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# Response of Cells to Hyperthermia under Acute and Chronic Hypoxic Conditions<sup>1</sup>

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## ABSTRACT

The lethal response of Chinese hamster cells heated to 42° was determined following 0 to 30 hr culturing under hypoxic conditions. Oxygenated and acutely hypoxic cells were equally sensitive to hyperthermia; however, sensitivity increased with the time of culturing under hypoxic conditions prior to treatment. Three hr at 42° resulted in a surviving fraction of ≈0.1 under acute hypoxic conditions and less than 0.001 for cells cultured for 30 hr under oxygen-deprived conditions before the heat treatment. The increased sensitivity to hyperthermia was due in part to a decrease in the pH of the medium which occurred as a result of cell metabolism; this could be reversed by increasing pH to 7.3 immediately prior to heat treatment. However, even under fully controlled pH conditions, prolonged oxygen deprivation increased hyperthermic cell killing by a factor of ≈5. This effect was not reversed by returning the cells to normal oxygen tension prior to treatment. These data demonstrate that tumorlike microenvironmental conditions (reduced O<sub>2</sub> tension and pH) substantially increase the sensitivity of cells to 42° hyperthermia.

## INTRODUCTION

The influence of the cellular microenvironment on the response of cells to hyperthermia is of fundamental importance to understanding the potential of hyperthermia for the treatment of the cancer patient. Variations in the concentration of metabolites such as oxygen, lactic acid, or hydrogen ions may affect the cellular response to either hyperthermia or radiation directly or may indirectly affect the response by altering the cell cycle distribution or proliferative fraction.

Recently a number of investigators have shown that hypoxic cells exhibit equal or greater sensitivity to hyperthermia than oxygenated cells (11, 16, 17, 23, 25). In addition, the pH of the cells during hyperthermia has been shown to be an extremely important determinant of cell sensitivity (9, 12, 13, 21). This paper describes our study of the effects of prolonged hypoxia and of reduced pH on the thermal sensitivity of Chinese hamster cells. Hopefully, studies of this nature will provide valuable assistance in planning the optimal hyperthermic treatment strategy.

## MATERIALS AND METHODS

**Cell Culture and pH Control.** CHO<sup>4</sup> cells were cultured in plastic flasks containing McCoy's Medium 5a plus 10% calf and 5% fetal calf serum. The medium was buffered with 26 mM NaHCO<sub>3</sub> and 5% CO<sub>2</sub> (pH 7.4 at 37°). Under these conditions, the population-doubling time for exponential phase cells was ≈14 hr.

The pH of the medium was adjusted by altering the NaHCO<sub>3</sub> concentration with 1 N HCl. The medium was equilibrated with 5% carbon dioxide plus humidified nitrogen or air by purging 15-sq cm glass or 25-sq cm plastic flasks containing attached cells and 1 or 5 ml fresh medium, respectively. The flasks were simultaneously gassed and agitated for 10 min with 120 three-inch reciprocal cycles/min. Purging was terminated by closing the outlet portal prior to the inlet portal in each flask. Changes in extracellular pH due to cell metabolism were controlled by adjusting the number of cells plated (5 × 10<sup>4</sup> to 1 × 10<sup>6</sup>) per ml medium. The pH was monitored throughout the course of the experiments by inserting a combination glass electrode (Corning Scientific Instruments, Medfield, Mass.) through a 1-cm diameter rubber closure into a 30-cu cm vessel which contained medium previously decanted from replicate treatment flasks. The medium was flushed with the appropriate gas mixture through inlet and outlet needles which were forced through the rubber closure. All pH values were determined at the appropriate temperature for each experiment. Variation in temperature between 37 and 42° resulted in a pH change of less than 0.03 unit. All pH values quoted are ±0.05 unit (S.E.).

