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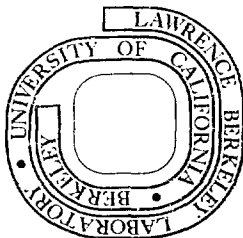
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Pepi Ross-Riveros
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Contents

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KINETICS OF RAT GLIOSARCOMA CELLS

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ABSTRACT

9L rat gliosarcoma cells were examined _in vitro_ for survival response to hyperthermic temperatures ranging from 39.0° to 45.0°C for graded exposure times. At 43.0°C, the split exposure response was also studied. The ability of a single cell to form a colony or to exclude trypan blue viability stain was used as an endpoint for survival.

Changes in cell cycle kinetics resulting from hyperthermia were compared for isosurvival levels achieved by appropriate exposure time to either 42.5°C or 43.0°C. After heat treatment, cells were held at 37.0°C for varying recovery periods. Cells were then either prepared for flow microfluorometry (FMF), or exposed to tritiated thymidine (^{3}HTdR) and prepared for autoradiography.

The survival studies indicated that the rate of change in cell killing for each increasing degree centigrade was greater for temperatures below 43.0°C than for temperatures above 43.0°C. An Arrhenius plot of the mortality rate vs. the inverse of the absolute temperature reveals inactivation energies of 509 kcal for temperatures below 43.0°C and 109 kcal for temperatures above 43.0°C. The shoulder width of the survival curves is maximal at 42.5°C. Although no specific biological function has yet been associated with the shoulder region of a hyperthermic survival curve, the shoulder width does represent an important parameter

since it describes a threshold time after which significant cell killing occurs. Thus both 43.0°C, the temperature at which mortality kinetics change, and 42.5°C, the temperature at which the shoulder width is maximum, represent critical temperatures for the 9L cells.

Cells remained attached to the growing surface of the tissue culture flask throughout the treatment time necessary to establish survival curves. However, prolonged treatment at temperatures of 43.0°C and higher causes cells to detach during treatment at a rate which corresponds to percent mortality as indicated by a trypan blue vital stain.

When 9L cells are given an *initial conditioning exposure* to 43.0°C, then *returned* to 37°C for 3 hours, followed by graded exposure intervals at 43.0°, the resulting survival curve indicates that cells require longer time for equal cell killing than for the single exposure condition, suggesting that the cells possess a capability to adapt to the higher temperature. In the exponential region, as the initial exposure is increased (resulting in lower surviving fraction) the thermal tolerance increases. For initial exposures resulting in survivals of 25%, 5%, and 1%, the thermal tolerance ratio was 2.3, 3.3, and 3.8 respectively. These values match those reported by other researchers using various cell lines for equivalent intitial survival levels.

Kinetic response using FMF, autoradiographic measures of labeling index (LI) and pulse labeled mitoses (PLM), and population growth parameters all indicated that cells experience two types of blocks in cell progression through the cell cycle immediately following hyperthemic treatment. First of all, cells appear to be blocked wherever they

may be in the cell cycle, resulting in a period of time which we have termed a period of "general arrest". Second, there appears to be a block in S which lasts longer than the general arrest. Thus when cells are released from the general arrest, they progress through the cell cycle only so far as the S block, resulting in a cohort of synchronized cells. The length of the general arrest is a function of both survival level and exposure temperature and is most prolonged for the longest exposure at the highest temperature. For example, when cells were treated to 25% survival using 43.0°C, cell numbers remained constant up to 29 hours after treatment. When cells were treated to the same survival using 42.5°C, the number of cells began to increase at about 18 hours post treatment and had nearly doubled at 30 hours post treatment. However, cells treated to 2.5% survival using 42.5°C did not increase their numbers until more than 21 hours post treatment and had only increased by a factor of 0.2 by 30 hours post treatment.

FMF analysis indicates that the general arrest occurs at all survival levels. Again, the general arrest increases as survival decreases or as temperature increases. For example, at 43.0°, delays of 9, 18, and 24 hours are seen for survival levels of 75%, 25%, and 2.5% respectively. In contrast, if 2.5% survival is achieved using 42.5°C the delay shortens to 9 hours. The delay is followed by an accumulation of cells with DNA content corresponding to S phase cells. These cells then progress through the cell cycle in a cohort.

An autoradiographic measure of the post treatment labeling index (LI) suggests that incorporation of tritiated thymidine is suppressed

below control levels during the period of general arrest. Following
the general arrest, kinetic rates appear to increase and the incorpor-
ation of ^{3}HTdR then rises above control levels. The general arrest as
measured by LI follows, with one exception, the same pattern established
by the cell count and FMF techniques. That is, at 43.0°C, the duration
of the general arrest increases with decreasing survival level and
for iso-survival levels the general arrest increases with increasing
temperature. At 42.5°C, the length of the general arrest appeared
constant for the three survival levels measured. This may have been
due to a variation in the general arrest which was smaller than the
three hour intervals at which samples were taken.

After heat treatment at 43.0°C, pulse labeled mitoses (PLM) curves
exhibit a lengthened G2+M phase in the initial portion of the curve.
When cells are treated to 75% survival level, G2+M is 3 times as long
as for control cells. When treated to 2.5% survival level, the PLM
curve shows that G2+M increases to 5 times the length of the control
value. For 75% survival the lengthening of G2+M corresponds to the
length of the general arrest indicated by previously mentioned techniques.
And the trend of increasing length of general arrest with decreasing
survival also appears to be preserved in the PLM data. The decrease
in labeling index during the general arrest, followed by the FMF indication
of an acumulation of cells in S, indicates that during the general
arrest, cells are blocked in S.

INTRODUCTION

I. BACKGROUND LITERATURE

Hyperthermia is under investigation at many laboratories both because of the clinical potential for using elevated temperatures in cancer therapy and because certain aspects about basic cell physiology might be learned from observations on cell and organ systems at elevated temperatures. Hyperthermia, as used in this thesis, refers to temperature states in cells or animals which are above the physiological temperatures normally existing under homeothermic control of the animal involved.

A. CLINICAL MOTIVATION

Anecdotal cases of partial and complete tumor regression following the high fever that results from infectious disease forms part of the basis for current clinical interest in hyerthermia as a treatment modality for cancer. The history of clinical observations on the effect of high fever or locally applied heat on tumors has been reviewed recently by Moricca et al.,[1] and others.[2,3,4]

The first significant clinical report on the effects of hyperthermia on tumors was made by Busch[5] in 1866. Rohdenburg discovered, upon reviewing case histories in 1918, that approximately half the recorded spontaneous tumor regressions were associated with high fevers, heat applications, or severe infections.[6] Artificially induced fever (41.5°C to 42.0°C for periods of several hours) was reported by Warren in 1935[7] and by Pettigrew in 1975[8,9] to induce tumor regression. Also, Cavaliere[10,11] has demonstrated partial and total regression of primary melanomas by regional perfusion with prewarmed blood of

tumors occurring in limbs of cancer patients. The tumors were raised to 41.5°C to 43.5°C for several hours.

B. CELL AND TISSUE

In parallel development with the clinical observations noted above, in vivo and in vitro laboratory work has supplied some scientific basis to support the clinical findings. The reader is referred to references 1,2,12-24 for recent reviews on cellular and tumor thermo-biology. Giovanella[13] and Moricca[1] give special attention to the historical development of hyperthermia research.

There are a few findings which form a framework for many of the present investigations in hyperthermia. They will be briefly stated here followed by more detailed discussion. The first is that tumor cells appear to be selectively sensitive to moderately elevated temperatures. That is, supranormal temperatures may damage tumor cells more than the surrounding, or corresponding, normal cells. Secondly, some reports indicate that oxygen deficient (hypoxic) cells may be injured by hyper-thermia as readily as are oxygenated cells. The importance of these findings is that tumors are thought to contain a significant fraction of hypoxic cells and that these hypoxic cells have been shown to have a greater resistance to conventional radiotherapy than do fully oxygenated cells. Thirdly, there is abundant evidence that hyperthermia treatment interacts synergistically with radiation and chemotherapy in the killing of cells. The mechanism by which synergistic interaction occurs is far from understood, but it is hoped that combined treatment studies will provide a clue to the mode of action of hyperthermia since there is some understanding of how x-rays affect cells. Finally, the mechanism

by which hyperthermia kills or injures cells and the manner in which
the injured cells repair and recover from this damage, may be quite
different from that for other modes of injury. For instance, surviving
cells once treated with hyperthermia are more resistant to killing
by subsequent treatments. And kinetic studies have been reported which
show that hyperthermic treatment induces delays, blocks, and synchronous,
subpopulations in the treated cells which are qualitatively different
from blocks and delays induced by x-rays. In the following sections
each of the above mentioned findings will be discussed in more detail.

1. Relative Sensitivity of Normal and Tumor Cells

One of the earliest cell culture studies which examined the relative
sensitivity of normal and malignant cells to heat was conducted by
Lambert in 1912.[30] He found that mouse and rat sarcoma cells were
more easily damaged by heat treatment than were normal mesenchymal
cells. Damage was measured by the cell's ability to migrate from
an explant of tissue that was grown in a plasma clot. In 1969, Chen
and Heidelberger[25] discovered that chemically transformed C3H cells
were killed more quickly at 43°C and 44°C than normal cells, although
at 45°C the cells were equally sensitive.

Overgaard and Overgaard[21,31,32] noticed ultrastructural changes
in tumor cells of mouse mammary carcinomas treated with hyperthermia,
while interspersed stroma and vascular cells in the same tumor were
not visibly altered.

Noting that the pH of interstitial fluid in human and rodent
tumors is 0.3 to 0.5 units lower than the normal tissue pH of about
7.4, Gerwick[33,34] postulated, and demonstrated in vitro, that the

acid environment could potentiate hyperthermic damage in the tumor
area resulting in increased tumor sensitivity. However, it has been
demonstrated that even when pH is carefully maintained at 7.4, malignant
cells are more thermosensitive than normal cells. Oxygen uptake of
Novikoff hepatoma and Ehrlich ascites carcinoma cells was found to
be suppressed at 42°C, relative to 38°C, while it remained unchanged
for normal and regenerating liver cells.[11,36] Giovarella's[35]
comparison of the survival of normal embyonal mouse tissues and trans-
formed, neoplastic mesenchymal cells after 43°C heat treatments in
tissue culture ". . . indicate that the acquisition of biological
malignant potential, both in vivo and in vitro, is accompanied by
increased thermosensitivity." This finding was observed even when
comparing a tumor-producing cell subline with the normal line from
which it was derived.

Looking for the biochemical cause of this differential sensitivity
of malignant cells, Mondovi et al.[37,38,39] found that temperatures
of 43°C were accompanied by reduced incorporation of labeled precursers
into DNA, RNA, and protein of Novikoff and Morris 5123 hepatoma cells
whereas in regenerating liver cells these same appeared unaffected.
The same authors also found that lysosomes were more labile in hepatoma
cells than in normal or regenerating liver cells.

There has been at least one case where cells transformed in vitro
by polyoma virus or SV 40 virus, and assayed in vitro did not indicate
a differential sensitivity to heat.[40] Sensitivity was measured by
monitoring growth curves of treated cells. However, if the transformed

cells were used to produce in vivo tumors, the cells derived from these tumors did display differential sensitivity.

2. Oxygen Effects

There are at least two important aspects of the "oxygen effect" in hyperthermia treatment of tumor cells. The first is that hyperthermia kills hypoxic cells as successfully as euoxic (normally oxygenated) cells.[21,41-44,47] The second aspect is that some evidence suggests that heat may sensitize hypoxic cells to x-irradiation more than it sensitizes euoxic cells.[45,46] This is in contrast to the fact that ionizing radiation alone does not damage hypoxic cells as much as it damages euoxic cells. Some research indicates that oxic and euoxic cells are equally sensitive to elevated temperatures,[41,48] but that the altered pH[21,34] and/or nutritional deficiencies[28] which are often associated with hypoxic tumor cells cause further sensitization. However, it remains that hyperthermia may be especially useful in treating the hypoxic fraction of tumor cells which escape normal x-ray treatment.

3. Synergistic Interactions

Synergism is observed when cells are treated with drugs while undergoing hyperthermic treatment[29,49] and when hyperthermic treatment is combined with radiation.[2,19,22,24,26,27,45,49-51]
The extent of interaction depends on the position of cells in the cell cycle,[27,51,52,58] oxygen concentration,[43] the temperature and length of heat treatment,[53] and the sequence in which the two modalities are administered.[19] The importance of each of these factors varies among cell lines.[54]

So far, a single coherent model to describe the synergistic interaction of hyperthermia with drugs or radiation has not emerged. For drugs, the mechanism is likely to be different for each drug used, depending on the mode of action of the drug. For radiation the synergism with hyperthermia may result from the fact that the two modalities injure predominantly different targets, as indicated by the S phase sensitivity to hyperthermia and S phase resistance to radiation for CHO cells.[55] For radiation, DNA (and perhaps the nuclear membrane) is known to be of primary importance but the critical target for hyperthermia is more likely to be a variety of macromolecules and/or cell membranes.

The range of results seen when hyperthermia is combined with radiation may be a function of the range of responses different cell lines have to hyperthermia only. For example, the final slope (T_o) of a hyperthermic survival curve varies by a factor of 10 among cell lines, while for radiation only, D_o remains within a factor of two.

Analytically, hyperthermia modifies the typical x-ray survival curve by decreasing the shoulder of the survival curve and by reducing the final slope of the survival curve. For some cell lines the primary effect is on the shoulder of the curve while for others the slope is primarily affected.[19] This may imply that for different cell lines, different intracellular systems may be involved in the hyperthermic-x-ray interaction.

Further variation in response among cell lines becomes apparent when examining the effect of sequence of hyperthermic and radiation treatments on survival. The work of Li and Kal[54] typifies the extent

of variation that is observed. They compared the response of HA-1
and EMT-6 cells to different sequences of treatment and varying lapses
of time between treatments. In HA-1 preheating resulted in greater
sensitization than postheating; for EMT-6 the opposite occurred. On
the practical side, such data present a serious problem in development
of clinical protocols. On the theoretical side, it is difficult to
develop a model to account for the observed findings unless there are
different targets in the cells. For instance if a radiation repair
enzyme is thermally unstable in the EMT-6 cells this would account for
postheating having a more lethal effect. However, Ben-Hur and Elkind
showed that the interaction between hyperthermia and x-irradiation
must be more than simple inactivation of repair processes, since cells
irradiated at 42°C have much lower survival rates than cells given
the same radiation dose at 0°C.[27,98]

In order to quantitate the radiation and hyperthermic tissue
response in situ, Robinson et al. has defined a thermal enhancement
ratio (TER) as ". . . the radiation sensitivity at an elevated tempera-
ture relative to that measured at (normal temperature)".[46] Obtaining
TER's both for normal tissue and for tumors, he is then able to combine
the concept of synergistic interaction with selective sensitivity by
defining a therapeutic ratio as the ratio of TER for tumor to TER for
normal tissue (i.e., Therapeutic Ratio = TER tumor/TER normal tissue).
Robinson has recently extended his scope to include hypoxic cells
and has found that TER of hypoxic cells increases more rapidly with
increasing temperature than does the TER for well oxygenated cells.
That is, hypoxic cells are not only more sensitive that euoxic cells

to hyperthermic treatment by itself; hypoxic cells also exhibit more synergism with radiation.

Very little has been done comparing interations of hyperthermia with radiations of different linear energy transfer (LET). Gerner and Leith[56] have examined the reproductive survival of CHO cells after 1 hour at 43ºC followed by x-ray or by 400 MeV carbon ions (peak region). They found that heat and x-radiation interacted synergistically while hyperthermia interacted in an additive manner with the carbon ion radiation.

