

Correlation between synthesis of heat shock proteins and development of thermotolerance in Chinese hamster fibroblasts

(hyperthermia/cell survival/sodium arsenite/ethanol/hypoxia)

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ABSTRACT Synthesis of a family of proteins called “heat shock” proteins is induced or enhanced in cells in response to various environmental stresses, suggesting that these proteins may perform functions essential to cell survival. Because a brief, non-lethal heat treatment can dramatically induce a transient resistance to a subsequent lethal heat treatment (thermotolerance), we examined the effect of heat treatment (41–46°C) on protein synthesis and cell survival in plateau-phase Chinese hamster fibroblast (HA-1) cells. After heat treatments that either drastically inhibited total protein synthesis (46°C) or did not suppress it (41°C), the synthesis of heat shock proteins was greatly enhanced over that in unheated cells, and cell survival was increased 10²- to 10⁶-fold when cells were challenged by a subsequent lethal heat treatment. The synthesis of heat shock proteins correlated well with the development of thermotolerance, and the stability of these proteins correlated well with the persistence of thermotolerance up to 36 hr. Sodium arsenite, hypoxia, and ethanol also induced both the synthesis of heat shock proteins and transient thermotolerance. A qualitative analysis of individual proteins suggests that the synthesis and persistence of polypeptides of *M_r* 70,000 or 87,000 most closely conformed to the kinetics of thermotolerance.

In the past few years, the induction or enhanced synthesis of a family of proteins in response to heat, other environmental stresses, and various chemical and mechanical treatments has been reported in cells from yeast to mammals (1–6). The function of these “heat shock” proteins is not well understood, but they may be essential to cell survival after certain kinds of environmental stress (3, 7, 8). Heat shock affects both transcription and translation in cells (7).

Mammalian cells exposed to a nonlethal heat treatment have been shown to acquire a transient resistance to subsequent heat challenge, as determined by an increase in cell survival. This phenomenon has been termed thermotolerance (9–11). The biophysical or biochemical basis of thermotolerance is not known. Enhanced synthesis of heat shock proteins has been associated with a transient, heat-induced thermal resistance in *Drosophila* and yeast (12, 13), but this phenomenon has not been well characterized.

To determine whether the development of thermotolerance is related to the synthesis of a set of specific proteins, such as heat shock proteins, we examined the effects of heat treatment (41–46°C) on protein synthesis in plateau-phase Chinese hamster (HA-1) fibroblasts. In parallel experiments, we determined the kinetics of the induction of thermotolerance by cell survival assay.

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MATERIALS AND METHODS

Cells and Culture Conditions. Plateau-phase cultures of Chinese hamster ovary fibroblasts, designated HA-1 (14), were obtained by plating 10⁴ cells per cm² in 35- or 60-mm Petri dishes. Beginning on day 4 after plating, the medium (Eagle’s minimal essential medium supplemented with 15% fetal bovine serum) was changed daily. Experiments were performed on day 2 of plateau phase (cell density, ≈ 1.2 – 1.5×10^6 cells per cm²). After each treatment, cells were trypsinized, counted with a Coulter Counter, and plated after appropriate dilutions were made. After 10 days of incubation at 37°C, colonies were stained and counted. Dishes containing 100–200 colonies were used for calculation of survival whenever possible. Trypsinization after heating had little effect on survival. Plating efficiency was 60–80%.

Heating. Thermotolerance can be induced in Chinese hamster ovary cells either by a short 45°C heat treatment, followed by an incubation period at near-physiologic temperatures, or by a prolonged incubation at <43°C (10). Heating of monolayers of cells by the protocols described in *Results* was carried out in hot waterbaths in incubators (15). The pH of the medium overlaying the cells was maintained at 7.2–7.4 by a regulated gas flow of a mixture of air and CO₂ and was monitored immediately before and after heating. The temperature of the waterbath was controlled to within 0.1°C.

Sodium Arsenite, Ethanol, and Hypoxia Treatments. Cells were treated with 50 μ M sodium arsenite in minimal essential medium with 15% fetal bovine serum at 37°C for 1 hr, rinsed, and incubated at 37°C for 6 hr before a 45°C heat challenge or labeling. Cells were treated with 6% (vol/vol) ethanol (16) for 1 hr at 37°C, rinsed, and incubated at 37°C for 5 hr before labeling. Cells were made hypoxic at 37°C for 8 hr (17) and then were reaerated for 6 hr at 37°C before labeling.

Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis. Cells were labeled with 10–20 μ Ci (1 Ci = 3.7×10^{10} becquerels) of [³⁵S]methionine in methionine-free minimal essential medium (specific activity, >1,000 Ci/mmol) per ml for 1 hr at 37°C. At the end of the labeling period, the medium was removed, cells were washed three times with cold phosphate-buffered saline, and sample buffer was added directly to the cells to give a final concentration of 10⁶ cells per ml. The sample buffer contained 1% 2-mercaptoethanol, 0.1% bromophenol blue, 50% (vol/vol) glycerol, 2% sodium dodecyl sulfate, and 25% (vol/vol) stacking gel buffer (0.5 M Tris-HCl, pH 6.8). The extracts were boiled for 3–5 min, and then equal amounts of proteins were loaded directly onto the polyacrylamide slab (5–15% gradient) at 20°C and electrophoresed (18). *M_r* markers—[¹⁴C]methylated filamin (240,000), myosin (200,000), ma-

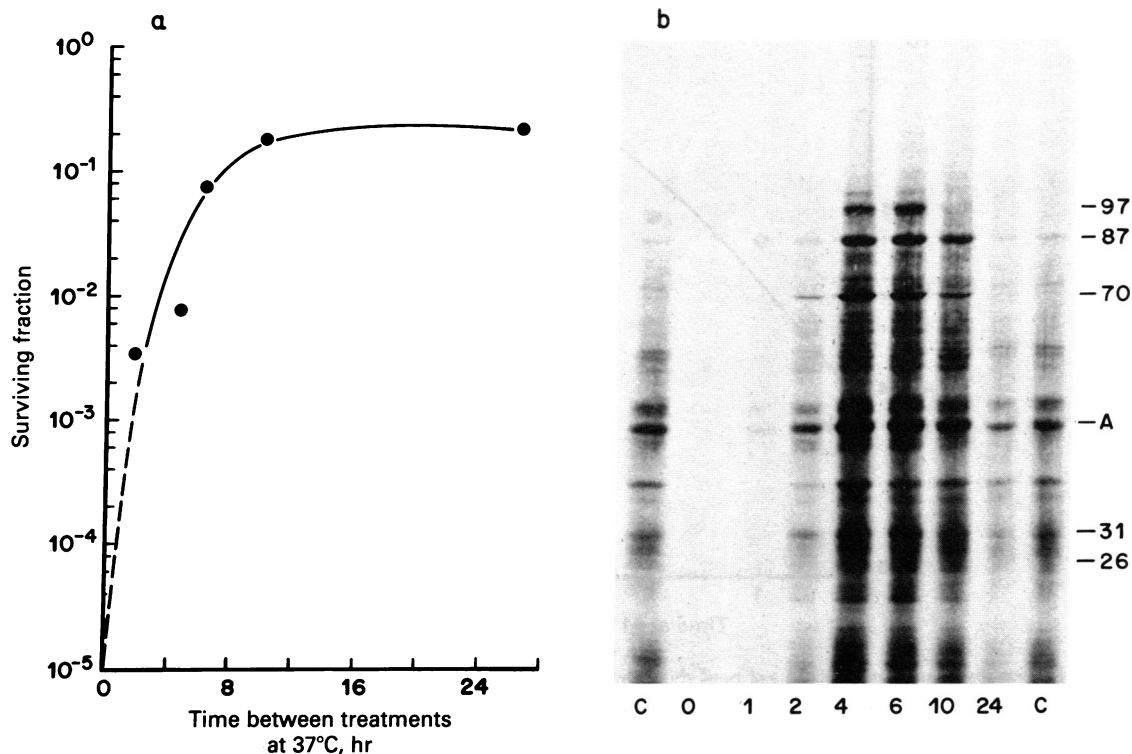


FIG. 1. Induced thermotolerance and synthesis of heat shock proteins in plateau-phase HA-1 cells exposed to 46°C for 6 min. After heat treatment, cells were incubated at 37°C for 0–24 hr before a second treatment at 45°C for 45 min or before labeling with [³⁵S]methionine. (a) Cell survival plotted as a function of time after the initial treatment (●—●). Treatment at 46°C for 6 min followed immediately by 45 min at 45°C yielded a survival of $<1 \times 10^{-5}$ (—). (b) Autoradiogram of a NaDodSO₄/polyacrylamide slab gel of ³⁵S-labeled protein from 46°C heat-shocked cells. Lanes: C, unheated control; 0–24, time (hr) of incubation before labeling. M_r s of proteins are shown $\times 10^{-3}$. A, actin (M_r , 43,000).

