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The pH of the Cytoplasm as an Important Factor in the Survival of *In Vitro* Cultured Malignant Cells after Hyperthermia. Effects of Carbonylcyanide 3-chlorophenylhydrazone*

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Abstract—*The effects of heat treatments at* 43°C *(hyperthermia), were studied on* in vitro *cultured cells, derived from a murine mammary carcinoma, at different* pH *values of the medium in the absence and presence of the proton conducting drug carbonylcyanide-3-chlorophenylhydrazone (CCCP).*

The survival of these cells after hyperthermia was always optimal at a pH *of the medium between* 7.75 *and* 8.0. *At this optimal* pH *the presence of CCCP hardly influenced the survival for treatment times up to* 90 min, *whereas there was a large effect of CCCP at lower* pH *values,* pH 6.5–7.5. *At higher* pH *values,* 8.0–9.0 *CCCP only had effect after at least* 1 hr *heating. For exposure times up to* 3 hr *CCCP only had a small effect at normal temperature,* 37°C, *except at very low* pH *values, below* 6.5, *where cell survival was impaired, even without CCCP.*

The large effect of CCCP in the pH *range* 6.5–7.5 *strongly suggests that the survival after hyperthermia is mainly determined by the cytoplasm* pH. *Changes in the* pH *of the cell medium may affect survival only after modifying the cytoplasm* pH.

In the pH *range* 6.5–8.0 *the cells are apparently able to maintain the optimal* pH *value inside, close to* 8.0. *The capacity to control* pH *is impaired by the addition of CCCP. Outside the range* 6.5–8.0 *the capacity to control* pH *appears to be insufficient or absent. Moreover it is presumed that there is a progressive deregulation of the* pH *control mechanism during a* 43°C *heat treatment. After* 2 hr *treatment at* 43°C *the cells apparently are no longer capable of maintaining their optimal cytoplasm* pH.

INTRODUCTION

THE INTEREST in hyperthermic treatment of tumours has its background in observations suggesting that a mild heat treatment (41°–44°C) causes a selective destruction of malignant cells [1–6]. Based on *in vitro* studies [7, 8] it was suggested that the relatively higher sensitivity of malignant cells to hyperthermia compared to normal cells could be considered as an intrinsic property. However malignant cells cultured *in vitro* appeared to be much less sensitive to hyperthermia than could be expected from *in vivo* experiments [5]. From this fact it has been concluded that the milieu of cells in a tumour plays an important role in the response of the tumour to heat.

Among other factors, as hypoxia [9, 10] and nutritional state [11], a lot of attention has been paid to pH. Compared to normal tissues, relatively low interstitial pH values have been observed in tumours [12–18] and several *in vitro* studies have shown that low pH

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Abbreviations—CCCP: carbonylcyanide 3-chlorophenylhydrazone; DMO: 5,5-dimethyl-2,4-oxazolidine; HEPES: *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; MES: 2-(*N*-morpholino)ethanesulfonic acid; MOPS: 2-(*N*-morpholino)propanesulfonic acid; Tricine: *N*-Tris (hydroxymethyl)methylglycine.

In a preliminary report, *Radiation Res.* **74,** 507 (1978), the effects of CCCP were erroneously ascribed to gramicidin. A description of the effects of gramicidin will be published elsewhere.

enchances heat induced cell lethality [19–24]. It has been proposed that lysosomes play a key role in hyperthermia induced cell death [6, 23] and biochemical studies have revealed that lysosomal enzymes can be activated from isolated lysosomes by a low pH of the medium or by heat [25–27]. Therefore one might assume that the pH of the cytoplasm should be a very important factor, but experiments of Dickson and Oswald [28] seem to contradict this assumption.

This investigation was undertaken to re-evaluate the role of the pH. Moreover, this study gives information about the effects of the drug CCCP. These effects of CCCP lead to the interpretation that the intracellular pH and the capacity of cells to control their intracellular pH play an important role in the response to hyperthermia.

MATERIALS AND METHODS

Responses of a cell line, designated M8013S, derived from a transplantable mammary carcinoma in $DBA_2 \times C57BL10$ mice, were investigated by the cloning technique for the assessment of cell reproductive capacity [29]. The cells were routinely cultured in Eagle's minimum essential medium with Hanks' salts which contained 45μM phenol red and was supplemented with 10% foetal calf serum and 100 i.u./ml penicillin, and were incubated at 37% in humidified air, containing 5% CO_2. For all experiments cells from an exponentially growing culture were trypsinized and plated as described by Joshi *et al.* [30].

