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EXPERIMENTAL ARTICLES

The Influence of Cyclic Heating and Cooling on *Escherichia coli* Survival

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Abstract—Repeated heating and cooling in lethal (2–52°C) and nonlethal (2–37°C) temperature ranges resulted in cell death of *Escherichia coli* B/r and *E. coli* B_{S-1} suspended in 0.01 M phosphate buffer, pH 7.0 at varying osmotic pressure, but not in cow's milk. The lethal effect increased with the rate of heating and cooling and with increasing suspension media tonicity; it may be caused by the temperature destabilization of cellular osmotic homeostasis.

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It has been previously found that when eukaryotic [1, 2] and prokaryotic [3–5] cells are heated at different rates to a lethal temperature and maintained at this temperature for a certain time period, they are damaged to a greater extent at a higher rate of heating. Furthermore, the damage to bacteria heated to a lethal temperature also increases with the rate of cooling of the cell suspension [6]. In both cases, the damaging effect depending on the rate of heating and cooling can be decreased or increased using suspension media with certain osmotic pressure [6, 7].

Based on the data cited, it seemed to be worthwhile to investigate the influence of repeated heating and cooling on bacterial survival in media with different osmotic pressure in the range of lethal and physiologically normal temperatures. The urgency of such research was prompted not only by scientific interest (analysis of the mechanism of the lethal effect of hyperthermia on cells), but also by the practical importance of the study of the influence of heating conditions on biological systems for the development of effective methods of application of the temperature factor in diverse fields of biology and medicine.

The goal of the present work was to determine the features and the nature of the damaging effect of repeated changing of heating and cooling conditions in lethal (2–52°C) and nonlethal (2–37°C) temperature ranges on *Escherichia coli* survival in liquid innutritious media with different osmotic pressure. With the same purpose and for comparison, the influence of cyclic changing of heating and cooling conditions on *E. coli* survival in the temperature range of 2–37°C in pasteurized whole milk was investigated.

MATERIALS AND METHODS

The objects of investigation were collection strains *Escherichia coli* B/r and *E. coli* B_{S-1} kindly provided by V.D. Zhestyanikov (Institute of Cytology, Russian Academy of Sciences). Bacterial cells were incubated in nutrient broth (1% peptone, 1% yeast extract, and 0.9% NaCl) for 18 h at 37°C up to the stationary phase of growth. The cells were washed from the medium by twofold centrifugation of the cell suspension, and the cell precipitate was resuspended up to the concentration 10^8 cell/ml in 0.01 M phosphate buffer, pH 7.0 (hypotonic buffer), in 0.01 M phosphate buffer pH 7.0 containing 6% NaCl (hypertonic buffer), in buffers with different NaCl concentrations, or in pasteurized whole milk.

To heat and cool the samples of cell suspensions at the average rate of 1.75 and 2.5°C per second, a medical syringe was used, which was connected by a clear cambric with a stainless steel tube 1 mm in diameter and 0.05 ml in volume. Bacterial samples were collected into the tube up to the cambric level using the syringe, and the outlet of the tube was sealed with molten wax. Then the tube was repeatedly immersed for 20 s (time of heating or cooling to reach the fixed temperature) into temperature-controlled vessels with water temperature 2, 37, or 52°C.

To heat and cool the samples at the average rate of 0.42 and 0.10°C per second, stainless steel test tubes 6 and 10 mm in diameter were used, with 0.5 and 1.2 ml

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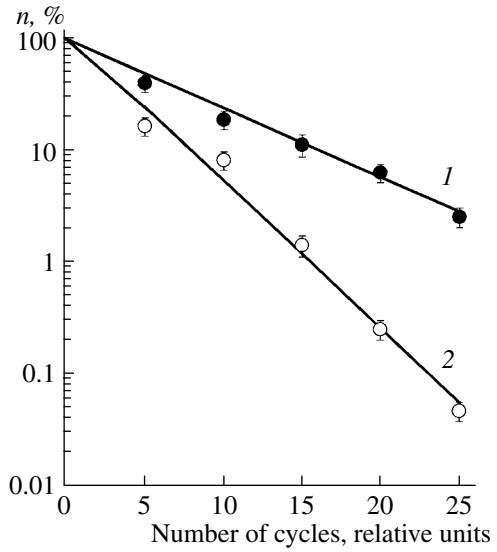

Fig. 1. The influence of the number of heating and cooling cycles within the temperature range of 2–52°C at the average rate of 2.5°C/s on *E. coli* survival in hypotonic (*1*) and hypertonic (*2*) phosphate buffer; *n*—fraction of surviving cells.

