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THERMOTOLERANCE IN 9L-GLIOSARCOMA CELLS

Gloria Wesenberg Hathaway
(Ph.D. thesis)

May 1982

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Gloria Wesenberg Hathaway

Ph.D. Thesis

May 1982

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Thermotolerance in 9L-Gliosarcoma Cells

GLoria Wesenberg Hathaway

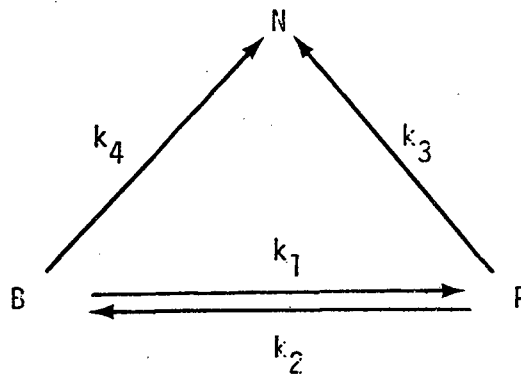
Abstract

Exposure of 9L-gliosarcoma cells to temperatures between 38°C and 40°C for up to 8 hours did not affect colony forming ability as compared to controls held at 37°C. However, treatment of cell populations with these non-lethal temperatures did decrease the rate of cell killing with further exposure at 43°C. The increased resistance (thermotolerance) to 43°C was not attributable to selective killing with the first heat exposure, since 100 percent viability was retained with the nonlethal conditioning exposure. Flow microfluorometry studies also did not reveal any significant redistribution throughout the cell cycle to account for thermotolerance. The development of sublethal thermotolerance was also not dependent upon protein synthesis as judged by studies with cycloheximide.

The magnitude of induced thermotolerance was dependent upon the conditioning temperature, time at the conditioning temperature and the 37°C interval between nonlethal and lethal exposures. For equal conditioning times of 4 hours, an increase in the conditioning temperature from 37°C to 40°C resulted in increased thermotolerance to 43°C. An increase in thermotolerance was also observed as the time at a particular conditioning temperature was increased. Finally, if cells were returned to 37°C after a sublethal conditioning treatment the resistance to elevated temperatures was lost with time. Maximum

tolerance occurred with zero interval between 40°C and 43°C exposures, and the T_0 decayed at the rate of .046 per hour with increasing 37°C interval between fractionations.

The kinetic values which were obtained in the sublethal studies were used to model cell inactivation for temperatures between 42°C and 45°C. An Arrhenius analysis of cell killing between 42°C and 45°C is not linear. Instead, a bending is observed suggesting that different reactions are more significant in different temperature domains. The change in the slope of the Arrhenius plot may result from the induction of thermotolerance. At lower temperatures (e.g., 42°C) tolerance is able to develop to a more significant extent as compared to 45°C, and cell killing occurs more slowly. A general reaction is proposed as follows:



The rate for tolerance induction, k_1 , is the rate at which cells enter the protected "P" state from the 37°C base "B" state, and k_2 is the back reaction. The rate for killing P cells is k_3 and the rate of killing B cells is k_4 . It is assumed that the back reaction from the nonviable "N" state to either P or B is negligible. The integrated survival equation for $B = B_0$, $P = 0$ and $N = 0$ at $t = 0$ is

$$S = \frac{(k_1 + k_4 + m_2)}{m_2 - m_1} e^{m_1 t} - \frac{k_1 + k_4 + m_1}{m_2 - m_1} e^{m_2 t} +$$

$$\frac{(k_1 + k_4 + m_1)(k_1 + k_4 + m_2)}{k_2(m_2 - m_1)} (e^{m_1 t} - e^{m_2 t})$$

$$m_1 = \frac{1}{2} \left\{ -(k_1 + k_2 + k_3 + k_4) - [(k_1 + k_2 + k_3 + k_4)^2 - 4(k_2 k_4 + k_1 k_3 + k_3 k_4)]^{1/2} \right\}$$

$$m_2 = \frac{1}{2} \left\{ -(k_1 + k_2 + k_3 + k_4) + [(k_1 + k_2 + k_3 + k_4)^2 - 4(k_2 k_4 + k_1 k_3 + k_3 k_4)]^{1/2} \right\}$$

By using kinetic data from the heat inactivation studies, the sublethal studies and best approximations, values for k_1 , k_2 , k_3 and k_4 can be obtained for temperatures between 42°C and 45°C. The terminal slope of the model survival curves generated from this equation are in close agreement with experimentally determined values. From this analysis it appears that tolerance is a sufficient condition to account for the bending of the Arrhenius plot of cell inactivation in the 42°C to 45°C range.

Edward Salzman

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INTRODUCTION

Hyperthermia has been used to refer to temperature states in single cells or animals which are above the physiological temperatures normally existing under homeothermic control of the organism involved.¹ Exposure of mammalian cells in culture to elevated temperatures (usually greater than 41°C) results in cell killing with time. If the fraction of cells capable of dividing and producing visible colonies is plotted on a logarithmic scale versus the time exposed to a particular temperature on an arithmetic scale, a cell survival curve is obtained. Depending on the cell line, the survival response may be strictly exponential as in the case of HeLa cells, or more commonly, the response consists of a shoulder region followed by an exponential portion.² A family of survival curves is depicted in Figure 1 for 9L gliosarcoma cells exposed to 41°C through 45°C temperatures.

The survival curves can be described by the parameters T_0 , T_q and n . For a given temperature T_0 is the inverse of the slope k or the time required to reduce the survival by a factor of $1/e$ in the exponential portion of the curve. T_q is the quasithreshold time, the time at which a back extrapolate of the exponential portion of the survival curve intersects a survival fraction of 1.0, and n is the extrapolation number, the point at which the back extrapolate of the exponential region intersects the ordinate. These parameters were derived from, and analogous to, the radiation survival curve parameters D_0 , D_q , and n , where D is dose as compared to T for time.

I. Dose

The concept of dose as it pertains to hyperthermia needs to be considered, especially as it may relate to mechanisms of cell killing. Heat itself is defined as that form of energy which passes from one body to another solely as the result of a difference in temperature.³ For cells heated in vitro this energy exchange occurs quite rapidly. Typically, cultured cells are heated in small flasks or vials by immersion into a temperature controlled water bath. Temperature measurements of 9L gliosarcoma cells heated in 4 ml of media in 25 cm² flasks have shown that temperature equilibration from 37°C to 45°C is complete within 5 minutes.¹ At this time the major energy transfer is complete, and the rest of the experiment takes place isothermally. At 43°C, however, 90 percent cell killing is not complete until 2 hours after initiation of 43°C treatment. Cell killing then is not solely a function of the initial energy deposition into the system; somehow, critical targets are affected by being kept at the elevated temperature.

Heat induced cell killing contrasts with ionizing radiation cell killing where energy is deposited which directly or indirectly through a series of radiochemical events alters critical targets leaving the cell incapable of further cell division.*

* Assuming no repair, the amount of cell killing caused by ionizing radiation is related to the total dose delivered rather than the dose rate.

In biological systems enzymatic processes undergo acceleration with rise in temperature but then decrease beyond a maximum temperature because of thermal destruction of the catalyst.⁴ (The rise in temperature results in increased kinetic energy and increased likelihood of molecules being in an activated state which is manifest in increased reaction rates.) The amount of acceleration of the enzymatic process is dependent upon the activation energy of that single reaction such that certain reactions have a more significant influence in some temperature range than others. For example, a reaction with a high activation energy, the rate of which is completely negligible at physiological temperature, may at somewhat higher temperatures become important. It could be suggested that altering the significance of certain reactions could result in either depletion of certain necessary precursor pools or the accumulation of toxins. Either process could result in loss of reproductive capability.

At higher temperatures thermal denaturation of the catalyst occurs. Landry and Marceau have reported that inactivation of many proteins including enzymes occurs in the 43°C to 48°C temperature range.⁵

Briefly, an increase in temperature results in two phenomena, acceleration of all the enzymatic processes, and at higher temperatures thermal denaturation of proteins, either of which may contribute to the loss of clonogenic potential.

II. Mechanisms of Cell Killing

Although we know that heat causes increased reaction rates, and denaturation of proteins, the critical target or series of events leading to cell death has not been elucidated. With sufficient

exposure to hyperthermia virtually every phase of cellular biochemistry becomes disrupted. These extensive perturbations of cell function confound all attempts to specifically identify alterations which directly cause cell death. Nonetheless, several hypotheses have been proposed to explain how heat causes cellular damage or death. These include changes in chromatin, alterations in the nucleolus and in RNA synthesis, increased lysosomal activity and membrane effects.

A. Effects on Chromatin

DNA itself does not appear to be the primary target for hyperthermic damage in the 40°C to 45°C temperature range. Firstly, the melting temperatures for DNA are sufficiently higher than those used in this research.* (Although pure calf thymus DNA has a T_M of 47°C, ionic interactions between DNA phosphates and positively charged neighboring macromolecules confer local stabilization such that calf thymus DNA complexed with various histones has a T_M greater than 74°C).⁷ In addition to melting, a different inactivation process, depurination, also takes place which involves heat induced degradation of the primary structure. Although the rate of depurination increases with temperature, the reaction rate is again not sufficient to account for loss of biological activity in the 40°C to 45°C temperature range. For example, inactivation of transforming *B. subtilis* DNA by depurination was not complete after 20 hours at 80°C.⁸

* The melting temperature is defined as the midpoint of the reversible denaturation curve.⁶

Corry reported that neither single strand breaks nor double strand breaks were formed in CHO cells exposed to temperatures in the 41°C to 46°C range.⁹ These relatively mild exposures did, however, inhibit repair of strand breaks caused by radiation. These results have recently been confirmed for the HeLa S3 system by Lunec and co-workers; hyperthermic treatments (44°C) did not produce any detectable DNA strand breaks, nor did preirradiation heat alter the initial levels of strand breaks induced by ionizing radiation.¹⁰ But again, the subsequent rate of DNA strand break repair was significantly reduced by heat treatment administered up to an hour before radiation. It is not clear in this case if hyperthermia is acting on the repair enzymes, or somehow altering the chromatin conformation rendering it less repairable. In either case heat is not directly damaging DNA, but is interacting with another damaging modality.

Heat exposure has been reported to cause chromosome-level aberrations in CHO cells. However, chromosomal damage could account for cell killing only when the CHO cells were in a very heat sensitive phase when subjected to 45.5°C treatments.*¹¹ The aberration frequency was very low and was not responsible for cell killing when the cells were heated in G₁ or M. Chromosomal damage cannot explain cell killing at lower temperatures or in all phases of the cell cycle.

* The 37 percent survival value for heated S phase cells was observed when there was about one aberration per cell as for X-irradiated cells.

Possibly, the increased chromosomal damage accounts for the increased sensitivity of S phase cells relative to other portions of the cycle, rather than being a direct mechanism of cell killing. For cells treated in G₁ or S there is an increased radiosensitization following hyperthermia, which may be due to an increased frequency of chromosomal aberrations.¹² Here again, interference with repair enzymes or structural proteins associated with DNA could explain the observed results.* The mechanism by which heat enhances radiation induced killing could be very different (and possibly unrelated) from the mechanism leading to cell death from heat alone.

Recent work has focused on alterations in the protein content of chromatin isolated from heated cells. Since the chromosomal proteins exist in close association with DNA, any heat induced changes in chromosomal proteins could affect DNA-dependent functions leading to cell death. Treatment of CHO cells at 45.5°C resulted in an increase in high molecular weight histone proteins isolated with DNA.¹⁴ The increase was proportional to heat duration and was 1.5 times the control value after 15 min. of heating at 45.5°C. The excess protein isolated with DNA may be cytoplasmic, nucleoplasmic or chromosomal in origin, or may be due to increased interaction of nonhistones normally

* Differential heat sensitivity of repair enzymes has in fact been reported. DNA polymerase- β , which is thought to be involved in DNA repair was found to be more heat sensitive than DNA polymerase- α , which functions in replication. In a variety of mammalian sources DNA polymerase- β activity declined 50 percent after 5 to 10 min of a 45°C treatment while DNA polymerase- α activity was not significantly reduced. At 40°C the T_{1/2} for DNA polymerase- β was 40-60 min while no loss of activity of DNA polymerase- α was observed for as long as 210 min.¹³

associated with DNA. Tomasovic et al. suggest that the increase in protein associated with DNA might lead to lethal aberrations and/or changes in gene expression.

Roti Roti et al. also observed an increase in the protein content of chromatin isolated from HeLa cells heated at temperatures between 40°C and 48°C.¹⁵ The heat-induced increase in chromosomal protein content was constant at the T_0 heating time between 44°C and 48°C.¹⁶ That is, for a particular survival level achieved by heating with different temperatures, the increase in chromosomal protein content was the same. Although the results appear consistent with the notion of increased chromatin protein associated with heat induced cell death, a number of problems remain unresolved with this interpretation.

First, it has been reported that no significant increase in non-histone to DNA ratios occurred following heating at temperatures below 43°C for time intervals that gave the same survival as heating at 45.5°C.¹⁷ Roti Roti et al. did find some increase in protein to DNA ratio in the 40°C to 43°C range, but the increase did not correspond to that found at equivalent survival levels for temperatures greater than 43°C.¹⁶ Furthermore, the kinetics for cell killing and increased protein content are not alike; the activation energy for the increase in chromosomal protein is approximately half the activation energy for cell killing, indicating that the protein increase does not constitute a rate limiting step.¹⁶

When cells are incubated at 37°C after heating there is a decrease in the protein to DNA ratios to control levels. For example, in HeLa

cells the protein to DNA ratio is restored to control values after 14 hours at 37°C following a 30 minute treatment at 45°C.¹⁵ Although the protein to DNA ratio returns to normal for the entire population, only 10 percent of these cells would be viable if tested by clonogenic assay as determined from established survival curves. There is no correlation between ultimate reproductive potential and protein to DNA ratios. Finally, it has been suggested that the restoration of the normal protein to DNA ratio plays a role in thermotolerance (*vide infra*, page 22).¹⁵ It is difficult to imagine how a return to control conditions would render the cell more resistant to further heat stress. Instead one might predict a reduced level of protein to DNA in the thermotolerant state relative to controls. This is not observed.

In addition to the protein to DNA ratio studies, Wartors et al have used the enzyme micrococcal nuclease to investigate alterations in chromatin structure.¹⁸ Micrococcal nuclease cleaves chromatin into nucleosomes. The rate at which the nuclease digests DNA is reduced in chromatin from HeLa cells heated to temperatures of 43.5°C or higher for 30 minutes. The authors suggest that the reduced rate of DNA digestion by micrococcal nuclease results from an increased mass of protein associated with the chromatin from heated cells. It could also be suggested that chromatin from heated cells serves as a poor substrate due to conformational changes, rather than due to an increase in protein associated with DNA.

There is no conclusive proof of the involvement of increased protein to DNA ratios in cell killing. The phenomenon could be

directly attributable to some other cellular change, such as altered permeability of the nuclear membrane. It should also be noted that this increased protein to DNA ratio may actually be due to an increase in ribonuclear protein in the nucleus as a result of heat rather than from a protein associated with DNA.

B. Increased Lysosomal Activity

Lysosomal changes may be important in hyperthermic cell damage, with heat reportedly increasing the number of lysosomes, their enzyme activity and their membrane permeability. In an ultrastructural study of HeLa cells, lysosome formation was the most prominent cytoplasmic change observed after the cell culture was heated at 45°C for 2 hr.¹⁹ Two separate effects of hyperthermia on mouse splenic lysosomes which depend on the severity of the treatment have also been reported.²⁰ Heating to temperatures below 42.5°C caused a transient increase in lysosomal acid phosphatase activity whereas heating to temperatures above 42.5°C caused an immediate increase in lysosomal membrane permeability. It is suggested that this second observation is related to the irreversible cell killing effect of severe hyperthermia.

Another study by Turano et al. concluded that although the special heat sensitivity of neoplastic cells is reflected at least partially at the level of the lysosomal membrane, it is probably not the primary mechanism of cellular damage by heat.²¹ Trypan blue, which has an inhibitory action upon the release and activity of hydrolytic enzymes contained in lysosomes, did not make hepatoma cells more resistant to higher temperatures when added at a concentration which almost

completely inhibits the lysosomal enzymes. Therefore, inhibition of these enzymes did not modify the effects of hyperthermia, and a direct effect of heat on lysosomes is not substantiated.

C. Changes in the Nucleolus and RNA Synthesis

Simard and Bernhard have reported that supranormal temperature selectively affects the nucleolus and intranucleolar chromatin in BHK cells.²² Heating from 42°C to 45°C for 15 to 60 minutes resulted in the disappearance of nucleolar DNA and conversion of the granular ribonuclear protein component of the nucleolus to a dispersed fibrillar component.* High resolution autoradiography confirmed that there was little incorporation of tritiated uridine into the nucleolus while extranucleolar sites continued synthesis. Without the intranucleolar DNA template, nucleolar RNA synthesis is blocked, possibly leading to cell death, since over 70 percent of nuclear RNA synthesis occurs in the nucleoli.²⁴ However, the nucleolar alterations appear to be readily reversible in both BHK and Hela cells when they are returned to 37°C. Based on a morphological criteria, the nucleolus appears to be a heat sensitive site, but it is not known how long cells can exist with the nucleolar lesions, or if these changes are responsible for loss of clonogenicity.

* These results have been confirmed for six other cell lines including 3 Hela strains and 3 normal cell lines; in electron micrographs, the granular component of the nucleolus almost completely disappeared and the nucleolus became largely fibrillar.²³ There was also a selective inhibition of nucleolar RNA synthesis.

The inhibition of nucleolar RNA synthesis by thermic shock may be related to certain phenomenon observed by McCormick and Penman.²⁵ In their studies the principle effect of incubation at 42°C on the protein synthesizing apparatus of Hela cells is a disaggregation of polyribosomes and a corresponding reduction in the rate of amino acid incorporation.

The decrease in protein synthesis with hyperthermia is secondary to a loss of RNA functions.²⁶ The loss of RNA function is not due to an irreversible destruction of any of the RNA components necessary for protein synthesis.²⁵ Instead it appears that the rate at which ribosomes commence translation (initiation) decreases while the rate of translation (elongation) is substantially unchanged. The temperature sensitive step probably involves the attachment of ribosomes to messenger RNA or initiation of polypeptide synthesis.* The block in the initiation of new protein chains with normal elongation of nascent polypeptides leads to a rapid disappearance of polyribosomes and a release of free mRNA.²⁸ The previously translated mRNA is retained in the cytoplasm as free ribonucleoprotein and as mRNP monoribosome complexes which are capable of recombining with ribosomes to form functional polyribosomes under the appropriate conditions.²⁸ Indeed with further incubation of Hela cells at 42°C there is a partial recovery in the number of functioning ribosomes and the rate

* Oleinik has confirmed the immediate cessation of initiation of protein synthesis in CHO cells exposed to 43°C from measurements of the incorporation of 35S-methionine into N-termini of growing peptides.²⁷

of amino acid incorporation.²⁵ This partial recovery does not occur when actinomycin is present, which suggests that the recovery of protein synthesis requires RNA synthesis. The newly synthesized RNA does not code for protein, since the reformation of polyribosome structures proceeds under conditions of complete protein synthesis inhibition. McCormick and Penman have suggested that an RNA species is produced in response to the depressed level of protein synthesis at 42°C, and that it acts to regulate the rate of protein synthesis by promoting the association of ribosomes with mRNA to commence translation. The RNA factor could be responsible for the partial recovery observed with prolonged incubation at 42°C. Furthermore, this factor may be produced not only in response to heat but to other agents which reduce protein synthesis. For example, preincubation of L5178Y murine leukemic lymphoblasts in the presence of various concentrations of cycloheximide decreased the adverse effects of high temperature upon protein biosynthetic activity.²⁶ The results were interpreted to indicate that inhibition of protein synthesis promoted the formation of a non-protein compound required for protein synthesis which enabled the cells to better withstand hyperthermia.

Briefly, the studies by Simard and Bernhard, McCormick and Penman and Love et al. may be interpreted as follows: heat causes a disruption of events in the nucleolus and inhibition of nucleolar RNA synthesis which leads to an interference of protein synthesis initiation. Alternatively, a heat sensitive step in protein synthesis could result in insufficient ribosomal protein available to form preribosomal

particles in the nucleolus. In either case, polyribosomes disaggregate, and the drop in protein synthesis triggers the formation of an RNA species (possibly extranucleolar) which promotes polyribosome reformation and resumption of protein synthetic activity. The cells appear to respond to a lack of protein synthesis by the production of a component that promotes initiation of polypeptide synthesis.

The implications of nucleolar lesions and polyribosome disaggregation for cell killing are unclear. The 42°C heat treatments used by McCormick and Penman are relatively mild, and it is not known if protein synthesis recovers with more severe heat treatments. The basis for increased sensitivity of nucleolar nucleic acids as compared to extranucleolar nucleic acids also remains unresolved. Finally, it has not been determined if the disruption in RNA synthesis is the rate limiting event leading to loss of reproductive potential. Further studies of the pathology of the nucleolus and in particular the synthesis and processing of ribosomal RNA in response to hyperthermia are needed.

New evidence has shown that temperatures above 40°C cause a reduction in ATP concentrations.²⁹ The extent of each reduction varies with the rise in temperature, and the higher the temperature the deeper the drop below control ATP levels. The shortage in energy supply could discriminatively undercut the rates of ATP-dependent biosynthesis (such as RNA synthesis) while concurrently allowing the breakdown to proceed at a quickened pace due to the positive effects of heat on reaction rates (*vide infra*). Since ATP is required for the

synthesis of RNA and is not required for the degradative pathway, the breakdown pathway will be favored at elevated temperatures corresponding to reduced ATP levels.

In summary, it appears that RNA functions are altered by hyperthermia. The importance of reduced energy supply in disrupting these functions and the ultimate involvement of these functions in cell death are still obscure. As we shall discuss later, it may be that RNA processes are more pertinent to cell recovery from hyperthermia rather than cell killing.

D. Membrane Effects

The plasma membrane has also been implicated as the critical target for cellular heat injury. A review of the plasma membrane structure offers insight into possible mechanisms of heat inactivation. The phospholipid bilayer with the polar head groups exposed to the aqueous phase and the hydrocarbon chains located in the interior of the bilayer is the structural determinant of all biological membranes. Proteins may be located on the surface of the membrane or may be immersed to varying degrees in the lipid bilayer.³⁰ A unique feature of pure phospholipid bilayers is their ability to undergo a reversible thermally induced gel to liquid crystalline phase transition which arises from a cooperative melting of the hydrocarbon chains. In the gel or solid state the hydrocarbon chains exist in a closely packed extended conformation which restricts molecular motion and renders the bilayer relatively impermeable. In contrast the liquid-crystalline bilayer is loosely packed, fluid, relatively permeable, and is

characterized by substantial amount of intra and intermolecular motion.³¹ A fluid, or at least partially fluid, lipid bilayer seems to be essential for cellular function.³²

The fluidity of the bilayer at a given temperature depends on a number of parameters including the length of the fatty acid chains and the degree of saturation of the chains.³³ Increasing the environmental temperature often (but not always) results in the production of membrane lipids having a greater average length. A comparison of thermophilic strains of *Bacillus* which may live in temperatures up to 89°C reveals that the mean length of the fatty acid molecule is 16 while in mesophiles living at 20-40°C it is 15 carbon atoms.³⁴ The increased chain length results in lipids with higher melting temperatures. The proportion of saturated fatty acids found in the membrane also tends to increase with the environmental temperature. An increase in the saturated fatty acid fraction is assumed to produce a more viscous membrane and one that undergoes transition from one lipid form to another at higher temperatures.

Escherichia coli incorporates increasing proportions of saturated and long-chain fatty acids into phospholipids as growth temperature is increased. In an electron spin resonance spin label study of an *E. coli* K-12 strain, W3102, it was found that the compositional variation resulted in phospholipids that have identical viscosities at the temperature of growth of the cells.³⁵ The variation of fatty acid composition of membrane phospholipids to produce membranes whose lipids have a constant fluidity at the temperature of growth has been termed homeoviscous adaptation.

