[CANCER RESEARCH 43, 1624–1627, April 1983]
0008-5472/83/0043-0000$02.00

Protection against Heat-induced Cell Killing by Polyols *in Vitro*[1]

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ABSTRACT

The polyols erythritol and adonitol reduced 45° heat killing in asynchronous Chinese hamster ovary cells. Heat protection by glycerol and erythritol increased with the apparent intracellular concentration, as inferred from cell volume measurements, and the number of hydroxyl groups per alcohol molecule. The nonlinear tetrahydroxy alcohol pentaerythritol did not protect but sensitized to heat killing. On cell survival curves, the reduced cell killing of protected cells was expressed by an increased D_0 for the pentahydroxy alcohol adonitol (0.3 M), whereas equimolar concentrations of glycerol increased primarily the D_q (quasithreshold dose) with little increase in D_0. The distribution of Chinese hamster ovary cells within the cell cycle was unaffected by the presence of 0.3 M glycerol in the culture medium. However, the polyols erythritol and sorbitol caused a small but significant loss of cells from the heat-resistant G_1 compartment. The cell cycle redistribution with prolonged incubation (6 hr) in polyol-supplemented medium is expected to increase the heat sensitivity of the perturbed cell population; the observed heat protection by polyols suggests that heat resistance in the presence of polyols is not an artifact of an asynchronous cell system. Instead, the data identify a family of heat-protective compounds that may occur naturally in mammalian cells.

INTRODUCTION

We reported previously that the trihydroxy alcohol glycerol can protect mammalian cells against heat killing, whereas mono- and dihydroxy alcohols sensitize to hyperthermia (3, 7). Other investigators have made similar observations (2, 8, 9). To better understand this effect, we tested a series of compounds for their ability to protect against heat killing that either differed from glycerol in specific substitutions in the 3-carbon molecule[3] or in the arrangement and the number of hydroxyl groups in a series of polyhydroxy compounds. We report in this paper the results of the study with heat protection by a series of polyols.[4]

MATERIALS AND METHODS

Asynchronous CHO[5] cells were routinely grown in monolayer in McCoy's Medium 5A supplemented with 5% fetal bovine and 10% bovine serum (Flow Laboratories, Inglewood, Calif.) as reported before (3, 7). Cells were trypsinized and plated in 25-sq cm Corning 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).

Stock solutions of each alcohol (reagent grade) were made up in fresh medium and sterilized by filtration. The cells were exposed to sugar alcohol solutions by aspirating the old medium, adding the appropriate new medium, and purging with 5% CO_2-95% air to maintain a pH near 7.4. At the end of the polyol exposure, the media were replaced with conditioned medium (resterilized medium after earlier 24-hr exposure to cell cultures), and the flasks were incubated for colony formation. The data were analyzed as reported before (3, 7).

The distribution of asynchronous CHO cells in the cell cycle was assessed by flow-cytometric analyses after a 6-hr incubation in either fresh medium or in 0.3 M polyol. After an overnight fixation in 70% ethanol, the ethanol was removed by centrifugation, and the fixed cells were resuspended in 0.2 ml of 50 mM $MgCl_2$-150 mM NaCl. Cells were stained with mithramycin (final concentration, 50 μg/ml) and propidium iodide (final concentration, 30 μg/ml) in Tris-buffered 0.9% NaCl solution, pH 7.2. After 5 min at room temperature, the cells were analyzed on a FACS III (Becton, Dickinson, & Co., Sunnyvale, Calif.) with an excitation wavelength of 458 nm (100 milliwatts) and fluorescence detection through a 640-nm long-pass filter. Data were stored and analyzed with a Tektronix 4052 computer.

Cell volumes were quantitated indirectly by measurements of modal cell volumes 0 to 2 hr after the addition of polyol media to cell suspensions. The initial exposure to hypertonic medium reduces the modal cell volume in response to the increased extracellular osmotic pressure; the entrance of the polyol solute into cells is then accompanied by a return of the modal cell volume toward control values. For glycerol, this method provides the same kinetic information as does the more direct measurement with radiolabeled glycerol (1). Measurements of modal cell volumes were confined to the first 2 hr after polyol exposure; longer exposures induce cell cycle redistributions which by themselves could change the modal cell volumes (data not shown).

