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Changes in cytoplasmic pH upon heat shock in embryonic and adult rat liver ceils

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Abstract. All living cells, when exposed to elevated temperatures, undergo physiological changes which result in the expression of a specific set of heat shock proteins. Study of the possible physiological changes in adult and embryonic rat liver cells indicated a change in intracellular pH upon heat shock. Using 2', 7'-bis (2-carboxyethyl)-5 (and -6) carboxyfluorescein acetoxymethyl ester, we demonstrate here that the intracellular pH of adult and embryonic liver cells is different and that there is an increase in relative fluorescence intensity in both adult and embryonic cells upon heat shock, which corresponds to about 0·2 to 0·3 pH units. We also show that in addition to heat, some of the inducers of heat shock like response in many systems also induce a change in intracellular pH and induce heat shock proteins at 37°C in fetal liver cells. The possible mechanisms of induction of heat shock proteins during heat shock and in the presence of inducers at normal temperature are discussed.

Keywords. Heat shock; intracellular pH; rat liver.

1. Introduction

Living organisms develop different protective mechanisms at the cellular level to survive under minor stressful conditions. Several types of stress, including a sudden increase in temperature have been shown to cause biochemical as well as morphological changes in cells and tissues (Lindquist 1986; Welch and Suhan 1985; Schlesinger *et al* 1982; Welch 1988; Morimoto *et al* 1990). Specifically, heat shock response is characterized by a rapid, vigorous and transient increase in the rate of expression of a small number of specific genes encoding the heat shock proteins (hsp). This is associated with a sudden decrease in the synthesis of other mRNA and the proteins (Ashburner and Bonner 1979; Atkinson and Waldew 1985). Interestingly, not only heat, but also a variety of other agents have been shown to cause a heat shock like response in many cell types (Ritossa 1962; Nover 1984). These agents include uncouplers of respiratory chain, chelating agents, steroid hormones, ionophores, etc., which have an effect on cellular mitochondria and energy metabolism. During heat shock response, a specific heat shock transcription factor (HSF) present in the cells was shown to be activated and its binding to specific regions (heat shock elements, HSEs) in the promoters of the hsps brings about the transcription of these genes (Pelham 1982; Wu 1984a,b). HSF has been cloned from yeast (Sorger and Pelham 1987), *Drosophila* (Wu *et al* 1987), tomato (Scharf *et al* 1990), mouse (Serge *et al* 1991) and He La cells (Rabindran *et al* 1991; Schuetz *et al* 1991). The interaction of these factors with DNA prior to heat shock is different among the different species studied (Sorger 1991).

In any system a temperature change of 5–8 degrees by itself is unlikely to alter

Abbreviations used: hsp, Heat shock protein; HSF, heat shock transcription factor; HSE, heat shock element; pH (i), intracellular pH; BCECF, 2', 7'-bis (2-carboxyethyl)-5 (and 6) carboxyfluorescein; AM, acetoxymethyl ester; DMEM, Dulbecco's modified Eagle's medium; 2,4 DNP, 2,4-dinitrophenol.

gene expression. Temperature is one of the parameters that regulates the ionization of solutions. In a living system, it is possible that a temperature increase of this magnitude may bring a physiological change leading to change in internal pH [pH (i)]. Also, a change in gene expression associated with change in pH(i) has been reported in many systems (Johnson *et al* 1976; Webb and Nuccitelli 1981; Ober and Pardee 1987; Dufresne *et al* 1988). Weitzel *et al* (1987) have shown a close correlation between change in cytoplasmic pH and induction of hsp 70. We have been studying the effect of heat on embryonic liver cells (Srinivas *et al* 1987) and the physiological changes that cells undergo during heat shock. During the course of our studies we have shown that heat shock causes prematurely enhanced expression of albumin and a specific degradation of alpha-feto protein mRNA. Not only heat but also Amiloride, an inhibitor of Na^+/H^+ exchange across the membrane (Grinstein 1987) caused a similar change in gene expression in embryonic liver cells at 37° C (unpublished observations).

