APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1995, p. 979–984 Vol. 61, No. 3
0099-2240/95/$04.00+0

Close Correlation between Heat Shock Response and Cytotoxicity in *Neurospora crassa* Treated with Aliphatic Alcohols and Phenols

ULF MEYER, PAOLA SCHWEIM, FRANCO FRACELLA, AND LUDGER RENSING*

Institute of Cell Biology, Biochemistry and Biotechnology, University of Bremen, 28334 Bremen, Germany

Received 16 May 1994/Accepted 14 December 1994

In *Neurospora crassa* the aliphatic alcohols methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, isobutanol, ethylene glycol, glycerol, and allyl alcohol and the phenolic compounds phenol, hydroquinone, resorcinol, pyrogallol, phloroglucinol, sodium salicylate, and acetylsalicylic acid were analyzed with respect to their capacities to induce heat shock proteins (HSP) and to inhibit protein synthesis. Both the alcohols and phenols showed the greatest levels of HSP induction at concentrations which inhibited the overall protein synthesis by about 50%. The abilities of the different alcohols to induce the heat shock response are proportional to their lipophilicities: the lipophilic alcohol isobutanol is maximally inductive at about 0.6 M, whereas the least lipophilic alcohol, methanol, causes maximal induction at 5.7 M. The phenols, in general, show a higher capability to induce the heat shock response. The concentrations for maximal induction range between 25 mM (sodium salicylate) and 100 mM (resorcinol). Glycerol (4.1 M) shifted the concentration necessary for maximal HSP induction by hydroquinone from 50 to 200 mM. The results reveal that the induction of HSP occurs under conditions which considerably constrain cell metabolism. The heat shock response, therefore, does not represent a sensitive marker for toxicity tests but provides a good estimate for the extent of cell damage.

Ritossa (19) described the aromatic compound 2,4-dinitrophenol as the first chemical inducer to elicit the heat shock reaction in *Drosophila melanogaster*. A large number of other chemical inducers have since been identified, including alcohols, amino acid analogs, and heavy metals, whose cellular effects cause perturbations of protein folding (for a review, see reference 16). Thus, one may expect to find structure-activity relationships among certain classes of inducers affecting protein folding, such as alcohols or phenols. Their inducing capacities may depend on their lipophilicities which in turn have a direct or indirect effect on protein conformation (14). Such a structure-activity relationship has already been described for alcohol toxicity (22). If this were also the case in the heat shock response, heat shock protein (HSP) induction could serve as a marker for protein-targeted pharmacological and toxicological effects. Neuhaus-Steinmetz et al. (14) showed that in rat glioma cells a heat shock response is elicited by alcohol concentrations causing damage visible in about 30% of the cells as estimated by neutral red uptake.

The two main questions were at which concentrations might different aliphatic alcohols and phenols induce the synthesis of HSPs and whether this inducing capacity depends on their lipophilicities. Because the relationship between the extent of cell damage and the onset of the heat shock response is important for toxicity tests, we made a detailed analysis of this relationship in *Neurospora crassa*, which has a well-established heat shock response (4, 7, 18) and has already been studied with respect to ethanol (20).

* Corresponding author. Mailing address: Institute of Cell Biology, Biochemistry and Biotechnology, University of Bremen, D-28334 Bremen, Germany. Phone: 421-218 2126. Fax: 421-218 4042.

MATERIALS AND METHODS

Cell culture. The bdA strain (catalog no. 1858; Fungal Genetics Stock Center, Kansas City, Kans.) of *N. crassa* was used in all experiments. Conidia of *N. crassa* were grown in Vogel medium (23) for 36 h at 25°C in constant light to homogeneous mycelial mats. Mycelia were then transferred for 1 h to fresh Vogel medium, to which the various chemical substances were added.

Protein labelling with [^{35}S]methionine. After a treatment for 1 h with alcohol or phenol, the mycelium was washed and labelled with L-[^{35}S]methionine (4 μCi/ml; specific activity, 0.5 GBq/mM; Amersham) during a 1-h recovery period in fresh Vogel medium. In spite of the high stressor concentrations the 1-h treatments were not lethal as shown by subsequent growth measurements. The HSPs were strongly labelled when L-[^{35}S]methionine was added during recovery from the treatment, whereas almost no labelling was observed when it was added during the stress treatment itself. For heat shock treatment, the mycelium was transferred into a water bath (43°C) for 1 h and was labelled with L-[^{35}S]methionine for the last 45 min of heat exposure.

