

Heat Fractionation and Thermotolerance: A Review¹

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Abstract

A rational approach to the design of clinical protocols combining fractionated hyperthermia plus X-irradiation or hyperthermia plus chemotherapy requires an understanding of the biology of fractionated heat alone. Mammalian cells growing *in vitro* can dramatically increase their tolerance to thermal damage (*i.e.*, reduce the cellular inactivation rate) after prior heat conditioning. Although the mechanism(s) for this cellular thermotolerance is still unknown, it is apparent that the thermal history, the heat fractionation interval, and the recovery conditions all modify significantly the degree of thermotolerance subsequently exhibited.

At the tissue level, the role of cellular thermotolerance is further complicated by host physiological mechanisms. Few data are available on heat fractionation *in vivo*, and the relative importance of physiological versus cellular effects remains to be defined.

Introduction

The potential benefit of hyperthermia in the treatment of human cancers has long been recognized, and several extensive reviews support this idea (5, 12, 13, 27). Renewed interest in hyperthermia research during the last few years stems largely from advances both in tissue culture assays and in the technology of heating animal tissues locally. Such interest has primarily revolved around the combined use of hyperthermia and X-irradiation or hyperthermia and chemotherapy either as single or multiple treatments. However, little is known about the biology of hyperthermia *per se* when applied in multiple fractions. This is unfortunate since clinically useful treatment protocols will depend, almost certainly, on fractionated regimens of heat alone or heat combined with other therapeutic modalities. In this regard, evidence for thermotolerance *in vivo* as it relates to fractionated hyperthermia protocols is important. Thermotolerance, as used here, is specifically defined as the reduced slope of the heat survival curve induced by prior heat conditioning (17, 26, 33). This review will be confined to human clinical data and to experimental studies of mammalian systems that are concerned with fractionated heat treatments to point out how little is actually known and to focus on areas that appear to have the most potential for future research.

In Vivo Studies

Clinical Experience. Clinical studies have frequently utilized hyperthermia delivered in multiple fractions, but the

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typical fractionation schedule followed was either an apparently arbitrary or random pattern determined by the individual tumor response. For instance, Pettigrew *et al.* (46) reported on the treatment of 51 patients with whole-body hyperthermia. The patients had a wide variety of tumors and were divided into 3 groups for different treatment protocols. One group was heated at weekly intervals for 4 hr to above 41°. A second group was initially heated for 90 min above 41° and then reheated similarly for 4 hr each on Days 3 and 6. A third group was heat treated like the second group but also received a bolus of a cytotoxic drug during the last treatment. Three of the 13 patients in this latter group had melanoma and were given melphalan, whereas the other 10 were given cyclophosphamide during the period of temperature rise and fluorouracil plus vincristine when their core temperature reached 41°. In this and similar studies, the lack of controls and the combination of hyperthermia with radiation, drugs, or surgery make it impossible to infer the relative efficiency of single versus fractionated heat applications (4, 40, 46, 57, 64). Observations such as the recurrence of heat-resistant tumors 3 months after hyperthermia treatments (46) or the diminution of the relative effectiveness of tumor heat treatments when given less than 7 days apart (49) are intriguing but only hint at the possible role of induced heat resistance in fractionated hyperthermia protocols and obviously do not differentiate between cellular and physiological effects.

Skin Studies. The first detailed thermal fractionation studies *in vivo* were published in 1947 by Moritz and Henriques. (42). Heating was produced by placing the open end of a brass cup onto the skin. Preheated water, which was pumped through the cup, maintained a constant temperature over the circular area of skin in contact with the hot water. They devised an epidermal scoring system for both human and porcine skin and reported that 9 min at 49° produced a complete and irreversible epidermal necrosis. In contrast, 2 heat fractions of 5 min at 49° each, separated by 240 min, caused only focal necrosis; and 3 fractions of 3 min at 49° each, with 120 min between each exposure, further reduced the injury to a severe vascular reaction without evidence of epidermal injury. Increasing the fractionation interval to either 4, 24, or 48 hr ameliorated the thermal damage to a mild vascular reaction. Similarly, 5 fractions of 2 min at 49° each, with either 30 or 60 min between fractions, resulted only in mild edema. The authors interpreted the reduction in injury as recovery from "latent" damage.

Hinshaw (35), in 1968, summarized his earlier work on the superimposition of 2 radiant burns. Pig skin was exposed to a 0.3-sec pulse from a 24-inch Army carbon arc search light, but the resulting skin temperatures were not given. The burns were scored histologically for the depth of dermal damage. A pulse of 2.04 cal/sq cm to the skin followed 1 min later by a second pulse of 5.86 cal/sq cm

produced skin damage, which extended from 0.2 to 0.4 mm into the dermis in 100% of the tests. Administration of the same 2 pulses 6 hr apart reduced the damage to a depth of 0.1 to 0.3 mm in 80% of the scored burns. The lack of sensitivity of this assay obfuscates the significance of these results.

Hahn *et al.* (23) used a 2-dose heat fractionation technique to determine tissue recovery of mouse tails after hyperthermia at 44°. One hr at 44° resulted in a 64% loss (*i.e.*, amputation) of the mouse tails but separating two 30-min fractions by 12 hr or more prevented the tail amputations. Also, 5 or 10 daily heat fractions of 30 min at 44° produced no apparent cumulative damage.

Experimental Tumor Studies. With the use of heat alone, in either single or fractionated applications, Allen (1) attempted to control Crocker sarcoma No. 39 tumors implanted in the rat tail with temperature-time combinations near the tolerance limit of the surrounding normal tissues. Rats were placed inside a heated cardboard box, but unfortunately only the air temperatures inside the box were regulated and documented. One of 16 tumors was controlled by a single heat treatment of 25 min at 74–79° (air temperature), whereas 2 other tumors were controlled by repeated heating, although each by a different treatment protocol. The small number of tumors treated with fractionated hyperthermia and the lack of intratumor temperature measurements limit the value of this study.

In 1963, Crile (9) published results on the response of S91 melanomas and Sarcoma 180's grown in the foot pads of mice. He used a water bath for producing hyperthermia and showed that heating for 90 min at 44° resulted in the complete destruction of the foot in 95% of the mice, whereas a pretreatment of 30 min at 44° 1 day prior to reheating (90 min at 44°) reduced the incidence of complete foot destruction to 13%. Similarly, the "cure" rate (defined as absence of tumor 3 weeks after treatment) for Sarcoma 180 was 80% following 30 min at 44°, but it decreased to 20% when the tumors were preheated 1 day earlier for 15 min at 44°. For both the implanted tumor and the normal foot, the induced heat resistance subsided by the second day after heating conditioning. On the third day after preheating, the foot loss was again 84% following 90 min at 44°, and the tumor cure rate returned to 80% after hyperthermia at 44° for 30 min.

Thrall *et al.* (62) implanted mouse mammary adenocarcinomas into the leg muscle and studied the heat-induced growth delay after heating at 44.5°, the highest temperature that did not result in the loss of the mouse leg. The tumor growth delay induced by 4 daily heat fractions of 15 min each at 44.5° was greater than that induced by a single heat treatment of 15 min at 44.5° but less than that that resulted from a single 39-min treatment.