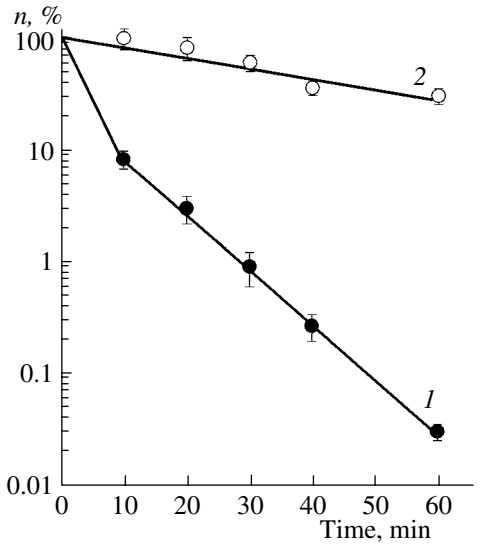

Fig. 2. The influence of the duration of exposure at 52°C on *E. coli* B/r survival in hypotonic (*1*) and hypertonic (*2*) phosphate buffer; *n*—fraction of surviving cells.

of the cell suspension, respectively. Afterwards, the tubes were repeatedly immersed into temperature-controlled vessels with water temperature 2 or 37°C for 1.5 and 2.0 min (time of heating or cooling to reach the fixed temperature). The average rate of heating was determined by means of a stopwatch and an electron thermometer equipped with a miniature temperature detector. The accuracy of temperature observation was ±0.1°C.

Cell survival was determined according to number of macrocolonies (CFU) after 18 h of incubation at 37°C of the transfers performed by the drop method [8].

RESULTS AND DISCUSSION

The curves of survival of *E. coli* B/r suspended in hypotonic and hypertonic phosphate buffers after repeated heating and cooling in the temperature range of 2–52°C at the average rate of 2.5°C/s are shown on Fig. 1. The data presented on the figure demonstrate that this temperature treatment induced intense cell death in spite of the fact that even at the maximum number of heating and cooling cycles, the overall exposure dose of heating at 52°C was too small to cause a lethal effect of a commensurable magnitude. This can be seen from the comparison of the data in Figs. 1 and 2.

The curves of survival of *E. coli* B/r heated at 52°C at the average rate of 1.75°C/s in the variants of bacterial suspension in hypertonic and hypotonic phosphate buffers are presented in Fig. 2. For instance, let us compare the values of exposure doses with equal effect during the heating of bacteria at 52°C in hypotonic medium (phosphate buffer), according to the data of Figs. 1 and 2. For this purpose, the first approximation of the dose corresponding to 25 cycles of heating and cooling was quantitatively calculated (Fig. 1). Due to the immediate change of heating and cooling conditions, on reaching the 52°C temperature, the cells were kept at this temperature for only several seconds (never longer than 5 s) per cycle of thermal treatment. Therefore, the maximum exposure dose of bacteria at 52°C may be considered to be close to 2 min for 25 cycles of heating and cooling. Cell survival at this dose of heating corresponding to 25 cycles of temperature treatment in the hypotonic buffer was 2.5% (Fig. 1, curve *1*). A similar survival level of cells occurs in the case of constant heating for 20 min at 52°C in the hypotonic buffer (Fig. 2, curve *2*).

Therefore, the values of equal-effect exposure doses of heating for continuous heating at 52°C in a hypotonic medium and for the heating and cooling treatment described above are hardly commensurable. Equal-effect doses for continuous thermal treatment of cells at 52°C and under conditions of repeated heating and cooling within the 2–52°C temperature range are all the more incommensurable for bacteria suspended in the hypertonic medium (phosphate buffer) (compare Fig. 1, curve *2* and Fig. 2, curve *2*). Comparison of the data presented in these figures with the calculated values of equal-effect exposure doses of heating at 52°C revealed that a 25-s exposure dose of heating over 5 cycles of heating and cooling in the temperature range of 2–52°C corresponded to a 60 min dose of continuous heating (Fig. 1, curve *2*); in both cases, death of 75% of cell population occurred.