For our pH studies we used the "zwitterion" buffers MES, MOPS, HEPES and Tricine [31, 32]. They were dissolved at a concentration of 50 mM in the medium used for culturing without serum and bicarbonate. The pH was adjusted by means of NaOH prior to sterilisation. Heat treatments (43.0°C ±0.05) were performed, always at least 4 hr. after plating, in plastic cluster dishes, Falcon 3004 (Becton Dickinson Co, U.S.A.) as described earlier [30]. Just before heat treatment the medium above the cells was replaced by 1 ml of buffer and immediately after the treatment the buffer again was replaced by 2.5 ml growth medium. CCCP could be added by means of a microliter syringe to two of the four wells in the cluster dish. In all experiments, except those described in Fig. 1 and 2 CCCP was only present during exposure to buffers of different pH or during the heat treatment. After treatment, the medium above the cells being replaced by 2.5 ml growth medium, the cells were incubated for 6 days.

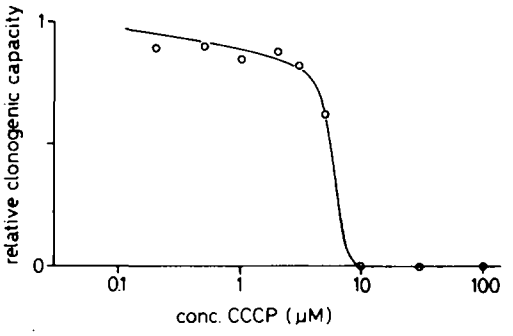

Fig 1. *The influence of CCCP present during the* 6 days *incubation after plating on the clonogenic of* M8013S *cells.*

The data given on clonogenic capacity are always relative to a control without the considered treatment. The plating efficiency of controls in the different experiments varied between 50 and 100% without an apparent influence on the reproducibility of the experiments. Most experiments proved to be fairly reproducible, and, if not otherwise stated, the data given are from one typical experiment. In cases with a poor reproducibility, 90 min treatment at 43°C in Fig. 7, 43°C treatment at pH 6.5 in Table 1 and in Fig. 8, data from 3 independent experiments were averaged. Culture media and foetal calf serum were obtained from Flow Laboratories (U.K.), MES, MOPS, HEPES and Tricine from Merck, Darmstadt (G.F.R.), CCCP from Serva GmbH (G.F.R.) and penicillin G from Mycofarm (The Netherlands).

Table 1. *Clonogenic capacity of M*8013*S cells after* 60 *min heat treatment at* 43.0°C *in culture medium with serum and bicarbonate*

	Without CCCP	With 10 μM CCCP
pH 8.1	0.65	0.58
pH 6.5	0.22±0.17(S. D.)	<0.002

The pH of the medium is altered by changing the CO_2 tension above the medium, the pH being monitored by small electrodes in a control dish coupled to a Radiometer PHM 62 pH meter. The data given for pH 6.5 are the average of three independent experiments.

RESULTS

CCCP, when continuously present in the culture medium after plating, inhibited cell proliferation at fairly low concentrations (Fig. 1). CCCP was described as an uncoupler of oxidative phosphorylation by Heytler [33]

and as such it is a potent and specific proton conductor [34]. The toxic effects (Fig. 1) may be due to uncoupling of oxidative phosphorylation by eliminating proton gradients across the mitochondrial membrane [34], or to deregulation of the ion balance of the cell caused by the free translocation of protons through the outer and other membranes. If the cells were exposed for only short times, 1–3 hr, to concentrations of CCCP in the growth medium which were toxic when continuously present, the effect on clonogenic capacity was small (Fig. 2). CCCP at a concentration of 10 μM was chosen for the experiments described below.

CCCP is used here as a tool to study effects of changes in the pH of the cell medium. The effects of exposure of the cells, after plating, to buffers of different pH at 37°C are shown in Fig. 3. Only small effects were obtained at a pH above 6.5. Below pH 6.5 the proliferative capacity of the cells was largely impaired if the exposure time was in excess of 30 min. The growth medium, as mentioned above, had a pH between 7.5 and 8.0. If CCCP was present during exposure to the different buffers, the effects below 6.5 were more pronounced (Fig. 4). Even a 30 min exposure had a large effect. At pH values above 6.5 the presence of CCCP in the buffer hardly influenced clonogenic capacity at all.

