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The Effect of Hyperthermia and Radiation on Lysosomal Enzyme Activity of Mouse Mammary Tumours*

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Abstract—*The effects of hyperthermia and radiation have been studied on the acid phosphatase and β-glucuronidase activities in lysosomes of C3H mice mammary tumours and of the spleen. Quantitative histochemical methods have been used.*

Hyperthermic treatment of both spontaneous and transplanted tumours caused an increase in the activity of both acid phosphatase and β-glucuronidase when measured immediately after treatment, but the activities returned to normal after 24 hr. *In contrast a radiation dose of* 3500 rad *did not cause an increase in activity of either enzyme immediately, but a large activation was observed after* 24 hr. *Combination of hyperthermic and radiation treatment caused increases in enzyme activities which were dependent on the time after treatment.*

Hyperthermic treatment of the lower body of mice bearing tumours also caused activation of lysosomal enzymes in the spleen. This may be hormone mediated.

It is considered that the increased lysosomal enzyme activity observed after hyperthermia may be a consequence of increased permeability of the lysosomal membrane caused by the hyperthermia.

INTRODUCTION

RECENTLY there has been great interest in the potential of moderate hyperthermia, in the range 40–43°C, as a treatment for cancer, either alone or in combination with radiotherapy [1–3]. Selective killing of neoplastic cells by elevated temperatures occurs in both murine and human cell cultures [4–6], and hyperthermia causes a delay of tumour growth, or complete cure of a number of animal tumours [7–9], and of some human tumours [10–12]. Heat also potentiates the therapeutic action of irradiation on a range of neoplasms [13–17].

Lysosomes, with their complement of hydrolytic enzymes, become involved as a consequence of cellular damage [18]. Lysosomal enzyme activation or enzyme release follows irradiation. Wills and co-workers [19–21] developed quantitative histochemical methods of studying lysosomes *in situ* and measured an increase in the amount of acid phosphatase after low radiation doses, in spleen, thymus and the C3H mammary adenocarcinoma. Paris and Brandes [22] also noted a rise in the activity of lysosomal acid phosphatase in mammary tumours after X-irradiation, using electron microscopy.

Few studies have been made of the effect of heat on lysosomes in any tissue, but Overgaard *et al.* have shown qualitative changes in the lysosomes of mammary tumours, [23, 24]. In this investigation the use of quantitative histochemical techniques has been extended to study lysosomal enzyme activity in tissues and tumours treated by hyperthermia.

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MATERIALS AND METHODS

Animals

Male and female C3H mice were used in all investigations. These were supplied by the Radiobiology Department of St. Bartholomew's Medical College.

(i) *Spleen.* Normal male C3H mice were used to study the effect of hyperthermia on lysosomes of the spleen.

(ii) *Spontaneous tumours.* Female C3H mice

bearing spontaneous mammary adenocarcinomas were used.

(iii) *Transplanted tumours.* Small pieces from a spontaneous tumour were transplanted subcutaneously into the thighs of male C3H mice. They were ready for use after 1–2 months, when the tumour had a diameter of about 1 cm.

Hyperthermia

(i) In vitro. Cubes of tumour tissue were covered in pre-warmed 0.25 M sucrose in 0.01 M phosphate buffer pH 7.4 and incubated in a water-bath at the chosen temperature. After heating the cubes were blotted dry and frozen (as below).

(ii) In vivo. The animals were warmed in a small Perspex water-bath around which water from a larger bath was circulated. The temperature of the latter was controlled by a toluene/mercury regulator, and was adjusted so that the smaller bath reached the desired temperature. This was monitored throughout the experiment using a thermometer accurate to 0.1°C and did not vary more than a maximum 0.3°C. The temperature recorded is the average over the experimental period.

