Protection against Thermal Cell Death in Chinese Hamster Ovary Cells by Glucose, Galactose, or Mannose

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ABSTRACT

The addition of α-glucose, α-galactose, or α-mannose to culture medium increased survival of heated Chinese hamster ovary cells in a concentration- and time-dependent manner. Heat protection by sugars was not immediate but required prior incubation in the sugar medium before hyperthermia. The degree of heat protection conferred by each sugar and its time dependence differed characteristically: galactose protection appeared rapidly (within 1 hr) and was proportional to the galactose concentration in the medium up to 0.3 M. Glucose and mannose were less effective heat protectors at 0.3 M concentrations when compared with galactose, but cell survival after 40 min, 45°, at concentrations below 0.1 M was similar in the three hyperosmotic sugar media. Heat protection by 0.3 M glucose under hyperosmotic conditions (600 mOsM) became apparent only after a preincubation period of at least 5 hr, 37°. Under isoosmotic conditions (300 mOsM, 10% medium-10 m w/ morpholinopropanesulfonic acid-250 mM glucose), heat protection by glucose appeared more rapidly (3 hr), but the time dependence of heat protection was not eliminated. Under the same isoosmotic conditions in galactose medium, the survival of heated cells was not measurable.

The 45° survival curve in hyperosmotic galactose medium (6 hr, 37° prior to heating, 0.3 M) was characterized by a D50 of 10 min (controls, 3 min) and a quasithreshold dose of 17 min (controls, 17.5 min). When galactose-loaded cells were returned to fresh medium, heat protection decayed rapidly; cell survival measured 1 or 6 hr later showed a small degree of residual heat protection. A 5-hr incubation period at 37° in galactose-supplemented medium resulted in a major intracellular accumulation of free galactose with smaller accumulations of glucose, sorbitol, and dulcitol. A similar incubation in glucose medium showed only minor intracellular elevations of polyols or sugars. A flow-cytometric analysis of the age distribution showed that incubation in the sugar-supplemented media slightly reduced the fractional cell population in G0 with concomitant gains in both the S- and G2-phase populations. Thus, heat protection by galactose or glucose is probably neither the result of a redistribution of cells in the cycle, nor does it always require the intracellular accumulation of free sugars and polyols. The greater degree of heat protection by galactose and its rapid manifestation under hyperosmotic conditions may be related to the intracellular accumulation of free galactose and/or its metabolites.

INTRODUCTION

The modulation of cellular heat sensitivity by the addition of exogenous compounds to the culture medium potentially provides an experimental approach to the study of mechanisms of thermotolerance and heat-induced cell death. Although many compounds are known that sensitize cells to heat killing (7), we know only a few with the ability to protect mammalian cells against thermal death. With the exception of the inorganic protector D2O (1, 3), all known heat protectors are part of a series of naturally occurring linear polyols (12, 13).

In a study of the polyol series glycerol through xylitol, the degree of heat protection at equimolar concentrations was proportional to the number of hydroxyl groups per molecule, given sufficient time for polyols to equilibrate between the intracellular and extracellular space (12). The time required for polyols to enter cells was also proportional to the number of hydroxyl groups, probably a reflection of polyol entry into cells by simple diffusion. This paper provides data to show that 3 biological sugars, like polyols, are part of a larger family of heat-protective compounds with the common characteristic of multiple hydroxyl groups.

MATERIALS AND METHODS

Asynchronous CHO cells were routinely grown in monolayer in McCoy’s Medium 5A supplemented with 10% fetal bovine serum (HyClone, Sterile Systems, Logan, UT) and found to be free of Mycoplasma contamination. Cells were trypsinized and plated in 25×cm2 Morison T-flasks at appropriate concentrations 16 hr before the beginning of a specific experiment. Cell survival was corrected for cellular multiplicity at the time of the experiment (typically near 2.0). Each survival value represents the mean of at least 2 experiments, each using 4 to 6 flasks with 20 to 350 colonies/flask.

Stock solutions of each sugar (reagent grade) were made up in fresh medium and sterilized by filtration. Osmotic pressure of the medium was measured routinely as a check on the sugar concentrations (Advanced Instruments; Model 3W). The cells were exposed to sugar solutions by aspirating the old medium, adding the appropriate new medium, and purging with 5% CO2-95% air to maintain a pH near 7.4. At the end of the sugar exposure, the media were replaced with fresh medium, and the flasks were incubated for colony formation. The treatment procedures and the data analyses were performed as reported before (8, 13).

The distribution of asynchronous CHO cells in the cell cycle was assessed by flow cytometric analyses of cell population samples after a 6-hr incubation in either fresh medium or in medium supplemented with 0.3 M sugar as described previously (12).