**Treatment under Hypoxic Conditions.** Glass flasks containing a known number of attached cells were rinsed, and the cell monolayer was covered with 1.0 ± 0.05 ml of medium (final volume) yielding an average medium depth of 0.067 cm. The 1-cm-diameter necks of the flasks were closed with specially designed sterile soft rubber stoppers which were stored in nitrogen for at least two weeks prior to use. The stoppers were forced through a beveled constriction in the flask neck which was matched to a groove in the stopper. Two 20-gauge stainless steel needles attached to a manifold were forced through each stopper and served as inlet and outlet valves. The 30-ml (15-sq cm) flasks were flushed with water-saturated N<sub>2</sub> (<5 ppm) plus CO<sub>2</sub> at a flow rate of 150 ml/min for 30 min. The flasks were agitated throughout the gassing procedure. The effluent O<sub>2</sub> concentration (C<sub>i</sub>) monitored with a Thermo probe (Thermo-lab Instruments, Inc., Pittsburgh, Pa.) was ≤15 ppm 10 min following initiation of the gassing procedure. The outlet

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<sup>4</sup> The abbreviation used is: CHO, Chinese hamster ovary.

needle was removed prior to the inlet needle, thus establishing an internal positive pressure of 0.2 kg/sq cm, which could be maintained for greater than 48 hr.

Given an average liquid depth ( $b$ ) of 0.067 cm and an oxygen diffusion coefficient ( $D$ ) of  $2 \times 10^{-5}$  sq cm/sec, the concentration of  $O_2$  at the cell depth for a nonagitated solution as a function of time  $C(b, t)$  is given by the equation (3, 6)

$$C(b, t) = C_1 + (C_0 - C_1) \frac{4}{\pi} \exp\left(-\frac{\pi^2 D}{b^2} t\right)$$

where  $C_0$  equals the original oxygen concentration in the air-saturated medium, and  $C_1$  equals the gas phase  $O_2$  concentration. Vigorous agitation eliminated the  $O_2$  gradient established at the glass-medium meniscus and increased the rate of  $O_2$  equilibration between the liquid and gas phase. Neglecting this latter effect, calculations indicate the liquid phase  $O_2$  concentration to be within 1% of the equilibrium value after 30 min of gassing. While these calculations provide reasonable estimates of the initial dissolved  $O_2$  concentration after gassing, they are less reliable for estimating  $O_2$  concentration after prolonged culturing under  $N_2$ . For long-term hypoxic experiments, hypoxia was demonstrated by comparing the radiation response of chronically hypoxic cells to the response of chronically hypoxic cells which were reoxygenated with air plus 5%  $CO_2$  prior to radiation.

**Treatment and Determination of Survival.** Twenty-four hr prior to treatment, an appropriate number of cells ( $5 \times 10^4$  to  $1 \times 10^6$ ) were inoculated in flasks containing fresh medium and placed in a  $37^\circ$  incubator containing air plus 5%  $CO_2$  (pH 7.4). Treatment was initiated by replacing the medium with fresh medium previously adjusted to the appropriate pH (6.7 to 7.4). The flasks were closed and gassed with air or nitrogen + 5%  $CO_2$  as previously described. The cells were then either heated or irradiated or placed in a  $37^\circ$  incubator for treatment at a later time. Cells were heated by total submersion of the flask in an insulated 30-liter water bath containing a heater-circulator (Model E-52; Haake, Inc., Saddle Brook, N. J.) maintained at the desired temperature  $\pm 0.1^\circ$ . Temperature equilibration in the culture flasks required  $\approx 2.5$  min. Following heat treatment, the cells were returned to the  $37^\circ$  incubator for 30 min prior to trypsinization. For radiation experiments, attached cells under oxygenated or hypoxic conditions were X-irradiated in glass flasks at room temperature with 280 kVp X-rays (half-value layer = 1.5 mm copper) at a dose rate of approximately 100 rads/min. Immediately following irradiation, the cells were placed in a  $37^\circ$  incubator for 30 min and then trypsinized.

Floating and attached cells, suspended with 0.1% trypsin, were pooled, counted, and inoculated in 5 replicate flasks containing fresh medium, pH 7.4. The total number and percentage of floating cells from control and treatment flasks did not vary significantly. The cells were incubated 7 to 11 days for colony development. Survival was determined by comparing the fraction of control to heated or irradiated cells which formed colonies as described previously (12). Culturing under hypoxia at  $37^\circ$  resulted in a decrease in

survival from the control values of 60 to 80% for 0 hr hypoxia, to 35 to 45% at 24 hr and 25 to 35% at 30 hr hypoxia. For all data plotted, survival is normalized to the control plating efficiency.