croglobulin (185,000), immunoglobulin G [159,000 (subunits, 50,000 and 30,000)], phosphorylase B (93,000), bovine serum albumin (69,000), carbonic anhydrase (30,000), lactoglobulin (18,400), and cytochrome *c* (12,300)—comigrated on the gel. For most of the initial studies, [³⁵S]methionine-labeled proteins from heat-shocked salivary glands of *Drosophila* (a gift of H. Mitchell and N. Petersen, California Institute of Technology) also comigrated on the gel for comparison.

RESULTS

Induction of Thermotolerance and Heat Shock Protein Synthesis After Initial Treatment at 46°C or 41°C. To examine induction of thermotolerance, we chose two nonlethal heating conditions: 41°C, at which total cell protein synthesis is unaffected, and 46°C for 6 min, at which cell protein synthesis is decreased by >95% after the heat treatment. Plateau-phase HA-1 cells were heated at 46°C for 6 min and incubated at 37°C for 0–24 hr before a second treatment at 45°C for 45 min, and then survival was assayed. Control cells were exposed to only one treatment at 45°C for 45 min, and the kinetics of thermotolerance development was measured by increases in survival above the control value ($\approx 8 \times 10^{-5}$) (Fig. 1a). Thermotolerance was fully developed by 4–6 hr of incubation at 37°C.

In parallel experiments, the effects of 46°C treatment on protein synthesis were examined. After 6 min of heating at 46°C, cells were incubated at 37°C for 0–24 hr before labeling with [³⁵S]methionine for 1 hr at 37°C. Protein synthesis was drastically inhibited by the heat treatment but recovered gradually during the 24-hr incubation period at 37°C, as seen by a greater incorporation of label at later time periods (Fig. 1b). Because amino acid transport remained unaltered by heat treatment (19), it is unlikely that the apparent decreases in protein

synthesis were due to changes in size of the amino acid pool. When the proteins synthesized after heat shock were compared with those synthesized by unheated cells, the synthesis of certain proteins was greatly enhanced. At 6 hr after heating, three proteins with apparent M_r s of 70,000, 87,000, and 97,000 were synthesized in greater amounts than in control cells. Other, less prominent proteins (M_r s of 59,000, 31,000, and 26,000) were also synthesized. The individual polypeptides had qualitatively different kinetics of synthesis. The rates of synthesis of the M_r 70,000 and 97,000 proteins reached maximum values 4–6 hr after heat shock and decreased to the control value by 8–10 hr. In contrast, synthesis of the M_r 87,000 protein was still appreciably enhanced by 10 hr after heating and returned to the control rate by 24 hr.

For the 41°C initial treatment, cells were exposed for 1–4 hr. One group of cells was immediately challenged by a second treatment at 45°C for 45 min, and cell survival was assayed. The rest of the cells were pulse-labeled immediately with [³⁵S]methionine for 1 hr at 37°C and protein synthesis was examined (Fig. 2). Thermotolerance was near its maximum by the end of each 41°C treatment. Incubation at 37°C between the two heat challenges did not result in an appreciable increase in survival (data not shown). Incubation at 41°C did not suppress total protein synthesis, but the synthesis of two proteins of M_r 70,000 and 87,000 was greatly enhanced in heated cells compared to unheated controls.