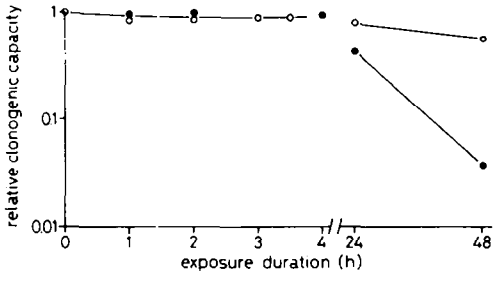

Fig. 2. The effect of exposure for different times to 10 μM (○—○) *or* 30 μM *CCCP* (● ●), *added to the culture medium after plating, on the clonogenic capacity of* M8013S *cells.*

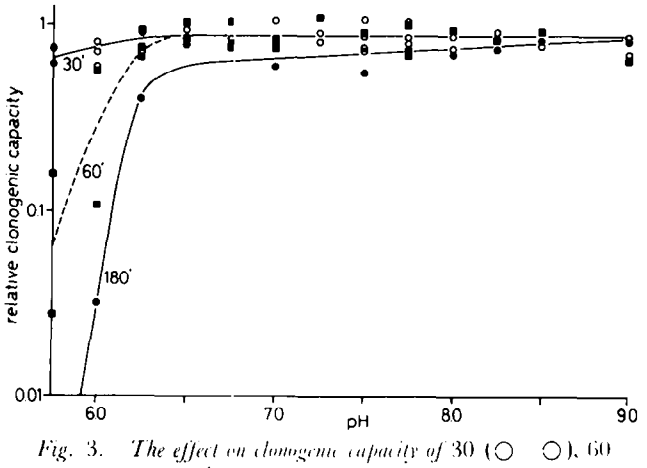

Fig. 3. The effect on clonogenic capacity of 30 (○ ○), 60 (■—■) *and* 180 min *exposure* (●—●) *at* 37°C *to overlapping series of buffers, MES* pH 5.75 6.50, *MOPS* pH 6.50–7.50, *HEPES* pH 7.25 7.75 *and Tricine* pH 7.75 9.00, *all dissolved in Eagle's minimum essential medium as described in Materials and Methods. After* 3 hr *exposure to MES buffer* pH 5.75 *clonogenic capacity was* <0.002, *this is considered in the drawing of the curves.*

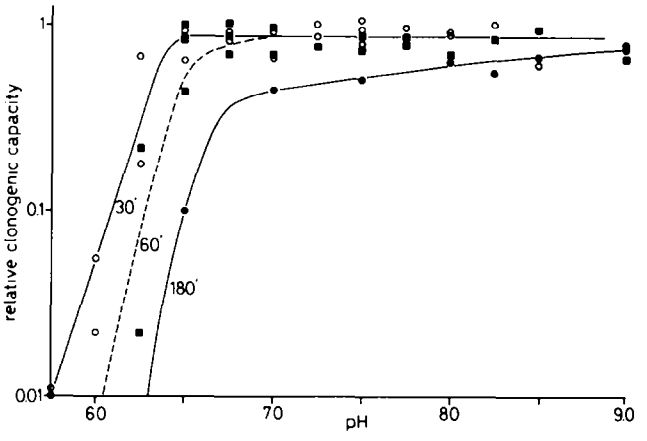

Fig. 4. Same conditions as for Fig. 3, but 10 μM *CCCP added with the different buffers,* 30 min *exposure* (○ ○), 60 min *exposure* (■—■) *and* 180 min *exposure* (● ●). *After* 3 hr *exposure at* pH 6.25 *the clonogenic capacity was* <0.003, *after* 60 min *exposure at* pH 6.00 *the clonogenic capacity was* 0.004, *which is considered in the drawing of the curves.*

If, however, exposure to different pH was combined with a heat treatment, CCCP had large effects at pH values above 6.5 (Figs. 5–7).

Without CCCP cells appeared to be rather resistant to changes in the pH for heat treatments up to 60 min. The survival curves in Figs. 5 and 6 show a plateau in the range 6.75–8.0 This plateau starts to disappear after 90 min heat treatment and it is absent after 120 min treatment (Fig. 7). The curve for 120 min treatment at 43°C without CCCP has a clear optimum at pH 8.0. Below pH 6.5 there is a very steep decrease in clonogenic capacity after heat treatments, 30 min treatment already being more effective than 3 hr exposure without heating (Fig. 5). The very low clonogenic capacities found at low pH could not be explained by detachment of the cells from the plastic. This was controlled by dark field microscopy: the cells stay attached to the cluster dishes under all conditions described.

