TIME-TEMPERATURE RELATIONSHIPS FOR HEAT-INDUCED KILLING OF MAMMALIAN CELLS*

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INTRODUCTION

Kinetic analyses of heat-induced cell killing *in vitro* have been used by numerous investigators in the hope of associating cell death at elevated temperatures with either general of specific molecular events.[1-6] Similarly, hyperthermic damage to tissues *in vivo* has been studied as a function of temperature with the aims of (1) demonstrating a differential response in tumor versus normal tissue, and (2) understanding these effects either in terms of cell killing and/or the perturbed physiological response to heat.[7-13] The motive for most of these studies has been the desire either to take advantage of some inherent greater heat sensitivity of tumor cells compared to the surrounding normal cells and, thus, achieve a therapeutic advantage, or to manipulate the biology of heat death so as to produce a differential effect. This paper will review the data, compare the kinetics of cellular and tissue damage, and examine the conclusions that can be drawn from these various sets of data.

IN VITRO STUDIES

Families of survival curves at different temperatures have been published for a number of mammalian cell lines.[5,6,14-21] A typical example is shown in FIGURE 1 for CHO cells.[6] These survival curves illustrate some general and particular feature of the cell lines studied to date. An exponential rate of cell killing is found for all cell lines and the exponential portion of the survival curve is preceded by a "shoulder" region. In part, this initial threshold reflects the temperature transient of the cells when culture flasks are transferred from 37°C to an elevated temperature. Some cell lines, notably HeLa cells,[5,19] do not show such a "shoulder" when either the temperature transient time is small compared to the total heating time, or when an appropriate transient correction has been made. The heating rate *per se* does not appear to affect the exponential portion of the survival curve when heating rates are of the order of degrees/sec.[5]

Survival curve families have been analyzed in terms of the Arrhenius equation

$$k = 1/D_0 = A \exp(-\mu/RT)$$

and sometimes by using the Johnson-Eyring modification as well.[1] The quantity A is the Arrhenius constant; μ, the activation energy; R, the universal gas constant; and T,

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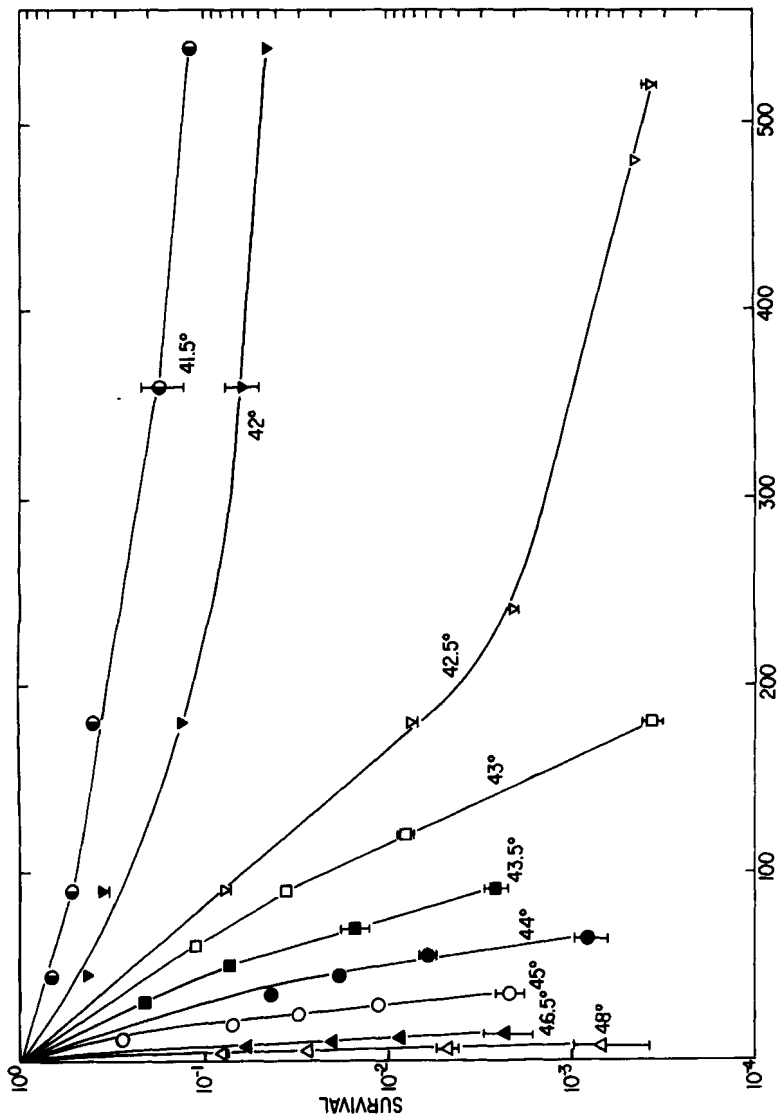

FIGURE 1. Family of survival curves for CHO cells at indicated temperatures. (From Bauer & Henle.[6] By permission of *Radiation Research*.)

the absolute temperature. In this formulation, the exponential slope of the survival curve is plotted as a function of the reciprocal negative absolute temperature, where the slope of the resulting line is a measure of the activation energy (μ) of the process(es) leading to cell death. The y-intercept (A) can be associated with entropy change by the use of the Johnson-Eyring form for A and is a requisite for the calculation of the free energy change in transforming a live cell into a heat-killed one.[22]

Analysis of Cell Survival Curve Parameters

The calculation of D_0 is generally based on a subjective choice of survival points, which are used in a linear regression analysis on the slope and the y-intercept of the survival curve. The subjective choice of survival points can be eliminated either by using an objective criterion for selecting the survival points to be included in the linear regression analysis,[21,23] or by using the complete set of survival points and determining the best fit to a mathematical survival equation after correcting the heating times for the temperature transient.[24] For an objective comparison of the various data sets, we have taken the published survival points, subtracted 2.5 min from the reported heating times[15,25] for a transient correction and calculated the survival curve parameters using a χ^2-minimization procedure.[24] TABLE 1 shows a comparison of calculated and