Delay in the Onset of Thermotolerance and Heat Shock Protein Synthesis. Li *et al.* (20) reported that a short 45°C treatment induced a subsequent delay in the onset of thermotolerance, as demonstrated by the shape of the two-fraction survival curve. To test the correlation between the onset of thermotolerance and the synthesis of heat shock proteins, we examined cell survival after a 45°C heat shock of 5 or 20 min

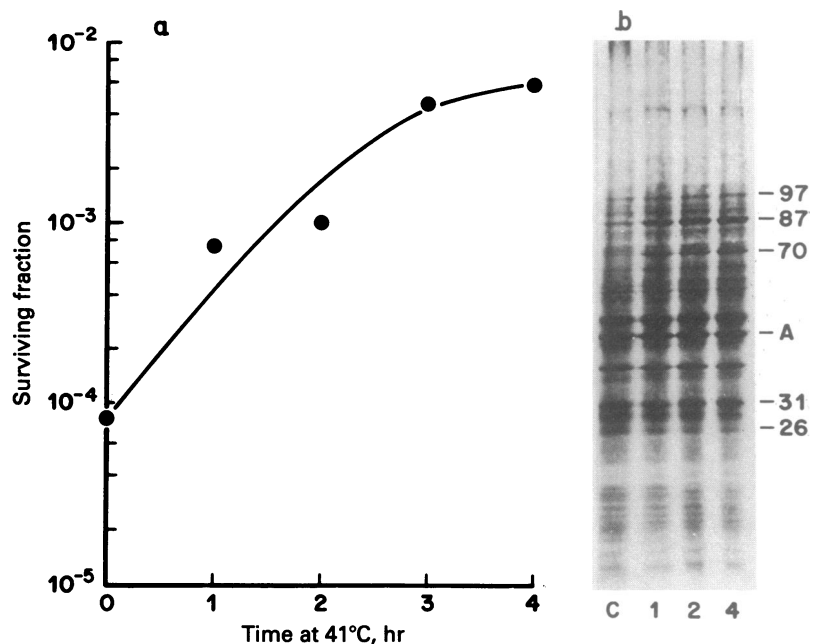


FIG. 2. Induced thermotolerance and synthesis of heat shock proteins in plateau-phase HA-1 cells exposed to 41°C for 1–4 hr. After heat shock, cells were immediately challenged by a second treatment at 45°C for 45 min or labeled with [³⁵S]methionine. (a) Cell survival plotted as a function of length of the initial treatment. (b) Autoradiogram of a NaDodSO₄/polyacrylamide slab gel of ³⁵S-labeled protein from 41°C heat-shocked cells. Lanes: C, unheated control; 1–4, time (hr) of exposure to 41°C before labeling. A, actin (*M_r*, 43,000). *M_s*s are shown × 10⁻³.

followed by 0–4 hr of incubation at 37°C and then a second treatment at 45°C for 40 or 25 min, for a total heating time of 45 min (Fig. 3). The induction of thermotolerance and the synthesis of heat shock proteins depended on the duration of the initial 45°C treatment. A 20-min initial exposure at 45°C introduced a subsequent delay of ≈2 hr in the onset of thermotolerance, as shown by the lag in the increase in survival values compared with a 5-min heat shock. Protein synthesis also was suppressed more drastically during the 2-hr interval after the 20-min heat shock than after the 5-min treatment. As protein synthesis recovered, the rate of synthesis of the *M_r* 70,000, 87,000, and

97,000 proteins was significantly enhanced over that of unheated cells. The rate of synthesis of the *M_r* 97,000 protein was temperature dependent; after the 45°C treatment, it was relatively lower than after the 46°C treatment, when compared at the same survival level (Figs. 1 and 3), and this change was progressive between 41°C and 47°C (data not shown).

Persistence of Thermotolerance and Heat Shock Protein Synthesis. Monolayers of cells were first exposed to 45°C for 20 min and then incubated at 37°C for 6 hr. The 6-hr incubation was chosen because thermotolerance was then close to its maximum value. Cells were then labeled with [³⁵S]methionine for

