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The Effect of Hyperthermia on Activation of Lysosomal Enzymes in HeLa Cells*

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Abstract—*The activity of lysosomal acid phosphatase has been studied by a quantitative cytochemical method in HeLa cells subjected to hyperthermic treatment, lysosomal enzyme activity has been related to cell survival.*

Incubation of cells for 0.5–5 hr *at temperatures greater than* 37°C *causes an increase in lysosomal enzyme activity with a very marked increase over the range* 42–44°C. *Enzyme activation occurred immediately after heat treatment and increased linearly with time over the period* 0–90 min. *The effect of heat on lysosomal enzyme activation was* pH *dependent and much more effective at* pH 5.6 *than at* pH 7.2.

An inverse relationship between lysosomal enzyme activation and cell survival has been demonstrated. It is concluded that activation of lysosomal enzymes is an important and early event in cellular injury caused by hyperthermia.

INTRODUCTION

THE MECHANISM by which hyperthermia causes tumour regression is not yet understood. Studies of cultured cells *in vitro* have demonstrated that hyperthermia treatment for various periods of time reduces cell survival [1, 2]. Overgaard [3, 4] and von Ardenne [5, 6] have postulated that hyperthermic activation of the lysosome plays an important role in subsequent cell destruction and the pH of the extracellular environment markedly influences the effects of hyperthermia [7, 8]. Under normal conditions the pH of tumours may be 1.0 pH unit below the surrounding normal tissue [5], and this may be an important factor in explaining the difference in the response of normal and neoplastic tissues to hyperthermia. Lysosomal enzyme activity is known to be increased by an acid environment [9], but it is not yet established whether hyperthermia could affect the pH of the cell.

Lysosomal enzyme activity can be measured using standard biochemical techniques but frequently during the preparative procedure the fragile lysosomal membrane is extensively damaged [10]. Horvat and Touster [11] showed that lysosomal enzymes were released from suspensions of lysosomes prepared from Ehrlich ascites cells and from rat liver when incubated at 37°C in 0.7 M sucrose, pH 5.0 for 0–250 min. The results of their experiments indicated a greater stability of Ehrlich ascites tumour lysosomes as compared to liver lysosomes. Turano *et al.* [12] compared suspensions of lysosomes prepared from Novikoff hepatoma cells and from rat liver incubated for 30 or 60 min at 38 or 43°C in 0.4 M sucrose. They showed that the lysosomes from neoplastic cells were considerably more fragile than those from normal liver, but were unable to establish whether lysosomal enzyme activiation was the primary mechanism of the cellular damage caused by the higher temperatures. These results using standard biochemical methods strongly indicate the need for a method which does not damage or influence the state of the lysosome membrane. Quantitative cytochemistry overcomes most of the problems and was therefore chosen for these experiments.

This present study has been designed to investigate the effect of hyperthermia on the stability of the lysosomal membrane and of the activity of the lysosomal enzymes in HeLa cells using well proven quantitative cytochemical methods. The effect of hyperthermia on lysosomal enzyme activity has been related to cell survival to determine whether a relationship exists between lysosome stability and cell viability, and to attempt to establish

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more precisely, the possible role of lysosomal enzyme activation in cellular regression initiated by hyperthermia.

MATERIALS AND METHODS

Cell type and method of culture

HeLa-S3 cells were maintained in Eagle's minimum essential medium supplemented with 20% newborn calf serum buffered at pH 7.3. with HEPES buffer (Gibco Bio-Cult). Stock cultures were grown in 20 oz glass medical flats in 25 ml medium at 37°C. The cells were sub-cultured every fifth day.

Determination of lysosomal enzyme activity

Exponentially growing cells were plated on to glass microscope slides and placed in complete medium in Petri dishes for 18 hr to allow recovery from trypsinisation before further treatment.