The tumour bearing mice were anaesthetised with 60 mg/kg of pentobarbitone sodium (Sagatal) injected intraperitoneally, and were placed in the bath in a vertical Perspex tube which fitted into a metal rack with 2 holes to accommodate the feet. When spontaneous tumours were used only those situated abdomenally were chosen, and the mouse was immersed up to the xyphisternum. Mice bearing transplanted tumours had only the hind legs below water. During the treatment the intratumoural temperature was measured by a probe attached to a Light Instruments electric thermometer, reading from 30° to 46°C to 0.05°C accuracy. This temperature rose, in 10 min, to within about 1°C of the water temperature and the exact temperature varied from mouse to mouse. At 43°C water temperature, the temperature within the tumour varied between 41.8° and 42.2°C. When spleen was heated the animal was again immersed up to the xyphisternum. A probe placed below the skin of the abdomen showed that the subcutaneous temperature reached the bath temperature after 15 min. All temperatures below 43.5°C to the lower body were well-tolerated by the animals.

Radiation

γ-Rays from a ^{60}Co source in a Gammabeam 50 unit were used. Animals were first anaesthetised as described, and placed in a Perspex box. The box was placed behind lead blocks, 10 cm thick, with a small conical hole 1–25 cm in diameter to permit irradiation of the tumours and shield the remainder of the body. The dose given to the tumour was 3500 rad, at a dose-rate of 350 rad/min, and the rest of the body received 3.5 rad.

Preparation of sections

Animals were killed by breaking the neck. The tissue was dissected out, washed, and cut into pieces about 3 × 3 × 3 mm, discarding the extremities and any necrotic areas of tumour. The blocks were frozen and mounted according to the method of Chayen and Bitensky [25]. Sections were cut at 8 μm on a Cambridge rocking microtome in a Bright's cryostat. The cabinet temperature was −20°C and the knife temperature −40°C. Sections were stored in the cryostat for 1–2 hr before use.

Cytochemical procedures

Acid phosphatase activity was measured in sections by the Gomori lead sulphide method described by Aikman and Wills, and Clarke and Wills [19–21, 26]. Incubation times of up to an hour were used. The absorbance was read on a Vickers M85 scanning microdenisitometer at 550 nm, × 40 objective and No. 2 spot size. Ten readings were taken from each of 3 sections for each incubation time and for the control. The mean density of the control was subtracted from the mean density produced at each incubation time; and average values were calculated from readings obtained from all tissue sections incubated for the same time. The progress curve obtained by plotting absorbance against time was used to determine rates of enzyme action [19].

β-Glucuronidase was determined by the method of Fishman and Goldman described by Chayen and Bitensky [25]. Readings were taken at 635 nm, × 40 objective and spot size 2 and plotted as for acid phosphatase.

RESULTS

(i) *The effect of hyperthermia on tumours* in vitro

Experiments *in vitro* were designed to study the effects of hyperthermia on tumours under conditions which prevented interference by other organs which could occur *in vivo*. A large transplanted C3H tumour was removed

from the mouse and cut into a number of blocks measuring about 3 × 3 × 3 mm. Half of these were incubated in 0.25 M sucrose in 0.01 M phosphate buffer, pH 7.4 in a water bath at room temperature, 22°C for 1 hr and the remainder were incubated in the same medium in a water bath at 43°C ± 0.3°C for 1 hr. The blocks were then frozen, sectioned and lysosomal enzyme activities were measured. The acid phosphatase activity was the same whether the tumours were incubated at 22°C or 43°C but was elevated considerably above the activity measured in 4 similar tumours which had not been incubated (Fig. 1). This increase was largest after 20 min