## RESULTS

The lethal response of cells to hyperthermia following 0 to 24 hr culturing under nitrogen is shown in Chart 1. Sensitivity to hyperthermia increases as a function of culturing time under hypoxic conditions prior to heating. This sensitizing effect is apparent after 12 hr culturing under nitrogen and becomes more marked at 24 hr. Prolonged hypoxia increases the slope of the initial portion of the response curve and delays the development of thermal tolerance which is observed under air or acute hypoxic conditions after about 3 hr heating. Reoxygenation of the cells with air + 5%  $CO_2$  for 10 min immediately (5 min) prior to treatment did not influence sensitivity; however, following 24 hr hypoxia, the sensitizing effect of hypoxia was diminished at a survival level below approximately 0.01 or with heat treatments greater than 3 hr. The pH of the medium was identical for cells heated under oxygenated or hypoxic conditions. When cells were irradiated rather than heated following 1 to 30 hr culturing under hypoxia, the dose of radiation required to reduce the surviving fraction

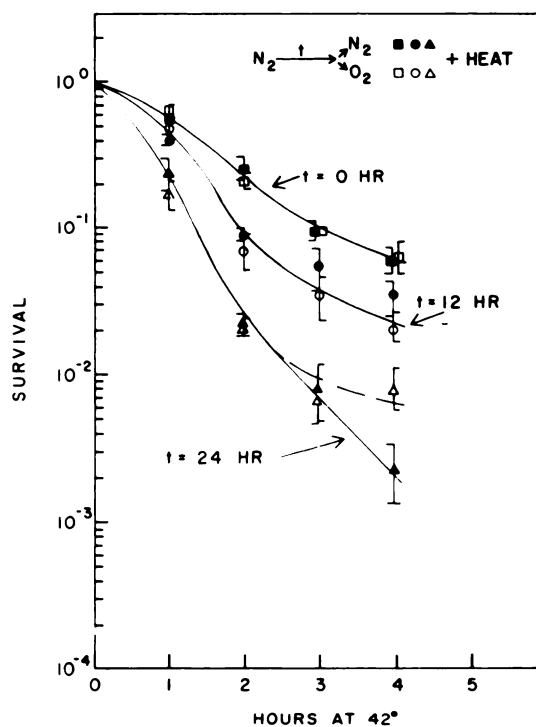


Chart 1. Approximately  $5 \times 10^5$  cells were gassed with nitrogen plus 5%  $CO_2$  and maintained for 12 or 24 hr at  $37^\circ$  prior to heating at  $42^\circ$ . Immediately prior to heat treatment, 5 replicate flasks containing cells were reoxygenated ( $\square$ ,  $\circ$ ,  $\triangle$ ) and heated simultaneously with cells maintained under hypoxia during treatment ( $\blacksquare$ ,  $\bullet$ ,  $\blacktriangle$ ). For the curve labeled  $t = 0$  hr, cells were heated under oxygenated conditions or under conditions of oxygen deprivation only during heat treatment. Hypoxic cells were reoxygenated with air plus 5%  $CO_2$  immediately after heating. The average cell number increased from  $5$  to  $6 \times 10^6$  during 24 hr culturing under hypoxic conditions, and the plating efficiency decreased from 71% to 43%. Mean  $\pm$  S.E. of pooled data from 2 to 4 experiments are plotted.

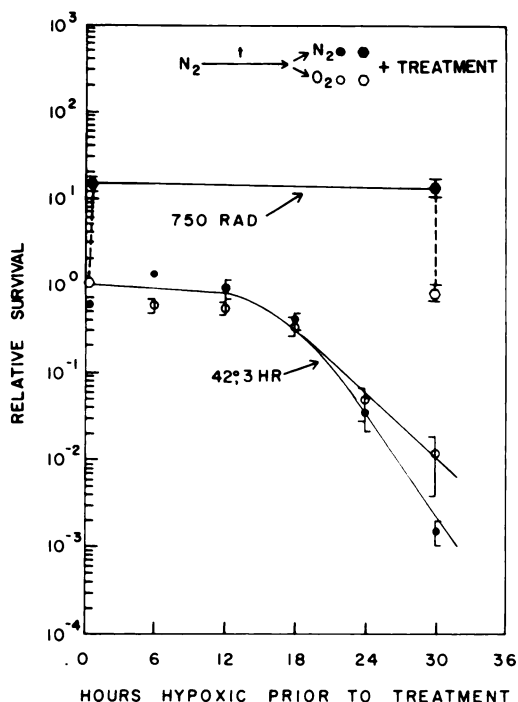


Chart 2. Approximately  $5 \times 10^5$  cells in 1 ml of medium were heated or irradiated under oxygenated (○, ○) or hypoxic (●, ●) conditions, following 0 to 30 hr culturing under hypoxic conditions at 37°. The data were normalized to the survival level obtained after heating or irradiation under oxygenated conditions without prior culturing under hypoxia. Mean  $\pm$  S.E. of the relative surviving fraction from 2 experiments are indicated.

to 0.01 increased by a factor of 2.9, when compared to cells which were reoxygenated prior to treatment (data not shown).