TABLE 1
SURVIVAL PARAMETERS

Reference	Calculated			Reported		D_0
	T(°C)	n	D_0(min)	n	D_0(min)	% Difference*
Bauer & Henle[6]	41.5	0.5	432.0		199.0	177
	42.0	0.5	144.9		86.2	68
	43.0	1.8	21.3		19.6	9
	44.0	3.1	8.0		8.1	−2
	45.0	1.2	5.7		4.6	25
	46.5	1.1	1.9		1.8	5
	48.0	1.0	0.6		0.6	0
Ross-Riveros & Leith[21]	42.0	1.7	248.2	1.1	323.0	−23
	42.5	4.1	62.1	13.5	48.8	27
	43.0	21.4	21.1	90.9	16.8	26
	44.0	33.9	9.1	34.8	9.3	−2
	45.0	2.8	6.7	8.6	5.6	20
Roti Roti *et al.*[5]	44.0	1.1	30.9		33.0	−6
	45.0	0.8	13.9		13.8	0
	46.0	1.4	6.5		6.5	0
	48.0	2.2	1.5		1.5	−1
Johnson & Pavelec[16]	41.0	2.7	418.2		526.3	21
	42.0	0.8	250.6		310.6	19
	43.0	2.4	57.0		75.4	24
	44.0	2.8	22.8		28.8	21
	45.0	1.8	14.1		12.3	−15

*$\{[D_0$ (reported) − D_0 (calculated)$]/D_0$ (reported)$\} \times 100$.

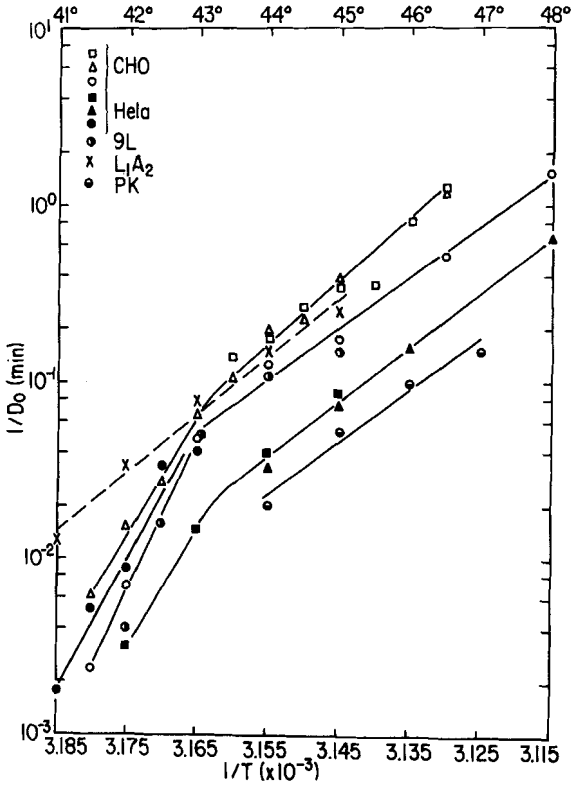

FIGURE 2. Arrhenius plot for D_0 from different cells lines. Values of D_0 were calculated by the χ^2-minimization procedure (see text) based on published survival data from References 6 (○), 1 (△), 15 (□), 17 (●), 5 (▲), 19 (■), 21 (◑), 14 (◒), and 27 (X).

published D_0 values for those data sets where D_0 values were specifically reported in the paper. Some discrepancies should be expected, since the heating procedures were not completely identical in these studies; thus the same transient correction is not fully justified in all cases. Nevertheless, in spite of the lack of complete information on the temperature transients, the calculated D_0 values (χ^2-minimization) were used as a first approximation in constructing the Arrhenius plots in FIGURE 2. The difference between reported versus calculated D_0 values appear random (TABLE 1), in most cases ≤ 25, and does not significantly affect the calculation of μ and A (TABLES 2 & 3).

Arrhenius Analysis of Survival Curve Parameters

The Arrhenius plots (FIGURE 2) are based on data sets which generally include survival determinations above and below 43°C. Specific cell lines can be characterized, in principle, by a value of μ and A. However, a check of the three data sets for both CHO and HeLa cells shows that μ and A can differ significantly in magnitude

TABLE 2

VALUES OF μ (KCAL/MOLE) ASSOCIATED WITH D_0

Cell Line	Reference	χ^2-Minimization		Reported		% Difference	
		$\mu(\geq 43°C)$	$\mu(\leq 43°C)$	$\mu(\geq 43°C)$	$\mu(\leq 43°C)$	≥43°C	≤43°C
CHO	6	135	399	131	302	−3	−32
	15	142	—	141	—	−1	—
	1	163	308	148	356	−10	16
CHL	16	140	199	185	185	24	−8
	18	142	395	—	—	—	—
HeLa	19	179	307	171	—	5	—
	5	150	—	154	—	2	—
9L	21	114	493	109	509	−5	3
PK	14	132	—	—	—	—	—
Average μ		144	347	148	304	7	15
± SD		18	93	25	135		

when survival data for the same cell line are obtained in different laboratories. Therefore, a specific value of μ and A may not absolutely characterize a particular cell line. FIGURE 2 also shows that the cellular inactivation rates can differ by factors up to 10, and this suggests that a general description of heat-induced cell death, based on a unit of hyperthermia dose calculated for a specific μ and A,[26] is of limited or no value.

The inflection point at 43°C, which has been observed for the majority of cells studied to date, is probably not a universal feature of mammalian cell killing by heat. The survival data for the L_1A_2 cell line (C3H lung)[27] shows either very little, or no inflection at 43°C, and similarly, data for one Chinese hamster lung line (CHL)[16] shows similar activation energies above and below 43°C (TABLES 2 & 3).

In general, neither the plot of $\ln(n)$ as a function of temperature, nor the plot of $\ln(1/n)$ as a function of the reciprocal negative absolute temperatures (FIGURE 3) can be fitted by straight lines. Three of the seven data sets suggest a peak of n between 43° and 45°C, but the remaining four sets of data do not support this suggestion. Thus, a temperature-dependent peak in n may exist for specific cell lines,[21] but apparently is not a universal feature of heat-induced cell death.