Sugars and polyols were assayed by gas-liquid chromatography (Hewlett-Packard; Model 5831A with flame ionization detector) after appro...
priate sample derivatization (14). The cells were grown on 25-mm-
diameter culture discs (Thermonox) to a density of approxi-
mately 10^6 cells each. Following incubation in the sugar medium, the discs were
rinsed in a series of 5 beakers filled with 10 ml of glucose-free Hanks’
balanced salt solution, each, during a total time interval of less than 1
min and then transferred to a conical plastic centrifuge tube containing
2 ml of cold, sugar-free Hanks’ balanced salt solution for sonication.
Following sonication at 0–4°, absolute ethanol was added to a final
concentration of 75%, and tubes were left overnight in the cold. Approp-
riate internal standards were added to the samples: 0.1 mg of o-
mannose or mannitol for sugar and polyol assays, respectively. Prior
chromatograms showed that these compounds are not detectable in
extracts of CHO cells. The protein precipitate was centrifuged and
discarded; the supernatant was removed and dried under nitrogen.

The aldononitrile acetate derivatives of intracellular sugars were
formed as described previously (15). Briefly, the dried material was
dissolved in 0.2 ml of 0.01 M hydroxylamine hydrochloride in pyridine
and sealed. Every step of the procedure was performed under nitrogen.
The tubes were heated to 90° for 30 min and cooled to room tempera-
ture, and 0.2 ml of acetic anhydride was added before reheating the
sealed flasks to 90° for 30 min. After cooling, the solution was dried,
and the residue was mixed with 1 ml of trichloromethane and 1 ml of 3
M HCl. The separation of the aqueous and nonaqueous layer was
completed by centrifugation, and the top aqueous layer was discarded.
The remaining trichloromethane solution was washed sequentially with
1 ml of distilled water, 1 ml of 10 mM NH_4OH, and 1 ml of water
to remove residual pyridine and acetic anhydride. The remainder
was transferred to a clean sample tube, together with an additional 0.1 ml of
trichloromethane rinse of the old tube and pipet. The trichloromethane
was evaporated gently under vacuum, leaving only the carbohydrate
derivatives. For analysis, the sample was redissolved in 0.1 ml of
trichloromethane, and sample volumes of 3 μl were injected.

Polysols were measured after forming the alditol acetate derivatives
(14). The dried sample was dissolved in 0.2 ml of pyridine, mixed with
0.2 ml of acetic anhydride, heated 10 min at 80°, and dried gently under
nitrogen. The acetate derivatives were dissolved in trichloromethane
from the sample, and the solution was evaporated under nitrogen to
dryness and reconstituted as above.

Samples were analyzed on a 1.8-m long, 2-mm (internal diameter)
glass column and packed with SP 2340, 100 to 120 mesh. The initial
oven temperature was 180°; the temperature was maintained constant
during the first 10 min after sample injection and programmed to increase
thereafter at 5°/min to a final temperature of 220°. The temperature of
the injection port and the flame ionization detector was 250°; the carrier
gas was nitrogen; the flow rate was 27 ml/min.

RESULTS

Asynchronous populations of CHO cells were exposed to
media supplemented with 0.3 M D-glucose, D-mannose, or D-
galactose and heated at 45° (35 min) 1, 3, 5, or 7 hr later (Chart
1). Control flasks were changed to fresh medium or to medium
supplemented with 0.3 M dulcitol which is not transported by the
sugar transport system into cells (6). As reported previously for
polysols (12), heating CHO cells shortly after their exposure to
hyperosmotic medium resulted in lower cell survival than in
medium controls. With prolonged incubation in sugar-supple-
mented medium, cell survival exceeded that of equally heated
control cells. Galactose required the shortest incubation time
before heat protection became apparent; cell survival was in-
creased over controls by a factor of nearly 10 after an incubation
period of only 1 hr. With longer incubation in the presence of
galactose, survival continued to increase but at a slower exponen-
tial rate of 0.14/hr (t1/2 = 4.8 hr). Glucose-supplemented
medium initially caused a potentiation of heat killing similar to
that achieved with the other media. Cell survival increased slowly
in both the glucose- and dulcitol-supplemented media; heat
protection appeared after a 5- and 7-hr incubation period. Cell
survival after heating in mannose medium increased within 1 hr
to that of controls but did not exceed controls until 5 hr later.