The method used to stain for acid phosphatase was based on that originally described [10, 13]. HeLa cells on glass slides were washed after the thermal treatment and then placed in a medium containing 0.3% β-glycerophosphate plus 4 mM lead nitrate in 0.05 M acetate buffer (pH 5.0). After incubation for 0, 30, 60 and 90 min, the slides were rinsed and placed in water saturated with H_2S to convert the lead deposit to a black lead sulphide stain, the formation of which is proportional to the enzyme activity [10]. The slides were mounted in Farrants medium.

Small areas of the cell cytoplasm were scanned with an M85 Vickers Scanning and Integrating microdensitometer, being careful to avoid the nucleus.

Measurements were made at a wavelength of 550 nm at ×400 magnification. A large number of cells were read each time to allow for minor variations in staining intensity as described in detail in the Results section. Sodium fluoride, a potent inhibitor of acid phosphatase, was added to the substrate medium at a concentration of 10 mM. This allowed a measurement of the background cytoplasmic activity to be made. Plotting absorbance readings obtained on the microdensitometer against incubation time gives a measurement of lysosomal enzyme activity [10].

Experimental techniques

The HeLa cells on glass slides were placed in Coplin jars containing fresh medium at 37°C. The Coplin jars were transferred to a waterbath set at the desired temperature, constant within ±0.1°C. The temperature of the medium equilibrated within 2–3 min. To study the effect of pH, Eagle's medium, without calf serum and HEPES buffer, was buffered with 0.02 M Tris–maleate and adjusted to pH 7.2, 6.6, 6.2, 6.0 or 5.6. If the lysosomal enzyme activity was not measured immediately after hyperthermic treatment the cells were returned to the complete growth medium and kept at 37°C.

Cell survivial

A well dispersed cell suspension was produced using 0.25% trypsin and 700 dispersed cells were added to each 25 cm^2 plastic flask (Nunc). The flasks were incubated for 20 hr to allow cell attachment and recovery from trypsinisation.

The cells were heat treated by submerging the flasks in a water-bath at the desired temperature (±0.1°C). After heat treatment the flasks were incubated at 37°C for a further 8 days to allow the single cells to form visible colonies.

The colonies were then fixed in 10% Neutral Buffered Formal Saline, stained with 0.5% crystal violet and colonies of 50 or more cells were counted to calculate the surviving fraction.

RESULTS

Effect of hyperthermia

The effect of temperature on lysosomal enzyme activity in HeLa cells was determined by incubating 16 slides covered with cells at each temperature (37, 40, 43, 44 and 45°C) for 90 min. The cells kept at each temperature were then incubated for 0–90 min in the β-glycerophosphate medium at 37°C. Four slides were used for each staining time and fifteen cells were read on each slide at random. A large number of cells were read to allow for the slight variations in staining intensity observed between cells similarly treated. Rates of lysosomal acid phosphatase activity were determined by plotting readings for absorbance at different staining times against incubation time. Temperatures above 37°C caused a marked increase in lysosomal enzyme activation with a very rapid increase in activity over the range 43–44°C (Fig. 1). Above 44°C the activation reached a maximum. Cell survival was determined after 1 hr incubation at each temperature (37, 39,