The multi-target, single-hit (n-D_0) model of cell killing is a unweighted model; i.e.,

TABLE 3

VALUES OF μ ASSOCIATED WITH SURVIVAL PARAMETERS (KCAL/MOLE)

Cell Line	Reference	α	β	$\beta^{1/2}$
CHO	6	152	210	106
	15	219	252	126
	1	184	363	182
HeLa	5,19	143	325	162
9L	21	210	443	222
PK	14	202	81	41
Average μ		185		
Average μ*		182	319	159
± SD		34	92	46

*Excepting the data from Reference 14.

it does not separate possible "high dose" from "low dose" effects. In radiation studies, a weighted model has been used successfully to distinguish single and double-hit events in producing cell death and this model is often called the α-β model.[28] When this survival model is also applied to heat-induced cell death, it can be shown that both the n-D_0 and the α-β model fit the survival data reasonably well, with a slightly better fit to the latter.[24] The Arrhenius analysis of α and β indicates that both parameters obey the Arrhenius law across the entire temperature interval studied without any

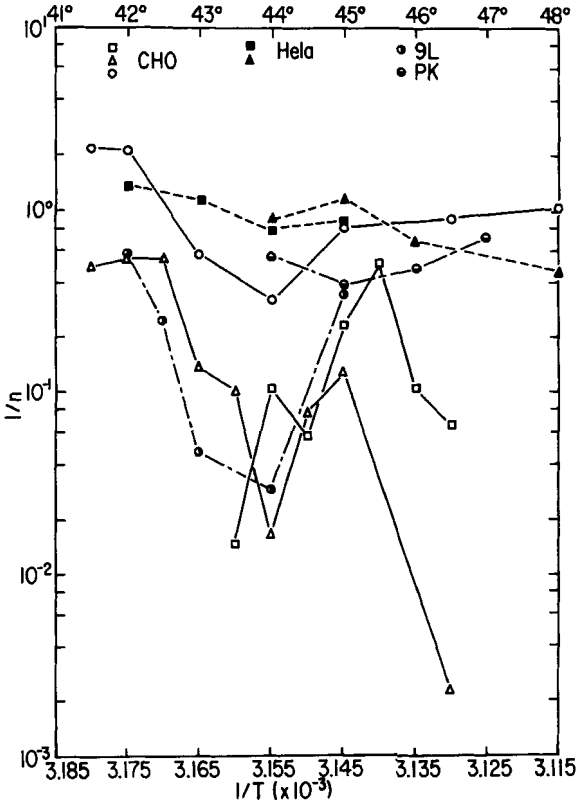

FIGURE 3. Arrhenius plot for n for different cell lines. Values of n were calculated by the χ^2-minimization procedure (text) and the source of the survival data is as in FIGURE 2.

evidence for an inflection point. This is shown in FIGURE 4 for three independent sets of CHO data. TABLES 2 and 3 list the activation energies associated with the parameters α and β for the CHO and other cell lines. The activation energy for β is similar to that of the D_0 below 43°C and, likewise, the activation energy for α is similar to that of the D_0 above 43°C. This suggests that the low activation energy of the D_0 above 43°C is associated with a dominance of single-hit lethal events (i.e., the $\exp(-\alpha t)$ term), and the high activation energy of D_0 below 43°C is dominated by the $\exp(-\beta t^2)$ term, where two events must interact to produce a lethal lesion. The

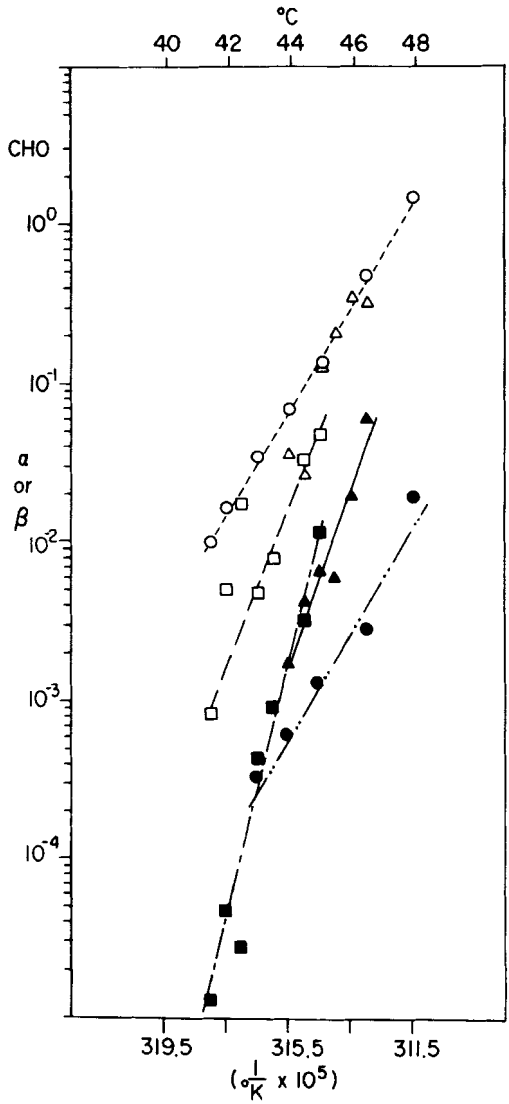

FIGURE 4. Arrhenius plot for α (open) and β (closed symbols). Data source for open symbols is as in FIGURE 2.

activation energy for $\beta^{1/2}$ however, is similar to that for α, which suggests that the energy requirements for both a single nonlethal and a single lethal lesion are comparable. In the case of radiation damage, the α and β are the probabilities of the occurrence of specific molecular events, namely, the production of double- and single-strand DNA breaks[29] (modified by repair and fixation processes), respectively. With heat damage, no such identification is possible as yet.

Step-Down Heating

The second important aspect of time-temperature relationships for heat-induced cell killing arises from the observation that marginally lethal or nonlethal temperatures become toxic when they are preceded by acute heat treatments at higher temperatures, such as 45°C.[30,31] This phenomenon is illustrated in FIGURE 5 for temperatures of 40° and 42°C with incubation periods up to 7 hr and may be called step-down heating. The upper two curves represent the control survival curves at 40° and 42°C, whereas the two lower curves represent the survival response to the same temperatures of cells that were preheated for 10 min at 45°C before transfer to either 40° or 42°C. The preheated cells are dramatically sensitized to these low temperatures, and on the Arrhenius plot (FIGURE 6) these data, though preliminary (Henle, K.J., manuscript in preparation), show clearly that the inflection point has been removed and that sublethally damaged cells are sensitized by extending the low activation energy for cell killing from the high-temperature region into the low-temperature region. One interpretation of this phenomenon in terms of the temperature dependence of α and β and their relative dominance above and below 43°C would be that sublethal damage is a nonlethal single-hit event that is normally repaired. At the low temperatures of approximately 39°–43°C, these lesions are not repaired and, thus, remain available for interaction with other single-hit events, normally nonlethal, to produce a lethal double-hit event.