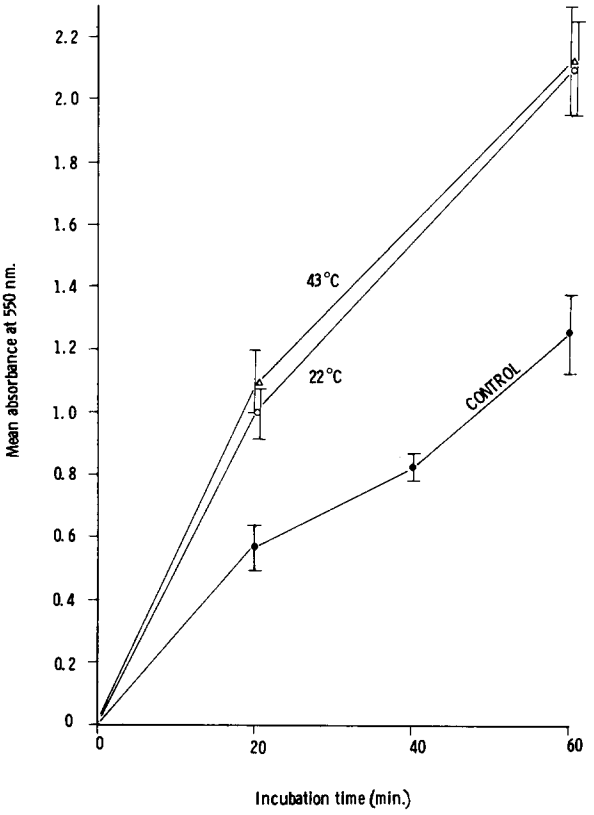

Fig. 1. Lysosomal acid phosphatase in blocks of C3H tumour treated in vitro. ●—*Blocks frozen immediately after removal from animal.* ±S.E.; ○—*Blocks incubated at* 22°C *for* 1 hr *in* 0.25 M *sucrose and* 0.01 M *phosphate buffer*, pH 7.4 *before freezing.* ±S.E.; △—*Blocks incubated at* 43°C *for* 1 hr *in* 0.25 M *sucrose and* 0.01 M *phosphate buffer*, pH 7.4, *before freezing.* ±S.E.

incubation in the reaction media, when the absorbance of the treated sections was 1.8 times that of the control sections. β-Glucuronidase activity was affected in a similar way; there was no difference between tumour pieces kept at 22°C and those kept at 43°C, but both increased in activity over untreated controls by approximately 1.4 times.

(ii) *The effect of hyperthermia on spontaneous tumours* in vivo

Seven C3H mice with spontaneous tumours were used. Three mice acted as controls and received 60 mg/kg of Sagatal and 1 hr later were killed and the tumour removed and sectioned. Two mice were placed, after anaesthesia, in a water-bath at 42.5°C for 1 hr, which produced an intratumoural temperature of about 41.5°C. The remaining 2 mice were also given hyperthermic treatment, one at 41.7°C water temperature and one at 43.5°C water temperature, both for 1 hr. All were killed immediately after heating, and tumour sections prepared for measurement of lysosomal acid phosphatase and β-glucuronidase activity.

The shape of the absorbance vs incubation time curve for spontaneous tumours was very similar to that obtained with transplanted tumour sections and the control rates were almost identical (Figs. 1 and 2). Heating at 42.5°C increased the activity of acid phosphatase in the tumour and the activity in the heated animals was about 1.4 times the activity in control animals. Treatment at 43.5°C increased the activity to a similar extent (Table 1). Treatment at 41.7°C water temperature, when the intratumoural temperature was 40.5–40.9°C, did not affect the activity measured after 60 min incubation and the

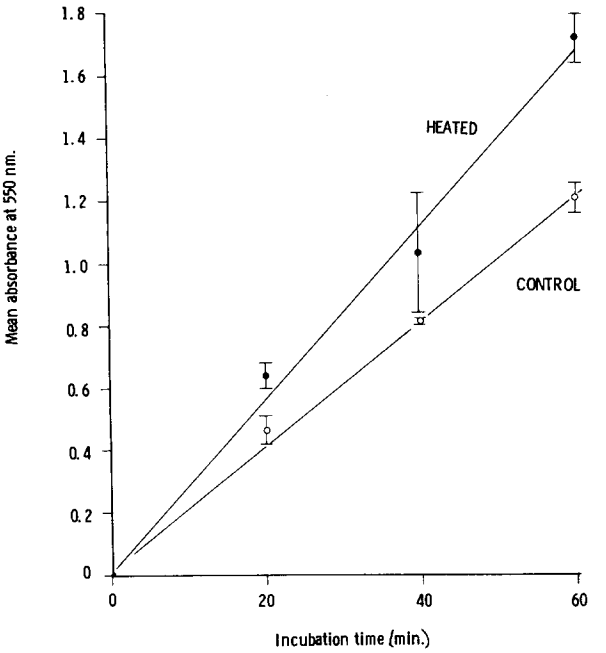

Fig. 2. Lysosomal acid phosphatase in spontaneous C3H tumours after hyperthermic treatment. ○—*Control-Mean absorbance reading from 2 untreated animals* ±*range;* ●—*Treated-Mean absorbance readings from 2 animals killed immediately after lower body hyperthermia of* 42.5°C *for* 1 hr ±*range.*

Table 1. *Effect of* 1 hr *at various temperatures on lysosomal enzyme activity in spontaneous C3H tumours*

Bath temperature °C	Acid phosphatase activity-ratio of heated to control after incubation of slices for: 20 min	60 min	β-Glucuronidase activity-ratio of heated to control after incubation of slices for: 20 min	60 min
41.7	0.2	1.2	1.6	2.2
42.5	1.4	1.4	1.4	1.9
43.5	1.6	1.5	1.3	1.5

activity over the first 20 min of incubation was less than that of the control (Table 1).