4 Mode of Injury and Repair

   a. Cell Survival

The effect of exposing cells to elevated temperatures varies in a complicated manner with the duration and temperature of the exposure. One of the most commonly used endpoints for hyperthermia work is that of proliferative capacity. After treatment, if a cell is capable of generating a colony of more than 50 cells within 12 days at 37ºC, the cell is said to have survived the treatment. For a constant exposure time, say of one hour, there is virtually no effect on survival (measured by proliferative capacity) up to a temperature of 41ºC, after which cell mortality increases with increasing temperatures. If, on the other hand, the temperature (>41ºC) is held constant while the duration of exposure is allowed to vary, most researchers have observed an initial period during which no or only slight cell death occurs followed by a period where cell survival appears to decrease according to an exponential relationship with time at temperature.

Thus for a particular cell line, a family of survival curves can be
obtained which will, in general, look like those in the figure below.

Time at temperature
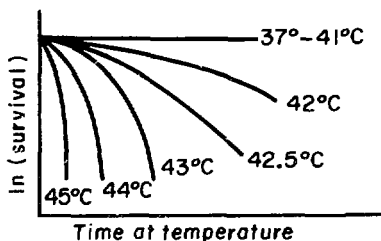
However, there is no simple function that will relate, for example,
1/2 hour at 44°C to 1 hour at 43°C. In fact the specific physiologic
response of surviving cells may be different at different temperatures.
A variety of parameters must be measured at each temperature; results
obtained for one temperature may not be generalized. This becomes
apparent as results from various laboratories are compared.

The kinetics of cell mortality due to hyperthermic stress has
been analyzed using the formalism Arrhenius originally developed in
1889 to meaure the influence of temperature on chemical reaction rates.
The mortality rate ("inactivation rate") on the exponential region
of the survival curve is substituted for the chemical reaction rate
and plotted as a function of the reciprocal of the absolute tempera-
ture.[55,59,61] The Arrhenius plot for a number of cell lines shows
a constant slope with a break in the slope occurring at 42.5°C (see
amplified discussion in discussion section).

There has been considerable effort to examine more subtle parameters of cell physiology than reproductive mortality. Specifically, biochemical properties, membrane changes, cell kinetics, and gross and molecular repair properties are currently under study in various laboratories.

b. Biochemistry

The effect of hyperthermia on several aspects of cell metabolism and biochemistry has been studied and reviewed extensively by Strom et al.[14] They observed that hyperthermic conditions resulted in reduced oxygen consumption and inhibition of DNA, RNA, and protein synthesis in tumor cells but not in normal cells. As a rule, the biosynethetic processes are inhibited by thermal treatment more than oxygen uptake, and formation of ribosomal RNA was preferentially inhibited compared to formation of other kinds of RNA.[39] Levine and Robbins also measured glucose incorporation into polysaccharides and incorporation of choline and linoleic acid into lipids for heat treated cells in culture.[62] They found these pathways unaffected.

When Palzer and Heidelberger used specific chemical agents to block DNA and/or protein synthesis, Hela cells became less sensitive to hyperthermia than control cells. In contrast, when RNA synthesis was blocked, there was enhanced cell killing by heat treatment.[58] Giovanella et al.[63] also found that suppression of RNA synthesis increased cell sensitivity to hyperthermia (L1210 leukemia cells).

One possible interpretation of these data would be that supranormal temperatures interfere with correct DNA and protein synthesis. If the normal progression through synthesis of these essential cellular macromolecules is suspended by other means, the cell is protected.

If, on the other hand, some RNA syn hesis can proceed normally under
elevated temperatures and is allowed to do so, the cell when returned
to 37°C can immediately begin manufacture of proteins necessary to
effect repair.

    c. Suggested Mechanisms for Hyperthermic Cell Damage

    The possible cellular constituents which may sustain hyperthermic
damage include the macromolecular systems of DNA, RNA, proteins, and
membranes. They will be discussed below in that order.

    Since in many cases hyperthermia interacts synergistically with
radiation, some inferences may be made concerning the cellular target
each modality interacts with. Synergism is defined as "the simultaneous
action of separate agencies which, together, have greater total effect
than the sum of their individual effects".[123] Now, if heat and
radiation each acted in the same manner on the same cellular target,
one would expect the combined effect to be simply the sum of their
individual effects. Similarly, if each modality interacted with an
entirely separate and independent target, one would again expect to
see a simple additive effect between the two modalities. The existence
of synergism between radiation and hyperthermia implies that each
modality acts in a different manner on the same target. For example,
while radiation may produce an effect directly on DNA (by either direct
action or by production of free radicals which in turn act directly
on the DNA), hyperthermia may act indirectly on the DNA by interacting
with the chain of events which lead to DNA synthesis (i.e., causing
membranes to become leady thereby depriving the cell of constituents
necessary to repair damage caused by radiation, causing conformational

changes which inactivate necessary enzymes, or by changing the structural rigidity of the protein-DNA complex and thereby interfering with resynthesis of damaged DNA). In this case, cellular changes produced by hyperthermia which may not have been lethal if hyperthermia alone was used, may become lethal when combined with radiation, yielding synergistic results.

A further argument against the direct role of DNA damage in hyperthermic cell killing is that the denaturation temperatures for DNA are well above the temperatures used in this research. For pH's ranging from 5 to 11, the denaturation temperatures are 55°C or greater.[72] In addition, some authors have compared DNA taken from thermophilic and mesophilic bacteria and have observed similar melting temperatures.[73,74] In contrast, proteins taken from thermophilic microorganisms have exhibited higher denaturation temperatures than analogous proteins taken from mesophilic microorganisms.[95,96]

Overgaard examined ultrastructural changes following local in vivo hyperthermia treatments to murine mammary carcinomas. He noticed shrinkage of cellular nuclei and condensation of heterochromatin within six hours of treatment along with decreased granulation in the nucleoli.[75] These observations could possibly be due to loss of function in structural proteins associated with the DNA or to minor conformational changes of the heterochromatin which result in progressive condensation.

Suppression of RNA synthesis has been observed to increase cell sensitivity to hyperthermia.[58,63] Yet hyperthermic treatment itself has been observed to inhibit RNA synthesis and formation of mature

ribosomal RNA's.[39,76] The function of the RNA molecule in cell damage sustained by elevated temperatures is not yet clear but provides an interesting area for further research.

Although proteins also denature at higher temperatures than those conventionally used in hyperthermia research, it is possible that minor conformational changes may occur between 42 to 45°C. Such small conformational changes could interfere drastically with the efficiency of important enzyme reactions, while not being observable in test tube evaluations of denaturation events. Damage at this level may possibly account for many of the effects seen in hyperthermic treatment. Loss of structural proteins could cause the chromatin condensation seen by Overgaard.[75,100] Loss of function in metabolic enzymes might inhibit biosynthetic pathways. And inactivated repair enzymes could provide the basis for synergistic interaction seen between hyperthermia and DNA specific agents such as x-ray and chemotherapeutic drugs.[29,49,2,19,27]

It has been suggested that the Arrhenius-type curve for cell mortality could yield inactivation energies that would correspond to particular target enzymes.[55,20] However, to date, this approach has not proved fruitful.

Additional evidence supporting the concept of proteins as a target is that proteins of thermophilic microorganisms have developed higher denaturation temperatures than analogous proteins from mesophilic microorganisms.[95,96]

It is very likely that membrane structure and function is altered by elevated temperatures. Membrane fluidity is an important membrane property which allows membrane proteins to move laterally and rapidly

in the plane of the membrane.[77,78] The fluidity of the membrane also affects protein transport,[78] glucoside transport,[79,80] and ion transport.[94] It may be that membrane fluidity will vary within a single membrane, as a function of lipid or protein clustering, which may in turn be necessary for specialized cell membrane or organelle functions.[81] In fact it may be because of different required functions that membrane composition varies from cell to cell and organelle to organelle.

As temperatures increase, cell membranes may undergo a critical phase transition resulting in cell injury. Several lipid components have transition temperatures in the range from 20 to 50°C.[84,14] Dep·· .ng on the composition of the membrane, a composite phase transition in the range from 41 to 45°C is possible. Since neoplastic and normal cells have different membrane properties,[82] this hypothesis may explain for the selective sensitivity of neoplastic cells to hyperthermia. Evidence in support of this concept was found by Reeves.[64] In a cloned, heat-resistant cell line, he observed less leakage of uridine containing materials through the plasma membrane than in the normal, sensitive, parent line. Gerner and Russell found that after 1 hour at 43°, CHO cells lost polyamines to the extracellular medium. Within a short time after treatment intracellular concentrations returned to normal.[106] Using ultra-structure techniques, Overgaard observed pronounced lysosomal activity along with disaggregation of polyribosomes within · few hours after treatment. However, the nuclear envelc e did not show damage up to 96 hours after treatment.[75]

Bacteria have been shown to alter their membrane components when incubated at high temperatures. They incorporate fatty acids with longer chain lengths and higher degree of saturation. It is known that these properties increase membrane viscosity. Perhaps a compensatory mechanism exists to protect the bacterial membrane from the melting effect at the elevated temperatures.[92,93]

There is evidence that cells in culture can develop transient resistance by hyperthermia if allowed to recover for 2 hours or more at 37°C between treatments,[85-87] or if treated at 42.5° continuously.[20] This property could reflect the cells' ability to incorporate protective lipids into their membranes.

Finally, it is possible for loss of membrane functions to occur due to damage to membrane-bound proteins. It might prove difficult to separate injury occurring exclusively to the lipid structure from injury occurring primarily to the membrane-bound protein. It seems that the research in this area has been limited to studies in the temperature range from 2 to 37°C. In this temperature range the fluidity of the membrane is affected by temperature and thereby appears to affect the structural support given the membranous enzymes.[88,89] That is, the thermostability of the lipoprotein complex may be more important than that of the lipid or protein by itself.[97]

The only evidence of membrane protein interaction at hyperthermic temperatures is indirect. Mondovi found that Ehrlich ascites cells which were exposed to 42.5°C for 3 hours stimulated an immune response when injected into host mice more effectively than did radiation-inactivated cells. He suggests that heat treatment unmasks or modifies

an antigen in some undefined way.[91] Studying multiple immunologic parameters in a clinical setting, DeHortius et al. stated that " . . . hyperthermia may favorably alter the immune balance between tumor and host in selected instances."[90] His findings suggested support for Moadovi's hypothesis.

d. Cell Kinetics

Cells vary in their thermal sensitivity during the cell cycle. As mentioned above, mid to late S-phase cells appear to be most sensitive to heat treatment,[55,57,58] with the exception of pig kidney cells where no differential sensitivity was found.[64] It is perhaps a result of the S-phase sensitivity that exponentially growing cells have greater sensitivity to heat than do plateau phase cells.[62,65]

Cell kinetics during and following hyperthermia has been studied using autoradiographic[66,67] and flow cytometry techniques.[68-71] Post-exposure proliferation rates have also been examined.[57,68] Kal et al. report that 1 hour at 43° delays growth of EMT-6 for 6 hours and causes a block in S as well.[67] Schlag observed a 4 hour growth delay in Chinese hamster lung cells treated for 30 minutes at $42^\circ C$. If he treated cells for an hour at $42^\circ C$ he observed with a cytofluorograph that cells appeared to be blocked in S for 4 hours.[70] One of the aims of this dissertation is to better detail what part of the cell cycle is affected by the growth delay at different temperatures and to compare kinetics for equal survival levels obtained by use of different temperatures.

e. Repair

Cellular repair of hyperthermic damage has been studied at
three levels: (1) the effect of hyperthermia on the ability of cells
to repair single- and double-strand DNA breaks caused by ionizing
radiation;[27,98-102] (2) survival studies designed to indicate repair
of sublethal and potentially lethal damage;[20,51,57,87] and
(3) survival studies designed to measure induction of a state of
thermotolerance.[20,61,64,85-87] The following discussion will consider
each of these approaches, respectively.

By incubating cells at supranormal temperatures between fractionated
x-ray doses and by varying the x-ray dose rate at elevated temperatures,
Ben-Hur and Elkind demonstrated that hyperthermia inhibits repair
of sublethal x-irradiation damage.[27,101] To see if the repair in-
hibitation was due to unrepaired single-strand breaks, they examined
the effect of hyperthermia on the rejoining rate of single-strand
DNA breaks and damage to the DNA complex.[98,99] They observed that
at 42°C, rejoining of x-ray induced single-strand DNA breaks (SSB)
proceeds more rapidly than at 37°C. However, they noted that incubation
at 41°C and 42°C after irradiation caused the DNA complex to repair
more slowly and atypically. They concluded that hyperthermia may
cause changes in the higher order chromatin structure (i.e., tertiary,
quaternary structure) or in the nucleo-protein or chromatin-nuclear
membrane structure, which interfere with repair of x-ray induced DNA
damage.[98-100]

Corry saw no significant difference between rejoining of x-ray
induced SSB in control cells and those pre-incubated at 43°C. However,

using very high doses, he did see a reduction in the rejoining rate
of double-stranded DNA breaks (DSB) when preheated at 43°C.[102]
Significantly, he mentions that "neither DSB nor SSB arise from the
heat treatment alone in the temperature range 41-46°C, and the fre-
quency of DSB induction by radiation remains unaffected by heat
treatment." However, the data for these observations is not presented
in the paper.

The concepts of subethal and potentially lethal damage to cells
treated with hyperthermia are exactly analogous to those developed
in radiation biology.[103] That is, the presence of a shoulder (D_q)
on the survival curve of heat treated cells indicates the capacity
of the cells to accumulate "sublethal" damage. When this capacity
is saturated, any further damage is "potentially lethal" resulting
in the exponential portion (slope--D_o) of the survival curve.

Repair of sublethal damage is observable by re-establishment
of the shoulder in a survival curve. Recovery from potentially lethal
damage is observable by an increase in survival, with time, at 37°,
after exposure. Recovery of potentially lethal damage (PLD) from
heat has been reported for HA1 cells and seems to be highly dependent
on nutritional factors.[28] Use of some drugs also appears to
stimulate repair of PLD.[57] Our experiments suggest that otherwise
untreated log phase cells may also show recovery from PLD due to hyper-
thermia. Recovery from heat induced sublethal damage has been well
demonstrated.[20,51,87] It is manifested in the reappearance of a
shoulder when cells are given an initial hyperthermic treatment, allowed
to recover at 37°C, and given a graded series of second doses.

In addition to a reappearance of the shoulder upon a second hyperthermic treatment, the final slope (T_o) of the exponential region of the survival curve is greater than the single exposure T_o. That is, after the cells' capacity to accumulate sublethal damage has been re-saturated, they accumulate lethal damage at a slower rate than they did for the single exposure condition. The term "thermotolerance" has been suggested to describe this phenomenon[61,85,86] and it has been observed in many cell systems (for review see Ref. 2). For temperatures less than 43°C, thermal tolerance can be induced during continuous treatment in some systems.[20,19,2] Gerner has termed this Type II thermotolerance while the thermotolerance which is dependent on split exposures is notated Type I.[105]

With one exception, the induced thermal resistance has been transient. Harris found that the thermal resistant trait persisted in the progeny of resistant cells and was stable for 15-24 serial passages (6 months) without additional heat treatment, after which a gradual decline in thermotolerance was observed.[61] Comparing thermotolerant clones of pig kidney cells to the sensitive parental line, Reeves[64] found the resistant lines leaked significantly smaller amounts of uridine-containing material through the cell membrane and that they began synthesis of macromolecular compounds more quickly after treatment than did the sensitive parental line. It is unfortunately not possible to generalize these results because of the transient nature of the thermotolerant state in other cell lines.

II. RATIONALE

The 9L-rat gliosarcoma system had been in use for several years at Lawrence Berkeley Laboratory when I started to examine the effects of hyperthermia. The fact that these cells can be cultured either *in situ*, *in vivo* or *in vitro* makes them extremely useful as a cellular biological tool.

During the initial characterization of the cell survival response (by proliferative capacity) to several temperatures (37.0-45.0°C) we noticed (qualitatively) a delay in the growth rate of surviving colonies which became progressively longer with increasingly severe heat treatments. This observation led to interesting questions concerning the manner in which heat affected growth of living cells. Parameters of interest include length of delay, changes in generation time, and induction and duration of blocks at certain phases of the cell cycle.