Sample preparation. After being washed and harvested, the mycelium was boiled for 5 min in Laemmli sample buffer (10), and the amount of trichloroacetic acid-precipitable radioactive proteins were determined in a scintillation counter (12).

Electrophoresis and fluorography. One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted as described by Laemmli (10). Lanes were loaded with equal amounts of radioactivity. Two-dimensional gel electrophoresis was conducted essentially as described by O'Farrell (17), with 10% polyacrylamide gels in the second dimension. Fluorography of the gels was carried out according to the procedure described by Bonner and Laskey (1).

Quantitative analysis. One-dimensional fluorographs were digitalized by using a video documentation and evaluation program (Intas, Göttingen, Germany) to quantitate the relative blackening grade of the protein bands.

The protein concentration was determined as described by Neuhoff et al. (15).

Antibody preparation. Rabbits were immunized with primary injections of 30 μg of dialyzed and lyophilized HSP70 isolated from *N. crassa* (4) in 0.25 ml of phosphate-buffered saline (PBS; pH 7.5) in a 1:1 mixture with Freund's complete adjuvant (Calbiochem) distributed to two subcutaneous sites on the back. Booster injections of 15 μg of HSP70 were given at 14-day intervals after primary injection in an identical manner except that incomplete Freund's adjuvant (Calbiochem) was used. For affinity purification the antiserum was applied to columns with immobilized HSP70. Specific antibodies were eluted with 0.1 M glycine (pH 2.7) and were neutralized and diluted (1:1,000) with 0.2% Tween 20 containing PBS (PBST).

Western blot (immunoblot) analysis. For Western blotting, the proteins were separated by one-dimensional SDS–10% PAGE and transferred to a nitrocellu-

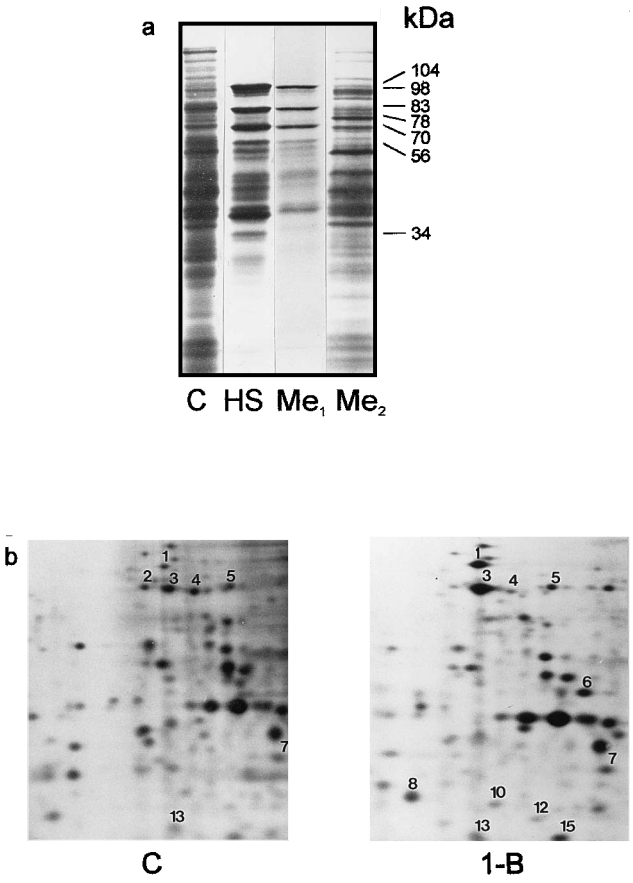

FIG. 1. Effect of alcohols on HSP induction. (a) Methanol treatment as analyzed by one-dimensional PAGE. Shown is a fluorogram of [^{35}S]methionine-labelled proteins. C, untreated control; HS, heat shock (1 h at 43°C); Me_1 and Me_2, methanol treatment (8.2 and 4.7 M, respectively, for 1 h, followed by 1 h of recovery). (b) 1-Butanol treatment as analyzed by two-dimensional PAGE. C, untreated control; 1-B, 1-butanol treatment (0.3 M for 1 h, followed by 1 h of recovery).

lose membrane (BA83; Schleicher & Schuell) with a Trans Blot Cell (Bio-Rad) (21). The blots were blocked with PBST for 1 h at 25°C. After removal of the blocking solution, the primary antibody (affinity-purified polyclonal rabbit anti-HSP70 against the major *Neurospora* HSP70 [4]) diluted in PBST was added and left for 1 h at 25°C. Subsequently, the blots were washed with PBST. The secondary antibody (goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate; Bio-Rad) diluted in PBST (1:5,000) was added, and after 1 h at 25°C the blots were washed with PBST. The immunocomplex was detected with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Boehringer, Mannheim, Germany).