In similar fractionation experiments on normal tissues, Suit (59) found that a single heat treatment of 173 min at 43.5° resulted in the loss of 50% of the heated mouse feet. If hyperthermia was given in equal daily fractions, the total heating time for 50% destruction of mouse feet was increased by a factor of 3.1 for 5 equal fractions and by 5.3 for 10 fractions. In other experiments (60),³ the effects of

heat fractionation on the TCD₅₀⁴ of a mouse fibrosarcoma were examined. The TCD₅₀ was the same for tumors either heated in a single treatment or treated with 2 equal heat fractions separated by up to 6 hr.³ However, separating the heat fractions by 22 hr increased the TCD₅₀ (control, 83 min at 43.5°) for 2, 5, and 10 fraction protocols by factors of 1.7, 2.8, and 4.4, respectively (60).

More recently, Overgaard and Suit (44) reported additional heat fractionation data both on the FSA fibrosarcoma grown in the feet of C3H mice and on the surrounding normal tissue. For temperatures between 42.5° and 44.5°, the TCD₅₀ increased by a factor of 1.2 to 1.4 as compared to a single heat treatment when 2 exposures were given 24 hr apart. Similarly, heat treatments of the skin given in 2 daily fractions increased the total heating time required for a skin response in 50% of the animals by about a factor of 1.3. This recovery ratio is much smaller than either the recovery ratios reported for cultured cells (see "In Vitro") or those for other *in vivo* systems discussed here. The difference could be due to physiological responses obviously absent in tissue culture or could result from the use of a conditioning heat treatment that is not conducive to the development of a high degree of thermotolerance (see "In Vitro Studies").

Experimental Whole-Body Studies. Wright (67) reported in 1976 that whole-body heating of mice also induced thermal resistance. He confined the animals to a constant-temperature box that was submerged in a water bath at 40.8° until he observed a thermoregulatory breakdown. Typically, the inadequate heat loss to the environment would raise the body temperature of the mice above the temperature of the water bath. In response, the mice attempted to thermoregulate until the whole-body temperature reached approximately 42°, when the thermoregulatory mechanism failed. Thereafter, the rectal temperature increased rapidly until the onset of convulsions near 44° and death. The temperature corresponding to the first sign of convulsions was defined as the CTM. Mice conditioned for 14 days at ambient temperatures of 15° had a lower CTM than mice acclimated for 2 weeks at 30°. When mice were conditioned acutely by confining them to the hot box until thermoregulatory breakdown (42°) either 24 or 48 hr before reheating, the time during which thermoregulation remained intact was increased by approximately 100%. However, the temperature for the onset of convulsions, the CTM, remained unchanged. By 72 hr after conditioning, the enhanced thermoregulatory capacity had returned to control levels. These data suggest that heat conditioning can also enhance physiological heat resistance. This factor may or may not have relevance to localized fractionated hyperthermia *in vivo*.

In summary, the few reports that provide experimental results on single *versus* multiple heat fractionation do demonstrate an altered heat response, *i.e.*, apparent resistance with heat fractionation. However, none of these studies was designed to distinguish either between tissue (*i.e.*,

of Sequence and Interval. Radiation Treatment Oncology Group Workshop, October 1976, Buffalo, N. Y. (Abstract).

⁴ The abbreviations used are: TCD₅₀, total treatment time at 43.5° that resulted in 50% tumor control; CTM, critical thermal maximum; dThd, thymidine; CHO, Chinese hamster ovary; TTR, thermotolerance ratio (see also Footnote 5).

physiological) versus cellular effects or between the cellular phenomenon of recovery from sublethal hyperthermic damage versus thermotolerance *per se*. A comparison of the biological effects of a single heat treatment with those resulting from a fractionated protocol is insufficient for evaluating these complex cellular phenomena. Invariably, the results from fractionation studies *in vivo* were interpreted in terms of cellular recovery from sublethal hyperthermic damage.

In Vitro Studies

Evidence for Induced Heat Resistance

In 1957, Selawry *et al.* (55) reported the induction of thermal resistance by multiple heat treatments in 3 lines of cultured human tumor cells (HeLa, HEP2, J96). Flasks containing 2×10^5 cells were heated from 7.5 to 15 hr at 42° during the first hyperthermic treatment followed by 6 to 11 additional heat fractions ranging from 24 to 168 hr at 42°. Heat treatments were separated by incubation periods at 36° and varied from 5 to 91 days. These treatments resulted in resistant sublines that survived higher temperatures, endured longer heating times, and recovered faster from heat-induced division delay than did unconditioned control cells. Resistance to hyperthermic treatment at 42–45° was also induced by growing cells continuously at elevated temperatures of 38–39°. The morphology of the heat-resistant cells did not differ from that of control cells.

Harris (28), in 1969, established heat-resistant sublines of pig kidney cells by isolating clones surviving either single or repeated heat treatments at 47°. The treatment times were 90 min long. The heat-resistant cells did not show abnormal morphology, cell size, growth rate, or chromosome patterns. The 46° heat survival curve of the thermotolerant subline 1375, which was isolated after 4 treatments of 90 min each at 47°, showed that the D_0 was increased by a factor of approximately 3 over that of the precursor cell line with little change in the extrapolation number. At least some degree of heat resistance remained after 24 passages (6 months at 37°) in 2 sublines with a gradual loss of resistance during that time. In contrast, a third resistant strain actually showed an increased heat resistance between passage 12 and 24. The appearance of resistant cells was independent of ploidy (29). Since such heat treatments reduced cell survival down to 10^{-5} to 10^{-6} , this procedure may well have selected for true mutants rather than a transiently thermotolerant cell population. At the molecular level, Reeves (47) found that "sensitive" pig kidney cells prelabeled with [^3H]uridine lost more acid-soluble uridine label through the plasma membrane during heat stress at 47° than did the so-called "resistant" pig kidney cells. The same differential was not observed when the cells were prelabeled with [^{14}C]dThd or [^{14}C]leucine. Macromolecular synthesis (incorporation of [^3H]dThd, [^3H]uridine and [^3H]valine) was equally inhibited in both cell lines; however, the duration of this inhibition was shorter and the subsequent rate of recovery, measured by cell number and precursor incorporation, was faster in the resistant line.

Palzer and Heidelberger (45) compared the killing of HeLa cells by single versus multiple heating. Two hr at 42°, in a

single treatment, reduced cell survival to 54%; while two 42° treatments of 1 hr each, separated by 6 hr, increased the surviving fraction to 70%. Longer fractionation intervals of 8 to 14 hr resulted in cell survival values between 60 and 65%. The authors attributed this apparent cellular recovery to the repair of sublethal damage. Similar fractionation experiments with CHO cells (33) resulted in a cell survival of 7.0% when 2 heat treatments of 17.5 min at 45° were separated by 12 hr. This compares to a 0.02% survival when the cells were given 1 exposure for 35 min at 45°.

Since these experiments used only a simple split-fraction protocol, it is not clear whether the observed heat resistance induced by heat conditioning was a result of recovery from sublethal heat damage or thermotolerance; however, these sets of data do suggest that the magnitude of resistance is dependent on the preheating conditions (*i.e.*, time and temperature) as well as the fractionation interval.

Development of Thermotolerance

Thermotolerance, *i.e.*, specifically the increase in D_0 , as defined earlier,⁵ can be induced both by heating for a short time at a higher temperature (>43°) followed by an incubation period at near-physiological temperatures (19, 33, 34) or by continuous heating at temperatures between 41.5° and 42.5° (12, 18, 20). In contrast, incubation of CHO cells at 40° for more than 2 generations resulted in no apparent cell lethality. Incubation at 40° for 7 hr did not alter significantly the slope of subsequent 45° heat survival curve, even through the D_q (the quasithreshold dose) was increased approximately 3-fold (34).

