Eur J Cancer Clin Oncol, Vol. 25, No. 11, pp. 1629–1635, 1989.
Printed in Great Britain

0277-5379/89$3.00 + 0.00

The Relationship Between Treatment Duration and Temperature for Hyperthermia Induced Lethality of Cultured Murine Cells

Influence of Medium Conditions

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Abstract—*The heat sensitivity and the time–temperature relationship of non-tolerant and thermotolerant M8013 cells treated at different pHs in either culture medium (including serum) or Hanks' salts solution (HBSS) were compared. The cells were growing asynchronously. Arrhenius plots for non-tolerant cells heated in culture medium pH 7.35 showed two linear parts below and above the transition temperature (T_{trans}). The inactivation energies below and above T_{trans} were respectively 2980 and 490 kJ/mole. With thermotolerant cells under the same conditions the inactivation energy was approximately constant over the range 42–46°C at 890 kJ/mole. The cells were more sensitive to heat treatment at low pH or in HBSS. Moreover, it appeared that the expression of thermotolerance was strongly dependent on medium conditions: the thermotolerance ratio (TTR, ratio between slopes of survival curves of thermotolerant and normal cells) was much lower at low pH or in cells heated in HBSS. Generally a high TTR observed in experiment with fractioned hyperthermia at temperatures above T_{trans} correlated fairly well with a high inactivation energy below T_{trans} from the Arrhenius plot derived from data from experiments with the same cells that were not made thermotolerant before treatment.*

INTRODUCTION

HEAT-INDUCED cell lethality has been shown to be dependent on both temperature and heating time. Arrhenius plots of cellular inactivation after exposure to temperatures in the range of 39–47°C appear to be composed of two linear parts below and above a transition temperature (T_{trans}) generally between 42 and 43°C [1–5]. A similar analysis from results on tumour and normal tissue responses to hyperthermia (reviewed by Field and Morris [6]) showed that the Arrhenius plots for the *in vivo* results resembled those from *in vitro* data. The general similarity of Arrhenius plots has been used to define the thermal dose [7], which might be of great practical value in the clinical application of hyperthermia [8]. The effects of hyperthermia are strongly influenced by thermotolerance. Thermotolerance in cells may develop after heat treatment during incubation at 37°C, but also during heat treatment. It has been proposed that thermotolerance development during heat treatment is responsible for the transition temperature in the Arrhenius plot: above T_{trans} cell inactivation occurs so rapidly that tolerance cannot be observed [9, 10]. More recent data indicate that thermotolerance development does not occur during treatment at temperatures above T_{trans} [11, 12], as it is inhibited at these temperatures.

Henle *et al.* [13], Nielsen and Overgaard [14] and Dikomey *et al.* [15] showed that the maximum extent of thermotolerance that could be obtained after priming heat treatment and subsequent incubation at 37°C was dependent on the level of damage following the priming treatment. When the priming treatment led to more cell killing, a longer interval at 37°C was required to obtain the (higher) maximum extent of thermotolerance in the surviving cells, at the same rate of induction.

Changes in the pH of the cellular medium have a profound influence on the survival of cells exposed to hyperthermia [e.g. 16, 17]. Several authors studied the influence of the development of the pH of the cellular medium. In these experiments the pH was generally lowered during a priming and a heat test treatment and also during the interval (where thermotolerance develops) between both treatments

Accepted 21 June 1989.

and sometimes even during a longer period including both heat treatments and interval. The results of these experiments were contradictory. In L1A2 cells [18] and in CHO cells [19, 20] a decreased maximum extent of thermotolerance at low pH was obtained in this way. Other results by Gerweck *et al.* [21, 22] with fractioned treatment of CHO cells at 42°C indicate that a higher extent of thermotolerance can be obtained at low pH. The results of Dikomey *et al.* [15], from experiments with CHO cells at different pH, indicate that the maximum extent of thermotolerance obtained was not dependent of the pH of the medium but on the effectiveness of the priming heat treatment (which in its turn was dependent on pH).

