Heat shock proteins and effects of heat shock in plants

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Abstract. Soybean seedlings when exposed to a heat shock respond in a manner very similar to that exhibited by cultured cells, and reported earlier [2]. Maximum synthesis of heat shock proteins (HSPs) occurs at 40 C. The heat shock response is maintained for a relatively short time under continuous high temperature. After 2.5 hr at 40 C the synthesis of HSPs decreases reaching a very low level by 6 hr. The HSPs synthesized by cultured cells and seedlings are identical and there is a large degree of similarity in HSPs synthesized between the taxonomically widely separated species, soybean and corn. Storage protein synthesis in the developing soybean embryo is not inhibited but is actually stimulated during a heat shock, unlike most other non-HSPs, whose synthesis is greatly reduced. Seedlings respond differently to a gradual increase in temperature than they do a sudden heat shock. There is an upward shift of several degrees in the temperature at which maximum protein synthesis occurs and before it begins to be inhibited. In addition, there appears to be a protection of normal protein synthesis from heat shock inhibition when the temperature increase is gradual. An additional function of the heat shock phenomenon might be the protection of seedlings from death caused by extreme heat stress. The heat shock response appears to have relevance to plants in the field.

Introduction

The exposure of cells of several animal species to a heat shock i.e., a sudden increase in the incubation temperature, results in the inhibition of synthesis of most cell proteins and in the new or increased synthesis of a relatively few proteins. This phenomenon has been studied extensively in *Drosophila* [9, 21], as well as for other insects [22] and in cultured avian and mammalian cells [10]. A heat shock response similar to that observed in animal cells has recently been shown to occur in cultured cells of tobacco and soybean [2]. The purpose of our work was: (1) to compare the heat shock response exhibited by cultured cells to that shown by intact plants. (2) To determine what effect a heat shock has on the synthesis of storage proteins in the developing seed (a group of proteins synthesized on a very prevalent class [7, 8] of messenger RNAs) and (3) to determine the possible function(s) of the heat shock response in enabling the plant to survive periods of heat stress. Some of these results have been briefly reported [1].

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Materials and methods

Plant material

Seeds of *Glycine max* L. Merrill, cultivar Tracy (kindly provided by Dr. Edgar Hartwig, Soybean Production Research, USDA, Stoneville, Miss.) were sterilized with full strength clorox and washed free of clorox with sterile water containing 400 μg/ml penicillin G. Seeds were germinated in the dark at 25 C for 3 to 4 days on a sterile layer of Whatman 3 MM filter paper soaked in a solution of 400 μg/ml of penicillin G in a sterile petri plate.

A callus culture line was isolated from developing cotyledons of Tracy and cultured as described [2].

Heat shock treatment and isolation of proteins

Tissue culture cells were incubated at 40 C for 30 min prior to labeling for 1 h with [^{35}S] methionine (specific activity 750 Ci/mmole; New England Nuclear), and proteins extracted and analyzed as described previously [2]. Seedlings of uniform size were selected and two seedlings were placed in a sterile 25 ml Erlenmeyer flask with 0.5 ml of 1/10 strength Hoagland's solution containing 50 μg/ml chloramphenicol. The flasks were incubated in the dark in a water bath shaker at 150 rpm. Prior to homogenization of the seedlings at the end of an experiment the cotyledons were removed and discarded. The protocol followed for temperature treatment and labeling in the individual experiments is provided in the legends to the figures. Seedlings were washed with ice cold H_2O and cut on ice into small pieces with a sharp scalpel, and 0.75 ml of 2 × sample buffer [13] was added. The preparation was immediately placed in a boiling water bath for 2 min prior to homogenization.

Developing soybean seeds (cultivar Tracy) approximately 150 mg in weight were isolated under sterile conditions and the seed coats removed. For the labeling of proteins the two cotyledons were separated and each placed on a 5 μl drop of 1/10 strength Hoagland's solution containing 25 μCi of [^{35}S] methionine (Amersham/Searle, 1300 Ci/mmol) on a piece of parafilm in a petri dish. An additional 5 μl (25 μCi) drop of the label was placed on top of each cotyledon. A filter paper disc soaked in sterile water was attached to the inside of the petri dish cover. After the appropriate incubation the cotyledons were washed with ice cold H_2O and cut with a razor blade. To the cut cotyledon pieces, 0.75 ml of 2 × sample buffer was added and further treatments were as described for seedlings.

Purified 11S protein (gift of Dr. A.H. Chen, Anderson Clayton Foods, Richardson, Texas) was used to prepare antiserum in rabbits. The 11S preparation was very slightly contaminated with 7S proteins. Immunoprecipitation of *in vivo* labeled proteins was carried out essentially as described by Kessler [11] except that the bound [^{35}S]-labeled 11S pelleted proteins

were solubilized in Laemmli sample buffer [13] and boiled for 3 min prior to electrophoretic analysis.