Fatty acid compositions have also been altered in mammalian systems. The lipids of mouse LM cells contained more unsaturated fatty acid when the cells were grown at 28°C vs. 37°C. Specifically, there was a significant decrease in 16:0 and increase in 18:1 fatty acids.³⁶

Change in the saturation of fatty acids often takes place by *de novo* synthesis. Of particular interest, however, is an efficient, rapid process for increasing membrane fluidity in certain bacteria and presumably all nonparasitic eukaryotic organisms. *Cis* double bond formation occurs by a direct, oxygen-dependent enzyme catalyzed desaturation of long-chain fatty acids.³⁷ The enzyme desaturase appears in bacterial cells of the *Bacillus* group when the temperature falls below 25°C.* The commonly observed inverse relationship between temperature and unsaturated fatty acid composition almost certainly represents an adaptation on the part of the organism to maintain the integrity and function of cell membranes.

The lipid liberation theory as originally proposed by Heilbrunn in 1924 suggested a correspondence between the temperature at which organisms die and the "melting point" of the membrane lipid.³⁸ It follows then that changes in the composition of the membrane would shift the temperature range over which the phase transitions occurred

* The reverse reaction, hydrogenation of the double bond to increase the saturated to unsaturated fatty acid ratio is a slow process involving strictly de novo fatty acid synthesis.

thereby changing the limits for maximum and minimum growth. Unfortunately, the relationship between phase transitions and cell death is not that simple. Although almost all organisms possess the ability to vary fatty acid composition and do make alterations in response to changes in the environmental temperature, the phase transition boundary itself does not directly determine the maximum and minimum growth temperature. For example, a temperature sensitive mutant of *Bacillus stearothermophilus* is capable of growth at temperatures well above those at which the gel to liquid crystalline phase transition is completed, and cell growth ceases at a temperature at which most of the membrane lipid still exists in a fluid state.³¹ A lack of correlation has also been noted in both *A. laidlawii* and *E. coli* where unsaturated fatty acid auxotrophs are capable of normal growth at temperatures above the upper boundaries of the membrane lipid phase transitions.³¹

Furthermore, discrete phase boundaries do not occur in the membrane of most eucaryotic organisms because of the presence of cholesterol and other sterols which are known to broaden or abolish the highly cooperative nature of the gel to liquid crystalline phase transition.³² Changes in lipid fluidity do occur in small regions of the membrane such that gel and liquid-crystalline phases exist simultaneously. A discrete phase transition of the entire membrane is not associated with the maximum growth.

Instead of an emphasis on phase transitions, McElhaney has suggested that the degree of fluidity or physical state of the membrane

could influence the maximum tolerated temperature in basically two ways.^{31,34} Firstly, the permeability properties of the membrane could be altered. An increase in the temperature would result in an increased rate of passive diffusion due to increased motion of the fatty acyl chains. The membrane might also become "leaky" to low molecular weight metabolites and even to intracellular macromolecules due to transient breakdowns in the permeability barrier. Secondly, it may be that membrane lipids which exist in an excessively fluid state are no longer capable of stabilizing certain membrane bound enzymes and transport systems in a fully functional state. Or as Bowler et al. stated, "Cellular heat injury is due to a change in the stability of lipoprotein complexes, or in enzymes whose activity is dependent on maintaining membrane structural integrity."³⁹ Evidence for the role of lipoprotein stability in cellular heat injury comes from a number of sources. For example, heat damage in the bacteria, *staphylococcus aureus*, was caused by an increase in the permeability of the cytoplasmic membrane and a consequent leakage of solutes.⁴⁰ The breakdown in control of passive permeability is also a feature of heat death in crayfish at 32°C although the details of these changes are dependent on the acclimatization temperature of the animal.³⁹

Exposure of CHO cells to 43°C causes a depletion of intracellular levels of spermidine and spermine, suggesting that an immediate aspect of thermal damage is a membrane defect resulting in the loss of molecules including these polyamines which are necessary for normal cell growth rates.⁴¹ Intracellular spermidine and spermine levels

seem to parallel RNA and DNA accumulation leading to the postulate that polyamine biosynthesis is involved in the control of both RNA and DNA synthesis. Thus, the inhibition of RNA synthesis and DNA synthesis caused by exposure to high temperatures may be due to the depletion of normal intracellular polyamine concentrations. Damage to the membrane could be indirectly responsible for many of the cellular changes accompanying heat exposure. Mondovi et al. also attributed the inhibition of DNA, RNA and protein synthesis in tumor cells exposed to heat to alteration of the membrane. In this study labelled precursors were not incorporated into various tumor cells while they were incorporated into "normal" cells. The authors concluded that cellular membranes of tumors which are different from normal cells may be damaged at different critical temperatures by exposure to heat resulting in leakage of substances necessary for DNA, RNA and protein synthesis.⁴² Hyperthermia has been observed to decrease cellular respiration and glycolysis in some cell lines.⁴³ Dickson and Shah suggested that these alterations are also secondary to damage at the RNA level, although a mechanism of interaction was not proposed.⁴⁴

In crayfish a dramatic breakdown in passive permeability to Na^+ and K^+ occurs early in death resulting in an exchange of these ions between extra and intracellular compartments. The breakdown in transport was correlated with temperature sensitivity of a membrane bound Mg^{2+} ATPase.⁴⁵ This enzyme was shown to have a temperature sensitivity the same as the whole animal.

A more extensive study of the Mg^{2+} ATPase of liver plasma membranes showed that decreased activity of the enzyme was related to increased fluidity of membrane lipids.⁴⁶ A high degree of membrane order is normally required for the optimal activity of this enzyme. Changing the crystalline to gel transition temperature by phospholipid and cholesterol manipulation and treatment of the membrane with a fluidizing agent, *cis*-vaccenic acid, confirmed the requirement for a certain degree of lipid order. The lipid order may be necessary to displace the Mg^{2+} ATPase enzyme to some extent so that substrate sites are available. Alternatively, ordering may influence the conformation of the enzyme and therefore its catalytic efficiency.⁴⁶ The influence of reduced energy supplies as reported by Lau also needs to be examined in regards to the loss of catalytic function of the Mg^{2+} ATPase system with increased temperature.

Plasma membranes have indirectly been implicated in cellular heat inactivation in a number of other studies. At 43°C Chinese hamster cells (HA1) exhibited a marked synergism in cell killing by hyperthermia and adriamycin while at 41°C the sensitivity of the cells to adriamycin was only mildly increased.⁴⁷ Hahn et al. have suggested that the increase in cell killing by adriamycin is due to increased quantities of the drug entering the cell at elevated temperatures. Once inside the cell the adriamycin acts at the level of the chromosome increasing the frequency of aberrations and exchanges.⁴⁸ At 43°C, fluorescent measurements showed much more adriamycin was able to enter

the cell than at lower temperatures.* Changes in membrane permeability in the 41°C to 43°C range and/or inhibition of active drug exclusion mechanisms could account for these results.⁴⁷

In other studies by Hahn et al. the polyene antibiotic amphotericin B was found to effectively inactivate Chinese hamster HA1 cells at 43°C but not at 41°C.⁴⁹ The drug acts by binding to accessible sterols (particularly cholesterol) within the membranes. The cytotoxicity of amphotericin B at 43°C implies an ability of the drug to reach membrane sites inaccessible at the lower temperatures and, hence a change in the structure of the membrane.

In another approach, ethanol was determined to be a heat analog because of the similar mode of cellular inactivation of the two agents.⁵⁰ The cytotoxicity of both ethanol and heat is enhanced by low pH and cysteamine and reduced by deuterium oxide. The deuterium oxide and cysteamine data strongly implicate the involvement of proteins since these molecules most likely are either protected by D₂O or have their function modified by sulfhydryl-rich compounds. The pH effects are consistent with fluidizing action of alcohols on phospholipids. The cellular membranes containing both lipids and proteins appear to be a likely target for both ethanol and heat.

At this time it seems likely that the cell membrane could be considered a "critical target" for heat inactivation. Disruption in

* Vig has pointed out that if hyperthermia increases the effectiveness of adriamycin by facilitating the influx of molecules into the cell, this cannot be considered true synergism as suggested by Hahn.⁴⁸ A higher degree of damage to cellular components is expected if there is an increased quantity of adriamycin in the cell.

normal membrane functions would in turn affect all aspects of cell function and account for the wide variety of cellular alterations observed with hyperthermic exposure.

III. Thermotolerance

A. In Vitro

The ability of cells to survive thermal stress can be greatly increased if cells are exposed to conditioning hyperthermia.* If cells are returned to 37°C following an initial heat exposure (greater than 43°C), cellular sensitivity to subsequent hyperthermic doses is reduced. The increased resistance is exhibited by an increased T_0 of the fractionated survival curve as compared to the unconditioned control survival curve. The thermotolerance ratio (TTR) has been defined as the ratio of the T_0 of the heat survival curve obtained at a time t after heat conditioning to the T_0 of the unconditioned control survival curve.⁵¹ For example, Gerner et al. reported a T_0 of .5 hour for HeLa cells continuously exposed to 44°C.² By comparison, cell inactivation rates for cultures previously treated with 44°C for either .5 or 1 hour followed by incubation at 37°C for 2 hour showed T_0 's of 1.1 and 1.5 hour, respectively, for subsequent thermal treatments at 44°C. The corresponding TTR ratios for the .5 and 1 hour heat treatments are 2.2 and 3.0 where a larger TTR corresponds to more resistance.

* The term conditioning treatment refers to the first heat treatment of a fractionated protocol which may be sublethal or cause some cell killing to a population of cells.

Thermotolerance (T.T.) has been induced in vitro in a variety of cell lines including HeLa, V79, CHO and 9L-gliosarcoma cells. In all cases the induced tolerance is dependent on, or modified by, a number of factors including the duration of the conditioning treatment and the length and status of the interval between fractionations.

For 9L gliosarcoma cells Ross-Riveros has shown that thermal tolerance increased with decreasing initial survival (in the exponential portion of the curve) for a fractionated 43°C exposure with a constant 3 hour, 37°C interval.¹ Initial survivals of 25 percent, 5 percent and 1 percent resulted in TTR's of 2.3, 3.3, and 3.8, respectively. For CHO cells both the kinetics and magnitude of the induction of thermotolerance are a function of the initial survival and interval at 37°C.⁵² At 45°C a longer conditioning treatment (decreased initial survival) resulted in a slower development of tolerance, but the maximum level of tolerance was greater than that achieved with shorter conditioning treatments. Following a conditioning treatment of 10 min at 45°C, maximal T.T. occurred after an 8 hour interval, whereas a conditioning treatment of only 5 min resulted in maximal T.T. after 2 hours. The TTR was 5.3 for the 10 min conditioning, and 3.4 for the 5 min conditioning treatment. The conditioning treatment determines the rate of the thermotolerance induction and the degree of tolerance. Or, in other words, depending on the conditioning dose a different interval between fractionations is needed for maximum

tolerance to be exhibited, and the magnitude of the tolerance is related to the conditioning dose.*

The development of thermotolerance after an initial lethal dose requires an incubation or interval period at or near physiological temperatures. In CHO cells an incubation period of 7 hours at 37°C, 39°C, 40°C or 41°C after conditioning of 10 min at 45°C resulted in similar thermotolerance ratios.^{52**} The development of tolerance was largely inhibited when the cells were kept at 20°C for the interval between fractionations, and temperatures of 0°C and 4°C completely blocked development of subsequent thermal resistance.^{51,52}

The inhibition of thermotolerance development by low interval temperatures was reversible in HeLa cells. When cells were returned to 37°C after 2 hours at 0°C between doses, survival increased rapidly and reached the same survival level 2 hours later as that for cells incubated at 37°C immediately after the initial thermal dose.⁵³ That is, 1 hour at 44°C + 2 hours at 0°C + 2 hours at 37°C + 1 hour at 44°C was equivalent to 1 hour at 44°C + 2 hours at 37°C + 1 hour at 44°C.***

* To date the fractionated studies have examined the kinetics and magnitude of tolerance for different survival levels obtained with a specific temperature; it would be interesting to compare isosurvival levels obtained with different temperatures to determine if the subsequence tolerance is based on the initial survival or the temperature used to obtain the survival.

** The survival curves were, however, displaced downward due to cell killing by post 45°C incubation at 39-41°C.

*** The change in T_0 cannot be determined as this example only assesses the survival of 1 point and not a change in slope.

CHO cells made tolerant by heat conditioning at 45°C for 10 minutes followed by 8 hours of incubation at 37°C did not lose tolerance during storage at 0°C over a period of 2 days.⁵¹ Thus, the development as well as the dissolution of thermotolerance can be arrested by cold storage. For fractionations using temperatures greater than 41°C, thermal resistance does not occur with elevated temperatures alone, but requires an incubation between fractionations for development. The specific requirements for the interval, namely that the temperature be near 37°C, implies that some enzymatic or metabolic activity is required for the development of thermotolerance.

In general, thermal resistance induced by heat treatment is transient and decays with time. CHO cells conditioned for 17.5 min at 45°C completely lost thermotolerance between 48 and 72 hours after conditioning.⁵⁴ For 5 and 10 min preconditionings, tolerance was maximal at 2 and 8 hour, respectively, but the T_0 's decayed thereafter to control levels in about 48 hours. Gerner verified the transient nature of the thermal tolerance of HeLa cells by determining that the viable progeny of previously heated cells had the same survival response as cells which received no prior thermal treatments.² In this experiment, cells were treated at 44°C for 1 hour, plated for colony formation and incubated for 13 days. Surviving colonies were trypsinized to harvest cells, grown for 20 hours, and again treated at 44°C. The progeny of heat treated cells did not have increased resistance to heat. The thermotolerant state of a cell population induced by a conditioning hyperthermic treatment is a transient phenomenon which eventually decays with time.

Heat resistance as a stable, and perhaps genetic variation, represents a separate phenomenon. Harris working with pig kidney cells isolated variant cell types with increased heat resistance which persisted for generations.⁵⁵ The resistant cells were obtained by a one-step heat exposure (47°C for 90 min) which reduced the plating efficiency to 10^{-5} or less.* Clonal sublines were isolated by removing single survivor colonies from the petri dishes. Certain sublines showed a level of heat sensitivity resembling controls while others exhibited increased resistance. The increased resistance persisted in the 358-7 line for at least 6 months as determined by periodic assay at 46°C. The heat resistant cells did not differ from their sensitive precursors in morphology, cell size, growth rate, or chromosome pattern.

Sublines with increased survival at elevated temperatures were obtained by repeated exposure of V79 Chinese hamster lung cells to 44.5°C.⁵⁶ Clonal isolates retained their resistance to thermal stress for at least 30 passages (3 months), and in this case, exhibited differences in growth rate and colonial morphology from precursor cells. Variants of this type arise by random and spontaneous alterations in a small fraction of the population, and whether conversion to the stable state occurs by sequential mutation or by some other process is not yet known. In hybrids constructed between resistant and sensitive V79 cells, survival was only slightly increased over levels obtained with sensitive cells, indicating that heat resistance is recessive or weakly condominant. Fusion of cytoplasts from sensitive

* Harris has noted, however, that termotolerance is greatest in cell strains derived by multiple heat exposures.⁵⁵

cells to resistant cells did not alter the thermal response, and it was concluded that heat resistance was a nuclear characteristic, controlled by genes at the chromosomal level.

Stable heat resistance with its apparent genetic change is distinct from thermotolerance. Thermotolerance refers to a transient resistance induced by heat to the population as a whole (or a significant portion of the population).

B. In Vivo

Although we have been primarily concerned with in vitro thermotolerance, it is important to note that thermotolerance has also been exhibited in in vivo systems. Crile observed in vivo tolerance in mice feet exposed to a 44°C water bath.⁵⁷ Heating for 90 min resulted in complete destruction of the foot in 95 percent of the mice; however, a pretreatment of 30 min at 44°C 1 day prior to the 90 min exposure reduced the incidence of complete foot destruction to 13 percent. In another series of experiments by the same investigator, Sarcoma 180 tumors were grown in the foot pads of mice. A 30 min treatment at 44°C resulted in an 80 percent cure rate, where cure rate was defined as the absence of tumor 3 weeks after treatment. When the tumors were heated for 15 min at 44°C 1 day prior to the 30 min treatment, the cure rate decreased to 20 percent. In both the normal foot and the implanted tumor the induced tolerance was transient and the heat resistance subsided by the second day after heat conditioning.

A more recent study by Law et al. used the mouse ear to investigate fractionated heat treatments.⁵⁸ In this system induced

thermal resistance was defined as the extra heating time above a single treatment required to cause necrosis. As with in vitro studies, both the magnitude and time course of the induced resistance depended on the duration and temperature of heating.

Heat treatments ranging from 41.5°C to 45.5°C in the mouse ear revealed the following relationships: as the duration of a particular priming treatment increased, the number of animals showing necrosis after a second treatment decreased.⁵⁹ With shorter priming treatments at a particular temperature induced resistance occurred earlier, but the magnitude of the tolerance was progressively less. Thermal resistance developed earlier after conditioning at lower temperatures for longer times than at higher temperatures for shorter times, although the same degree of resistance could ultimately be achieved by adjusting the treatment time.

The in vivo thermotolerance results have particular significance in regard to clinical application of hyperthermia as an anti-tumor agent. Since any therapeutic protocol will more than likely depend upon multiple fractionations of heat, the phenomenon of induced tolerance cannot be ignored.

It has only been in the last two decades that hyperthermia has been seriously considered as a treatment modality, despite a smattering of encouraging results dating back as far as 1866.⁶⁰ The renewed interest in the clinical use of hyperthermia has arisen from two recent findings. First, some tumor cells may be more sensitive to heat than normal tissue, because solid tumors act as a heat reservoir and

retain heat due to abnormal vascularity and relatively poor blood flow.⁶¹ Second, hyperthermia may be uniquely effective against larger tumors.⁶² Metabolic deprivation with consequent reduction in pH could account for this increased sensitivity (see p. 31).

Hyperthermia has also been evaluated in conjunction with other therapeutic agents. Chemotherapy and hyperthermia protocols have been investigated, since heat may facilitate drug uptake by altering membrane permeability.⁶² Increased enzymatic rates might also enhance the efficacy of certain chemicals such as alkylating agents although no data is available on this point.

Hyperthermia has most commonly been used as an adjuvant to radiation therapy. The effectiveness of this combination may be related to differences in the cell killing patterns of these two agents. Cells tend to be most sensitive to hyperthermia during S phase, which usually is the most radioresistant phase.⁶³ In addition, the tumor hypoxic fraction, which can be extremely radioresistant, may be equally sensitive to hyperthermia as euoxic cells.⁶⁴ It has also been reported that heat interferes with repair of sublethal radiation damage.⁶⁵

The usefulness of hyperthermia as an adjunct to radiotherapy depends upon achieving a greater thermal sensitization of tumors than of normal tissues. The Thermal Enhancement Ratio (TER) has been defined as the ratio of the x-ray dose without heat to the x-ray dose with heat to achieve the same level of damage.⁶⁶ The Therapeutic Gain Factor (TGF) is defined as the ratio of the TER of the tumor to

the TER of normal tissue.⁶⁷ Maximizing the TGF is a primary concern in the combined modality treatment regimes.

It becomes apparent in multiple fractions of any protocol involving heat that induction of thermotolerance can act as a double edged sword. Ideally, T.T. would be maximized in normal tissue and minimized in tumors.

It is beyond the scope of this paper to review the numerous clinical results which have been obtained in the past few years. There are many studies, giving details of these investigations.^{64,65,68-72} Perhaps one point should be emphasized at this time, however. Namely, that the problems facing the clinician working with hyperthermia are quite different from the cell or molecular biologist working in the same area. The biologist is interested in elucidating the mechanism of heat killing and tolerance induction. The clinician doesn't need to know how heat inactivates cells in order to use it effectively. Similarly, mechanisms of thermotolerance are unimportant as long as treatments are designed to account for the transient resistance. The most pressing problems in hyperthermia therapy at this time are uniform heating of tumors and thermal dosimetry. Although some methods of tumor heating, including ultrasound and certain invasive radio-frequency techniques can be highly localized when administered, the in vivo heat distribution does not remain uniform. The production of hot spots is extremely painful to the patient, and treatments cannot be continued. Regional hyperthermia also cannot act against unlocalized microfoci of disease or metastases, and consequently, heat alone cannot

be considered as a "cure" for cancer. The clinical success of hyperthermia acting with other agents will ultimately depend on the development of techniques for producing, controlling and measuring thermal fields. Although still in the anecdotal stage, excellent therapeutic results have been obtained with heat and radiotherapy, and indicate that solving the problems of in vivo heat administration would be most worthwhile.

C. Modification of the Hyperthermic Response

Cell culture studies have identified several factors, including cellular acidity, ambient oxygen concentration, nutritional status and cellular growth stage, which influence the thermal response.

Enhanced cell killing was observed in CHO cells exposed to reduced pH and elevated temperatures.⁷³ At a pH of 7.4 a 4 hour exposure to 42°C reduced survival to 10 percent while the same exposure at pH 6.7 resulted in .01 percent survival. For CHO cells, increased heat sensitivity at low pH was manifest as a reduction in T_0 . The pH sensitivity was most prominent at temperatures which were barely lethal at pH 7.4. This increased cell killing of CHO cells was correlated with simultaneous exposure to heat and reduced pH as opposed to heating before or after pH alteration. At higher temperatures, 45.5°C, Freeman reported that survival of CHO decreased from 1 percent at pH 7.35 to .003 percent at pH 6.65 after a 20 minute exposure.⁷⁴ Increased extracellular acidity was also associated with markedly reduced viability in L1A2 ascites cells.⁷⁵ In this case the reduced survival was due to a change in the shoulder of the survival curve as

reflected by a decreased extrapolation number (n) with no significant change in the final slope (T_0).⁷⁶ L1A2 cells treated at 42.5°C at pH 6.4 had an increased lysosomal activity, and intense cell lysis resulted in lethal damage to the entire cell population within 6 hours of treatment. Overgaard concluded that, for cells maintained in an acid milieu, membrane lesions combined with increased lysosome activity may be important mechanisms of heat induced damage.⁷⁵

Thermal sensitivity remained constant from pH 7.35 to 7.85 for CHO cells.⁷⁴ The importance of cytoplasmic pH in hyperthermia treatments has also been investigated in conjunction with the proton conducting drug carbonylcyanide-3-chlorophenylhydrazone (CCCP).⁷⁷ With addition of CCCP the pH of the internal cytoplasm equilibrates with the pH of the medium. The survival of mouse mammary carcinoma M80135 cells at 43°C was optimal when the pH of the medium was between 7.75 and 8.0. At this optimal pH the presence of CCCP hardly influenced the survival for treatment times up to 90 min, whereas there was a large effect of CCCP in the 6.5 to 7.5 pH range. The authors suggest that in the 6.5 to 8.0 pH range the cells are able to maintain the optimal intracellular pH. The capacity to control cytoplasmic pH is impaired by the addition of CCCP. Outside the 6.5 to 8.0 range the capacity to control pH appears to be insufficient or absent even without CCCP.

To insure consistent results in fractionated hyperthermia experiments, it is essential to consistently maintain the pH of the system. Theoretically, it would be possible for the second fraction of a split dose treatment to be administered at an increased pH

resulting in an increased survival. A tolerance-like effect can be simulated by a change in pH.

In addition to the sensitization of heat induced cell killing, reduction in extracellular pH has been reported to alter the recovery of hyperthermic damage and the development of thermotolerance. Nielsen and Overgaard have shown that a low extracellular pH in the interval between fractionated exposures of L_1A_2 cells results in reduced thermotolerance.⁷⁶ Thermotolerance ratios were determined for a first heat treatment of 1.5 hour at 42°C followed by a 10 hour at 37°C interval and then graded doses at 42°C with alterations in pH's at various stages. The same tolerance (TTR = 2.4) was observed when pH 6.5 was maintained throughout the whole experiment and when a pH of 7.2 during the first heat treatment was combined with a pH of 6.5 in the remaining experiment. A pH of 7.2 during both heat treatments (interval pH of 6.5) also did not change the 2.4 TTR. However, when the pH was 7.2 during the first treatment as well as in the interval, a higher tolerance was observed. (The TTR was 4.1 for all 3 phases at 7.2, and the TTR was 4.0 for a 7.2 pH first treatment and interval followed by a pH of 6.5 for the second treatment.)⁷⁶ The development of maximum tolerance appears to depend on optimal pH conditions during the first fractionation and the interval. The pH during the second treatment is less critical.