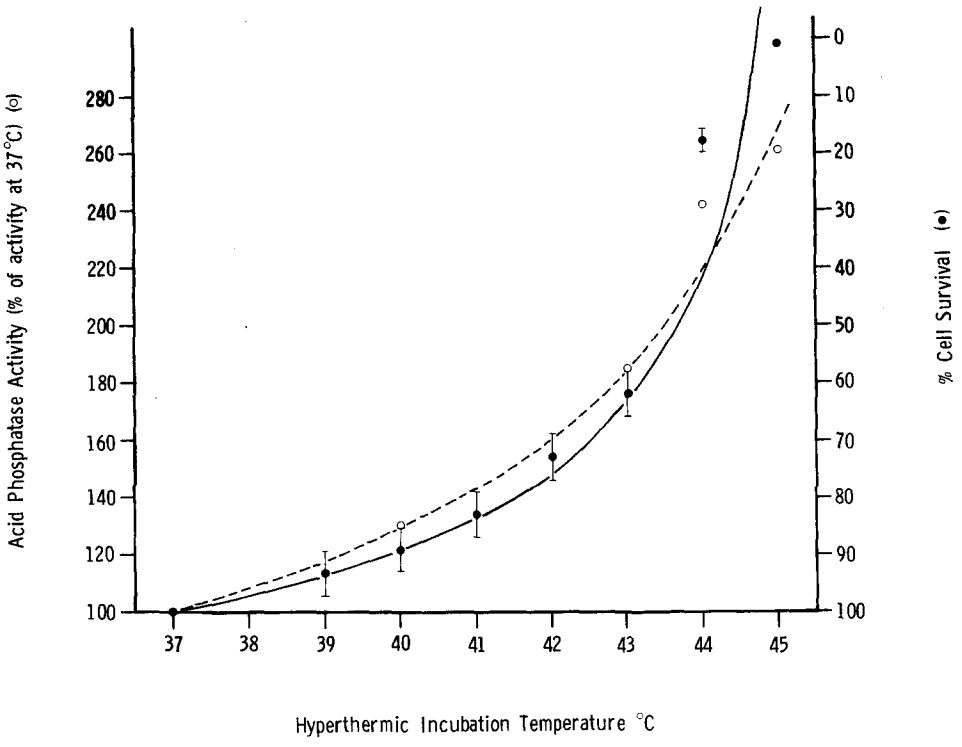

Fig. 1. *Effect of temperature on lysosomal enzyme activity and cell survival in HeLa cells after incubation for* 1 hr. *Acid phosphatase activity is expressed as a* % *increase in the rate of lysosomal enzyme activation, taking activity at* 37°C *as* 100. *Cell survival is expressed as a* % *of the unheated control. All figures show mean* ± S.E.

40, 41, 42, 43, 44 and 45°C). At temperatures up to 42°C there was only a small loss of cell viability, but at temperatures of 43°C or above cell survival decreased rapidly (Fig. 1).

Effect of time of exposure to elevated temperatures

The effect of time at elevated temperatures was investigated by subjecting the cells on slides to temperatures of 37, 42, 43 and 44°C for periods of 0, 30, 60 or 90 min. All the cells were then incubated in glycerophosphate medium for 60 min to determine acid phosphatase activity. The absorbance readings obtained from the microdensitometer were plotted against time at the elevated temperature. There is a linear increase in enzyme activity over the period 0–90 min. (Fig. 2). Cell survival was measured by heating the flasks at 43°C for periods of 0, 0.5, 1, 2, 3, 4 and 5 hr. Cell viability was steadily lost over the time period studied (Fig. 3).

Recovery after hyperthermic treatment

To investigate lysosomal enzyme activity in the period following hyperthermia, cells were kept at 43°C for 1 hr and then incubated at 37°C for periods of 0, 3, 6, 14, 18 and 24 hr before measurment of enzyme activity. Cells kept at 37°C throughout the whole period served as controls. A very rapid increase in enzyme activity was observed immediately after heating which fell to a low value after 6 hr. Activity then slowly increased up to 24 hr (Fig. 4).

Effect of pH

The effect of the pH of the cell environment on lysosomal enzyme activation induced by hyperthermia was studied by incubating cells in media buffered at different pH values. Tris–maleate buffer (0.02 M) was used to give a range of solutions at pH 7.2, 6.6, 6.2, 6.0 or 5.6. A minimum of four slides covered in cells were incubated at each pH for 90 min at 42°C. Cells treated in a similar manner but incubated at 37°C were used as controls. All the cells including controls were incubated for 60 min in the β-glycerophosphate medium to determine enzyme activity. Lysosomal enzyme activation rapidly increased as pH fell (Fig. 5) but at pH 5.6 cells became detached from the slides making no readings possible. At 37°C the lysosomes were slightly more labile at low pH than normal, but this increase was small when compared to the increase at 42°C, which appears to indicate that the observed effects are due to pH and not the use of Tris-maleate buffer.