It is impossible to study an influence of pH on the development of tolerance separately from the influence of pH on its expression during the test heat treatment, when the pH is changed during both priming, test heat treatment and interval. Therefore, in our experiments we changed pH (and other medium conditions) only during the test heat treatment. Based on the presumption of an inhibition of development of thermotolerance during test heat treatment at temperatures above T_{trans} these experiments will give data on the altered expression of thermotolerance dependent on the pH or altered medium conditions. Thermotolerance was induced by mild heating 30 min at 43°C 5 h before test heat treatment to obtain maximum thermotolerance as described previously [23]. Furthermore we investigate the relation between temperature and heating time on cell survival and the transition temperature in this relationship at different conditions of the pH of the cellular medium (pH 7.35 vs. 6.55 in complete culture medium and pH 7.5 vs. 6.6. in Hanks' salts solution).

MATERIALS AND METHODS

The M8013 cell line was grown in Eagle's minimum essential medium with Hanks' salts which was supplemented with 10% foetal calf serum and 100 IU/ml penicillin, as reported earlier [17, 24]. In all experiments exponentially growing cells were used, doubling time 12.0 ± 0.2 h. Absence of mycoplasma infection was checked at regular intervals. When the cells were heated they were kept either in culture medium or in Hanks' salts solution (HBSS) buffered by 25 mM MOPS (3-morpholinopropanesulfonic acid). The pH of the buffered Hanks' salts solution was adjusted to pH 6.6 or to pH 7.5 (± 0.01) by means of concentrated NaOH using a Radiometer PHM 62 pH meter, prior to sterilization. The Hanks' salts solution was pipetted over the cells after washing with this solution once immediately before heating. For thermal exposure dishes (diameter 6 cm) with cells were placed on grids in a thermostatically controlled (± 0.05°C) waterbath. When the cells were heated in culture medium the pH of the medium was maintained at pH 6.55 ± 0.05 or at pH 7.35 ± 0.05 by enhanced CO_2 pressure in the air space above the grid in the waterbath. When the cells were treated in culture medium of low pH, medium at this pH was pipetted over the cells immediately before heat treatment. The temperature in control dishes was measured with thermocouples. When control dishes were placed in the waterbath they reached the desired temperature to 0.02°C within 1 min. To induce thermotolerance, dishes with cells were preheated, 30 min at 43°C, 5 h before the test treatment. Between priming and test treatment cells were kept at 37°C in an incubator. Changes in the cellular medium (pH, serum) were only made during the test treatment. Priming treatment for induction of thermotolerance and the incubation at 37°C after priming was always in complete medium at normal pH (pH 7.35 ± 0.05). We previously showed [23] that the 5 h interval after priming led to maximum development of thermotolerance.

After final treatment cells were trypsinized immediately. The cells were first washed with Hanks' salts solution (without MOPS buffer, pH approximately 7.6) and then trypsinized for 5 min in Ca^{2+} and Mg^{2+} free medium with 0.5 g/l trypsin. Thereafter the cells were plated at appropriate dilutions in culture medium and placed in an incubator for 6 days to allow colony formation. The plating efficiency of untreated cells trypsinized in the same way varied between 80 and 100%. To obtain information about the general shape of the heat survival curves, typical data from the different experimental conditions were plotted and curves, fitted by eye, were drawn (Figs. 1–3).

For further analysis curves were fitted to the data on survival of cells (S) relative to that of unheated control cells (S_0) according to the formula

$$S/S_0 = 1-[1-\exp(-t/t_0)]^N.$$

The procedure has been described previously [25]. t represents the heating time at a specific temperature, t_0 is the heating time which would kill 63% of the cells if the extrapolation number, N, were equal to one. t_0 determines the, reciprocal, final slope of the heat survival curve. Each independent series of experiments at a certain temperature was analysed separately, and in all cases a 1 min temperature transient correction is made (*cf.* Ref. [26]). The formula to describe the heat survival curve has been proposed by Roti Roti and Henle [26] and is chosen because of the general similarity between heat survival curves (non-tolerant cells) and X-ray survival curves. The use of the formula has the advantage that it allows for an easy comparison of heat responses at various temperatures by using t_0. More-