The main emphasis of our work is the comparison of the effect of different temperatures and exposure times on perturbations in cell cycle kinetics. Although other researchers have looked at perturbations in cell cycle kinetics at one or another temperature, we are not aware that anyone has yet compared cell kinetics resulting from cells treated at several temperatures and exposure times. It is difficult to develop the basis for comparison since there is no clear concept of dose in hyperthermic treatment. That is, how long at, say, 43°C, corresponds to 1 hour at 42.5°C?

For our work we sought to use iso-survival levels as the basis for comparison. For example, survival curves indicate that either 75 min. at 42.5°C or 45 minutes at 43°C reduce cell survival to 75%. By

repeating these exposure conditions for kinetic studies we aimed to reduce survival to an iso level at the time the kinetic measurements were made.

Other researchers have observed that a state of tolerance to hyperthermic exposure can be induced in cells if they are allowed to recover at 37°C for some time between heat exposures (see introduction and discussion sections). We were also able to observe a thermotolerant effect in 9L cells using heat exposures that yielded the iso-survival levels used in the kinetic studies. Because many cells are differentially sensitive to heat in various phases of the cell cycle, we hoped to correlate post hyperthermic treatment kinetics with the interval that cells rested at 37°C between split exposure treatments. For example, if cells accumulate in a heat-resistant phase of the cell cycle in response to the initial hyperthermic exposure, it would not be surprising to see increased resistance to killing by high temperatures.

Clinically, many glial cell tumors represent a class of malignancies which are resistant to successful treatment using conventional therapies.[109] A glial cell tumor system therefore provides a good system on which to study the effects of a new treatment modality such as hyperthermia. The 9L tumor model has the ability to be grown and treated in vivo, in situ, and then assayed quantitatively in vitro. Because of the difficulty in producing controlled hyperthermia conditions in the brain at the beginning of this work, I have dealt entirely with cells maintained in vitro. However, the recent development of hyperthermia producing equipment[107] should make the extension to

in vivo-in vitro experiments possible. In fact, some work has already begun along these lines.[108]

MATERIALS AND METHODS

Tumor cells used in these experiments were originally obtained
from a malignant tumor produced in the brain of CD Fischer rats by
weekly intravenous injection of N-nitrosomethylurea. The KT-9 tumor
(from which 9L cells were derived) was histologically classified as a
high-grade astrocytoma which was stable over many passages in tissue
culture. The tumor was " . . . extremely cellular with moderate
pleomorphism and nuclei varying in shape from oval to round with a
diffuse chromatin pattern and rare prominent basophilic nucleoli. The
interstitium was finely fibrillar and composed of glial fibrils.
Numerous mitoses were present, a few of which were atypical. Stellate
areas of necrosis were bordered by palisading tumor cells. There was
also prominent palisading of cells about vessels. Increased vascularity
and marked endothelial proliferation were also present."[116] Cells
were supplied to us by the Brain Tumor Research Center, University
of California, School of Medicine, San Francisco, California, where
the cells were developed into an in vivo-in vitro model. The cells
are now designated 9L tumor cells, and in solid tumors they histo-
graphically appear as a mixed gliosarcoma.

The experiments described in this dissertation used cells grown
in vitro. Stock cultures were maintained in monolayer growth in 75 cm^2
plastic tissue culture flasks (#3002, Falcon Plastics, Oxnard, CA)
with 15 ml of Eagle's minimum essential medium (Grand Island Biological
Co., Grand Island, NY) with Earle's Salts and 20% fetal calf serum.
Initially all cultures were grown in medium containing antibiotics
(penicillin, 50 U/ml; streptomycin, 50 μgm/ml). However, it was found

that alternating between antibiotic free medium and medium containing both antibiotics and an anti mycoplasmic agent (1% Knamyc:n) resulted in better protection against m:coplasma. Two culture series were carried forward simultaneously, alternating in staggered fashion between the media, so that Kanamycin free cells were always available for experiments. Thus possible toxic interaction between Kanamycin and hyperthermia was avoided.

Cells for experiments were seeded from log phase stock cells at 10^5 cells in 5 ml of medium per 25 cm^2 culture flask (Falcon Plastics), 2 days prior to an experiment. Medium used in experiments contained antibiotics which have been reported not to interact with hyperthermia in the concentration mentioned above;[117,118] anti mycoplamic agents were not used in experimental media. This procedure yielded about 5 x 10^5 asynchronous, log phase cells per flask in monolayer on the day of the experiment.

I. HYPERTHERMIC TREATMENT

Cells were subjected to hyperthermic treatment in the 25 cm^2 flasks after sealing with Parafilm (Mara Products, Neenoh, WI). The flasks were layed on the bottom and middle shelf of a standard test tube rack and immersed in a horizontal position in a thermostatically controlled, constant temperature, stirred water bath accurate to $\pm 0.05^oC$ (Lo-Temptrol Model 154, Precision Scientific Co.) for 1 to 12 hours (Plate 1).

Upon removal from the water bath, the supernatant media was removed and the number of cells in the supernatant that had detached from the plastic surface were counted (Coulter Counter, Model B, Coulter

Electronics, Hialeah, FL). In separate experiments, survival characteristics of the cells in the supernatant were determined.

The pH of the supernatant media was monitored as a function of time and temperature (Beckman Model G or Corning Digital 109 pH meter) in order to rule out secondary stress on the cells due to pH changes with time at high temperatures. At all temperatures, pH remained constant at 7.4 ± 0.1 units.

Cells were removed from the surface of the flask with 0.03% trypsin-EDTA (3-5 minutes), pelleted by centrifugation, resuspended, diluted, counted on a hemacytometer, and seeded into 60 mm diameter plastic petri dishes (Falcon Plastics) at an appropriate concentration. Five replicate dishes were seeded for each dilution. At high survival levels, the total number of cells per dish was kept at 10^4 by addition of the appropriate number of heavily irradiated (3600 rad of 150 kV x-rays) feeder cells. Cells were incubated at 37°C in a 5% CO_2 environment for 10 to 12 days.

Survival was determined by counting colonies of more than 50 cells after staining dishes with Hematoxylin or Methylene Blue. Each stainable colony was assumed to represent the progeny of a single cell which has survived the heat treatment. A colony size of 50 healthy cells was chosen for ease of comparison with the data of other investigators who also used this criteria. Elkind and Sutton showed that counting essentially all stained areas rather than those colonies with 150 or more cells shifted the survival curve by only a small amount.[104] Their data suggests that the exact lower limit used as criteria for a surviving cell is not too critical.[103]

Each experiment consisted of two replicate flasks per point. Each flask was seeded into 2-3 dilution sets of five dishes and each experiment was repeated at least twice so that most points on the survival curves represent an average of four flasks (30-40 dishes).

Hyperthermic effects were also determined by the cell's ability to exclude trypan blue vital stain (0.4% by volume) mixed v:v with 0.5 ml of cell suspension immediately after hyperthermic treatment. The percent of cells that took up the stain was determined after incubation for 5 minutes at 37°C. Approximately 100 cells were counted.

In order to observe the thermotolerant effect, flasks were initially treated at 43.0°C to one of four survival levels (75%, 25%, 5%, 1%), allowed to recover for 3 hours at 37.0°C, and then given graded second exposures to hyperthermia. The cells were then assayed for proliferative capacity as described above.

II. THERMAL DOSIMETRY

The sensitive portion of a 22 gauge thermistor needle probe (resistance 1200 ohms at 37°C) was placed at the media--plastic interface of a 25 cm^2 Falcon flask containing 5 ml of complete media. The flask was then placed into the water bath and heating and cooling curves were measured for temperatures from 37.0°C to 45.0°C under conditions identical to the experimental exposure conditions.

The change in resistance of the thermistor with temperature was measured with a Keithley digital electrometer (Model 616), Keithley Instruments, Inc., Cleveland, Ohio, and permanent tracings were made by recording the d.c. output of the electrometer on a Grass Model 7 Polygraph (Grass Instruments, Inc., Qunicy, Mass.).

Sixty-three percent of the temperature change was reached within 1 min. for the highest experimental temperature (45.0°C). At the lowest temperature (39.0°C), 63% of the temperature change was reached within 0.5 min. For all temperatures, the full change in temperature was achieved within 5 min.

III. FLOW MICROFLUOROMETRY

Flasks of cells were seeded at 10^5 cells per flask two days prior to heat treatment. The cells were then subjected to hyperthermia using 42.5°C or 43.0°C for times appropriate to reduce survival to 75%, 25%, or 2.5%. Upon removal from the water bath, flasks were dried, caps loosened, and flasks placed back into a 37° incubator for varying periods of time. Cells were prepared for FMF according to the method of Crissman,[112] and stained with Chromomycin A_3 or Propidium iodide (PI). Samples were analyzed on a locally constructed flow microfluorometer. The electronics were of the Steinkamp, et al. design[113] and the flow cell was after Holm's design.[114] A laser excitation wavelength setting of 514 nm for Propidium iodide or 457 nm for Chromomycin A_3 stained cells was used.

The stains used bind to the DNA of the cell and fluoresce when illuminated by light and an appropriate excitation wavelength. The intensity of the fluorescence is taken to be proportional to the amount of bound stain which is, in turn, a function of the amount of DNA in the cell. Chromomycin A_3 belongs to a class of antibiotics which has a high specificity for helical DNA but not for RNA. Chromomycin A_3 appears not to cause uncoiling of the DNA double helix[119] and does not intercalate into DNA.[120] Used at a concentration of 100 µg/ml,

Chromomycin A_3 essentially stains to full intensity within 20 min.[111] Propidium iodide is an analogue of ethidium bromide which quantitatively intercalates into double-stranded regions of DNA and RNA[121] (so RNAase is used in the preparation of this stain).

The intensity of the fluorescent signal is measured with a photomultiplier tube and a frequency histogram of the light output for individual cells is constructed and stored. Since DNA content is presumed to be directly related to the position of a cell in the cell cycle, the histograms can be interpreted to represent the distribution of cells in various stages of the cell cycle.

Several computer programs were considered for analysis of the FMF histograms. However, existing, available programs failed either to accomodate the perturbed S populations or to account for overlap of the S region into the G1 and G2+M regions. So, DNA histograms were analyzed by use of a mechanical planimeter. The G1 and G2 peaks of the histogram were represented by one leg of a right triangle. The hypotenuse represented the leading edge of the G1 peak or the trailing edge of the G2 peak; the base of the triangle was formed by the zero level (see Fig. 1). The area of the G1 and G2 peaks were computed by doubling the area of the appropriate triangle. To allow for the fact that S phase cells extend into the G1 and G2 regions, the hypotenuse of the triangle is aligned with the upper portion of the histogram. The extent to which there is increased spread at the base of the triangle is attributed to S phase cells by inclusion of the spread in the total area of the histogram and exclusion of the spread from the calculation of G1 and G2 regions.

The placement of the G1 and G2 peaks bears some comment. Immediately upon viewing the histogram, the two maxima are discernible; these are referred to as the apparent G1 and G2 peaks. However, closer examination of the histogram reveals that the apparent G2 peak usually does not occur at twice the intensity of the apparent G1 peak, although cells in G2+M should have twice the DNA content of cells in G1. The asymmetric shape of the S contribution which adds to these peaks results in shifting the apparent peaks toward the center of the histogram. An attempt was made to place the peaks at their true position by use of the following protocol.

The location of the apparent G1 peak ($G1_A$) on the abscissa was doubled to indicate the expected location of the G2 peak. The true G2 peak ($G2_T$) was placed midway between this value and the apparent G2 peak ($G2_A$). The true G1 peak ($G1_T$) was then placed at 1/2 the true G2 value (Fig. 1). That is,

$$G2_T = G2_A + \frac{2 \times G1_A - G2_A}{2}$$

$$G1_T = \frac{G2_T}{2}$$

Planimeter analysis then resulted in repeatable values for G1, S, and G2+M. Surprisingly, the S value remained relatively insensitive to the exact positioning of the true peaks as long as $G2_T$ was constrained to be 2 x $G1_T$. Of course, the G1 and G2 + M values were dependent on the exact positioning of the true peaks.

IV. AUTORADIOGRAPHY

A. *LABELING INDEX (LI)*

Flasks of cells were subjected to hyperthermic treatment according to the same regimen as for the FMF work. However, after spending varying periods of time at 37.0°C after heat treatment, cells were exposed to 2 ml of medium containing 1 µCi/ml of tritiated thymidine (^{3}H-TdR) (specific activity, 6.7 Ci/mM, New England Nuclear). Possible dilution of thymidine pool in the cell was checked by repeating the experiment using an alternate precursor molecule, tritiated deoxyuridine (spec. act. 15-30 Ci/mM, New England Nuclear). After 15 minutes incubation at 37°C with ^{3}H-TdR, cells were washed twice with Earle's basic salt solution (BSS). Harvested cells were pelleted and resuspended in 5 drops of BSS and one drop was spread on each of four microscope slides. The slides were air dried, fixed in absolute methanol for 15 min., rinsed in distilled water for 1/2 to 1 hour and air dried. Slides were then dipped in Kodak NTB-2 nuclear emulsion and exposed at 4°C in light tight boxes containing Drierite for 8 days. The autoradiographs were developed for 6 min. in Kodak D-170 developer and stained for 10 min. in Hematoxylin. Cells with more than 5 grains were counted as positive. This procedure closely follows the work of Nomura et al.[114].

B. PERCENT LABELED MITOSES (PLM)

Media was removed from flasks, pooled, and supernatant cells removed by centrifugation. Then cells were exposed to 2 ml medium containing 1 µCi/ml ^{3}H-TdR for 15 minutes prior to heat treatment. The monolayers were washed twice with BSS and 5 ml of the pooled

original medium in which they had been growing was replaced (so that
nutrient depletion in the medium at the time of heating would not
be different from other experiments). Cells were treated at $43.0^{o}C$
to 75% and 2.5% survival. After hyperthermic treatment, flasks were
replaced in a $37.0^{o}C$ incubator. Each 1-1/2 hours autoradiographs
were prepared. Finally, the percent labelled mitoses were scored
for each point.

RESULTS

I. SURVIVAL CURVES

The effect of temperatures from 39.0°C to 45.0°C on survival of 9L cells grown in monolayer is shown in Fig. 2. At 42.0°C, the shoulder region is not unequivocably distinguishable. Exposure to temperatures of 42.5°C and above resulted in distinct shoulder and exponential regions of the survival curve. Temperatures of 39.0°C or 41.0°C for as long as six hours produced no detectable effect on clonogenic cell survival. All survival curves were normalized to the control plating efficiency, determined individually for each experiment. Control plating efficiencies were typically 70 percent.

The survival curves in Fig. 2 can be described by the parameters T_o, T_q, and n, analogous to the radiation curve parameters D_o, D_q, and n. For a given temperature, T_o is the time required to reduce survival by a factor of 1/e in the exponential region of the survival curve and is determined by a least square fit to data points where survival is less than 30%. 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. n is the extrapolation number, the point at which the back extrapolate of the exponential region intersects the ordinate. The manner in which these parameters vary with temperature is shown in Table I and Fig. 3. The quasi-threshold time (T_q) and the extrapolation number (n) both show a discontinuity at 42.5°C and 43.0°C respectively (Fig. 3).

An Arrhenius-type plot of the rate of inactivation ($1/T_o$) vs. the reciprocal of the absolute temperature in degrees Kelvin or in

degrees centigrade is shown in Fig. 4. From the Arrhenius equation:

$$d(\ln k) = (\mu/2) \ (dT/T^2)$$

where k is the rate of cellular inactivation; T is the temperature
in $^{\circ}$K; and   is the macroscopic activation energy for the sum of the
chemical reactions that produce cell inactivation. Integration yields:

$$\ln(k_1/k_2) = \mu/2 \ (1/T_2 - 1/T_1)$$

and the activation energy between any two temperatures is then

$$= 2 \ \ln \ (k_2/k_1) \ \cdot \ (1/T_1 - 1/T_2) \ .$$

The activation energy is also obtained from the slope of the Arrhenius
plot, $\mu/2$. Our data clearly shows a significant change in μ at 43.0°C
(Fig. 4).