The kinetics for development of thermal sensitization or radiation resistance as a function of culturing time under hypoxia are shown in Chart 2. Approximately  $5.5 \times 10^5$  cells/ml medium were exposed to single doses of 750 rads or 3 hr heat under air, acute or chronic hypoxic conditions, or following reoxygenation of cells previously cultured under hypoxia. The data were normalized to the survival level obtained when cells were treated under aerobic conditions without prior culturing under hypoxia. Sensitivity to hyperthermia increases substantially as hypoxic culturing is prolonged; for example, after culturing under hypoxia for 30 hr, the relative survival of hypoxic cells decreased by a factor of 500. In contrast to these results, hypoxia protects cells against the lethal effects of X-irradiation, an effect which is constant for up to 30 hr culturing under nitrogen. Reoxygenation prior to radiation resensitizes cells to radiation.

From the foregoing data, it is not clear if prolonged oxygen deprivation sensitizes cells to hyperthermia directly or by effecting changes in the microenvironment due to cell metabolism (e.g., accumulation of lactic acid and reduction in pH) which subsequently influences sensitivity. The results in Table 1 indicate that an accumulation or depletion of essential metabolites is involved in sensitization, and that these changes occur more rapidly under anaerobic conditions. For the data presented in Table 1, cells were maintained under nitrogen or oxygen (air) for 24 hr prior to heat

treatment for 3 hr. Under hypoxic conditions, cell survival decreases rapidly with increasing cell density, while under air, survival decreases very little even at significantly higher cell densities. Extracellular pH at the time of heat treatment is also indicated in Table 1. The decrease in survival correlates with decreasing extracellular pH, which is more pronounced under anaerobic conditions. However, it should also be noted that under equivalent pH conditions, (7.10 under  $N_2$  and 7.11 under  $O_2$ ) cells cultured under  $N_2$  for 24 hr are more sensitive than are cells cultured aerobically. Therefore, chronic oxygen deprivation also appears to sensitize cells to hyperthermia in a pH- and cell density-independent manner.

This possibility was investigated in more detail by culturing cells under hypoxic conditions for 27 hr, which resulted in a decrease in pH from 7.40 to 6.90. Replicate flasks were then heated for 3 hr under hypoxic or reoxygenated conditions at pH 6.90 or following readjustment of pH to 7.3. The pH was adjusted by gassing (while agitating) the flasks with air or nitrogen containing a reduced concentration of carbon dioxide ( $\approx 1.7\%$ ) for 15 min, immediately prior to heat treatment. The results, which are presented in Table 2, clearly indicate that metabolic acidification increases the sensitivity of cells to hyperthermia and that this effect is in large part reversible by adjustment of the pH. Readjustment of pH from 6.90 to 7.31 increased survival from 0.0085 to 0.049. However oxygenated or acutely hypoxic cells (under  $N_2$  for 3.5 hr) were somewhat more resistant (survival,  $\approx 0.1$ )

Table 1

*Influence of cell density on sensitivity to hyperthermia*

Exponential-phase cells were gassed with air or nitrogen plus 5%  $CO_2$ . Twenty-four hr later, the cells were heated for 3 hr at 42°. The average number of cells/ml medium during the 24 hr of culturing at 37° and pH at the time of treatment are indicated.

$N_2$			$O_2$		
Cells/ml $\times 10^6$	Survival following 42°, 3 hr	pH	Cells/ml $\times 10^6$	Survival following 42°, 3 hr	pH
0.21	0.10 $\pm$ 0.005 <sup>a</sup>	7.29	0.34	0.18 $\pm$ 0.01	7.33
0.42	0.048 $\pm$ 0.005	7.10	0.64	0.17 $\pm$ 0.01	7.11
0.63	0.0019 $\pm$ 0.0008	6.75	1.80	0.12 $\pm$ 0.007	7.12

<sup>a</sup> Mean  $\pm$  S.E. of the surviving fraction.

