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Effect of pH on Single or Fractionated Heat Treatments at 42–45°[1]

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ABSTRACT

The temperature dependency of the pH-sensitizing effect was determined in cells exposed to single or fractionated heat treatments over the temperature range of 42–45°. Chinese hamster ovary cells were exposed to single graded heat treatments or to an initial heat treatment followed 10 hr later by second heat treatments. Sensitization was quantitated by comparing survival curve terminal slopes of cells heated at pH 7.4 or pH 6.7.

Reduction in pH increased the sensitivity of cells exposed to single or fractionated treatments. The magnitude of this sensitizing effect was most pronounced at 42°, regardless of the fractionation scheme and decreased with increasing temperature. At survival levels below approximately 0.15, low pH sensitization was greater by a factor of 2 in cells exposed to fractionated compared to single heat treatments over the temperature range of 43–45°. The increased sensitivity of cells exposed to fractionated heat treatments occurred as a result of reduction in medium pH between heat treatments which inhibited the development of thermotolerance.

INTRODUCTION

Hyperthermia, alone or in combination with other forms of cancer therapy, is currently being evaluated in a number of institutions. The rationale for hyperthermic therapy is based in part on reports that the regional variation in tumor pH to values ranging from less than pH 6.6 to 7.4 may be expected to increase the sensitivity of the low-pH tumor cells to hyperthermia (1, 3, 5, 6, 8, 10, 11, 17, 22, 23). Equally important to the present study, however, are the reports that these pH differences between tumor and normal tissue may become pronounced during and following heat treatment (2, 4, 20). *In vitro* studies have shown that reduction in medium pH to "tumor-like" values, *e.g.*, pH 6.7, increases the sensitivity of cells to single doses of hyperthermia. Studies in Chinese hamster ovary cells (6), human glial cells (9), and mouse mammary tumor cells (16) indicate that the pH-sensitizing effect is more pronounced at temperatures which are only moderately lethal at normal pH. However, there is uncertainty regarding the relative clinical efficacy of single *versus* fractionated heat treatments. Several factors may influence tumor and normal tissue response to single as opposed to fractionated heat treatments. In this paper, we consider one of these; the relative magnitude of the pH-sensitizing effect in cells exposed to single and fractionated heat treatments over the temperature range of 42–45°. The pH values used are pH 7.4 (normal tissue pH value) and 6.7 (1, 3, 11, 17). At 37°, an extracellular pH of 6.7 slows the cell proliferative rate but does not affect cell viability (6).

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MATERIALS AND METHODS

Cell Culture and pH Control. Chinese hamster ovary cells were cultured as monolayers in plastic flasks containing McCoy's Medium 5A at 37° in air plus 5% CO_2 (pH 7.4). The medium was supplemented with 10% calf serum and 5% fetal calf serum plus the antibiotics penicillin, streptomycin, and neomycin sulfate. Under these conditions, the population-doubling time was 13 to 14 hr during the exponential growth phase, and the plating efficiency was 85 to 95%. The medium pH was adjusted by varying the gas phase CO_2 concentration in 30-ml treatment flasks 30 min prior to heat treatment. Thirty min prior to the first heat treatment, attached cells in 4 ml of medium were sparged with humidified air plus 5 or 27% CO_2 through manifold-attached 20-gauge stainless steel needles. The flasks were flushed for 7 min at a flow rate of 150 ml/min and sealed. The sealed flasks were stored in incubators at a CO_2 concentration approximating the CO_2 concentration inside the flask. This latter procedure was necessary to eliminate the small pH change which occurred due to diffusion of CO_2 through the plastic flasks over a period of several hr. The medium pH was monitored throughout the course of the experiment by inserting a temperature-compensated combination glass microelectrode through the neck of replicate treatment flasks. The medium pH did not vary significantly over the 10- to 12-hr treatment interval and was 7.4 or 6.7 ± 0.05 at 37°. At the treatment temperatures, 42–45°, the pH was approximately 0.05 to 0.08 unit higher. Following heat treatment, flasks containing 27% CO_2 were sparged with 5% CO_2, and the cells were cultured at 37° for colony formation.