The possible relationship of the above-mentioned phenomenon with the pH effect[32,33] is intriguing. Data published by Gerweck[33] can be graphed on the Arrhenius

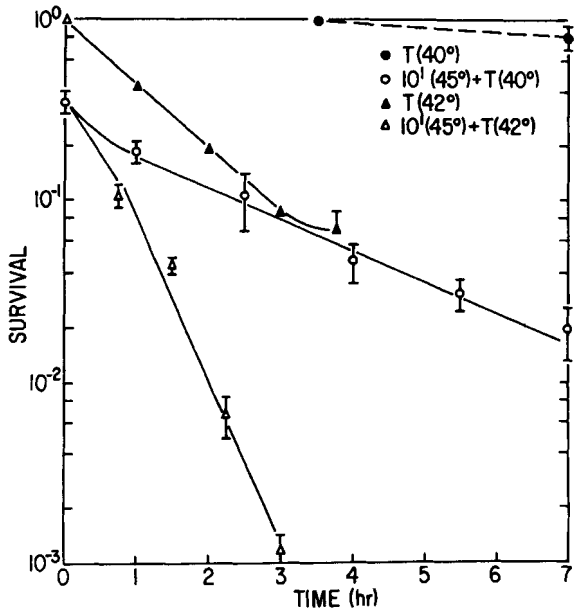

FIGURE 5. Survival curves of CHO cells heated at 40° or 42°C with and without preheating at 45°C for 10 min. (Henle, K. J., manuscript submitted.)

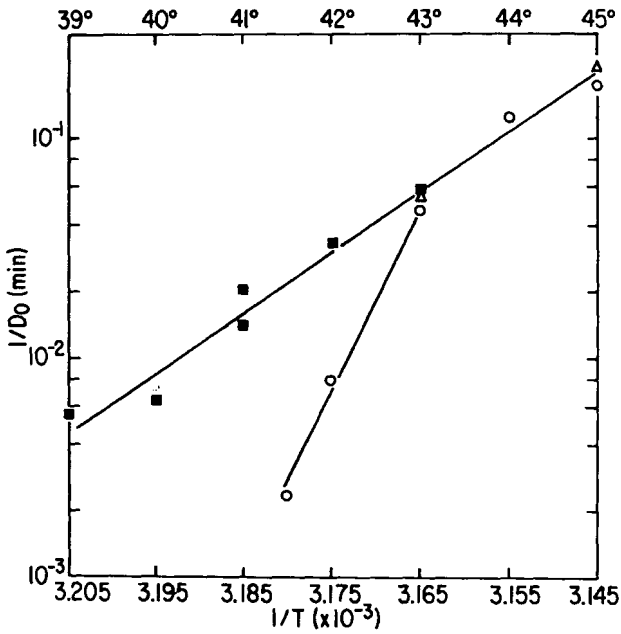

FIGURE 6. Arrhenius plot of D_0 for CHO cells heated either at a single temperature (open symbols) or by step-down heating (solid squares, see text). Open circles represent data taken from Reference 6.

plot (FIGURE 7) and suggest that a pH of 6.7 has a sensitizing effect similar to that produced by acute heat conditioning at 45°C. This is further supported by new data on the pH effect in L_1A_2 cells,[27] which indicate no sensitization of the D_0 at pH 6.5 or 6.3. The Arrhenius plot for the heat response of these cells does not have an inflection point at 43°C and the activation energy for heat killing is similar to that of other cell lines above 43°C (FIGURES 2 & 7). This suggests that L_1A_2 cells do not have the same capacity to accumulate and/or repair sublethal heat damage as most other cell lines. As a result, the sensitization by the lowered pH would not be expected. This new approach to the study of sublethal heat damage by its interaction with environmental factors could eventually lead to new insights into the critical target(s) for heat-induced cell killing and the relevant cellular repair systems.

Summary of in Vitro Studies

The study of the kinetics of heat-induced cell killing may continue to provide insights into possible mechanisms of heat damage. The failure of the extrapolation number, n, to obey the Arrhenius law suggests that the capacity to repair and/or accumulate sublethal damage cannot be analyzed in the same way as the D_0. This, of course, assumes that n is an adequate measure of these phenomena in hyperthermic killing. The identification of hyperthermic sublethal damage on the Arrhenius plot provides new possibilities for its study and specifically its interaction with and

modification by environmental factors. The kinetic analysis of heat survival curves in terms of α and β shows intriguing relationships and will become more useful if probabilities of specific molecular events can be associated with each of these parameters.

In Vivo Studies

The quantitation of cell killing in tissues is significantly more difficult than that in cell culture. Generally heat damage to tissues is quantified either in terms of a

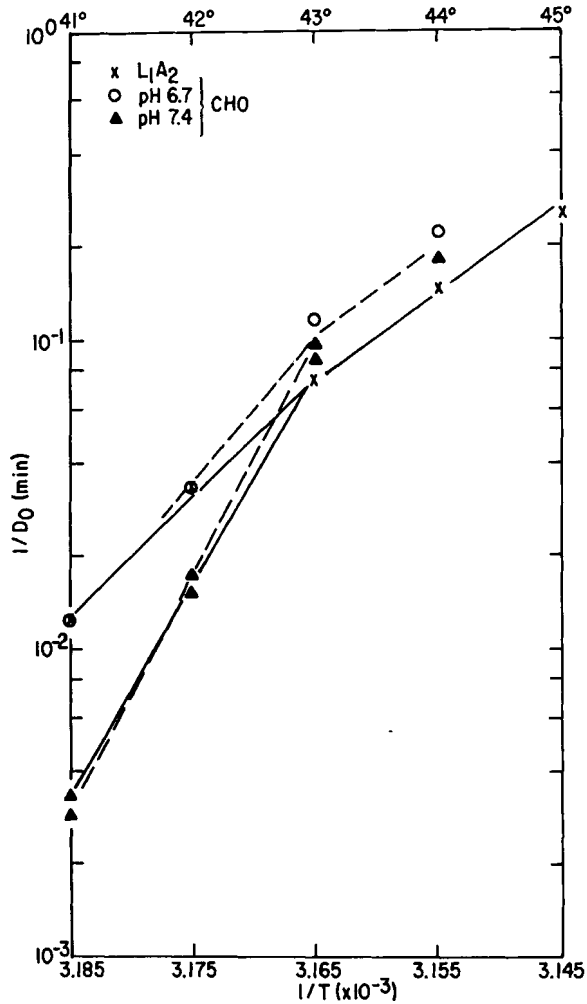

FIGURE 7. Arrhenius plot of D_0 for L_1A_2 cells[27] and CHO cells[33] at acid and normal pH values.

subjective scoring system of tissue response, or by measuring a 50% all-or-none response with an end point of animal death, leg loss, tail or ear necrosis, or tumor control. Each of these assays may represent a different (unknown) degree of cell killing and/or a modification of the normal tissue physiology after hyperthermia. In many cases, the types and even the location of the target cells that determine the measured response is unknown. Since the shape of the heat survival curve for the target cells cannot be defined, an Arrhenius analysis, comparable to the data obtained *in vitro* is impossible without major assumptions.