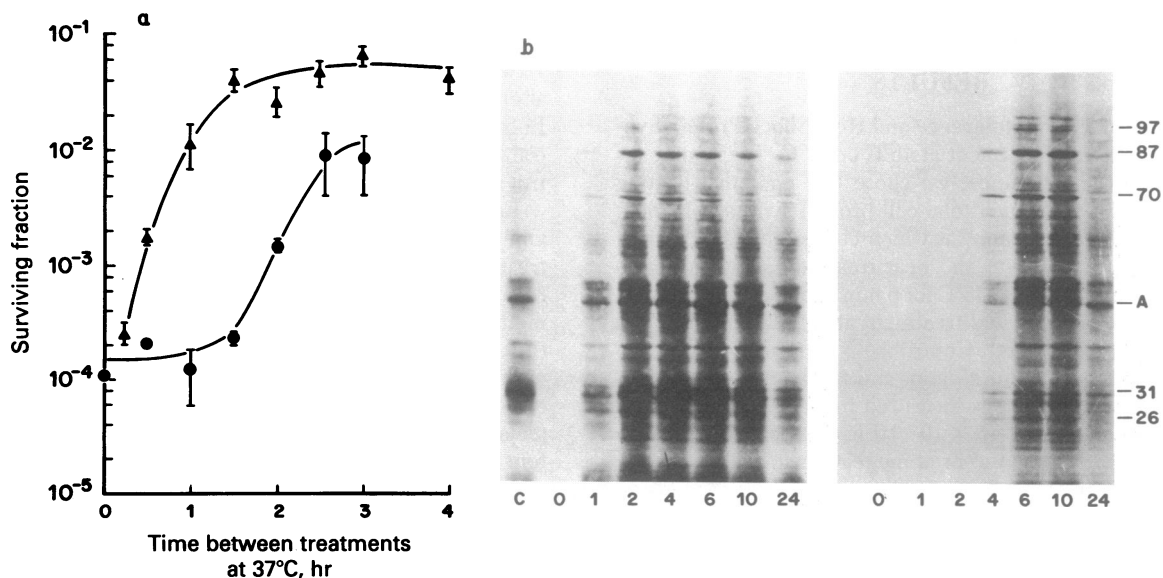


FIG. 3. Onset of thermotolerance and the synthesis of heat shock proteins in plateau-phase HA-1 cells. After heat shock (45°C for 5 or 20 min), cells were incubated at 37°C for 0–4 hr before a second treatment at 45°C for 40 or 25 min, for a total heating time of 45 min. (a) Cell survival plotted as a function of time between initial heat shock and second heat treatment. ▲, Five-minute initial treatment; ●, 20-min initial treatment. (b) Autoradiogram of a NaDodSO₄/polyacrylamide slab gel of ³⁵S-labeled protein from cells heat-shocked at 45°C for 5 min (Left) and 20 min (Right). Lanes: C, unheated control; 0–24, time (hr) of incubation before labeling. A, actin (*M_r*, 43,000). *M_s*s are shown × 10⁻³.