A reduced TTR in log phase CHO cells exposed to fractionated 45°C hyperthermia at low pH has also been recently reported.⁷⁸ The thermotolerance ratios declined from 4.2 at a pH of 7.2 to 1.5 at a pH of 6.3. During these experiments the pH adjustment was maintained

during the 10 minute heat shock, the 7 hour interval and the final heat treatment. These studies show that alteration in the pH not only affects cell killing, but also the magnitude of the induced resistance. The kinetics of thermotolerance induction with altered pH have only been characterized for the L_1A_2 system. Nielsen and Overgaard found that maximum tolerance was achieved 10 hours after a 1.5 hour exposure at 42°C for both a 6.5 and 7.2 pH treatment although the magnitude of the tolerance was reduced for the lower pH. For other cell lines it would be interesting to determine if lowered pH delays the development of maximal tolerance thereby contributing to the reduced TTR. Varying the 37°C interval in conjunction with alteration in pH would be necessary to define any kinetic effects.

Another factor, oxygen tension, which plays a significant role in radiation killing, has a minimal effect in hyperthermic cell killing. In one experiment flasks of CHO cells were made hypoxic by N_2 gassing for 10 min. Heat survival curves determined 30 min after gassing showed the cells to be at least as sensitive as oxygenated cells.⁷⁹ In another study hamster V79 and mouse EMT6 cells were rendered extremely hypoxic, without being subjected to metabolic stress, using a chamber technique that relies upon passive gas exchange.⁸⁰ At 43°C, hyperthermia killed euoxic and hypoxic cells equally. Consistent with Powers' results, Gerweck et al. determined that oxygenated and acutely hypoxic cells (≤ 10 hour culturing under hypoxia) were equally sensitive to hyperthermia.⁸¹ However, prolonged oxygen deprivation of up to 30 hours increased CHO cell killing by a factor of 5 for a 3 hour

treatment at 42°C in low density cultures in the absence of change in extracellular pH. The effect was not reversed by returning the cells to normal oxygen tension prior to treatment. Under chronic oxygen deprivation the capacity of cells to repair sublethal heat damage which may occur during heat treatment, and which is likely to be an enzymatic and energy dependent process, may be impaired. Changes in internal cellular pH may also occur with chronic oxygen deprivation, altering the sensitivity to heat.

Studies by Hahn using the HA1 cell system indicate that the sensitivity of cells to hyperthermia may be related to their nutritional history and to a lesser extent on their milieu at the time of heat exposure.⁸² The absence of nutrients sensitized the HA1 cells to 43°C heat treatment while the converse was not true. That is, the presence of full medium did not by itself protect unfed cultures. However, when unfed cultures were given fresh medium with serum 2 to 3 hours prior to 43°C treatment, their heat resistance returned to equal nutrient supplied controls. In exponentially growing cells fresh medium, even without serum, protected the cells to a considerable degree when it was administered immediately prior to transfer to 43°C. When time was allowed to elapse between the administration of serum free medium and transfer to 43°C, the sensitivity to heat increased and the role of serum was crucial. In the continued absence of serum the cells killed by heat lysed either during or shortly after heat exposure. The role of specific media components in providing protection has not been elucidated, but it appears to be quite complex and time dependent.

Variations in available glucose concentrations did not appear to be a major determinant in the response of HA1 cells to 2 hour incubation at 43°C as determined by 2-deoxy-D-glucose inhibition studies.*⁸² However, glucose deprivation combined with moderate hypoxia resulted in selective killing of HeLa S-3 cells at temperatures as low as 41°C.**⁸³ No enhancement of cell killing was observed with hyperthermia under oxidic conditions in the absence of glucose.

Not only are the cell requirements for specific media components complex but the interaction among various factors may play a more significant role in determining cell survival.

The heat sensitivity of plateau-phase cells has been investigated in several studies. A widely variable response was found, and both fed and unfed plateau-phase cells were either more sensitive or more resistant than similar exponentially growing cells. No consistent trends regarding thermotolerance induction are apparent either.

At pH 7.2 PNJ mouse ascites cells in plateau phase were more sensitive (100 fold increase after 2 hours) to 42°C treatment than exponential cells. At a lower pH, 6.4, plateau phase cells were still more sensitive than the exponential population, but only slightly more so.⁸⁴ Plateau phase HA1 cells were also more sensitive to 43°C than

* Deoxy-D-Glucose competitively inhibits the transport of glucose across the cellular membrane and therefore reduces the availability of glucose to the cells.

** In this study "glucose-free" medium was used.

exponential cells.⁸² Exponentially growing mouse leukemia L1210 cells were more resistant to 43°C and 44°C than were stationary phase cells.⁸⁵ This difference in sensitivity could not be explained by cell cycle distribution differences. S phase cells declined from 70 percent in an exponentially growing population to about 46 percent in a stationary population, and S phase cells were found to be the most sensitive to hyperthermia.

After a single heat treatment at 42°C the sensitivity of exponentially growing and unfed plateau phase L1A2 cells was similar, despite differences in pH and cell cycle distribution.⁸⁶ Although no difference in the heat response was found after a single treatment, the recovery demonstrated during two-dose hyperthermia fractionation was reduced in unfed cells relative to cells in exponential growth. In both cultures separation of two hyperthermic treatments with variable 37°C intervals resulted in increased survival up to 10 hours, but the survival of unfed cells was less than that of exponential cells, suggesting a reduced recovery. Furthermore, the degree of induced thermotolerance with fractionated exposures was reduced in unfed plateau phase cells relative to cells in exponential growth. In this study the unfed plateau L1A2 cells were heated at pH 6.8 in nutritionally depleted medium. Manipulation of pH did not effect differences between exponential and plateau phase cultures, and the authors attributed the dissimilar response to metabolic depletion. In contrast, Gerner observed the same degree of thermotolerance using fractionated heat doses in plateau phase EMT6 cells treated in

sufficient medium and at a pH of 7.4 relative to exponentially growing cells.⁸⁷

In other reports plateau phase cells have been observed to be more heat resistant than exponentially growing cells. In particular, both fed and unfed plateau phase V79 cells were less sensitive to 43°C than exponential cells.⁸⁰ There was no difference in killing between euoxic and hypoxic V79 cells in either the exponential or plateau phase. In an embryonic lung fibroblast line, WI-38, Kase and Hahn also found that plateau phase cells (treated 9-11 days after seeding) survived better at 43°C than the exponential cells (treated 1-2 days after seeding).⁸⁸ It should be noted that in this particular experiment, the plating efficiency was as low as 5 percent. The difference in heat response could be due to a selection of a heartier subpopulation in the case of the plateau cells, or it could be due to a feeder cell effect.

Thus, no conclusive trends can be found in these studies on the sensitivity of plateau versus exponential cells. It may be that the actual density state of the culture is not very critical to the determination of heat sensitivity. Instead, the interplay of various attendant factors such as pH, local oxygen concentration, nutritional status, cell cycle distribution, and the cell line could be more critical in determining the final survival outcome. Although the thermotolerance response cannot be attributed to a specific change in any of these factors they must be held constant so as not to influence the survival response.

D. Suggested Mechanisms of Thermotolerance

1. Sublethal Repair

A 1973 study by Palzer and Heidelberger attributed the increase in HeLa cell viability associated with a fractionated heat treatment to sublethal repair.⁸⁹ A 2 hour treatment at 42°C reduced survival to 54 percent while two 1 hour exposures separated by 6 hours at 37°C resulted in 70 percent survival. Since this experiment used only a simple split fraction protocol (i.e., comparison of 1 point per treatment) it is not possible to determine if there is recovery from sublethal damage or induction of thermotolerance. Recovery from sublethal damage during fractionated exposures would be indicated as a reinstatement of the shoulder region of the survival curve and resumption of the single dose slope. However, in a number of cell lines, including HeLa, the survival slopes for single and double heat treatments are different. The change in rate of cell killing observed with the second exposure cannot be explained as recovery from sublethal damage. Furthermore, in some cell lines, such as the HeLa lines used by Gerner et al., there is no shoulder on the single dose survival curve, which implies no capacity for sublethal damage accumulation, although there is increased survival with the fractionated exposures.² In V79 cells prolonged exposure (7 hours) at 42.5°C greatly enhanced thermal resistance to subsequent treatment at 45°C.⁹⁰ In this experiment by Harisiadis et al. no interval was allowed between the 42.5°C and 45°C exposure, and consequently repair of sublethal damage cannot be responsible for the observed increase in survival.

In brief, the thermotolerance response cannot be attributed to repair of sublethal damage. First it does not explain the change in slope (i.e. the change in rate of cell killing)) observed with the second fractionation, secondly it does not account for increased resistance in cell lines with no shoulder and finally it does not account for increased resistance in fractionated exposures at different temperatures with no interval for recovery.

2. Cell Cycle Effects

Investigators have also questioned the role of selective killing within the cell cycle and cell cycle redistribution in the development of thermotolerance. Hyperthermic killing of both CHO and Hela cells is cell cycle phase dependent, as determined by heating synchronous cell populations. For CHO cells the M and S phases were the most sensitive, as indicated by the smaller shoulders on the survival curves compared with the curve for cells heated in the resistant G_1 phase.⁹¹ Slight differences in sensitivity as compared to CHO cells were observed for Hela cells, with late S and early G_2 being the most sensitive, and G_1 and late G_2 being up to 7 times more resistant.⁹²

The variation in hyperthermic response with cell age has been determined in other cell lines as well. In L1210 mouse leukemic cells the heat sensitivity increased with increasing percent of cells in S phase;⁸⁵ EMT6 and V79 cells were most sensitive in mid S phase to a 2 min 52°C heat pulse.⁹³

When considering the thermotolerance response, it could be suggested that the first heat treatment kills off the more sensitive cells (mid to late S) leaving a more resistant population (G_1 and late G_2) for the second fractionation. The final slope of the survival curve for the asynchronous population would be determined by the sensitivity of the resistant cells, which would be killed more slowly. However, work by Sapareto et al has shown that this variation in cell cycle sensitivity cannot completely account for the thermotolerant response.⁹⁴ Firstly, the magnitude of the thermotolerant response in asynchronous CHO populations is much greater than the survival response of synchronous G_1 cells, which are in the most resistant phase. Secondly, fractionated exposures of synchronous G_1 cells leads to thermotolerance. A 3 to 4 fold increase in T_0 was observed in synchronous G_1 cells exposed to heat 20 hours after an initial 45.5°C treatment for 15 min.⁹⁴ The final slope in a split exposure is not totally due to the inherent resistance of a certain subpopulation since the magnitude of the inherent resistance does not correspond to the tolerance response and since the sensitivity of the resistant subpopulation also varies according to conditioning treatments.

Redistribution of cells in the cell cycle in response to the initial heat treatment does not contribute to the thermotolerance response either. Studies by Ross-Riveros using asynchronous 9L-gliosarcoma cells showed that the initial heat stress induced a general arrest of the cell cycle.¹ The length of the arrest is dependent on the temperature used and the time at the temperature. In general, the lower the initial survival or the higher the temperature

used to obtain the same survival the longer was the arrest. For example, cell cycle delays of 9, 18 and 24 hours were observed for 45°C treatments, which reduced survival to 75, 25, and 2.5 percent, respectively. In contrast, if 2.5 percent survival is achieved using 42.5°C, the delay is shortened to 9 hours. At the end of the general arrest, released cells progressed through the cell cycle until they reached a block in S phase which had not yet been released. Following the release of the S phase block the cells progress through the cell cycle in a cohort. In the case of a fractionated dose the general arrest and S phase accumulation argue against a phase dependent contribution to increased survival of the second treatment. Firstly, tolerance is apparent with a three hour 37°C interval between 43°C treatments. This time period is less than the general arrest period during which cells cease to progress. Secondly, when cells do begin to progress, they tend to accumulate in the S phase, which has been reported to be the most heat sensitive phase of the cell cycle, and these cells would not be expected to exhibit resistant qualities.

Although selective killing within the cell cycle and redistribution effects do not play a significant role in thermotolerance, it has yet to be established whether all phases of the cycle are equally as capable of being brought into a state of thermotolerance. At temperatures greater than 43°C it may be that an initial heat stress followed by an interval at 37°C are required for thermotolerance, but different phases of the cycle have different thermotolerance ratios for the same initial treatment and 37°C interval. Hypothetically, the maximum

tolerance achieved could also be related to cell cycle effects if different phases of the cycle have different tolerance potentials. The severity of the initial treatment would determine what subpopulations were left. If these subpopulations varied in their ability to exhibit tolerance they would in turn determine the maximum tolerance achievable.

3. Thermoresistant Subpopulations

In certain cell lines, such as CHO and V79, continuous exposure to temperatures between 40°C and 42.5°C results in biphasic survival curves. The biphasic response may be indicative of a resistant subpopulation characterized by an increased T_0 , which would explain the decreased killing with a fractionated exposure. A 42°C exposure of CHO cells for up to 2.5 hours resulted in a T_0 of 50 min while longer heating was characterized by a T_0 of 475 min.⁹⁵ In V79 the changing slope of the survival curve occurred after 3.5 to 4 hours of exposure to 42.5°C. Exponential killing was observed for temperatures greater than 42.5°C.⁷¹ If indeed there are two populations of varying sensitivities, a biphasic response would be expected at all temperatures. Furthermore, prolonged exposure of V79 cells to 42.5°C offered protection from subsequent treatment at 45°C.⁹⁰ This finding cannot be explained by the selection of a thermoresistant remnant in the cell population, because a 7 hour exposure at 42.5°C followed immediately by an additional 60 min exposure at 45°C actually killed fewer cells than did a 60 min exposure at 45°C without preconditioning.

Since the biphasic response does not occur at higher temperatures, it could be that at lower temperatures the change in slope is caused

by the induction of thermal tolerance. That is, for temperature exposures below 42.5°C a 37°C interval is not required for induction of protection in certain cell lines.

IV. Rationale

Although a distinct heat resistant subpopulation does not account for thermotolerance, an inhomogeneous population of cells with varying heat sensitivities could contribute to a tolerance response. In order to more fully assess the role of subpopulations in thermotolerance it occurred to us that a sublethal conditioning treatment could be administered prior to a lethal dose. Here the term sublethal refers to a nonlethal conditioning dose which enhances survival to lethal temperatures. With this method, no selection or preferential killing can occur with the first fractionation, as there is still 100 percent viability after the conditioning dose.

In this study the sublethal thermotolerance response in 9L gliosarcoma cells was characterized. This included examination of the relationships between the specific conditioning temperature, time at the conditioning temperature and interval between sublethal and lethal exposures.

In addition to characterizing the sublethal thermotolerance response the kinetic data has been used to evaluate cell killing at various temperatures. In particular, we wish to see if the non-linearity of the Arrhenius plot in the 42°C to 45°C range can be attributed to the phenomenon of thermotolerance. Since Arrhenius plots are determined from the exponential portion of the survival

curve we will restrict our analysis to this region. The "shoulder" of the 9L gliosarcoma cells is also interesting and may reflect the ability of cells to accumulate sublethal thermal damage. However, this parameter has no significance in an Arrhenius analysis and its examination will be left to future investigators.

MATERIALS AND METHODS

I. Cell Cultures

The 9L gliosarcoma cells used in these experiments were derived from a chemically induced rat brain tumor. Tumor number 9 developed in a CD Fischer rat 26 weeks after injection of N-nitrosomethyl urea at 5 mg/kg for 36 weeks. The tumor was classified as a high-grade astrocytoma as indicated by the positive phosphotungstic acid hematoxylin stain demonstrating glial fibrils. The individual nuclei of the tumor varied in shape from a polygon to round, and an occasional multinucleated cell was present. Numerous mitoses were observed, including a few atypical (tripolar or quadrapolar) ones. Generally, the nuclear chromatin was evenly dispersed and nucleoli were infrequent.⁹⁶

The tumor was successfully propagated in both subcutaneous tissue and tissue culture. A frozen aliquot of the original number 9 tumor was acquired by the Brain Tumor Research Center at the University of California at San Francisco and designated number 9L. The cells were developed as an in vivo-in vitro model, and in solid tumors they appear histologically as a mixed gliosarcoma. The 9L cells used in this work are periodically (approximately every 3 to 4 months) obtained from the tumor bank at UCSF.

The stock cells are maintained in monolayer growth in 75 cm² plastic tissue culture flasks with 15 ml of Eagle's minimum essential medium with Earle's Salts and 20 percent fetal calf serum. The cells are maintained in a 37°C incubator with a 5 percent CO₂/95 percent air mixture.

Antibiotics were not added to any of the stock or treatment flasks. Some antibiotics such as amphotericin B are known to greatly enhance cell killing by hyperthermia.⁴¹ Hahn reported that at 43°C small doses of amphotericin B are highly cytotoxic to cells while no effect or survival was noted for temperatures up to 41°C over the range of 0 to 5 µg of the drug.

It has also been noted that routine passaging with antibiotics is associated with an increased mycoplasma contamination. Specifically, Barile noted a correlation between the incidence of mycoplasma contamination in cell cultures maintained with antibiotics (72 percent) versus those cultured without (7 percent).⁹⁷ Apparently, the use of antibiotics which inhibits contamination with bacteria can create conditions selective for mycoplasma since these organisms are, for the most part, antibiotic resistant.

In addition to the described handling procedures, cultures were routinely screened for mycoplasma using Hoechst 33258 stain according to the method of Chen.⁹⁸

II. Hyperthermic Treatment

Small flasks (25 cm²) were seeded at 1×10^5 cells in 4 ml of media plus fetal calf serum from stock cultures approximately 48 hrs prior to an experiment and held at 37°C. Before an experiment, the cells were viewed under a light microscope to assure log phase conditions and morphological integrity. The flasks were tightly sealed with caps, wrapped in parafilm and placed horizontally on a test tube rack. The rack was immersed in a thermostatically controlled,

constant temperature, stirred water bath accurate to $\pm 0.05^{\circ}\text{C}$ (FS 2 constant temperature circulator, Haake and Lo-temptrol Model 154, Precision Scientific Co.) for the appropriate time. The temperatures of the baths were calibrated to a National Bureau of Standards thermometer. For the fractionated treatments, the flasks were removed from the water bath, wiped dry, and placed in a 37°C incubator with the caps loosened. After the appropriate 37°C interval the flasks were reimmersed as previously described.

Following heat treatment, the supernatant media was suctioned off. The cells were rinsed with Earle's Balanced Salt Solution (Gibco) and detached from the surface with trypsin-EDTA Solution (Gibco) or protease (*Bacillus polymyxa* Type IX, Sigma, 6 mg/ml in Spinner Media with 8 percent serum). The protease treatment more readily dispersed the cells into a single cell suspension as compared to trypsin which frequently resulted in cell clumps which had to be broken up by forceful pipetting. No difference in plating efficiency for either control or heat treated cells was noted between the two agents. Single cell suspensions were verified under a light microscope. Media was added to stop further action of the enzyme and aliquots of cells were suspended in isotone to be counted (Coulter Counter, Model Z, Coulter Electronics). The Coulter counts were frequently verified with hemacytometer counts. The cells were seeded into 60 mm dishes or 25 cm^2 flasks with an appropriate number of feeder cells (see results). At least two dilutions with five replicate dishes were seeded for each point. Cell concentrations were plated to yield 30 to

100 viable cells. The dishes were incubated at 37°C in a 5 percent CO₂/95 percent air incubator for 10 to 12 days and then fixed and stained with ethanol plus methylene blue. Survival was determined by counting colonies with more than 50 cells.

A. pH

Since alteration in pH is known to significantly alter cell survival,⁷³⁻⁷⁸ the pH was closely monitored. In replicate flasks pH measurements were made (Corning Digital 109 pH meter) at various times during the treatment protocol. At all temperatures, even with extended treatment times, pH remained constant at 7.4 ± 0.2 units.

B. Protein Inhibitors

In particular experiments cycloheximide (Grand Island Biological Company) was used to inhibit protein synthesis. A concentration of 5 µg/ml was made up in Eagle's minimum essential medium with 20 percent fetal calf serum and added to the cells for appropriate time intervals.

III. Flow Microfluorometry (FMF)

The rapid flow-microfluorometry technique permits the analysis of DNA in a large population of monodispersed cells. Fluorescent stains are used to bind to cellular DNA. The intensity of the fluorescence is proportional to the amount of bound stain which in turn is a function of DNA content. The relative amount of DNA is indicative of position in the cell cycle since the DNA content doubles as the cell readies for division. The FMF studies reveal the frequency of cells in the various phases of the cell cycle and also provided information on cell cycle kinetics.

In these studies flasks of cells were heat treated as described in section II. Identically treated cells were pooled to yield a minimum of 1×10^6 cells per sample and then fixed (15 mM $MgCl_2$ in 25 percent ETOH) for at least 30 min. The cells were treated with RNAase (.2 ml of 1 mg/ml for 1 hr at 37°C) and then stained with propidium iodide (2 ml of .05 mg/ml).^{*} The cell samples were resuspended in saline for FMF analysis (Chemical Biodynamics, University of California at Berkeley).

The DNA histogram which is obtained is an accumulation of the light outputs from individual cells. The intensity of the fluorescent signal from each cell is measured with a photomultiplier tube and this information is collected and stored. A typical DNA histogram for a control population is illustrated in Fig. 2 and represents the distribution of cells in various stages of the cell cycle. The area under the left hand peak is primarily G_1 cells, while the second peak is formed primarily from $G_2 + M$ cells. The difficulty in FMF analysis arises from the contribution of S phase cells to the G_1 and $G_2 + M$ peaks. Cells which have just initiated DNA synthesis have approximately 2n DNA content while cells completing a round of replication have approximately 4n. Although these cells are in S phase they contribute to the G_1 and $G_2 + M$ peaks, respectively. The S phase contribution to the peaks acts to shift the

^{*} Propidium iodide quantitatively intercalates into double stranded regions of DNA and RNA which explains the necessity of an RNAase treatment.

peaks toward the center. In order to compensate for the S phase contribution we have chosen to analyze the data with a mechanical planimeter using certain approximations. The protocol for FMF analysis was as follows: from the apparent G_1 maximum, as it appears visually, a $2 \times G_1$ value was calculated. This value ($2 \times G_1$) was to the right of the apparent G_2 peak as it appears in the histogram. The midpoint between the G_2 apparent and the $2 \times G_1$ value value was taken as the true G_2 . One half of G_2 true was used as the G_1 true. The G_1 true and G_2 true peaks, established in this manner, each formed one leg of a right triangle when extended to the base of the histogram. The hypotenuse was formed from the leading edge of the G_1 peak or the trailing edge of the G_2 peak. The hypotenuse of each was aligned with the upper portion of the histogram because of the increased spread at the base due to S phase cells. The 0 level formed the base of the triangles, and a 90° extension from the G_1 true and G_2 true peaks to the base formed the third side of each triangle. The areas of each of the two triangles was computed, doubled, and then compared to the total area to give the percentage of cells in G_1 and $G_2 + M$. The area not accounted for by G_1 and $G_2 + M$ was attributed to the S phase population. A control analysis is illustrated in Figure 2.

RESULTS

I. Survival ParametersA. Feeder Cell Effect

The 9L gliosarcoma cell line requires feeder cells for optimal plating efficiency.* It is thought that feeder cells provide short-lived diffusable factors that are necessary for cell growth, but these factors have not been identified.⁹⁹ Heavily irradiated (~5000 rad) 9L cells which were incapable of forming colonies were used as the feeder layer. Figure 3 shows the plating efficiency of control cells versus the feeder cell concentration. Total cell numbers** ranging from 100 to 10,000 per 60 mm dish resulted in approximately 45 percent plating efficiency. Increasing the feeder concentration to 10^5 cells increased the plating efficiency an additional 10 percent. Further increases in cell number beyond 10^5 cells caused a sharp reduction in the plating efficiency. At 10^6 cells per dish the plating efficiency had dropped to 28 percent in contrast to the 56 percent maximum level.

The effect of feeder cell concentrations on heat stressed cells was also examined, the rationale being that perhaps damaged cells are even more dependent on feeder cells than controls. The plating

* The plating efficiency is the ratio of the number of colonies counted to the number of viable cells seeded.

** The total cell number includes the number of feeder cells plus the number of test cells. The lower limit is established by the number of test or control cells seeded without the addition of feeder cells.

efficiency of cells exposed to 43°C for 2 hours and then plated with different concentrations of feeder cells is shown in Figure 4a. A similar dependence on feeder cell concentration is apparent with the heat stressed cells as with control cells. A maximum plating efficiency is obtained with approximately 10^5 cells per dish.