Several techniques are now available to measure pH (i) of cells. These include the equilibrium distribution of a radioactively labelled weak acid (like [^{14}C] 5,5-dimethyl-2,4-oxazolidine dione (Roos and Boron 1981) or a base across plasma membranes, measuring the shift in the position of the inorganic phosphate peak by nuclear magnetic resonance (NMR) (Moon and Richards 1973) and study of fluorescence of a pH sensitive dye trapped inside the cell (Thomas *et al* 1979). Of these, the fluorescence dye technique is more sensitive and suitable for studying pH(i) changes in live cells. Thomas *et al* (1979) have shown that the spectral signal from the dye, 6-carboxyfluorescein, trapped in Ehrlich ascites tumor cells could report pH(i). Later, Rink *et al* (1982) have shown that an analogue of 6-carboxyfluorescein, 2', 7'-bis (carboxyethyl) 5, 6-carboxyfluorescein (BCECF) is better for studying pH (i) as it does not leak out of cells because of the two carboxyl groups. Using BCECF-acetoxymethyl ester (AM) to measure pH (i), Takasu *et al* (1990) have reported an alkalinization in porcine thyroid cells in the presence of insulin like growth factor 1. To get an insight into the changes that occur in cells during heat shock, we have measured the internal pH using BCECF-AM before and after heat shock.

We report here that the pH (i) of adult and embryonic liver cells is different and upon heat shock there is an increase in pH (i) in both adult and embryonic liver cells. We also show that some of the inducers that cause heat shock like response in many systems, also cause a change in pH(i) and induce hsps at 37° C in fetal liver cells.

2. Materials and methods

Adult liver tissues of Wistar rats or Wistar rat embryos of 12–13 days of gestation were used m all the experiments reported. The optimum temperature for incubation of adult and fetal liver Cells for the induction of heat shock response without loss of cell viability was 42° C. In all experiments reported in this communication, cells from fetal liver and adult liver obtained after perfusion were incubated at 37 and 42°C for 30–60 min in Dulbecco's modified Eagle's medium (DMEM) without serum. BCECF-AM was from Molecular Probes, Eugene, Oregon, USA. Alpha-[^{32}P] dATP was supplied by Jonaki, Hyderabad. All other chemicals used were from Sigma Chemical Co., St. Louis, Missouri, USA.

2.1 *Cell preparation*

Fetal and adult liver cells were obtained by gentle homogenization of fresh livers from the animal. The homogenate was centrifuged and the pellet washed several times (by centrifugation and resuspension in 20–30 pellet volumes of PBS) till a clean liver cell suspension was obtained. The purity of the cell suspension was checked under the microscope. Adult liver was perfused before homogenization by inserting a thin tube into the hepatic portal vein that is connected to the liver and passing PBS into the liver lobe from a syringe with gentle pressure. Perfusion removes 80% of the blood cells from liver suspension and helps in obtaining a clean preparation of cells with fewer washings.

2.2 *Measurement of intracellular pH using fluorescent indicator dye*

Adult and fetal liver cells were resuspended in DMEM buffered with MOPS (pH 7·5, final concentration of MOPS 25 mM) and the ionic balance and the external pH of the cells was kept constant in all the experiments. For measuring, pH (i) cells were incubated with BCECF-AM (final concentration of 500 nM containing 2×10^6 cells/ml) for 30 min in DMEM buffered with MOPS at 37 and 42° C. Preliminary experiments indicated that 30 min incubation with BCECF-AM was optimum for measuring fluorescence in whole cells. The fluorescence signal was less during shorter incubation times and longer incubation did not increase it. Therefore in all experiments reported here cells were loaded with BCECF-AM for 30 min at 37 and 42° C, centrifuged, washed in phosphate buffered saline [PBS phosphate, 2 mM (pH 7·5); NaCl 0·2 M; KCl, 2·5 mM] and resuspended in PBS at the same concentration (2×10^6 cells/ml). Two ml of these cells were used for fluorescence measurements.