Acute versus Chronic Conditioning. The 45° survival curve of control CHO cells (*i.e.*, no preheating) is characterized by a D_0 of 3.3 min. However, if the cells were first preheated for 17.5 min at 45° and then returned to 37° for 12 hr before reheating at 45°, the 45° heat survival curve showed a D_0 of 14.5 min (Chart 1; Ref. 33). HeLa cells conditioned for either 0.5 or 1.0 hr at 44° and then tested at 44° increased their D_0 from 0.5 to 1.1 and 1.5 hr, respectively, after a incubation period of 2 hr at 37° (17). Continuous hyperthermia at 42° for up to 2.5 hr resulted in a D_0 of about 50 min (12, 20). For longer heating periods, the heat survival curve became biphasic, indicating the presence of a resistant cell fraction characterized by a D_0 of approximately 475 min (Chart 2). In contrast, a resistant population did not appear for continuous heating above 42.5°, at least for survival down to 10^{-4} . Harisiadis *et al.* (26) also reported the appearance of a resistant cell population when Chinese hamster V79 cells were heated at 42.5° for periods in excess of 3.5 hr. In the same study, they showed that V79 cells heat conditioned at 42.5° for either 3.5 or 7 hr immediately prior to hyperthermia at 45° increased the D_0 on the 45° survival

⁵ Thermotolerance, corresponding to a fractionation interval t , can be expressed in terms of a ratio of the D_0 of the heat survival curves:

$$\text{TTR}_t = \frac{D_0(t)}{D_0(0)}$$

where $D_0(t)$ is the D_0 of the heat survival curve obtained at time t (hr) after heat conditioning, and $D_0(0)$ is the D_0 of the unconditioned control survival curve. With multiple heat conditioning treatments, time t also refers to the time after the first conditioning treatment and TTR_{*n*} can be expressed as $\text{TTR}_1 + \text{TTR}_2 + \dots$ where $t = t_1 + t_2 + \dots$

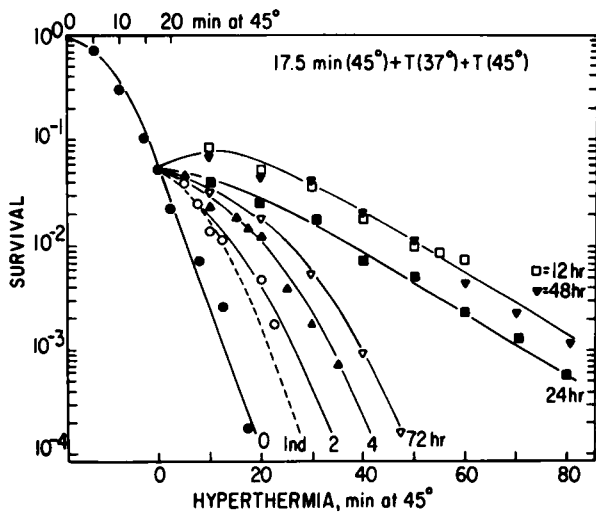


Chart 1. Development of thermotolerance in asynchronous CHO cells is illustrated by a series of survival curves obtained at various times after heat conditioning at 45° for 17.5 min. *Top abscissa*, duration of hyperthermia for the single treatment control curve; *bottom abscissa*, that for second treatment survival curves. The fractionation intervals are indicated in hr. The independent (*Ind*) curve represents the unconditioned control curve displaced downward for comparison (data from Ref. 33).

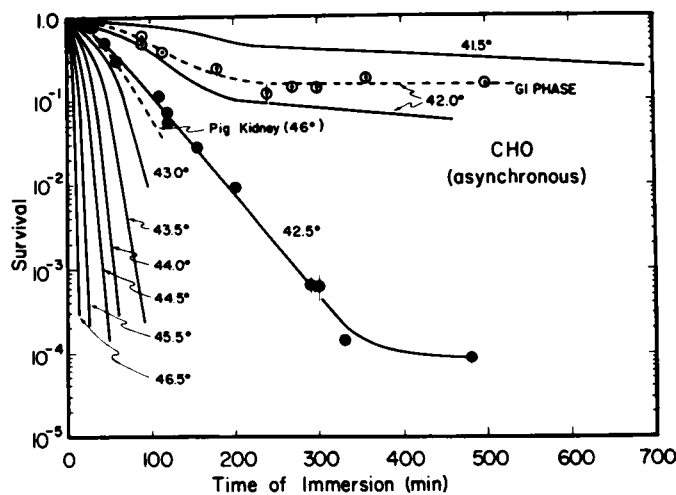


Chart 2. Development of thermotolerance in asynchronous CHO cells at 41.5-42.5° is illustrated by the appearance of biphasic survival curves during continuous hyperthermia. The survival curve for synchronized G₁ cells at 42° is similar to that for asynchronous cells [data with permission of the authors (12)].

curve from about 2 min (control) to 22 and 45 min, respectively. More recently, Bauer and Henle⁷ have induced "optimal" thermotolerance (See "Kinetics of the Development of Thermotolerance") in CHO cells by a treatment of 10 min at 45° plus 8 hr at 37°; then they reheated these tolerant cells at temperatures between 42° and 48°. One would expect that reheating maximally thermotolerant cells would establish simple exponential survival curves, with or without shoulders, and that the D_0 of these curves would reflect the degree of thermotolerance. However, the reheating of such thermotolerant cells resulted in biphasic survival

⁷ K. D. Bauer and K. J. Henle. The Relationship of Thermotolerance to the Arrhenius Inactivation Energy, submitted to *Radiation Research*.

curves in the range of 42-44°, indicating the presence of at least 2 subpopulations among these thermotolerant cells. Also, it is apparent that acute heat conditioning prior to chronic hyperthermia enables a group of cells to develop additional thermotolerance between 42° and 44° that is in excess of that induced by either acute or chronic conditioning alone. Although circumstantial, data from the above studies suggest that the thermotolerance induced by continuous heating at 42.5° may be casually related to the thermotolerance that follows acute conditioning at 45°. It is also possible that each effect is independent but capable of interacting with the other.

Kinetics of the Development of Thermotolerance. The kinetics of thermotolerance can be best analyzed in terms of a graph of the D_0 's as a function of the fractionation interval. Such a graph indicates that both the magnitude and the kinetics of the induction of thermotolerance are a function of the conditioning heat dose (Chart 3; Ref. 34). For instance, following a conditioning heat treatment of 10 min at 45°, the D_0 of the CHO hyperthermia survival curve increased from 3.3 to a maximum of 17.6 min at 45° in 8 hr, corresponding to a TTR_2 ⁵ of 5.3. For longer fractionation intervals, thermotolerance decayed at a rate of 0.13 min/hr of incubation at 37°. On the other hand, a conditioning treatment of only 5 min at 45° allowed a much faster development of thermotolerance. The maximum D_0 of 11.5 min, *i.e.*, $TTR_2 = 3.4$, occurred 2 hr after conditioning. Thermotolerance thereafter decayed at a rate of 0.24 min/hr of incubation at 37°. The 2 decay rates may be similar; however, considering the amount of data collected and the variation therein, this assumption must be tentative.

