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Heat Protection by Glycerol *in Vitro*[1]

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ABSTRACT

Heating of either Chinese hamster ovary or HeLa cells in medium containing glycerol protected against thermal killing. Above glycerol concentrations of 100 mM, protection of Chinese hamster ovary cells increased in a concentration-dependent manner. Exposure to glycerol only, before or after heating at 45°, did not protect against cell killing. Glycerol protection against thermal damage was also expressed at the subcellular level. The fractional increase in the protein:DNA ratio for nuclei from heated HeLa cells (15 min, 48°) was 1.6 with heating in 1 M glycerol, compared to 2.0 for medium controls.

Both glycerol (1 M) and heat-induced thermotolerance (4 hr, 41.5°) partially reversed the sensitizing effects of pH 6.4 and stepdown heating at 41.5°. The partial deprivation of nutrients achieved by incubating cells in Hanks' balanced salt solution-sensitized Chinese hamster ovary cells against 45° hyperthermia. Glycerol reversed this sensitization but only when nutrient deprivation was short term (75 min). With a long-term, 8.5-hr exposure to glycerol in Hanks' balanced salt solution, cells were significantly more sensitive to heat killing at 41.5° than were cells heated for an equal period in Hanks' balanced salt solution alone.

The similarities in the characteristics of glycerol protection and heat-induced thermotolerance suggest a common mechanistic basis for the two phenomena, although the ability of glycerol to act as a sensitizer in nutrient-deprived cells is not understood.

INTRODUCTION

Studies with globular proteins have shown that the temperature at which 50% of the proteins are denatured can be significantly increased by the presence of glycerol (1, 5). Furthermore, bacteria heated in the presence of high concentrations of glycerol can be protected against thermal killing (18), and these observations were recently extended to mammalian cells (4, 8, 17). The present study was designed to characterize the nature of glycerol protection of mammalian cells *in vitro* as a basis for comparing heat-induced thermotolerance (9) with glycerol protection. The biological variables chosen for this study were those that are presently considered important to sensitizing tumor cells *in vivo*, namely, low pH of the culture medium and nutrient deficiency of the medium (3). In addition, sensitization to low hyperthermic temperatures by stepdown heating (11) was studied for its interaction with glycerol protection.

MATERIALS AND METHODS

Asynchronous CHO[3] and HeLa cells were routinely grown in monolayer and tested for *Mycoplasma* contamination every 6 months. The medium for CHO cells was McCoy's Medium 5A with 10% bovine and 5% fetal bovine serum; HeLa cells were cultured in minimal Eagle's medium (Grand Island Biological Co., Grand Island, N.Y.) with 10% fetal bovine serum. Cells were trypsinized and inoculated into 25-sq cm Corning plastic T-flasks at appropriate concentrations 16 hr before survival experiments. Cellular multiplicities at the beginning of experiments were 1.8 to 2.0 for CHO and 1.3 to 1.5 for HeLa cells.

Exposure to glycerol (reagent grade) was accomplished by aspirating the medium in which the cells had been plated and replacing it with fresh medium containing the stated concentrations of glycerol (0 to 2 M). A pH of 7.4 was maintained by purging each flask with 5% CO_2:95% air after every medium change, except for the nutrient deprivation experiments. The NaCl solution used for the nutrient deprivation experiments was HBSS (Grand Island Biological Co.; Catalog No. 402) with a $NaHCO_3$ concentration of 0.15 g/liter. This concentration of bicarbonate produced a pH of 7.4 in HBSS without purging the flasks. For the experiments with a medium pH of 6.4, we omitted the 2.2-g/liter addition $NaHCO_3$ that is normally added to pH 7.4 medium and adjusted the osmotic pressure to 290 mOsm with additional NaCl (Advanced Instruments Model 3W osmometer). The osmotic pressure of glycerol-supplemented medium or HBSS was checked routinely; in 1 M glycerol medium, it was 1300 ± 15 (S.E.) mOsm. Unless otherwise stated, heating in glycerol was preceded by a 30-min incubation period at 37° to allow glycerol to equilibrate between the intra- and extracellular space (8).