Fluorescence measurements were performed with a Hitachi.F-4000 steady state spectro-fluorometer using a 1 cm path length quartz cuvette. Excitation and emission slits with a nominal band pass of 3 nm were used for all experiments. All spectra were recorded using the corrected spectrum mode. The excitation wavelength used was 500 nm in all cases. Emission spectra were recorded between 510 and 600 nm at 37° C or 42° C or as indicated in figure legends.

To measure changes in pH(i) in the presence of inducers of hsps, the same number of cells with the same concentration of BCECF-AM as above were incubated at 37° C in the presence of inducers; 8-OH quinoline (final concentration 10^{-5} M), 2, 4-dinitrophenol (2, 4 DNP) (final conc. 10^{-3}M), dexamethasone (final conc. 10^{-4} M), tunicamycin (final concentration 5×10^{-8} M) and valinomycin (final concentration 10^{-5} M) for 30 min. Cells were washed to remove both the dye and the inducer, resuspended at 2×10^6/ml and spectra were recorded as described above.

For generating a standard curve of the pH-dependent fluorescence of BCECF-AM taken up by adult and fetal liver cells, 20 ml of 2×10^6/ml of cells were loaded with BCECF-AM, then washed and resuspended in 20 ml of PBS and lysed with 0·1 % Triton X-100 and homogenized. The homogenate was spun at 1,200 *g* in a HB-4 rotor for 10 min and the pH of the supernatant was adjusted to different values between 6–8. Fluorescence spectra of the cell lysate at different pH values were recorded and standard curves were generated for adult and fetal liver cells separately.

2.3 *Dot blot hybridization*

It was reported earlier that 30 min of incubation at elevated temperature induces the synthesis of mRNA for heat shock proteins (Srinivas *et al* 1 987). For dot blot hybridization, total cellular RNA was isolated as described previously (Chomczynski and Sacchi 1987) from fetal liver cells incubated with inducers (concentration of inducers is the same as used for fluorescence measurements), 8-OH quinoline, 2, 4 DNP, dexamethasone, tunicamycin or valinomycin at 37° C for 30 min and blotted on nitrocellulose paper in a dot blot apparatus. The blots were baked for 2 h at 80° C. Plasmid DNA from pHS 811, 709 and 208 (coding for hsps 89, 70 and 27 respectively), a gift from E Hickey and L A Weber, Biology Department, University of South Florida, Tampa, Florida, USA, was isolated using alkaline lysis method (Maniatis *et al* 1982) and was nick translated using [α^{32} P]-labelled dATP under conditions described in Maniatis *et al* (1982). RNA blotted onto nitrocellulose paper was hybridized with each of these probes (2-4× 10^6 cpm/ml) as described by Thomas (1983), washed, dried and exposed to X-ray film for 24–36 h.

3. Results

3.1 *Changes in intracellular pH upon heat shock*

Internal pH of the cells was measured using the pH dependent fluorescent dye bis carboxyethyl carboxyfluorescein. In the acetoxymethyl ester form, the dye is incorporated into liver cells where it is hydrolyzed by the esterases inside (Rink *et al* 1982; Rotman and Papermaster 1966), generating a fluorescent, impermeable ionized form, BCECF. Thomas *et al* (1979) have reported that the spectral signal from the dye trapped inside the cell could report pH (i). The dye is taken up by the cells within 30 min at both temperatures. To measure pH (i), cells were loaded with BCECF-AM for 30 min at 37 and 42° C (to equilibrate the dye inside and outside), externally bound dye was washed off and the cells were resuspended in the same volume of PBS buffered with MOPS in a fluorescence cuvette. In figure 1 are shown the corrected fluorescence spectra of the dye taken up by the cells at 37 and 42° C. There is an increase in relative fluorescence intensity at 42° C as compared to 37° C. To take into account for possible differential uptake of the dye at these two temperatures, cells were loaded with BCECF-AM at 37° C, external dye was washed off and the cells were incubated at 37 and 42° C for 30 min. The spectra shown in figure 2 indicate an increase in relative fluorescence intensity of the dye in cells at 42° C, clearly indicating a change in pH (i) of cells at 42° C.