β-Glucuronidase activity was also raised in the tumours given hyperthermic treatment at 42.5°C (Fig. 3). After 20 min incubation of the slices the absorbance of the heat treated tumours was 1.4 times that of the control and this increased to 1.9 times after 60 min incubation. Treatments at 41.7° and 43.5°C also increased the β-glucuronidase activity, but the increase was not proportional to the temperature of the hyperthermic treatment (Table 1).

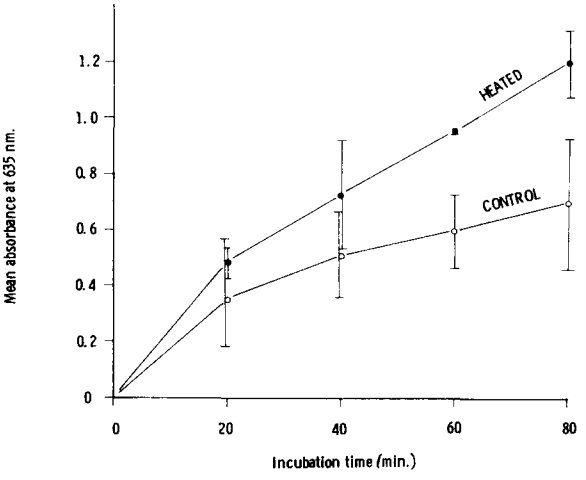

Fig. 3. *Lysosomal β-glucuronidase in spontaneous C3H tumours after hyperthermic treatment.* ○—*Control-Mean absorbance reading from* 3 *untreated animals* ±*range;* ●—*Treated-Mean absorbance readings from* 2 *animals killed immediately after lower body hyperthermia of* 42.5°C *for* 1 hr ±*range.*

(iii) *The effect of hyperthermia on spleen* in vivo

Spleen was used to investigate whether the lysosomes of a normal tissue also would show an activation in response to hyperthermia. Six male C3H mice were used for these experiments. Two controls were anaesthetised with 60 mg/kg Sagatal and killed 1 hr later. The spleen was removed and sections cut. The remaining 4 were anaesthetised and immersed up to the xyphisternum in a water-bath at 42.0°C for 1 hr, killed and spleen sections prepared. In addition two C3H mice with mammary tumours transplanted into their thighs were given hyperthermic treatment at 43°C water temperature for 1 hr to the tumour region only. The intratumoural temperatures in these animals were 41.8° and 42.1°C. After warming, the mice were immediately killed and sections cut from their spleens. Acid phosphatase and β-glucuronidase activities were measured in the lysosomes of all the spleen sections.

In the heated animals, acid phosphatase activity in the spleen after 20 min incubation was 1.5 times control and the activity after 60 min incubation was similarly increased (Fig. 4a). β-Glucuronidase activity after 20 min was increased by 2.3 times, and the activity after 60 min by rather less (Fig. 4b).

Hyperthermic treatment of the tumour region only also increased the lysosomal enzyme activity in spleen (Fig. 4). The activity of acid phosphatase in spleen was increased 1.2 times over control after 60 min incubation. β-Glucuronidase activity after 60 min was elevated to 1.5 times control by tumour hyperthermia. Lysosomal enzyme activity in spleen is thus affected by heat at a remote site.