It is of interest to know when this thermotolerance is again abolished, *i.e.*, when the D_0 returns to control levels. However, this has not been studied in detail, mainly due to the technical difficulties incident to the resumption of cell proliferation following heat-induced division delay. To assay for single cell survival under these conditions requires either trypsinization and replating of cells that could selectively lyse heat-damaged cells (25) or the use of a multiplic-

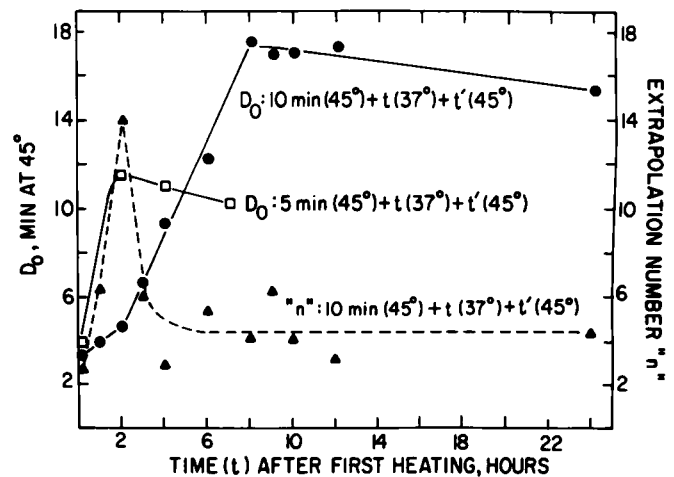


Chart 3. Kinetics of the development of thermotolerance in asynchronous CHO cells following heat conditioning at 45° for 5 or 10 min is shown in the plot of the D_0 of survival curves obtained with various fractionation intervals. - - -, corresponding extrapolation number, n , of survival curves after conditioning of 10 min at 45° (data from Ref. 34).

ity correction factor (56) that is open to question at high multiplicities. In spite of this uncertainty, a multiplicity correction factor has been used with CHO cells conditioned for 17.5 min at 45° (33). These heat-conditioned cells lost thermotolerance between 48 and 72 hr after conditioning. During this interval, the majority of surviving cells have entered the second division cycle after heat arrest. HeLa cells conditioned for 1 hr at 44° and subcultured 13 days later showed a heat response identical with that of unconditioned control cells (17). Also, Harisiadis *et al.* (26) reported that the biphasic survival curve at 42.5° reverted to a simple exponential curve 20 hr after an initial heat treatment of 4 hr at 42.5°. However, these data cannot be directly compared to our data, since the cells had been incubated at 17° for 20 hr and this temperature is known to modulate the kinetics of thermotolerance (31).

Modification of Thermotolerance during Recovery at 0–41°

In general it appears that the development of thermotolerance after heat conditioning at 45° requires a development period at near-physiological temperatures (17, 33, 34). Specifically, an incubation period of 7 hr at 37°, 39°, 40°, or 41° after conditioning of 10 min at 45° resulted in similar thermotolerance ratios, *i.e.*, TTR₇'s of 4.3, 4.2, 4.1, and 3.7, respectively (34). However, an equal development period at 20° reduced the TTR₇ nearly by 70% from that at 37° (31). The development of thermotolerance was fully inhibited when CHO (34) or HeLa (19) cells were stored at 0° following conditioning hyperthermia. This inhibition was reversible, and thermotolerance developed at the control rate when HeLa cells were returned to 37° following 2 hr at 0° (19). Also, CHO cells rendered thermotolerant by heat conditioning with 10 min at 45° and 8 hr of incubation at 37° did not lose thermotolerance during storage at 0° over a period of 2 days.⁶ The same degree of thermotolerance was achieved for the treatment sequence [10 min (45°) + 2 days (0°) + 8 hr (37°) + test heating (45°)] as for the treatment sequence [10 min (45°) + 8 hr (37°) + test heating (45°)].⁶ Thus, the development as well as the dissolution of thermotolerance can be arrested for long periods by cold storage (19).⁶

Even though incubation at 39–41° did not significantly inhibit the development of thermotolerance, survival *per se* was dramatically reduced by a parallel downward shift of the thermotolerant survival curves (Chart 4). The downward shift was proportional to the incubation temperature and was a factor of 300 at 41°. This sensitization did not occur when cells were allowed to recover at 37° prior to reheating at moderate temperatures of 42° (see "Acute versus Chronic Conditioning").⁷ It has been suggested that this additional lethality may be due to the fixation of sublethal hyperthermic damage that remained after heat conditioning (34). Thus, the mechanism(s) for protection of the preheated cells against further thermal damage is still functional when cells are sufficiently traumatized to reduce cellular survival.

Thermotolerance and Multiple Heat Fractionation

It is not likely that a 2-fraction hyperthermia protocol will be used in the clinic; thus, the development of thermotolerance following a multiple-fraction regimen has also been studied (2). Three heat fractions were used in the following

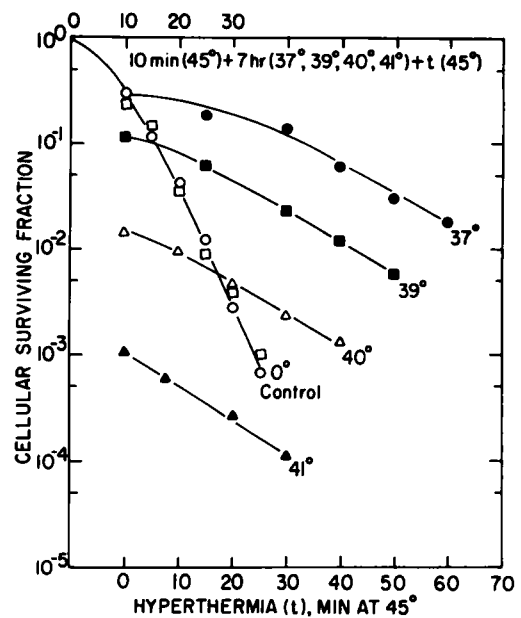


Chart 4. The effect of low hyperthermia (39–41°) and 0° on thermotolerance following 10 min at 45° and a 7-hr incubation period at the indicated temperatures (data from Ref. 34). *Top abscissa*, heating times for the control curve; *bottom abscissa*, heating times, *t*, for the second heat treatment, reflecting a conditioning treatment of 10 min at 45°.

sequences: 10 min (45°) + 4 hr (37°) + 15 min (45°) + 4 hr (37°) + test heating (45°); and 10 min (45°) + 4 hr (37°) + 30 min (45°) + 4 hr (37°) + test heating. In both cases TTR₄₊₄ was reduced to 3.4 as compared to a TTR₈ of 4.9 for single conditioning [*i.e.*, 10 min (45°) + 8 hr (37°) + test heating (45°)]. As mentioned above, the 8-hr incubation period resulted in maximal thermotolerance after a single conditioning of 10 min at 45°; for double conditioning, maximal thermotolerance also appeared 8 hr after the second conditioning treatment with a TTR₄₊₈ of 5.7; *i.e.*, double conditioning increased both the magnitude and the total development time required for maximal thermotolerance.

For clinical purposes, a 4-hr fractionation interval may be impractical, whereas 24-hr fractionation intervals are more likely to be considered. Therefore, we also examined the effect of daily conditioning heat treatments on the development of thermotolerance (2). After correcting for cell proliferation during the fractionation intervals, we found that the TTR₂₄ of 2.3, induced by a conditioning treatment of 10 min at 45°, remained near 2.0 when daily fractionation was continued up to 4 days. However, increasing the daily fraction to 20 min at 45° after the initial heating of 10 min at 45° increased the TTR₂₄ to 2.8 on the third and to 3.5 on the fourth day. Superficially, this suggests that the level of thermotolerance from multiple heat treatments is similar to that following a single conditioning treatment insofar as it is determined by both the magnitude of the fractions and the fractionation intervals. It can be more complicated, however, because increasing the heating time in the course of multiple heat fractionation may induce the cells to increase their level of thermotolerance.