To express the increased fluorescence intensity in units, spectra of the dye in the cell lysates adjusted to different pH values were generated as described in §2. Figure 3 shows the corrected spectra of calibration curves obtained at 37° C with adult and embryonic cell lysates adjusted to different pH values. As can be seen, the fluorescence of BCECF-AM taken up by the cells is also pH dependent. Spectra of lysates prepared from the same number of cells incubated at 37 and 42° C were recorded on the same graphs. Based on relative fluorescence intensities, pH(i) of adult liver cells at 37° C appears to be lower (6·9) than the pH (i) of fetal liver cells (pH 7·0) and upon heat shock there is an increase of pH (i) in both embryonic and adult liver cells by 0·2–0·3 pH units making it 7·2 in adult liver cells and 7·3 ± 0·1 in fetal liver cells.

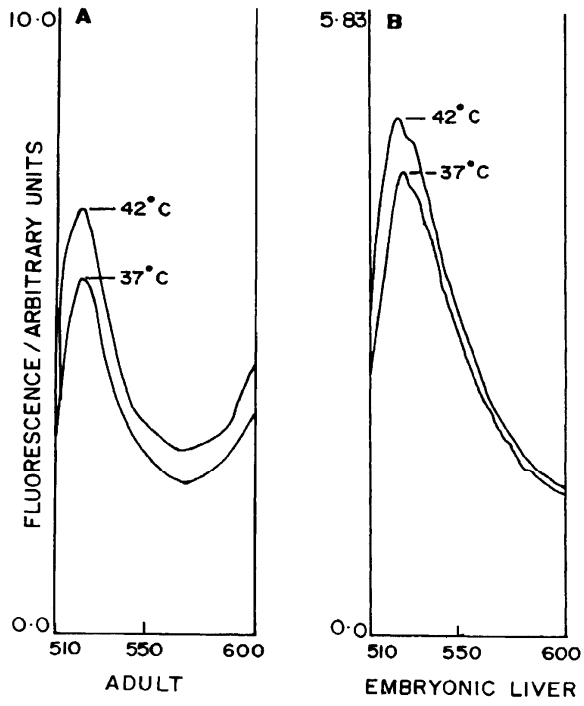

Figure 1. Fluorescence spectra of BCECF-AM taken up by cells at 37 and 42° C in adult and fetal liver cells. Adult and fetal liver cells were incubated with BCECF-AM in DMEM buffered with MOPS for 30 min, centrifuged and washed and resuspended in 2 ml of PBS buffered with MOPS. Spectra of the cells recorded at 37° C, keeping the excitation wavelength at 500 nm.

To correlate the increase in relative fluorescence intensity of same amount of dye with change in pH (i), we have directly measured the fluorescence in live cells. For this both types of cells were loaded with BCECF-AM at 37° C, cells were pelletted and washed to remove the external dye and resuspended in the same volume of DMEM buffered with MOPS. Fluorescence of BCECF (taken up by the cells at 37° C) during heat shock was monitored in the time mode over a period of 20 min while increasing the temperature from 37 to 42° C. The temperature of the cell suspension reached 42° C in 5-6 min and the recording was done for 20 min from the time the temperature reached 42° C. During the 20 min recording cells were kept in suspension by slow stirring without cell damage. Figure 4 is the time scan of adult and embryonic liver cells respectively, which indicate an increase in fluorescence upon heat shock. Since the spectral signal of the dye trapped inside the cells is a measure of the internal pH of the cells and since increase in fluorescence was directly recorded during heat shock, the heat shock is causing an increase in pH (i).