Heat Treatment and Determination of Surviving Fraction. Cells exposed to single or fractionated heat treatment were inoculated in flasks 18 to 24 hr prior to the initiation of treatment. A sufficient number of cells were plated in 4 replicate flasks for each unique pH, temperature, and treatment duration, so that 5 to 200 clonogenic cells/flask survived treatment. For the single-dose experiments, cells were exposed to graded single treatments at pH 6.7 or 7.4. Cells heated at pH 6.7 were maintained at pH 6.7 for a period of time equal to the time that cells exposed to fractionated heat treatments were maintained at low pH, *i.e.*, 10 to 12 hr after the initiation of heat treatment. This procedure was used to ensure that post-heat low-pH enhancement of hyperthermic cell killing was equivalent in cells exposed to single or fractionated heat treatments (5). The medium pH was then adjusted to 7.4. In split-dose experiments, cells at pH 6.7 or 7.4 were exposed to an initial heat treatment of 120, 45, 20, or 10 min at 42°, 43°, 44°, or 45°, respectively. Following the initial treatment, cells were maintained at 37° and then exposed to graded second treatments, usually 10 hr later. Following the second treatment, the medium pH of cells heated and maintained at pH 6.7 between treatments was adjusted to pH 7.4. To minimize known and unknown variables affecting cell response to hyperthermia, most experiments at pH 6.7 and 7.4 were performed simultaneously.

Cells were heated by submersion of the flasks in an insulated water bath containing a heater circulator which maintained the selected temperature within ±0.05°. Within 3 min or less (depending on the treatment temperature), the medium temperature was within 0.05° of the water bath temperature. Upon removal of the flasks from the water bath, they were quickly dried with towels in a 37° warm room to minimize evaporative cooling.

The surviving fraction was calculated by comparing the fraction of cells giving rise to colonies in heated and control flasks 7 to 10 days after treatment, after correcting for cell multiplicity. Cell multiplicity, at

the time of the first time treatment (approximately 1.8) was determined by phase-contrast microscopy in a 37° warm room. The cells were reexamined at the time of the second heat treatment. For the fractionation intervals used, there was no change in the total cell number (attached or floating) or multiplicity between treatments. An additional multiplicity correction for cells exposed to second heat treatments was therefore not required.

The multiplicity-corrected surviving fraction data when plotted on log (surviving fraction) *versus* linear (dose) paper yielded survival curves the terminal slopes of which were fit to straight lines by linear regression analysis. For single-dose 43–45° experiments, all surviving fraction data below a survival level of 0.15 was used for calculation of slope. For the fractionated (second dose) experiments, all surviving fraction data arising from cells exposed to second heat treatments was used in the calculation of the slope. Reduction in medium pH influenced the sensitivity of cells at survival levels above 0.15. However, inadequate data precluded a determination of the relative magnitude of this effect in cells exposed to single *versus* fractionated heat treatments throughout the shoulder region. The terminal slopes were expressed as D_0[3] values. The pH ER was obtained from the D_0 ratio at pH 7.4 *versus* pH 6.7. The term "thermotolerance" was used to indicate the increased resistance to hyperthermia occurring as a result of a prior heat treatment, as evidenced by an increased D_0. The dose-response curve at pH 6.7, 42°, was biphasic and exhibited 2 exponential inactivation regions. Two slopes were therefore calculated from survival levels greater or less than 10^{-4}. Each survival curve experiment was repeated 3 or more times, and the means (± S.D.) of the D_0s and D_0 ratios were calculated.

RESULTS

Initial experiments were performed to determine the time interval between treatments required for the development of maximal thermal resistance. Results of an experiment performed at 44° are shown in Chart 1. A priming heat treatment of 20 min was followed by second heat treatments of 20 or 40 min for cells heated and maintained at pH 6.7 or 7.4, respectively. Maximal resistance to second heat treatments occurred approximately 10 hr after the initial treatment regardless of the pH during and between treatments. Similar kinetics was observed at 43° and 45°. At 42°, maximal resistance developed in 4 to 6 hr following a 120-min treatment and remained unchanged for fractionation intervals of 10 hr and more (7). Therefore, all fractionated heat survival curve experiments were performed with a time interval of 10 hr between treatments.