RESULTS

Monolayer cells were exposed to either glycerol, erythritol, or pentaerythritol for 90 min at 37° and then heated to 45° for 30 min. A plot of the logarithm of cell survival *versus* the logarithm of concentration of the added polyhydroxy compound showed similar slopes for glycerol and erythritol, but at any fixed concentration the tetrahydroxy alcohol erythritol was approximately twice as effective in reducing cellular heat killing as was the trihydroxy alcohol glycerol. In contrast to the linear polyhydroxy alcohols, the nonlinear tetrahydroxy alcohol pentaerythritol sensitized CHO cells to 45° hyperthermia with increasing concentrations (Chart 1). Immediately after the addition of the hypertonic media (approximately 600 mOsm) the modal cell volumes decreased to nearly 50% of controls. Within 30 and 90 min after the addition of glycerol and erythritol-supplemented media, respectively, the modal cell volumes had returned to approximately 90% of controls (Chart 2). Modal cell volumes after the addition of pentaerythritol followed the same kinetics as those after

[1] This investigation was supported by USPHS grants CA-33405 and CA-33406 awarded by the National Cancer Institute, Department of Health and Human Services. A polyol is defined by the generalized formula $CH_2OH\ (CHOH)_nCH_2OH$: glycerol, $n = 1$; erythritol, $n = 2$; adonitol, $n = 3$; sorbitol and its stereoisomers, $n = 4$.

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[3] K. J. Henle and L. Rice, unpublished data.

[4] These data were presented in preliminary form as a poster during the 29th Annual Meeting of the Radiation Research Society, May 31 to June 4, 1981, Minneapolis, Minn.

[5] The abbreviations used are: CHO, Chinese hamster ovary; D_0, reciprocal slope of the exponential portion of the survival curve; D_q, quasithreshold dose.

Received August 23, 1982; accepted January 3, 1983.

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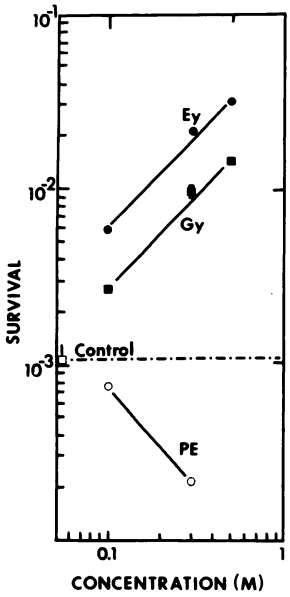

Chart 1. Modification of 45° heat killing by polyhydroxy alcohols. Asynchronous CHO cells were incubated for 90 min at 37° in fresh medium (control) or medium supplemented with glycerol (*Gy*), erythritol (*Ey*), or pentaerythritol (*PE*). After the 90-min equilibration time, the flasks were heated for 30 min at 45°. The medium was replaced in all flasks after hyperthermia, and surviving cells were allowed to form colonies. Unheated controls showed that the plating efficiency was not significantly reduced by exposures to polyhydroxy alcohols in concentrations up to 0.5 M.