Time-Temperature Relationships for Tissue Damage

For tissue damage, the time-temperature relationship can be viewed in terms of a semilogarithmic plot of temperature vs heating time for a fixed end point.[10,34] FIGURE 8 shows such a plot based on data from normal and malignant tissues where the tissue response was measured at several temperatures. The source of each data set is indicated in the graph. The dominant exponential slope is $-0.7\ °C^{-1}$ with some exceptions. This is equivalent to saying that for every degree increment, the heating time must be reduced by $e^{-0.7} = 0.5$ for the same biological effect. In contrast, the dotted line in FIGURE 8 indicates a possible biphasic fit with an inflection point near 43°C and exponential slopes of $-1.0\ °C^{-1}$ and $-0.6\ °C^{-1}$ below and above 43°C, respectively; however, neither fit is statistically superior to the other.

Temperature Measurements and Gradients

The spread in the apparent heat sensitivities of tissues requires an examination of heating methods. The predominant method for inducing hyperthermia is conduction heating based on aqueous media, primarily because of its relative simplicity and reproducibility. Local hyperthermia production in small animals requires heating near the air-water interphase where steep temperature gradients may be encountered. The specific shape of this gradient depends principally on the circulation of the heating medium and, to a smaller extent, on evaporation (Henle, K.J. & L.A. Dethlefsen, manuscript in preparation).

FIGURE 9 shows our experimental set-up for conduction heating of either murine small intestine in medium or mouse feet with and without tumors in saline. Variable horizontal flow in the treatment pan can be used to minimize temperature gradients near the surface. These temperature gradients were measured with a YSI 511 thermistor in two different orientations (FIGURE 10). The temperature of the precision-controlled reservoir was maintained at 45.15°C resulting in 45.0°C inside the treatment pan at a flow rate of 3.4 liters/min through the heat exchanger. The temperature gradient with a vertical thermistor orientation extends to a depth of 10 mm because of an artifactual heat conduction along the thermistor leads. The gradient measurement with the thermistor in the horizontal-looped configuration represents, in part, an artifact in the other direction due to heat conduction along the thermistor leads from a deeper water layer and the meniscus formation near the surface. The double line represents the temperature variation near the surface due to fluctuating water levels. The true temperature gradient near the water surface probably lies

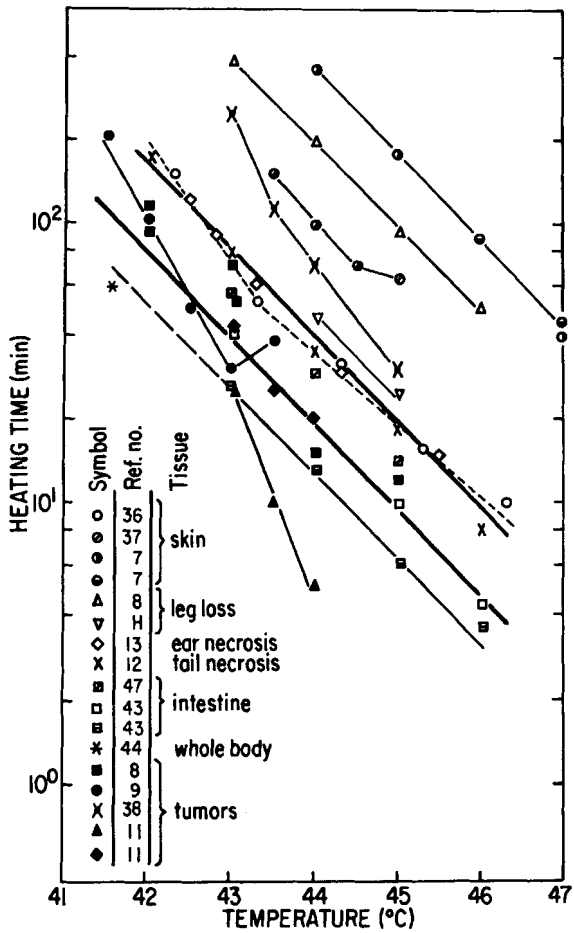

FIGURE 8. Time-temperature plot for normal and malignant tissues. The two heavy lines define the time-temperature and band referred to in the text. The short dashed line represents an alternate fit to the resistant edge of the time-temperature response band and the long dashed line represents an extrapolation from the LD_{50} data for heating of the murine jejunum to the LD_{50} for whole-body hyperthermia of rats.

somewhere between the two measurements. When the circulating pump was turned off, the new equilibrium temperature in the pan 10–15 min later had decreased significantly (FIGURE 10) in spite of a constant temperature in the reservoir. Temperature measurements over the uncovered reservoir showed a gradient similar to that in the covered circulated pan, but shifted approximately 1 mm into the water bath, depending on the number of circulating pumps in the reservoir (data not shown). When a heat sink is introduced into the water layer near the air-water interphase, the gradient is expected to shift deeper into the water, depending on the thermal characteristics of the cool object and the available water-bath circulation.

With the heating of normal and tumor-bearing mouse feet, for example, we find

FIGURE 9. Experimental set-up for heating of murine small intestine and leg.

that the temperature transients in the tumor are much longer than in the normal foot, probably because of the greater mass of the tumor-bearing foot and increased circulation to the tumor. But even the measurement of the temperature distribution within the submerged object is beset by potential artifacts. Heat conduction along thermistor leads could only be avoided if an isothermal path for the leads could be found, and FIGURE 10 illustrates that artifactual heat conduction errors can be of the order of a degree. Therefore, reported temperatures of the heating medium can differ significantly from actual tissue temperatures, and this may explain apparent heat resistance in some data, particularly when details of the heating procedure and tissue temperature measurements and/or methods are not provided. With electromagnetic heating, temperature gradients of up to 3° within tumors have been reported,[35] indicating a large degree of uncertainty for the biologically relevant temperatures. For

tumor heating, the relevant temperatures would be the lowest temperatures within the tumor volume since this may determine the regrowth of the tumor, whereas in normal tissue, the highest tissue temperature may be relevant in determining the onset of detectable tissue necrosis.