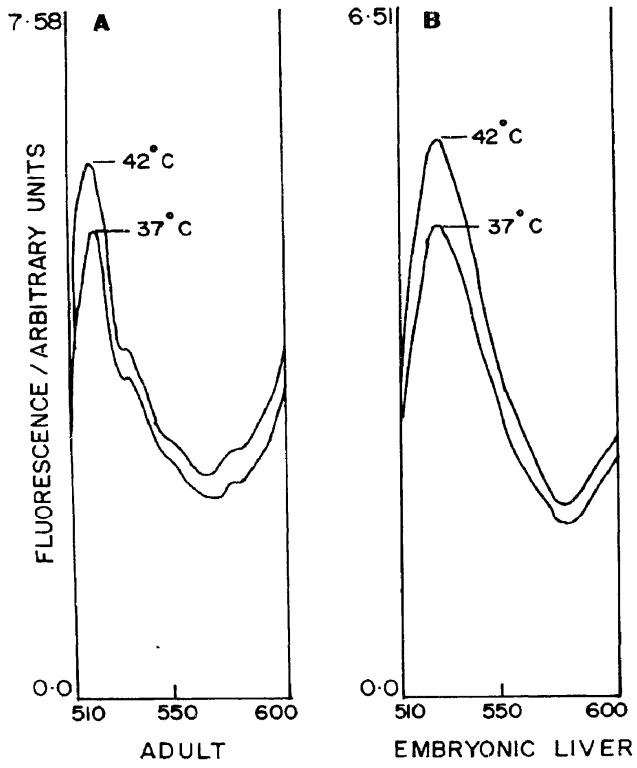

Figure 2. Corrected spectra of BCECF-AM taken up by cells at 37° C. Legend is the same as in figure 1 but the dye was removed and duplicate samples were incubated at 37 and 42° C. Spectra were taken as described above.

3.2 *Change in pH(i) with inducers of heat shock response at 37° C*

As stated earlier, in addition to heat, a variety of chemicals were shown to induce a heat shock like response at normal temperature in many systems (Ritossa 1962; Atkinson and Waldew 1985). To study whether a similar change in pH(i) occurs in the presence of these inducers, we have measured the pH (i) of fetal liver cells in the presence of some inducers.

Fetal liver cells were incubated at 37° C in the presence of inducers and BCECF for 30 min, and were washed to remove the inducer as well as the dye and fluorescence spectra of the dye taken up by the cells was recorded. As shown in figure 5, similar to heat shock, there is an increase in relative fluorescence intensity of BCECF in fetal liver cells incubated in the presence of the inducers at 37° C. The relative fluorescence intensity increases by 8-10% at 42° C and ranged between 5-20% with inducers suggesting an increase in pH (i) in the presence of inducers of heat shock response. The change in pH(i) is largest in the presence of