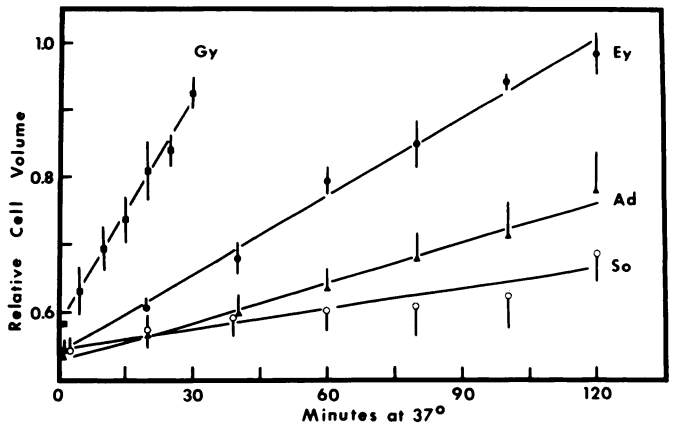

Chart 2. Modal cell volumes as a function of time after cell exposures to glycerol or polyols. CHO cells in suspension were mixed in equal volumes with media supplemented with 0.6 M glycerol (*Gy*), erythritol (*Ey*), adonitol (*Ad*), or sorbitol (*So*) for a final concentration of 0.3 M polyol. The modal cell volume was determined on a Coulter channelizer at various times up to 2 hr after polyol exposure. Between measurements, the counting vials were kept in a forced air-CO_2 incubator at 37° with gentle agitation to prevent cells from settling out. The data represent the means from 4 separate experiments and are expressed as the perturbed cell volumes relative to unperturbed control volumes; *bars*, S.D. Linear regressions of the data yield rate constants of 0.0112, 0.0039, and 0.0020 min^{-1} with $r > 0.99$ for glycerol, erythritol, and adonitol, respectively. The rate constant for sorbitol was 0.00099 min^{-1} with $r = 0.95$.

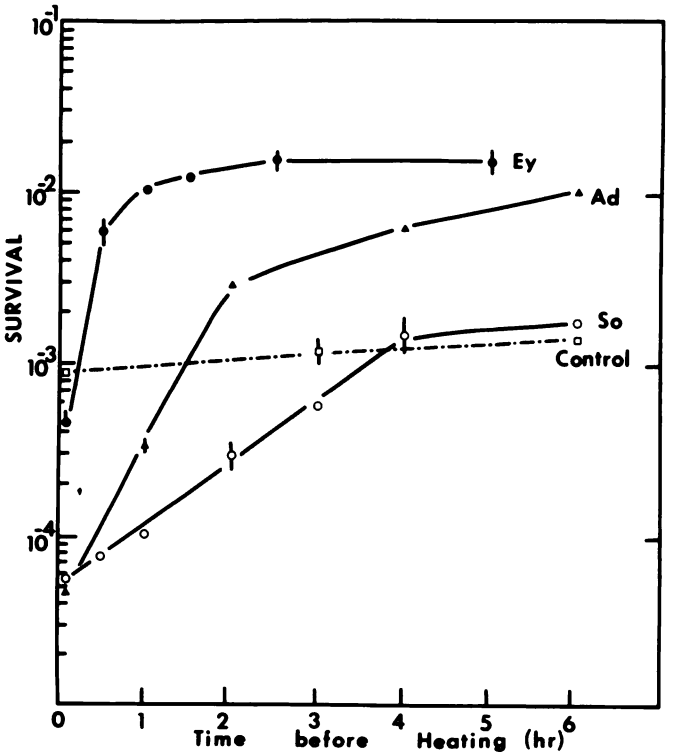

Chart 3. Kinetics of heat protection by polyols. Cell survival was measured as a function of time after cell exposure to 0.3 M erythritol (*Ey*), adonitol (*Ad*), or sorbitol (*So*). Control cultures were exposed to fresh medium. After various incubation periods, the culture flasks were heated for 30 min at 45°; following the complete experiment (approximately 7 hr), the media were replaced in all flasks.

erythritol addition (data not shown). The pentahydroxy polyol adonitol caused a more prolonged reduction in modal cell volumes with an apparent equilibration rate of half of that for erythritol (Chart 2). Similarly, the modal volume after sorbitol addition increased at one-half the rate of that for adonitol (Chart 2). The slow increase in modal volume during continuous osmotic stress from sorbitol is not fully understood.

The apparently limited ability of the longer-chained polyols to equilibrate with the intracellular space also correlated with their ability to protect cells against heat-induced killing. Chart 3 shows the survival of cells that were heated equally (30 min at 45°) at various times after the addition of polyol-supplemented media to culture flasks. Cells heated shortly after polyol addition survived less well than did medium controls, but with prolonged exposures to polyols before heating cell survival increased above the medium controls. The rate at which heat protection appeared decreased with increasing number of hydroxyl groups per polyol molecule while the required time for reaching a plateau in survival increased (Chart 3). Erythritol showed heat protection within 30 min, and cell survival reached a plateau after approximately 90 min. Heat protection by adonitol did not appear until 2 hr, and cell survival did not reach a plateau within 6 hr. Cell survival in the presence of sorbitol increased even more slowly to reach but not to exceed the medium control levels only after 4 hr. Sorbitol is reportedly incapable of diffusing through the plasma membrane (11), although slow diffusion of sorbitol into the cell during 6 hr cannot be ruled out.