Details of the heating procedure have been reported before (8). At specified times after hyperthermia, the medium in each flask was aspirated; conditioned medium (previously exposed to cells) was added, and the flasks were incubated for 6 to 8 days at 37° until macroscopic colonies were visible. Colonies were then fixed, stained, and counted. Survival curves in the charts show single-cell survival, corrected for cellular multiplicity (20), calculated from the mean of at least 2 separate experiments. Each experiment had 4 to 6 flasks/experimental point with 40 to 350 colonies/flask. Standard errors of the mean are indicated in the charts when larger than the graphical symbols.

Protein:DNA ratios were determined for nuclei from heated and unheated HeLa cells (19). After 4 washes with phosphate-buffered saline (NaCl, 8 g/liter-KCl, 0.2 g/liter-Na_2HPO_4 1.15 g/liter-KH_2PO_4 0.2 g/liter), the cells were lysed in a solution of 1% Triton X-100, 200 mM EDTA, and 80 mM NaCl, pH 7.2. Three washes with the lysis solution was followed by 3 washes with 0.15, 0.25, and 0.15 M NaCl, respectively. This procedure leaves nuclei without visible cytoplasmic contamination (19). The protein and DNA content of the isolated nuclei were analyzed by the method of Lowry *et al.* (16) and the diphenylamine reaction (2), respectively.

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[3] The abbreviations used are: CHO, Chinese hamster ovary; HBSS, Hanks' balanced salt solution; D_0, reciprocal slope of the exponential portion of the survival curve; D_q, quasithreshold dose.

RESULTS

Glycerol Protection in CHO Cells. Cell killing by 45° hyperthermia was reduced by heating in the presence of glycerol. In medium supplemented with 1 M glycerol, the D_0 on the 45° survival curve increased from 3.0 min (fresh medium control) to 7.2 min without a significant change in D_q (Chart 1). The degree of heat protection by glycerol was concentration dependent (Chart 2). With constant heat treatments of 30 min at 45°, cell survival increased linearly with glycerol concentrations in the range of 0.1 to 1.0 M. Above 1 M concentrations, glycerol caused a decrease in cell survival even at 37°, from 70% at 1 M to 5% at 2 M. However, increasing the exposure time to glycerol from 1 to 2 hr did not kill additional cells, with or without subsequent hyperthermia (Chart 2). Cell killing by glycerol at 37° probably resulted from osmotic shock (8). When cell survival was corrected for 37° toxicity, glycerol protection above and below 1 M concentrations showed the same concentration dependence (Chart 2).

The presence of glycerol during hyperthermia was required for protection (Chart 3). The highest survival values after 30 min at 45° were obtained when glycerol (1 M) was present both before and during hyperthermia. The presence of glycerol for 1 additional hr after heating did not further increase cell survival. When cells were heated within 3 to 5 min after exposure to glycerol, survival was lower by a factor of 2 to 3 when compared to cells that had been exposed to glycerol for 1 hr before hyperthermia but nevertheless higher than in unprotected cells. Exposure to glycerol either before or after but not during hyperthermia resulted in lower cell survival than in the controls, in which old medium had been replaced by fresh medium prior to heating (Chart 3).