II. SUPERNATANT CELLS

    For various heat treatments, the number of cells found in the
supernatant (Fig. 5) did not increase over a period of 6 hours during
treatment at temperatures of 39.0°C to 42.0°C. For cells grown in
monolayer culture, the release of cells into the supernatant medium
is usually a sign of cytotoxicity or approaching cell death. After
four hours at 43.0°C, the number of cells in the supernatant had doubled
and by 6 hours had quadrupled (evident in unnormalized data). However,
the 43.0°C survival curve illustrated in Fig. 2 was not affected since
survival was assessed within 3 hours at 43.0°C. At 44.2°C, the percentage
of total cells appearing in the supernatant increased rapidly after
2 hours. Within one hour at 45.0°C, supernatant cells had increased

by a factor of 8 and continued to increase to 80 times the control
value by 6 hours at 45.0°C. It seems likely that the increase in super-
natant cells represents cells which have detached from the growing
surface of the tissue culture flux during heat treatment. It is worth
noting that cells did not begin to detach until after the time at
each temperature necessary for construction of the survival curves
shown in Figure 2.

An attempt was made to construct a separate set of survival curves
for supernatant cells at each temperature. The control plating efficiency
of supernatent cells averaged 17% ± 2.9% (Standard Error), indicating
a low clonogenic potential for suspended cells even when not heated.
The low control plating efficiency prevented construction of statistically
significant survival curves.

III. DYE EXCLUSION MEASUREMENTS

In addition to measuring cell survival by proliferative capacity,
the exclusion of trypan blue dye was used as an indicator of cell
viability for attached cells. Exclusion of trypan blue proved a much
less sensitive indicator of cell integrity than did colony formation
and data did not correlate well between the two assays. Harris[115]
reported a similar lack of correspondence between mortality measured
by trypan blue exclusion and colony forming ability for pig kidney
cells. However, the number of dead cells as indicated by trypan blue
viability studies correlated extremely well with the percent of cells
detaching from the surface of the culture flask (Fig. 6). Since both
trypan blue exclusion and cell detachment provide a measure of one
aspect of cell membrane integrity, defects in this membrane function

appear to influence survival only at severe exposures, where survival
is less than 0.1% by colony formation. Fig. 60 shows that these effects
become important after 3 to 3.5 hr at 43.0°C and after 1.5 to 2 hr
at 44.2°C. There is no effect for up to 12 hours of continuous exposure
at 42.0°C.

IV. DOUBLE HYPERTHERMIC EXPOSURE

When cells are given a single initial exposure to hyperthermia
and then returned to 37.0°C for three hours, followed by graded second
exposures to the same hyperthermic temperature, the final slope of
the survival curve is greater than that from the single exposure condition.
Our results for 4 different initial exposure times at 43.0°C are shown
in Fig. 7. T_o for the single exposure condition is 16.8 minutes.
If cells are initially treated to 75% survival (S_i = 75%) the T_o for
the subsequent exposure is 108 minutes. For initial survival levels
of 25%, 5%, and 1%, the second exposure T_o's are measured at 39, 55,
and 64 minutes respectively. Although the last three values may not
be statistically different, they follow a trend established by other
data (2) which suggests that the second exposure T_o increases with
decreasing initial survival (Fig. 7) in the exponential region. There
does not appear to be a re-establishment of the shoulder region of
the curve although there appears to be some increased cell survival
(resembling PLD) for re-exposure after an S_i of 5% or 1%.

The increase in T_o indicates that cells are not killed as readily
by hyperthermic treatment upon a second exposure. The extent of this
increased resistance to heat treatment can be indicated by taking
the ratio of T_o resulting from a single hyperthermic exposure to T_o

of the split exposure condition. This ratio has been termed Thermo-
tolerance (TT).[2] That is:

$$TT = \frac{T_o \ (\text{split exposure})}{T_o \ (\text{single exposure})}$$

A subscript can be used to denote the survival level (S_i) that the
split dose population was initially reduced to. A superscript indicates
the temperature used in centigrade degrees. For example:

$$TT_{75\%}^{43} = \frac{108 \ \text{min.}}{16.8 \ \text{min.}} = 6.42$$

where S_i = 75% and the temperature was 43.0°C.

When S_i was high enough to remain within the shoulder region
of the single exposure curve, the resulting thermal tolerance was
relatively high. When S_i was in the exponential portion of the single
exposure survival curve, thermal tolerance increased with decreasing
S_i. For example, for S_i = 25%, 5%, and 1%, TT = 2.32, 3.26, and 3.83.
This observation has been made on the exponential region for other
cell lines as well (Fig. 8).[2]

V. KINETIC STUDIES

A. FLOW MICROFLUOROMETRY

Sample histograms resulting from flow microfluorometric analysis
are shown in Figs. 9 and 10. Two temperatures and three survival
levels were emphasized. 43.0°C and 42.5°C temperatures were chosen
because the break in the Arrhenius plot occurred at 43.0°C and the
shoulder width was maximal at 42.5°C. In addition, these temperatures
lie within the clinically important range. The survival levels of

75%, 25%, and 2.5% represent the various regions of the survival curve.
Table II shows the hyperthermic exposure interval required to reduce
survival to the required level for each temperature.

For each temperature and survival level, the first change noticeable
in the histogram is a relative accumulation of cells in the S region
of the histogram. The length of time that passes before this accumulation
is noticeable varies depending on the survival level to which the cells
were treated and the temperature used to achieve that survival level.

At 43.0°C, for 75% survival, the accumulation of cells in the S
region is apparent 6 hours after treatment. For 25% survival level, an
accumulation of cells in the S region becomes noticeable at 9-12 hours
post treatment. For 2.5% survival, the relative increase in S phase
cells is not observable until 24 hours after hyperthermic treatment has
terminated. That is, for a constant temperature (43.0°C in this case),
as survival decreases, the post treatment interval lengthens before a
change in the FMF histogram distribution is noticeable.

This pattern is repeated for 42.5°C. For the 75% survival level,
the accumulation of cells in the early S region is apparent 3 hours
after treatment. For 25% survival, a mid-S accumulation is noticeable
at 6 hours whereas for 2.5% survival the cell accumulation is still
in early S at 6 hours post treatment.

A graphical representation of the G1, S and G2+M populations is
shown for 75% survival using 43.0°C temperatures in Fig. 11. Control
values are 50.0%, 34.1% and 15.8% for S, G1, and G2+M respectively.
The accumulation of cells in S is accompanied by a relative depletion
of cells in G1 and G2+M. By 12 hours the cohort of accumulated cells

have moved to G2+M and after 30 hours the distribution is approaching
normal. This pattern of redistribution through the cycle following
the accumulation of cells in S is seen for both 43.0°C and 42.5°C
for all survival levels measured.

Since the accumulation of cells in S leads the progression of
cells through the rest of the cell cycle, it can be useful to compare
only the changes in the S fraction for different survival levels achieved
with the use of either 43.0°C (Fig. 12) or 42.5°C (Fig. 13). This
presentation emphasizes that with decreasing survival fraction, the
length of dely increases (i.e., the time increases before the expected
accumulation in S is observed).

To assure that the cell cycle histograms were not being sufficiently
biased by population increases or decreases during the observation
periods, cell counts were taken throughout the post treatment time
during which kinetic studies were conducted (Figs. 14 and 15). At
43.0°C cell populations remained constant at 30 hours post treatment
for the 25% survival level. For 75% survival, cell numbers began
to increase rapidly after 10 hours post treatment. This may explain
the sudden drop in percent cells in S at the 75% survival level in
Fig. 12. For 2.5% survival a 37% decrease in cell numbers occurred
by 30 hours after treatment.

At 42.5°C the cell population count remained constant for 9 hours
post treatment at the 75% survival level. At 25% survival, the cell
numbers did not change until more than 21 hours post treatment, while
for the 2.5% survival level, a 20% drop in cell numbers was observed

21 hours after treatment and a 20% increase in cell counts was noted
by 29 hours post treatment (Fig. 15).

A control growth curve indicates a population doubling time of
13-1/2 hours.

Figures 16-18 illustrate the difference in kinetic response seen
for 3 iso-survival levels achieved using either 42.5° or 43.0°C. For
all survival levels, the accumulation of cells in S occurs sooner for
the lower temperature exposure. Before the accumulation of S phase
cell starts to be seen, the FMF histogram appears to remain constant.
We have termed this interval which occurs prior to the discernible
kinetic response, an interval of general arrest.

Even when the clonogenic survival response to various exposure
conditions appears to be constant, the kinetic response may be different.
Figure 16 shows that for cells treated to a 75% survival level, those
treated at 43.0°C develop a maximum S region population 6 hours later
than those treated at 42.5°C. Figure 19 shows that this pattern is
consistent when temperatures are extended to include 42.0°C and 44.0°C.
The interval between peak S populations increases with decreasing
survival (i.e., 12 hours for 25% survival (Fig. 17) and 15 hours for
2.5% survival (Fig. 18)).

In summary, the FMF results indicate 1) For a constant temperature
the length of the general arrest increases for decreasing survival
(Figs. 12 and 13). 2) For iso-survival levels the length of the general
arrest increases with increasing exposure temperature (Figs. 16-18).
Data from post treatment cell counts indicate delays in cell population
growth that supports the FMF results.

B. AUTORADIOGRAPHIC STUDIES WITH DNA LABELS

1. Labeling Index

The labeling index (LI) for [3]HTdR was followed for 36 hours after
hyperthermic treatment. Two temperatures (42.5°C and 43.0°C) and 3
survival levels at each temperature (75%, 25%, and 2.5%) were evaluated.
The data indicated a post treatment kinetic pattern which closely
followed the FMF results.

The control LI was 49% ± 1%. Since the LI measures the fraction
of cells in S, this value is to be compared with a control FMF value
of 50%. Figure 20 shows that using 43.0°C exposure treatment, peak
LI values occurred 9 hours after treatment at 75% survival, 12 hours
after treatment for 25% survival, and 30 hours after treatment for
2.5% survival. In addition the LI was lower than control value for
the earlier time points.

When cells were treated at 42.5°C, the maximum labeling index
value was measured at about 6 hours post treatment for all survival
levels (Fig. 21). However, a biphasic response was observed for 25%
and 2.5% survival which can also be seen in the FMF data for these
survival levels and exposure temperature.

Iso-survival levels are compared in Figure 22. Once again, the
pattern can be observed that for equal survival response, the time
required for the LI to peak increases with increasing temperature.

That is, for conditions which yield equivalent clonogenic survival
responses, the kinetic response, as measured by autoradiographic label-
ing index, varies with temperature. On the other hand, for constant
temperature, the kinetic response varies with survival level.

2. Pulse Labeled Mitoses

PLM curves for control cells and cells treated at 43.0°C to 75% and 25% survival levels are illustrated in Figure 23. For the control population the maximum in the percentage of labeled mitoses occurred 3-1/4 hours after treatment; this period is generally taken to include G2+M. For treated cells this interval lasted 10-1/2 hours for cells treated to 75% survival and 14 hours for cells to 25% survival. In the case of treated cells we suggest that this interval represents G2 plus the interval of general arrest referred to earlier in regard to the FMF findings.

The duration of S, calculated as the time between the two points on the first wave where 50% of the rise in labeled mitoses has occurred, was 4-1/2 hours for controls, 5-1/4 hours for cells treated to 75% survival and 7-1/2 hours for cells treated to 2.5% survival.

The cycle time for control cells was 12-1/2 hours. This value compares favorably with the population doubling time of 13 1/2 hours obtained from growth curves. For cells treated to 75% survival the cell cycle time was 10-1/2 hours, not significantly different from the control value. However, at the 2.5% survival level, the duration of the first post hyperthermic cell cycle increased to 17 hours.

DISCUSSION

I. SURVIVAL DATA

The survival data for 9L cells indicate that loss of clonogenic potential occurs for temperatures of 42.0°C and higher (Fig. 2). The extent of cell killing is a complex function of both the exposure temperature and the exposure time.

In terms of the fraction of cells which survive exposure to hyperthermic conditions, 9L cells appear to be about 4 times more resistant than CHO cells[19] and as much as 60 times more sensitive than pig kidney cells.[61] When compared to Palzer and Heidleberger's data, 9L cells in our hands seem to be more resistant than Hela cells,[57] but the data of Gerner et al. would indicate that 9L cells are equally or more sensitive to hyperthermia than Hela cells.[85]

Another measure of cell sensitivity to hyperthermia is the rate at which cells are killed on the exponential region of the survival curve. That is, as the value for T_o decreases, sensitivity increases. In terms of this parameter, 9L cells again appear less sensitive than CHO cells and more sensitive than Hela or pig kidney cells.

The shape of the hyperthermic survival curve bears a superficial resemblance to radiation survival curves inasmuch as the shoulder and exponential regions are prominent features of both curves.   In fact, the parameters of the multitarget radiation model can be used to describe hyperthermic survival curves quite adequately (Table I) (Figs. 3 and 4). However, there are serious problems in the meaningful interpretation of these parameters for hyperthermic survival curves.

One difficult aspect in the interpretation of hyperthermic survival curves is that time at temperature, or, in other words, rate of cell killing, is not a function of energy transfer into or out of the culture system. The rate of energy transfer into the system is a function of the temperature difference between the culture system and the water bath according to the formula $\Delta Q = mc\ t$ where:

Q is energy transferred, in Calories;

m is the mass of the culture system (cells, flask, media, trapped air);

c is the heat capacity of the culture system; and

t is the temperature difference.

Since for all temperatures studied, thermal equilibrium at the growing surface of the flask is achieved within 5 minutes of immersing the culture flask into the water bath, the major energy transfer to the culture system is complete within these first few minutes. Since the rest of the experiment is conducted isothermally, there can be no further net transfer of energy except for that involved in chemical reactions. Yet significant cell killing does not occur until after exposure of 1 hour or more for temperatures up to $44.0^{\circ}C$. It is difficult to think of ΔQ having an effect other than to raise the internal energy of the culture system (according to the first law of thermodynamics $\Delta Q = \Delta U + W$ where ΔU represents the change in internal energy, and W represents work done). Yet somehow, there is a delay, on the order of hours, before the critical targets in the cell are affected by the increase in internal energy.

The increase in internal energy due to increased temperature most likely consists of increased kinetic energy and increased likelihood of molecules being in an activated state.[122] Both these factors result in increased reaction velocities which have an important effect on cellular biochemistry. In general, the speed of a reaction is doubled for each 10 degree rise in temperature. (It is interesting to note that mutation rates also double for every 10 degree rise in temperature). The rate of increase reaction velocity with temperature was theoretically derived by van't Hoff in 1884 (Ref. 59) and is given by

$$\frac{d(\ln k_1)}{dT} - \frac{d(\ln k_2)}{dT} = \frac{\Delta Q}{2T^2}$$

where k_1 and k_2 are the forward and backward reaction rates respectively, and T is the temperature. Rearranging the terms yields

$$d\left(\ln \frac{k_1}{k_2}\right) = \frac{Q}{2} \frac{dT}{T^2}$$

which is of the same form as the Arrhenius equation. It is surprising, and may well be significant, that in spite of the complex array of chemical reactions that are in process in a cell, the kinetics of cell killing by hyperthermia follow the kinetics of the Arrhenius equation (Figs. 4 and 24). In fact, ΔQ in the equation above can be correlated with μ, the activation energy. Then after integration

$$\ln(k) = \frac{\mu}{2}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

where, for cellular inactivation, k is the net reaction constant and represents the net activation energy for the sum of reactions which

cause cell death. Conveniently, ∴ can be calculated from the slope
of the Arrhenius curve.