over $1/t_0$ may be used for Arrhenius analysis [4]. We also tried to fit curves to our data using the formula $S/S_0 = \exp(-\alpha t - \beta t^2)$, proposed by Roti Roti and Henle [26]. However, the α-values we obtained were negative in about 50% of cases. Apparently this formula does not give a good approximation of the survival curves of M8013 cells. In the Arrhenius plots (*cf.* Fig. 4) the position of T_{trans} and the slopes were determined by non-linear least square fitting. The 95% confidence limits of T_{trans} were then determined from the residual sum of squares by linear least square fitting.

RESULTS

Figure 1 shows survival curves of M8013 cells treated in culture medium, including foetal calf serum. The pH in these experiments was maintained at pH 7.35 ± 0.05. When the cells were heated at 42.5°C and when no priming heat treatment was given survival curves showed a long resistant tail presumably as a result of development of tolerance during treatment (not shown). At temperatures below 42.5°C survival curves did not clearly show the 'tail' presumably as this would require very long heating times. At temperatures of 43°C and above, the cells showed survival curves with a rather broad shoulder ([23], Fig. 1). Priming heat treatment (30 min at 43°C) led to thermotolerance in M8013 cells as described previously [23]. The 5 h interval was chosen as this led to the maximum extent of tolerance after priming. The survival curves at temperatures of 43°C and higher of thermotolerant cells are biphasic (Fig. 1): the first part of the curves, down to a survival level of approximately 5×10^{-2}, is linear, beyond this first part cell survival drops far more rapidly. We did not observe biphasic survival curves for thermotolerant cells at 42.5°C or lower; however, we did not follow the curves beyond 30 h exposure duration.

When the pH of the medium is kept at 6.55 during the test heat treatment both non-tolerant and thermotolerant cells are sensitized. The general shape of the survival curves of non-tolerant cells at pH 6.55 is not very difficult from those at pH 7.35 (Fig. 2). Thermotolerant cells treated at temperatures of 43°C and above at low pH did not show the characteristic biphasic pattern in the survival curves as was observed at pH 7.35. Figure 2 shows some examples of survival curves of thermotolerant cells heated at low pH: especially at temperatures above 43°C the curves have a shoulder and below a survival level of approximately 2×10^{-1} a well-defined 'final slope'. At temperatures below 43°C (not shown) the curves, plotted in the same way as in Figure 2, were approximately linear down to a survival level of about 10^{-2}. Due to the fact that very long heating times are involved (up to 50 h at 41°C) to reach this level, it was practically impossible to obtain further information on the shape of the curve below this survival level.

When cells were heated in buffered Hanks' salts solution without serum (HBSS) they were always more sensitive to heat treatment. This observation confirms our earlier results [23] with the same cell line and those of Hahn [27] with Chinese hamster HA-1 cells. Typical examples of survival curves of M8013 cells treated in HBSS at pH 7.5 are shown in Fig. 3. It is clear from these results that cells, and especially thermotolerant cells, are more sensitive to heat treatment in HBSS. The first linear part of the survival curves for thermotolerant cells in Fig. 1 is absent in these curves. Survival curves of the cells treated at pH 6.6 (not shown) had, qualitatively, the same general shape as at 7.5.

For the Arrhenius analysis the first linear part of the survival curve is taken, sometimes occurring after an initial shoulder and nearly always within 4 h of treatment duration. Within 4 h, presumably,