Under low cell density conditions the heat stressed cells appear to be more dependent on feeder cells for increased viability as evidenced by the rapid change in plating efficiency in the 10^3 to 10^5 cells per dish range. Normalizing the heat stress data to the control with the same number of feeder cells emphasizes the increased dependence of damaged cells on the feeder layer (Fig. 4b). At low cell densities the plating efficiency is still low while at higher concentrations the fluctuations from maximum survival were minimal once normalized to the control.

These studies show the importance of maintaining a constant cell density since variation in cell number can, by itself, alter the survival response. For example, a survival level of .1 percent requires seeding 1×10^5 cells in order to count ~100 viable colonies. Control dishes only require seeding 100 cells. If the heat stressed cells were normalized to controls without feeders the survival would appear higher than if normalized to controls with feeders. In our experiments total cell concentrations were kept between 5×10^4 and 10^5 cells per 60 mm dish. At high survival levels feeder cells were added to yield these concentrations, and at extremely low survival levels larger plating surfaces were used.

B. Plating Efficiency versus Number of Viable Cells

Further study of the 9L gliosarcoma system has revealed that the plating efficiency of control cells is not linear with the number of test cells seeded even when the total cell number (feeders plus test cells) are at approximately 10^5 per dish. That is, seeding twice the number of test cells does not necessarily result in twice the number of colonies. A general trend of decreasing plating efficiency with increasing cell number seeded was observed. As shown in Figure 5, certain ranges of cell numbers had equal plating efficiencies. For all survival studies we attempted to keep the colony counts per 60 mm dish above 30 and below 90 by doing a number of dilutions per point. In this colony count range the plating efficiency remained at 55.0 percent (with a standard deviation of 4.24). At higher cell densities colonies may overlap and be counted as a single colony leading to a reduced plating efficiency. Competition for "growth factors" in the medium, serum and feeder layer could also result in reduced viability. At extremely low cell densities the increased plating efficiency may be due to an excess of these factors.

II. Survival Studies

The response of 9L-gliosarcoma cells to varying times at 43°C is shown in Figure 6. The survival curve is characterized by a shoulder region with a T_q of 54 min. followed by an exponential region of cell killing. The slope of the exponential portion is .04 per min. corresponding to a T_0 of 25.0 min.* The 95 percent confidence interval

* A biphasic fit can also be applied to this data, but the slope in the 2 to 3 hour range is not significantly different from the slope in the 3 to 5 hour range.

for the T_0 is 24.1 min. to 26.0 min. Counts from over 400 dishes were used to obtain this survival curve. In the next series of experiments the effect of conditioning heat treatments on the 43°C response is examined.

A. Variation of Sublethal Temperature

Temperatures between 37°C and 40°C are not lethal to cells for treatment times up to 10 hours. However, exposure to these sublethal temperatures does alter subsequent response to 43°C. 9L gliosarcoma cells were exposed to 38°C, 39°C or 40°C for 4 hours followed immediately by graded exposures to 43°C. Figure 7 shows that as the conditioning temperature increases the resistance or tolerance to 43°C also increases. The slopes of the 43°C survival following each of the pretreatment protocols are shown with the 95 percent confidence interval in Figure 8. Thermotolerance ratios of 1.26, 3.24 and 4.63 were obtained with 4 hour pretreatments at 38°C, 39°C and 40°C, respectively.

It was also determined that the water bath handling alone did not alter the survival response. The 43°C survival curve for cells exposed to a 37°C water bath for 4 hours was not significantly different from cells held in a 37°C incubator prior to graded doses at 43°C. The slope for the water bath treated cells was $.036 \pm .003$ as compared to $.0398 \text{ min} \pm .002$ for controls.

B. Duration of Sublethal Exposure

In the last series of experiments I observed that for equivalent exposure times, increasing the nonlethal temperature conferred more protection to lethal exposure. In these experiments the effect of

increasing duration at a particular sublethal temperature on a subsequent 43°C challenge was examined. As shown in Figure 9, survival remained at 100 percent for treatment times up to 8 hours at 40°C. However, changes not measured by viability were occurring with the increasing exposure to 40°C as evidenced by increased resistance to 43°C. As the time at 40°C increased from 1 to 4 hours, the sensitivity to 43°C decreased (Figure 9). The thermotolerance values for 1, 2 and 4 hour pretreatments at 40°C were 2.32, 3.42 and 4.63. The 95 percent confidence intervals for the T_0 's were 58.02 min $\pm$ 2.93 min for 1 hour, 85.4 min $\pm$ 6.42 for 2 hours and 115.8 $\pm$ 6.6 min for 4 hour initial treatment at 40°C prior to 43°C. The 43°C survival curve is also illustrated in Figure 9 for visual comparison.

C. Effect of 37°C Interval Between Sublethal and Lethal Exposures

The effect of increasing the 37°C interval between sublethal (40°C) and lethal (43°C) exposures was also examined. For 1, 2 and 4 hour pretreatments at 40°C tolerance to 43°C was maximum immediately following the conditioning treatment (i.e., for 0 interval). As the interval between the 40°C and 43°C exposure increased the resistance to 43°C decreased. Figure 10 shows the rate of decrease of T_0 with increasing 37°C interval following a 4 hour conditioning treatment at 40°C. Each point in Figure 10 represents the inverse of the slope of the 43°C survival curve after the conditioning treatment (4 hours at 40°C) and the appropriate 37°C interval. The error bars represent the 95 percent confidence interval from two or more experiments. The T_0 decreased at the rate of .046 per hour which is equivalent to the rate at which thermotolerance is lost. (If each T_0 is divided by the

43°C control T_0 of .417 hour the loss of TT is obtained which has the same slope as the T_0 decrease.) This data can also be interpreted as the rate of readaptation to 37°C instead of a loss of thermal resistance.

It has not been determined if the adaptation to the lower temperature (37°C) occurs by reversal of pathway(s) involved in adaptation to elevated temperature, or if these two processes occur by completely different pathways. Nonetheless, adaptation to lower temperatures is accompanied by a loss of thermal resistance, and the kinetics for loss of T.T. or adaptation to lower temperatures are equivalent.

A family of survival curves is depicted in Figure 11 which illustrates the loss of thermal resistance with increasing 37°C interval following a 4 hour at 40°C conditioning treatment. All of the survival curves are not included for purposes of clarity, but the inverse of the slopes of the curves have been shown in Figure 10.

Loss of thermal resistance to 43°C was also determined for 1 and 2 hour conditioning treatments at 40°C. As seen in Figure 12, the decay of T_0 with increasing 37°C interval was approximately the same as with a 4 hour at 40°C pretreatment. A T_0 or T.T. decay rate of .041 per hour was obtained with a 1 hour preconditioning while a decay rate of .047 per hour was observed with a 2 hour conditioning. For a 40°C pretreatment the magnitude of induced resistance is dependent upon the time at the conditioning temperature (with no 37°C interval) and this resistance will decay at a rate of $.045 + .003$ with increasing 37°C interval regardless of the magnitude of induced T.T.

In brief, the foregoing experiments have shown the following relationships. First, the magnitude of induced thermal resistance increases with increasing temperature of the sublethal treatment. The magnitude of thermal resistance also increases with increasing duration at the nonlethal conditioning temperature. Therefore, a 2 hour conditioning treatment at 40°C is approximately equivalent to a 4 hour conditioning treatment at 39°C as determined by subsequent resistance to 43°C. Finally, as the 37°C interval between nonlethal and lethal exposures increases thermal resistance decreases. The loss of thermotolerance occurs at the same rate regardless of the magnitude of induced resistance. Consequently, some degree of thermal resistance persists for longer periods of time in populations which are rendered more tolerant by the initial conditioning.

III. Flow Microfluorometry (FMF)

Since differences in cell killing throughout the cycle have been reported, I have examined the cell cycle distribution in response to sublethal temperatures. If conditioning at the nonlethal temperatures causes a redistribution of cells into a more heat resistant phase, thermotolerance to the second fractionation would not be surprising. For a 40°C pretreatment maximum thermal resistance to 43°C is observed immediately after the conditioning treatment. If cell cycle redistribution contributes to T.T. it should be apparent with 40°C exposure. Planimeter analysis (as discussed in Materials and Methods) of the DNA distributions is illustrated in Figure 13. It is apparent that significant cell cycle redistribution does not occur with the 40°C

exposure. A slight increase in S phase cells with a corresponding decrease in $G_2 + M$ cells was observed at 40°C after at least 4 hours of exposure, but our experiments did not use exposures of this length.

Although it has been determined that significant redistribution does not occur with nonlethal exposure, it is of interest to examine the age distribution response of these conditioned cells to lethal temperatures. The 9L-gliosarcoma cells were treated at 40°C for 4 hours followed immediately by 43°C exposure for up to 6 hours. The planimeter analysis is shown in Figure 14. The cell killing results in an increased proportion of cells in S phase as compared to 37°C controls and a corresponding decrease in G_1 and $G_2 + M$. It should be noted that even with the conditioning treatment which increases the survival many of the cells contributing to the FMF histogram are reproductively nonviable. For example, following 4 hours at 40°C plus 3 hours at 43°C only 27 percent of the cells are capable of producing colonies, and after the conditioning treatment plus 6 hours at 43°C only 7 percent have this ability. Shifts in the age distribution can be caused by an accumulation of nonviable cells in a relatively heat sensitive phase, a block in viable cell progression or a combination of these two events.

IV. PROTEIN SYNTHESIS INHIBITION AND THERMOTOLERANCE

In these experiments the role of protein synthesis in the induction and development of thermotolerance was examined. Cycloheximide which acts at the translational level of protein synthesis by limiting the termination step was used to inhibit protein synthesis.²⁷ In

9L-gliosarcoma cells exposure to 5 $\mu\text{g/ml}$ of this drug for 15 minutes reduces protein synthesis to 20 percent of controls, and a 2 hour exposure at this concentration reduces protein synthesis to 7 percent of controls.* If, protein synthesis is required for the development of thermotolerance, exposure to cycloheximide during the conditioning heat treatment should block the increased heat resistance observed with the second (lethal) fractionation. Exposure to cycloheximide during the conditioning treatment (4 hours at 40°C) did not alter this increased resistance as determined by survival values at 2, 3 and 5 hours at 43°C. Figure 15 shows the survival values obtained at 43°C for each of the three conditions, 40°C heat treatment, 40°C heat treatment plus cycloheximide and no pretreatment. The survival of cycloheximide treated cells falls within 1 standard deviation of the cells conditioned at 40°C only, and both of these values are significantly different from the 43°C controls. Exposure to 5 $\mu\text{g/ml}$ of cycloheximide for 4 hours at 40°C did not alter the plating efficiency as compared to controls which did not receive the drug treatment.

I also observed that preincubation with cycloheximide without the conditioning heat treatment conferred considerable resistance to 43°C exposure. In this experiment cells were exposed to 5 $\mu\text{g/ml}$ of cycloheximide for 4 hours prior to lethal heat treatment. Cycloheximide pretreated cells had a survival of 61.2 percent following 2 hours at 43°C while cells which received no drug treatment and 2 hours at 43°C had a survival of 11.37 percent. The difference in survival was more

* P. Ross personal communication. The results were determined from ^3H -leucine uptake studies.

dramatic after 3 hours at 43°C when cycloheximide pretreated cells had a survival of 21.95 percent versus .82 percent for untreated controls. Although survival is enhanced with cycloheximide pretreatment no definite conclusions on the degree of resistance can be made with just these two data points.

In summary, cycloheximide does not inhibit development of thermotolerance when administered in conjunction with the conditioning nonlethal temperature. Furthermore, exposure to cycloheximide without heat conditioning, by itself confers protection to 43°C heat treatment.

DISCUSSION

I. Kinetics

The use of non-lethal temperatures for the first fractionation of a split hyperthermia exposure shows conclusively that thermotolerance is not due to selective killing by the initial heat treatment. In these studies exposure of 9L gliosarcoma cells to temperatures of 38°C, 39°C and 40°C resulted in 100 percent viability as compared to 37°C controls, yet these exposures altered the subsequent response to 43°C. For gliosarcoma cells the altered response was exhibited as a decrease in the cell survival slope (or increase in T_0) at 43°C following pretreatment. Cells held at 37°C and then exposed to 43°C were inactivated at the rate of 2.38 hr^{-1} . Following 4 hour conditioning treatments at 38°C, 39°C and 40°C, the rate of cell killing at 43°C decreased to 1.89 hr^{-1} , $.737 \text{ hr}^{-1}$ and $.515 \text{ hr}^{-1}$, respectively. Increasing the nonlethal exposure temperature for a given time increases the resistance to lethal exposure as seen by the decrease in k (or increased T_0).

This change in T_0 with exposure to nonlethal temperature was not reported by Henle et al for CHO cells exposed to 40°C followed by 45°C exposure.⁵² CHO cells grew normally at 40°C without any evidence of cell killing for more than two generations; incubation at 40°C for 0 to 7 hrs prior to acute heating at 45°C produced only small changes in the 45°C T_0 .* The T_q of the 45°C heat survival curve was, however,

* The authors reported no change in the subsequent T_0 , but evaluation of their data shows a small T.T.R. of 1.34 for a 7 hr 40°C pretreatment.

increased approximately 3 fold by 40°C preincubation for 7 hrs. The Tq increased from 8.3 min for the 45°C control to 22.8 min for the pretreated cells. (The Tq for 9L gliosarcoma cells decreased slightly from 54 min for the 43°C control to 44 min for cells pretreated at 40°C for 4 hours.)

It should be noted that in the CHO experiments of Henle, Karamuz and Leeper, 45°C was used for the second fractionation. It may be statistically more difficult to demonstrate tolerance because of the steepness of the 45°C T_0 . The difficulty with this rationalization, however, is that fractionated 45°C treatments in CHO cells results in significant thermotolerance with a sufficient 37°C interval.⁵⁴ From these experiments it appears that the slope of a 45°C survival curve can be shifted by induced thermotolerance. At this time the differences obtained between the 9L gliosarcoma system and the CHO cell system used by Henle et al. cannot be resolved.

In further investigation of the CHO response to fractionated hyperthermia, Joshi and Jung stated that T.T. to 43°C is considerably higher than that observed for 45°C when each is preceded by a 40°C treatment.*¹⁰⁰

As with the gliosarcoma cells, Joshi and Jung found that a 40°C pretreatment of CHO cells altered the slope of the 43°C heat response curve more strongly than the shoulder width. The T_0 of the CHO 43°C

* Joshi and Jung came to this conclusion from evaluation of the Henle, Karamuz and Leeper studies.^{52,100} The investigators own experiments consisted of a 1 hr pretreatment at 41°C (not 40°C) followed by 45°C. A relatively small T.T.R. of 1.38 was obtained. Extrapolation from 43°C results predict that 41°C is less efficient than 40°C in inducing tolerance and the relatively short 41°C treatment (1 hr) should also result in less tolerance, so this result is not surprising.

survival curves increased by factors of 1.1, 1.9, and 3.5 for 1, 3 and 5 hr pretreatments at 40°C. These values are consistent with those found in this study where 1, 2 and 4 hour exposures to 40° resulted in TTR's of 2.32, 3.42 and 4.63. Dramatic results were reported for a 9 hr pretreatment of CHO cells to 40°C. Following this lengthy conditioning, a 90 min exposure to 43°C resulted in approximately 90 percent survival, whereas a 90 min control exposure (no pretreatment) reduced survival to 1.55 percent.¹⁰¹ In both the CHO and 9L-gliosarcoma cell lines increasing the duration of exposure to the nonlethal conditioning temperature resulted in increased survival to 43°C.

Thermotolerance induced in CHO cells by 40°C pretreatment was maximal at the end of the conditioning treatment and was not increased by further incubation at 37°C.¹⁰¹ When the two thermal doses were separated by incubation at 37°C, the magnitude of thermotolerance remained constant for about 2 hours followed by a slow decline.¹⁰¹ Thermotolerance decayed at approximately $.045 \text{ hr}^{-1} \pm .003$ in 9L gliosarcoma cells exposed to 40°C regardless of the length of the initial pretreatment.

Thermotolerance induced by fractionated exposures to higher temperatures (>43°C) requires several hours of incubation around 37°C to develop.⁵² For instance, following a conditioning heat treatment of 10 min at 45°C the T_0 of the CHO hyperthermia survival increased from 3.3 min to a maximum of 17.6 min with an 8 hr 37°C interval (T.T.R. = 5.3). A shorter conditioning treatment of only 5 min at 45°C required a 2 hr 37°C interval for a maximum T.T.R. of 3.4. In another

study HeLa cells exposed to two one hour 44°C treatments separated by varying time intervals at 37°C showed maximum survival with at least a 5 hr interval.²

These results show that temperatures of 44°C and 45°C (at least in the cell lines studied) require a 37°C interval for induction of protection. The sublethal thermotolerance studies and a 42.5°C fractionated study in V79 cells indicate that, with temperatures lower than 43°C, maximal tolerance is exhibited immediately following the conditioning treatment.*⁹⁰

From these reports, it could be suggested that at the higher temperatures, (>43°C) cell killing proceeds too rapidly as compared to tolerance induction. The manifestation of thermotolerance might require the stimulus of elevated temperature in addition to an adequate amount of time for the kinetic response. In the fractionated lethal treatments the tolerance mechanism is stimulated with the first heat treatment, and if the cell population is returned to 37°C they can enter the protected state. For a sublethal conditioning treatment, tolerance can be stimulated and developed during the initial heat exposure.

The present experiments show that sublethal thermotolerance is not a strictly temporal response. That is, the magnitude of induced protection does not depend upon the total combined time of the preconditioning treatment and interval at 37°C. For example, the

* In Chinese hamster V79 cells as the interval between two equal 4 hr exposures at 42.5°C increased, the cell survival fraction decreased, indicating that thermotolerance was being eliminated.⁹⁰

T.T.R. for 1 hr at 40°C plus 3 hrs at 37°C followed by 43°C does not equal 2 hrs at 40°C plus 2 hrs at 37°C followed by 43°C, and these two protocols do not equal 4 hrs at 40°C plus 0 hr at 37°C followed by 43°C. The T.T.R.'s for each of these conditions are 1.85, 3.19 and 4.63, respectively. In this example, all cells had at least a 1 hr exposure to 40°C combined with a 37°C interval as needed to total 4 hrs, prior to 43°C treatment. Although the total duration prior to 43°C and the temperature of the preconditioning treatment were equivalent, the magnitude of the thermotolerance ratios were not. Evidently, the degree of resistance does not depend on the stimulus of a certain temperature combined with an interval for a specified time for tolerance development.

The dependence of thermal resistance upon conditioning temperature, time at the conditioning temperature and interval between fractionated exposures is indicative of a rate process of sublethal events as opposed to an on/off phenomenon. If tolerance for a population were an on/off event, any exposure above a certain minimum would be expected to result in one defined tolerant state. Instead, a continuum in the degree of induced protection is observed. This graded response of the population could be the result of one of two types of events. Firstly, there could be two states available to a particular cell, unprotected and protected. Transition from one state to another would be dependent on a certain probability function, and lengthening exposure to elevated temperatures would result in increased probability of transition to the protected state. The second alternative would be a continuous

tolerance response for individual cells with upper and lower limits physiologically determined for the heat protected and unprotected states. For either a discrete cell transition or a continual cell response, the graded thermotolerant response characteristic of cell populations would result.

It might also be noted that different subpopulations of cells might possess different capabilities in regard to maximum achievable tolerance. For example, different portions of the cell cycle may have different T.T.R.'s for equivalent fractionated protocols.*

The sublethal thermotolerance studies have characterized the relationships between the conditioning temperature, time at the conditioning temperature and interval between nonlethal and lethal exposures. The molecular events corresponding to changes in cellular heat sensitivity are still unresolved.

II. Mechanisms of Thermotolerance Development

A. Membranes

As with cell killing, the specific mechanism(s) responsible for the development of thermotolerance remain unknown. From a number of studies, including this one, it can be concluded that thermotolerance is not completely attributable to repair of sublethal damage, redistribution within the cell cycle, or selective killing with the first heat fractionation.

* This is not to be confused with differences in heat sensitivity throughout the cell cycle. The most heat resistant cycle (usually G₁) could have a relatively large or small T.T.R. as compared to other portions of the cell cycle for a given fractionated protocol.

The cytoplasmic membrane has been suggested as a site for cellular changes related to heat protection. These changes include alteration of membrane cholesterol content, variation in lipid composition and alterations in membrane permeability.

Cress et al. reported a high degree of correlation between the membrane cholesterol content and the rate of cell killing by hyperthermia for four different cell lines.¹⁰² There was a linear relationship between cholesterol levels (80 to 400 μg per 2×10^6 cells) and the T_0 for cell death. In addition, the cholesterol concentration in the particulate fraction was shown to decrease with time at the elevated temperature. Experiments were in progress to determine if the cholesterol concentration is elevated in thermotolerant subpopulations.

Using bacteria, Yatvin et al. have shown that systematic variation of growth temperature leads to predictable changes in cell lipid composition and to changes in survival of the cells after hyperthermia (see page 14).¹⁰³ Extending these studies to V79 Chinese hamster lung cells the investigators cultured the cells at 33°C and 37.5°C.¹⁰⁴ Survival to subsequent hyperthermic insult was positively correlated with growth temperature (greater in 37.5°C grown cells vs. those grown at 33°C). The authors suggest that a critical alteration of membrane lipid structure occurs at a temperature a few degrees above normal and that this can be systematically altered by changes in lipid composition. Possibly protection to the cell is conferred by specific changes in lipid composition. The lipid

composition may in turn influence the activity of membrane bound proteins and/or the permeability across the membrane.

Interesting findings were reported by Reeves who compared a clonal strain of pig kidney cells characterized by heritable and stable resistance to heat shock with the heat sensitive parental line.¹⁰⁵ Sensitive cells pre-labeled with ³H-uridine released significantly more label-containing materials during heat stress than did pre-labeled resistant cells. This differential release of uridine containing substances was not paralleled by a generalized leakiness to other compounds. The resistant cells were also found to regain the capacity to synthesize vital molecules sooner and at initially faster rates, than the sensitive cells. The leakage of nucleosides, nucleotides, and other small uridine containing molecules by the heat sensitive cells during thermal stress, leaves them at a metabolic disadvantage relative to the less permeable resistant cells, and excessive loss of RNA precursor molecules might well be expected to block the recovery cycle. It is not surprising then, that functions return to control levels faster in the resistant line as compared to the sensitive line. Reeves suggested that permeability changes causing decreased leakage of uridine containing materials during heat stress may be important cellular mechanisms in resistance to heat shock. Reeves results could have some relation to transient thermotolerance if the heat resistant pig kidney line represents a stable and heritable shift of a response which is normally transient. That is, transient and permanent heat resistance could be due to the same cellular changes, only in one case

the cell gradually reverts to its "normal" state, while in the other rare instance the alteration is fixed. If permanent and transient tolerance are related, the nature of the cellular alterations and their mediation remains to be elucidated.

B. Heat Shock Proteins

In any current discussion on the possible mechanisms of thermo-tolerance, the significance of the heat shock proteins cannot be ignored. Many organisms, including drosophila, yeast and mammalian cells exhibit increased synthesis of a small set of proteins upon elevation of growth temperature.*¹⁰⁶⁻¹⁰⁸ Within a few minutes of heat treatment, production of messenger RNAs at previously active regions is reduced and initiated at a small number of quiescent sites. Consequently, protein synthesis is redirected from the production of a broad spectrum of proteins, characteristic of the various tissues and cultured cells, to the production of a small number of non-tissue specific heat induced proteins.¹⁰⁹ The fact that the patterns of protein synthesis are altered with a marginal shift in temperature has implied to some investigators an essential cellular function for the newly synthesized proteins.^{107,108} Since heat conditioning also results in protection from subsequent heat stress, it has further been suggested that the heat shock proteins may play a role in tolerance acquisition.¹⁰⁷

* These proteins have been termed heat shock proteins although they are produced following exposure to a number of agents besides heat.

The heat shock protein response has been most widely studied in *Drosophila*. If *Drosophila* larvae or their excised tissues are subject to heat shock, puffs are induced at a few specific sites on the polytene chromosomes.¹⁰⁶ A specific set of RNAs are transcribed from the genes forming the heat shock puffs, and in turn some of these RNAs are preferentially translated into the heat shock polypeptides.* The synthesis of most other proteins ceases.