Above pH 8.0 the cells appeared to be rather resistant initially, but after prolonged heating, longer than 60 min, there was a considerable decline in clonogenic capacity.

Heat treatments in the presence of CCCP

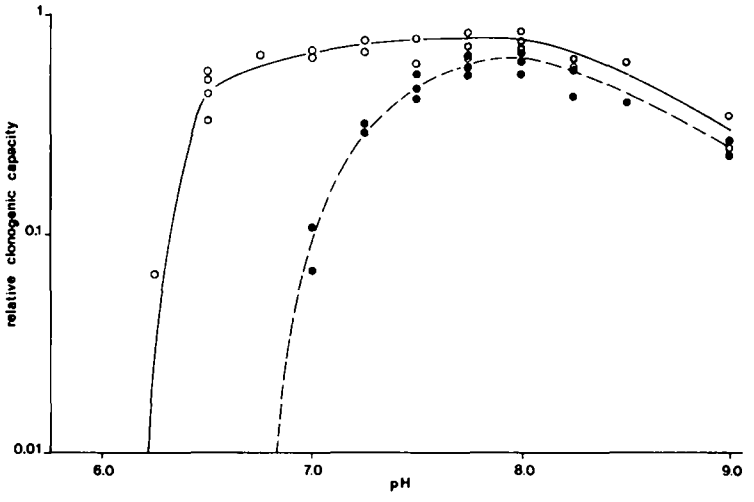

Fig. 5. The effect on clonogenic capacity of 30 min *hyperthermic treatment at* 43.0°C *during exposure to different buffers with* (●—●) *and without* (○ ○) 10 μM *CCCP.*

At pH 6.0 *without CCCP the relative clonogenic capacity was* <0.001. *With CCCP at* pH 6.5 *and* 6.75 *the clonogenic capacities were respectively* 0.006 *and* 0.007. *The values below* 0.01 *are considered in the drawing of the curves.*

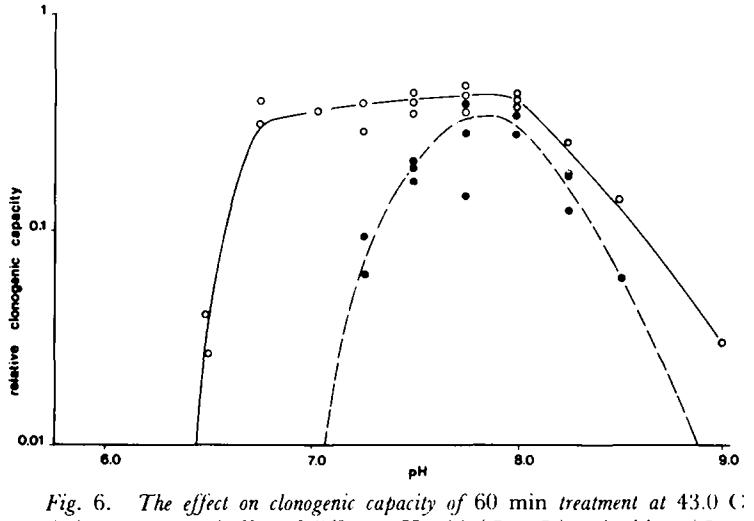

Fig. 6. The effect on clonogenic capacity of 60 min *treatment at* 43.0 C *during exposure to buffers of different* pH *with* (●—●) *and without* (○— ○) 10 μM *CCCP.*

At pH 6.25 *without CCCP the relative clonogenic capacity was* <0.001. *With CCCP at* pH 7.0 *and* 9.0 *clonogenic capacities were respectively* <0.002 *and* <0.001. *The values below* 0.01 *are considered in the drawing of the curves.*

result in survival curves with an optimum between pH 7.75 and 8.0 (Figs. 5–7). At pH values below this optimun the cells were much less resistant to 43°C heat treatment than without CCCP and there is no question of a plateau in the curve. At pH values around the optimum and above, effects of CCCP developed only after prolonged heat treatment (Figs. 6 and 9). After heat treatments longer than 90 min with CCCP, there was a sharp decline in clonogenic capacity at the optimal pH (Fig. 9), which is not observed after treatments without CCCP (Fig. 8).