In light of this Arrhenius type analysis, it is necessary to
address two problems. First. since the reaction rates increase as
soon as the system reaches thermal equilibrium (on the order of minutes),
how can the increased reaction rates lead to cell death which occurs
hours later? And second, how can the Arrhenius analysis, which was
developed for specific reactions, be interpreted meaningfully for
a cellular system?

The first question can be answered by considering the effect
of increased reaction rates on the chemistry of the cell. Pool sizes
for certain key precursors may begin to deplete. This depletion would
not occur instantly, but according to the complicated pathways that
surround it. For example, a pathway that builds the pool may still
be intact but the velocity constant for the reaction(s) that build
the pool may not have increased as much as the velocity constant for
the reactions that deplete the pool. Alternatively, or in addition,
toxins may begin to accumulate at a higher rate than at $37.0^{\circ}C$. And
proteins, nucleotides and membranes may undergo conformational changes
that prevent proper function. There may even be competition within
a specific reaction between an increased reaction constant and decreased
availability of the functional molecule due to denaturation. The
important point is that the velocity constants for the myriad reactions
in the cell may be affected to different extents, resulting in new
product concentrations, which may be lethal to the cell, but which
could take on the order of hours to achieve.

The problem of interpreting Arrhenius curves for biological systems has been a subject of controversy for 50 years and Johnson et. al. present a thorough discussion of the pertinent points. The Crozier theory encompasses most of the important points, as listed below from Johnson's book.[59]

(1) a catenary series of reactions, each with a definite val , temperature characteristic, or critical thermal increment, underlies every physiological process; (2) the overall process is limited, within definite ranges of temperature, by a slowest step or master reaction (3) at a certain critical temperature a different master reaction may assume control, and a sharp "break" will be apparent in the slopes of lines in an Arrhenius plot of the data; and (4) it might be possible to identify corresponding master reactions in different physiological processes.

The criticisms of Crozier's and similar theories center on the following concepts. First, the designation of which reaction is a master reaction is arbitrary, since the net results at all temperatures depend on contributory reactions. It may be more accurate to say that some reactions have more influence within a particular temperature range than others.

Second, the accuracy with which a break can be determined on the Arrhenius plot may not be great enough to associate any specific reaction with the shift in slope. Thus the contribution made to changes in the slope by differences in temperature characteristics for specific catenary reactions cannot be determined.

Lastly, the assumption that rate limiting reactions may be recognized by their measured μ values ignores the extent to which these values may vary as a function of their chemical environment (particularly

in interspecies comparisons). It may be that the variation in chemical environment can be largely controlled in cell culture.

In fact, for the cell culture system, the conformity of many cell lines to the Arrhenius relation and the large changes in the slope by a factor of 5 for 9L cells supports the assumption that the process of cell death by hyperthermia is predominantly limited by one set of reactions below $43.0^{\circ}C$ and by another predominant set of reactions above $43.0^{\circ}C$.

In addition, the sensitivity of many cell lines can be easily compared if composite Arrhenius graphs are made. For instance, at a glance, Fig. 24 shows that Chinese hamster lung cells are more sensitive to heat than Chinese hamster ovary cells. It is also readily apparent that the entire range of cell inactivation rates for any one temperature lies within a factor of 10.

Figure 23 shows the change in the shoulder width (T_q) and extrapolation number (n) with temperature. Ben-Hur and co-workers demonstrated that hyperthermia interferes with cellular ability to accumulate sublethal radiation injury[27,98] by reducing the shoulder on the radiation survival curve. And Elkind and Whitmore have associated the shoulder region of the radiation survival curve with accumulation of sublethal radiation damage.[103] To date, there has been no such interpretation for the shoulder of hyperthermia survival curves. It seems likely, however, that the shoulder region has some biological significance in terms of postponing the lethality of the hyperthermic insult.

II. KINETICS

Flow microfluorometry (FMF) and autoradiographic techniques were used to study the cell cycle kinetics when cells were returned to 37.0°C after treatment. The FMF data presented in Fig. 19 show a clear difference in the cell cycle progression of 9L cells treated at 42.0°C, 42.5°C, 43.0°C or 44.0°C although the fraction of surviving cells were chosen to be the same. Initially, cell progression through the cell cycle appeared to be stopped at all points in the cell cycle. This interval which we termed "general arrest", was followed by an accumulation of cells in S. We suspect that at the end of the general arrest, released cells progressed through the cell cycle until they reached a block in S which had not yet been released, causing cells to accumulate. The block in S took four times as long to appear at 44.0°C than at 42.5°C. When treated at 43.0°C the S phase block took 3 times as long to develop as when cells were treated at 42.5°C. Figures 16-19 show that the length of the general arrest increases with increasing temperature for constant surviving fractions. Figures 12 and 13 show that the general arrest also increases with decreasing survival achieved by use of a single temperature. The lower survival levels, however, do not appear to represent a selected population of non-growing cells, since the kinetics for a particular survival level, say, 2.5% survival level differ significantly depending on the temperature used. In fact, the duration of the general arrest for cells treated to 2.5% survival at 42.5°C is about equivalent to cells treated at 43.0°C to 75% survival.

Thus the length of the general arrest may constitute a more sensitive index of physiologic stress to the cell than simple reproductive mortality. The exact nature of the stress is open to speculation at this point. Some possibilities include, ·.pletion of important precursors due to altered rate kinetics or due to membranes which have become leaky, heat inactivation of key enzymes or other macromolecules, or acceleration of non-essential or damaging chemical reactions which compete with necessary reactions. The introduction of thermal noise which could interfere with the stereo spec·ficity of key biochemical events is not likely to be important for the 6 centigrade degree temperature shift considered in this research.

Following the release of the S phase block at 9 hours post treatment, the accumulated cohort of cells entered the G2+M phase. This is indicated for 43.0°C, 75% survival condition, in Fig. 11. It appears that the cells which had previously been in G2+M have also been released and have moved into G1 as indicated by the gradual increase in the percent cells in G1 beginning at 9 hours. This time correlates well with the time when the number of cells starts to increase (9-12 hours post treatment) (Fig. 14).

The pattern of an S phase block followed by progression to G2+M and G1 as a result of hyperthermic treatment has been observed for other cell lines.[67,69,70] We observed this same qualitative pattern for each exposure condition. The major qualitative difference we observed was in the length of the general arrest which varied quantitatively with exposure condition. The kinetics of cell redistribution

in the cell cycle may be clinically important in designing mixed
modality treatment schedules.

The labeling index (LI), measured by autoradiography, of cells
treated identically to those used for the FIF studies revealed almost
the same pattern of post treatment kinetics for cells in S. At 25%
survival it took 3 to 4 times as long for the S phase cell accumulation
to develop when 43.0°C was used rather than when 42.5°C was used.
That is, the general arrest increased with increasing temperatures,
for iso-survival levels. At 43.0°C, the general arrest increased
with decreasing survival level. In addition, the LI was considerably
below control values during the time of the general arrest. This
data suggests that the cells have actually stopped incorporating thymidine
during the interval of the general arrest. This could be due to the
same reasons speculated on above, during the discussion of the FMF
data.

The LI data for cells treated at 42.5°C also shows reduced numbers
of cells incorporating thymidine during the time of the general arrest.
The constant duration of the general arrest, as indicated by a maximum
incorporation of tritiated thymidine occurring at 6 hours post treatment
for all survival levels, may be associated with the location of this
temperature on the initial portion of the Arrhenius plot. The mechanism
of recovery from the general arrest may be different. The biphasic
response seen at 42.5°C but not at 43.0°C also indicates a different
dominant mechanism. Or the explanation may merely be that, at 42.5°C
the difference in duration of the general arrest is less than three
hours, while we took measurements at 3 hour intervals. The biphasic

response at 75% survival for 42.5°C may have been suppressed by the increase in cell numbers which occurs sooner for this exposure condition.

The pulse labeled mitoses (PLM) curves illustrated in Fig. 23 further confirm the existence of a state of general arrest which increases in duration as the severity of exposure increases. The interval between the end of the pulse and the first peak of labelled mitoses is usually taken to represent the G2+M phase of the cell cycle. However, with treated cells, the increase in this interval may be caused by the period of general arrest combined with the period of G2+M. Such a supposition is supported by the fact that the pattern of lengthening of the G2+M interval as measured in the PLM curves follows the pattern of lengthening of the general arrest as measured by FMF (Fig. 11) and LI (Fig. 21). That is, the general arrest increases with increasing severity of treatment.

It should be noted that the peak of the PLM curve, even in the case of the control, does not reach 100% labeled mitoses. We cannot completely explain this fact; however, we can speculate on a few contributing factors. First, there may be a variance in the cell cycle time among cells, since they are classified as a mixed gliosarcoma. There may be a subpopulation of cells in G2+M which are slowly released. In addition we counted both prophase and metaphase cells as mitotic which tends to yield a broader and lower peak than if only labeled metaphase are counted. However, the shape of the curves is definite enough to extract extensive information on the parameters of the cell cycle. The fact that the peaks are lower for experimental conditions than for control conditions may indicate that some cells die in mitoses

during treatment but continue to be observed as unlabeled mitotic
cells for the duration of the observation period.

The control cell cycle period of 12 1/2 hours is consistent with
the population doubling time of 13 1/2 hours. The population doubling
time is longer due to some cell death than would be expected in colony
growth. It is also expected that the duration of S will be shorter
when calculated from the PLM curve than when calculated from the FMF
histogram. This is because the FMF histogram includes all cells which
have a DNA content corresponding to S region cells while the LI and
PLM curves count only that subpopulation which is actively taking
up thymidine at a great enough rate at the time of the pulse to see
on the autoradiographs. Thus a cell with DNA content corresponding
to S phase cells but which is not taking up thymidine at the time
of the pulse will be counted as S by FMF but not by PLM analysis.

For the 2.5% survival conditions the PLM curve indicated a
lengthening of the cycle period by approximately 40%, along with a
concomitant increase in the duration of S. It appears that the cells
continue to grow approximately 40% more slowly than control cells
even after the general arrest has terminated. The mitoses which occur
at about 18 hours to not correlate with an increase in cell population
as shown in Fig. 14. It is possible that at this stage dead cells
begin to lyse at about the same rate that new cells are generated.
At 75% survival the first mitotic peak correlates well with an increase
in cell counts indicated in Fig. 11.

We have shown that 9L cells exhibit thermal tolerance (as evidenced
by a 2 to 6 fold increase in T_o) when treated with a split exposure

to 43.0°C temperatures. Thermal tolerance does not appear to be caused by redistribution of cells within the cell cycle in response to the initial hyperthermic treatment. First of all, our data shows that cells cease to progress during a general arrest period which, at most survival levels observed, is greater than the three hours that cells are allowed to spend at 37.0°C between treatments. Thus redistribution could not occur during this time. Secondly, when cells do begin to progress, they tend to accumulate in the S phase, which has been reported to be the most sensitive phase of the cell cycle to heat[19,55] and so would not be expected to exhibit resistant qualities. Studying other aspects of cell kinetics and thermal tolerance, Sapareto has also arrived at these conclusions.[69]

Treatment schedules which are similar to those used here have been reported to produce thermal tolerance in other cell lines.[2,69,85,87] Leith[2] has suggested that thermal tolerance (TT), defined as the ratio of the resistant mortality rate to the single exposure mortality rate, may be dependent mainly on survival level resulting from the initial exposure, with the thermal tolerance increasing as the initial survival level decreases. Figure 8 illustrates the extent to which 9L data matches other data Leith has plotted.[2] Although it is difficult to say whether the increase in TT is significant for 9L cells, it is suggestive that for initial survivals of 25%, 5%, and 1% treated at 43.0°C, the 9L data fits directly on the log-linear plot which represents thermal tolerance of Hela and CHO cells. At 75% survival the 9L data is outside the range initially considered in Leith's analysis and the thermal tolerance ratio sits considerably

off the line that the other points form. This variation may have a physiological basis in the fact that it is the only initial survival level taken well on the shoulder of a single exposure survival curve. On the other hand, the thermal tolerance ratio resulting from 1% initial survival level falls directly on the line formed by Hela and CHO cells thus extending the range of the log-liner relationship between S_i and TT.

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SURVIVAL CURVE PARAMETERS

Temperature (measured) °C	Slope, k, (95% Confidence) calculated by method of least squares min.$^{-1}$ ($\times 10^{-3}$)	T_O calculated $T_O = 1/k$ min.	n calculated for least square fit	T_Q calculated $T_Q = T_O \ln n$ min.
42.0	-3.13 ± 0.11	320	1.1	16
42.5	-20.5 ± 3.8	49	14	130
43.0	-59.5 ± 15.4	17	91	76
44.0	-107 ± 71	9.3	35	33.0
45.0	-178 ± 71	5.6	8.6	12

TABLE II

Percent Surviving Cells	Time at 42.5°C	Time at 43.0°C
75	1 hr 15 min	45 min
25	3 hr 15 min	1 hr 38 min
2.5	5 hr 7 min	2 hr 18 min

Times required to reduce cell survival to three levels using either 42.5°C
or 43.0°C.
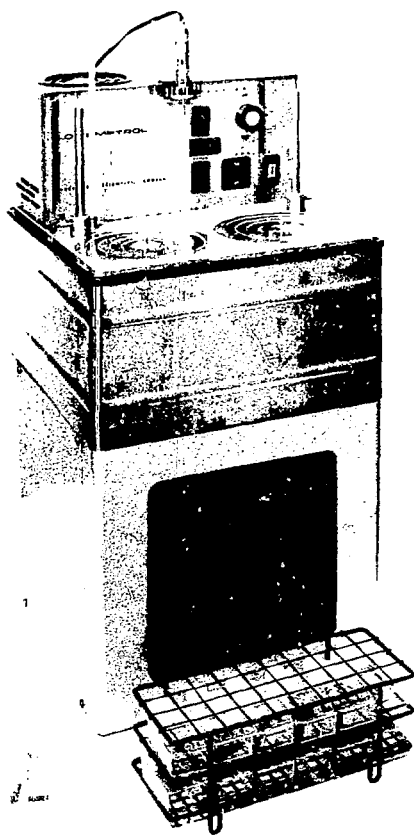
CBB 765-4328