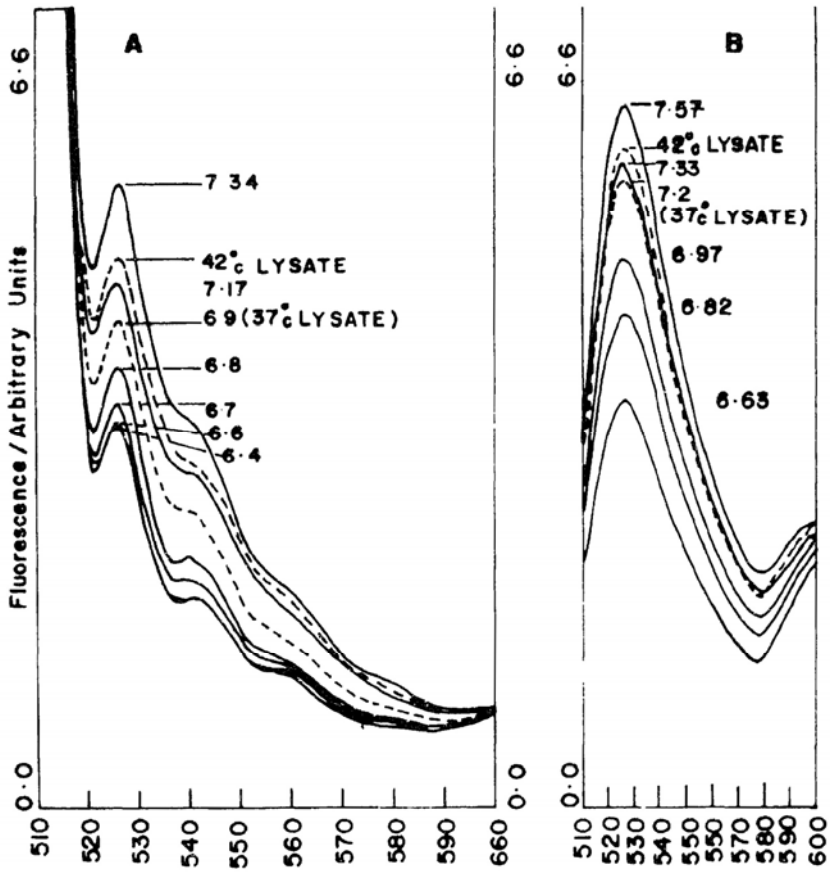

Figure 3. Calibration curves with adult and embryonic liver cell lysate at known pH values. Cell lysates from adult and fetal liver cells were prepared as described in §2 and pH of lysate was adjusted to different pH values. Emission spectra were recorded with excitation at 500 nm. **(A)** Adult liver. **(B)** Embryonic liver.

dexamethasone (corresponding to about 0·4 pH units) and is followed by 2, 4 DNP, tunicamycin, heat shock and valinomycin.

3.3 *Induction of hsps in the presence of inducers*

The expression of heat shock genes in the presence of inducers at 37° C (during which there was a change in pH (i)) was confirmed by dot hybridization studies. For these studies total RNA was isolated from fetal liver cells incubated at 37° C in the presence of inducers for 30 min and spotted on to nitrocellulose paper and hybridized to nick traslated hsp probes, pHS 811, 709 and 208 as described in §2. Figure 6 shows the dot hybridization pattern of RNAs isolated from fetal liver cells incubated in the presence of different inducers. Hybridization is concentration

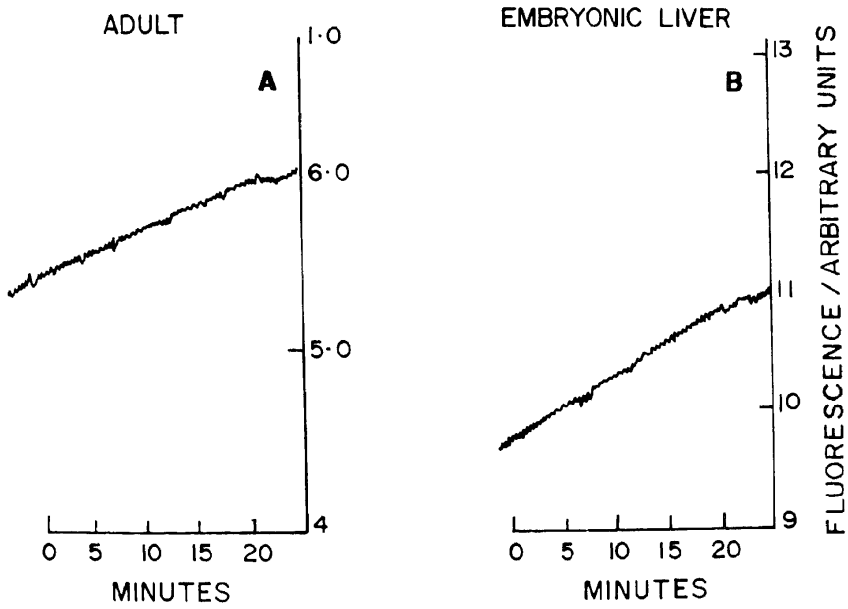

Figure 4. Scans of adult and embryonic liver cells in "time mode". Cells were incubated with BCECF-AM at 37° C as described in figure 2. Cells (2 ml) were taken in a cuvette and increase in fluorescence was recorded, while the temperature of the cells was increased in the cuvette from 37 to 42° C. Excitation 500 nm. Emission 528 nm and slit width 5 nm for both excitation and emission.