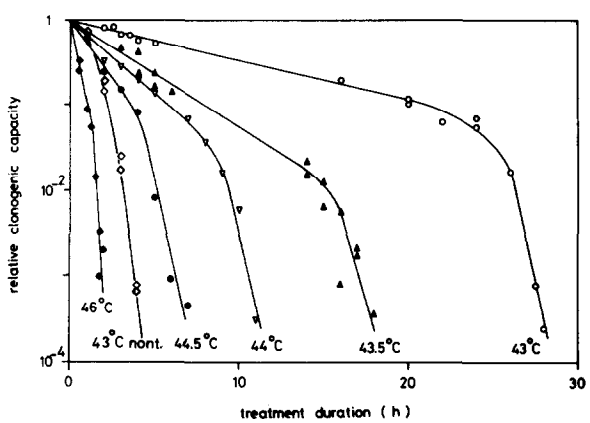

Fig. 1. Typical examples of heat survival curves of thermotolerant M8013 cells treated in complete medium with serum pH 7.35 ± 0.05. Thermotolerance was induced by priming, 30 min at 43°C, treatment. Priming was done 5 h before the test treatment to obtain the maximum extent of thermotolerance. One example of a survival curve (43°C) for non-tolerant cells is also given (nont.)

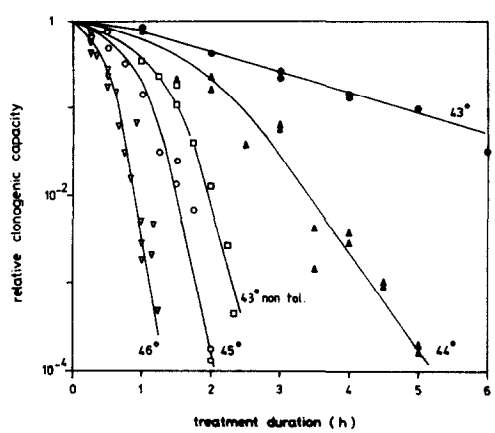

Fig. 2. Typical examples of heat survival curves of thermotolerant M8013 cells treated in complete medium with serum pH 6.55 ± 0.05. Thermotolerance was induced by priming treatment, 30 min at 43°C, at pH 7.35, 5 h before the test treatment. For comparison one curve for non-tolerant cells under the same medium conditions is drawn (43°C non tol.).

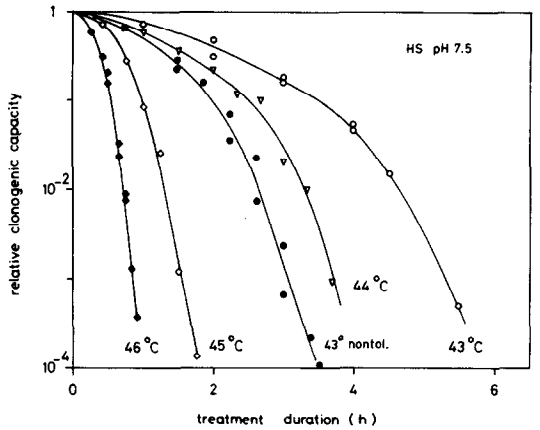

Fig. 3. Typical examples of heat survival curves of thermotolerant M8013 cells treated in buffered HBSS pH 7.5. In one curve ('43° nontol.') cells were not thermotolerant. Thermotolerance was induced by priming, 30 min at 43°C, treatment in complete culture medium 5 h before test treatment.

the shape of the curve is not much influenced by changes in cell cycle distribution that still might occur during heating [28]. Thermotolerant cells treated in Hanks' salts solution seem rather characterized by a broad shoulder rather than a decreased final slope, however, TTR values calculated from D_q values are about equal to those calculated from D_0 values, e.g. TTR (Dq) values for cells in HBSS pH 6.6 at 43°C and 46°C are 2.1 and 2.4 (*cf.* Table 1).

In Fig. 4 Arrhenius plots of data are shown derived from survival curves for cells treated at different pH or without or with serum. The results show that the sensitizing effect of low pH in non-tolerant cells is especially clear at temperatures below the transition point and in Hanks' balanced salts solution. The highest extent of thermotolerance is observed when cells are treated in complete culture medium (including serum) at pH 7.35. Thermotolerance ratios (TTR, ratio between 'final' slopes of thermotolerant and non-tolerant cells) are given in Table 1. TTR values are nearly always lower at low pH. The only case were the TTR at low pH is higher than at normal pH is at 42°C. There are no significant differences in the transition temperatures (T_{trans}) in the plots for non-tolerant cells, although there is some indication of a shift to a lower temperature at low pH (see Table 1). Above T_{trans} the inactivation energy calculated from the data in Fig. 4A and B for non-tolerant cells is in the range 395–490 kJ/mole (Table 2). With thermotolerant cells heated in complete medium the inactivation energy is higher: 890 kJ/mole (Table 2). The highest inactivation energy is observed below T_{trans} in cells heated in complete medium at pH 7.35: 2980 kJ/mole (Table 2).