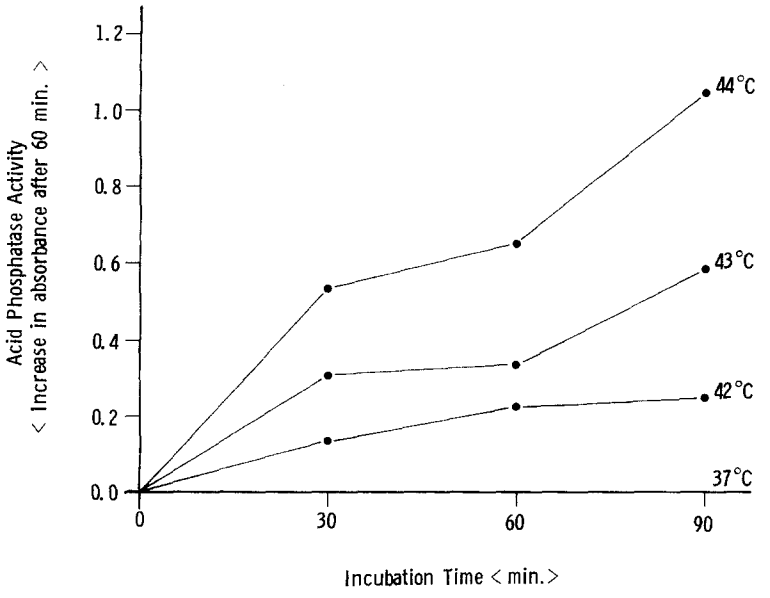

Fig. 2. Effect of hyperthermic incubation time on enzyme activity in HeLa cells. Acid phosphatase activities after incubation at 42, 43 and 44°C are shown after subtraction of activities at 37°C.