The induction of messenger RNA and protein synthesis in *Drosophila* by heat treatment is so vigorous that after 60 min at 37°C, 90 percent of the [³H] leucine incorporation is into heat shock proteins.¹⁰⁹

In *Drosophila melanogaster* the apparent molecular weights of the heat shock proteins have been estimated from SDS-acrylamide gels to be approximately 82,000, 70,000, 68,000, 36,000, 27,000, 26,000, 23,000, and 22,000 daltons.¹⁰⁹ Several of the heat shock proteins appear to have individual induction characteristics with respect to the temperatures at which they are maximally induced, the range of temperatures over which they are synthesized, and the kinetics of their induction. At least in *Drosophila* the control of the synthesis of heat shock proteins, while similar, is not necessarily coordinate.¹⁰⁹

Preliminary findings have been reported for yeast systems with regard to heat shock proteins.¹⁰⁷ A rapid shift in cultivation temperature of *Saccharomyces cerevisiae* yeast from 23°C to 36°C

* In vitro translation studies show that preexisting messages remain stable in a certain elevated temperature range partially imposed by media conditions. This would indicate a great deal of translational regulation after heat shock if "normal" messages were present simultaneously with the heat shock messages.

results in a transient protection of the culture from death due to exposure to 52°C. The thermal resistance correlates extremely well with the cellular level of a 100,000 M.W. heat shock protein. A mutant strain of this yeast (ts 136) is temperature sensitive at 36°C for RNA transport from the nucleus to the cytoplasm. The mutant strains fail to synthesize heat shock proteins when shifted from 23°C to 36°C and also fail to acquire an increase in thermal resistance with the shift. However, when cultures of strain ts 136 which have been previously shifted from 23°C to 36°C are subsequently returned to 23°C, synthesis of heat shock proteins begins, and thermal resistance is also acquired. From this data, it appears that conditions which allow cells to synthesize heat shock proteins are also permissive for the acquisition of thermal resistance.

The pattern of proteins synthesized by chicken embryo fibroblasts changes dramatically with incubation at 45°C or with the addition of certain amino acid analogues (e.g., canavanine or hydroxynorvaline).¹⁰⁸ A rapid increase in the synthesis of three proteins with molecular weights of 22,000, 76,000, and 95,000 daltons to levels which dominate the cells biosynthetic capacity is observed. In this study cycloheximide blocked the induction of heat shock proteins induced by analogues but not by heat shock. Kelley and Schlesinger suggest that this result is consistent with a model in which an aberrant protein is required to signal induction of heat shock proteins.¹⁰⁸ In this case, both treatments affect the structure and function of proteins, but in order for analogues to alter protein structure they must be incorporated into

the polypeptide chain, and, therefore, are blocked by cycloheximide. The defective protein(s) required for induction could be a repressor acting at a promoter site on the cell genome or an enzyme or surface receptor whose malfunction indirectly signals the actual transcriptional and translational events.

As with other systems, the function of the heat shock proteins from chick embryo fibroblasts has not been elucidated. Preliminary analyses indicate that they are major proteins of the cell membrane and that they are possibly associated with glucose metabolism and hexose transport.¹⁰⁸ Partial proteolytic digests of these proteins show they are not structurally related to each other, or to the major cytoskeleton proteins actin and desmin.¹⁰⁸

In mammalian culture systems heat shock proteins with molecular weights of 120,000, 95,000/93,000 and 76,000 have been reported for mouse L cells, BHK cells and HeLa cells following 5 min at 45°C plus 2 hrs at 37°C. A slightly different heat shock protein profile has been found in Chinese hamster HA-1 cells.¹¹⁰ The three proteins whose synthesis is enhanced by heat shock at 45°C have molecular weights of 59K, 70K and 87K. The kinetics of thermotolerance development and decay correlated well with the induction and disappearance of heat shock proteins. Furthermore, other agents which induced heat shock proteins such as arsenite and hypoxia also conferred transient thermal resistance.¹¹⁰

In this section some of the data on heat shock protein production and the possible relevance to thermal resistance has been noted. It should be emphasized that at this time the only relationship between

these two events (heat shock protein production and thermal resistance) is entirely circumstantial with the kinetic data suggesting a temporal coincidence. No specific function has been attributed to any of the heat shock proteins. Indeed, the heat shock proteins could be involved in cell protection, cell killing or they may have no functional consequence, merely produced in large quantities under appropriate conditions. With sufficient temperature elevation increases in certain enzymatic reactions could result in increases in certain proteins. Alternatively, heat shock protein production could result from a loss of feedback control due to conformational changes in particular proteins.

The questions of whether the heat shock protein play a role in the development of thermotolerance and, if so, how the protection is mediated, has not been answered. We are still a long way from understanding how heat damages a cell, and how resistance to this damage is conferred.

C. Cycloheximide and Thermotolerance

Current speculation about heat shock proteins and their possible relevance to thermotolerance may have some bearing on results obtained with cycloheximide studies. For 9L gliosarcoma cells, it was found that cycloheximide did not block sublethal tolerance induction when it was administered prior to the second heat treatment (i.e. cycloheximide administered concurrent with the sublethal exposure did not effect the subsequent T.T. ratio). Furthermore, if cycloheximide was administered prior to a single heat treatment, a resistance to the heat was observed.

These results were not confirmed by Leeper et al.¹¹¹ Cycloheximide at 1 $\mu\text{g/ml}$ during a 7 hr interval reduced T.T. by 60 percent, and if present for only 2 hrs prior to the graded hyperthermia, reduced T.T. by 45 percent. However, cycloheximide treatment prior to a heat treatment resulted in protection. It is not at all clear how the drug could act in these two very different ways, inhibiting tolerance in one instance and inducing it in another, depending on whether there had been an initial heat exposure. The results in 9L cells are consistent with one mode of action of the drug which somehow results in increased heat resistance.

In other studies, cycloheximide exposure was found to reduce hyperthermic cell killing. Cell viability was higher in HeLa cells heated at 42°C in the presence of 1 $\mu\text{g/ml}$ cycloheximide as compared to cells exposed without inhibitors.⁹² The effect of cycloheximide was cell cycle dependent, protecting against hyperthermia in G_1 and S phase but not in G_2 . Cycloheximide mediated protection against hyperthermic cell killing was also dose dependent. Viability was highest in HeLa cells heated to 42.0°C for 2 hr with 10 μM cycloheximide (95 percent inhibition of ^3H -leucine) and lowest in cells heated with a .1 $\mu\text{g/ml}$ dose of drug (corresponding to 46 percent inhibition of ^3H -leucine incorporation).⁹²

Cycloheximide has been reported to alter the effects of heat on protein biosynthesis. Preincubation of L5178Y murine leukaemic lymphoblasts in the presence of varied concentrations of cycloheximide decreased the adverse effects of high temperature upon protein

biosynthetic activity as measured by ^3H -leucine exposure.²⁶ The longer the cells were preincubated in the presence of .1 mM cycloheximide, the more pronounced was their rate of recovery after the heat shock and removal of the inhibitor. The relative increase in activity during the recovery phase, caused by the addition of cycloheximide immediately before heat shock, suggests a protective effect of the inhibitor.

In HeLa cells treatment with 100 $\mu\text{g/ml}$ of cycloheximide prior to 42°C heat exposure resulted in a significantly increased ability to incorporate radioactive amino acids.⁹² As with the L5178Y system the amount of protection (as determined by protein synthesis recovery) afforded by pretreatment with cycloheximide increased with longer preincubation. McCormick and Penman also reported that the number of ribosomes associated with polyribosome structures at 42°C increased with the length of preincubation with cycloheximide.²⁵

The results of Fuhr et al. and also McCormick and Penman have been interpreted to indicate that inhibition of protein synthesis promoted the formation of a non-protein compound required for protein synthesis which enabled the cells to better withstand hyperthermia.* If heat and cycloheximide exposures trigger a similar cellular response by reducing protein synthesis, it may not be surprising that conditioning doses of either agent result in thermotolerance.

* Other studies with actinomycin have shown the protein synthesis recovery to be dependent upon RNA synthesis.²⁵

It should also be mentioned that even at concentrations of 100 $\mu\text{g/ml}$ not all protein synthesis is blocked by cycloheximide.⁹² Instead, protein synthesis is reduced to 2 percent of normal. A 25 $\mu\text{g/ml}$ cycloheximide exposure of chick embryo fibroblasts reduced protein synthesis by 97 percent.¹⁰⁸ Interestingly, the induction of the heat shock proteins in chick embryo fibroblasts is not altered when cycloheximide is administered during the 45°C heat shock. In this study, cycloheximide was removed prior to adding label so it is not possible to discern if the heat shock proteins are all translated following cycloheximide treatment or if some of the residual 3 percent protein synthesis represents production of these proteins during drug treatment.

From these experiments, it might be inferred that a reduction in protein synthesis by any agent (e.g., chemical or heat) leads to a protective state. This transition could be mediated by proteins (heat shock proteins), nonprotein compounds, or a combination of the two. The fact that cycloheximide does not block T.T. when administered during the conditioning treatment does not rule out protein involvement. New proteins could be selectively produced during cycloheximide treatment or could be translated once the inhibitor was removed. Heat treatment may result in a generalized inhibition of protein synthesis which stimulates production of a few select proteins. It also appears that nonprotein compounds may be produced in response to protein synthesis inhibition and that these substances may aid in the recovery of protein synthesis, either generalized or select.

III. IMPLICATIONS OF SUBLETHAL THERMOTOLERANCE ON THE ARRHENIUS ANALYSIS OF HEAT INACTIVATION

In the preceeding experiments it has been shown that some alteration is occurring with exposure to sublethal temperatures which confers protection to subsequent lethal exposure. In regards to this phenomenon of thermotolerance it is interesting to evaluate the heat inactivation curves for 9L gliosarcoma cells shown in Figure 1. The extent of cell killing is a complex function of both the exposure temperature and time at the temperature. There is no simple relationship to equate a survival level achieved at one time and temperature to the same survival achieved with a different temperature and time. Qualitatively, it appears that cells are killed very slowly below 43°C and at a much faster rate above this temperature.

In the following discussion we will develop a model based on the induction of thermotolerance to explain the changing slope of the Arrhenius plot with increasing temperature.

A. The Arrhenius Analysis

Arrhenius observed that the rate of a reaction could be related to the temperature by

$$k = Ae^{-E_a/RT} \quad (1)$$

where k represents the rate constant, A the pre-exponential factor,*

* The pre-exponential factor is the number of molecules colliding per second in unit volume, or in a monomolecular reaction it is regarded as the frequency of vibration of the activated bonds in the reacting molecules.³

E_a the activation energy,* R the gas constant and T the absolute temperature in degrees K.¹¹² The plot of $\ln K$ vs. $1/T$ should yield a straight line with the slope of $-E_a/R$ in accordance with the Arrhenius equation. For a number of complex physiological processes the slope of this relationship remains linear over a considerable temperature range.⁴ However, the apparent activation energy for cell killing with increasing temperature is not linear from 42°C through 45°C. For a number of cell lines, including CHO, HeLa and gliosarcoma, the $\ln$ of the cell inactivation rate versus $1/T$ reveals an inflection point at approximately 43°C.^{91,113}

The break in the Arrhenius plot has been interpreted as reflecting different mechanisms of cell killing above and below the inflection point. Landry and Marceau examined the inactivation rate of HeLa cells for temperatures ranging from 41°C to 55°C.⁵ The process of heat induced cell killing was postulated to be the result of a series of reactions, each of them being successively rate limiting within a certain temperature range. In the case of HeLa cells three sequential reactions were sufficient to explain cell killing in the 41°C to 55°C range, and comparison with known biochemical reactions suggested possible rate-limiting events. The kinetics for temperatures between 48°C and 55°C closely resembled nucleic acid degradation; for temperatures from 43°C and 48°C the kinetic parameters were in the range of those measured for the denaturation of proteins; at temperatures near

* The activation energy represents the energy that a mole of the reactant must have before a reaction is possible.³

the physiological range (41-43°C) the authors suggest that the rate at which a cell loses its proliferative capacity may be limited by the rate at which some organized intracellular structures are disassembled, implying damages at the level of whole organelles.*

The notion of identifying master reactions in different physiological processes by correspondence in their measured E_a values as Landry and Marceau have done was originally proposed as part of the Crozier theory. The Crozier theory embodied the following chief points. "1) every physiological process is characterized by a series of reactions, each with a definite E_a value; 2) the overall process is limited by the slowest step or master reaction at that temperature; 3) at "critical" temperatures a different master reaction assumes control and is characterized by a break of the Arrhenius plot; and 4) it may be possible to identify master reactions in different physiological processes by correspondence in their measured E_a values."⁴

In a thorough review, Johnson et al. discuss some of the drawbacks of the Crozier theory.⁴ Firstly, particular reactions do not completely assume control at one precise temperature. Although many

* In this study direct comparison of various activation energies was not made, but rather the activation enthalpy $\Delta H^\ddagger$ and the activation entropy $\Delta S^\ddagger$. According to the Eyring theory for the calculation of the absolute rate of a reaction

$$k = \frac{RT}{N_0 h} e^{\Delta S^\ddagger/R} e^{-\Delta H^\ddagger/RT}$$

where N_0 equals Avogadro's number and h is Planck's constant. For liquid and solid systems $E_a \approx \Delta H^\ddagger + RT$.¹¹⁴

biological processes are dependent upon a series of reactions and are largely limited in certain temperature ranges by some reactions more than others, it is important to consider that all the reactions in a process are influenced by an increase in temperature. Depending upon the activation energies of the particular reactions, some reactions may have more influence within a particular temperature range than others. For example, a reaction with a high activation energy that is negligible at lower temperatures may at higher temperatures become significant. Nonetheless, the other reactions are still influenced in accordance with their own activation energies, and the net result at all temperatures remains dependent upon all the contributory reactions.

Secondly, the physical reality of sharp breaks in the Arrhenius plot remains questionable although changes in slope do occur. When graphing the Arrhenius plot, a continuous variation in E_a may be misconstrued as successive linear relations when the units on the ordinate are relatively close together. Figure 16 illustrates this difference in the Arrhenius plot for 9L-gliosarcoma cells. The two linear portions seemingly present in curve 16A are revealed as a continuous variation in 16B, when the same data are plotted on the wider scale of the ordinate. Landry and Marceau have also shown that a progressive bending in the Arrhenius plot is apparent for HeLa cell killing when a wider range (41°C-55°C) of temperatures is studied.⁵

Changes in the Arrhenius slope may also be associated with changes in the state or structure of the system. These could include changes in viscosity or diffusion with temperature that might effect reaction

rates or changes in the enzyme itself. Therefore, a change in E_a may not be due to a different enzymatic process assuming control but rather a change in the molecular configuration which affects the catalytic properties of the enzyme.*

The assumption that rate limiting reactions may be recognized by comparison of activation energies ignores the extent to which E_a values may vary in different cellular and chemical environments. Changes in the observed E_a have been found to occur under the influence of various factors including constituents of the culture media, age of the culture, the presence of inhibitors and even disease.

Perhaps more important than a change in E_a with environment is the fact that the calculated E_a may not be the result of a single reaction. In a numerical example, Burton showed that a two-step chain could reveal an activation energy which was an admixture of the two values of E_a that were actually involved.¹¹⁵ Burton concluded that a straight line through observed points on an Arrhenius plot could result from dominance shared by a number of reactions of different temperature characteristics.

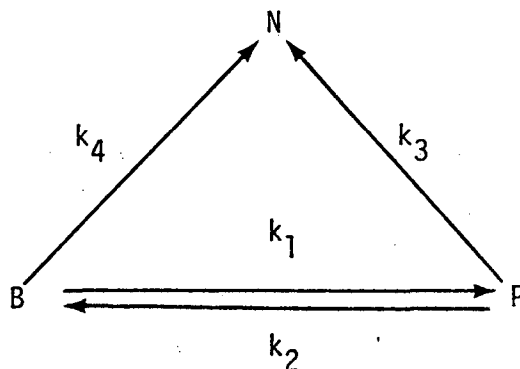
In light of the foregoing discussion, we need to be cautious in interpreting the Arrhenius plot for cell killing. Obviously, a change in E_a is occurring with increasing temperature, but a gradual bending of the slope rather than a sharp break may be more representative of

* Johnson et al propose that the net rate of the process under consideration can be accounted for on the basis of three reactions involving the same enzyme: 1) the catalytic reaction itself, where there must be a certain energy E_a for the reaction to occur; 2) the reversible inactivation of the enzyme and finally, 3) the irreversible inactivation of the molecule.⁴

physiological actions. The calculated E_a may not be due to one rate limiting reaction, and the E_a for the same reaction may vary from system to system.

B. Model for Heat Inactivation

It is apparent that changes in the activation energy for heat killing of 9L gliosarcoma cells occur with increasing temperature. Part of this change in E_a could be related to the contribution of thermotolerance at the lower temperatures.* In specific, we suggest that cell killing in the 42°C to 45°C temperature range can be represented by the following reaction scheme:



k_1 represents the development of thermotolerance, or the rate at which cells go from the 37°C base (B) state to the "protected" (P)

* In a model recently proposed by Lepock and Kruuv the "break" at 43°C in the Arrhenius plot was attributed to thermotolerance.¹¹⁷ By assuming the induction of thermotolerance with time in the surviving population, and that 43°C was the maximum temperature at which tolerance can be induced the authors generated model survival curves which predicted the "43°C" break. Although similar in concept to the model proposed here, actual rate constants were not derived by Lepock and Kruuv and the model survival curves predicted survival values up to three orders of magnitude less than experimentally determined curves.

state. The back reaction of P to B is given by k_2 . The rate of killing P cells is k_3 , and the rate of killing B cells is k_4 . The back reactions from the nonviable "N" state to either B or P is taken as negligible. At low temperatures ($<43^\circ\text{C}$) cell killing would occur primarily through k_3 pathways while at higher temperatures ($>43^\circ\text{C}$) k_4 would dominate. If k_3 is characterized by a higher activation energy and thus a slower reaction rate as compared to k_4 significant differences in cell killing rates at different temperatures would be expected. The relationship between the various reaction rates (k_1 through k_4) at any one temperature would determine the net inactivation for cell killing that is measured experimentally. The rate equations for the proposed reaction scheme are as follows:

$$\frac{dB}{dt} = -(k_1 + k_4) B + k_2 P \quad (2)$$

$$\frac{dP}{dt} = -(k_2 + k_3) P + k_1 B \quad (3)$$

$$\frac{dN}{dt} = k_4 B + k_3 P \quad (4)$$

The integrated rate equations for the boundary conditions $B = B_0$,

$P = 0$ and $N = 0$ at $t = 0$ are

$$B = \frac{(k_1 + k_4 + m_2)(B_0)}{m_2 - m_1} e^{m_1 t} - \frac{(k_1 + k_4 + m_1)(B_0)}{m_2 - m_1} e^{m_2 t} \quad (6)$$

$$P = \frac{(k_1 + k_4 + m_1)(k_1 + k_4 + m_2)(B_0)}{k_2(m_2 - m_1)} \left(e^{m_1 t} - e^{m_2 t} \right) \quad (7)$$

where

$$m_1 = \frac{1}{2} \left\{ -(k_1+k_2+k_3+k_4) - [(k_1+k_2+k_3+k_4)^2 - 4(k_2k_4+k_1k_3+k_3k_4)]^{1/2} \right\}$$

$$m_2 = \frac{1}{2} \left\{ -(k_1+k_2+k_3+k_4) + [(k_1+k_2+k_3+k_4)^2 - 4(k_2k_4+k_1k_3+k_3k_4)]^{1/2} \right\}^*$$

The surviving fraction is given by $B+P/B_0$ or

$$S = \frac{(k_1+k_4+m_2)}{(m_2-m_1)} e^{m_1 t} - \frac{(k_1+k_4+m_1)}{(m_2-m_1)} e^{m_2 t} +$$

$$\frac{(k_1+k_4+m_1)(k_1+k_4+m_2)}{k_2(m_2-m_1)} \left(e^{m_1 t} - e^{m_2 t} \right) \quad (8)$$

By using kinetic data from the heat inactivation studies, the sub-lethal studies and best fit approximations in keeping with various boundary conditions, values for k_1 , k_2 , k_3 and k_4 can be estimated for temperatures in the 42°C to 45°C range. Thus, it is possible to test the model to a first approximation by comparing final slopes of the model survival curves to the slopes of the exponential portion of the experimentally determined heat inactivation curves.

1. Determination of k_4

The value for k_4 at various temperatures was determined from an Arrhenius extrapolation of killing rates at 44°C and 45°C. At these temperatures it is assumed that k_4 predominates and that very few cells enter the protected state. This assumption is supported by the

*See Appendix A for derivation

observation that fractionated 45°C exposures require a significant 37°C interval between heat treatments for tolerance to develop.^{51,52,54} Because of the 37°C interval requirement, Li and Hahn have suggested that a thermotolerance free period is created.¹¹⁷ Step-down heating experiments by Henle also suggest that a tolerance free period is created with 45°C conditioning treatments.¹¹⁸ They found that cells preheated at 45°C had a much faster rate of cell killing at low temperatures (i.e., below 42°C) than cells which did not receive the conditioning treatment.¹¹⁸ Possibly the cells which had the 45°C exposure were not capable of becoming tolerant at the lower temperature and as a result were killed much more rapidly.

For 9L gliosarcoma cells the k_4 activation energy is calculated from the slope between the 44°C and 45°C points in the Arrhenius plot shown in Figure 16A. A value of 104 kcal was obtained.* The rate of exponential cell killing at 45°C equals 17 min. which we assume to be primarily by the k_4 path. The values of k_4 at other temperatures can be determined from the integrated Arrhenius equation:

$$\ln \frac{k_4(T_2)}{k_4(T_1)} = \frac{E_a}{R} \frac{(T_2 - T_1)}{T_1 T_2} \quad (9)$$

Table 1 gives the values of k_4 at temperatures of interest in the 42°C to 45°C range.

* The plot of $\ln k$ versus $1/T$ gives a line with a slope of E_a/R .

Table 1.

T°C	T°K	k ₄ (hr ⁻¹)	k ₄ (min ⁻¹)
42	315	2.11	.035
42.5	315.5	2.75	.046
43	316	3.57	.06
44	317	5.98	.10
45	318	10.08	.17

We assume linear Arrhenius kinetics in this temperature range only.

At temperatures below 41°C k₄ equals 0.

2. Determination of k₃

At 43°C the rate of killing a maximally protected population (k₃) was found to be .27 hr⁻¹.^{*} By definition protected cells are killed more slowly than control cells, and consequently, the activation energy of k₃ should be greater than the activation energy of k₄. Since both pathways (k₃ and k₄) represent cell inactivation by heat, the activation energies should also be similar. For purposes of developing the model a value of 200 kcal was given to the k₃ activation energy.^{**} Table 2 shows the k₃ values for 42 through 45°C as determined from the integrated Arrhenius rate equation (9) using k₃ at 43°C (.27 hr⁻¹), and Ea equals 200 kcal.

* Data not shown

** Other investigators have shown that maximum T.T. at 45°C decreased the slope approximately 5 fold.^{51,52} Using this estimation for k₃ at 45°C, Ea is about 200 kcal.

Table 2

T°C	T°K	k ₃ (hr ⁻¹)	k ₃ (min ⁻¹)
42	315	.098	.0016
42.5	315.5	.163	.0027
43	316	.27	.0045
44	317	.737	.0123
45	318	2.0	.0333

Again we assume Arrhenius relations only in the 42°C to 45°C range, and k₃ approaches 0 at temperatures less than 41°C.

3. Determination of k₂

In this section we do not assume strict Arrhenius kinetics for the induction of protection. In keeping with observed effects of increasing temperature on biological reactions we predict that the reaction $B \rightleftharpoons P$ increases, reaches a biological optima and then decreases with increasing temperature. Or as Johnson stated "implicit in the familiar fact that at either the hot or cold limits of a relatively narrow range of temperatures, all biological processes practically cease is the consequence that some intermediate range of temperature must be the most favorable."⁴

Although the net reaction ($B \rightleftharpoons P$) will have an optimum and hence varying activation energies with temperature, we will assume Arrhenius kinetics for the back reaction k₂.*

*By fixing k₂, we can use best fit approximation for k₁.

Values for k_2 at 37°C and 40°C are experimentally available. At 37°C it was observed that thermotolerance decayed at the rate of .046 per hour which is equivalent to a k_2 at 37°C of .046 hr⁻¹. At 40°C the value of k_2 can be derived from setting up two simultaneous equations from experimental data and solving for k_1 and k_2 .