If the cells were heated in the medium used for culturing (with serum and bicarbonate), without an enhanced CO_2 tension above the medium, the pH of the medium readily attained the value of approximately 8.1. The survival in this case (Table 1) was slightly higher than in buffer pH 8.0 (Fig. 8) and CCCP hardly influenced the survival. If the heating was performed under enhanced CO_2

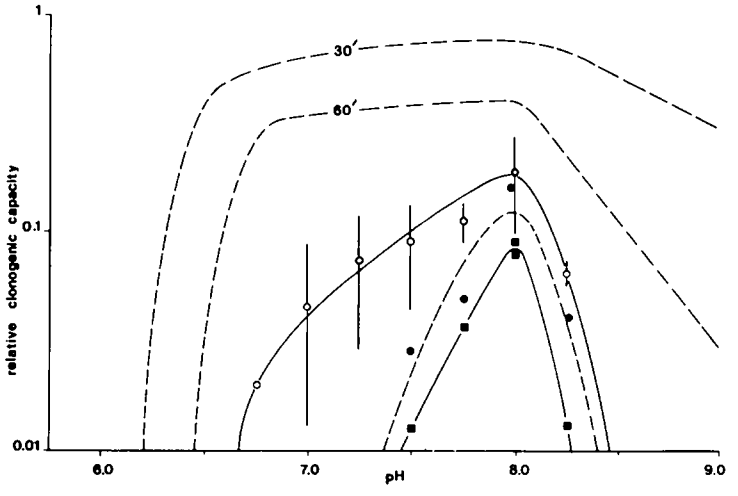

Fig. 7. The effects of 90 (○—○) *and* 120 min *treatment* (■—■) *at* 43.0°C *at different* pH *without CCCP and* 90 min *at* 43.0°C *with CCCP* (●—●) *on the clonogenic capacity of M*8013*S cells. The data for* 90 min *treatment without CCCP are from three independent experiments, the bars represent standard deviation. At* pH 6.5 *clonogenic capacity was* <0.001 *and at* pH 8.5 *and* 0.008. *The values below* 0.01 *are considered in the drawing of the curves.*

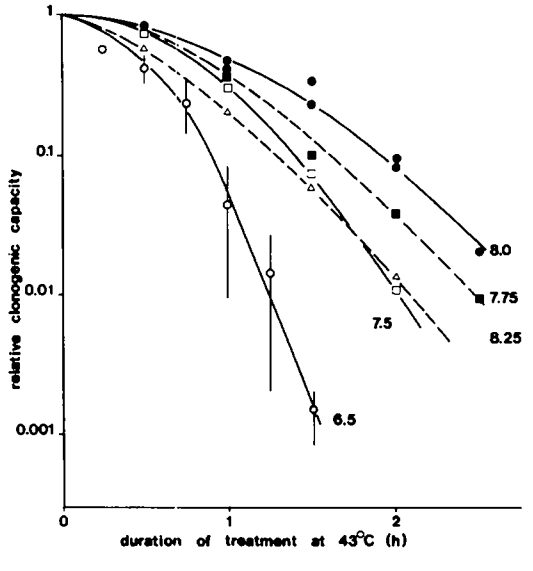

*Fig. 8. Survival curves for M*8013*S cells treated at* 43.0°C *during exposure to buffers at different* pH. *The data given for* pH 6.5 *are the average of three independent experiments, the bars represent standard deviation.* pH 8.25 (△—△), pH 8.0 (●—●), pH 7.75 (■—■), pH 7.5 (□—□), pH 6.5 (○—○).

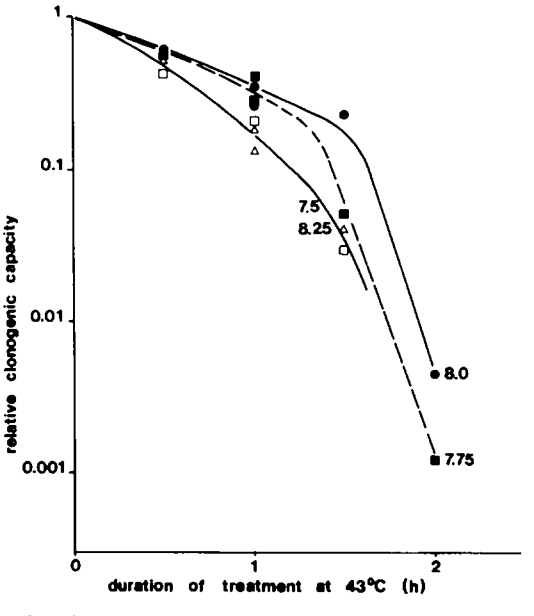

Fig. 9. Same conditions as for Fig. 8, *but* 10 μM *CCCP present during treatment.* pH 8.25 (△—△) pH 8.0 (●—●), pH 7.75 (■—■), pH 7.5 (□—□). *Clonogenic capacities after* 2 hr *treatment at* pH *values not shown in this figure were all* <0.001.