Mechanism(s) of Thermotolerance Development

Recovery of Macromolecular Synthesis following Hyperthermia. Although the specific mechanism(s) for the induc-

tion of thermotolerance remains unknown, the appearance of maximal thermotolerance is correlated with the recovery of protein synthesis as measured by the incorporation of labeled amino acids into the acid-insoluble fraction 6 to 8 hr following conditioning of 10 min at 45° (31, 38). Postconditioning incubation in the presence of cycloheximide (either 1 or 10 µg/ml) reduced thermotolerance in proportion to the concentration of the drug. Specifically, the presence of 1 µg/ml during 7 hr of incubation at 37° reduced the TTR₇ from 6.5 (no drug) to 3.1 (39). This concentration of cycloheximide reduced the incorporation of tritiated amino acids to 25% of the control levels. Furthermore, the recovery of protein synthesis at 6 to 8 hr did not appear with chronic heat conditioning at 40°, and a single heat treatment at 40° did not induce thermotolerance (34). This would suggest that the development of thermotolerance requires the synthesis of new proteins.

The inhibition of protein synthesis by cycloheximide, either prior to (39) or during (45) hyperthermia, also desensitized cells to heat injury, reducing the slope of the heat survival curve. This type of thermotolerance could be an entirely different effect. Thermotolerance at the survival level induced by heat conditioning requires hours to develop, whereas the protective effect of cycloheximide occurs rapidly. In general, the inhibition of protein synthesis prior to heat treatment, either by cycloheximide (16, 39, 41), puromycin (16), essential amino acid starvation (48), or conditioning hyperthermia (39), reduced the inhibition of cellular protein synthesis as compared to heated control cells. In the presence of actinomycin D, the development of this resistance to heat disruption was blocked, and the authors suggested, therefore, that either a RNA molecule or a short-lived protein is synthesized that would mediate heat resistance by stabilizing the polysome (21), in particular the heat-sensitive initiation phase of translation.

The role of protein synthesis in thermotolerance remains obscure and is complicated by the lack of understanding of the relationship (if any) between polysome stabilization and thermotolerance assayed at the survival level after heat fractionation. Furthermore, the correlation between the resumption of protein synthesis and the development of thermotolerance after heat conditioning at 45° (39) may be entirely fortuitous, especially in the light of the finding that this correlation breaks down when hyperthermia at 45° is followed immediately by incubation at 40°. Under these conditions, thermotolerance developed fully (Chart 4), but the resumption of protein synthesis was delayed by an additional 12 hr.⁶

Incubation in the presence of hydroxyurea (1 to 10 mM) after 10 min at 45°, on the other hand, did not inhibit the development of thermotolerance (39). Furthermore, the recovery of DNA synthesis, as measured by the incorporation of [³H]dThd, which became apparent as early as 4 hr after conditioning, was accompanied by a long-term depression in the cellular rate of DNA synthesis. Even 30 hr after conditioning, DNA synthesis remained at only 30% of control levels, while the cellular labeling index was near that of unheated cells (31, 38). These findings suggest that

DNA synthesis is not directly involved in thermotolerance.

The recovery of RNA synthesis, as measured by the incorporation of [³H]uridine, followed rather than preceded the recovery of protein synthesis and did not occur until 8 hr after hyperthermia at 45° for 10 min (31, 38). This might be expected in the light of the work of Schochetman and Perry (53), who documented the reassembly of heat-disrupted polysomes (ribosomes and mRNA) upon return to 37° into functional units without the need for new mRNA synthesis. In any case, the recovery of RNA synthesis could not be correlated with the appearance of thermotolerance (31, 38), and the inhibition of RNA synthesis (50% of control) with lucanthone (5 µg/ml) failed to inhibit the development of thermotolerance (39). At this level of inhibition, the primary effect would be on rRNA synthesis which makes it difficult to assess the overall role of RNA synthesis in thermotolerance.

Cell Cycle Effects. The development of thermotolerance as a specific function of the cell cycle stage has not been studied in detail. However, Sapareto *et al.* (51) observed thermotolerance in terms of the development of a resistant cell population during continuous heating between 41.5° and 42.5° for both asynchronous CHO cells and synchronized G₁ cells (see also Chart 2). However, the capacity for development of thermotolerance in the heat-sensitive S-phase cells remains unknown.

Heat conditioning at 45° for 17.5 min killed more than 95% of asynchronous CHO cells; thus, the surviving cells had to be predominantly the relatively heat-resistant G₁ or G₂ cells (33), and these cells must be primarily responsible for the observed thermotolerance. During recovery at 37° and the appearance of thermotolerance, these cells could move only into a more sensitive phase of the cell cycle. Also, the incubation of cells in hydroxyurea following heat conditioning blocks the movement of G₁ cells into the S phase, and this did not inhibit the development of thermotolerance (26, 39). It is unlikely, therefore, that thermotolerance is the result of cellular redistribution within the cell cycle.

Proliferative Status and pH. The proliferative status and the nutritional status are both significant modulators of the heat sensitivity of mammalian cells (24, 37); however, little is known about their influence on the cellular capacity to develop thermotolerance. The relative sensitivity of both exponentially growing and plateau-phase cells appears to depend on several factors such as the cell type (24, 37), the viral transformation (37), and the cellular distribution within the cell cycle (37, 52). Preliminary results from this laboratory suggest little difference in the magnitude of thermotolerance that developed in exponential *versus* plateau-phase CHO cells 16 hr after heat conditioning at 45° for 10 min.⁶

Recent data by Gerweck (20) show that the thermotolerance that appeared with continuous heating of confluent CHO cells at 42°, pH 7.4, at an absolute survival of 10⁻¹ was no longer apparent when the pH was lowered to 6.7. However, recent work by M. L. Freeman (personal communication) indicates that, at 42° and pH 6.65, a biphasic part also appeared, but below a survival level of 10⁻⁴. These results indicate the complexity that could occur *in vivo* with large tumors that are more acidic than normal surrounding

⁶ K. J. Henle and D. B. Leeper. Recovery of Macromolecular Synthesis in CHO Cells following Hyperthermia at 45°. Modifications at 40°, submitted to *Cancer Research*.

tissues. Fractionated hyperthermia then could protect normal tissues relative to the tumor tissue since thermotolerance would not develop effectively in the latter.

Tonicity. In cell-free systems (8) and in bacteria (3, 43), thermal denaturation can be ameliorated by elevating the concentration of salts in solution. Attempts to mimic thermotolerance by incubation of CHO cells in hypertonic medium were unsuccessful. Instead, both normal and thermotolerant cells incubated in either hypo- or hypertonic medium were sensitized to thermal killing. Sensitization was optimal when heating commenced at the same time the cells were undergoing the rapid volume change associated with the altered tonicity of the extracellular medium (32). Sensitization by hypo- or hypertonic conditions were also observed by Hahn *et al.* (25).