Table 2

*Influence of  $O_2$  and pH on heat sensitivity of chronically hypoxic cells*

Approximately  $5 \times 10^5$  cells/ml medium were heated under oxygenated or acute (3.5 hr) hypoxic conditions, or following 27 hr culturing under hypoxia. Cells maintained under hypoxia were either treated under hypoxic conditions or reoxygenated immediately prior to treatment. In replicate flasks, the medium pH was adjusted to 7.31 immediately prior to treatment as indicated.

Treatment condition	pH	Survival following 42°, 3 hr
Acute $N_2$ or $O_2$	7.38	0.125 $\pm$ 0.023 <sup>a</sup>
Chronic $N_2$	6.90	0.0085 $\pm$ 0.0024
Chronic $N_2$ (pH adjusted)	7.31	0.049 $\pm$ 0.018
Chronic $N_2$ (reoxygenated)	6.85	0.0082 $\pm$ 0.0017
Chronic $N_2$ (reoxygenated pH adjusted)	7.30	0.047 $\pm$ 0.0076

<sup>a</sup> Mean  $\pm$  S.D.

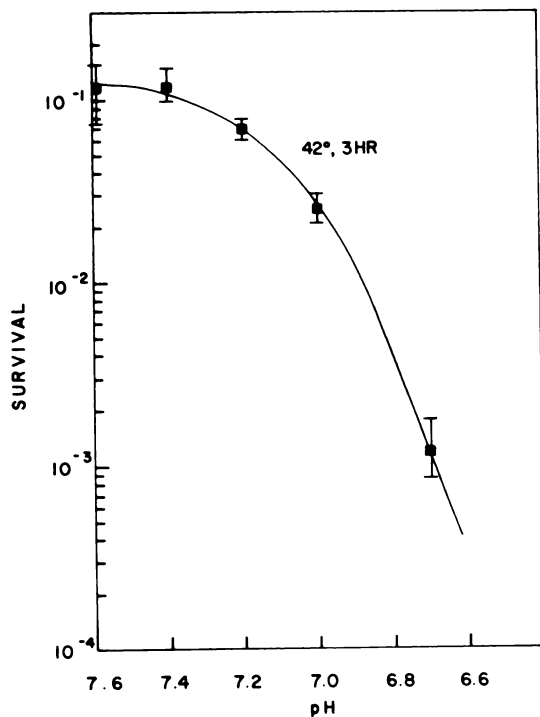


Chart 3. Exponential-phase cells were overlain with fresh pH adjusted medium gassed with air + 5% CO<sub>2</sub> and maintained at 37° for 1 hr prior to treatment. One hr following treatment, cells were trypsinized and plated for clonal growth.

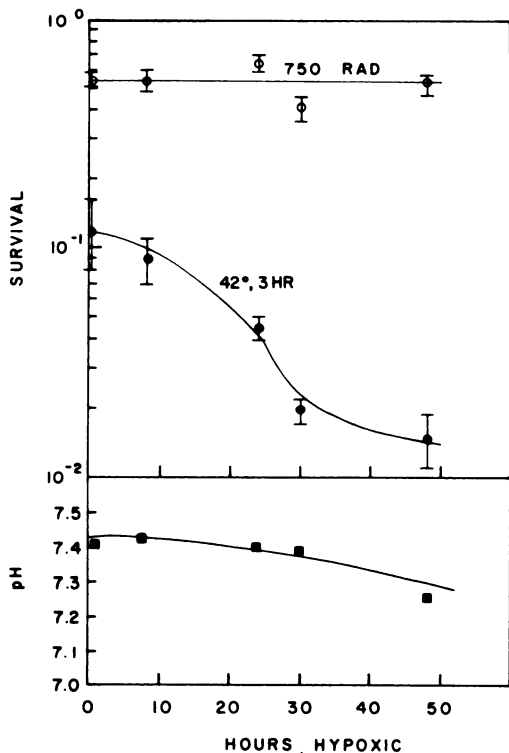


Chart 4. Upper Panel, the response of low-density hypoxic cells to 750 rads or 3 hr heating at 42°. The cells were cultured under hypoxic conditions for up to 48 hr prior to treatment. The cell density was  $1.1 \times 10^5$  cells/ml medium at "0" hr hypoxic and  $1.8 \times 10^5$  cells/ml medium at 48 hr. Lower panel, the change in pH due to cell metabolism.

