 (v/v) glacial acetic acid : methanol (must be prepared fresh for each use).

d. Clean microscope slides (used as-is from box).

4. Reagents for *in situ* denaturation of chromatin

a. 0.07N NaOH in 70% ethanol (35 ml 70% ethanol : 15ml 0.07N NaOH.

b. 95, 70, and 50% ethanol solutions.

5. Antibody staining reagents

a. Tris-HCl buffer, pH 7.6 (to make 1 liter:

6.1 g Tris base

37 ml 1N HCl

Dilute to 1 liter, adjust pH to 7.6 with HCl or NaOH.

b. Antibody diluent:

Tris-HCl, pH 7.6

0.5% bovine serum albumin (0.5 g per 100 ml)

0.5% Tween-20 (0.5 ml per 100 ml)

(used to dilute both antibodies).

c. Anti-BrdUrd primary monoclonal antibody - used at 1:50,000 to 1:100,000 dilution (for our experiments we used antibody developed at Lawrence Livermore Labs by Dr. Frank Dolbeare. Other sources of antibody may require lower dilution; see note below in Section 3). 400 μl per slide is required.

d. Sheep anti-mouse IgG (whole molecule) peroxidase-conjugated polyclonal secondary antibody (affinity isolated antigen-specific antibody available from Sigma #A-5906) - usually diluted 1:100. 400 μl per slide is required.

6. Diaminobenzidine-hydrogen peroxide (DAB-H_2O_2) substrate buffer (made fresh right before use)

a. 100 mg DAB tetrahydrochloride (electron donor) in 200 ml Tris-HCl buffer.

b. 0.01% H_2O_2 (substrate for peroxidase) - use 4.67 ml of 1:100 dilution of 30% stock peroxide solution (Sigma) added to Tris-HCl - DAB solution from above.

c. After mixing, adjust pH to 5.0 using HCl.

NOTE: DAB is a suspected carcinogen; care should be taken to avoid breathing dust or allowing DAB solution to come in contact with skin.

7. Slide counterstaining reagent

a. 0.5% methylene blue in 70% ethanol, filtered after preparation (0.5 g per 100 ml ethanol).

PROCEDURE:

1. Labeling Conditions

CHO cells are pulse labeled for 15 min in full medium using 10 μM BrdUrd at 37°C (e.g., for 4.5 ml culture medium volume, use 90 μl 0.5 mM BrdUrd stock). After the pulse, 2 rinses using 4.5 ml each of warm, full medium (McCoy's 5a - 10% fetal calf serum) are followed by the addition of 4.5 ml warm McCoy's 5a which is supplemented with 10 μg/ml stable thymidine.

2. Slide Preparation

Colcemid is used to accumulate metaphase cells; the timing of its use depends on the design of the experiment. Accumulation for longer than 3-4 hrs is usually not recommended, as some of the mitotic cells begin to move past the drug-induced block after about 4-6 hrs of Colcemid treatment. 90 μl of Colcemid stock solution is added per 4.5 ml culture flask (final concentration is 0.2μM), and the cultures are incubated for the appropriate interval at 37°C.

At the end of the accumulation period, cultures are either trypsinized or gently shaken to detach mitotic cells; all medium and trypsin rinses (if appropriate) are collected in a 15 ml centrifuge tube, and centrifuged at 800 x g for 5 min (we use an IEC Lab Top centrifuge set at 1/3).

After the centrifugation step, the supernatant is discarded and the pellet is gently resuspended by finger vortex, and 10 ml of 0.075 M KCl hypotonic solution is added, the tube is inverted to mix the cell suspension, and the tube is incubated for 8 min at room temperature (hypotonic composition and incubation time may need to be altered for cells other than CHO). Following the incubation period, the cell suspension is centrifuged again as before, and the supernatant is discarded. The pellet is resuspended in residual liquid (finger

vortex), and 10 ml of fresh Carnoy's fixative is slowly added, and the cell suspension is allowed to fix for 10 min (cells can be stored overnight at this point).

To make slide preparations, recentrifuge samples as before, discard supernatant, and resuspend cell pellet in residual fixative. Add enough fresh fixative to achieve a slightly milky color (usually 0.5 - 1.0 ml for 5 X 105 starting cell number) in order to produce an appropriate cell density on the slide. Slides (directly from the box) are then wetted by dipping into ice-cold distilled water, and the runoff of water is examined by holding the slide up to the light. If the water fails to runoff smoothly, the slide is discarded and a new one is tested. Immediately after wetting, the slide is placed horizontally onto a dry paper towel and 1-2 drops of cell suspension is applied to the center of the slide. After the slides appear dry they are placed into slide boxes and allowed to dry overnight.