(iv) *Effect of combining hyperthermic and radiation treatment on transplanted tumours* in vivo

Twenty-one mice with tumours transplanted into the thigh were used for these experiments. Four mice were anaesthetised and killed either 1 hr or 1 day later. Lysosomal enzyme activity was not effected by the time which had elapsed between anaesthetising and killing. Eight animals were placed in the heating bath with the tumour immersed in water at a mean temperature of 43°C for 1 hr. Six of these were killed immediately, and the remaining two were killed 24 hr after treatment. Three of the mice received 3.5 krad of γ-irradiation to the tumour. One of these was

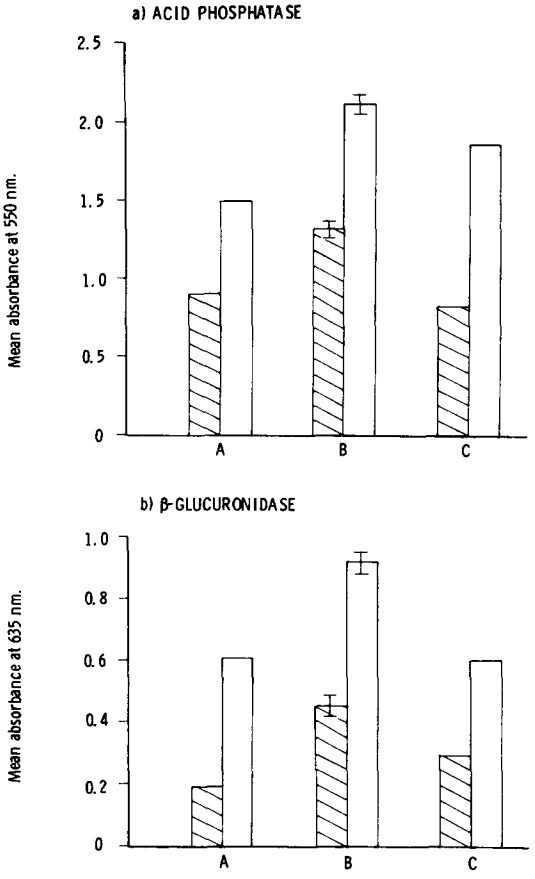

Fig. 4. Lysosomal enzymes in C3H spleen after hyperthermic treatment. □*—Mean absorbance reading after* 20 min *incubation in medium* ±S.E. *when appropriate.* ▨*—Mean absorbance reading after* 60 min *incubation in medium* ±S.E. *when appropriate. A—Control,* 2 *animals; B—Spleen treated–lower body hyperthermia of* 42°C *for* 1 hr, *killed immediately,* 4 *animals; C—Tumour treated-tumour hyperthermia of* 43°C *for* 1 hr, *killed immediately,* 2 *animals.*

killed 1 hr later and the others 25 hr later. A further 6 mice were irradiated with 3.5 krad to the tumour and then transferred rapidly to the heating bath for 1 hr of hyperthermic treatment of the tumour at 43°C water temperature. Three of these were killed immediately, and 3, 24 hr post-hyperthermia. After death the tumours were removed, sections were prepared and lysosomal acid phosphatase and β-glucuronidase activities were measured.

Immediately after hyperthermic treatment the activity of both lysosomal enzymes was elevated over control values after 20 min incubation of the slices. [Fig. 5 (a and b), Columns A and B]. the activity of β-glucuronidase was 1.1 times control, and the activity of acid phosphatase was 1.3 times the control value. Twenty-four hours after hyperthermic treatment neither lysosomal enzyme showed an increase in activity over control values and the β-glucuronidase activity was a little lower than that of the control [Fig. 5 (a and b), Column E].

One hour after irradiation of the tumour the lysosomal enzyme activities were depressed by a small amount [Fig. 5 (a), (b), Column C]. Twenty-five hours after irradiation the acid phosphatase activity was 1.6 times control but the β-glucuronidase activity was only 1.1 times control [Fig. 5(a), (b), Column F].

The immediate result of combining hyperthermic treatment at 43°C with a dose of 3.5 krad to the tumours was to induce enzyme activities similar to those obtained after hyperthermia alone [Fig. 5 (a), (b), Column D]. The activity of acid phosphatase was the same as after heat treatment only (Fig. 5 (a) Colume B] but β-glucuronidase activity was increased to 1.3 times the control value which was an enhancement over the effect of heat alone [Fig. 5 (b) Column B]. Twenty-four hours after the combination treatment β-glucuronidase activity was similar to control [Fig. 5 (b) Column G], while the activity of acid phosphatase was raised to 1.4 times control [Fig. 5 (a) Column G]. These activities were lower than those measured 25 hr after irradiation only [Fig. 5 (a), (b), Column F].