DISCUSSION

In the present study we compared the heat sensitivity and the time–temperature relationship of normal and thermotolerant M8013 cells treated at different pH in either culture medium (including serum) or HBSS. The results show that M8013 cells are more sensitive to heat treatment when treated at low pH or in HBSS in the absence of serum confirming our previous observations [17, 23]. Thermotolerance was induced by heating the cells for 30 min at 43°C, 5 h before test treatment, always in culture medium at pH 7.35. Also the subsequent incubation at 37°C was performed under standard conditions (complete medium at pH 7.35). In this way the maximum extent of thermotolerance development was obtained (*cf.* Ref. [23]). Just before test heat treatment, after induction and development of thermotolerance, the medium could be changed (pH, HBSS) and the altered medium conditions were maintained during test treatment. As the induction and development of thermotolerance occurred in the same way in all cases, the results of this kind of experiments give information on the way in which medium conditions influence the degree of expression of thermotolerance during the test heat treatment, this at temperatures above T_{trans} where it is assumed that no further development of thermotolerance takes place [11,

Table 1. Thermotolerance ratios (TTR ± S.E.M.) and transition temperatures (95% confidence limits in parentheses) derived from data shown in Fig. 4

Temperature (°C)	CM pH 7.35	CM pH 6.55	HBSS pH 7.5	HBSS pH 6.6
42	1.1 ± 0.1	4.6 ± 0.7	0.6 ± 0.1	4.0 ± 0.4
43	17.3 ± 1.5	7.4 ± 1.1	3.8 ± 0.5	2.3 ± 0.3
44	16.4 ± 2.2	4.1 ± 0.5	4.1 ± 0.6	2.2 ± 0.3
45	7.1 ± 0.9	4.6 ± 0.7	3.1 ± 0.5	2.4 ± 0.2
46	7.4 ± 0.9	3.2 ± 0.5	2.9 ± 0.5	2.6 ± 0.4
T_{trans}	43.2°C (42.7–43.4)	42.8°C (42.4–43.4)	43.1°C (42.7–43.7)	42.5°C (42.1–43.0)

CM: complete medium including serum; HBSS: buffered Hanks' salts solution. The transition temperatures are obtained from plots for non-tolerant cells.