Comparison of these results revealed that the killing effect under conditions of repeated heating and cooling

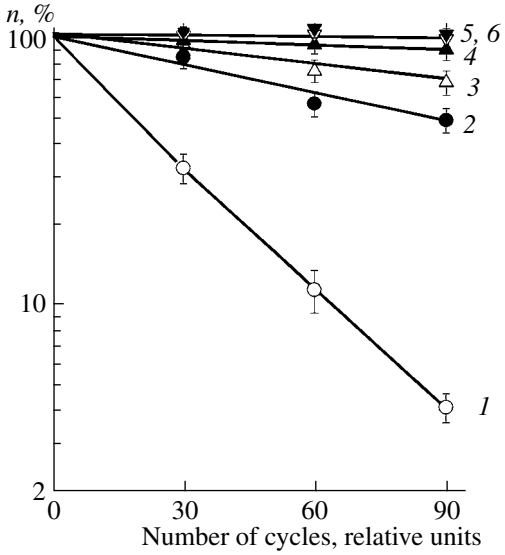

Fig. 3. The influence of the number of heating and cooling cycles within the temperature range of 2–37°C at the average rate of (*1, 2*) 1.75, (*3, 4*) 0.42, and (*5*, *6*) 0.10°C/s on *E. coli* B/r survival in hypertonic (*1, 3, 5*) and hypotonic (*2*, *4*, *6*) phosphate buffer; *n*—fraction of surviving cells.

within the 2–52°C temperature range was substantially higher than under conditions of continuous heating of the cell suspension at 52°C. The nature of cell damage caused by repeated heating and cooling within the 2–52°C temperature range is difficult to interpret in terms of the existing hypotheses of the mechanisms of thermal damage; thermal death of cells is usually believed to be the result of thermogenic damage of cell lipids [9], proteins [10, 11], and membrane structures [12]. This conclusion is supported by the data shown in Fig. 3. The figure presents the survival curves of *E. coli* B/r after repeated heating and cooling within a nonlethal range of 2–37°C under different heating and cooling rates in media with different osmotic pressures. It can be seen that this treatment induces noticeable cell death even in the range of certainly nonlethal temperatures at moderate rates of heating and cooling, 0.4 and 1.75°C/s. In this case, interpretation of the reasons for cell death using the term "hyperthermia" make no sense as the upper level of the temperature used, 37°C, is the physiological optimum for *E. coli* growth, and the lower temperature level, 2°C, is the temperature for storing the transferring cultures for many weeks and even months [13–15].

Table 1. The influence of the number of heating and cooling cycles at the average rate of 1.75°C/s within the temperature range of 2–37°C on *E. coli* B_{S-1} survival in media with different tonicity

Number of heating and cooling cycles, relative units	Fraction of the cells surviving after the temperature treatment in hypotonic and hypertonic media, % $\bar{X} \pm S_{\bar{X}}$	
	0.01 M phosphate buffer pH 7.0 with 6% NaCl	0.01 M phosphate buffer pH 7.0
0	100 ± 7.0	100.0 ± 7.0
30	23.5 ± 2.4	68.7 ± 6.3*
60	5.2 ± 0.6	54.9 ± 6.1*
90	0.9 ± 0.1	31.6 ± 3.9*

Note: Each average value was obtained as a result of three determinations.

* The difference from the value obtained in the hypotonic medium is reliable ($p < 0.01$).

The similar results presented at Table 1 reveal that cells of the mutant *E. coli* B_{S-1} were also killed by repeated heating and cooling in the nonlethal temperature range of 2–37°C.

Thus, the data obtained indicate that the death of cells under repeated heating and cooling of cell suspensions was caused by the cyclic changing of conditions (heating and cooling), the rate of changing of temperature treatment, and the value of osmotic pressure of the medium in which the cells were suspended, rather than by the thermal dose. The last conclusion is also confirmed by the data in Fig. 1 and 3 illustrating an increase in the rate of cell death with increasing tonicity of the suspension media and of the rates of heating and cooling of bacterial suspensions during the cyclic changing of conditions of temperature treatment.

Apparently, the nature of the damage causing cell death under repeated heating and cooling can be interpreted according to our conception that osmotic homeostasis is one of the critical systems in thermogenic cell damage [3, 5].