DISCUSSION

Lysosomes are very sensitive to mechanical damage and are easily lysed in hypotonic buffer. Hence the need for non-disruptive techniques for studying lysosomes. Cytochemical procedures used in the investigation are particularly useful here as they allow the enzyme activity to be measured *in situ* without having to resort to homogenisation to isolate the organelles.

For both acid phosphatase and β-glucuronidase in tumours the rate of increase in absorbance over the first 20 min was always larger than the rate over the subsequent period (Figs. 1–3). This initial period of incubation is important as here the activity measured is determined largely by the permeability of the lysosomal membrane to substrate [19, 20]. The rate of change of absorbance from 20 min incubation onwards depends more on the activity of the lysosomal enzymes than on the entry of substrate into the lysosome. It is the initial rate that is most sensitive to the radiation and hyperthermic treatments investigated above, particularly with acid phosphatase activity, Fig. 5 (a). Thus the increased rates observed are likely to

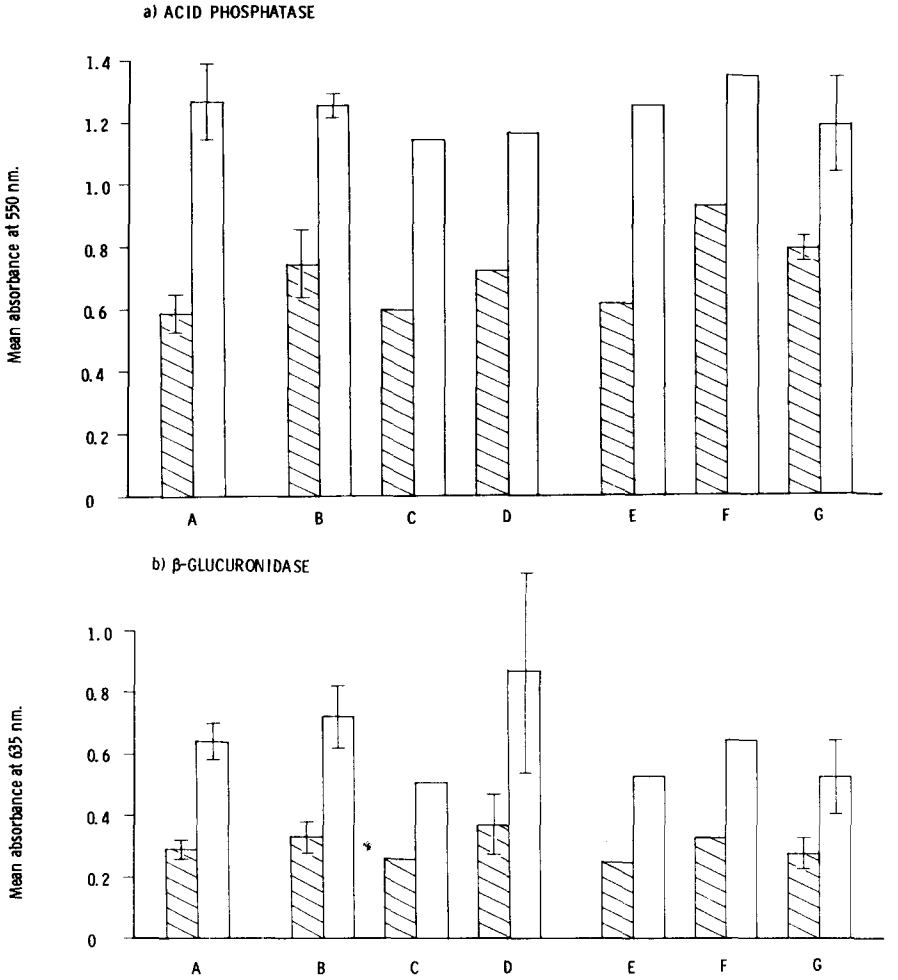

Fig. 5. Lysosomal enzymes in transplanted C3H tumours after hyperthermic and radiation treatment. ▨—*Mean absorbance reading after* 20 min *incubation in medium* ± S.E. *when appropriate.* □—*Mean absorbance reading after* 60 min *incubation in medium* ± S.E. *when appropriate. A—Control, 4 animals; B—Hyperthermia* 43°C *for* 1 hr *killed immediately 6 animals; C—Irradiation*-3.5 krad; *killed* 1 hr *later*, 1 *animal; D—Irradiation*-3.5 krad *followed by hyperthermia*, 43°C *for* 1 hr, *killed immediately*, 3 *animals; E—Hyperthermia*, 43°C *for* 1 hr *killed* 24 hr *later*, 2 *animals; F—Irradiation* 3.5 krad, *killed* 25 hr *later*, 2 *animals; G—Irradiation* 3.5 krad *followed by hyperthermia*, 43°C *for* 1 hr, *killed* 24 hr *later*, 3 *animals.*

demonstrate an increase in the permeability of the lysosomal membranes. The two enzymes, acid phosphatase and β-glucuronidase behaved in a similar, if not identical, way to the various treatments imposed. This is evidence for the effects being on the lysosomes or lysosomal membrane as a whole, rather than on individual enzymes. Incubating tumour tissue *in vitro* for an hour caused a large increase in lysosomal enzyme activity (Fig. 1). The enzyme activities were not different after incubation at 22° or 43°C. The damage caused by such incubation treatment may have masked any hyperthermic activation at 43°C; or the result may indicate that isolated tumour tissue is not affected directly by heat.