The most heat-resistant tissues in FIGURE 8 are represented by the data for human and porcine skin.[7] These skin reactions were measured as a function of the water temperature in a brass head that was held against the skin for conductive heating. The temperatures of the various skin layers can be expected to be significantly lower than that of the heating medium, which would shift the data towards greater heat sensitivity. This is underscored by the work of Okumura and Reinhold[36] who studied rat skin reactions as a function of temperature and heating time. They injected 30 ml of air subcutaneously to form an air pouch, which was heated by immersion in water. A 44°C water-bath temperature heated the skin and subcutis to 43°C within 5 min; thereafter, the skin temperature rose to only 43.3°C within an hour. The air temperature in the pouch remained approximately 3° lower than that in the skin. We have plotted the scored skin reactions (moderate to severe) against the reported water temperature after subtracting 0.7° and this may still overestimate true skin tempera-

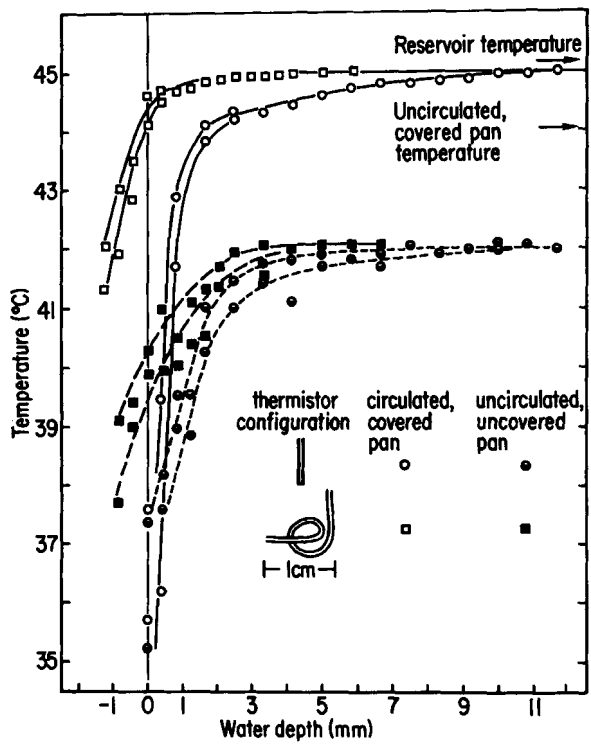

FIGURE 10. Temperature gradients near the water surface in the uncirculated (broken line) and circulated (solid line) treatment pan. Circles correspond to measurements with vertical thermistor orientation and squares to horizontal-looped thermistor orientations in the treatment pan in FIGURE 9.

tures, particularly for higher water temperatures, when heating times were less than one hour and the temperature gradients may have been steeper.

The data by Crile[8] on the leg-loss response may entail heating in a poorly circulated bath; details of the heating method were not given. Our own data on the temperature response of the mouse foot using the experimental set-up in FIGURE 9 indicate a substantially greater sensitivity (Henle, K.J. & L.A. Dethlefsen, manuscript in preparation); in fact, the time-temperature requirement for a 50% leg loss is lower by a factor of 2 from that reported by Robinson[37] for a response score, TET_{50}, of murine leg skin and still lower than the $TCD_{50/180}$ for his mammary tumors.[38] Most of the remaining data for both normal and neoplastic tissues appear to fall into a relatively narrow heat response band, where the resistant edge is well defined by several normal tissues.

Immunogenicity

Immunogenic tumors, such as the EMT-6,[11] KHJJ carcinoma,[11] Sarcoma-180,[8] Yoshida sarcoma,[36] and so forth, appear heat sensitive when compared to normal tissue. In general, the response of such tumors is probably based on "artifactual immunogenicity," which tends to overstate the therapeutic efficacy of any agent.[39] On the other hand, the mammary carcinoma data by Overgaard, which probably represent a nonimmunogenic tumor system, had to be evaluated in terms of a TCD_{10} for lack of high tumor-control rates. Corresponding values of TCD_{50} would be expected to shift the data towards greater heat resistance. In our laboratory, the heat response of a slowly growing and a rapidly growing mammary carcinoma line is being evaluated; these tumors were established by Mendelsohn and Dethlefsen[40,41] from spontaneous tumors and are apparently nonimmunogenic (Dethlefsen, L.A. & R.M. Riley, unpublished observation). Neither tumor line can be controlled with a single heat treatment below the 50% leg loss time at 45°C, even though with fractionated hyperthermia tumor control becomes possible to a limited extent (Henle, K.J. & L.A. Dethlefsen, manuscript in preparation). Similar results were reported by Hahn *et al.*[42] for the nonimmunogenic KHT mouse mammary carcinoma, which showed regressions after hyperthermia up to five treatments of 30 min at 44°C, but no cures. Other data for nonimmunogenic tumors have been reported that would place the TCD_{50} far above normal tissue tolerance as defined the heat response band in FIGURE 8,[35,38] suggesting either that parts of the tumor remained much colder than expected, or that the tumor was so resistant to hyperthermia that tumor control was achieved by sterilizing the normal tissue tumor bed. Thus, FIGURE 8 can represent the heat response of heat-sensitive tumors, but the heat resistance of nonimmunogenic tumors can often not be measured adequately.

Noncellular and Cellular Heat Effects

The most sensitive tissue to heat damage, the small intestine, does not represent a true cellular heat response, but a breakdown of intestinal barriers to bacterial infections.[43] The cellular response, as measured by the crypt survival assay, can be correlated with the LD_{50} response when mice are maintained on high doses of

gentamicin.[43] It is interesting to note in FIGURE 8 that an average time-temperature value for the death of rats by whole-body heating[44] is approximately equivalent to a jejunal heat treatment that results in bacterial septicemia and death. Average temperature values, of course, are difficult to interpret biologically, but superficially the low tolerance to whole-body heating suggests either the existence of an exquisitely heat-sensitive cellular subpopulation that is vital to the animal, or an effect that is not based on cell killing such as the noncellular damage to the small intestine. If the latter were true, the heat sensitivity of tumors would need to be much greater than that of the small intestine for whole-body hyperthermic therapy, at least for single treatments, to succeed without killing the host.

The LD_{50} values for gentamicin-treated mice represent crypt cell survival and can be considered the sensitive edge of the temperature response band for normal tissues. Thus, the heat response of a large variety of tissues differs only by a factor of approximately 2. This is in contrast to the results *in vitro* (FIGURE 2), which shows differences in inactivation rates by up to a factor of 10.