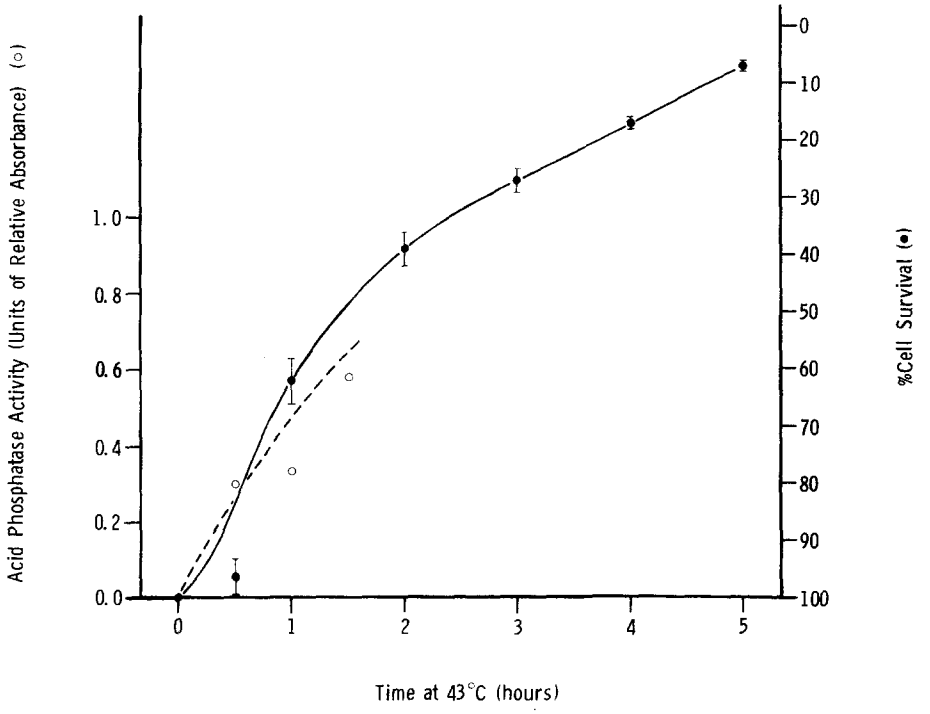

Fig. 3. Effect of hyperthermic incubation time on lysosomal enzyme activity and cell survival in HeLa cells kept at 43°C. Acid phosphatase activity expressed as rate of increase of absorbance which is plotted against time at the elevated temperature. Cell survival is expressed as a % of the unheated control. All figures show mean ± S.E. where applicable.

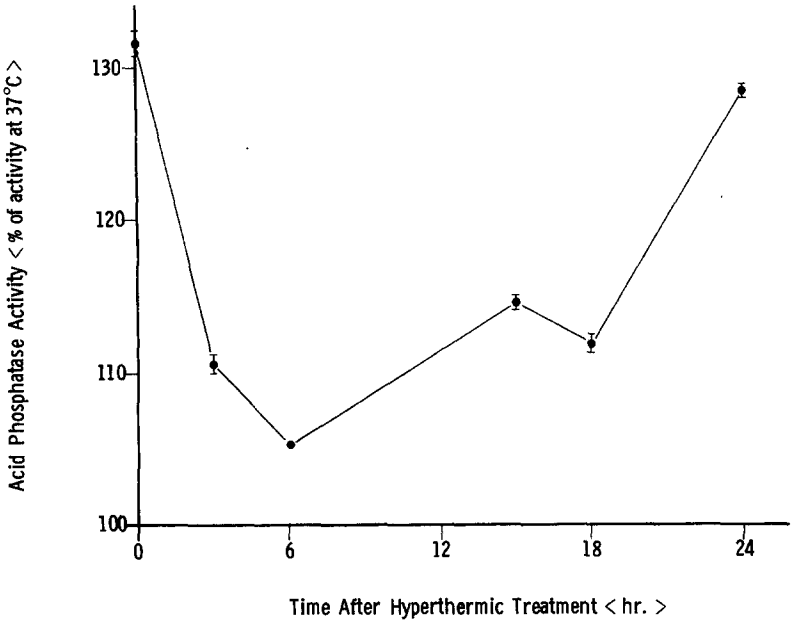

Fig. 4. *Lysosomal enzyme activity in HeLa cells measured at intervals after hyperthermic treatment* (43°C *for* 1 hr). *Results are expressed as a* % *increase in the rate of lysosomal enzyme activation, taking activity at* 37°C *as* 100. *All figures show mean* ± S.E.

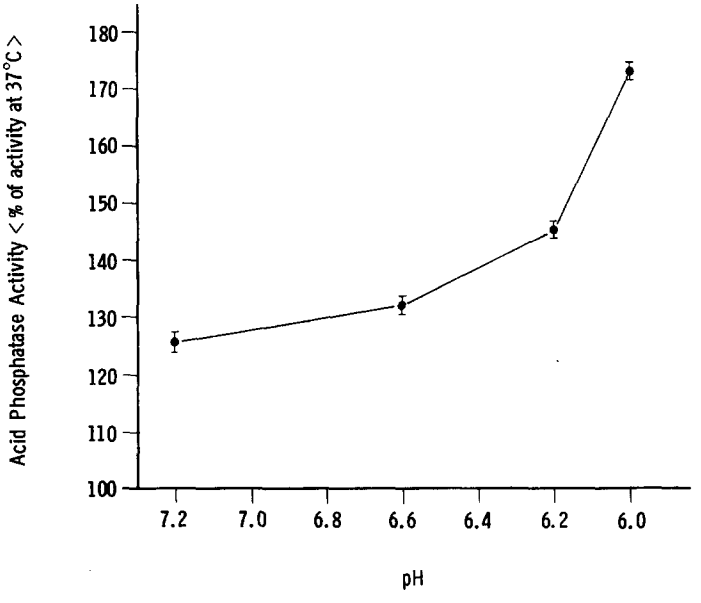

*Fig.*5. *Influence of environmental* pH *on lysosomal enzyme activation in HeLa cells exposed to* 42°C *for* 90 min. *Results are expressed as a* % *of the acid phosphatase activity at* 37°C. *Standard errors are shown.*