The initial sensitization to hyperthermia with heating shortly
after the medium change may be related to the hyperosmotic
stress caused by the sugar-supplemented media. Chart 2 shows
data for cells that were exposed and heated (40 min, 45°) in
high-glucose concentrations under isoosmotic conditions. We
diluted fresh medium to 10% with distilled water and added 10
mM morpholinopropanesulfonic acid buffer for pH stability (pH
7.4) and 0.25 M glucose or galactose for a final osmotic pressure
of 300 to 305 mOsm. The cells were incubated in these media
for a total period of 6 hr and heated at the times indicated (Chart
2). The plating efficiency of unheated cells in glucose or galactose
was 58 and 37%, respectively, similar to those in hyperosmotic
media (Chart 3). Comparison of Charts 1 and 2 shows that heat
protection by glucose appeared more rapidly under isoosmotic
stress than under hyperosmotic conditions. However, heat protection
by glucose was neither immediate nor constant over the pre-
heating incubation periods, as might be expected if the osmotic
stress were responsible exclusively for the time dependence of
heat protection. Curiously, all cells heated 40 min at 45° under
parallel conditions in galactose failed to survive, regardless of
the preheating incubation period, suggesting that high concen-
trations of galactose combined with nutrient deprivation can
sensitize cells to hyperthermia in a manner reminiscent of glycerol (13). This surprising phenomenon is the subject of a separate study in progress.

The concentration dependence of heat protection by glucose, mannose, and galactose is shown in Chart 3. Hyperthermia of 40 min, 45°, was preceded by a constant incubation period of 6 hr, 37°. Sugar concentrations of 0.1 and 0.3 m showed dose-dependent cytotoxicity; for high concentrations of 0.3 and 1 m, the cytotoxicity of galactose was significantly less than that of glucose (latter data not shown). Concentrations below 5 mM were not included, since glucose is normally present in medium at this level. Survival of heated cells was similar for the 3 sugars over the concentration range of 5 to 100 mM. Above 0.1 m, galactose continued to increase survival; with a correction for the enhanced 37° toxicity, cell survival with galactose protection can be fitted by a straight line over the entire range. Both glucose and mannose showed either equal or lower cell survival above 0.1 m, even with a correction for 37° cytotoxicity. Taken together, the data in Charts 1 and 3 show that heat protection by glucose and mannose is significant and that optimal sugar concentration for protection is near 100 mM.

Chart 4 shows the 45° survival curves for cells incubated 6 hr prior to heating in media with glucose and galactose concentrations of either 5 or 300 mM. The medium control (5 mM glucose) is characterized by a D50 of 3 min, 45°, and D50 of 17.5 min, 45°. High glucose (0.3 mM) protected cells against thermal death but much less than equimolar galactose. The survival curve of the latter cells showed a D50 of 10 min, 45°, and a D50 similar to that of controls.

When cells are incubated in galactose-supplemented medium (0.3 mM) for only 1 hr at 37° (Chart 5), heat protection is less than that after 6 hr of incubation (Chart 4). The 45° survival curves obtained with galactose-loaded cells 1 or 6 hr after return to fresh medium show that most of the heat protection has disappeared by 1 hr (Chart 5); a small residual degree of protection remains and is similar for the 2 latter survival curves.

Chart 6 shows the age distribution of an asynchronous cell population after a 6-hr incubation in 0.3 mM glucose-, galactose-, or dulcitol-supplemented medium, as measured by flow cytometry. Dulcitol was included as a control for galactose; although it is not transported by the same carrier system and does not accumulate intracellularly like galactose, the perturbation of cell progression through the cycle was remarkably similar for the 2 compounds. The same is true for glucose; similar DNA histograms result after incubation with sorbitol (12) or its sugar (Chart 6B). Neither of the 2 sugars (Table 1) nor their respective alcohols (12) caused a significant accumulation of cells in the heat-resistant G1 phase, ruling out the possibility that increased cell survival after hyperthermia in sugar-supplemented media is based on a population redistribution.

The intracellular sugar and polyol content after an incubation of 5 hr at 37° in 0.3 mM glucose- or galactose-supplemented medium is shown in the chromatograms in Charts 7 and 8, respectively. Chart 7B shows that control cells contain free intracellular glucose and galactose. The survival curve of the latter cells showed a D50 of 10 min, 45°, and a D50 similar to that of controls.
HEAT PRODUCTION BY SUGARS

Chart 4. Cell survival curves for sugar-protected CHO cells. Following a 6-hr, 37°C incubation period in medium supplemented with 0.3 M glucose (●), 0.3 M galactose (▲), 5 mM galactose (△), or control medium (●), the flasks were heated at 45°C during the indicated incubation interval. After the complete experiment, the sugar media were replaced with fresh medium.

represent approximately 2 to 4 μg/10⁶ cells. Integration of the areas under the peaks indicates that intracellular free glucose and galactose are not significantly different from control cells. In contrast, a similar incubation in galactose medium increased free galactose to 30 to 50 μg/10⁶ cells and largely obscured the glucose peak. A rigorous quantitation of the 2 intracellular sugars is difficult under these conditions and will be attempted in a separate in-depth study.