Cell Membrane Lipid Composition. In 1924, even before it was established that cell membranes contained lipids, Heilbrunn (30) postulated that organisms adapt to temperature changes by altering their plasma lipid composition and that heat resistance was related to the melting temperature of the lipids. Today it is known that mammalian cells, during adaptation to growth at low temperatures, do decrease the degree of saturation of membrane lipids (15) and, conversely, that acclimation to high ambient temperatures is accompanied by an increase in saturated fatty acids. An altered composition of membrane lipids can also be achieved by controlling the proportion of saturated fatty acids in the diet, which in turn partially determine the physical state of the membrane as a function of temperature (15, 61, 66). The degree of fatty acid saturation has been related both to membrane fluidity and the temperature-activity characteristics of enzymes associated with the membrane. Numerous membrane-bound enzymes are functional only in a fluid-membrane environment (22), and their temperature-activity characteristics are apparently determined by the degree of unsaturation, or the fluidity of the membrane lipids, rather than by the enzymes themselves (61). Therefore, lipids may regulate physiological parameters either by their specific interaction with membrane proteins or by an alteration of the physical state of the membrane (54). In bacteria, thermal death has been correlated with membrane fluidity (14) or membrane lipid composition (68), but heat-induced membrane alterations in mammalian cells appear reversible and may not correlate with thermal death (47, 58). The Arrhenius plot of the diffusion coefficient for ascites tumor cell membranes did not show an inflection point in the range of 24–46° as does the Arrhenius plot for thermal killing. The data suggested an activation energy of 20 kcal/mol (58) which is much smaller than the activation energy for thermal cell inactivation (see "Thermodynamics and Arrhenius Plots"). Thus the relationship between the cell lipid composition and the heat-induced death of mammalian cells is still unclear.

Although a relationship between membrane lipid composition and long-term temperature acclimation has been known for some time (15), a recent finding by Li and Hahn⁹ also suggests a correlation of membrane lipids with thermotolerance. Exposure of HA-1 cells to ethanol followed by

incubation under physiological conditions in alcohol-free medium up to 20 hr induced tolerance to subsequent alcohol damage, tolerance to Adriamycin damage, and thermotolerance. Likewise, heat conditioning at 43° induced not only thermotolerance but also tolerance to alcohol damage.

Chromatin. Both DNA and chromosomal proteins are affected by hyperthermia above 40°; however, neither single-strand breaks, nor double-strand breaks (7), nor DNA base damage (65) are induced by hyperthermia alone, suggesting that DNA repair and thermotolerance are not closely correlated. However, *in situ* melting curves of Sprague-Dawley rat thymus cell DNA suggest that a thermolabile DNA portion separates into single strands at temperatures between 38° and 50° (10). These thermolabile DNA regions can be stabilized with NaCl (11) or with divalent cations (10). However, our failure to mimic thermotolerance in medium supplemented with NaCl, KCl, or CaCl₂ suggests that the thermolabile DNA is not directly involved in the development of thermotolerance (32).

The nonspecific adsorption of nonhistone chromosomal proteins to DNA following hyperthermia has been documented recently (50, 63). This adsorption is rapid as are the sensitization to radiation by hyperthermia (12, 33) and the inhibition of repair of radiation (5) and chemical damage (27). Furthermore, the reduction in the rate of micrococcal nuclease digestion of DNA in chromatin from heated HeLa cells suggests that the restricted access to DNA is responsible, at least in part, for the reduced radiation repair capacity of heated cells, but probably not for thermotolerance, which takes a longer time to develop (see Chart 3).

Thermodynamics and Arrhenius Plots. The Arrhenius plot for thermal inactivation of a variety of mammalian cells has an inflection point at 42.5–43.0° (6, 12). It is interesting that 42.5° also marks the limit where thermotolerance can be induced by continuous heating (51). The specific determination of the inactivation enthalpy below 42.4° is uncertain, since the calculated inactivation rate is based on a survival range of a decade at best (51) and the exponential and shoulder regions on the survival curves cannot be clearly distinguished. Nevertheless, the inactivation enthalpy below the inflection point appears approximately 2.5 times that above the inflection point (12, 51). The induction of thermotolerance by acute heat conditioning (10 min at 45° + 8 hr at 37°) shifted both the Arrhenius inflection point and the temperature limit for biphasic survival curves from approximately 43° to 45°. ⁷

The Arrhenius analysis for heat killing of thermotolerant cells suggests 3 thermodynamically distinct categories of tolerance. ⁷ (a) Thermotolerance developed during heating in excess of 3 hr at temperatures below 43° and expressed in terms of the larger D_0 on the biphasic survival curve (Thermotolerance 1) was characterized by an inactivation enthalpy, ΔH , of 420 kcal/mol and an inactivation entropy, ΔS , of 1265 cal/mol (°K). These are approximately 1.4 times the values calculated from the initial D_0 on the same biphasic survival curve [$\Delta H = 305$ kcal/mol; $\Delta S = 900$ cal/mol (°K)]. The greater values of ΔS and ΔH in Thermotolerance 1 could be interpreted as a greater structural order as well as increased internal bonding in tolerant cells. (b) After acute heat conditioning, the resistant subpopulation represented

⁹ G. C. Li and G. M. Hahn. Thermotolerance and Tolerance to Adriamycin Induced by Ethanol, submitted to *Nature*.

by the terminal portion of the biphasic survival curves that appeared below 45° (Thermotolerance 2) resulted in ΔH and ΔS not significantly different from those of nontolerant cells killed at temperatures below 43°. In this case, thermotolerance appeared to be directly associated with the shift from 43° to 45° in the inflection point on the Arrhenius plot. For Thermotolerance 2, therefore, cell killing proceeds at a high ΔH of approximately 300 kcal/mol. (c) Thermotolerance either to 45–48° (monophasic survival curves) or to 42–44°, in terms of the initial portion on the biphasic curve after acute conditioning (Thermotolerance 3), was characterized by a ΔS of 321 cal/mol (°K) that was reduced relative to that of control cells [$\Delta S = 351$ cal/mol (°K)] with no significant change in ΔH ($\Delta H \approx 125$ kcal/mol). The heat resistance of the strain of pig kidney cells isolated by Harris (28) was also associated with a reduced ΔS but an unchanged ΔH . Low ΔS values are often associated with reversible systems and may indicate that "critical targets" are not stabilized as in Thermotolerance 1 but rather that damage is more reversible. The Gibbs free energy, ΔG , for heat killing of both normal and all thermotolerant cells fell in the range of 19.6 to 22.6 kcal/mol. Values in this range have been described as indicative of protein denaturation (36). Thus, the "critical target" in the heat death of normal and tolerant cells may remain the same.

Discussion

The thermal history of cellular systems is an important determinant of their response to hyperthermia. Previous temperature studies of living organisms were limited primarily to microorganisms that are capable of growth over the temperature range of approximately 0–100°. In homeotherms, superficial tissues are routinely exposed to smaller temperature fluctuations, which are tolerated well when limited to relatively short periods. The large increase in heat resistance after heat conditioning, however, was unexpected for mammalian cells, and its underlying mechanism should be of great interest to basic cell biology. At present, however, the role of thermotolerance in the combined use of hyperthermia and radiation or chemotherapeutic agents in cancer therapy needs to be clarified. Existing clinical treatment protocols already call for the fractionation of combined hyperthermia and radiation or drugs with fractionation intervals of 72 hr. This fractionation interval is based on tissue culture data that may not be relevant at all at the tissue or organ level. The existence of thermotolerance *in vivo* is strongly suggested but has yet to be documented as a phenomenon separate from cellular repair or physiological factors. Furthermore, its role in the fractionation of hyperthermia combined with other therapeutic modalities is completely unknown. As with other therapeutic modalities, the final and most important clinical question is whether thermotolerance can be manipulated to produce a significant differential tumoricidal effect.