Results of a fractionated heat treatment experiment performed at 42° are shown in Chart 2. Dashed lines are drawn through the single dose survival data; solid lines indicate the response of cells to the second heat treatments administered 10 hr after the initial 120-min heat treatment. In cells exposed to single heat treatment, there is an initial relatively rapid decrease in survival followed by a substantially reduced rate of cell killing under both medium pH conditions. However, both the initial and final rates of cell killing are substantially increased at low pH. The terminal slope D_0 (surviving fraction less than 10^{-4}) of 60 ± 17 min was approximately 6 times greater than the pH 7.4 terminal slope D_0. The initial rate of cell inactivation at pH 6.7 (surviving fraction greater than 10^{-4})

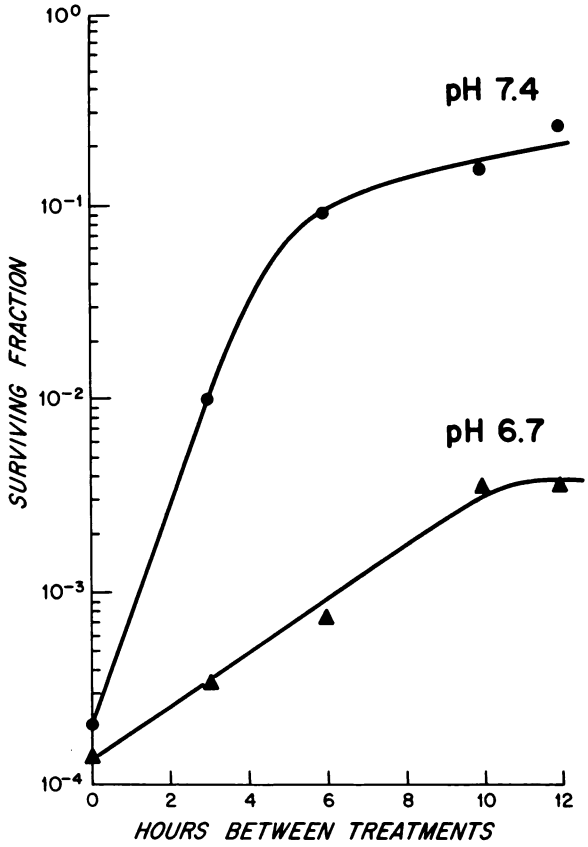

Chart 1. Influence of pH on the development of thermal resistance in cells exposed to fractionated 44° heat treatment. Cells were heated and maintained at pH 7.4 (●) or 6.7 (▲) during and between heat treatment. An initial 20-min heat treatment was followed by second heat treatments of 40 or 20 min for cells heated and maintained at pH or 7.4 or 6.7, respectively.

yielded a D_0 of only 28 ± 3.5 min. Cell exposed to second heat treatments yielded a single-component survival curve. In cells exposed to second heat treatments, the calculated D_0 values varied with pH. The second dose D_0s were similar to the D_0s obtained from the terminal position of the single-dose curves. Experiments performed at 43, 44, and 45° are shown in Chart 3. The abscissa scale is identical for all panels, emphasizing the increasing rate of cell killing at increasing temperature. Regardless of temperature, reduction in medium pH enhances the response of cells to single heat treatments. Reduced pH also increases the sensitivity of cells previously exposed to hyperthermia compared to cells maintained at pH 7.4 throughout treatment. Nevertheless, thermotolerance clearly develops under these reduced pH conditions.