Plate 1.   Water bath with culture flasks arranged
on test tube rack for immersion.
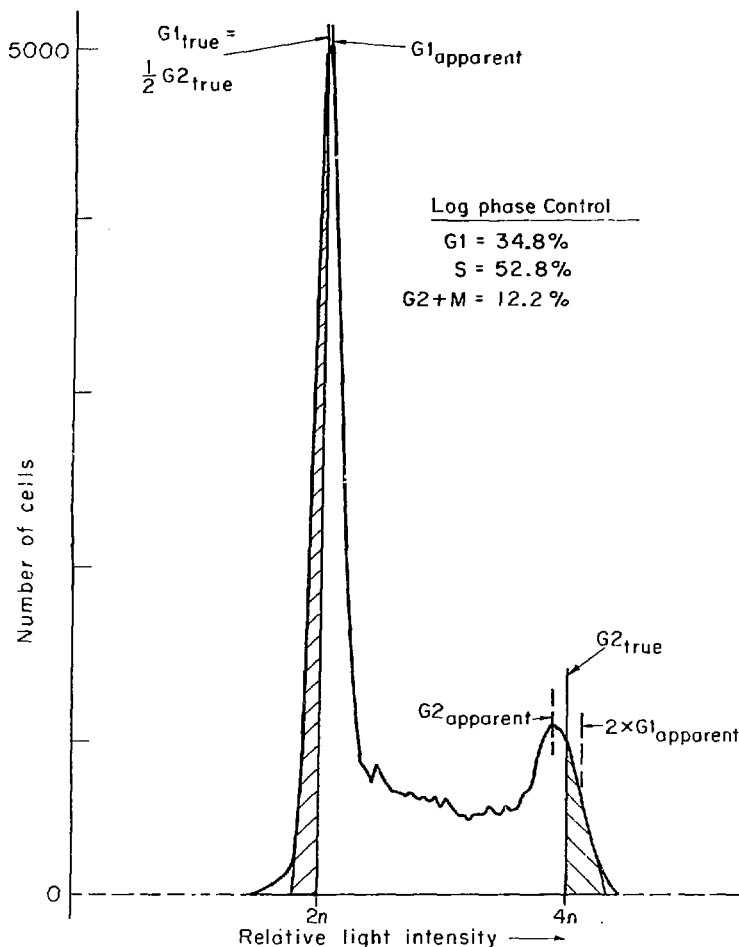
Fig. 1. Example of analysis of flow microfluorometry (FMF) .togram.
The area of the envelope representing the total cell population
is measured with a planimeter. The subpopulation, G1 and
G2+M are represented by twice the area of the appropriate
right triangle. The S population is represented by the
remainder when G1 and G2+M are subtracted from the total
population.
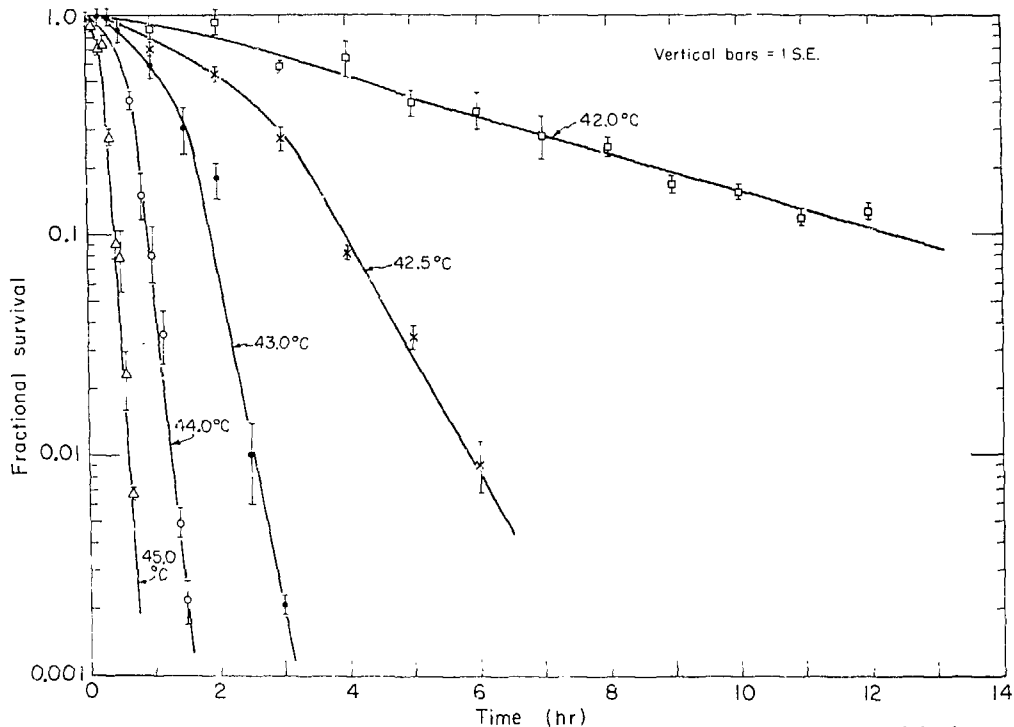
Fig. 2.   Survival of asynchronous, exponentially growing 9L cells exposed to different temperatures for varying lengths of time. Exponential portions of curve are fitted by the method of least squares. Standard error of the control is propagated through normalization.
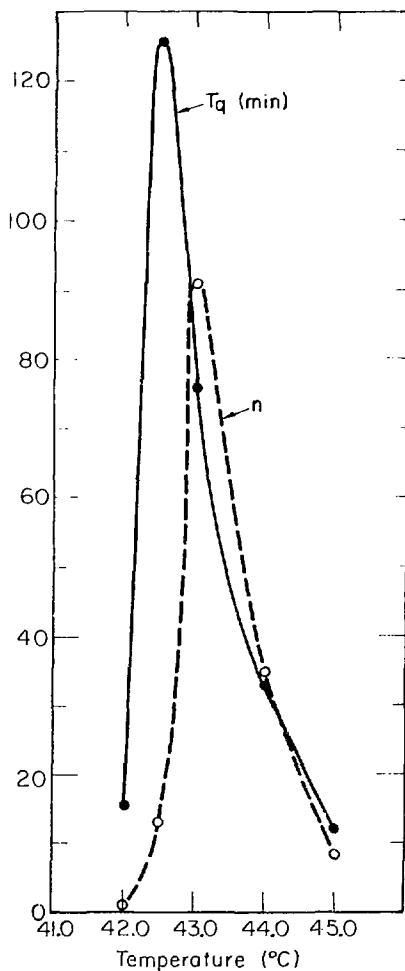
XBL775-3496

Fig. 3. Variation of T_q (min) and extrapolation
number "n" with temperature showing dis-
continuity at 42.5°C and 43.0°C, respectively.
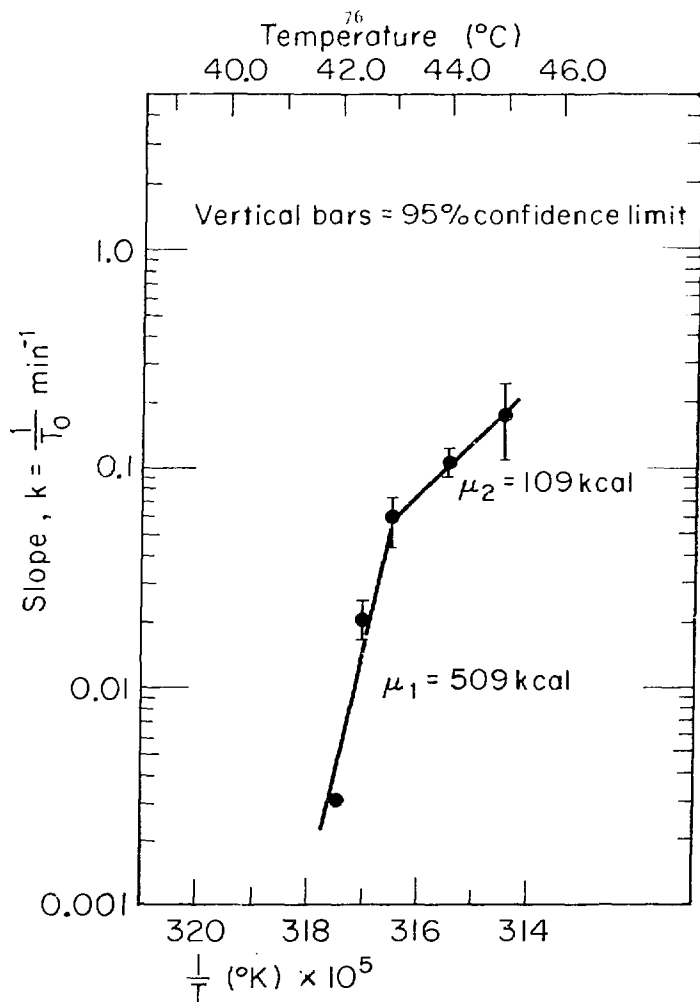
Fig. 4. Arrhenius analysis· Variation of slope (k = 1/T_o) with
temperature showing discontinuity at 43.0°C. Confidence
limit calculated from error in least squares determination
of slope.

XBL787-3337
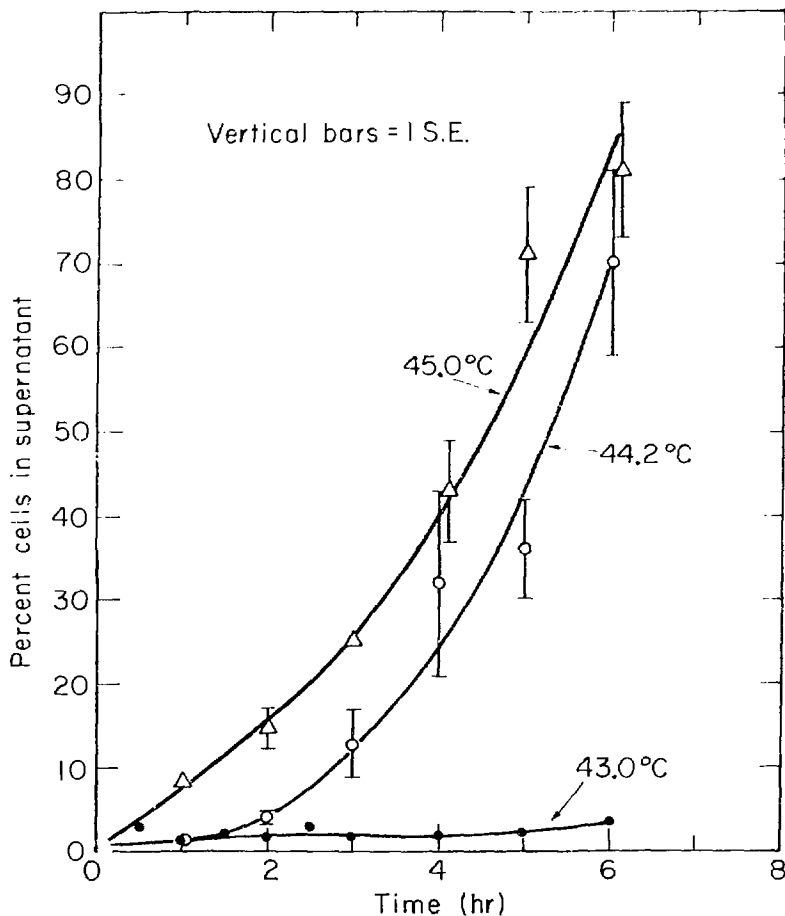
Fig. 5. Percent cells in the supernatant as a function of time at
temperature. The percent cells in the supernatant =

$$\frac{\text{\# supernatant cells}}{\text{\# supernatant cells} \div \text{\# attached cells}} \times 100.$$ Data is
normalized to the zero hour value.

XBL765-5501
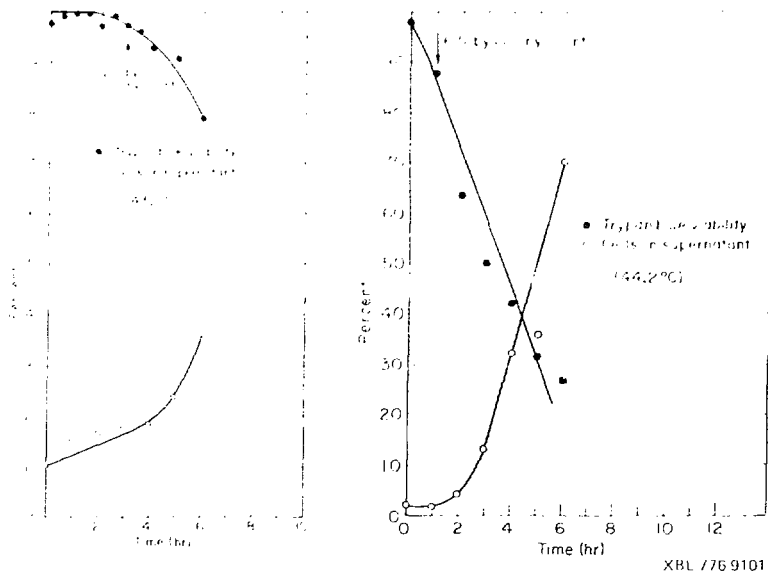
Fig. 6. Correlation between viability as measured with Trypan Blue and cell detachment from the growing surface of the flask, at 43.0°C and 44.2°C. Percent cells in the supernatant =

$$\frac{\#\ \text{supernatant cells}}{\#\ \text{supernatant cells} + \#\ \text{attached cells}} \times 100.$$

XBL 776 9101

THERMAL TOLERANCE
43.0°C
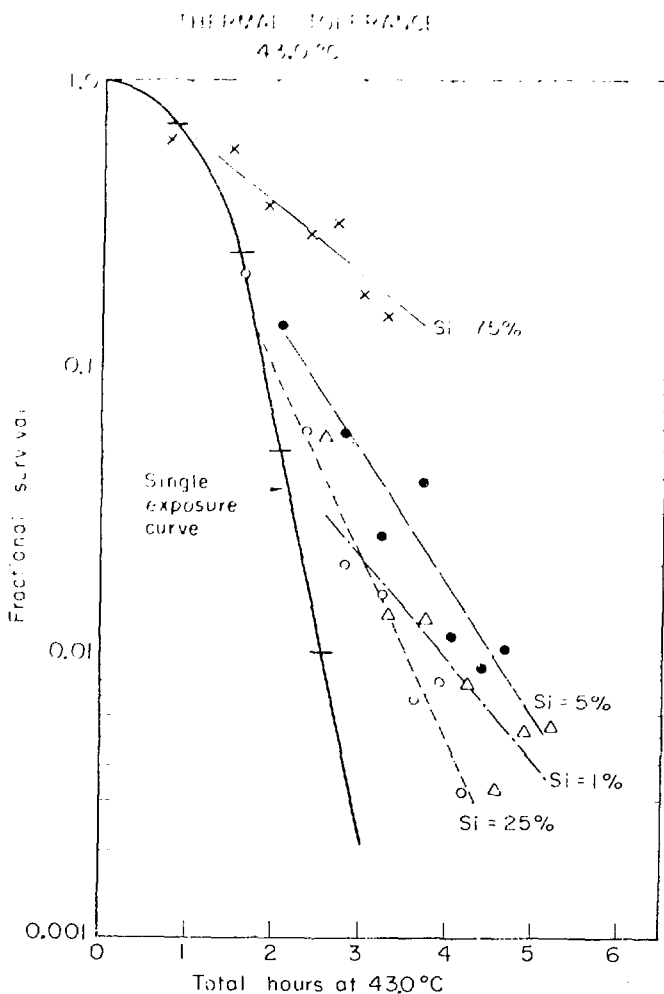
Fig. 7. Continuous curve represents the cell survival response to single exposure to 43.0°C. Other lines represent survival response when cells were returned to 37.0°C for 3 hours before receiving a graded second exposure. The initial exposure was calibrated to reduce survival to 75% (x), 50% (o), 5%(●), or 1% (Δ).
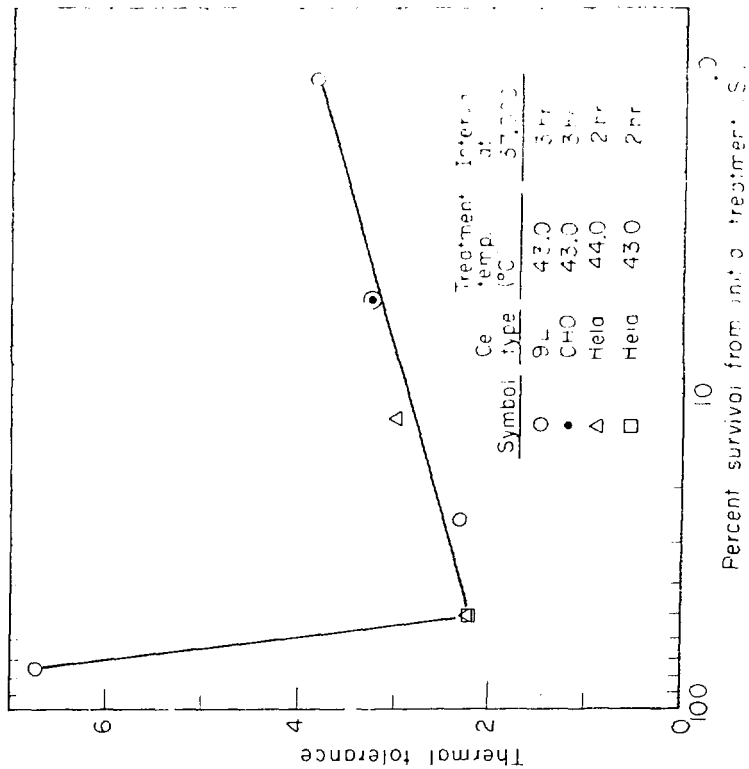
Fig. 8. Plot of the thermal tolerance ratio as a function of the first level resulting from the first exposure ... Hela Cells. See text for definition of thermal ...