All experimental treatments with the alcohols and phenols were reproduced two to five times and revealed similar results.

RESULTS

Aliphatic alcohols. The saturated monohydroxy alcohols induced high-molecular-mass HSPs of 104, 98, 83, and 70 to 72 kDa (Fig. 1a). These HSPs are identical with the HSPs induced during heat shock. After treatment with the saturated alcohols the low-molecular-mass HSPs, i.e., those of 43, 34, and 24 kDa, were not detectable on one-dimensional gels. Allyl alcohol induced the high-molecular-mass HSPs as well as HSPs of lower molecular mass at concentrations around 10 mM. Concentrations of ethylene glycol up to 4.4 M and glycerol up to 4.1 M did not induce the heat shock response or influence the general protein pattern (data not shown). The 40- through 42-kDa band—consisting mainly of actin—appears to be induced or maintained at almost the same synthesis rate for all treatments.

In addition to the usual high-molecular-mass HSPs, 4.7 M methanol induced a protein with an apparent molecular mass of about 78 kDa (Fig. 1a). This protein is not synthesized under normal or heat shock conditions.

Two-dimensional gel electrophoresis (Fig. 1b) revealed that the major HSP of the HSP70 family (spot 3) is also strongly induced after alcohol treatment. In the case of spots 2 and 4, a decline in synthesis due to 2-butanol treatment was apparent, whereas the synthesis of spot 5 in comparison to the synthesis of the control was nearly constant. An increase in the synthesis rate is also readily seen in the 83-kDa HSP (spot 1). A new protein in the 45-kDa range is induced after 2-butanol treatment (spot 6). Spot 7 appears to be slightly induced and may correspond to HSP34. Spots 8 through 15 are also induced but less strongly than by the phenolic compounds (see Fig. 3b). These results are similar to those obtained after exposure to methanol, ethanol, and 1-propanol (data not shown). Spots 10, 12, and 15 may be present in the controls at low intensities (Fig. 1).

Alcohol concentration dependency. HSP induction by methanol required the highest concentrations (4 to 8 M), whereas the different butanols induced HSP synthesis at concentrations between 0.2 and 0.7 M (Fig. 2a). This concentration-activity relationship is paralleled by a lipophilicity-activity relationship, as determined on the basis of the partition coefficient of octanol/water as a measure of alcohol lipophilicity (Fig. 2c). In a double logarithmic plot the alcohol concentrations necessary to induce maximal HSP synthesis decrease with increasing octanol/water partition coefficients. The straight line drawn through the datum points (Fig. 2c) reveals a strong correlation ($r = 0.97$). It is noteworthy that the concentration range of induction is also a function of alcohol structure: the higher the inducing capacity, the smaller the range. This means that lipophilic alcohols reach toxic concentrations within a shorter range than the less lipophilic alcohols.

The effects of different alcohols on total protein synthesis were determined by following the same protocol. Dependent again on their lipophilicities, the alcohols showed a structure-activity relationship (Fig. 2b): with methanol, 50% inhibition of protein synthesis occurred at a concentration of about 5 M, whereas with 1-butanol and isobutanol, 50% inhibition was already reached at about 0.35 M (a 50% inhibition of total protein synthesis was also achieved by a 1-h heat shock treatment at 43°C). Allyl alcohol was the only unsaturated alcohol tested, and it showed a 50% inhibition of protein synthesis at a concentration of less than 0.01 M (data not shown). The double logarithmic plot of the alcohol concentrations leading to 50% protein synthesis inhibition versus the octanol/water partition coefficients reveals a strong correlation ($r = 0.94$) similar to that in HSP synthesis induction (Fig. 2c).

Phenolic compounds. Except for phloroglucinol and acetylsalicylic acid, all phenolic compounds induced the synthesis of HSPs similarly to aliphatic alcohols and heat shock (Fig. 3a). In contrast to the aliphatic alcohols tested, most of the phenols (phenol, hydroquinone, resorcinol, and pyrogallol) showed a strong induction of the low-molecular-mass HSPs of 34 and 24 kDa. Also, a protein of 45 kDa and four proteins in the 25.5- to 29.5-kDa range were induced. Sodium salicylate, in constrast, induced the high-molecular-mass HSPs only.