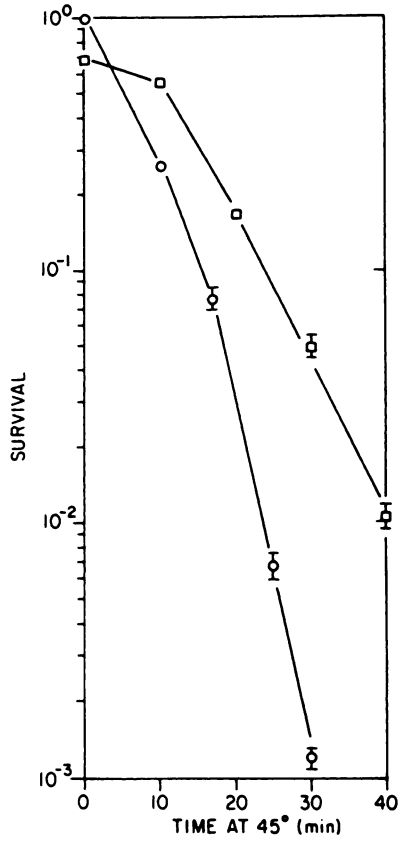

Chart 1. Survival of asynchronous CHO cells after heating at 45° in fresh medium (○) or in medium supplemented with 1 M glycerol (□). Old medium was aspirated and replaced with fresh medium. Flasks were then incubated at 37° for 30 min to allow glycerol to equilibrate with the cells before heating. After the maximum heating time of 40 min, the media were again aspirated, and conditioned medium was added to the flasks. Curves are fitted by eye, and cell survival after hyperthermia was not corrected for 37° glycerol toxicity. *Bars*, S.E.

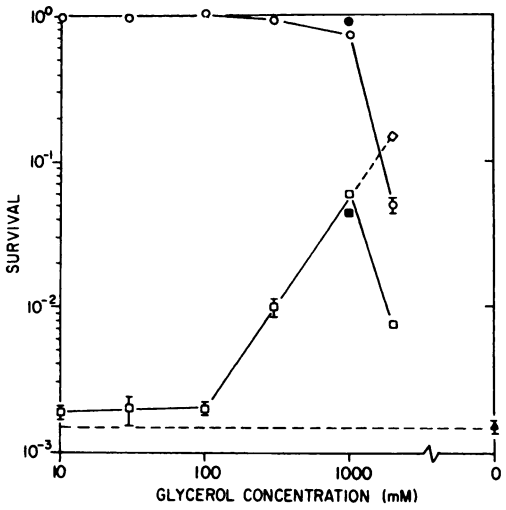

Chart 2. Concentration dependence of glycerol protection against the heat killing of CHO cells. After a 30-min incubation period at 37° in fresh medium (controls) or fresh medium with glycerol (○, □), cells were either heated for 30 min at 45° (■, □, ▲) or maintained at 37° (○, ●). After the treatment, all media were replaced by conditioned medium. ■, ●, cell survival for cells that had been equilibrated for 90 min at 37° in glycerol before treatment. *Dashed horizontal line*, survival level of controls. □– – –◇, survival value after 30 min at 45° in 2 M glycerol with the correction for loss of survival at 37°. *Bars*, S.E.