DISCUSSION

The results of this investigation have demonstrated that the degree of lysosomal enzyme activation is dependent on the duration of hyperthermia, the temperature attained and the pH of the cell environment. Below 42°C the degree of lysosomal enzyme activation is small but this rapidly increases as the temperature rises (Fig. 1). The curve for cell survival follows a similar pattern, with a rapid fall in cell viability around 42°C.

The duration of hyperthermia is also important (Fig. 2, 3) the lower the temperature the longer the time required to cause values for lysosomal enzyme activation and decreased cell survival which are equal to those resulting from high temperatures for short periods.

Below 42°C, even after a long period of heating, lysosomal enzyme activation is increased only slightly.

After heating at 43°C cell viability was gradually lost over the 5 hr heating period. The steep gradient in Fig. 3 over the first 1.5 hr appears to indicate that the lysosomal enzymes are activated early in the heating process.

The observation that there is variation in the degree of lysosomal enzyme activation in different cells after hyperthermia, is probably due to the stage of the cells in the cell cycle. Similar observations were made on lysosomal enzyme activity in HeLa cells treated by irradiation [13]. Hyperthermic treatment causes a block in the S and G_2+M phases of the cycle [14] while the block in G_2+M is transient, most of the cells blocked in S die in that phase [15]. These effects on the cell cycle may explain the shape of the curve shown in Fig. 4. Cells in S phase may die immediately causing the rapid increase in activation seen at zero time while lysosomal enzyme activation in cells at other stages of the cell cycle may be delayed until 24 hr (Fig. 4). A repair process, possibly re-organisation of the lipid and/or protein components of the membranes or rapid regeneration of new lysosomes may occur [16]. Lysosomal activity in cells subjected to hyperthermia increased as the pH of the cell environment fell (Fig. 5). Overgaard [8] using L1A2 ascites cells subjected to hyperthermia under similar conditions showed that cell viability decreased as the pH was lowered. The enzyme activity of lysosomes in suspension is known to increase as the pH decreases [9]. The reaction although primarily intracellular is affected by the pH of the extracellular environment. Since the relationship between extracellular and intracellular pH is unclear [17], intracellular pH cannot be assumed to be identical to the pH of the extracellular environment. Von Ardenne [18, 19] has shown how hyperthermia can cause a reduction in tumour pH by altering the microcirculation. In cell cultures, any metabolic changes can only lead to a minimal change in pH due to the buffering capacity of the medium used.

The effects of hyperthermia on lysosomes could be explained as a result of effects of hyperthermia on the lysosomal membrane. The rapid fall in cell viability around 42°C may be due to a lipid transition occurring in the lysosomal membrane causing a structural change. Some lecithins undergo a phase transition at 42°C [20] and as the membranes contain about 30% lecithins this transition may cause increased membrane fluidity. As a consequence the lysosome membrane could become more permeable to substrates and possibly eventually cause release of lysosomal enzymes into the cell.

Membrane fluidizing drugs have been shown to act directly to decrease hyperthermic temperatures and exposure times required to kill cells. Yatvin [21] concluded that the membrane lipids are the likely target for hyperthermia while Kwock *et al.* [22] hypothesised that heat causes an irreversible unfolding of membrane protein.

The results presented here demonstrate that activation of lysosomes and lysosomal enzymes correlates closely with cell survival showing that the lysosomes are of primary importance in the development of cellular injury, following a period of hyperthermia. Lysosomal enzyme activation being potentiated by an environment with a low pH and under these conditions lower temperatures are as effective as high temperatures at normal pH.

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