A two-dimensional analysis of the effects of hydroquinone on protein synthesis revealed results in a high-molecular-mass range comparable to that of aliphatic alcohols (Fig. 3b). Readily visible is the induction of a 70- and an 83-kDa HSP (spots 3 and 1), while spot 2 showed no change and spot 5

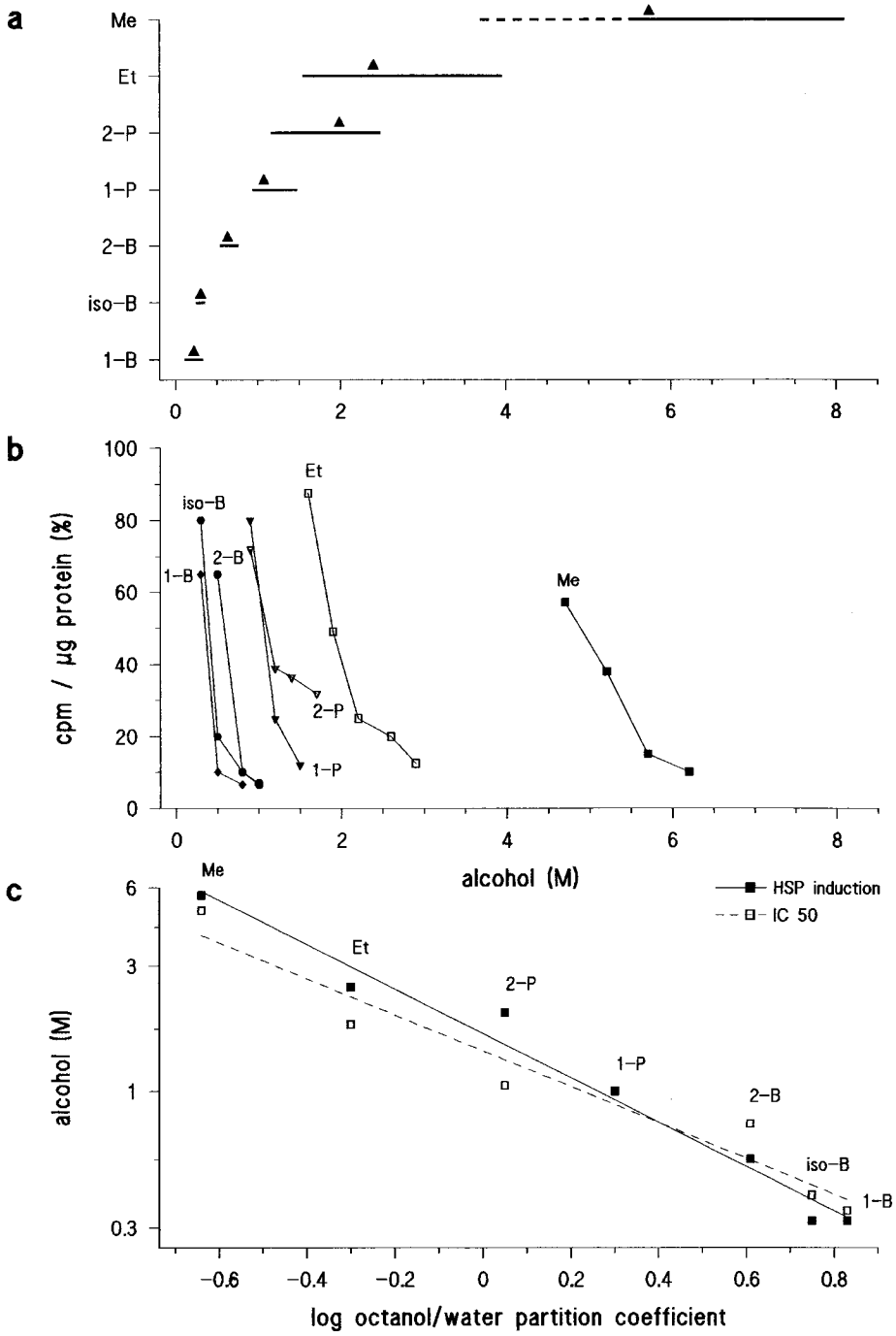

FIG. 2. Induction of HSP70 and inhibition of total protein synthesis by exposure to different alcohols. (a) Concentration (molar) range (horizontal lines) at which HSP70 is induced above control levels (triangles show maximum induction). The dotted line indicates the concentration range within which methanol induced a 78-kDa protein. HSP98 is induced within the range indicated by the dotted and solid line. Alcohols (Me, methanol; Et, ethanol; 2-P, 2-propanol; 1-P, 1-propanol; 2-B, 2-butanol; iso-B, isobutanol; 1-B, 1-butanol) are on the ordinate. (b) Protein synthesis inhibition. Abbreviations of alcohols are the same as for panel a. (c) Correlation between concentration (logarithmic scale on the ordinate) necessary to induce maximal HSP70 synthesis or to inhibit total protein synthesis by 50% (IC 50) and the logarithm of the octanol/water partition coefficients of the different alcohols (taken from reference 6).

showed a decline in its synthesis rate. Hydroquinone treatment elicited one new spot at 45 kDa (spot 6). As already indicated by one-dimensional SDS-PAGE, several changes in protein synthesis in the low-molecular-mass range were observed. Spot 7 possibly corresponds to HSP34, which seems to be present only