First, however, the fraction of protected cells following a 1 hour at 40°C and also a two hour at 40°C conditioning treatment must be determined. Following a 40°C pretreatment the total rate of killing at 43°C (R_t) is equal to the rate of killing protected cells (R_p) times the fraction protected (F_p) plus the rate of killing without conditioning (R_B) times the unprotected fraction (F_B).

$$R_T = R_p(F_p) + R_B(F_B) \quad (10)$$

$$F_p + F_B = 1 \quad (11)$$

$$R_T = R_p(F_p) + R_B(1-F_p) \quad (12)$$

At 43°C R_p equals .27 hr⁻¹, and R_B equals 2.38 hr⁻¹. Following a 1 hour conditioning at 40°C R_T is 1.03 hr⁻¹ which corresponds to a .64 F_p by equation 12. A two hour 40°C conditioning has an R_T of .7 or an F_p of .8. These values can now be used to determine k_1 and k_2 at 40°C.

At 40°C k_3 and k_4 equal 0 (i.e., no cell killing), and the entire reaction is given by a simple first order reversible reaction:



The rate equations are given by:

$$\frac{dB}{dt} = k_1 B + k_2 P \quad (14)$$

$$\frac{dP}{dt} = k_1 B - k_2 P \quad (15)$$

The integrated rate equation for B at $t = 0$, $B = 1$ and $P = 0$ is

$$B = \frac{k_2}{k_1 + k_2} + \left(\frac{k_1}{k_1 + k_2} \right) e^{(-k_1 - k_2)t} \quad (16)$$

at $t = 1$ hour, $P = .64$ and $B = .36$

$t = 2$ hour, $P = .80$ and $B = .20$

(from equation 12).

By substitution into equation 16:

$$.36 = \frac{k_2}{k_1 + k_2} + \left(\frac{k_1}{k_1 + k_2} \right) e^{(-k_1 - k_2)^1} \quad (17)$$

or

$$.36 - .64 \frac{k_2}{k_1} = e^{(-k_1 - k_2)} \quad (18)$$

* See Appendix B for derivation.

and at $t = 2$

$$.2 = \frac{k_2}{k_1 + k_2} + \left(\frac{k_1}{k_1 + k_2} \right) e^{(-k_1 - k_2)2} \quad (19)$$

or

$$.2 - .8 \frac{k_2}{k_1} = e^{(-k_1 - k_2)2} \quad (20)$$

substituting 18 into 20

$$.2 - .8 \frac{k_2}{k_1} = \left(.36 - .64 \frac{k_2}{k_1} \right)^2 \quad (21)$$

Rearranging

$$0 = -.07 + .34 \frac{k_2}{k_1} + .41 \left(\frac{k_2}{k_1} \right)^2 \quad (22)$$

Solving for the positive root

$$\frac{k_2}{k_1} = .17 \quad (23)$$

$$k_2 = .17 k_1 \quad (23)$$

By substituting for k_2 in equation 18 we find

$$.36 - .64 (.17) = e^{(-k_1 - .17k_1)} \quad (24)$$

$$\begin{aligned} k_1 &= 1.18 \\ k_2 &= .2 \end{aligned} \quad (25)$$

By substituting k_2 equals $.046 \text{ hr}^{-1}$ at 37°C and $.2 \text{ hr}^{-1}$ at 40°C into equation 9, E_a is found to be 94,450 cal. Table 3 gives the rest of the k_2 values at different temperatures using this activation energy.

Table 3

$T^\circ\text{C}$	$T^\circ\text{K}$	$k_2(\text{hr}^{-1})$
37	310	.046
40	313	.2
42	315	.525
42.5	315.5	.666
43	316	.846
44	317	1.36
45	318	2.18

4. Determination of k_1 and Model Survival Slopes

Using a best fit (to the final slope of the survival curve) approach we have estimated k_1 values in the 42°C to 45°C temperature range. These values are shown in Table 4 along with the previously derived rate constants.

Table 4

$^\circ\text{C}$	$k_1(\text{hr}^{-1})$	$k_2(\text{hr}^{-1})$	$k_3(\text{hr}^{-1})$	$k_4(\text{hr}^{-1})$
42	9	.525	.098	2.11
42.5	.5	.666	.163	2.75
43	.04	.846	.27	3.57
44	<< .04	1.36	.737	5.98
45	<< .04	2.18	2.0	10.08

The slopes for the survival equation (8) using these values for k_1 through k_4 are shown in Table 5. (Time points to generate the model survival curves were chosen in keeping with experimental protocol.)

Table 5

T°C	k(hr ⁻¹)	k(min ⁻¹)	ln K.
42	.19	.00361	-5.75
42.5	.857	.0143	-4.25
43	2.24	.0373	-3.28
44	5.98	.01	-2.3
45	10.08	.168	-1.78

Figure 16 shows that the Arrhenius plot of the modelled heat inactivation curves closely match the experimentally determined curves. Instead of different mechanisms of cell killing above and below 43°C the cells inherent sensitivity may be altered. The changing activation energy for hyperthermic cell killing can be attributed to shifts in relative importance of individual reactions (based on their own activation energy) at different temperatures. For temperatures below 43°C tolerance is developed to a significant extent resulting in a reduced rate of cell killing. At higher temperatures killing occurs rapidly and the development of tolerance is insignificant. The close fit of the modelled survival curves to experimentally determined curves suggests that the development of thermal tolerance is a sufficient condition to cause a progressive bending of the Arrhenius plot.

APPENDIX A

Derivation of Survival Equation

$$\frac{dB}{dt} = - (k_1 + k_4)B + k_2P \quad (1A)$$

$$\frac{dP}{dt} = - (k_2 + k_3)P + k_1B \quad (2A)$$

Solve for 2 variables, B and P, with 2 simultaneous equations.

Differentiate (2A)

$$\ddot{P} = - (k_2 + k_3)\dot{P} + k_1\dot{B} \quad (3A)$$

Substitute (1A) to eliminate $\dot{B}$ in (3A)

$$\ddot{P} = - (k_2 + k_3)\dot{P} + k_1[-(k_1 + k_4)B + k_2P] \quad (4A)$$

or

$$\ddot{P} = - (k_2 + k_3)\dot{P} + k_1k_2P - (k_1 + k_4)(k_1B) \quad (5A)$$

from (2A)

$$k_1B = \dot{P} + (k_2 + k_3)P \quad (6A)$$

(5A) becomes

$$\ddot{P} = -(k_2+k_3)\dot{P} + k_1k_2P - (k_1+k_4)[\dot{P} + (k_2+k_3)P] \quad (7A)$$

or

$$\ddot{P} = -(k_1+k_2+k_3+k_4)\dot{P} - (k_1k_3+k_2k_4+k_3k_4)P \quad (8A)$$

$$\text{let } K = k_1+k_2+k_3+k_4 \quad (9A)$$

$$K' = k_1k_3+k_2k_4+k_3k_4 \quad (10A)$$

then

$$\ddot{P} = -K\dot{P} - K'P \quad (11A)$$

or

$$\ddot{P} + K\dot{P} + K'P = 0 \quad (12A)$$

$$\text{let } d/dt = D \text{ then} \quad (13A)$$

$$(D^2 + KD + K')P = 0 \quad (14A)$$

Use the quadratic formula to find m_1 and m_2 such that:

$$D^2 + KD + K' = (D - m_1)(D - m_2) \quad (15A)$$

let m_1 and m_2 be the roots of the quadratic

$$m = \frac{-K \pm \sqrt{K^2 - 4K'}}{2} \quad (16A)$$

$$(D - m_1)(D - m_2)P = 0 \quad (17A)$$

The general solution to equation 17A is

$$P(t) = (\text{const}_1)e^{m_1 t} + (\text{const}_2)e^{m_2 t} \quad (18A)$$

The boundary conditions are $P = 0$ when $t = 0$. Therefore,

$$\text{const}_1 = -\text{const}_2 \equiv X \text{ and}$$

$$P(t) = X(e^{m_1 t} - e^{m_2 t}) \quad (19A)$$

Equation (1A) becomes

$$\dot{B} = -(k_1 + k_4)B + k_2 X(e^{m_1 t} - e^{m_2 t}) \quad (20A)$$

$$\text{Multiply by integrating factor } e^{(k_1 + k_4)t} \quad (21A)$$

$$\int_{Be}^{(k_1 + k_4)t} + (k_1 + k_4)Be^{(k_1 + k_4)t} = \int k_2 X e^{(k_1 + k_4)t} (e^{m_1 t} - e^{m_2 t}) \quad (22A)$$

$$Be^{(k_1 + k_4)t} = k_2 X \int e^{(k_1 + k_4)t} (e^{m_1 t} - e^{m_2 t}) dt \quad (23A)$$

$$B e^{(k_1+k_4)t} = k_2 X \left(\frac{e^{(k_1+k_4+m_1)t}}{k_1+k_4+m_1} - \frac{e^{(k_1+k_4+m_2)t}}{k_1+k_4+m_2} \right) \quad (24A)$$

$$B = k_2 X \left(\frac{e^{m_1 t}}{k_1+k_4+m_1} - \frac{e^{m_2 t}}{k_1+k_4+m_2} \right) \quad (25A)$$

$$\text{at } t = 0 \quad B = B_0$$

$$B_0 = k_2 X \left(\frac{1}{k_1+k_4+m_1} - \frac{1}{k_1+k_4+m_2} \right) \quad (26A)$$

$$X = \frac{B_0 (m_1+k_1+k_4)(m_2+k_1+k_4)}{(m_2-m_1)k_2} \quad (27A)$$

$$P(t) = \frac{(k_1+k_4+m_1)(k_1+k_4+m_2)B_0}{(m_2-m_1)k_2} \left(e^{m_1 t} - e^{m_2 t} \right) \quad (28A)$$

$$B(t) = \frac{B_0 (k_1+k_4+m_2)}{m_2-m_1} e^{m_1 t} - \frac{B_0 (k_1+k_4+m_1)}{m_2-m_1} e^{m_2 t} \quad (29A)$$

$$\text{Survival} = \frac{B+P}{B_0} \quad (30A)$$

$$S = \frac{(k_1+k_4+m_2)}{m_2-m_1} e^{m_1 t} - \frac{(k_1+k_4+m_1)}{m_2-m_1} e^{m_2 t} + \frac{(k_1+k_4+m_1)(k_1+k_4+m_2)}{(m_2-m_1)k_2} \left(e^{m_1 t} - e^{m_2 t} \right) \quad (31A)$$

from (16A)

$$m_1 = \frac{1}{2} \left\{ -(k_1+k_2+k_3+k_4) - [(k_1+k_2+k_3+k_4)^2 - 4(k_2k_4+k_1k_3+k_3k_4)]^{1/2} \right\} \quad (32A)$$

$$m_2 = \frac{1}{2} \left\{ (k_1+k_2+k_3+k_4) + [(k_1+k_2+k_3+k_4)^2 - 4(k_2k_4+k_1k_3+k_3k_4)]^{1/2} \right\} \quad (33A)$$

Appendix B

$$\frac{dB}{dt} = -k_1 B + k_2 P \quad (1B)$$

$$B + P = 1 \quad (2B)$$

Substitution and rearrangement gives

$$\frac{dB}{dt} = (k_1 + k_2) B = k_2 \quad (3B)$$

Multiply by integrating factor $e^{(k_1 + k_2)t}$

$$e^{(k_1 + k_2)t} \left[\frac{dB}{dt} + (k_1 + k_2)B \right] = k_2 e^{(k_1 + k_2)t} \quad (4B)$$

$$\left[B e^{(k_1 + k_2)t} \right]' = k_2 \int e^{(k_1 + k_2)t} dt \quad (5B)$$

$$B e^{(k_1 + k_2)t} = \frac{k_2 e^{(k_1 + k_2)t}}{k_1 + k_2} + \text{const.} \quad (6B)$$

$$B = \frac{k_2}{k_1 + k_2} + \text{const} e^{(-k_1 - k_2)t} \quad (7B)$$

at $t = 0$ $B = 1$ therefore

$$\text{const} = 1 - \frac{k_2}{k_1 + k_2} = \frac{k_1}{k_1 + k_2} \quad (8B)$$

$$B = \frac{k_2}{k_1 + k_2} + \frac{k_1}{k_1 + k_2} e^{(-k_1 - k_2)t} \quad (9B)$$

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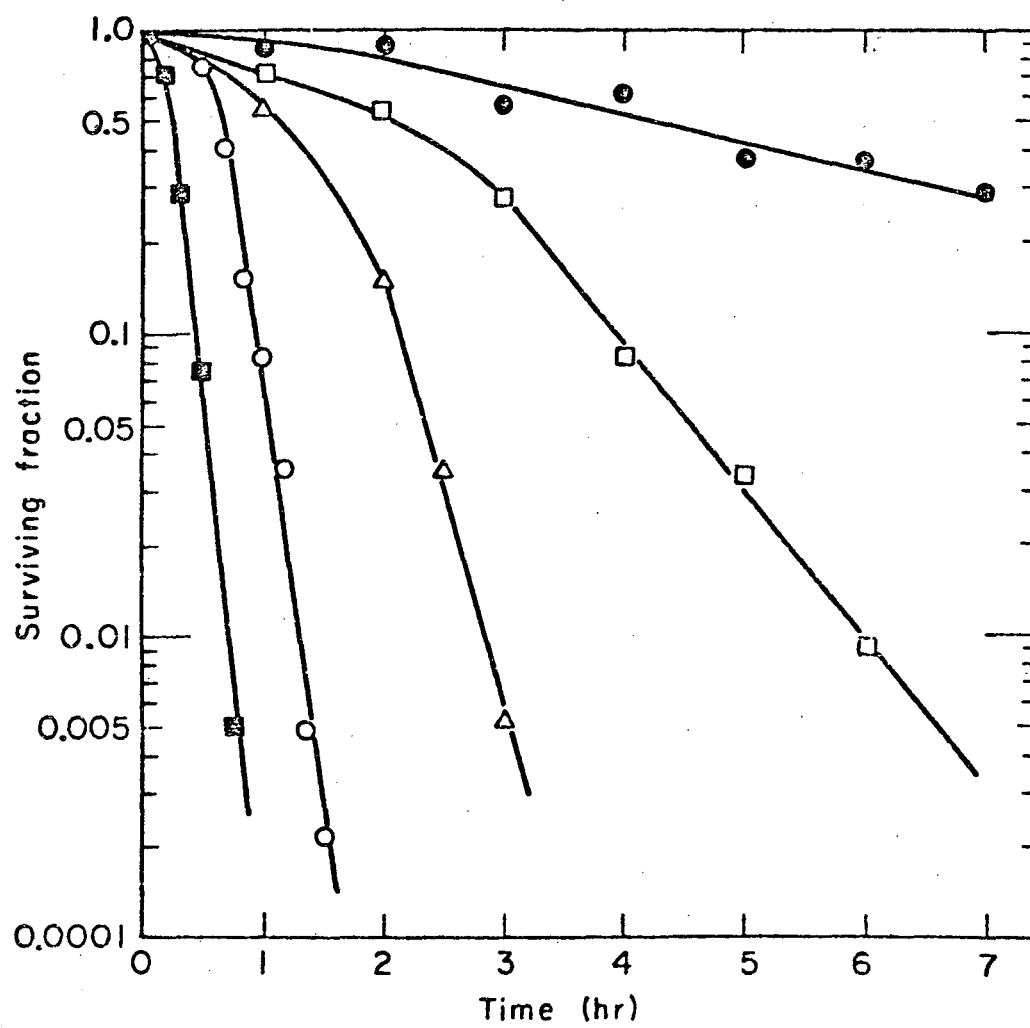
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FIGURES AND LEGENDS

Figure 1. Survival of asynchronous, exponentially growing 9L gliosarcoma cells exposed to different temperatures for varying lengths of time. Exponential portions of the curves are computer fit by the methods of least squares:

Temperature °C	slope (hr ⁻¹) ± 95% Concentrated
● 42°C (1)	.19 ± .01
□ 42.5°C (1)	.89 ± .11
▲ 43°C	2.5 ± .10
○ 44°C (1)	5.98 ± .58
■ 45°C	10.08 ± 1.0

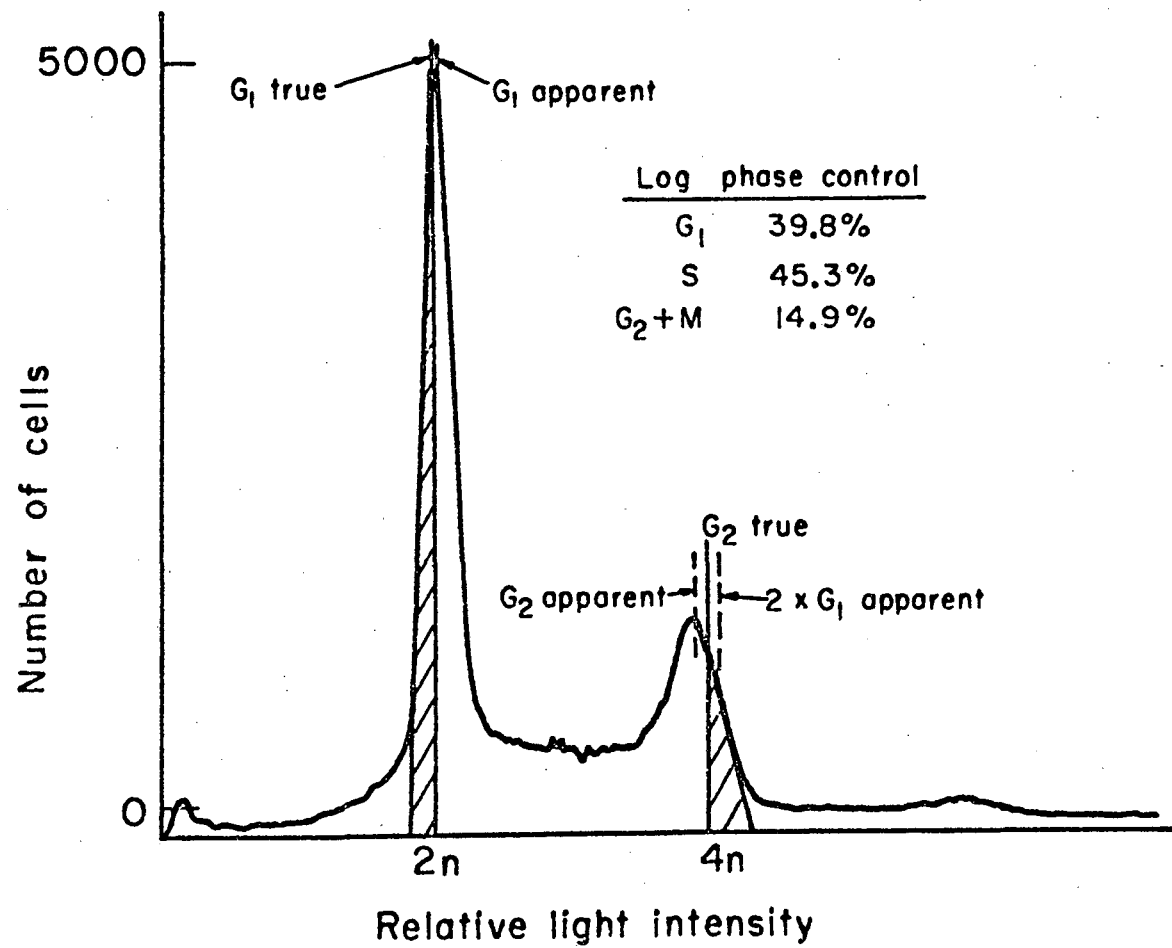
[Ross-Riveros¹ and Hathaway]



XBL8110-4306

Figure 1

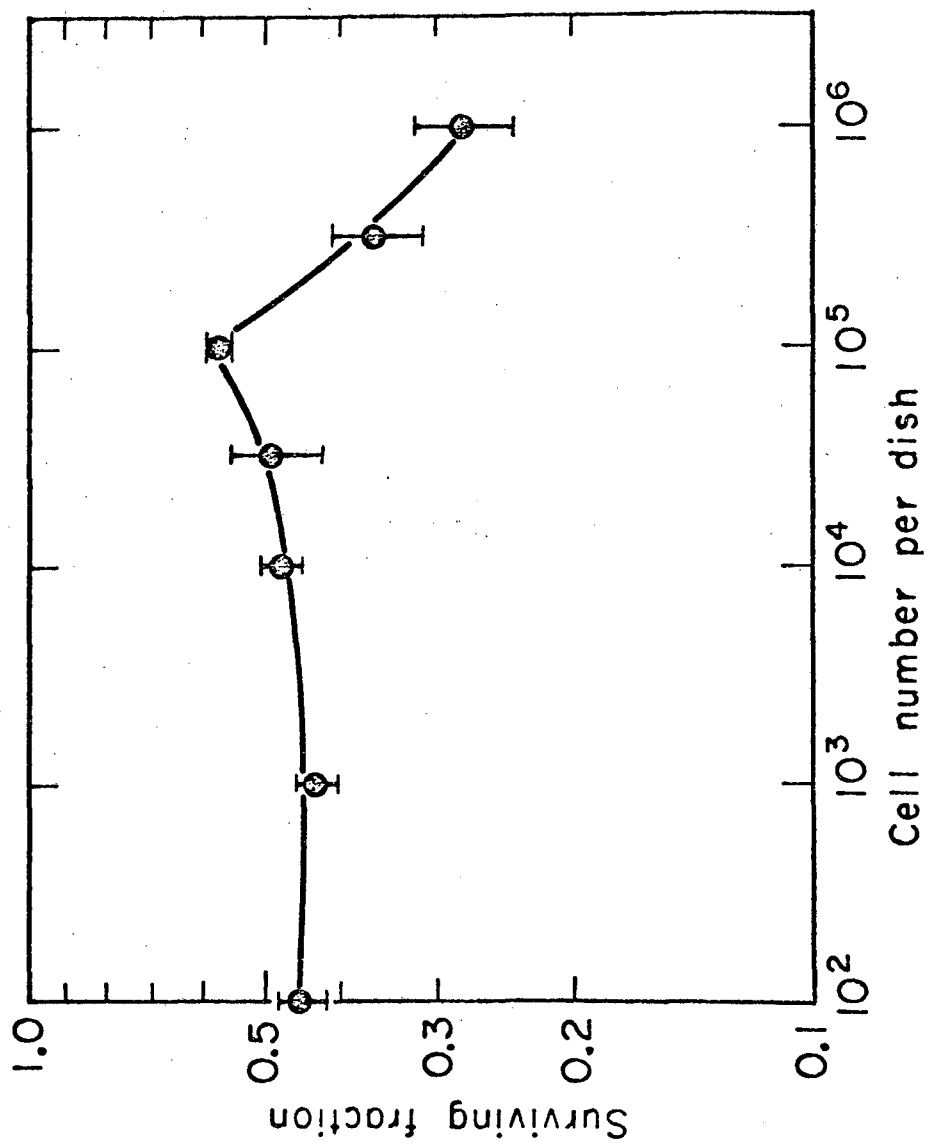
Figure 2. Planimeter analysis of a flow microfluorometry histogram obtained from a population of 9L gliosarcoma cells held at 37°C. The percentage of cells in G_1 and G_2+M are represented by twice the areas of the appropriate right triangles. The percentage of cells in S is determined by subtracting the G_1 and G_2+M populations from the total population. Details are given in the text for placement of the G_1 true and G_2 true peaks.



XBL 8110-4304

Figure 2

Figure 3. Feeder cell effects on the control plating efficiency. As the total number of cells per dish (heavily irradiated feeders plus test cells) increased, the plating increased to a maximum of 56 percent at 10^5 cells per dish. With further addition of feeder cells the plating efficiency rapidly decreased. The error bars represent the standard deviation of all the plates counted per point.