From experiments similar to those shown in Charts 2 and 3, the mean ± S.D. of the terminal slope D_0 values from 3 or more experiments was calculated (Table 1). The pH ERs were calculated from simultaneous experiments in which cells were heated at pH 6.7 and 7.4. For the single heat treatments, pH enhancement is most pronounced at 42°, yielding a terminal slope pH ER of 5.9 ± 1.9. It should be pointed out that, if the pH 7.4 terminal slope is compared with pH 6.7 initial slope (survival levels greater than 10^{-4}), the slope ratio would increase to 15 ± 5. At higher temperatures, the single-dose pH ERs decrease to 1.7 ± 0.3 at 43° and 1.4 ± 0.1 at 45°.

The split-dose (second dose) D_0s are also shown in Table 1.

[3] The abbreviations used are: D_0, min of heat treatment that reduces the surviving fraction by a factor of e (67%) on the exponential portion of the survival curve; pH ER, pH enhancement ratio, obtained by dividing the D_0 at pH 7.4 by the D_0 at pH 6.7.

Again, the pH enhancement effect is more pronounced at the lower temperatures, ranging from 5.6 ± 1.4 at 42° to 2.5 ± 0.6 at 45°. Except at 42°, the pH-sensitizing effect is greater by a factor of approximately 2 in preheated cells than in cells exposed to single heat treatments. At 42°, the terminal slope pH enhancement ratios are identical in cells exposed to prolonged single heat treatments (5.9 ± 1.9) or to second heat treatments (5.6 ± 1.4). The relationship between the pH ER and treatment temperature is illustrated in Chart 4.

To determine if the greater pH-sensitizing effect observed at the time of the second heat treatments was due to a more pronounced pH sensitization of preheated cells or to an inhibition of thermotolerance development between treatments, additional experiments were performed at 44°. Preheated cells were maintained at pH 6.7 during the second heat treatments only, between heat treatments only, or both between and at the time of the second treatment. The results in Table 2 show that reduction in medium pH between heat treatments only or reduction in pH at the time of the second heat treatment only increases the sensitivity of cells to second heat treatments. Sensitivity is further increased when pH is reduced both between treatments and at the time of the second treatment. In these limited studies, performed at 44° only, reduction in pH between treatments contributes most to the sensitization of preheated cells.

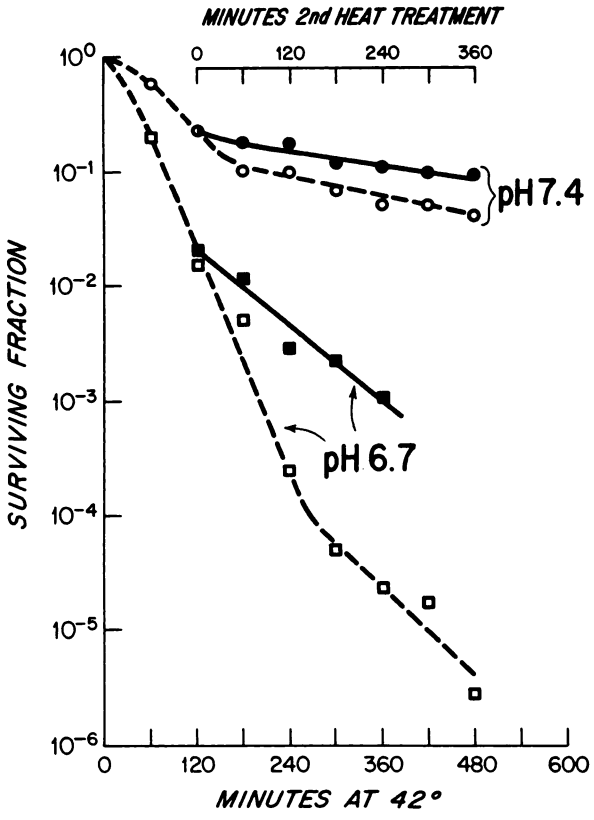

Chart 2. Influence of pH on the lethal response of cells exposed to single or fractionated 42° heat treatments. - - - -, response of cells exposed to single graded heat treatments at pH 7.4 or 6.7. Cells were also exposed to second heat treatments 10 hr after an initial 120-min heat treatment. ——, response of cells to graded second heat treatments at pH 7.4 or 6.7. The medium pH during and between heat treatments was 7.4 or 6.7. The temperature between treatments was 37°.