in small amounts in the control. Spots 8 through 12 seem to correspond to the protein bands in the 25.5- to 29.5-kDa range seen in the one-dimensional SDS-PAGE (Fig. 3a). At least three spots (spots 13 to 15) are visible with an apparent mass of 24 kDa but with different isoelectric points.

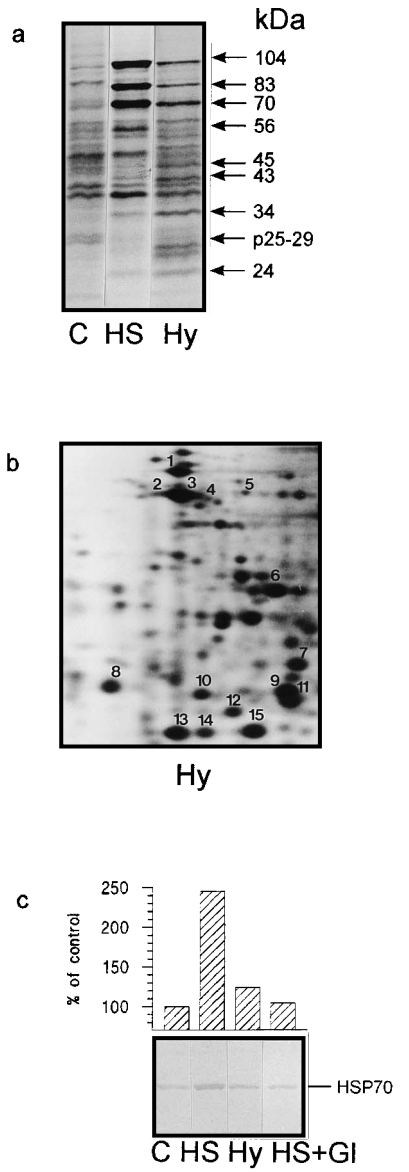

FIG. 3. Effect of hydroquinone treatment on protein synthesis. (a) One-dimensional analysis. C, untreated control; HS, heat shock treatment (1 h at 43°C); Hy, hydroquinone treatment (50 mM for 1 h, followed by 1 h of recovery). (b) Two-dimensional analysis of the [^{35}S]methionine-labelled proteins. Hy, hydroquinone treatment (50 mM for 1 h, followed by 1 h of recovery). (c) Effects of different treatments on the level of HSP70 as revealed by Western blot analysis. C, untreated control; HS, heat shock (1 h at 43°C); Hy, hydroquinone treatment (50 mM for 1 h, followed by 1 h of recovery); HS+GL, heat shock (1 h at 43°C) in the presence of 4.1 M glycerol.

Further examined were the effects of hydroquinone on the amount of HSP70 by means of Western blotting. The results corroborate the findings revealed by fluorography (Fig. 3c).

Phenol concentration dependency. The lowest concentrations of the phenols necessary for the induction of high-molecular-mass HSPs range between 20 and 90 mM (Fig. 4a). Salicylate is the most effective inducer among the phenolic compounds, while resorcinol is the least. Both cover only a small inductive concentration range (window), whereas pyrogallol and hydroquinone show a somewhat larger range.