The figure shows a plot with "Thermal tolerance" on the vertical axis (values 2, 4, 6) and "Percent survival from init. a treatment %" on the horizontal axis (logarithmic scale 0.1, 10, 100).

Symbol	Cell type	Treatment temp. (°C)	Treatment interval
○	9L	42.0	3 hr
●	CHO	43.0	3 hr
△	Hela	44.0	2 hr
□	Hela	43.0	2 hr
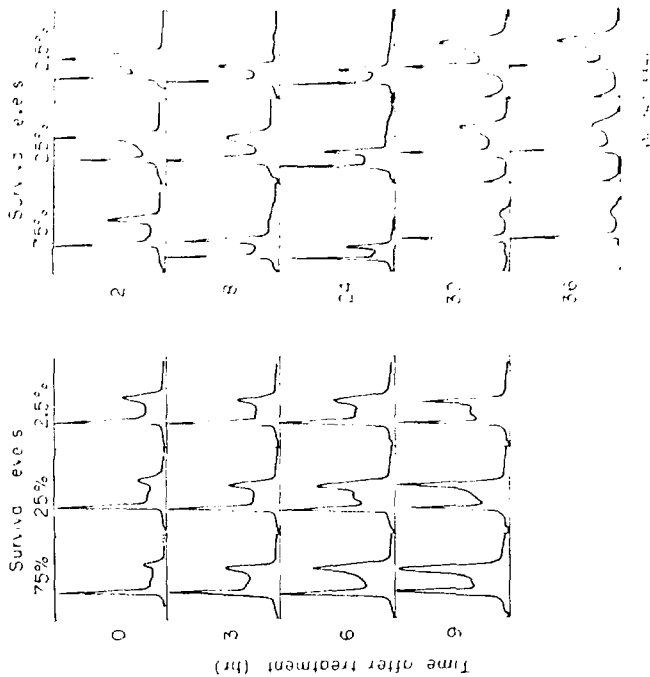
Fig. 9. Examples of DNA histograms obtained from PHA cultures of cells treated at 42.5°C. Columns represent a general increase [illegible] represent the amount of time cells were incubated at 37°C between treatment and fixation. The profiles are made up of cells which survive sooner for cells treated for [illegible] survival than [illegible] [illegible]
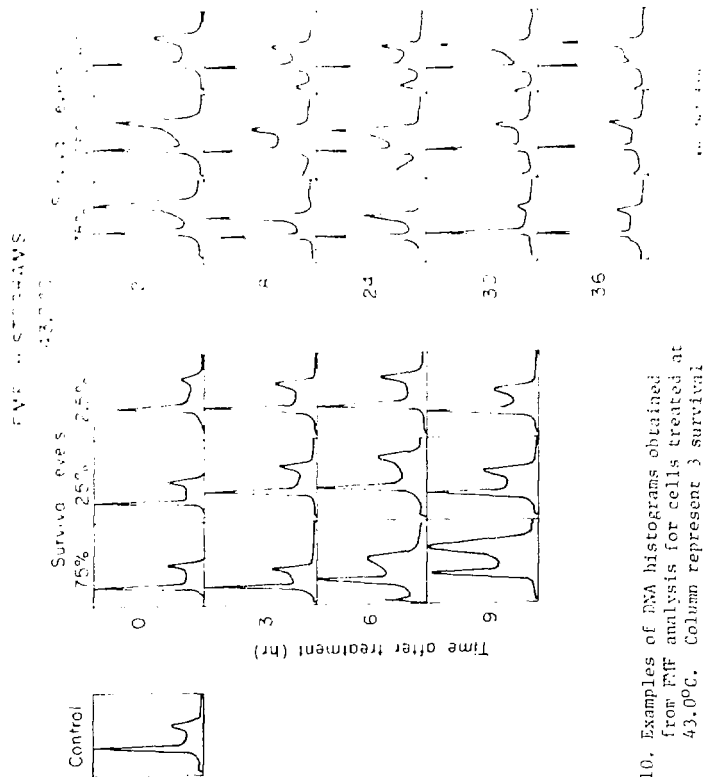
Fig. 10. Examples of DNA histograms obtained
from FMF analysis for cells treated at
43.0°C. Column represent 3 survival
levels. Rows represent the amount of time cells
were incubated at 37.0°C between treatment and fixation. [text unclear]
of cells appears sooner for cells treated to 75% survival than the lower
survival levels. Also, note that for each survival level, the accumulation in S
cells in S develops later at 43.0°C than at 42.5°C.

CELL CYCLE ANALYSIS

75% Survival
43.0 °C

---△ G1
——○ S
—·—● G2+M

Control values

Percent of cells

Hours after treatment
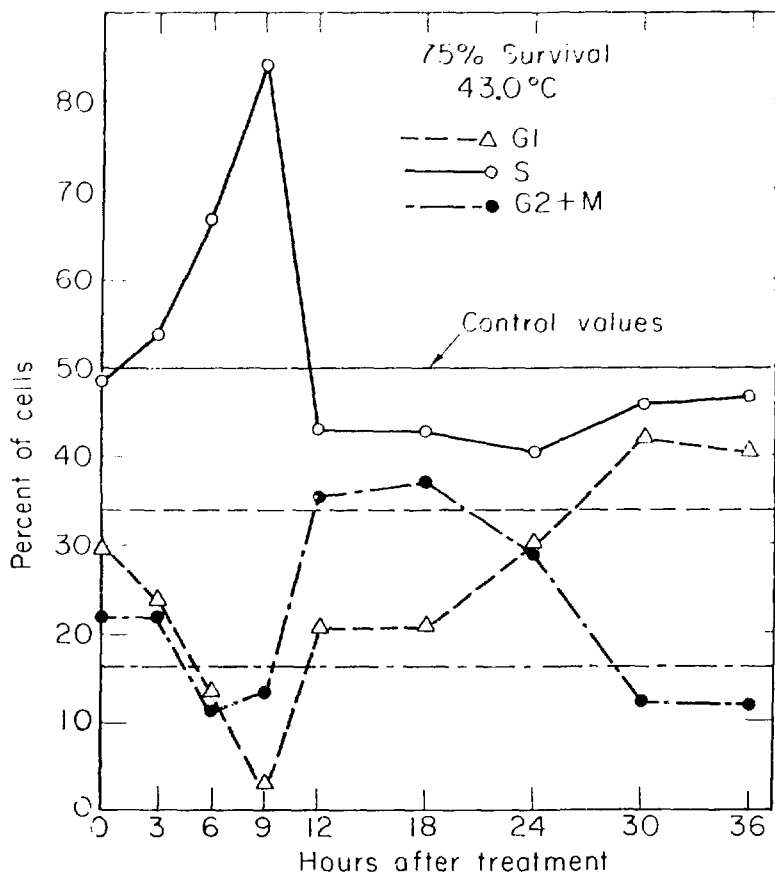
XBL7711-3979

Fig. 11.   Graphical result of planimeter analysis of cells treated to
75% survival at 43.0°C.   The cohort of cells accumulated in
S appears to move into G2+M and then into G1.   This pattern
was also observed for all other temperatures and survival
levels studied.

PLANIMETER ANALYSIS OF FMF HISTOGRAMS

43.0 °C

75% Survival

Control value

Percent cells in S

← 9hr →   25% Survival

← 6hr →

2.5% Survival

Hours after treatment
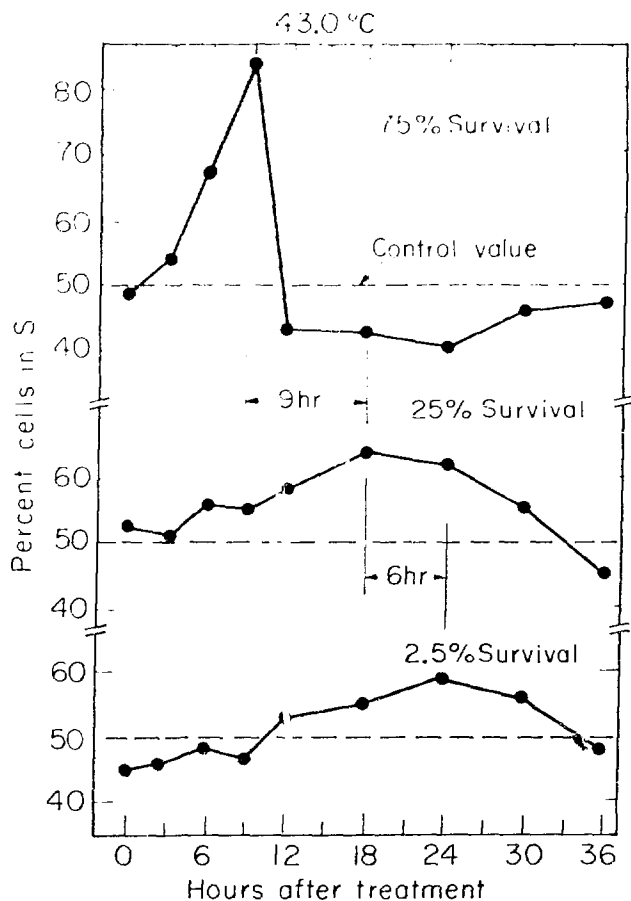
Fig. 12.   Comparison of the time it takes cells to accumulate in
S depending on the survival level to which they are
treated using 43.0°C. Notice that length of general
arrest increases with decreasing survival.
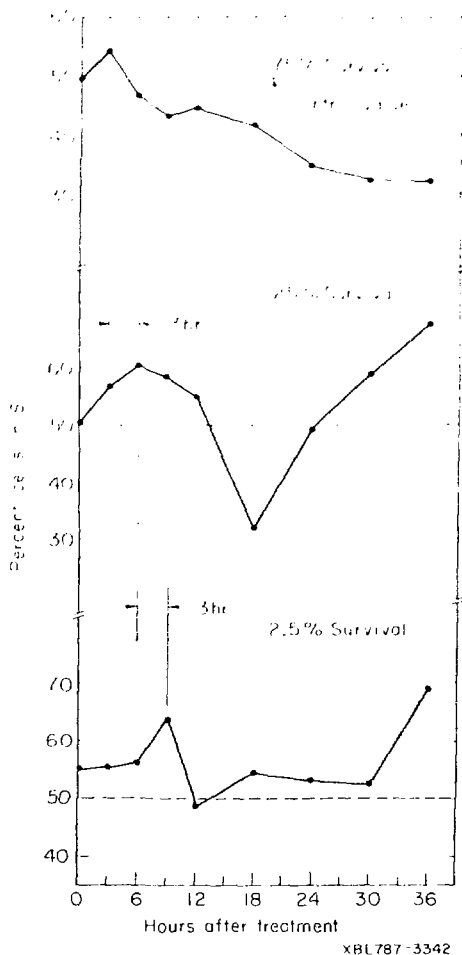
Fig. 13. Comparison of the time it takes cells to accumulate in
S depending on the survival level to which tye are
treated using 42.5°C. Notice that length of general
arrest increases with decreasing survival.
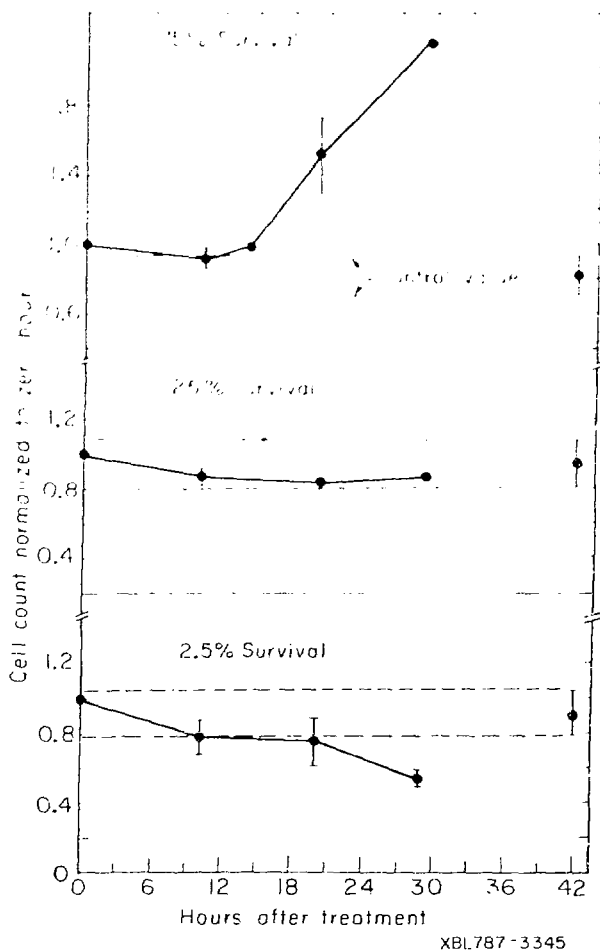
Fig. 14. Rate of increase in cell population with time at 37.0°C after treatment at 43.0°C to 75%, 25%, and 2.5% survival levels. Notice that length of growth delay increases with decreasing survival.

POST TREATMENT CELL COUNTS
42.5 °C
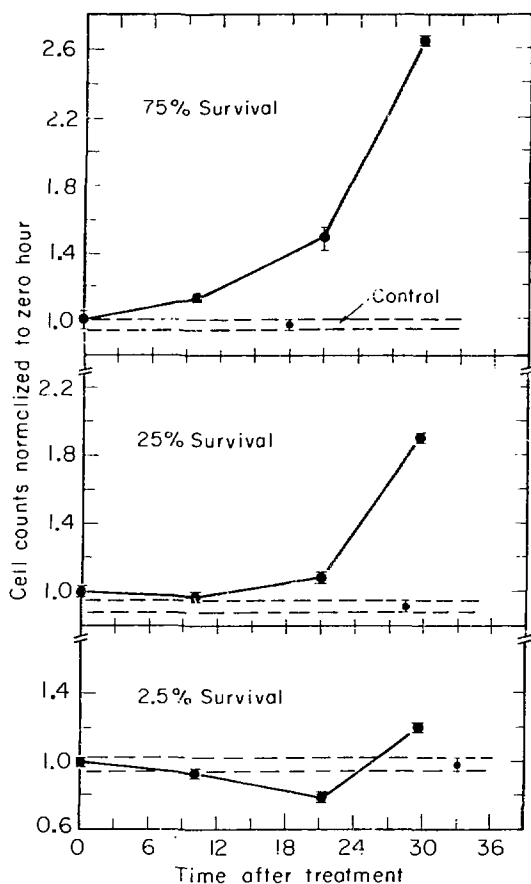
Fig. 15.   Rate of increase in cell population with time at 37.0°C
after treatment at 42.5°C to 75%, 25%, and 2.5% survival
levels.   Notice that length of growth delay increases
with decreasing survival.
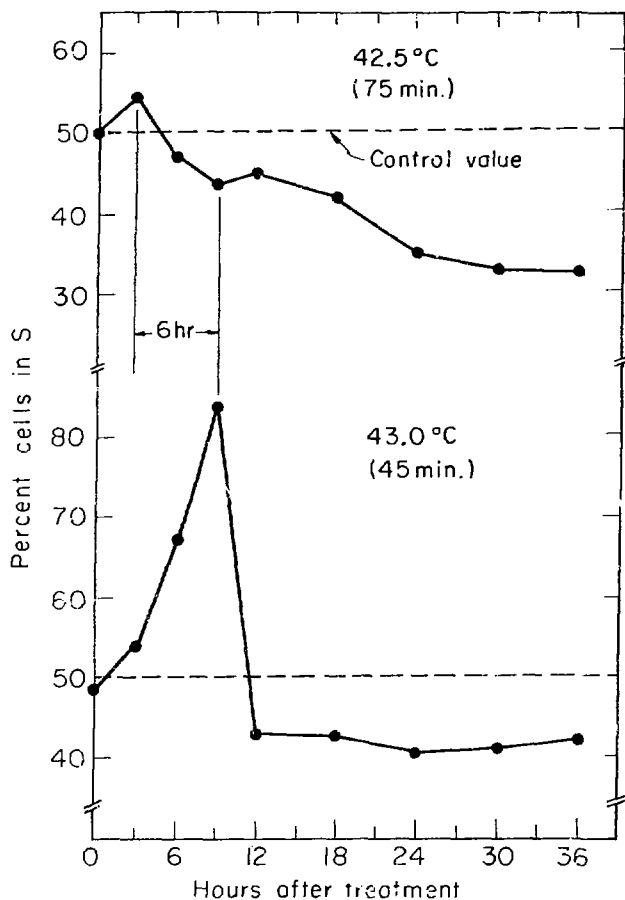
Fig. 16. Comparison of time it takes cells to accumulate in
S for iso-survival of 75% achieved by testing for
either 75 min at 42.5°C or 45 min at 43.0°C.