DISCUSSION

The increased sensitivity of cells to single heat treatments at low pH has been observed in several cell lines and was not unexpected (5, 6, 9, 12, 16, 19, 23). In studies involving 3 cell lines treated at 2 or more hyperthermic temperatures (6, 7, 16), it has been shown that the pH-sensitizing effect is greater at the lower temperatures. The present study shows that the pH effect is temperature dependent in cells exposed to fractionated as well as single heat treatments (Table 1). As the cell inactivation rate increases with temperature, the pH enhancement effect becomes relatively less pronounced. In this context,

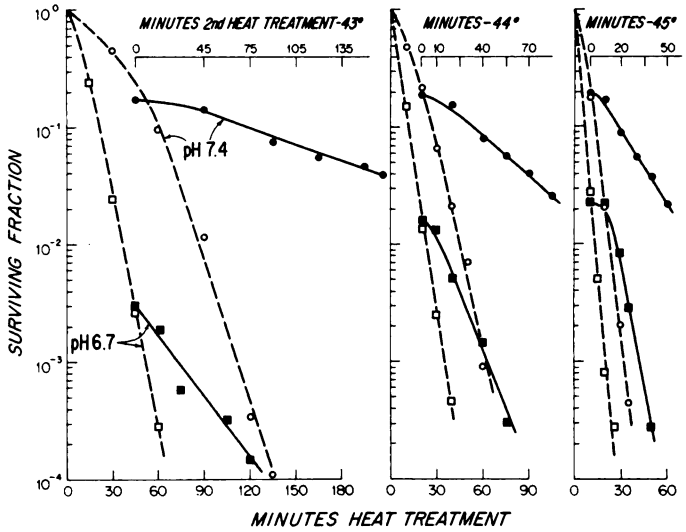

Chart 3. Influence of pH on the fraction of cells surviving single or fractionated heat treatments at 43, 44, or 45°. The *abscissa* scale is identical in all panels. - - - -, single treatments; ——, second heat treatments. For cells exposed to second heat treatments, the initial heat treatment duration was 45, 20, or 10 min at 43°, 44°, or 45°, respectively.

Table 1

pH-enhanced sensitivity to single and fractionated heat treatments

	Single heat treatments			Second heat treatments		
	D_0[a] (min)			D_0[a] (min)		
Temperature	pH 7.4	pH 6.7	pH enhancement ratio[b]	pH 7.4	pH 6.7	pH enhancement ratio[b]
42°	423 ± 17[c]	71 ± 22	5.9 ± 1.9	469 ± 96	84 ± 12	5.6 ± 1.6
43	12 ± 2	6.9 ± 1.1	1.7 ± 0.3	97 ± 8	26 ± 6	3.7 ± 0.6
44	6.4 ± 0.8	4.1 ± 0.7	1.6 ± 0.2	38 ± 9	13 ± 5	3.2 ± 0.6
45	3.3 ± 0.4	2.2 ± 0.2	1.4 ± 0.1	19 ± 4	7.4 ± 2.6	2.5 ± 0.4

[a] Time of heat treatment required to reduce the surviving fraction by a factor of e (67%) on the terminal portion of the survival curves. Further details provided in "Materials and Methods."

[b] Ratio of D_0s at pH 7.4 and pH 6.7.

[c] Mean ± S.D. of 3 or more experiments.