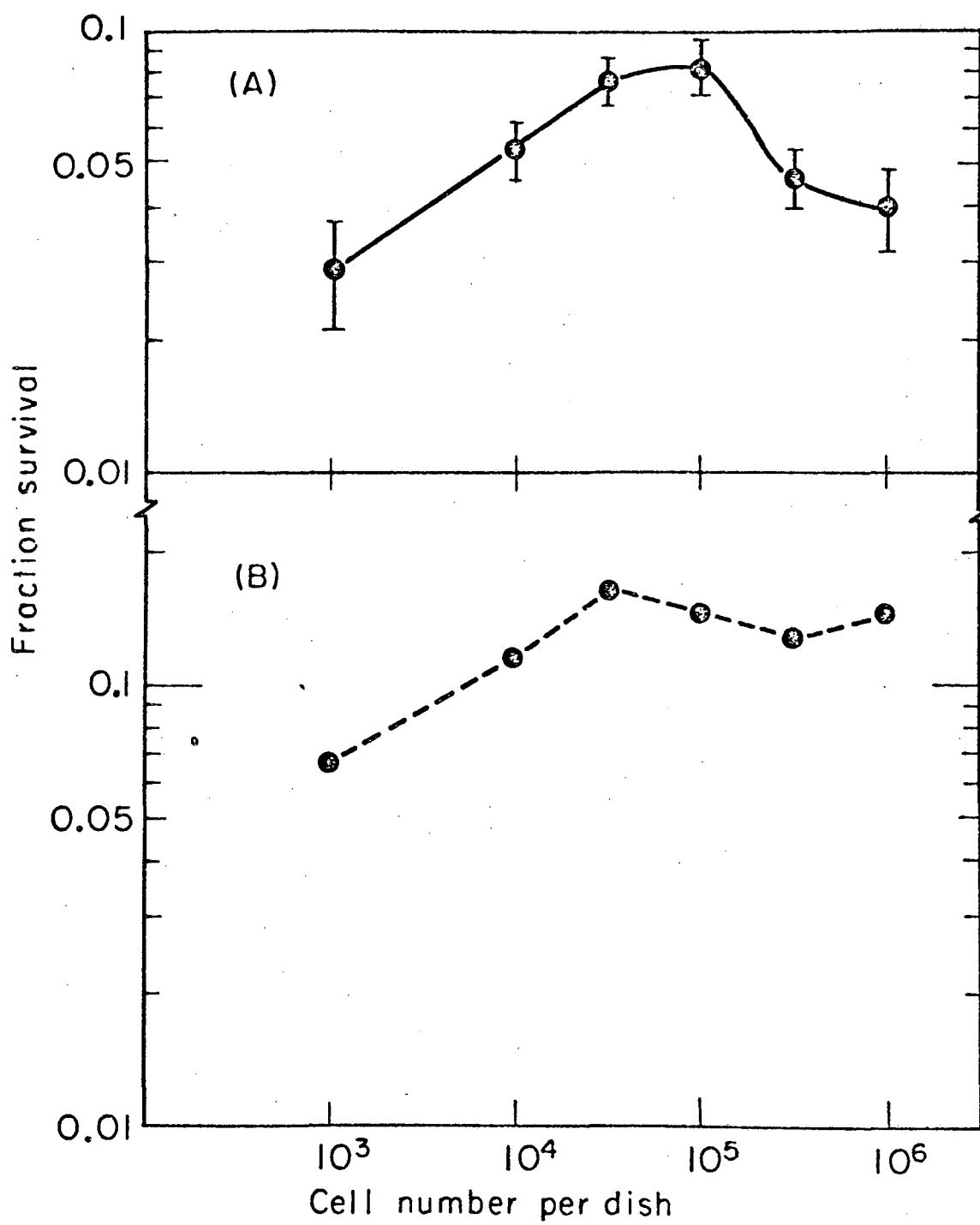


XBL8110-4299

Figure 3

Figure 4A. Feeder cell effects on the surviving fraction of heat stressed cells (43°C for 2 hours). A maximum surviving fraction was observed when the total cell number per dish was in the 10^4 to 10^5 range. The error bars represent the standard deviation of all of the plates counted per point.

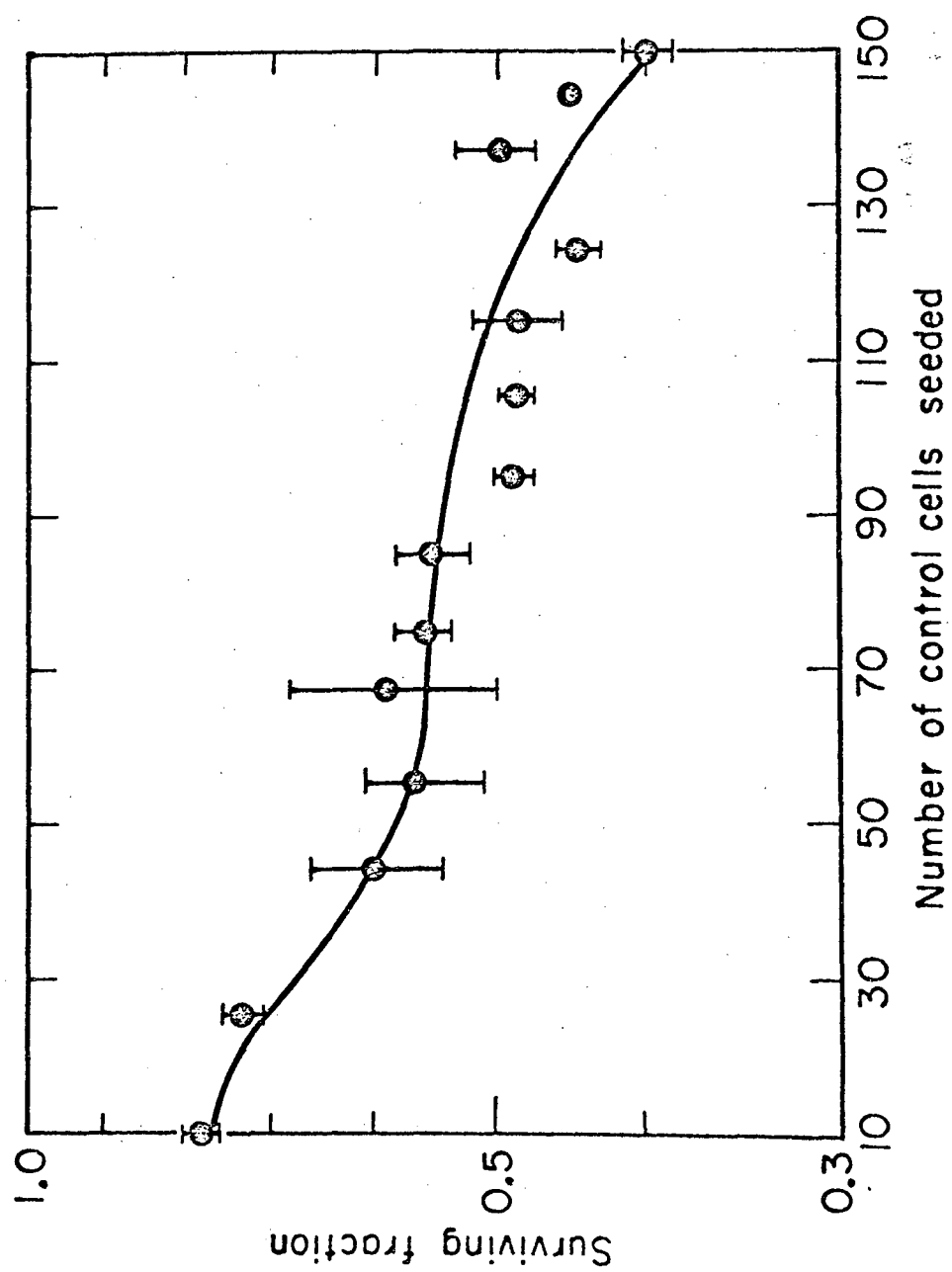
Figure 4B. Normalization of the heat stressed surviving fraction shown in Figure 4A to the corresponding 37°C control plating efficiency illustrated in Figure 3. An increased dependence of heated cells on feeder cell concentrations is apparent at the lower cell densities.



XBL8110-4300

Figure 4

Figure 5. The plating efficiency of control cells versus the number of test cells seeded with a constant feeder cell concentration. In general, as the number of test cells increased, the surviving fraction decreased. The error bars represent the standard error of the mean between groups (5 replicate dishes per group with up to 20 groups per point).



XBL8110-4296

Figure 5

Figure 6. Survival of 9L gliosarcoma cells after varying times at 43°C. A computer program was developed to determine the slope of the exponential portion of the curve by the method of least squares and to also determine values for T_0 , T_q , and n . At 43°C T_0 was 25.0 min. and T_q was 54 min. Error bars represent the standard error.

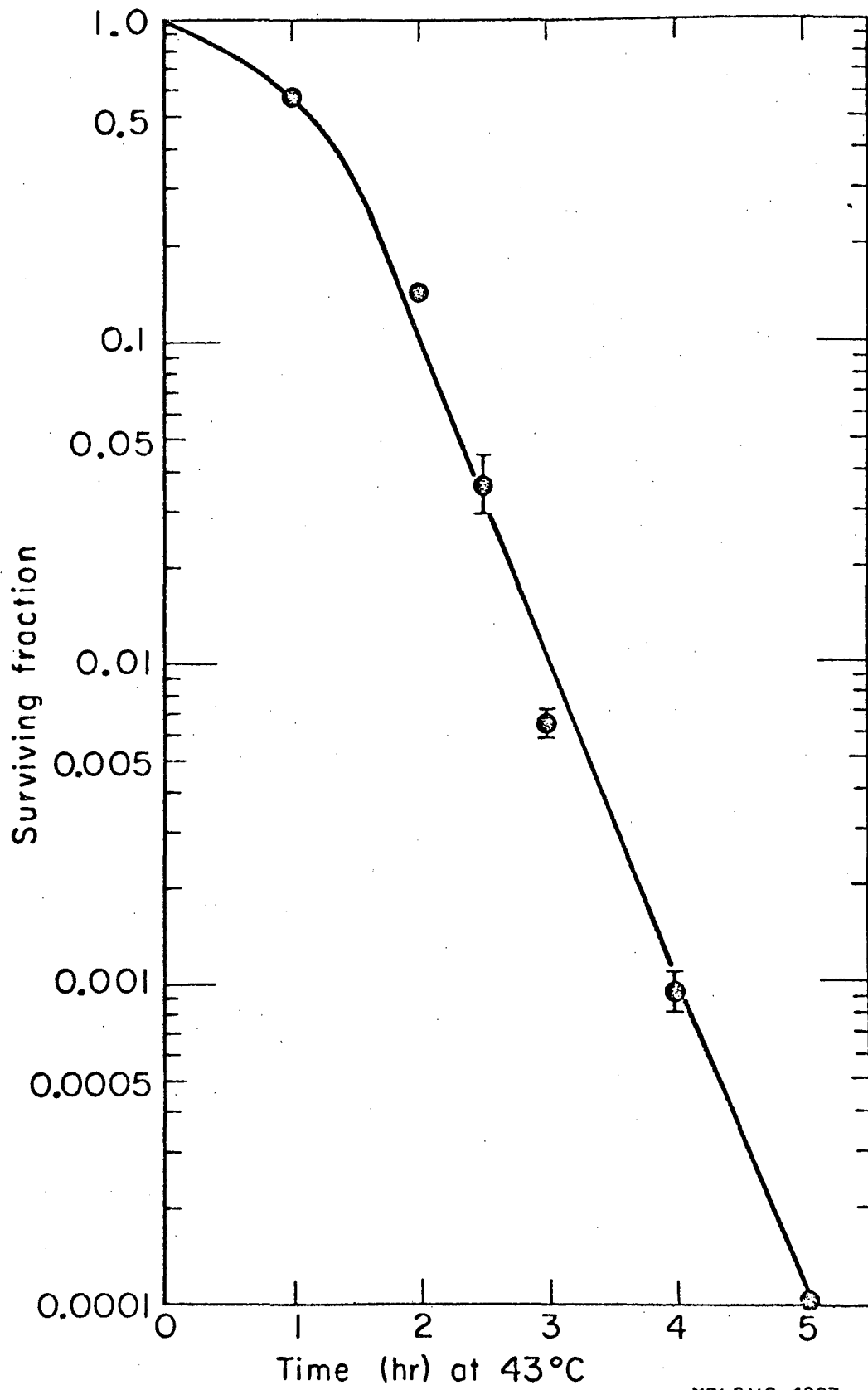
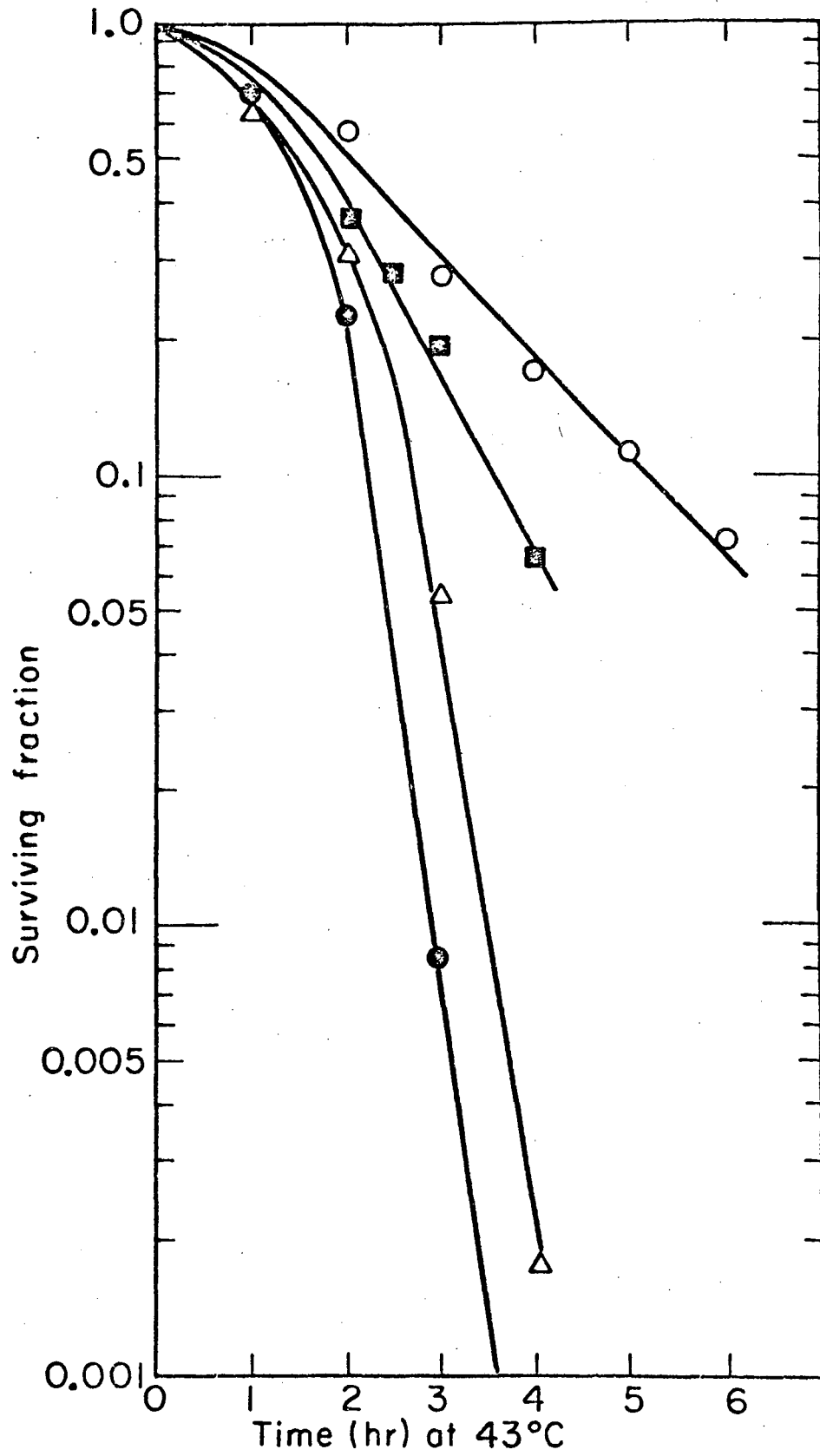


Figure 6

Figure 7. Nonlethal heat pretreatment effect on 43°C survival.

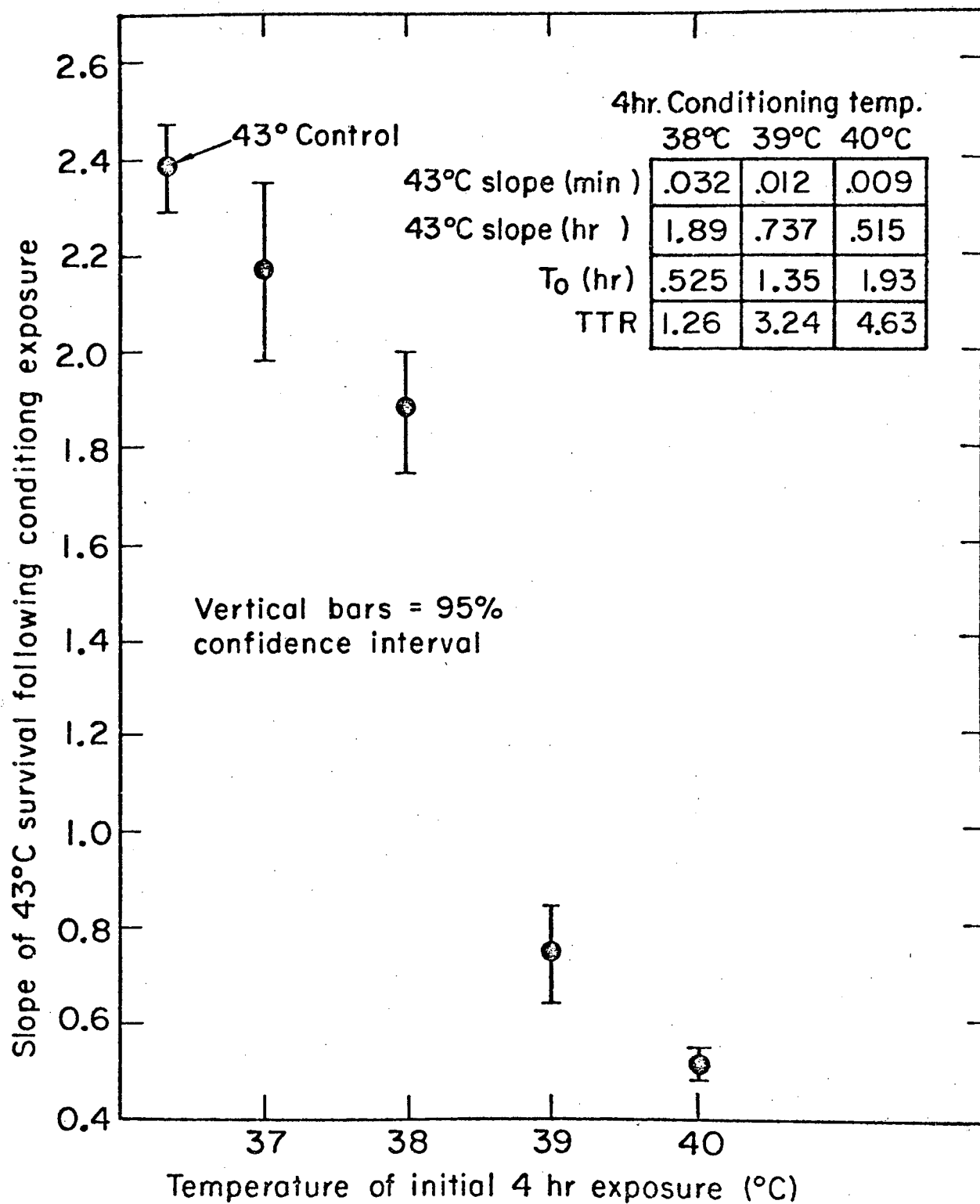
Survival of 9L gliosarcoma cells to 43°C following 4 hour pretreatments at 40°C (○), 39°C (■) and 38°C (Δ). Survival at 43°C for cells held 4 hours in a 37°C water bath is also shown (●). Error bars are omitted for purposes of clarity, but the 95 percent confidence intervals for each of the slopes are shown in Figure 8.



XBL8110-4295

Figure 7

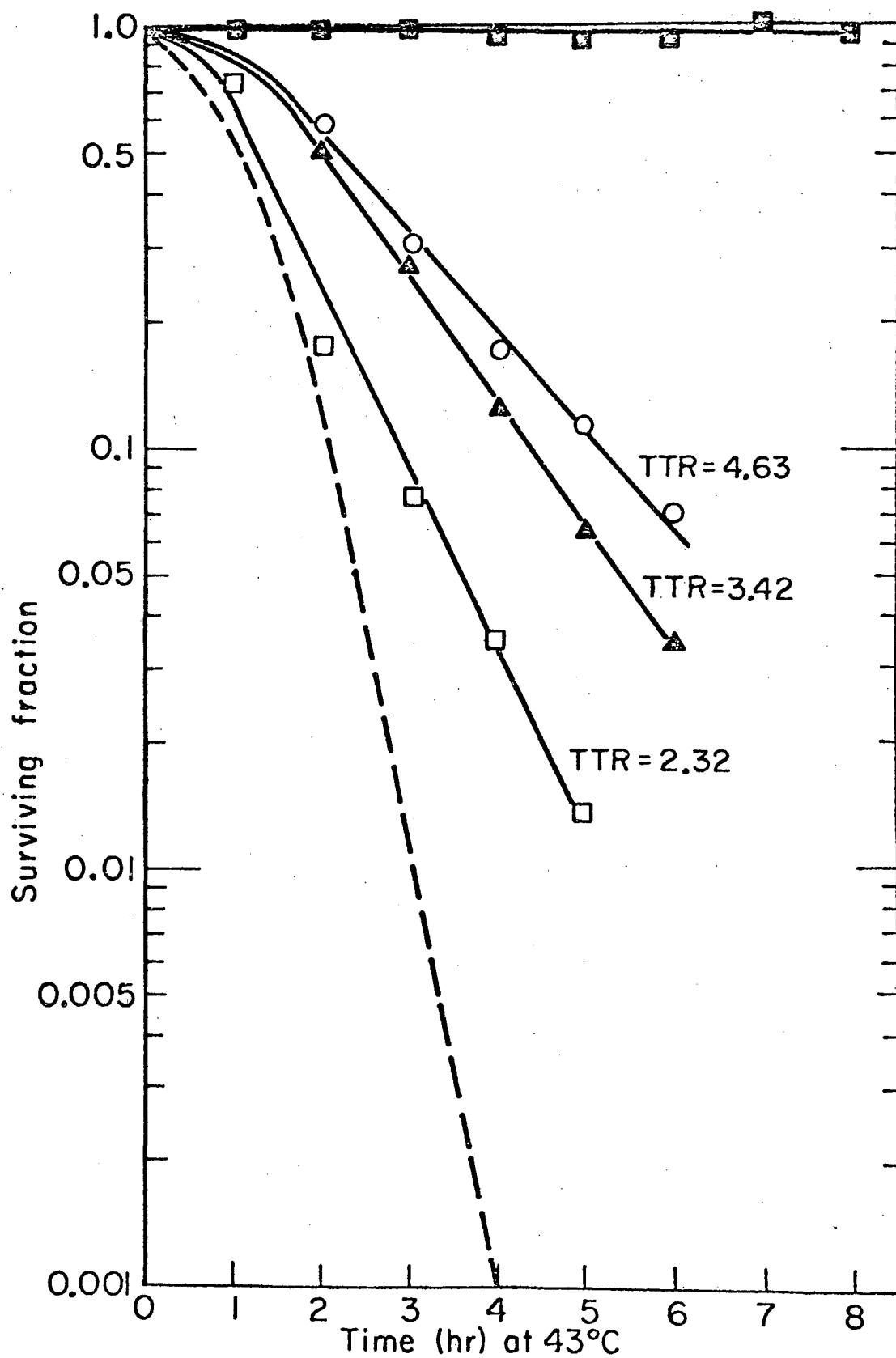
Figure 8. Cell survival slopes at 43°C following 4 hour nonlethal conditioning treatments at 37, 38, 39, and 40°C. The error bars represent the 95 percent confidence interval for the 43°C slopes after pretreatment. Thermotolerance ratios of 1.26, 3.24 and 4.63 were found for 38, 39 and 40°C pretreatments, respectively.