Cell survival curves at 45° are shown in Chart 4 for cells incubated for a constant 6 hr at 37° in media containing 0.3 M glycerol, erythritol, pentaerythritol, and adonitol. These curves show that heat protection per linear polyol molecule increases with increasing polyol chain length and that heat protection by adonitol is characterized primarily by an increased D_0 on the survival curve. In contrast, heat protection by glycerol and erythritol is expressed in an increase of the D_q with a smaller effect on D_0. A quantitative comparison of heat protection by adonitol with that by glycerol and erythritol is not possible since adonitol probably did not equilibrate fully between the intra- and

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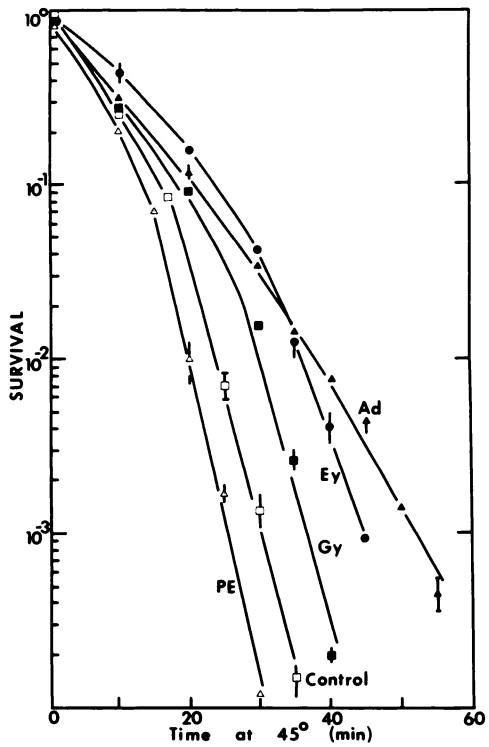

Chart 4. Cell survival curves with 45° hyperthermia in the presence of polyhydroxy alcohols. Cells were exposed to fresh medium (control), pentaerythritol (*PE*), glycerol (*Gy*), or polyols for 6 hr at 37° and then heated to 45° for various intervals. *Ey*, erythritol; *Ad*, adonitol; *bars*, S.D. The concentrations of the polyhydroxy alcohols were 300 mM.

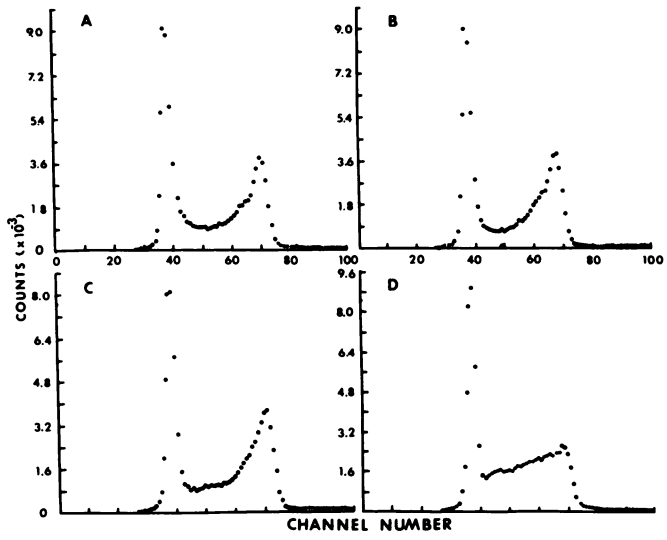

Chart 5. DNA histograms of cells incubated for 6 hr at 37° in fresh medium (controls; *A*), 300 mM glycerol (*B*), 300 mM erythritol (*C*), or 300 mM sorbitol (*D*). For details, see text.