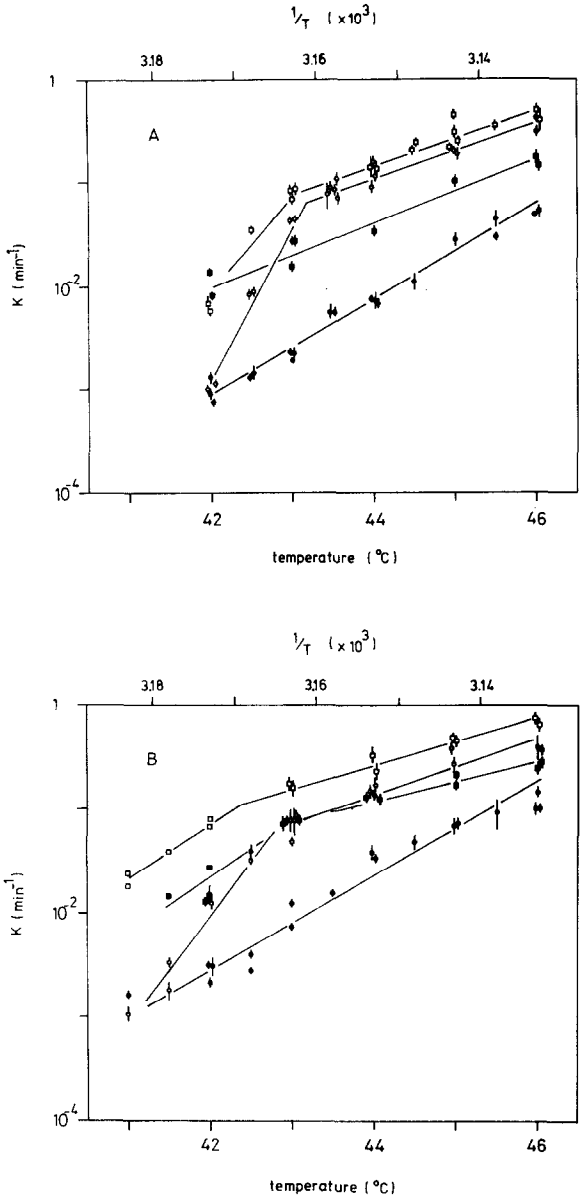

Fig. 4 (A and B). Arrhenius plots of the inverse of the heat survival curve slopes vs. the inverse of the absolute heating temperature. A: (○) *Non-tolerant cells and* (●) *thermotolerant cells heated in culture medium pH* 7.35. (□) *Non-tolerant and* (■) *thermotolerant M*8013 *cells treated in buffered HBSS, pH* 7.5 ± 0.01. *Every individual data point represents the results of analysis of a complete survival curve (examples given in Figs.* 1–3). *B:* (○) *Non-tolerant and* (●) *thermotolerant cells heated in culture medium, pH* 6.55 ± 0.05. (□) *Non-tolerant and* (■) *thermotolerant cells heated in buffered HBSS, pH* 6.6 ± 0.01.

12] during the test heat treatment. It appeared that the expression of thermotolerance was strongly dependent on medium conditions: TTR was much lower at low pH or in cells heated in buffered HBSS (see Table 1 and Fig. 4A and B). The transition temperature T_{trans} in the plots of Fig. 4 reflects the maximum temperature permitting development of thermotolerance during continuous heating [11, 12]. The TTR value of 17.3 observed with cells in complete medium (CM), pH 7.35 at 43°C (Table 1) is very high compared to other cell lines in view of the fact that only a mild heat treatment was used to induce thermotolerance. Dikomey *et al.* [15] report high TTR values in CHO cells but only after more severe priming treatment. The high TTR value observed in CM, pH 7.35 may explain why biphasic survival curves can be observed in thermotolerant M8013 cells under these conditions. When the TTR is not so high (as at low pH) the survival level would drop too fast in the initial part of the curve and the second phase of the curve cannot emerge. The abrupt drop in survival level after prolonged heating of thermotolerant cells (CM, pH 7.35; Fig. 1) indicates that thermotolerance in the cells does not decline gradually during heating as at 37°C but is maintained up to a certain point where it more or less abruptly collapses. Similar observations have been made by Read *et al.* [28]. Read *et al.* showed a decline in survival after 24–26 h continuous heating at 42°C of CHO cells which was related to progression of cells, after a first delay in G_1, into S phase. The decline in thermotolerance after about 25 h of heating at 43°C in the M8013 cells might also be related to cell cycle phenomena. The decline in thermotolerance is however also observed after about 1 h at 46°C and it seems highly unlikely that cell cycle phenomena would play a role in this case. Further research is required with respect to this.

The rather high inactivation energy (890 kJ/mole) for thermotolerant cells heated in complete medium seems to be a particular property of the M8013 cell line. With other cell lines the inactivation energy for thermotolerant cells derived from

Table 2. Inactivation energy (IE) values (kJ/mole) and the corresponding compensation factor (k, ± S.E.) per degree (°C) obtained from the Arrhenius plots in Fig. 4*