References

- Allen, F. M. Biological Modification of Effects of Roentgen Rays. II. High Temperature and Related Factors. *Am. J. Roentgenol.*, 73: 836–848, 1955.
- Bauer, K. D., Henle, K. J., and Dethlefsen, L. A. Induction of Thermotolerance in CHO Cells by Multiple Heat (45°C) Fractions. *Radiation Res.*,

- 70: 631, 1977.
- Beuchat, L. R., and Worthington, R. E. Relationships between Heat Resistance and Phospholipid Fatty Acid Composition of *Vibrio Parahaemolyticus*. *Appl. Environ. Microbiol.*, 31: 389–394, 1976.
- Birkner, R., and Wachsmann, F. Ueber die Kombination von Roentgenstrahlen und Kurzwellen. *Strahlentherapie*, 79: 93–102, 1949.
- Bronk, B. W. Thermal Potentiation of Mammalian Cell Killing: Clues for Understanding and Potential for Tumor Therapy. *Advan. Radiation Biol.*, 6: 267–324, 1976.
- Connor, W. G., Gerner, E. W., Miller, R. C., and Boone, M. L. M. Prospects for Hyperthermia in Human Cancer Therapy. Part II. Implications of Biological and Physical Data for Applications of Hyperthermia to Man. *Radiology*, 123: 497–503, 1977.
- Corry, P. M., Robinson, S., Getz, S. Hyperthermic Effects on DNA Repair Mechanisms. *Radiology*, 123: 475–482, 1977.
- Crabb, J. W., Murdock, A. L., and Ameluxen, R. E. A Proposed Mechanism of Thermophily in Facultative Thermophiles. *Biochem. Biophys. Res. Commun.*, 62: 627–633, 1975.
- Crile, G. The Effects of Heat and Radiation on Cancers Implanted on the Feet of Mice. *Cancer Res.*, 23: 372–380, 1963.
- Darzynkiewicz, Z., Traganos, F., Sharpless, T., and Melamed, M. R. DNA Denaturation *in situ*. Effect of Divalent Cations and Alcohols. *J. Cell Biol.*, 68: 1–10, 1976.
- Darzynkiewicz, Z., Traganos, F., Sharpless, T., and Melamed, M. R. Effects of 0.25 M Sodium Chloride Treatment on DNA Denaturation *in situ* in Thymus Lymphocytes. *Exptl. Cell Res.*, 100: 393–396, 1976.
- Dewey, W. C., Hopwood, L. E., Sapareto, S. A., and Gerweck, L. E. Cellular Responses to Combinations of Hyperthermia and Radiation. *Radiology*, 123: 463–474, 1977.
- Dietzel, F. *Tumor und Temperatur*. Munich: Urban and Schwarzenberg, 1975.
- Esser, A. F., and Souza, K. A. Correlation between Thermal Death and Membrane Fluidity in *Bacillus Stearothermophilus*. *Proc. Natl. Acad. Sci. U. S.*, 71: 4111–4115, 1974.
- Ferguson, K. A., Glaser, M., Bayer, W. H., and Vagelos, P. R. Alteration of Fatty Acid Composition of LM Cells by Lipid Supplementation and Temperature. *Biochemistry*, 14: 146–151, 1975.
- Fuhr, J. E., Overton, M., and Leisy, M. Protective Effect of Cycloheximide upon Protein Synthesis by L5178Y Cells Exposed to Hyperthermia. *Cytobios*, 11: 107–113, 1974.
- Gerner, E. W., Boone, R., Conner, W. G., Hicks, J. A., and Boone, M. L. M. A Transient Thermotolerance Survival Response Produced by Single Thermal Doses in HeLa Cells. *Cancer Res.*, 36: 1035–1040, 1976.
- Gerner, E. W., Costa, M., Fuller, J. M., and Russell, D. H. Biochemical Aspects of Hyperthermic Damage. The Effects of Elevated Temperatures on Two Enzymes Involved in Cell Proliferation. *Radiation Res.*, 70: 610, 1977.
- Gerner, E. W., and Schneider, M. J. Induced Thermal Resistance in HeLa Cells. *Nature*, 256: 500–502, 1975.
- Gerweck, L. W. Modification of Cell Lethality at Elevated Temperatures. The pH Effect. *Radiation Res.*, 70: 224–235, 1977.
- Goldstein, E. S., and Penman, S. Regulation of Protein Synthesis in Mammalian Cells V. Further Studies on the Effect of Actinomycin-D on Translation Control in HeLa Cells. *J. Mol. Biol.*, 80: 243–254, 1973.
- Grisham, C. M., and Barnett, R. E. The Role of Lipid-Phase Transitions in the Regulation of the (Sodium + Potassium) Adenosine Triphosphatase. *Biochemistry*, 12: 2635–2637, 1973.
- Hahn, E. W., Feingold, S. M., and Kim, J. H. Repair of Normal Tissue Injury from Local Hyperthermia. *Radiation Res.*, 70: 634, 1977.
- Hahn, G. M. Metabolic Aspects of the Role of Hyperthermia in Mammalian Cell Inactivation and Their Possible Relevance to Cancer Treatment. *Cancer Res.*, 34: 3117–3123, 1974.
- Hahn, G. M., Li, G. C., and Shiu, E. Interaction of Amphotericin B and 43° Hyperthermia. *Cancer Res.*, 37: 761–764, 1977.
- Harisiadis, L., Sung, D., and Hall, E. J. Thermal Tolerance and Repair of Thermal Damage by Cultured Cells. *Radiology*, 123: 505–509, 1977.
- Harkedar, I., and Bleehen, N. M. Experimental and Clinical Aspects of Hyperthermia Applied to the Treatment of Cancer with Special Reference to the Role of Ultrasonic and Microwave Heating. *Advan. Radiation Biol.*, 6: 229–266, 1976.
- Harris, M. Growth and Survival of Mammalian Cells under Continuous Thermal Stress. *Exptl. Cell Res.*, 56: 382–386, 1969.
- Harris, M. Mutation Rates in Cells at Different Ploidy Levels. *J. Cell Physiol.*, 78: 177–184, 1971.
- Heilbrunn, L. V. The Colloid Chemistry of Protoplasm IV. The Heat Coagulation of Protoplasm. *Am. J. Physiol.*, 69: 190–199, 1924.
- Henle, K. J. The Effects of Hyperthermia and Radiation Damage in Chinese Hamster Cells, *In Vitro*. Ph.D. Thesis, University of Pennsylvania Philadelphia, Pa., 1976.
- Henle, K. J., and Dethlefsen, L. A. Sensitization to Hyperthermia (45°C) of Normal and Thermotolerant CHO Cells by Anisotonic Media. *Radiation Res.*, 70: 632, 1977.
- Henle, K. J., and Leeper, D. B. Interaction of Hyperthermia and Radiation