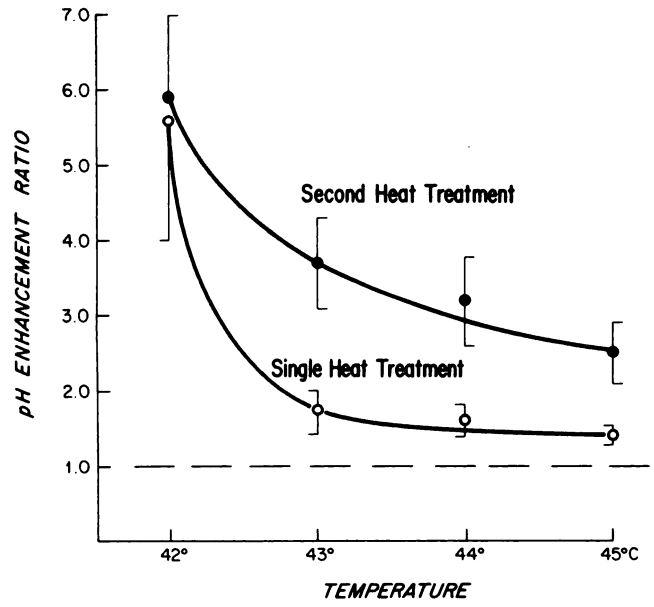

Chart 4. Temperature dependency of the pH-sensitizing effect in cells exposed to single or fractionated heat treatments. The terminal slope ratios of cells heated at pH 7.4 or 6.7 are indicated as the pH ERs. The terminal slopes of cells exposed to single or second heat treatments were calculated from data such as those shown in Charts 2 and 3 and are summarized in Table 1. *Bars*, S.D.

Table 2

Influence of pH reduction sequence on the response of cells to fractionated 44° heat treatments

pH[a]			
First treatment[b]	Interval[b]	Second treatment[b]	pH enhancement ratio[c]
7.4	7.4	7.4	
7.4	7.4	6.7	1.4 ± 0.3[d]
7.4	6.7	7.4	1.9 ± 0.2
7.4	6.7	6.7	2.3 ± 0.4

[a] Medium pH was varied by adjusting the CO_2 concentration immediately following the first heat treatment and immediately prior to the second heat treatment. Following the second heat treatments, the pH was adjusted to 7.4 for colony formation.

[b] The initial heat treatment of 20 min at pH 7.4 was followed by 10 hr of culturing at 37° at the indicated pH. The cells were then exposed to second heat treatments at pH 7.4 or 6.7.

[c] Calculated from the second heat treatment D_0s.

[d] Mean ± S.D.

it is worth noting that heating in Hanks' balanced salt solution increases the sensitivity of plateau-phase cells to hyperthermia compared to the response observed when cells are heated in complete medium containing serum (15). Furthermore, Li *et al.* (15) reported that variation in pH (6.7 or 7.5) did not influence cellular response to single heat treatments in Hanks' balanced salt solution.

The response of cells to graded single heat treatments at 42° is somewhat more complex than is observed at higher temperatures. Due to the relatively slow inactivation rate at this temperature, the survival response could be quantitated following up to 480 min treatment at both pH values. At 42°, the response curves are characterized by a significant decrease in slope beginning approximately 180 to 300 min (pH 7.4 or pH 6.7) after the initiation of treatment, and the inactivation rates become similar to those obtained when cells are exposed to fractionated heat treatments. Both the kinetics of this resistance development and the resultant inactivation parameters suggest that these cells develop thermotolerance during heat treatment identical to the tolerance which develops when cells are exposed to fractionated treatment. This is further supported by the similar pH ERs of cells exposed to single or fractionated treatments (Table 1). Tolerance development during single continuous heat treatments was not observed at the higher temperatures, *i.e.*, 43–45°. While it may develop after 3 to 5 hr of continuous treatment, the survival level at which it may occur precludes an evaluation of this possibility.