The slopes of the curves showing the inhibitory effects on protein synthesis differ among the phenolic compounds; the steepest decrease in the synthesis rate is observed with salicylate and phenol, which are also the strongest HSP-inducing compounds. At higher concentrations, at which protein synthesis is inhibited by about 80 to 90%, HSP synthesis is also inhibited.

Normally, hydroquinone induced HSPs maximally at 50 mM. In the presence of 4.1 M (30%) glycerol this concentration is shifted to 200 mM. Surprisingly, glycerol had no inhibiting effect on the synthesis of the low-molecular-mass HSP34 and HSP24, which were heavily labelled even at 50 and 100 mM.

Phloroglucinol, at concentrations between 20 and 80 mM, and acetylsalicylic acid, at 10 mM, had no inhibitory effect on protein synthesis, nor did they have an inducing effect on HSP synthesis.

DISCUSSION

Roychowdhury and Kapoor (20) examined the effect of ethanol on *N. crassa* but only up to a concentration of 1%. No typical heat shock response was observed at this concentration. Only HSP80 and HSP34 were synthesized at higher rates. In our experiments, a heat shock response with ethanol as the stressor was observed when cells were exposed to about 1.9 M (11%). Roychowdhury and Kapoor described a protein, which they called alc 80. This protein was induced only when the mycelium was subjected to two simultaneously applied treatments: treatment with either ethanol, acetate, lactate, or low sucrose concentrations and treatment with heat. We found a protein within the range of molecular mass of alc 80 when the mycelium was shocked with methanol or 1-butanol without additional heat treatment. In *Saccharomyces cerevisiae*, aliphatic alcohols were found to induce lower-molecular-weight HSPs to a higher extent than heat at concentrations similar to those applied in *N. crassa* (5). Even in mammalian cells the HSP-inducing concentrations of alcohols are surprisingly high (14).

As mentioned above, the octanol/water partition coefficients for the alcohols is strongly correlated with their HSP-inducing capacities. This relationship is not as evident in the phenolic compounds. Hydroquinone, for example, shows a slightly lower octanol/water partition coefficient than resorcinol (0.64 and 0.8, respectively). However, hydroquinone is more toxic and induces HSP synthesis at lower concentrations. This is perhaps due to the fact that the electrophilic quinones react with proteins. Diphenols are easily converted to their corresponding quinones by enzymes like laccase, tyrosinase, and polyphenol oxidase (2) and can act as quinoid-type, Michael-type acceptors binding to nucleophilic moieties like sulfhydryl groups (11). Such electrophilic substances are always more toxic than one would predict by the corresponding baseline narcosis model (11). This may be the reason why allyl alcohol showed a higher toxicity and inducing capacity than predicted by this model. Allyl alcohol is a proelectrophilic compound and can be converted to its corresponding aldehyde acrolein, which is electrophilic and thus able to react like quinones (11). In contrast to the aliphatic alcohols, the phenol derivatives showed strong induction of the low-molecular-mass HSP34 and HSP24 together with the high-molecular-mass HSPs.

Evidence that protein misfolding occurs during exposure to alcohols or phenols comes from the experiments in which 4 M glycerol given together with heat shock or hydroquinone inhibits the heat shock response. Glycerol is known to be a "compatible solute," belonging to a class of substances which stabilizes the structure of macromolecules (for a review, see reference 9). Glycerol suppresses the aggregation of proteins