- in CHO Cells: Recovery Kinetics. *Radiation Res.*, **66**: 505-518, 1976.
34. Henle, K. J., Karamuz, J. E., and Leeper, D. B. Induction of Thermotolerance in Chinese Hamster Ovary Cells by High (45°) or Low (40°) Hyperthermia. *Cancer Res.*, **38**: 570-574, 1978.
 35. Hinshaw, J. R. Early Changes in the Depth of Burns. *Ann. N. Y. Acad. Sci.*, **150**: 548-553, 1968.
 36. Joly, M. A Physico-chemical Approach to the Denaturation of Proteins, p. 207. New York: Academic Press, Inc., 1965.
 37. Kase, K. R., and Hahn, G. M. Comparison of Some Responses to Hyperthermia by Normal Human Diploid Cells and Neoplastic Cells from the Same Origin. *European J. Cancer*, **12**: 481-491, 1976.
 38. Leeper, D. B., Karamuz, J. E., and Henle, K. J. Hyperthermia-induced Alterations of Macromolecular Synthesis. *Radiation Res.*, **70**: 610, 1977.
 39. Leeper, D. B., Karamuz, J. E., and Henle, K. J. Effect of Inhibition of Macromolecular Synthesis on the Induction of Thermotolerance. *Proc. Am. Assoc. Cancer Res.*, **18**: 139, 1977.
 40. Mackenzie, A., McLeod, K., and Cassel-Smith, A. J. Total Body Hyperthermia: Techniques and Patient Management. *In: Proceedings of the International Symposium on Cancer Therapy by Hyperthermia and Radiation*, American College of Radiology, 1975, pp. 272-281.
 41. McCormick, W., and Penman, S. Regulation of Protein Synthesis in HeLa Cells: Translation at Elevated Temperatures. *J. Mol. Biol.*, **39**: 315-333, 1969.
 42. Moritz, A. R., and Henriques, F. C. Studies of Thermal Injury. II. The Relative Importance of Time and Surface Temperature in the Causation of Cutaneous Burns. *Am. J. Pathol.*, **23**: 695-720, 1947.
 43. Novitsky, T. J., and Kushner, D. J. Influence of Temperature and Salt Concentration on the Growth of a Facultatively Halophilic "Micrococcus" Sp. *Can. J. Microbiol.*, **21**: 107-110, 1975.
 44. Overgaard, J., and Suit, H. D. Hyperthermia *in Vivo*: Time-Temperature Relation and Split Dose Effect. *Radiation Res.*, **70**: 634, 1977.
 45. Palzer, R. J., and Heidelberger, C. Studies on the Quantitative Biology of Hyperthermic Killing of HeLa Cells. *Cancer Res.*, **33**: 415-421, 1973.
 46. Pettigrew, R. T., Galt, J. M., Ludgate, D. M., and Smith, A. N. Clinical Effects of Whole Body Hyperthermia in Advanced Malignancy. *Brit. Med. J.*, **4**: 679-682, 1974.
 47. Reeves, R. O. Mechanisms of Acquired Resistance to Acute Heat Shock in Cultured Mammalian Cells. *J. Cellular Physiol.*, **79**: 157-170, 1972.
 48. Reichman, M., and Penman, S. Stimulation of Polypeptide Initiation *In Vitro* after Protein Synthesis Inhibition *In Vivo* in HeLa Cells. *Proc. Natl. Acad. Sci. U. S. A.*, **70**: 2678-2682, 1973.
 49. Rohdenberg, G. L., and Prime, F. Effect of Combined Radiation and Heat on Neoplasms. *Arch. Surg.*, **2**: 116-129, 1921.
 50. Roti Roti, J. L., and Winward, R. T. The Effects of Hyperthermia on the Protein to DNA Ratio in Isolated HeLa Chromatin. *Radiation Res.*, **74**: 159-169, 1978.
 51. Sapareto, S. A., Hopwood, L. E., Dewey, W. C., Raju, M. R., and Gray, J. W. Effects of Hyperthermia on Survival and Progression of Chinese Hamster Ovary Cells. *Cancer*, **38**: 393-400, 1978.
 52. Schlag, H., and Luecke-Huhle, C. Cytokinetic Studies on the Effect on Hyperthermia on Chinese Hamster Lung Cells. *European J. Cancer*, **12**: 827-831, 1976.
 53. Schochetman, G., and Perry, R. Characterization of the Messenger RNA Released from L Cell Polyribosomes as a Result of Temperature Shock. *J. Mol. Biol.*, **63**: 577-590, 1972.
 54. Schroeder, F., Holland, J. F., and Vagelos, P. R. Physical Properties of Membranes Isolated from Tissue Culture Cells with Altered Phospholipid Composition. *J. Biol. Chem.*, **251**: 6747-6756, 1976.
 55. Selawry, O. S., Goldstein, M. N., and McCormick, T. Hyperthermia in Tissues-Cultured Cells of Malignant Origin. *Cancer Res.*, **17**: 785-791, 1957.
 56. Sinclair, W. K., and Morton, R. A. X-ray and Ultraviolet Sensitivity of Synchronized Chinese Hamster Cells at Various Stages of the Cell Cycle. *Biophys. J.*, **5**: 1-25, 1965.
 57. Stehlin, J. S. Regional Hyperthermia and Chemotherapy by Perfusion. *In: Proceedings of the International Symposium on Cancer Therapy by Hyperthermia and Radiation*. American College of Radiology, 1975, pp. 266-271.
 58. Strom, R., Santoro, A. S., Crifó, C., Bozzi, A., Mondovi, B., and Rossifanelli, A. The Biochemical Mechanism of Selective Heat Sensitivity of Cancer Cells. IV. Inhibition of RNA Synthesis. *European J. Cancer*, **9**: 103-112, 1973.
 59. Suit, H. D. Hyperthermia in the Treatment of Tumors. *Proceedings of the International Symposium on Cancer Therapy by Hyperthermia and Radiation*. American College of Radiology, 1975, pp. 107-114.
 60. Suit, H. D. Hyperthermic Effects on Animal Tissues. *Radiology*, **123**: 483-487, 1977.
 61. Tanaka, R., and Teruya, A. Lipid Dependence of Activity-Temperature Relationship of (Na⁺, K⁺)-activated ATPase. *Biochim. Biophys. Acta*, **323**: 584-591, 1973.
 62. Thrall, D. E., Gillette, E. L., and Bauman, C. L. Effect of Heat on the C3H Mouse Mammary Adenocarcinoma Evaluated in Terms of Tumor Growth. *European J. Cancer*, **9**: 871-875, 1973.
 63. Tomasovic, S. P., Turner, G. N., and Dewey, W. C. The Effect of Hyperthermia on Non-Histone Protein Isolated with DNA. *Radiation Res.*, **73**: 535-552, 1978.
 64. Von Ardenne, M. Selective Multiphase Cancer Therapy: Conceptual Aspects and Experimental Basis. *Advan. Pharmacol. Chemotherapy*, **10**: 339-380, 1972.
 65. Warters, R. L., Winward, R. T., and Roti Roti, J. L. Effects of Hyperthermia on Chromatin Composition and Structure. *J. Cell Biol.*, **75**: 126a, 1977.
 66. Williams, R. E., Wisnieski, B. J., Rittenhouse, H. G., and Fox, F. C. Utilization of Fatty Acid Supplements by Cultured Animal Cells. *Biochemistry*, **13**: 1969-1977, 1974.
 67. Wright, G. L. Critical Thermal Maximum in Mice. *J. Appl. Physiol.*, **40**: 683-687, 1976.
 68. Yatvin, M. B. Influence of Membrane Lipid Composition on Hyperthermic and Radiation Killing of *E. Coli* Cells. *Radiation Res.*, **70**: 610, 1977.