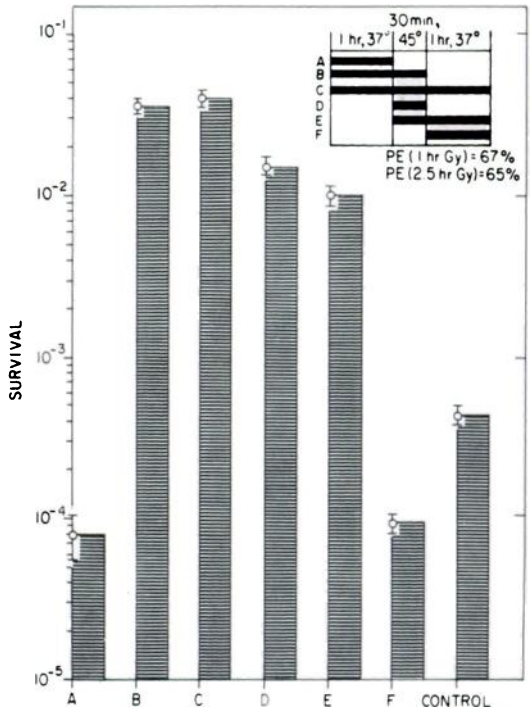

Chart 3. Sequencing of glycerol exposure and 45° hyperthermia. The sequencing protocols *A* to *F* are indicated in the *inset* where the *solid black band* indicates the presence of glycerol (1 M). The 37° plating efficiency was 67 and 65% for cells exposed to glycerol for 1.0 and 2.5 hr, respectively. In Groups *A*, *D*, and *E*, approximately 3 to 5 min elapsed between the exposure to the new medium and the start of hyperthermia. The control group was changed to fresh medium prior to heating. *Bars*, S.E.

To assess the possible contribution of metabolic processing of glycerol at 37° before heating to protection against heat killing, cells were exposed to glycerol for 1 hr prior to heating at either 37° or 0–4°. Table 1 shows that glycerol protection was similar under those 2 conditions. However, metabolic processing of glycerol during hyperthermia cannot be ruled out (see "Discussion").

Glycerol Protection in HeLa Cells. HeLa cells are less sensitive to hyperthermia than are CHO cells (11). With heating in 1 M glycerol (Chart 4), the D_0 of the 48° survival curve for HeLa cells increased from 2.8 to 3.2 min, and the D_q increased from 5.2 to 7.6 min. After a correction for temperature transients (13), the D_q values were 3.0 and 5.4 min, respectively. Glycerol protection against heat damage to HeLa cells also was expressed at the subcellular level. An increase in protein content of nuclei is the only nuclear alteration that has been clearly documented in heated cells, and most of the original studies were conducted with HeLa rather than with CHO cells. We have measured the protein and DNA content in HeLa cells to compare our results with those in the literature (19) and to demonstrate glycerol protection in terms of a characterized subcellular end point. The fractional increase in nuclear protein content after heating for 15 min at 48° was 2.0 in fresh medium but only 1.6 with equal heating in 1 M glycerol (Chart 4). Heating in the presence of 1 M ethylene glycol had no significant effect on the protein content of nuclei from heated cells (Chart 4). Ethylene glycol slightly sensitizes CHO cells to heat killing at this concentration (8).

Table 1

Equilibration temperature and cell survival in glycerol

	Plating efficiency (%)
1 hr, 37° / 1 hr, 4° } medium control	91.1 ± 3.2[a] / 104.1 ± 1.9
1 hr, 37° + 30 min, 45° / 1 hr, 4° + 30 min, 45° } medium	0.15 ± 0.01 / 0.09 ± 0.01
1 hr, 37° + 30 min, 45° / 1 hr, 4° + 30 min, 45° } 1 M glycerol in medium	6.84 ± 0.16 / 8.85 ± 0.38

[a] Mean ± S.E.

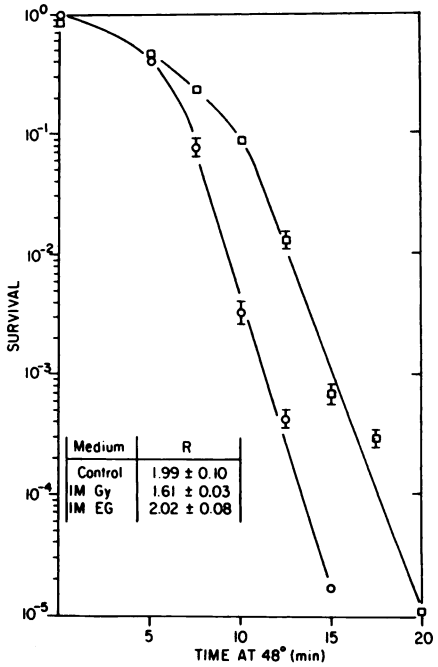

Chart 4. Survival of asynchronous HeLa cells after 48° hyperthermia. Old medium was replaced 30 min prior to hyperthermia by fresh medium (○) or by fresh medium with 1 M glycerol (□). Heating times are not corrected for temperature transients. The protein:DNA ratios were determined for cells heated in medium for 15 min at 48° and divided by the protein:DNA ratio of unheated cells (*R*). Similarly, the *R* values were determined for cells heated in 1 M glycerol (*Gy*) and 1 M ethylene glycol (*EG*) (*inset*). For details, see text. *Bars*, S.E.