dependent and specific. Results confirm the expression of hsps at 37° C in the presence of inducers.

4. Discussion

One of the physiological changes correlated with a change in gene expression is the internal pH of the cell (Johnson *et al* 1976; Webb and Nuccitelli 1981; Ober and Pardee 1987; Dufresne *et al* 1988). pH(i) was also shown to be affected by cell-cell contact (Galkina *et al* 1992). Since a temperature dependent change in gene expression takes place during heat shock, it is possible that temperature may change the pH (i) of cells. Change in gene expression associated with change in pH has been reported earlier. Wadsworth and Riddle (1988) have shown that there is a lowering of pH (i) during larval development of *Caenorhabditis elegans.* Lowering of pH(i) was also shown to occur during heat shock in yeast cells (Weitzel *et al* 1987). Nishimura *et al* (1989) reported that rapid changes in extra cellular pH in cultured astrocytes leads to the induction of major hsp, hsp 68, which is due to the change in pH (i) of these cells.

The relative increase in BCECF fluorescence intensity induced by heat shock was reproducible. This relative increase in fluorescence intensity cannot be due to difference in cell number or due to differential loss of cells during washings as there

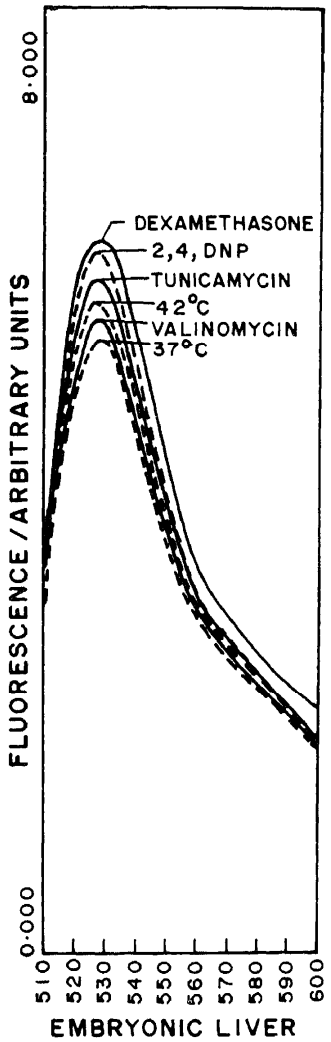

Figure 5. Fluorescence spectra of embryonic liver cells incubated with BCECF-AM and inducers of heat shock response. Embryonic liver cells were incubated with BCECF-AM in the presence of the inducers for 30 min at 37° C, washed and resuspended at the same concentration. Corrected spectra were recorded as described in figure 1.

will be a decrease in fluorescence due to cell loss. Also the data in figure 4 rule out such a possibility as the relative fluorescence intensity from a constant number of cells increases over 20 min when the temperature was increased from 37 to 42° C. These observations with BCECF conclusively prove the change in pH (i) upon heat shock. Results obtained with total cell lysates (figures 3 and 4) and the direct measurement of fluorescence in whole cells during temperature shift (figure 4) from 37 to 42° C indicated an increase in pH (i) in both adult and embryonic liver cells upon heat shock. Change in pH(i) of 0·2 to 0·3 units, as seen by increase in relative fluorescence intensity, is probably sufficient for the activation of HSF. Induction of hsps in adult and fetal liver cells and the premature expression of albumin in early