XBL8110-4298

Figure 8

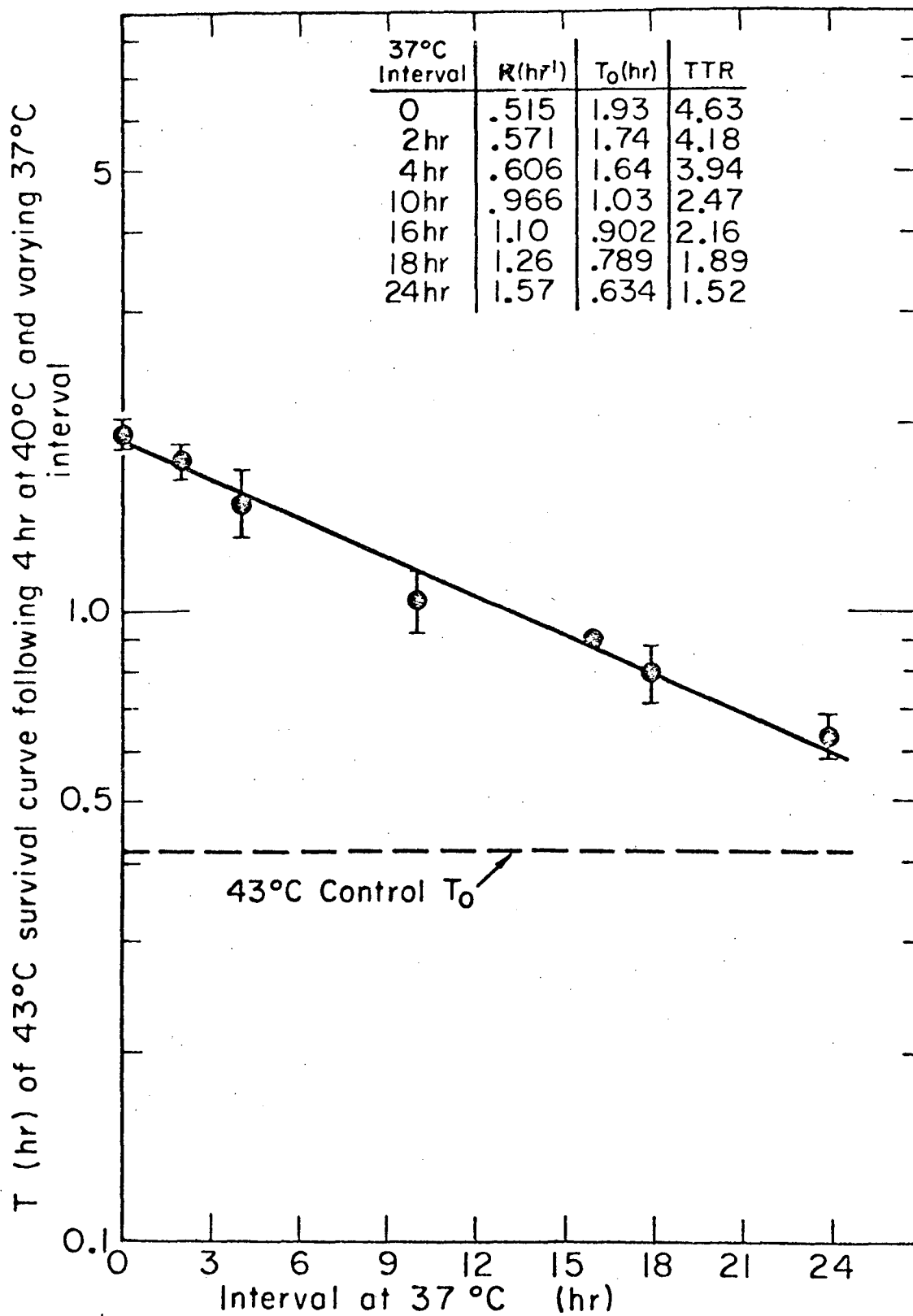
Figure 9. Survival to 43°C following varying times of exposure to 40°C. Pretreatments were 1 hour at 40°C (□), 2 hours at 40°C (▲) and 4 hours at 40°C (○). For comparison the dashed line represents 43°C survival for cells previously held at 37°C. Exposure to 40°C for up to 8 hours does not result in loss of viability as shown by the solid squares (■). The 95 percent confidence intervals for the T_0 's were 58 min. $\pm$ 2.9 min. for 1 hour, 85.4 min. $\pm$ 6.42 for 2 hours and 115.8 $\pm$ 6.6 min. for 4 hour conditioning treatments at 40°C prior to 43°C exposure.



XBL8110-4294

Figure 9

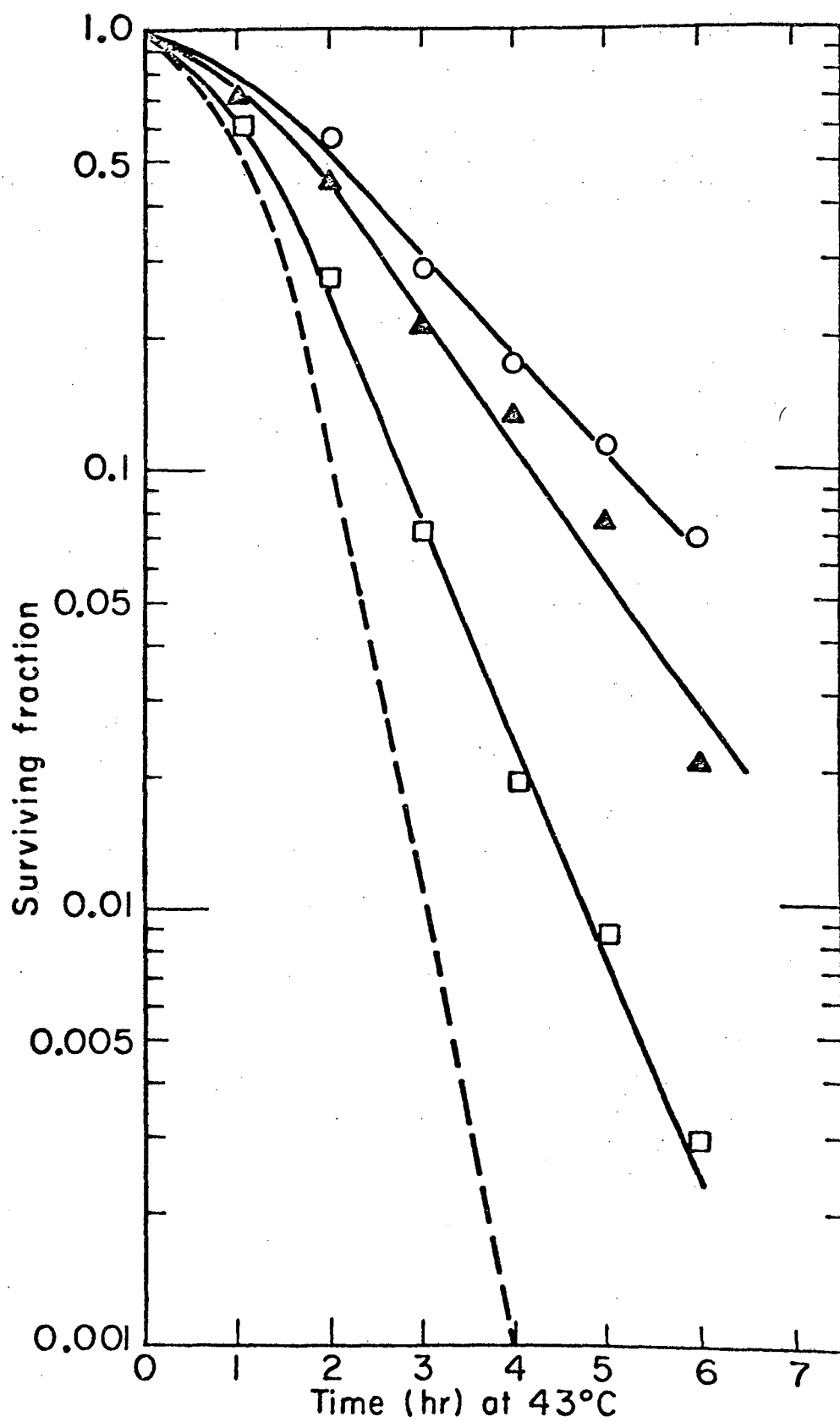
Figure 10. Change in thermal resistance with increasing 37°C interval between 40°C and 43°C exposures. For a 4 hour conditioning exposure at 40°C the T_0 (or inverse of the slope) at 43°C was maximum immediately following the pretreatment (i.e., with 0 interval at 37°C). With increasing 37°C interval between the 40°C pretreatment and graded 43°C exposure the T_0 decreased at the rate of .046 per hour. The T_0 decreased at the rate of .046 per hour. The T_0 for the 43°C exposure without pretreatment is shown by the dashed line. The error bars represent the 95 percent confidence interval from two or more experiments.



XBL8110-4303

Figure 10

Figure 11. Survival at 43°C following 4 hours at 40°C and varying 37°C intervals. All of the survival curves corresponding to the slopes in Figure 10 are not shown for purposes of clarity. Illustrated 43°C curves include pretreatments of 4 hours at 40°C plus 0 interval at 37°C (○), 4 hours at 40°C plus 4 hours at 37°C (▲), and 4 hours at 40°C plus 16 hours at 37°C (□). Survival at 43°C with no pretreatment is indicated by the dashed line. The 95 percent confidence intervals for the inverse of the slopes were shown in the preceeding Figure 10.



XBL 8110-4301

Figure 11

Figure 12. The effect of the 37°C interval on 43°C survival following 1 or 2 hour pretreatments at 40°C. For both 1 and 2 hour conditioning treatments tolerance is lost with increasing 37°C interval prior to 43°C exposure. Although the magnitude of the induced resistance is dependent upon the time at 40°C, the rate of decay of tolerance was similar. The T_0 decayed at the rate of .041 for 1 hour conditioning treatment at 40°C and at the rate of .047 for a 2 hour pretreatment. These values are in close agreement with the T_0 decay rate of .046 per hour found for a 4 hour conditioning exposure at 40°C (Figure 11).

1 Hour at 40°C → 37°C Interval → 43°C

37°C interval (hr)	k/hr	T ₀ (hr)	TTR
0	1.03	.9672	2.32
3	1.29	.7704	1.85
7	1.57	.6322	1.52
16	2.05	.4859	1.17

Rate of change
of T₀ = .041/hr

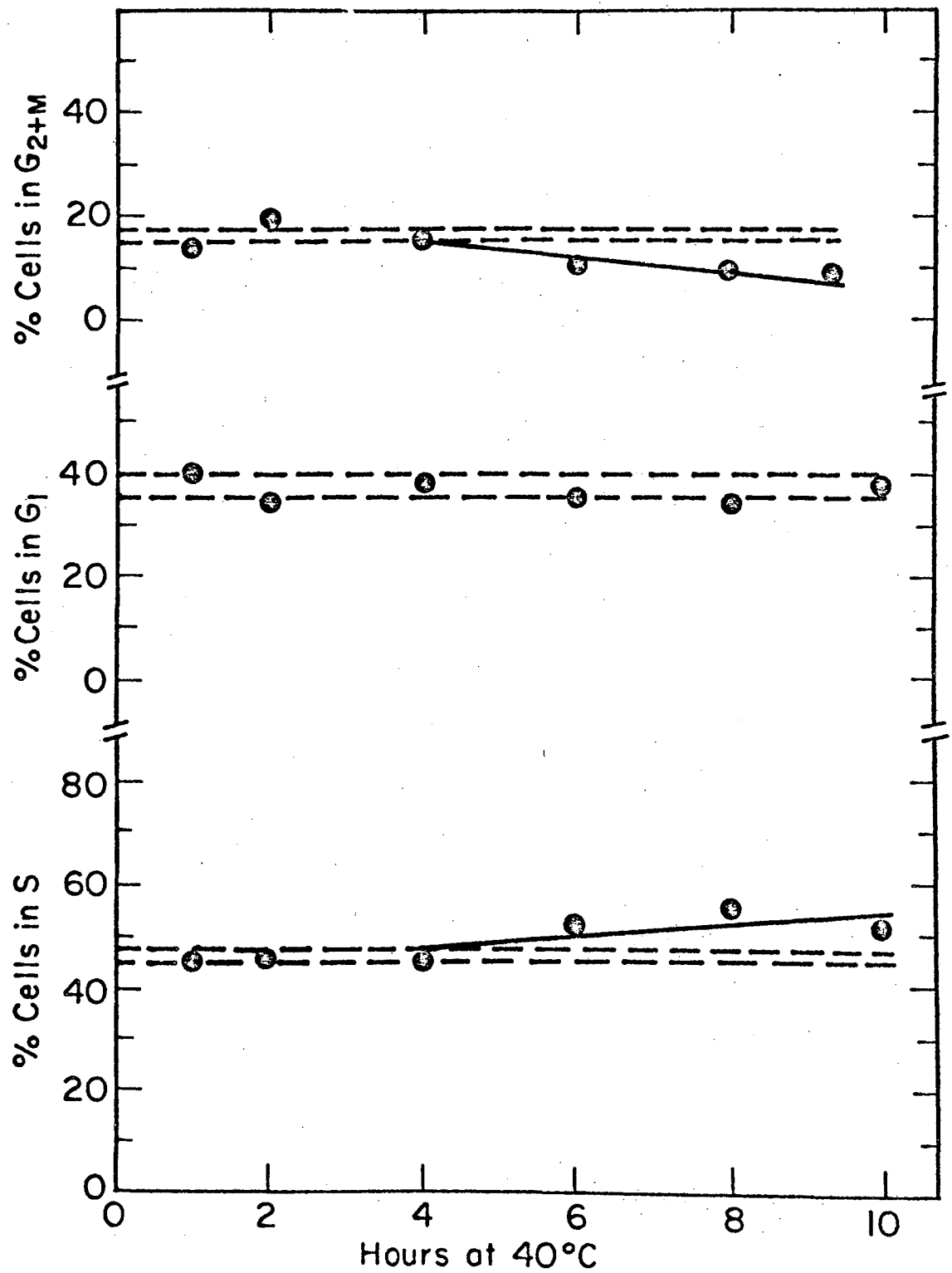
2 Hours at 40°C → 37°C Interval → 43°C

37°C interval (hr)	k/hr	T ₀ (hr)	TTR
0	.698	1.423	3.41
2	.75	1.33	3.19
4	.844	1.178	2.83

Rate of change
of T₀ = .047/hr

XBL 8110-4305

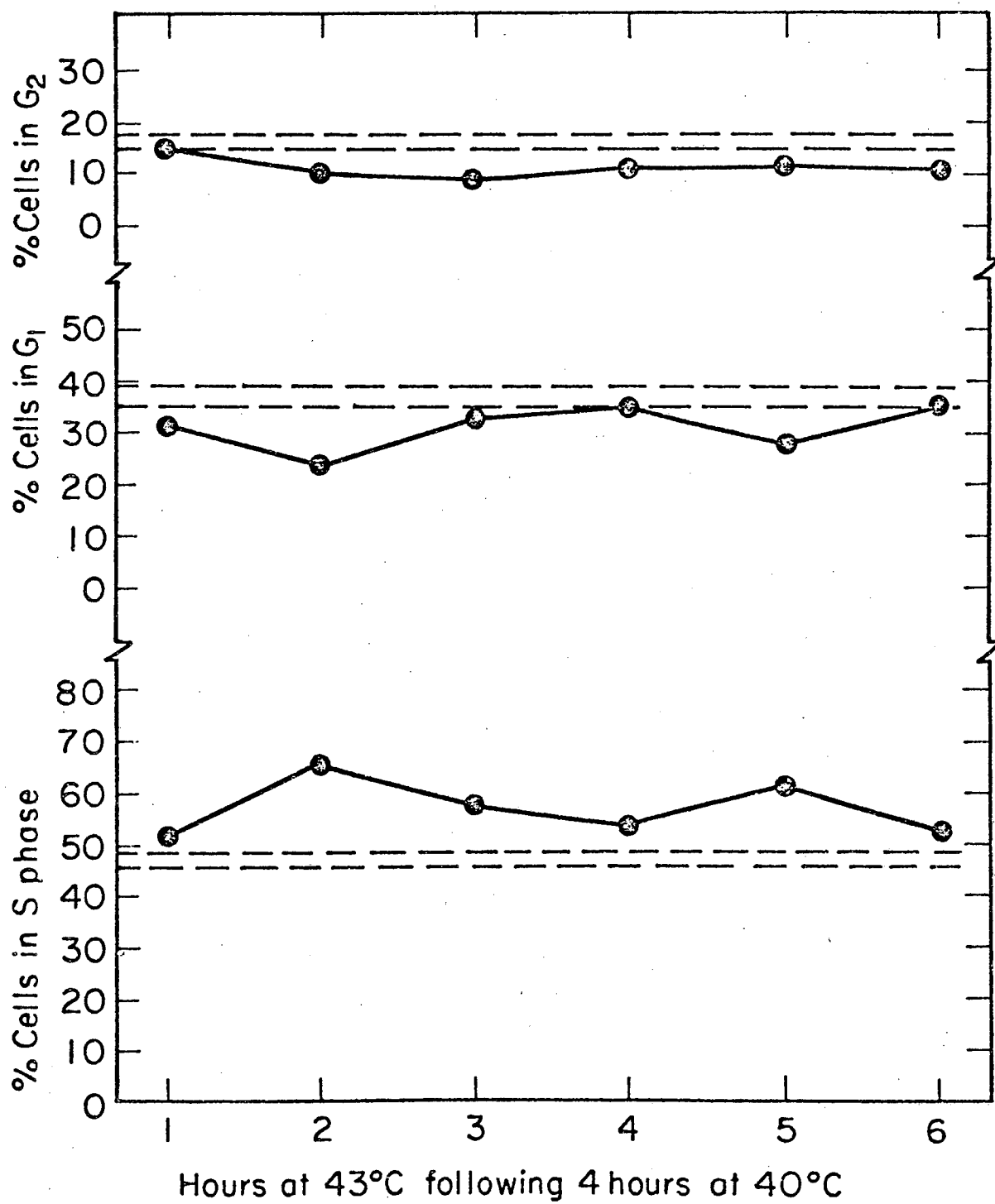
Figure 13. Planimeter analysis of flow microfluorometry histograms obtained from 9L gliosarcoma cells continuously exposed to 40°C. The dashed lines represent two 37°C control values for G_2^+M , G_1 and S, respectively. For the first 4 hours treated populations have identical cell cycle distributions as compared to the controls. Therefore, redistribution into a more heat resistant portion of the cell cycle during the conditioning treatment does not account for thermo-tolerance. After 4 hours at 40°C a slight decrease in G_2^+M and a corresponding increase in S is apparent. Pretreatments of this duration were not used.



XBL8110-4302

Figure 13

Figure 14. Planimeter analysis of flow microfluorometry histograms obtained from 9L gliosarcoma cells exposed to 40°C for 4 hours followed by graded doses at 43°C. The dashed lines represent the cell cycle distribution of two 37°C control values for G₂+M, G₁, and S, respectively. Following 4 hours at 40°C there is no change in cell cycle distribution (Figure 13). However, additional exposure to 43°C results in a slight increase in S phase cells and a corresponding decrease in G₁ and G₂+M cells.



XBL822-3619

Figure 14

Figure 15. The effect of cycloheximide on thermotolerance as measured at 43°C. Four conditions were compared: 1) no pretreatment; 2) 4 hours at 40°C prior to 43°C exposure; 3) exposure to 5 µg/ml of cycloheximide during the 4 hour at 40°C pretreatment; and 4) pretreatment for 4 hours with 5 µg/ml of cycloheximide. Exposure to the protein inhibitor during the nonlethal conditioning did not interfere with the development of thermotolerance. Furthermore, cycloheximide treatment alone (no conditioning heat treatment) conferred considerable protection to subsequent 43°C heat challenge.

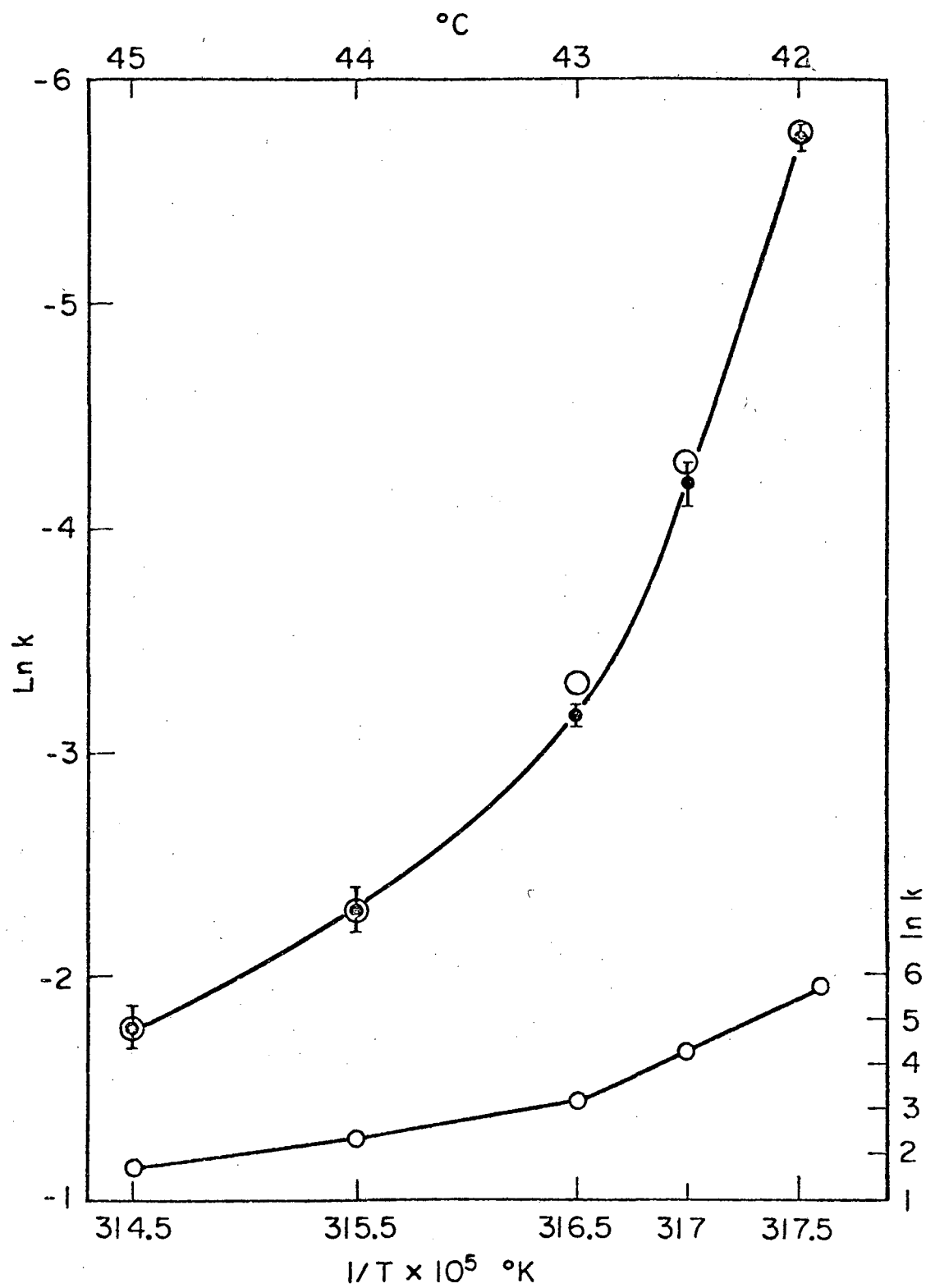
43° C SURVIVAL (%)

Pretreatment	2 hrs	3 hrs	5 hrs
0	15.9	.73	.01
4 hrs at 40° C	56.6	29.3	5.67
Heat+Cycloheximide	53.6	30.9	12.6

Pretreatment	2 hrs	3 hrs
0	15.9	.73
5 ug/ml Cycloheximide	61.2	21.9

Figure 15

Figure 16. Arrhenius plot of heat inactivation of 9L gliosarcoma cells in the 42°C to 45°C range. The bottom plot (○) shows a linear relationship in E_a with a "break" at 43°C when the units on the ordinate (right hand scale) are relatively close together. When $\ln K$ versus $1/T$ is plotted on an expanded scale as in the top curve (●), a gradual bending with a continuous variation in E_a is observed. The error bars represent the 95 percent confidence intervals for $\ln K$. Also shown with the top curve are the values of $\ln K$ derived from the mathematical model (○). These values are in close agreement with the experimentally obtained results.



XBL 822-3620

Figure 16

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