Glycerol Protecton with Low pH and Stepdown Heating. Thermal killing is enhanced when cells are heated at low pH or when low-temperature hyperthermia is preceded by an acute high-temperature shock (stepdown heating) (11). Cell survival curves at 41.5° illustrate the degree of heat sensitization of CHO cells by stepdown heating (Chart 5, 10 min at 45° followed immediately by 41.5°) and by low pH (Chart 6). For the low-pH experiments, cells were heated in medium at pH 6.4 which was replaced with medium at pH 7.4 at the end of the experiment. The sensitization by low pH and stepdown heating reduced the D_0 of the control survival curves by factors of approximately 5 to 6; specific values for the D_0 on the control curve could not be determined because exponential regions cannot be clearly defined. Sensitization by either pH 6.4 or stepdown heating was reversed with heating in medium containing 1 M glycerol (Charts 5 and 6). In addition, glycerol-protected cells that were not subjected to stepdown heating showed a heat sensitivity to 41.5° similar to that of cells that had developed thermotolerance after more than 3 hr at 41.5° (Chart 5), even under low-pH conditions (Chart 6).

Glycerol Protection and Nutrient Deprivation. To study glycerol protection under partial nutrient deprivation (amino acids, vitamins, and serum, but not glucose), cells were exposed to HBSS for 30 min at 37° prior to heating at either 45° with a total exposure to HBSS of 75 min or at 41.5° with a total exposure to HBSS of 8.5 hr. With acute nutrient deprivation

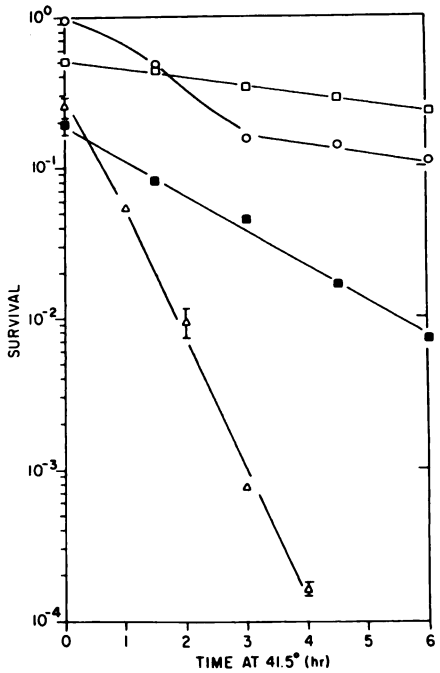

Chart 5. Interaction of stepdown heating and glycerol. CHO cells were heated for 10 min at 45° and transferred immediately to 41.5°. (△, stepdown heating). □, control 41.5° survival curves with heating in medium; □, 41.5° survival curves with heating in 1 M glycerol. ■, stepdown heating in glycerol. *Bars*, S.E.