The relationship between cell killing and a 50% tissue necrosis heat treatment is

TABLE 4

In Vitro CELL SURVIVAL CORRESPONDING TO THE HEAT-SENSITIVE AND HEAT-RESISTANT EDGE OF THE HEAT RESPONSE BAND IN FIGURE 8

	Cell Line*			
	PK[14]	9L[21]	HeLa[5,19]	CHO[1,6,15]
Lower limit heat-sensitive edge	0.6	0.3	0.25	0.03
Upper limit heat-resistant edge	0.9	0.7	0.6	0.4

*Superscript refers to the respective references.

not clear for most tissues. Marmor *et al.*[11] reported that a "curative" heat treatment for the EMT-6 tumor *in vivo* corresponded to *in vitro* cell survival of 10%–80%, and this is much higher than cell survival associated with a radiation TCD_{50} dose (<0.1%). They suggested a possible selective destruction of tumor blood vessels by hyperthermia, or a tumor-directed immune response. On the assumption that the time-temperature response band in FIGURE 8 defines the heat sensitivity of adequately heated normal tissue with no immune involvement, one can try to correlate the tissue response with the reduction in cell survival in a variety of cultured cells after equivalent heat treatments. TABLE 4 shows upper- and lower-limit survival values for heat treatments, which correspond to the resistant and the sensitive edge of the response band in FIGURE 8, respectively, for four cell lines that cover a wide range of heat sensitivities. The table shows that cell survival after these heat treatments is always higher than 0.1% for all cell lines. Therefore, it appears that the destruction of normal tissues by heat does not require extensive cellular killing throughout the tissue volume, but that the heat response of tissue may be dictated either by the sensitivity of cellular subpopulations or by a physiological reaction to relatively low levels of cell killing that impairs the viability of the whole tissue.

The heat sensitivity of tumor microcirculation has been studied by Reinhold *et*

al.[45] at 42.5°C with the average time for cessation of circulation reported to be 140 ± 60 min. This heat treatment lies near the resistant edge of the heat-response band (FIGURE 8) and, thus, suggests that microcirculation is not differentially sensitive to heat.

Temperature Transients and Corrections

Comparisons of the heat response of various tissues are complicated by relatively long temperature transients with conductive heating. This difficulty could be largely obviated by measuring temperature transients and calculating the biological time equivalent at a constant temperature. This calculation is possible with the equation[46]

$$t_T = \int_{t_0}^{t_f} e^{b\Delta T(t)}\, dt,$$

which is based on equivalent biological effects from FIGURE 8. The equivalent time, t_T, at a target temperature T is thereby related to $\Delta T(t)$ (the temperature difference between actual and target temperature, t_0 and t_f (the initial and final heating time), and b (the slope of the line FIGURE 8, generally equal to $-0.7\ ^\circ C^{-1}$). In most cases, $\Delta T(t)$ is an exponential function, so that

$$t_T = \int_{t_0}^{t_f} \exp[b\Delta T_0 \exp(ct)]\, dt,$$

where c is the exponential slope of the temperature transient and reflects the thermal characteristics of the system; and ΔT_0 is the temperature difference at time t_0. This equation is evaluated in terms of an analytical solution

$$t_T = \frac{1}{c}\left[ct + b\Delta T_0 e^{ct} + \frac{(b\Delta T_0 e^{ct})^2}{2 \cdot 2!} + \frac{(b\Delta T_0 e^{ct})^3}{3 \cdot 3!} + \cdots\right]_{t_0}^{t_f}.$$

In fact, within the limitations of the data in FIGURE 8, there is no inherent biological advantage in maintaining a constant tissue temperature rather than a variable temperature that is well defined in time.

Summary of in Vivo Studies

An inherent greater heat sensitivity of tumors is not supported by the data presented here. The possibility remains, however, of utilizing environmental factors, such as pH, or fractionation schedules which might take advantage of possible differences in tissue physiology and/or different repair capacities to achieve an increase in the therapeutic ratio for cancers treated either by hyperthermia alone, or in conjunction with other therapeutic agents.

Assuming that *in vitro* data on pH and step-down heating are relevant to tissues, the temperature range of 39°–43°C should be particularly interesting, and the lower end of this temperature range would promise the greatest differential effects.

However, an optimal therapeutic protocol may also require an acute conditioning treatment at higher temperatures.

On the other hand, if the time-temperature response of some tumors should have a steeper slope than the normal tissues, as suggested by the tumor data in References 9, 11, and 38, then the temperatures above 43°C should promise a greater therapeutic ratio. Undoubtedly, more data (which extend over a wide temperature interval) are required to answer this question.

References

1. Dewey, W. C., L. E. Hopwood, S. A. Sapareto & L. E. Gerweck. 1977. Radiology **123:** 463–472.
2. Connor, W. G., E. W. Gerner, R. C. Miller & M. L. M. Boone. 1977. Radiology **123:** 497–503.
3. Johnson, H. A. 1974. Am. J. Pathol. **75:** 13–25.
4. Landry, J. & N. Marceau. 1978. Radiat. Res. **75:** 573–583.
5. Roti Roti, J. L., K. J. Henle & R. T. Winward. 1979. Radiat. Res. **78:** 522–531.
6. Bauer, K. D. & K. J. Henle. 1979. Radiat. Res. **78:** 251–263.
7. Moritz, A. R. & F. C. Henriques. 1947. Am. J. Pathol. **23:** 695–720.
8. Crile, G. 1963. Cancer Res. **23:** 372–380.
9. Overgaard, K. & J. Overgaard. 1972. Europ. J. Cancer **8:** 65–78.
10. Dickson, J. A. 1977. *In* Selective Heat Sensitivity of Cancer Cells. A. Rossi-Fanelli, R. Cavaliere, B. Mondovi & G. Moricca, Eds. pp. 43–111. Springer Verlag, New York, N.Y.
11. Marmor, J. B., N. Hahn & G. M. Hahn. 1977. Cancer Res. **37:** 879–883.
12. Morris, C. C., R. Myers & S. B. Field. 1977. Br. J. Radiol. **50:** 576–580.
13. Law, M. P., R. G. Ahier & S. B. Field. 1978. Br. J. Radiol. **51:** 132–138.
14. Harris, M. 1969. Exp. Cell Res. **56:** 382–386.
15. Westra, A. & W. C. Dewey. 1971. Int. J. Radiat. Biol. **19:** 467–477.
16. Johnson, H. A. & M. Pavelec. 1972. Am. J. Pathol. **66:** 557–564.
17. Palzer, R. J. & C. Heidelberger. 1973. Cancer Res. **33:** 415–421.
18. Robinson, J. E. & M. J. Wizenberg. 1974. Acta Radiol. **13:** 241–248.
19. Gerner, E. W., R. Boone, W. G. Connor, J. A. Hicks & M. L. M. Boone. 1976. Cancer Res. **36:** 1035–1040.
20. Sapareto, S. A., L. E. Hopwood, W. C. Dewey, M. R. Raju & J. W. Gray. 1978. Cancer Res. **38:** 393–400.
21. Ross-Riveros, P. & J. T. Leith. 1979. Radiat. Res. **78:** 296–311.
22. Johnson, F. H., H. Eyring & B. J. Stover. 1974. The Theory of Rate Processes in Biology and Medicine. p. 22. Wiley and Sons, New York, N.Y.
23. Henle, K. J., S. P. Tomasovic & L. A. Dethlefsen. 1979. Radiat. Res. In press.
24. Roti Roti, J. L. & K. J. Henle, 1980. Radiat. Res. In press.
25. Henle, K. J. & D. B. Leeper. 1976. Radiat. Res. **66:** 505–518.
26. Atkinson, E. R. 1978. IEEE Trans. Microwave Theory Tech. MTT-**26:** 595–598.
27. Nielsen, O. S. & J. Overgaard. 1979. Cancer Res. **39:** 2772–2778.
28. Kellerer, A. M. & H. H. Rossi. 1971. Radiat. Res. **47:** 15–34.
29. Chadwick, K. H. & H. P. Leenhouts. 1973. Physics Med. Biol. **18:** 78–87.
30. Henle, K. J., & D. B. Leeper. 1976. Radiol. **121:** 451–454.
31. Henle, K. J., J. E. Karamuz & D. B. Leeper. 1978. Cancer Res. **38:** 570–574.
32. Overgaard, J. 1977. Cancer **39:** 2637–2646.
33. Gerweck, L. E. 1977. Radiat. Res. **70:** 224–236.
34. Suit, H. D. 1977. Radiology **123:** 483–487.
35. Dickson, J. A. 1978. *In* Clinical Prospects for Hypoxic Cell Sensitizers and Hyperthermia. W. L. Caldwell & R. E. Durand, Eds. pp. 207–227.