The stability of the osmotic homeostasis of cells is known to be one of the significant conditions for their normal functioning [15]. This stability can be changed within a certain range; beyond this range, cell survival decreases up to their death. Disturbance of cellular osmotic homeostasis can be caused by changing of the ratio of the concentrations of osmotically active elements of cell and of the environment. A similar phenomenon occurs in cell suspension upon temperature change because of initial differences of the electrolyte composition of the cells and of the suspension liquid. Unlike suspension media (for instance, Ringer's solution and other biological liquids), cells contain not only strong electrolytes with high levels of electrolytic dissociation weakly depending on the solution temperature, but also weak electrolytes such as proteins, nucleic acids and amino acids, and other compounds with a relatively low level of electrolytic dissociation, which, on the contrary, depends heavily on solution temperature [4]. These electrolytes dissociate upon temperature increase due to molecular heat motion and, consequently, they associate upon temperature decrease [4]. Therefore, osmotic homeostasis is disturbed by temperature changes, even under conditions

of the initial equality of osmotic pressures of the cell contents and the suspension medium; this process is examined in more detail in the works [4–6, 16]. The osmotic pressure of the cells increases with heating and decreases with cooling. This conclusion is indirectly confirmed by the data of studies showing that hypertonic suspension media protect the cells from the damage upon heating [16], and, conversely, increase the extent of damage upon cooling [6].

Increase (or decrease) of the intracellular osmotic pressure relative to the osmotic pressure of the medium in the course of heating (or cooling) can induce disturbance in the cell membrane permeability and cause cell death. In this case, increased difference between the heating and cooling temperature and increased rate of temperature change will increase the depth of osmotic (hypertonic and hypotonic) shock, and thereby cause more intense cell death even in a nonlethal temperature range than can be caused by continuous thermal treatment; this result is demonstrated in the present work.

The conclusion that death of microorganisms upon repeated heating and cooling is caused by destabilization of the cellular osmotic homeostasis is also confirmed by the data of Table 2, representing the results of the influence of this treatment in a temperature range of 2–37°C on the survival of *E. coli* B/r suspended in pasteurized whole cow's milk. As may be seen from Table 2, repeated heating and cooling did not have a lethal effect on bacteria in this version of the experiment, although the average rate of heating and cooling was the same as in the case of similar temperature treatment in phosphate buffer with varying NaCl content. In the latter case, changing of the temperature conditions induced the death of *E. coli* B/r cells (Fig. 3, curves *1*, *2*). The lack of lethal effect in the variants with cells suspended in milk can be explained by the stable balance between the intracellular osmotic pressure and that of the medium during the thermal treatment of the suspension, i.e., by the preservation of cellular osmotic homeostasis. The constancy of osmotic homeostasis of the cells under these conditions can be explained by the presence of both strong (e.g., NaCl) and weak electrolytes (proteins, amino acids, and other compounds with low levels of electrolytic dissociation) among the electrolytes contained in the cells and in milk. These compounds react in a similar way to temperature fluctuations by association and dissociation to osmotically active elements and have a similar effect on the osmotic pressure both of the cells and of the medium; the cells can therefore maintain osmotic homeostasis even under variable temperature conditions. Obviously, this is a possible reason for these drastic differences in bacterial response to the cyclic temperature changes in media with different electrolytic composition such as buffer with different NaCl content and cow's milk (see the data of Fig. 3, curves *1–3*, and Table 2).

The reasons for increased cell damage under conditions of repeated heating and cooling at the average rate of 1.75 and 2.5°C/s in media with increased osmotic pressure (Figs. 1 and 3) can be explained thus: the cells suffer from hypotonic shock upon heating and from hypertonic shock upon cooling, with all the ensuing consequences, including cell death [3]. Obviously, these effects should increase during repeated heating and cooling, and they depend on the value of osmotic pressure in the suspension media. It seems likely that increased thermal death of microorganisms in hypertonic media under temperature changes is the result of this dependence and is caused by the overall osmotically stabilizing, protective (upon heating), and osmotically destabilizing, damaging (upon cooling) effect of hypertonic conditions on cell physiology.

Table 2. The influence of the number of heating and cooling cycles within the temperature range of 2–37°C at the average rate of 1.75°C/s on *E. coli* B/r survival in pasteurized cow milk

Number of heating and cooling cycles, relative units	Fraction of the cells surviving after the temperature treatment, % $\bar{X} \pm S_{\bar{X}}$
0	100 ± 11.1
30	99.8 ± 9.9
60	102.7 ± 12.5
90	89.6 ± 9.1

Note: Each average value was obtained as a result of three determinations.

Thus, repeated heating and cooling at a certain rate even within a nonlethal temperature range causes death of *E. coli*, particularly in suspension media with increased osmotic pressure. The assumption was made that processes of destabilization osmotic of cell homeostasis underlie the lethal effect of this temperature treatment of microorganisms. The data obtained and their interpretation contribute significantly to the modern conception of the nature of cell thermal damage and can be used in various fields of biology and medicine.

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