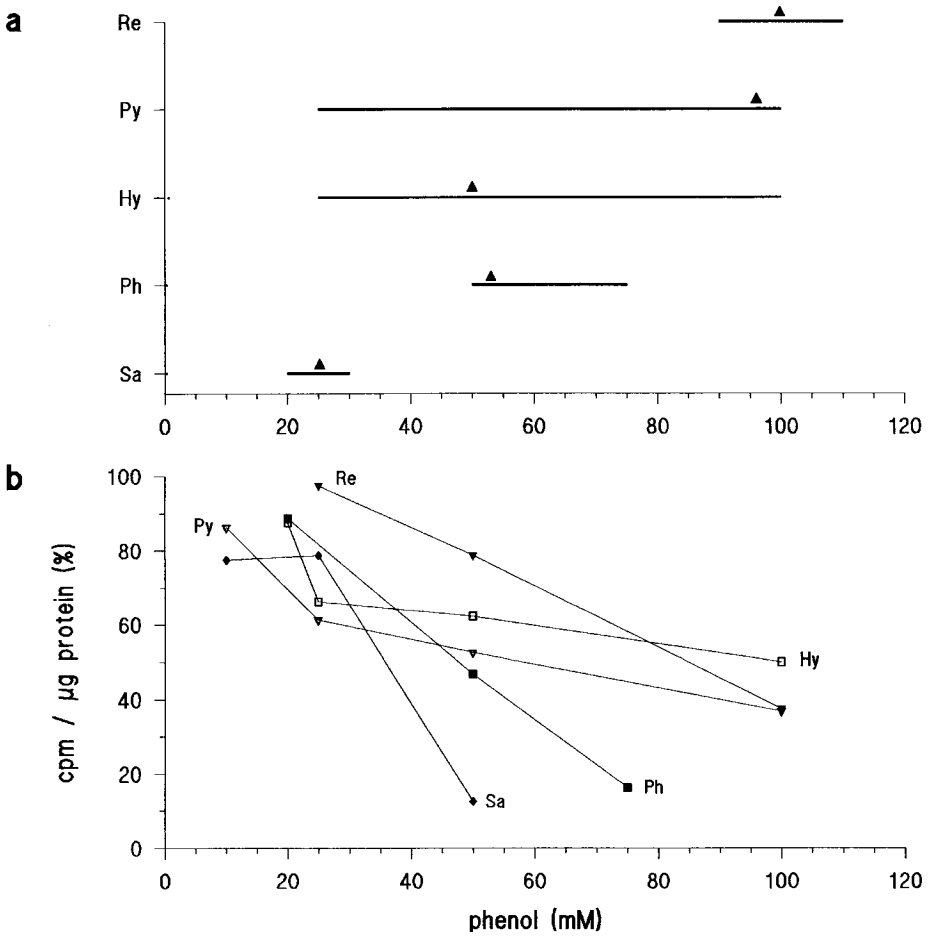

FIG. 4. Induction of HSP70 and inhibition of total protein synthesis by 1 h of exposure to different phenolic compounds. (a) Concentration range (horizontal lines) at which HSP70 is induced above control levels (triangles show maximum induction). Phenols (Re, resorcinol; Py, pyrogallol; Hy, hydroquinone; Phe, phenol; Sa, salicylate) are on the ordinate. (b) Protein synthesis inhibition in indicated percentage of untreated controls (ordinate). Abbreviations of phenols are the same as for panel a.

which occurs with heating (8). Glycerol thus prevents the immediate need for additional HSPs, which act to disaggregate proteins. These results are in agreement with the observation that 1 M glycerol inhibits the heat shock response of chicken embryo fibroblasts after heat shock (3). Moreover, Mosser et al. (13) described an in vitro inhibition of heat shock factor activation due to glycerol.

The reducing agent mercaptoethanol induces HSP34 (24). This effect is similar to that of some of the polyhydroxyphenols, which also have reducing properties. Phenol induces HSP34, although it lacks a reducing capacity. However, in microorganisms the metabolic pathway of phenol includes intermediates which have this property.

From the experimental results we conclude that the heat shock response—induced by different alcohols or heat shock—occurs under conditions that have considerable cytotoxic effects. Alcohols affect membranes, ion homeostasis, and protein conformation. Experiments with rat glioma cells, analyzing the effects of different alcohols on membrane properties as measured by a neutral red assay, further support this assumption (14). This membrane damage leads to drastic changes of the intracellular ion concentration, in particular to a pH decrease of up to 0.4 units in glioma cells (13a). Furthermore, the results described above reveal a rather small window in the range of concentrations of alcohols and phenols within which the heat shock response is triggered. This window is always observed at stressor concentrations leading to a 50% inhibition of protein synthesis, which may suggest a causal connection between the two processes in the sense that the extent of protein synthesis inhibition is interrelated with the heat shock factor-activating system. Higher alcohol concentrations lead to inhibition of the heat shock response and probably to irreversible changes and eventually to cell death. HSP induction is thus not a sensitive test for subtle pharmacological or toxicological effects but contributes to a more detailed estimation of the extent of cell damage on the level of protein synthesis inhibition and/or proteotoxicity.

ACKNOWLEDGMENT

We thank M. Vicker for critically reading the manuscript.