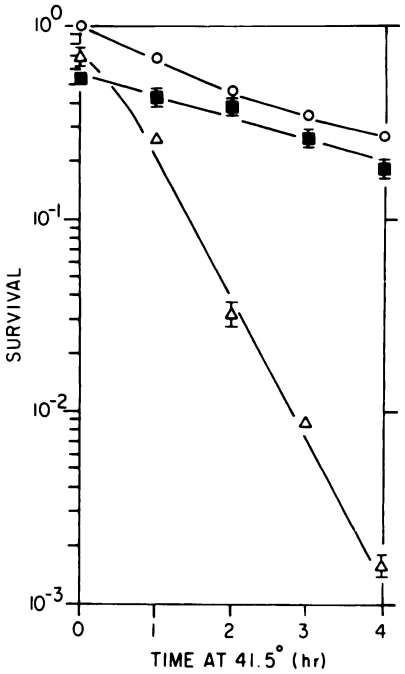

Chart 6. Interaction of pH 6.4 and glycerol. CHO cells were exposed to medium at pH 6.4 and heated at 41.5° (△). ○, controls at pH 7.4. Glycerol added to the acidic medium did not change the pH but increased cell survival (■). *Bars*, S.E.

and heating at 45°, cell killing was enhanced by a factor of approximately 2; the increased cell killing was primarily a "shoulder" effect with a reduction in D_q of about 2.5 min (Chart 7*A*). The addition of 1 M glycerol to either medium or HBSS similarly increased the D_0 by a factor of 2.8 over the nonglycerol controls (Chart 7*A*). Moreover, with a small correction for the 37° plating efficiency in HBSS *versus* medium, survival was identical in the 2 experimental (glycerol-protected) groups. Thus, glycerol protected against the small degree of heat sensitization by acute nutrient deprivation.

Long-term nutrient deprivation (8.5 hr in HBSS) with heating up to 8 hr at 41.5° also showed enhanced cell killing compared to heating in fresh medium (Chart 7*B*). As in Charts 5 and 6, glycerol-protected cells in medium exhibited a 41.5° heat sensitivity similar to medium control cells after the development of thermotolerance (second part of the biphasic survival curve). However, long-term nutrient deprivation in the presence of 1 M glycerol did not protect against cell killing at 41.5° but actually sensitized to 41.5° hyperthermia (Chart 7*B*). In addition, cell killing in HBSS plus glycerol was enhanced at 37°. This indicates that glycerol protection against heat damage requires an unknown nutritional factor, the absence of which does not sensitize to 41.5° hyperthermia by itself.

Thermotolerance and Low-pH, Stepdown Heating, Nutrient Deprivation. When CHO cells were made thermotolerant by continuous heating for 4 hr at 41.5°, the sensitization to heat killing by a medium pH of 6.4, stepdown heating, and heating in HBSS was largely reversed (Chart 8). In these experiments, the medium was replaced after thermotolerance induction (4 hr at 41.5°) with the appropriate new medium, and 41.5° hyperthermia was continued for up to 4 additional hr. For the stepdown heating sequence, the thermotolerant cells were heated for 10 min at 45° before the second 41.5° heat treatment. The results show that thermotolerant cells, like glycerol-protected cells, are largely resistant to heat sensitization by low pH and stepdown heating (Chart 8). In addition,