Several factors could account for the relatively greater pH sensitization of preheated compared to nonpreheated cells. Reduced pH could increase the time at 37° required for maximum development of thermal resistance to greater than 10 hr. However, in several experiments similar to those shown in Chart 1, this was not observed. A second possibility is that the initial heat treatment induces changes in critical biomolecules which result in their being relatively pH sensitive at elevated temperatures. The data presented in Tables 1 and 2 indicate that this is not the case, at least at 44°; *i.e.*, the single-dose pH ER was 1.6 ± 0.2 (Table 1), whereas the pH ER of previously heated cells maintained at pH 7.4 except during the second treatment was 1.4 ± 0.3 (Table 2). Rather, the increased split-dose pH enhancement effect is due to an inhibition in the magnitude of thermotolerance development by low pH during the 37° time interval between treatments. Reduction of pH to 6.7 between split heat treatments at pH 7.4 increased the sensitivity of cells to the second heat treatment by a factor of approximately 1.9 compared to cells maintained at pH 7.4 throughout. Studies by other investigators suggest that the development of thermotolerance may result from the synthesis of heat shock proteins which occurs following an initial heat shock (13, 14, 21). It is not known if reduction in medium pH inhibits the synthesis of these heat shock proteins.

In general, the results presented here are similar to those observed by Nielsen and Overgaard (18) in L_1A_2 cells at 42°. In these relatively heat-sensitive cells (thermotolerance did not develop during single treatments at pH 7.2), reduction in medium pH from 7.2 to 6.5 increased the sensitivity of cells to fractionated treatments more than to single treatments. Also in the L_1A_2 studies, this occurred secondary to pH reduction between treatments. Alteration in the kinetics of tolerance development by pH variation was not observed. Goldin and Leeper (10) compared the response of cells exposed to single 45° heat treatments or fractionated treatments over a pH range of 6.3 to 7.2. In the fractionated heat treatments, Chinese hamster ovary cells were exposed to an initial heat treatment of 10 min and then to second heat treatments 7 hr later. Cells were maintained at the specified pH throughout treatment. Their studies indicate that the relatively greater pH sensitization of cells exposed to fractionated *versus* single treatments becomes more pronounced at lower pH. Their report and the present studies therefore indicate that the degree of pH sensitization in cells exposed to fractionated heat treatment increases with (*a*) decreasing pH and (*b*) decreasing temperature.

These observations are relevant to the uncertainty regarding the fractionation number or fractionation interval most likely to elicit the maximum diffential response between tumor and normal tissue in the clinical applications of hyperthermia. Accumulating evidence shows that hyperthermia may substantially compromise vascular function, further reducing tumor pH

and oxygen concentration for 24 hr or more following hyperthermia (2, 4, 20). Under these conditions, daily hyperthermia fractionation may be suggested, since the results presented here show that the pH enhancement effect is greater in cells exposed to fractionated heat treatment. However, there are several reasons why a single heat treatment or weekly heat treatments (*i.e.,* following the decay of thermotolerance) may be more appropriate than daily treatments: (*a*) one must consider the possibility that vascular damage induced by frequent hyperthermia treatments may result in tumor hypoxia and low pH and reduce the efficacy of concomitant radiation or chemotherapy; (*b*) it is not presently known if the higher-temperature heat treatments, *e.g.,* 45° for 30 min, cause greater pH and pO_2 changes in tumor tissue compared to normal tissue. If not, both tissues may be equally sensitive to subsequent frequent heat treatments. Additional caution is suggested from the results obtained in this paper. Chart 2 clearly indicates that a single continuous heat treatment at 42° results in the largest surviving fraction difference at pH 7.4 *versus* pH 6.7. In view of these multiple considerations, the employment of single heat treatments (or a limited number of treatments at fractionated intervals greater than the thermotolerance decay times) should be considered for use in combination with other treatment modalities. Regardless of the fractionation scheme, however, treatments which are only moderately lethal under low pH conditions, *e.g.,* 42 to 43°, may be expected to spare normal tissue and be relatively damaging to low pH tissue. In summary: (*a*) reduction in extracellular pH increases the sensitivity of cells exposed to single or fractionated heat treatments; (*b*) the pH-sensitizing effect is most pronounced at temperatures which are only moderately lethal at normal pH, and it decreases with increasing temperature; (*c*) at 43–45°, the pH-sensitizing effect is greater in cells exposed to fractionated heat treatment than it is in cells exposed to single heat treatments; and (*d*) at 43° and above, reduction in medium pH during and between heat treatments increases thermal sensitivity more than does reduction of pH during heat treatment only or between heat treatments only.

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