REFERENCES

1. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46:**83–88.
2. **Cain, R. B.** 1988. Aromatic metabolism by mycelial organisms: actinomycete and fungal strategies, p. 101–144. *In* S. R. Hagedorn, R. S. Hanson, and D. A. Kunz (ed.), Microbial metabolism and the carbon cycle. Harwood Academic Publishers, New York.
3. **Eddington, B. V., S. A. Whelan, and L. E. Hightower.** 1989. Inhibition of heat shock (stress) protein induction by deuterium oxide and glycerol. Additional

support for the abnormal protein hypothesis of induction. J. Cell. Physiol. **139:**219–228.
4. **Fracella, F., S. Mohsenzadeh, and L. Rensing.** 1993. Purification and partial amino acid sequence of the major 70,000 Dalton heat shock protein in *Neurospora crassa*. Exp. Mycol. **17:**362–367.
5. **Groepper, T., and L. Rensing.** 1993. Inhibitors of proteases and other stressors induce low molecular weight heat shock proteins in *Saccharomyces cerevisiae*. Exp. Mycol. **17:**46–54.
6. **Hansch, C., and A. Leo.** 1979. Substituent constants for correlation analysis in chemistry and biology. John Wiley & Sons, New York.
7. **Kapoor, M.** 1983. A study of the heat-shock response in *Neurospora crassa*. Int. J. Biochem. **15:**639–649.
8. **Kim, D., and Y. J. Lee.** 1993. Effect of glycerol on protein aggregation. Quantitation of thermal aggregation of proteins from CHO cells and analysis of aggregated proteins. J. Therm. Biol. **18:**41–48.
9. **Kirst, G. O.** 1990. Salinity tolerance of eukaryotic marine algae. Annu. Rev. Plant Physiol. Plant Mol. Biol. **41:**21–53.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
11. **Lipnick, R. L.** 1989. Narcosis electrophile and proteophile toxicity mechanisms. Application of SHR and QSAR. Environ. Toxicol. Chem. **8:**1–2.
12. **Mans, R. J., and G. P. Novelli.** 1961. Measurement of the incorporation of radioactive amino acids into protein by a filter-paper disk method. Arch. Biochem. Biophys. **94:**48–53.
13. **Mosser, D. D., P. T. Kotzbauer, K. D. Sarge, and R. I. Morimoto.** 1990. *In vitro* activation of heat shock transcription factor. DNA-binding by calcium and biochemical conditions that affect protein conformation. Proc. Natl. Acad. Sci. USA **87:**3748–3752.
13a. **Neuhaus-Steinmetz, U.** Unpublished results.
14. **Neuhaus-Steinmetz, U., C. Xu, F. Fracella, B. Oberheitmann, C. Richter-Landsberg, and L. Rensing.** 1994. Heat shock response and cytotoxicity in C6 rat glioma cells. Structure activity relationship of different alcohols. Mol. Pharmacol. **45:**36–41.
15. **Neuhoff, V., K. Philipp, H. G. Zimmer, and S. Mesecke.** 1979. A simple, versatile, sensitive and volume-independent method for quantitative protein determination which is independent of other external influences. Hoppe-Seyler's Z. Physiol. Chem. **360:**1657–1670.
16. **Nover, L.** 1991. Heat shock response. CRC Press, Boca Raton, Fla.
17. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250:**4007–4021.
18. **Plesofsky-Vig, N., and R. Brambl.** 1985. Heat shock response of *Neurospora crassa*: protein synthesis and induced thermotolerance. J. Bacteriol. **162:**1083–1091.
19. **Ritossa, F.** 1962. A new puffing pattern induced by heat shock and DNP in *Drosophila*. Experientia **18:**571–573.
20. **Roychowdhury, H. S., and M. Kapoor.** 1987. Ethanol and carbon source starvation enhance the accumulation of HSP80 in *Neurospora crassa*. Can. J. Microbiol. **34:**162–168.
21. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
22. **Veith, G. D., D. Y. Call, and L. T. Brooke.** 1983. Structure-activity relationship for the fathead minnow, *Pimephales promelas*: narcotic industrial chemicals. Can. J. Fish. Aquat. Sci. **40:**743–748.
23. **Vogel, H. J.** 1956. A convenient growth medium for *Neurospora* (medium N). Microb. Genet. Bull. **13:**42–43.
24. **Zoeger, D., C. Scholle, A. Schroeder-Lorenz, D. Techel, and L. Rensing.** 1992. Some starvation-induced proteins in *Neurospora crassa* are related to glucose-regulated proteins. Exp. Mycol. **16:**138–145.