than were chronically hypoxic cells at essentially equivalent pH values, and reoxygenation immediately prior to heating did not reverse this residual chronic hypoxic effect. These results are similar to those shown in Chart 1, *i.e.*, reoxygenation prior to treatment did not substantially influence sensitivity unless survival was below 0.01. The effect of pH alone was determined by heating exponential phase aerobic cells in fresh medium for 3 hr as indicated in Chart 3. Variation in pH between 7.6 and 7.2 did not markedly influence survival; however, below pH 7.0, survival decreased rapidly with decreasing pH. As previously reported (13), the reproductive capacity of CHO cells was not influenced by variation in pH from 7.6 to 6.7 at 37°.

The results shown in Chart 4 show the effect of prolonged oxygen deprivation on cellular sensitivity in the absence of significant changes in the concentration of extracellular metabolites. For these studies, low-density cultures ( $1 \times 10^5$  cells/ml) were gassed with nitrogen and heated for 3 hr under hypoxia 0 to 48 hr later. Chronic oxygen deprivation alone increases cell killing by a factor of approximately 5 after 30 hr culturing under nitrogen. This 5-fold reduction in survival is significantly less than is the 500-fold reduction in survival shown in Chart 2, which was observed after culturing cells at 5 times higher cell density.

The rate of extracellular pH change due to cell metabolism under hypoxic conditions at 37° is indicated by the lower curve of Chart 5. During the first 6 hr culturing under

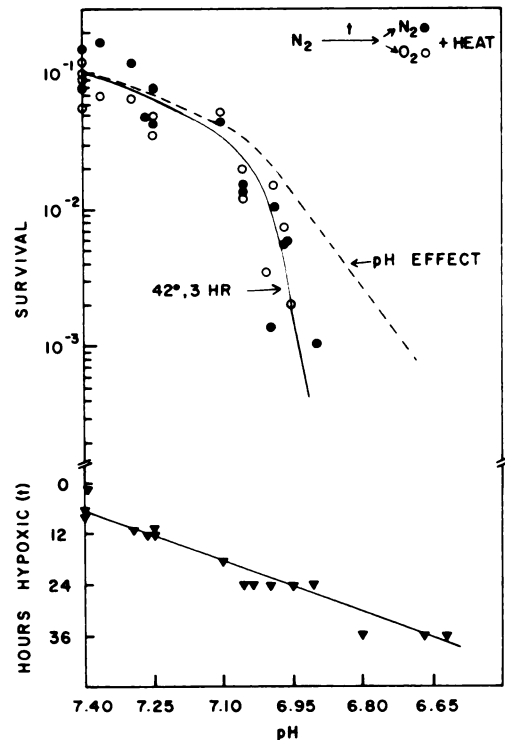


Chart 5. Lower ordinate and abscissa, decrease in extracellular medium pH due to cell metabolism when  $\approx 5.7 \times 10^5$  cells in 1 ml medium are cultured under hypoxia for up to 36 hr. The cells were heated at the indicated pH ( $\blacktriangle$ ), and the resultant cell survival is shown (upper solid curve,  $\circ$  and  $\bullet$ ). Some cells were reoxygenated immediately prior to treatment ( $\circ$ ). Plating efficiencies varied from the control value of 70% at 0 hr to 35% following 30 hr culturing under hypoxia. Influence of reduced pH is replotted from Chart 3 (---). The results from 4 experiments are plotted; error bars have been omitted for clarity.

hypoxia, no measurable pH change was observed; however, during the following 30 hr, pH decreased linearly with time to 6.65 after 36 hr culturing under hypoxia. The total number of cells/flask (in 1 ml medium) increased from  $5 \times 10^5$  cells to  $6.5 \times 10^5$  cells during the 36 hr culturing under hypoxia. Identical culturing conditions did not influence the normal proliferative rate of oxygenated cells. The *solid curve* in the upper section of Chart 5 represents the survival of these cells which were heated for 3 hr at the indicated pH. For example, 24 hr culturing under hypoxia reduced medium pH to 7.08 to 6.9: the surviving fraction of cells heated under these conditions ranged from  $\approx 10^{-2}$  to  $10^{-3}$ . Reoxygenation immediately prior to treatment (*open circles*) did not influence survival. The *upper dashed curve* labeled "pH Effect" indicates the change in cell response due to variation in extracellular pH and is replotted from Chart 3. The difference in the survival level for the 2 curves increases with the time of culturing under hypoxia and is probably due to the pH-independent sensitizing effect of prolonged hypoxia as shown in Chart 4.