3. Staining of Slides

After the slides have dried, the chromatin must be denatured *in situ* in order for the antibody to bind to the BrdUrd-substituted regions of the chromosomes. In order to accomplish this, a rapid alkali-alcohol treatment is performed. The time of this treatment may have to be modified for cell types different than CHO.

In order to avoid swelling of the chromosomes, the water content of the cell preparations is adjusted by treatment of slides with graded concentrations of ethanol. An effective method for this involves dipping the dried slides sequentially into Coplin jars containing 95, 70, and 50% ethanol (2 - 3 dips per concentration). After this step, the slides are denatured for 3 min, by gently dipping the slides into a Coplin jar filled with 0.07N NaOH in 70% ethanol (total ethanol concentration is about 50%), followed by 2 dips in 50% ethanol. At this point the slides are ready to prepare for staining with the primary antibody.

Prior to antibody staining, the slides are rinsed in about 200 ml of Tris-HCl buffer at 37°C. A good way to perform this step is to use glass 3 x 4 inch staining dishes (or equivalent) into which a magnetic stir bar has been placed. The slides are then placed into a glass staining rack which is located over the stir bar in such a way as to allow the stir bar to

gently agitate the buffer. The apparatus is then placed on a magnetic stirrer. This rinsing setup is important for the rinse steps in between the antibody staining steps, as agitation is necessary to ensure adequate removal of unbound antibody.

For the application of the primary antibody, a humidified container is necessary. A plastic box with a tight-fitting lid is convenient for this purpose. Wetted paper towels are layered onto the bottom of the box and the slides are then placed onto a small flat tray that fits into the container on top of the wetted towels. Slides are placed horizontally onto the tray, the tray is placed into the humidified box, and 400 μl of the primary antibody at the working dilution (in antibody diluent - see note below) is then carefully applied to the slide, so as to effect an even layering of antibody on the slide, without disruption of the fragile cell spreads. The cover is loosely placed on the box which is then placed into a 37°C incubator or warm room, and allowed to incubate for 30 min.

After the staining period, the slides are gently rinsed, using a pasteur pipette filled with Tris-HCl, and loaded into the glass slide staining rack, followed by 2 x 5 min rinses at 37°C in Tris-HCl buffer, using the magnetically-stirred glass staining dish. The secondary antibody is applied to the slides in a manner identical to that for the primary antibody (400 μl - 30 min at 37°C), followed by pipette rinses and 2 x 5 min stirred rinses as before.

The DAB-H_20_2 substrate is easily applied by immersing the glass slide rack into the glass staining dish filled with 200 ml of the DAB-H_20_2 solution. The reaction is allowed to commence for 10 min at 37°C, followed by 2 x 5 min rinses of Tris-HCl, as after the antibody staining steps. The slides are then allowed to dry overnight.

Counterstaining of the slides is accomplished by staining with methylene blue for 10 min, followed by a minimal water destain. After staining, the dried slides can then be permanently mounted with coverslips, using Permount or an equivalent mounting compound.

NOTES ON ANTIBODY DILUTIONS:

By far the most variable aspect of this procedure involves the determination of an optimal antibody titer for both staining steps. The dilutions stated in **MATERIALS** were found to be optimal for our use, and may be thought of as a starting point in the application of this technique to other cell types (and labeling protocols). To aid in the determination of the optimal dilutions for other systems, the following recommendations are offered:

1. The first antibody step is the most critical for good band resolution. Usually, three or four two-fold stepwise dilutions should be made for each replicate slide, as this will ensure good resolution. A sequence of 1:50,000, 1:100,000, and 1:200,000 dilution is routinely used in our application. Of course, if you are not using a high-affinity antibody (such as the Lawrence Livermore antibody), these dilutions may be out of the optimal range for good staining.

2. Dilution of the secondary antibody, though not as critical as the primary, is important in order to avoid non-specific staining of the chromosomes. In order to be certain that this complication does not occur, the optimal dilution of this reagent should be titrated upon the application of the technique to a new system, and when a new lot of secondary antibody is used. The best way to determine this dilution is to use pre-immune serum on the primary step (at an appropriate concentration - usually near the midpoint of the expected dilution sequence of the anti-BrdUrd primary). Since it is usually difficult to obtain the appropriate pre-immune sera, the usual practice is to simply omit the primary staining step, and use the secondary alone in the staining procedure. Accordingly, secondary antibody dilution series of 1:50, 1:100, and 1:200 were found to be acceptable for our purpose.

3. Another important negative control involves staining unlabeled preparations. These slides should show no peroxidase activity whatsoever. The most likely explanation for any observed staining is impurity in the second antibody (confirmed by omitting the first antibody step) or non-specific binding of primary or secondary antibodies; both are usually alleviated by further dilution of the corresponding antibody.