36. OKUMURA, Y. & H. S. REINHOLD. 1978. Europ. J. Cancer **14:** 1161–1166.
37. ROBINSON, J. E., R. A. SLAWSON & W. A. MCCREADY. 1978. *In* Cancer Therapy by Hyperthermia and Radiation. C. Streffer, Ed. pp. 245–246. Urban & Schwarzenberg, Baltimore, Md.
38. ROBINSON, J. E., R. A. SLAWSON & W. A. MCCREADY. 1978. *In* Cancer Therapy by Hyperthermia and Radiation. C. Streffer, Ed. pp. 242–244. Urban & Schwarzenberg, Baltimore, Md.
39. HEWITT, H. B. 1978. Adv. Cancer Res. **27:** 149–200.
40. MENDELSOHN, M. L. & L. A. DETHLEFSEN. 1973. *In* Recent Results Cancer Res. **42:** 73–86. M. L. Griem, E. V. Jensen, J. E. Ultmann & R. W. Wissler, Eds. Springer Verlag, Berlin, E. Germany.
41. MENDELSOHN, M. L. & L. A. DETHLEFSEN. 1975. Lawrence Livermore Lab Report #UCRL-51798: 1–13.
42. HAHN, G. M., G. C. LI & J. B. MARMOR. 1978. *In* Clinical Prospects for Hypoxic Cell Sensitizers and Hyperthermia. W. L. Caldwell & R. E. Durand, Eds. pp. 199–206.
43. HENLE, K. J. 1979. 70th Annual Meeting. Am. Assoc. Cancer Res., New Orleans.
44. DICKSON, J. A. & H. A. ELLIS. 1974. Nature **248:** 354–358.
45. REINHOLD, H. S., B. BLACHIEWICZ & A. BERG-BLOK. 1978. *In* Cancer Therapy by Hyperthermia and Radiation. C. Streffer, Ed. pp. 231–232. Urban & Schwarzenberg, Baltimore, Md.
46. HENLE, K. J. & J. L. ROTI ROTI. 1980. Radiat. Res. In press.
47. MILLIGAN, J. A. & D. B. LEEPER. 1978. Radiat. Res. **74:** 529 (Abstract).

DISCUSSION OF THE PAPER

C. STREFFER: Dose effect curves of melanoma cells frequently show a broad shoulder, which may be an explanation for the relatively high radioresistance of these cells after doses of 200-500 R. Such cells are good candidates for a combined modality like irradiation plus hyperthermia. A human melanoma cell line was cultured *in vitro*. Radiation doses of 200 and 400 R, 24 hours after the incubation had started (24-hour-old cells), showed no or only little effect on the growth curve measured by the DNA content. Also, incubation of the cells at 40° or 42°C for 3 hours diminished the cell growth not at all or only to a small extent. However, when the melanoma cells were incubated for 3 hours at 40° or 42°C just after irradiation with 400 R a strong sensitizing effect was observed for the growth of the cells. This was manifested especially by a high cell loss 96–144 hours after treatment.

The biochemical determination of the incorporation of ^{3}H-labeled thymidine into the DNA yielded also a synergistic action for x-irradiation and incubation at 42°C. The DNA synthesis was inhibited up to 24 hours after combined treatment. However, at later periods it was quite at the normal level in the surviving cells.

In further experiments the distribution of the melanoma cells in the cell generation cycle was determined by measuring the DNA content of isolated cell nuclei with microscope photometer after staining with ethidium bromide. Simultaneously the labeling index of the same cell nuclei was measured after incubation with ^{3}H-labeled thymidine and autoradiography. By these methods it was possible to measure the DNA content of each single cell nucleus and to check whether the cell nucleus was labeled or not.

In 27-hour-old cells (27 hours after starting the incubation) most of the untreated cells (56%) were in the S phase of the generation cycle and almost all (93%) were labeled. Only few G_1-phase cells were labeled. After x-irradiation, and even more so after irradiation plus heating for 3 hours at 42°C, the number of S-phase cells

decreased and that of labeled G_1-phase cells increased. Under these conditions a new class of cells occurred, which have less DNA content than the diploid genome and which are called hypoploid cells. This effect is much enhanced in 168-hour-old cells. It is synergistic for x-irradiation and elevated temperature.

Cytofluorometric investigations showed that chromatin is released from the cell nuclei and micronuclei are formed. These processes show the apparent involvement of chromatin damage in the extreme cell loss that was observed in the combined treatment, which has also been demonstrated by other authors with other cell lines and other techniques. It further underlines the synergistic effect on the combination on radioresistant tumor cells.