## DISCUSSION

The results of this study show that the response of CHO cells to 42° hyperthermia is not altered by acute hypoxia, *i.e.*,  $\leq 10$  hr, and thus are in agreement with the results reported by Power and Harris (23) under well-defined nutrient conditions, and by Gerweck et al. (11). In a recent report, Bass et al. (2) obtained similar results with CHO-K1 cells; however, HeLa S<sub>3</sub> cells appeared to be somewhat protected by acute hypoxia. Other investigators (16, 17, 25) observed substantial sensitization under acute hypoxic conditions; however the method used for obtaining hypoxia (respiration depletion of oxygen by very high density cultures) may have rapidly resulted in significant changes in nutrients and pH. Studies of the effect of prolonged oxygen deprivation on sensitivity to hyperthermia have not been previously reported to our knowledge. The results presented here indicate that prolonged oxygen deprivation increases the sensitivity of cells to hyperthermia in low-density cultures in the absence of change in extracellular pH (Chart 4). Chronic oxygen deprivation may limit the capacity of cells to repair sublethal heat damage which may occur during heat treatment and is likely an enzymatic and energy-dependent process (10).

The experiments described in this report indicate that prolonged oxygen deprivation prior to heat treatment increases hyperthermic sensitivity, and that the gas phase at the time of treatment influences response only under specific conditions. Cells maintained hypoxic for  $\leq 27$  hr and then heated for 3 hr under oxygenated or hypoxic conditions were equally sensitive to hyperthermia (Charts 1 and 2; Table 2). However an oxygen sparing or protective effect was observed when the total time under hypoxia at 37° plus 42° was  $>30$  hr (Chart 2). The reason for this sparing effect is not known. If the pH of cells heated under hypoxia decreased by 0.01 to 0.03 units more than cells heated under oxygenated conditions, a greater sensitivity of cells heated under hypoxia may have occurred. These pH-related effects would become apparent at pH values below 6.9

(Chart 3) where sensitivity is strongly influenced by pH. However no significant differences in extracellular pH were consistently noted between cells heated under the 2 gas phases, although small changes undetected by the pH measuring technique ( $\pm 0.03$  pH units) may have occurred. Other mechanisms are possible, however, and further studies will be required before these possibilities are resolved. Nevertheless, it is quite clear that chronic oxygen deprivation increases hyperthermic sensitivity regardless of the pH at the time of treatment (Chart 4; Tables 1 and 2).

In addition to the sensitizing effect of prolonged oxygen deprivation, which was apparent at pH 7.35 to 7.4 (Chart 4), the primary factor controlling sensitivity to hyperthermia under the relatively high cell density hypoxic conditions used in these experiments was a decrease in pH due to cell metabolism. This pH-dependent increased sensitivity could be abolished by readjustment of pH immediately prior to heat treatment (Table 2). Nevertheless, reversal of pH to 7.3 (or reoxygenation) immediately prior to treatment did not totally reverse the sensitizing effect of prolonged culturing under hypoxia as compared to acute hypoxic cells (Table 2). The possibility exists that differences in the cell-cycle distribution (18) may account for differences in heat sensitivity. However, this does not seem likely, as Sapareto et al. (24) have shown that cycling exponential-phase CHO cells are only slightly more sensitive to 42° hyperthermia than cells in G<sub>1</sub>.

The substantial increase in hyperthermic sensitivity at low extracellular pH reported in this paper has also been observed by Overgaard (21) in L1A2 ascites tumor cells at 42.5° and in cultured human glioblastoma cells at various temperatures.<sup>5</sup> It therefore appears that, in general, low extracellular pH sensitizes cells to hyperthermia. However, the magnitude of this effect at a specific pH may vary from cell line to cell line. The cellular target of hyperthermic lethality has not been identified, and the mechanism of pH sensitization is not known (12). Variations in extracellular pH are reflected intracellularly and *vice versa*, although the relationship is dependent on the buffer system, pH range in question (28), and possibly the cell line studied. Reduced pH could increase cell lethality by either directly sensitizing the cellular target of hyperthermia, or by suppressing repair of sublethal heat damage, or both, as previously discussed (12).