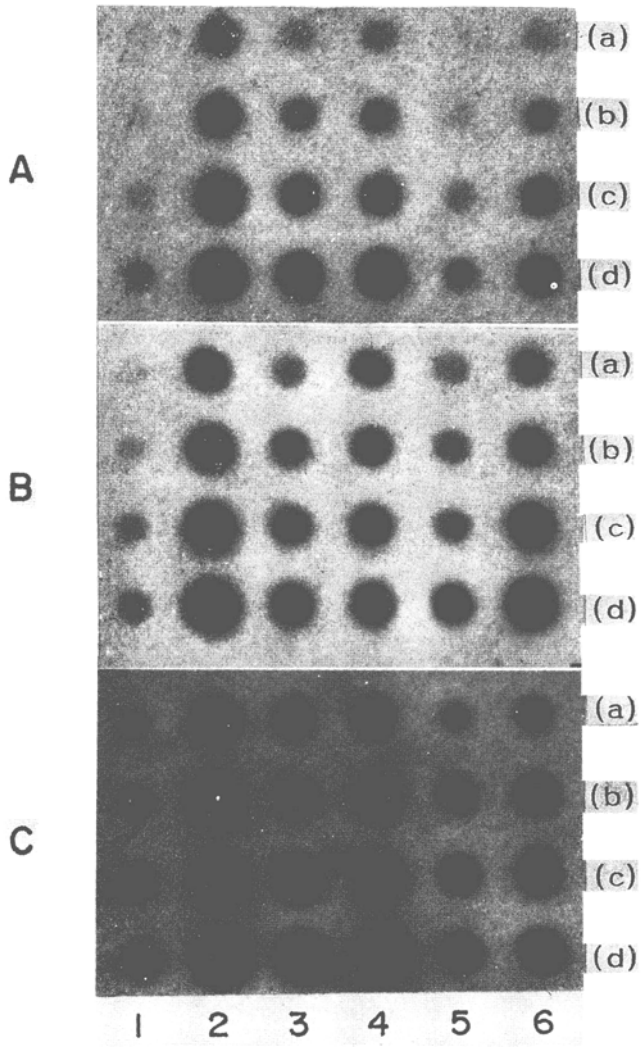

Figure 6. Induction of hsps in the presence of inducers at 37° C in fetal liver cells. Total RNA, isolated from fetal liver cells incubated at 37° C in the presence of inducers, was hybridized with DNA probes for hsps as described in §2. **(A)** RNA probed with plasmid pHS 811, **(B)** pHS 709 and **(C)** pHS 208; these code for hsp 89, 70 and 27 respectively. (a), (b), (c), (d) refers to concentrations of RNA 0·5, 1, 2 and 4 μg respectively. (1) Fetal liver 37° C; (2) 8-OH quinoline; (3) 2, 4 DNP; (4) dexamethasone; (5) tunicamycin; (6) valinomycin.

embryonic rat liver cells upon heat shock have been reported earlier (Srinivas *et al* 1987). The present data indicate that during heat shock there is a physiological change i.e., change in pH(i) in parallel to changes in gene expression. We have observed an induction of albumin gene expression in fetal liver cells at 37° C in the presence of amiloride, an inhibitor of Na^+/H^+ antiporter which is known to change pH(i). A change in pH(i), as seen during heat shock, was also observed in the presence of other inducers of hsps at 37° C. Since many of the inducers of heat

shock response disturb the membrane potential or energy metabolism, the change in pH (i) is not unexpected or unusual. Induction of mRNA for hsps and an increase in relative fluorescence intensity in the presence of these inducers at 37° C suggest that the mechanism of activation of heat shock genes under these conditions is similar. It will be interesting to see if HSF binds to heat shock elements in the presence of these inducers. Experiments are in progress to study whether the change in pH(i) in response to heat shock is a common phenomenon and whether HSF and HSE are involved in the activation of hsps in the presence of these inducers.

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