Table 1

Distribution in the cell cycle (percentage) after 6 hr 37° incubation in 0.3 M polyol

	G_1	S[a]	G_2-M	Q[a]
Control (medium)	33.9	56.3	7.8	2.0
Glycerol	33.7	55.6	7.7	3.0
Erythritol	32.4	55.4	9.5	2.7
Sorbitol	32.7	59.6	7.7	0.0

[a] In the fitting procedure for analyzing DNA histograms, "Q slots" are used to represent the cohort of cells in late S that appears as small bumps. Thus, the total number of S-phase cells equals S + Q.

extracellular space within the 6-hr incubation period before heating ("Discussion").

The age distribution within the cell cycle was measured after a 6-hr, 37° exposure to 0.3 M glycerol, erythritol, or sorbitol (Chart 5). The flow-cytometric analysis of these cell populations show that both glycerol and erythritol cause only insignificant perturbations in cell cycle distribution; sorbitol, however, significantly altered the DNA histogram (Chart 5*D*) with an apparent accumulation of cells in middle-to-late S phase. Overall, the fraction of cells in G_1, S, and G_2-M changed but little (Table 1).

DISCUSSION

We have hypothesized previously that heat-induced thermotolerance is mediated by intracellular protector molecules that are synthesized in response to a conditioning heat treatment (3, 5, 6). Based on the finding that glycerol can protect against heat-induced cell killing, we postulated that naturally occurring protector molecules synthesized by thermotolerant cells are polyhydroxy compounds (3). The data in this paper show that linear polyols constitute a family of heat-protecting compounds and that they may reduce heat killing only when they are present inside of the cells (see below). The nonlinear tetrahydroxy alcohol pentaerythritol is not a naturally occurring compound and cannot protect against heat damage (Charts 1 and 3). The mechanism of heat protection by polyols is likely different from that involved in the protection by glycerol against freezing and free radical damage (3) and may be based on altered hydrophobic interactions. In this study, we could not compare directly the degree of heat protection per intracellular molecule of polyol since adonitol and sorbitol showed limited (Chart 2) or negligible diffusion (11) across the plasma membranes, respectively. However, the possible comparison of glycerol with erythritol suggests that the additional hydroxyl group in erythritol increased heat protection by a factor of 2 at equimolar concentration (Charts 1 and 3).

In the review of this paper, one of the referees suggested an alternate hypothesis for the apparent requirement that polyols be present intracellularly in order to protect against thermal death. According to this reasoning, extracellular polyols may protect heat-sensitive sites on the plasma membrane, but simultaneously osmotic pressure differences across the membrane sensitize cells to heat killing. Thus, cellular exposure to high polyol concentrations in the medium initially would result in an overall decrease in cell survival but, as the polyols enter the cells and thereby reduce the osmotic pressure differences, cell survival increases to reflect eventually heat protection by polyols alone. The data in this paper cannot be used to distinguish between these alternate interpretations.

Glycerol and erythritol did not perturb the age distribution of the cell cycle (Chart 5, *A* and *B*). Therefore, heat protection by these 2 polyols cannot be ascribed to a redistribution artifact. Although sorbitol caused an apparent redistribution in the cell cycle (Chart 5*C*), the heat-resistant G_1 phase (10) was depleted in a minor way with a concurrent accumulation of cells in the heat-sensitive S phase (Table 1). Qualitatively, this redistribution would serve to underestimate heat protection by sorbitol and support the argument that polyol-mediated protection against heat damage cannot be explained by a net movement of cells

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out of sensitive and into heat-resistant parts of the cell cycle.

Heat protection by adonitol is difficult to quantitate for the above-mentioned reasons; nevertheless, heat protection by adonitol yields a hyperthermia survival curve that resembles more closely that of thermotolerant cells (4) than heat survival curves with the smaller polyols, glycerol and erythritol. Heat protection by glycerol increases primarily the D_q of the cell survival curve (Chart 3; Ref. 7) in contrast to protection by adonitol which increases primarily the D_0 with little change in D_q. Similarly, accumulations of free intracellular galactose increases primarily the D_0 on the 45° hyperthermia survival curve (5). These observations are consistent with the hypothesis that heat-induced thermotolerance is mediated by endogenous polyhydroxy compounds (6).

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