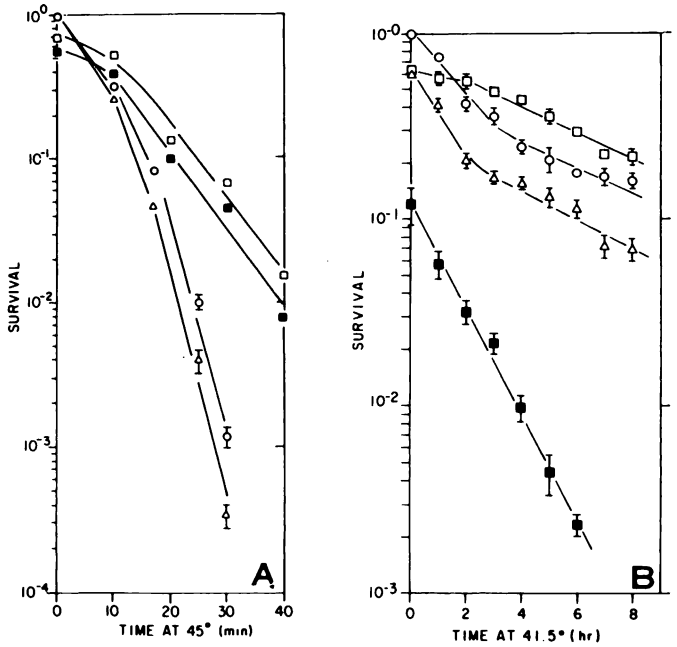

Chart 7. Interaction of nutrient deprivation and glycerol. *A*, short-term nutrient deprivation with a total incubation period in HBSS of 75 min and 45° hyperthermia. Medium was replaced 30 min prior to heating with HBSS (△), fresh medium (○), HBSS supplemented with 1 M glycerol (■), or medium supplemented with 1 M glycerol (□). After heating, cells were grown in conditioned medium. *B*, long-term nutrient deprivation with a total incubation period in HBSS of 8.5 hr and 41.5° hyperthermia. Medium was changed 30 min prior to heating, as in *A*. *Symbols* are as in *A*. *Bars*, S.E.

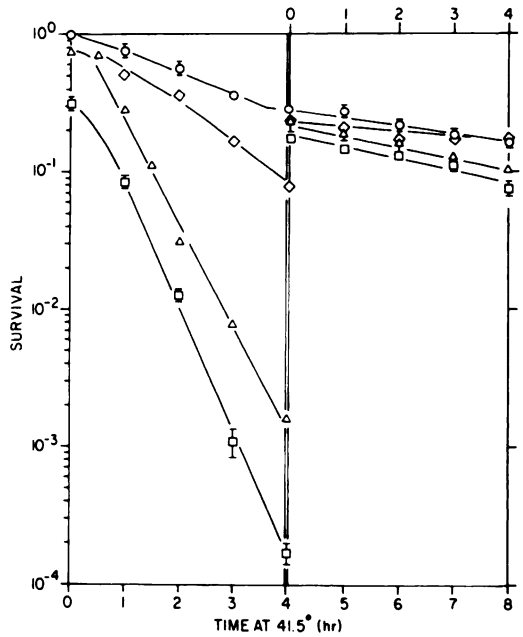

Chart 8. Sensitization by heating at 41.5° in HBSS (◇), heating in pH 6.4 medium (△), or by stepdown heating (□) compared to medium, pH 7.4 controls (○). *Left*, media were changed prior to heating at 41.5°. *Right*, cells were heated for 4 hr at 41.5° to induce thermotolerance before changing the media. Stepdown heating was initiated by "heat conditioning" for 10 min at 45° after the medium was changed to fresh medium. The effects of HBSS, pH 6.4, or stepdown heating in thermotolerant cells were then determined by heating up to an additional 4 hr at 41.5°. *Bars*, S.E.

thermotolerant cells heated in HBSS were less sensitive to 41.5° hyperthermia than were nonthermotolerant-control cells (Chart 8).

DISCUSSION

Several generalizations can be made about protection against cellular heat killing by glycerol. *(a)* Glycerol protection is not limited to a specific cell line but is observed in CHO cells (8) (Chart 1), HeLa cells (Chart 4), Ehrlich ascites tumor cells (4), BP-8 (14), and V-79 Chinese hamster cells (17). *(b)* Protection against heat killing requires glycerol to be present intracellularly during heating (8) (Chart 3). It is unlikely that heat protection is mediated by a metabolic product of glycerol, since cell survival in glycerol medium was similar when cells were equilibrated with glycerol at either 37° or 0–4° (Table 1). Although the temperature independence of glycerol equilibration does not eliminate the possibility that heat protection is mediated by a glycerol metabolite that is formed during hyperthermia, the observation that glycerol can sensitize long-term nutritionally deprived cells (Chart 7*B*) suggests that simple glycerol metabolism at hyperthermic temperatures does not produce a protective metabolite. Nevertheless, it is still possible to postulate a cofactor for glycerol metabolism that is not contained in HBSS and thus prevents the formation of the protective metabolite under long-term nutrient deprivation. *(c)* Significant protection requires glycerol concentrations well above the physiological range (15). Glycerol is much less toxic than glucose (8) at molar concentrations, and glycerol toxicity was primarily a function of its concentration in the medium (Chart 2) rather than the time of exposure; toxicity at 37° was similar for a 1.0-, 2.5-, and 8.5-hr exposure (Charts 1, 3, and 7). The time independence of glycerol toxicity suggests that cell killing by glycerol is due to an osmotic shock, rather than to a chemical toxicity of glycerol.