The clinical importance of these naturally occurring microenvironmental differences will be dependent on the tissue fluid hydrogen ion and oxygen concentrations and on the volume of tumor tissue affected. A common feature of neoplastic tissue is an elevated rate of lactic acid production under oxygenated conditions (4, 29). Hypoxia further increases the rate of lactate production commonly by a factor of 2 to 3 or more (7). Gullino et al. (14) compared the composition of serum, tumor tissue fluid, and normal s.c. tissue interstitial fluid in 3 rat hepatomas, a fibrosarcoma, and a carcinoma. The only consistently observed differences between normal and tumor tissue fluid were decreased glucose content and elevated lactate and carbon dioxide content in the tumor fluid. No significant differ-

<sup>5</sup> Unpublished observations.

Table 3  
Tumor and normal tissue pH

The interstitial fluid pH from human and rodent tumors and normal tissues (s.c., muscle, and liver) was determined with glass electrodes. Blood sugar levels were elevated in the patients examined by Naeslund and Swenson. The pH values obtained by Meyer *et al.* were determined 10 to 90 min after surgical removal of tissue from the patient.

Investigator	Tumor types	Tissue fluid pH		Assay conditions
		Normal	Tumor	
Gullino <i>et al.</i> (15)	Rat: carcinomas, fibrosarcomas, hepatomas	7.33 ± 0.00 <sup>a</sup>	7.05 ± 0.09	<i>In vivo</i>
Eden <i>et al.</i> (8)	Rat: carcinomas, sarcomas, hepatomas	7.39 ± 0.12	7.03 ± 0.12	<i>In vivo</i>
Ashby (1)	Human melanomas	7.43 ± 0.08	6.78 ± 0.09	<i>In vivo</i>
Naeslund and Swenson (20)	Human cancer of colon	7.57 ± 0.25	6.89 ± 0.28	<i>In vivo</i>
Meyer <i>et al.</i> (19)	Human, various	6.60 ± 0.38	6.21 ± 0.29	Surgical specimens

<sup>a</sup> Mean ± S.D. for all tumors studied by each investigator.

ences were observed between the tumor and the normal tissue fluid protein, amino acid, cholesterol, or lipid phosphorus content. Comparable results have been obtained in several other solid and ascitic mouse tumors (5).

Gullino *et al.* (15) determined the interstitial fluid pH in a variety of rat tumors. The tumor fluid pH ranged from 7.19 to 6.95 which was 0.2 to 0.4 units lower than the blood and s.c. fluid pH. The pH difference was due to the elevated levels of lactate and carbon dioxide. The abnormally low pH in tumor interstitial fluid has also been demonstrated by Eden *et al.* (8) in rats and by Naeslund and Swenson (20) in mice. Similar observations were made in several varieties of human tumors by Ashby (1), Meyer *et al.* (19), and Naeslund and Swenson (20). In these investigations micro pH gradients were not determined. These data are summarized in Table 3.

In addition to reduced pH, several lines of investigation suggest or demonstrate that tumors contain foci of hypoxic cells (26). The presence of these radioresistant foci may account for the failure to control certain tumors locally by radiotherapy (27). The results obtained in this study indicate that hyperthermia may be effective for eliminating acute and especially chronic hypoxic cells even in the absence of changes in extracellular pH. Sufficient information is not available to predict if the immediate and long-term effects of hyperthermia on blood flow, respiration, or glycolysis will tend to enhance or diminish these naturally occurring differences which may also affect the response of cells to radiation. Clearly, these questions require additional attention. Nevertheless, it is unlikely that all the neoplastic cells of a tumor reside in a chronically hypoxic and acidic environment even at elevated temperatures. The adjunctive use of radiation would appear useful for eliminating the relatively heat-resistant and radiosensitive oxygenated cells existing at normal pH.

In summary, tumorlike environmental conditions (reduced pH and oxygen) sensitize cells to hyperthermia. This microenvironmentally induced sensitization is prominent at clinically tolerable temperatures (22). Hyperthermia should, therefore, be useful whether used alone or in combination with radiation for the treatment of cancer.

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