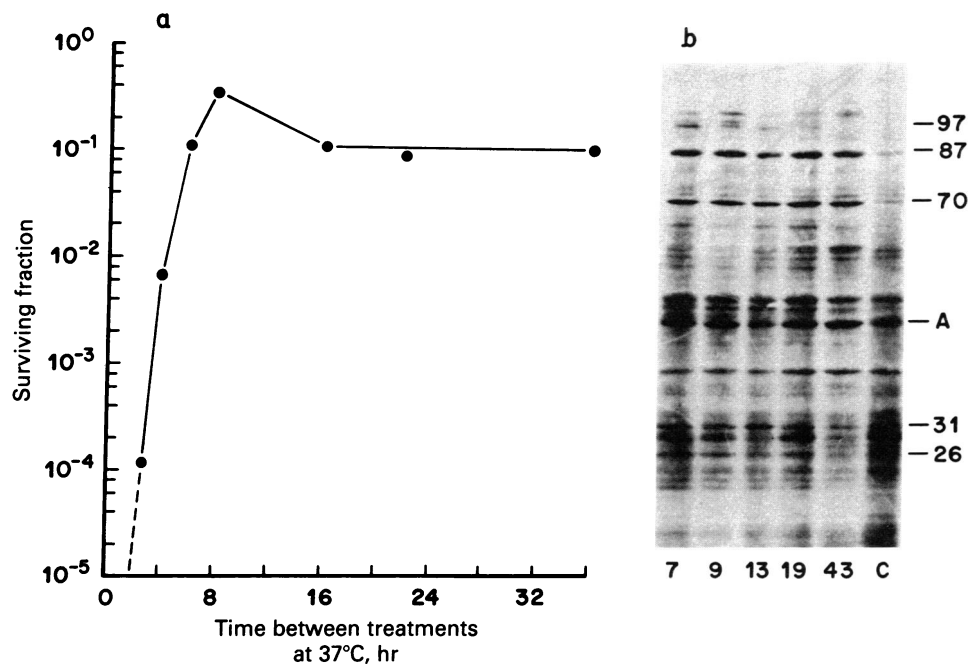


FIG. 4. Persistence of thermotolerance and synthesis of heat shock proteins in plateau-phase HA-1 cells. After heat shock (45°C for 20 min), cells were incubated at 37°C for 0–36 hr before a second heat treatment (45°C for 45 min), and then the survival was assayed. To study the persistence of heat shock proteins, cells were incubated after heat shock at 37°C for 6 hr, labeled with [³⁵S]methionine for 1 hr, and incubated at 37°C for an additional 0–36 hr. (a) Cell survival plotted as a function of time after the initial heat shock (●—●). Cell survival of <10⁻⁵ after initial heat shock at 45°C for 20 min followed immediately by a second treatment at 45°C for 45 min (---). (b) Autoradiogram of a NaDodSO₄/polyacrylamide slab gel of ³⁵S-labeled proteins from heat-shocked cells. Lanes: 7–36, total time (hr) of incubation at 37°C after heat shock; C, unheated control. A, actin (M_r, 43,000). M_rs are shown × 10⁻³.

1 hr at 37°C and incubated at 37°C for 0–36 hr. The rate of decay of thermotolerance was much slower than the rate of induction (Fig. 4). After 36 hr, cell survival decreased only from 30% to 10%. These survival results correlate well with the persistence of the heat shock proteins synthesized between hours 6 and 7 after heating. Up to 36 hr after pulse-labeling, no significant decay of the M_r 70,000 and 87,000 proteins was detected. In contrast, the M_r 97,000, 31,000, and 26,000 proteins decayed more rapidly.

Induction of Thermotolerance and Heat Shock Protein Synthesis by Agents Other than Heat. In various biological systems, other agents, such as sodium arsenite, induce some proteins similar to those induced by heat (6, 7). It was our interest to test whether those agents also induced thermotolerance and synthesis of heat shock proteins. We compared exponentially growing HA-1 cells exposed to 45°C for 15 min with cells treated with sodium arsenite (Fig. 5). After an initial exposure to either heat shock or sodium arsenite, the cells acquired a tolerance to the subsequent heat challenge, as evidenced by the increase in survival values. Six hours after either treatment, proteins of M_r 70,000, 87,000, and 97,000 were synthesized in greater amounts than in untreated cells. Ethanol and hypoxia, which also induce thermotolerance (refs. 16 and 21; unpublished data), were also examined for induction of heat shock protein synthesis (Fig. 5b), and the same results were observed.

DISCUSSION

Our data suggest that heat shock proteins may play a pivotal role in development of thermotolerance. Transcription is strongly affected by the heat shock response; it seems likely that heat shock proteins, through their association with the nuclear matrix (22), may protect the genome from the adverse effects of heat and other environmental stresses. Several lines of evidence

in plateau-phase HA-1 cells implicate heat shock proteins in the development of thermotolerance. (i) Heat treatment enhanced the synthesis of heat shock proteins and induced a transient thermotolerance at 41–46°C, under conditions in which the initial heat treatment either did not suppress total protein synthesis or drastically inhibited it. (ii) When thermotolerance was fully developed, the rate of synthesis of most heat shock proteins returned to control values. (iii) The delay of onset of thermotolerance correlated well with the delay in the induction or enhanced synthesis of heat shock proteins. (iv) There was good correlation between the persistence of some heat shock proteins and thermotolerance. (v) Agents known to induce thermotolerance induced synthesis of heat shock proteins, and agents known to induce synthesis of heat shock proteins induced thermotolerance. It seems reasonable to hypothesize that heat shock proteins may play a role in providing cells with an additional measure of heat resistance. However, because we do not know the precise functions of heat shock proteins, it is also possible that the effect of heat shock on RNA or protein synthesis may simply reflect the state of cells after heating.