The activity of lysosomal acid phosphatase and β-glucuronidase was increased in both spontaneous and transplanted C3H tumours immediately after they had received hyperthermic treatment *in vivo*. The activation is not confined to neoplastic cells, as the lysosomal enzymes in spleen were activated after lower-body hyperthermia. The latter activation occurred at a lower temperature than that used with tumours, since the water temperature was only 42°C and spleen, being a deep tissue, was probably several degrees cooler. There is a possibility that part of this activation was due to indirect stimulation by hormones. Clarke and Wills [26] obtained lysosomal enzyme activation in spleen and thymus after administration of various steroid hormones, particularly cortisone and hydrocortisone. The hormones might therefore be produced in response to hyperthermic stress

and influence the lysosomes of other tissues. An indirect effect on spleen was found to occur when the tumour only was given hyperthermic treatment (Fig. 4), but the increase in enzyme activity was not so large as that after lower body hyperthermia. When spontaneous tumours were given hyperthermic treatment the whole abdomen had to be immersed. Treatment of spontaneous tumours at 42.5°C for 1 hr (Table 1) caused a higher percentage increase in enzyme activity than did treatment of transplanted tumours at 43.0°C for 1 hr [Fig. 5(a and b)]. This may have been due to a larger hormone-mediated-activation when more of the body was subjected to hyperthermia.

The activation of lysosomal enzymes due to hyperthermic treatment *in vivo* differs from the activation caused by irradiation in the time of its appearance. Whereas the radiation effect takes several hours to develop, the effect of hyperthermia appears immediately, and is completely reversed after 24 hr (Fig. 5). Thus the mechanism of damage must be different for the two modalities. Aerobic respiration is inhibited in tumour cells at supranormal temperatures [9]. This leads to an accumulation of lactic acid and a fall in pH within the tumour. The increased acidity may promote the release and activity of the acid hydrolases within the lysosomes if the period of hyperthermia is not excessive. This inhibition could be reversed when the pH of the cell returned to normal values.

The immediate response of transplanted tumour lysosomes to hyperthermic treatment was not potentiated by tumour irradiation prior to heating. Nor was the lysosomal activation 24 hr after irradiation increased if the radiation was followed by 1 hr at 43°C. The combination treatment used is one that has produced a large percentage of cures in the C3H tumour system [7]. The individual heat and radiation regimes alone would not cause a high percentage of cures, so some interaction between the modalities must occur. In contrast, hyperthermia and irradiation do not appear to be synergistic in their effects on lysosomes as measured by these cytochemical techniques. Lysosomal activation may not therefore be a primary event in tumour regression which results from combination therapy. Nevertheless lysosomes are probably very important in the hyperthermic response and must be involved in all types of tumour cell destruction, which involves autolysis, but as a secondary agent triggered by some other biochemical event.

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