Both glycerol protection against heat killing and the thermotolerance that is developed at 41.5° are similar in that both reduce the sensitizing effects of pH 6.4 and stepdown heating (Charts 5, 6, and 8). At the subcellular level, the fractional increase in protein content in nuclei from heated HeLa cells is reduced both in glycerol-protected (Chart 4) and in thermotolerant cells (3). Furthermore, both glycerol-protected and thermotolerant CHO cells show a similar final slope on the 41.5° heat survival curve (Charts 5 and 7*B*). The similarities in protection characteristics and sensitivity to 41.5° hyperthermia suggest but do not prove a common mechanistic basis for glycerol protection and thermotolerance.

Glycerol protection and thermotolerance also show dissimilarities. The 45° survival curve for CHO cells in 1 M glycerol has no clear exponential region but continues to bend downward between 40 and 60 min at 45° (8). In both HeLa and CHO cells, glycerol protection against high temperatures (45 and 48°) is more effective with short rather than with long and highly lethal heat treatments (Charts 1 and 4). Generally, this is not observed in thermotolerant cells, in which protection is equally effective for long and short heat treatments, as expressed by a single exponential survival curve (6, 7, 12). However, an adequate comparison of glycerol protection and thermotolerance on the basis of survival curves is not possible without a specific study of the effects of hyperosmotic conditions on the expression of thermotolerance.

The osmotic shock that accompanied the addition of glycerol to medium probably enhanced cellular heat sensitivity (10). However, the glycerol-mediated pressure difference across plasma membranes is transient (8) and should enhance cell killing only when hyperthermia is initiated shortly after the addition of glycerol. This effect was indeed observed (Chart 3); furthermore, a glycerol-induced osmotic shock immediately prior to or following hyperthermia equally reduced cell survival in a heated population. The apparent heat sensitization by osmotic shock may be ascribed to a greater fragility of cellular membranes during and after hyperthermia, although this interpretation is speculative.

The most surprising finding in this study was the sensitization to 41.5° hyperthermia by glycerol under prolonged nutrient deprivation conditions (Chart 7*B*). The sensitization was in contrast to the protection by glycerol to 45° hyperthermia under short-term nutrient-deprivation conditions (Chart 7*A*). This implies that glycerol protection requires nutritionally intact cells, although at present we cannot speculate what nutrient is involved in the sensitization. The unknown nutrient is probably available in intracellular pools that become depleted with prolonged incubation in HBSS.

Mechanisms of thermotolerance are still completely unknown. Glycerol protection against heat damage has been ascribed to its ability to increase hydrophobic interactions between solvent and solute macromolecules, such as globular proteins (1, 5). The similarities in the characteristics of glycerol protection and thermotolerance shown in this paper are consistent with the possibility that heat-induced thermotolerance is mediated by an intracellular protector that is capable of strengthening hydrophobic interactions. However, such protector molecules need to be synthesized before thermotolerance can be expressed, and cells require a longer time for thermotolerance development than for heat protection by glycerol, which is limited only by glycerol diffusion into cells. Both naturally occurring intracellular protectors and glycerol would then prevent cell death by stabilizing a wide spectrum of cellular macromolecules against thermal denaturation (8). Direct support for this hypothesis is not yet available, although the larger polyols appear to possess the postulated properties of a physiological, intracellular protector.[4]

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