tension so as to obtain a pH of approximately 6.5, the survival of the cells was significantly lower and CCCP had a very large effect. The drawbacks of these experiments are that only a pH range of approximately 6.5–8.2 can be obtained and that pH may vary slighty during heating. Nevertheless the data given in Table 1 indicate that the same phenomena are observed under these conditions as described above.

DISCUSSION

The large effects of CCCP when present during heat treatments in the pH region 6.5–7.75 may be explained by its proton conducting properties [34]. We assume that, with CCCP, the pH of the cytoplasm inside the cell equilibrates with the pH of the medium outside. As, at pH 8.0 CCCP has only a small effect during heat treatments up to 90 min, we suppose that uncoupling of the mitochon-

dria and the resulting lack of energy (ATP) does not interfere with the effects of CCCP during treatments shorter than 90 min. However, after a treatment at 43°C with CCCP longer than 90 min at pH 8.0 (Fig. 9), clonogenic capacity drops rapidly. This may mean that energy supply becomes necessary at that point, maybe to carry out some repair processes.

The plateau in the survival curves without CCCP (Figs. 5 and 6) may be explained by assuming that cells in the absence of CCCP are capable of controlling their internal pH and to keep it near the "optimum" pH close to 7.75. This implicates that the pH outside the cell is not as important for cell survival after hyperthermia as the cytoplasm pH. The disappearance of the plateau in the survival curves after a hyperthermic treatment longer than 90 min should mean that the pH control capacity of the cells is impaired by the heating. Outside the range 6.5 8.0 the cells are apparently not capable of maintaining the "optimal" pH inside. Without heating the cells are able to withstand 30 min exposure in buffers of pH below 6.5 but longer exposure impairs clonogenic capacity. Probably an increased permeability of the outer membrane to protons at 43°C interferes with the pH control capacity of the cells. The fact that 30 min treatment at 43°C below pH 6.5 is about as effective as 3 hr exposure below pH 6.5 at 37°C in the presence of CCCP may be explained in this way. Apparently this increased permeability to protons is not enough to bypass the pH control mechanism if no CCCP is added (Figs. 5 and 6).

The considerable decrease in proliferative capacity in all cases where it may be assumed that the internal pH of the cell is low, even without heat treatment, is compatible with the hypothesis that lysosomal enzymes are activated under these conditions [3, 6, 18, 35]. Dickson and Oswald [28], using the labeled 5,5-dimethlyl-2,4-oxazolidine (DMO) distribution method, concluded that changes in intracellular pH are not important for hyperthermia-induced cell death, but in their discussion they state that the intracellular pH calculated from the DMO method is an "overall" pH which perhaps may not reflect the pH of the microenvironment of the lysosomes. The "optimal" cytoplasm pH found in this study is relatively high compared with the values generally obtained using the DMO method [36].

The lysosome hypothesis cannot account for the hyperthermia-induced cell death if the intracellular pH is not low. Hyperthermia-induced cell death however still exists at pH 8.0. Moreover the extent of it increases further at a pH above 8.0. Maybe thermal uncoupling of the mitochondria plays an important role under these conditions. The fact that a drop in clonogenic capacity occurs, after treatments in the presence of CCCP, which can be thought to be due to uncoupling (Fig. 9), points in this direction. Maybe progressive thermal uncoupling occurs faster at higher pH and interacts with the uncoupling by CCCP. Phosphorylation in chloroplasts has been proved to be very sensitive to heat treatments [37] and the same may be the case with mitochondria. An inhibition of cellular respiration by heat treatments has been reported [38] but further research is required to elucidate the role of thermal uncoupling.

The present results may be important to gain more insight into the mechanism of selective hyperthermia-induced cell death in tumours. Very probably the pH of the milieu of the cells in a tumour is not the most important factor to explain selective sensitivity to hyperthermia but rather the metabolic state of the cells which are chronically deprived of oxygen which may lead to a low intracellular pH. Further research concerning the physiological factors playing a role *in vivo* is still necessary.

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