Condition	Non-tolerant *k*	Non-tolerant IE	Thermotolerant *k*	Thermotolerant IE
CM pH 7.35 above T_{trans}	1.8 ± 0.3	490	2.9 ± 0.3	890
CM pH 7.35 below T_{trans}	35.2 ± 2.3	2890	2.9 ± 0.3	890
CM pH 6.55 above T_{trans}	1.8 ± 0.2	490	2.9 ± 0.2	890
CM pH 6.55 below T_{trans}	11.4 ± 0.6	2035	2.9 ± 0.2	890
HBSS pH 7.5 above T_{trans}	1.8 ± 0.6	490	2.0 ± 0.2	580
HBSS pH 7.5 below T_{trans}	13.3 ± 2.1	2165	2.0 ± 0.2	580
HBBS pH 6.6 above T_{trans}	1.6 ± 0.1	395	1.6 ± 0.1	395
HBBS pH 6.6 below T_{trans}	3.5 ± 0.3	1024		

*The compensation factor indicates how much longer a heat treatment must be maintained to obtain an equal effect at 1°C lower temperature.

Arrhenius plots is approximately the same as for non-tolerant cells above T_{trans} (e.g. Ref. [29]). In Arrhenius plots, curves for thermotolerance and normal cells run generally parallel above T_{trans}. This may be related to the unusual high TTR values observed with the M8013 cell line. In Fig. 4B, however, the part of the curve of thermotolerant cells (lowest curve) above 44°C might have been drawn with a slope more or less equal to that of the curve for normal cells.

Below T_{trans} continuous induction and development of thermotolerance is possible (*cf.* Refs [10, 11]). Our results support this hypothesis. A high TTR at temperatures above T_{trans} correlates to some extent with a high inactivation energy below T_{trans} (*cf.* Tables 1 and 2). The low inactivation energy below T_{trans} at low pH (or with HBSS) may be the result of decreased expression of thermotolerance with these medium conditions; however, a slower (*cf.* Ref. [15]) or a reduced induction of tolerance [22] at low pH during heating may also play some role. The low inactivation energy and the corresponding shallow slope in the Arrhenius plot below T_{trans} at low pH explains that at 42°C (Table 1) a rather high TTR may still be observed. In one case (HBSS, pH 7.5, 42°C) we even obtained a TTR below 1. In spite of the interval of 5 h between 'priming' and test treatment we seem to have the sensitizing effect observed in 'step down' heating. The correlation observed between a high TTR above T_{trans} and a high inactivation energy below T_{trans} in cells that were not made tolerant by means of a priming heat seems to extend to other cell lines as well (Table 3). This might have important repercussions for the 'thermal dose' concept: a high TTR (above T_{trans}) presumably predicts more or less a high inactivation energy below T_{trans} and thus a high compensation factor per degree Celsius. Further research into this aspect *in vivo* is certainly required.

In solid experimental tumours a relatively low pH is often found [30] and other nutritional conditions may be impaired as well, although this may be limited to certain areas of solid tumour. Thermotolerance has been shown to occur in experimental tumours (e.g. Ref. [31]). The present results indicate that impaired conditions in the cellular environment may lead to a decreased expression of thermotolerance.

Acknowledgement—The skilful technical help of Mrs. M. Krämer-Luinenburg is gratefully acknowledged. This investigation was supported in part by a grant from the IRS (Interuniversitair Institut voor Radiopathologie en Stralenbescherming).

Table 3. Comparison of TTR values above T_{trans} and inactivation energies are derived from Arrhenius plots at temperatures below T_{trans} for various cell lines. The TTR values are obtained from experiments where thermotolerance is induced by priming heat treatment several hours before test treatment at a temperature above T_{trans}. The data on M8013 cells concern experiments in complete medium (including serum)

Cell line	TTR	Inactivation energy (kJ/mole)	Reference
L1A2	4.5	923	Nielsen [12]
CHO	4.3	1262	Bauer and Henle [1]
GM 3440	2.7	960	Raaphorst and Azzam [29]
CHO	17.8	1440	Dikomey *et al.* [32]
R1H	10.8	1610	Dikomey *et al.* [32]
M8013 (pH 7.35)	17.3 (43°C)	2800	Present results
M8013 (pH 6.55)	7.4 (43°C)	1990	Present results

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