PLANIMETER ANALYSIS OF FMF
HISTOGRAMS
25% Survival
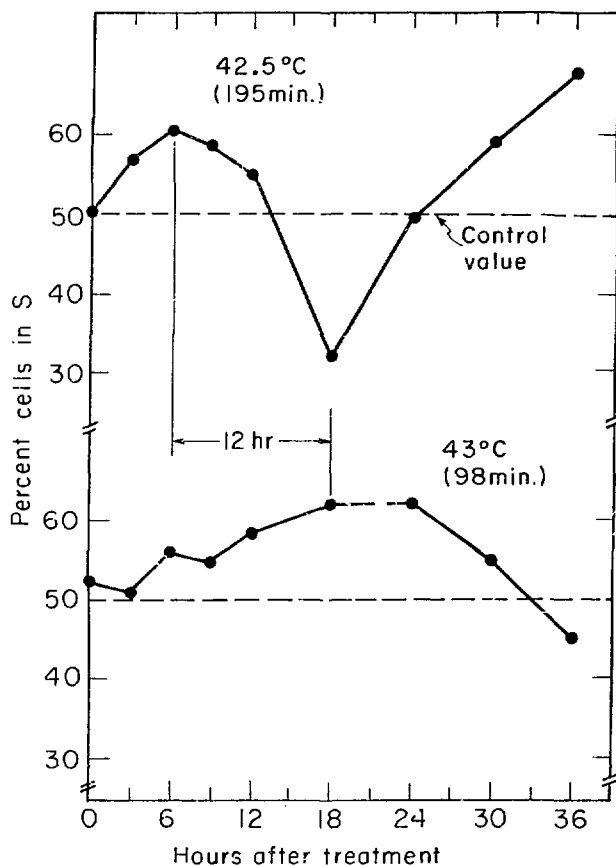
Fig. 17. Comparison of time it takes cells to accumulate in S
for ios-survival of 25% achieved either by treating
at 42.5°C for 195 min or at 43.0°C for 98 min.
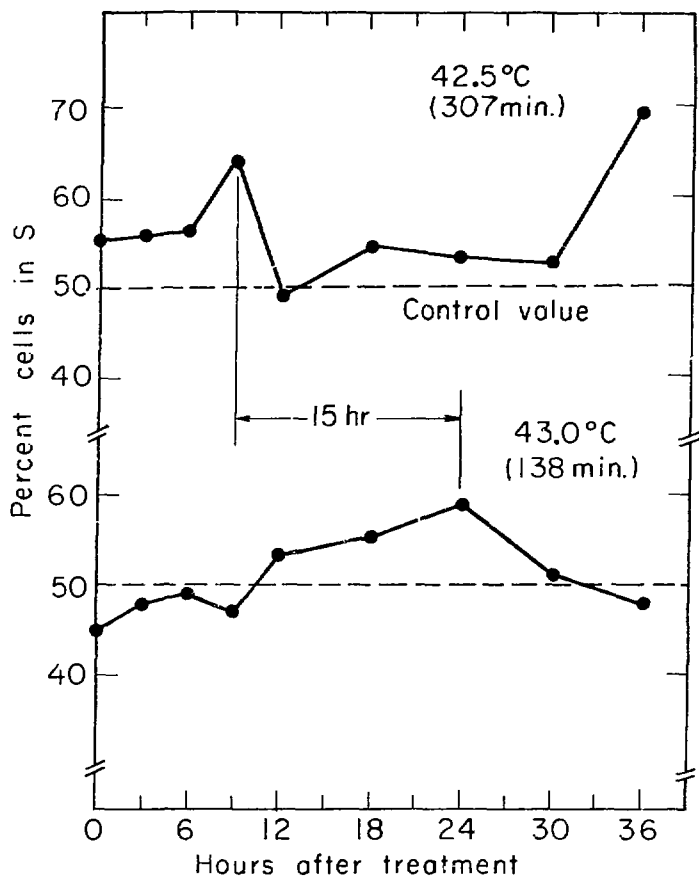
Fig. 18. *Comparison of time it takes cells to accumulate in S for iso-survival of 2.5% achieved either by treating at 42.5°C for 307 min or at 43.0°C at 138 min.*

PLANIMETER ANALYSIS OF FMF
HISTOGRAMS USING FOUR TEMPERATURES
25% Surviving
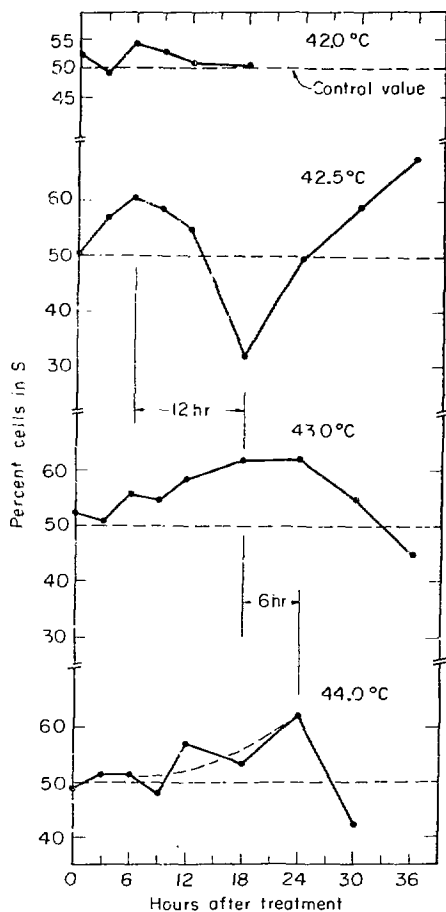
Fig. 19.   Illustration of the general trend that the length
of the general arrest increases with increasing
temperature for iso-survival.
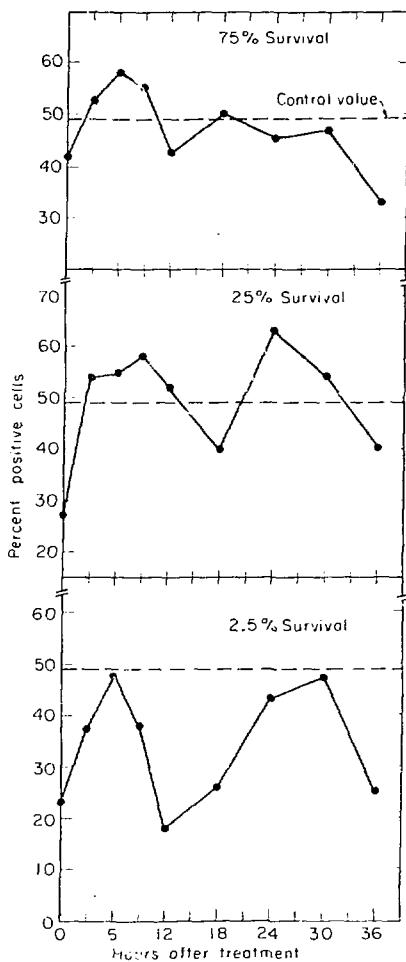
Fig. 20. Labelled population as a function of time at 37.0°C
after treating cells at 42.5°C to survival levels
of 75%, 25%, and 2.5%.
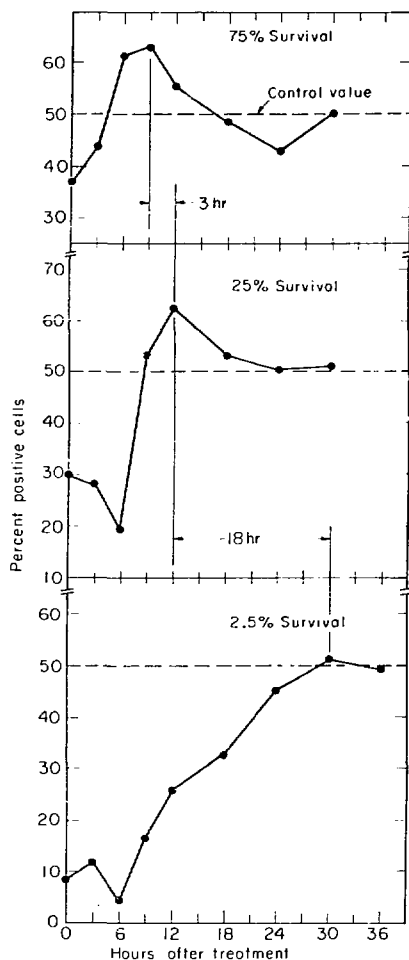
Fig. 21. Labelled population as a function of time at 37.0°C after treating cells at 43.0°C to survival levels of 75%, 25%, and 2.5%. The length of the general arrest increases with decreasing survival.
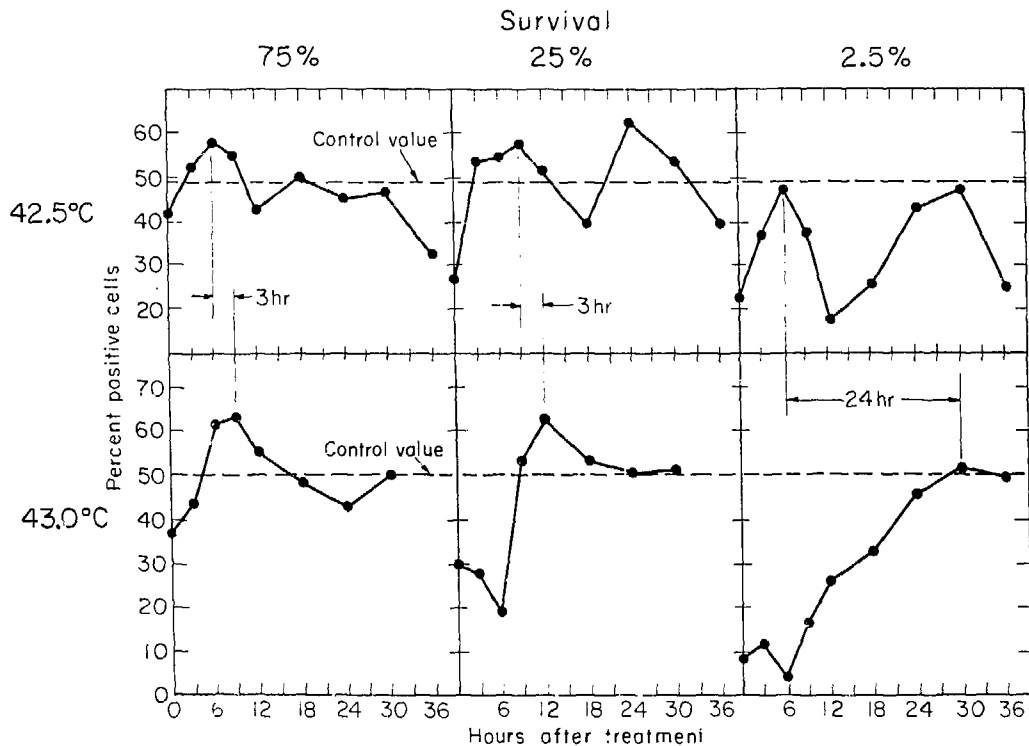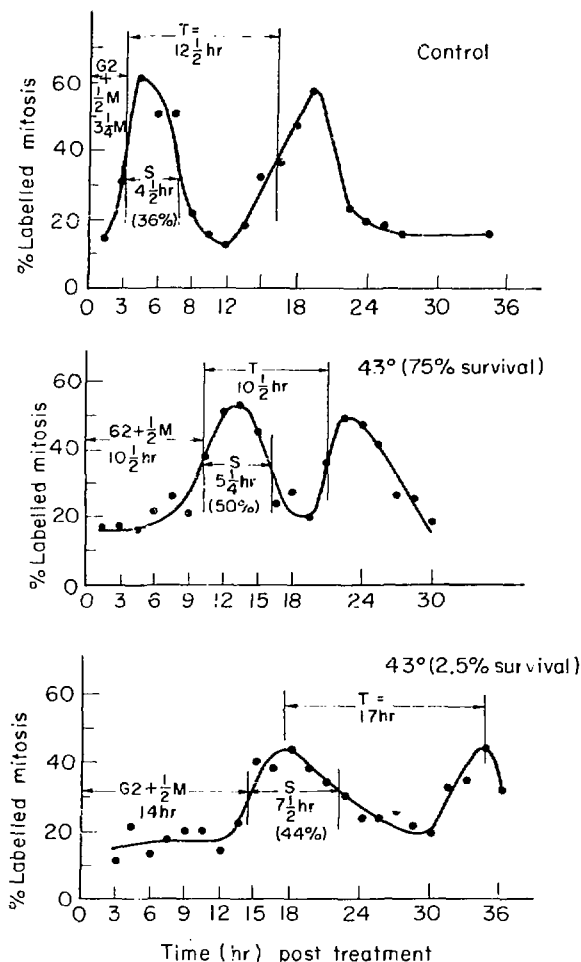
Fig. 22. Labelled populations for iso-survival at two temperatures. Notice that the length of general arrest increases with increasing temperature.

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XBL787-3379

Fig. 23. Pulse labelled mitoses curves for control cells and cells
treated at 43.0°C to 75% survival and 2.5% survival. The
progressive increase in the initial G2+M phase corresponds
to pattern in which the general arrest increases.
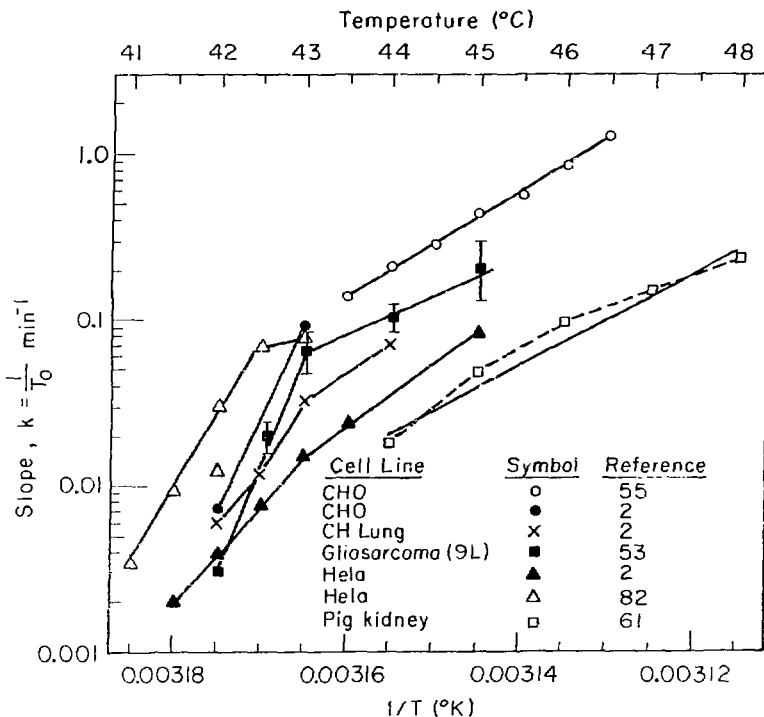
Fig. 24. Arrhenius plot for cell inactivation of several cell lines.
Approximately parallel slopes may indicate similar inactivation
mechanisms for many cell lines.