The synthesis of each protein increased and decreased at specific times after the initial heat treatment, depending on the duration and temperature of the initial treatment (e.g., the rate of synthesis of the M_r 97,000 protein tended to be greatly enhanced after cells were exposed to higher temperatures and for longer times). When the proteins induced by heat shock, ethanol, sodium arsenite, or hypoxia were compared, the most prominent polypeptide induced by all agents was the M_r 70,000 protein. The rate of synthesis of the M_r 87,000 protein was enhanced by sodium arsenite, 41–46°C heat shock, and ethanol, but less so by hypoxia. The fact that synthesis of the M_r 97,000 protein was significantly enhanced only at a high heating temperature, longer heating time, or higher arsenite concentration implied that its synthesis might be related to the severity of the

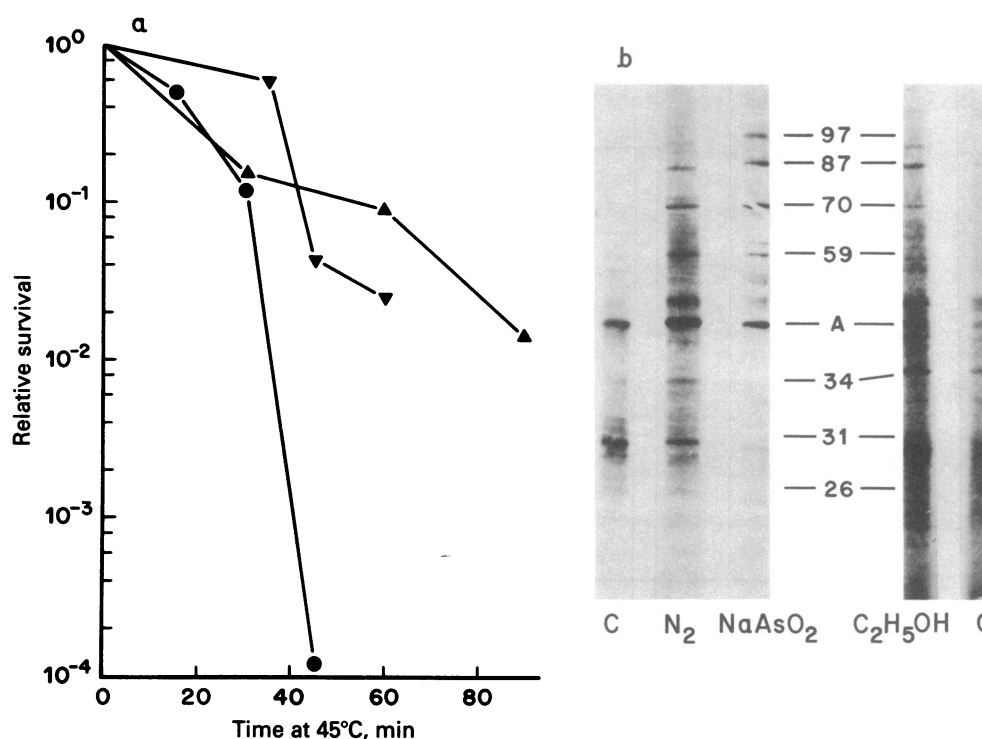


FIG. 5. Induced thermotolerance and synthesis of heat shock proteins by agents other than heat. (a) Cells were initially exposed to 45°C for 15 min or to 50 μ M sodium arsenite for 1 hr at 37°C and incubated at 37°C for 6 hr before a 45°C heat treatment. Relative survival of exponentially growing HA-1 cells plotted as a function of the length of the 45°C heat challenge. ▲, Initial heat treatment; ▼, sodium arsenite treatment; ●, control (no pretreatment). (b) Autoradiogram of a NaDodSO₄/polyacrylamide slab gel of ³⁵S-labeled proteins from cells treated with sodium arsenite, hypoxia or ethanol and incubated at 37°C for 5–6 hr before labeling. Lanes: C, control; N₂, 8-hr hypoxia; NaAsO₂, 50 μ M sodium arsenite; C₂H₅OH, 6% ethanol. A, actin (M_r, 43,000). M_rs are shown $\times 10^{-3}$.

external stresses. Thus, the proteins that correlated less well with development of thermotolerance may well have functions distinct from protecting heat-sensitive targets. Alternatively, they may represent a mosaic of events whose overall effect is thermotolerance. The mechanisms by which these proteins might protect cells from lethal heat treatments remain unknown. Correlation studies using endpoints other than survival might be powerful tools for the identification of the roles of individual proteins, although definitive cause/effect relationships between these proteins and survival await future studies.

The practical importance of understanding the function of these proteins in the induction of thermotolerance is emphasized by recent studies showing that thermotolerance can be induced in certain tumors and normal tissues (23), and that heat shock proteins can be induced in normal tissues (24) and mouse tumors (unpublished data).

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