The large galactose peak is not an artifact of galactose contamination from the medium. In a separate study, we measured residual galactose in each rinsing beaker to determine the number of rinses required to reduce extracellular galactose to submicromolar levels. The data show that, after the fifth rinse, galactose in the rinsing solution was below 2 μg/ml (11 pm). In addition, a similar rinsing procedure with glucose (Chart 7C) did not result in a high glucose peak, suggesting that the chromatograms represent true intracellular sugar levels.

The chromatograms in Chart 8 show intracellular polyols. Control cells showed no detectable sorbitol or dulcitol (Chart 8B) but an unidentified peak with a retention time of 23.3 min. Incubation in high glucose medium reduced the unidentified peak but showed no evidence of accumulating polyols (Chart 8C). In contrast, galactose-loaded cells were characterized by the complete absence of the RT23.3 peak, but both sorbitol and dulcitol accumulated to levels of 40 to 50 μg/10⁶ cells (Chart 8D). The latter data represent evidence that the polyol pathway exists in CHO cells. A comprehensive quantitative study of intracellular polyol accumulations in sugar-supplemented media will be presented in a separate publication.

DISCUSSION

We have shown previously that the linear polyols glycerol, erythritol, and adonitol can (a) reduce killing in heated cell populations (12, 13) when added to the culture medium prior to heating and (b) that the degree of protection was related to both the equilibration time (entry of polyol into cells) and the chain length of the polyols (number of hydroxyl groups). In this paper, we have examined 3 common hexitols with respect to their ability to protect CHO cells against heat killing. These sugars differ from each other only in the position of the hydroxyl group on C-2 or C-4; i.e., mannose is the C-2 epimer, and galactose is the C-4 epimer of glucose. Unlike the polyols, these sugars are generally transported in the pyranose ring form across the plasma membrane by facilitated diffusion, binding to a stereospecific transport molecule that accepts only the β-series sugars (configuration of C-5) (6). With high extracellular sugar concentrations, phosphorylation by hexokinase often becomes rate limiting, rather than sugar transport (6). These conditions favor free intracellular sugar and can lead to intracellular concentrations of nonmetabolizable sugar (2-deoxy-β-glucose) that exceed the extracellular concentration (4). To our knowledge, the extremely high intracellular accumulations of galactose have never

Chart 6. Age distribution of CHO cells after incubation in supplemented media. The distribution of DNA content was measured in cell populations following a 6-hr, 37° incubation in control medium (A), medium supplemented with 0.3 M d-glucose (B), 0.3 M d-galactose (C), or 0.3 M dulcitol (D).

Table 1

<table>
<thead>
<tr>
<th>All measurements were made after 6 hr at 37° in medium supplemented with 0.3 M sugar or dulcitol.</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Fractional distribution of cells in the cycle (%)</td>
</tr>
<tr>
<td></td>
<td>G_1</td>
</tr>
<tr>
<td>Medium controls</td>
<td>34.9</td>
</tr>
<tr>
<td>Glucose</td>
<td>33.9</td>
</tr>
<tr>
<td>Galactose</td>
<td>26.0</td>
</tr>
<tr>
<td>Dulcitol</td>
<td>26.8</td>
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</table>

been observed before.

The initial sensitization to hyperthermia after a medium change may be related to the hyperosmotic stress induced by the sugar-supplemented media. For example, if heat protectors acted on a component of the plasma membrane exposed to the medium, we might expect instantaneous and constant heat protection independent of prior incubation. Cell survival might then be modified only by a time-dependent osmotic pressure difference across the plasma membrane (9). In order to determine whether the time dependence of heat protection was entirely due to osmotic stress, we heated cells under isoosmotic conditions in 250 mM sugar medium. Although the low-salt concentrations of the diluted isoosmotic medium may also stress the cells, we observed heat protection by glucose which was neither immediate nor constant for variable incubation intervals before heating. Thus, the kinetics of heat protection does not simply reflect a cellular reaction to hyperosmotic pressure gradients.

As with polyols, the mechanism of heat protection by sugars is mediated probably through increased hydrophobic interactions (2); these are considered important modulators of macromolecular heat stability, for example, in membranes and globular proteins. We have demonstrated that galactose at high extracellular concentrations provides the highest level of heat protec-
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**Chart B.** Intracellular polyol content. Alditol acetate derivatives were made from the water-soluble extracts of CHO cells following incubation of 5 hr. 37°C in control medium (A), medium supplemented with 0.3 M glucose (C), or galactose (D). A shows the chromatogram of standards: erythritol (Ey, retention time 6.1 min); ribitol (Ri, 15.1 min); arabinitol (Ar, 15.7 min); mannitol (IS, 21.3 min); dulcitol (Du, 22.5 min); sorbitol (So, 24.2 min); and inositol (In, 26.5 min). The peak marked X (RT 23.3) is an unidentified cell component with a retention time of 23.3 min.

HEAT PRODUCTION BY SUGARS

Heat production by sugars and sugar analogues, submitted for publication.

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**REFERENCES**

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