Further procedures including the electrophoretic analysis of proteins were similar to those used previously for tissue culture cells [2]. The following molecular weight standards were used: phosphorylase A, 92 500: bovine serum albumin, 67 000; γ-globulin, 50 000 and 25 000; actin 43 000; lysozyme, 14 400; cytochrome C, 12 300 daltons.

For the determination of viability of cells 3 day old seedlings (Tracy) were placed in a 25 ml erlenmeyer flask with 0.5 ml of 1/10 strength Hoagland's solution and then exposed to various temperature treatments. After the final treatment the hypocotyls were sectioned with a razor blade and their viability determined by staining for 20 min in 0.5% Evans blue in 4% sucrose [20]. The sections were then washed in H_2O twice and examined under the microscope. For each temperature treatment the cortex cells of at least 5 sections (with a total of about 4000 cells) were counted and statistically evaluated.

Results

Seedlings were preincubated for 1 hr at different temperatures ranging from 25 to 45 C and then labeled for 1 hr with [^{35}S] methionine at the same temperature. Seedling extracts were prepared and analyzed by polyacrylamide gel electrophoresis (Fig. 1). The pattern of newly synthesized polypeptide bands seen at 37–43 C when compared to the proteins synthesized at 25 C is very similar to that reported earlier with cultured cells [2]. The molecular weights of the bands are indicated in Fig. 1. These bands, the heat shock proteins (HSP's) are first faintly detected at 31 C although some of the proteins are present in small amounts at the control temperature as is true for cultured cells [2]. By 34 C their synthesis can be clearly seen. In some gels the 94 K, 21 K, and 16 K bands can each be resolved into doublets. The optimum temperature for maximum induction of the HSP's occurs at 40 C. If equal amounts of tissue rather than equal cpm (as has been done in the analysis in Fig. 1) are loaded in each lane of the gel very little label is seen in the heat shock bands at 43 C compared to 40 C (data not presented). Above 40 C the synthesis of HSP's and all other proteins are drastically reduced (see Fig. 7). There is a significant reduction in normal protein synthesis during a heat shock.

When seedlings are incubated at 40 C for increasing lengths of time from 1–5 hr prior to labeling for 1 hr, the relative amounts of most of the HSP's increase during the first 2.5 hr of incubation (Fig. 2). One HSP (16 K) decreases continuously after the first 2 hr. At periods of incubation between 2.5 and 6 hr the synthesis of all heat shock bands progressively decreases, becoming very low after 6 hr at 40 C. Total protein synthesis as measured by the incorporation of [^{35}S] methionine into trichloroacetic acid insoluble

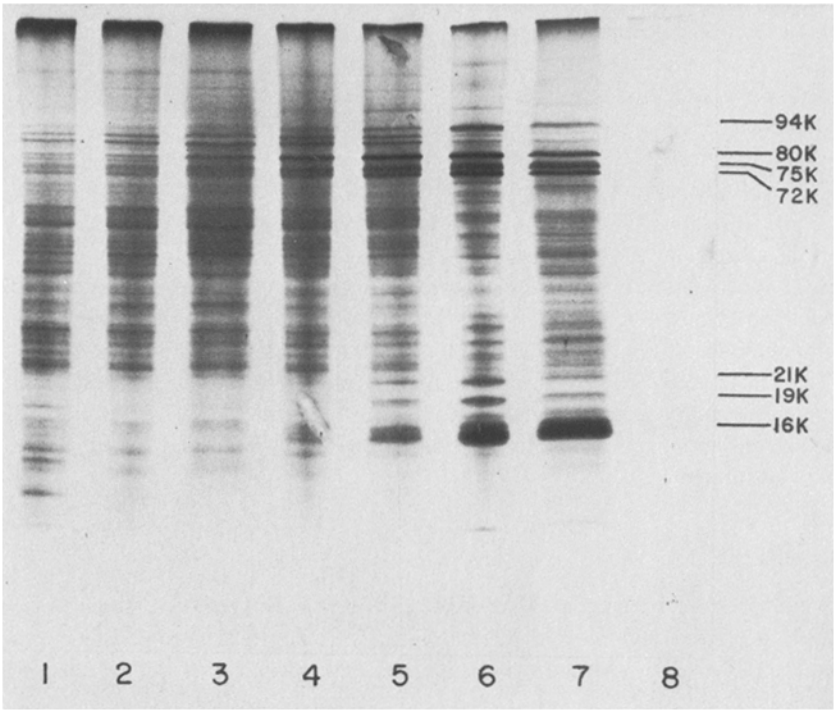

Figure 1. Protein synthesis in soybean (cv. Tracy) seedlings during a heat shock at various temperatures. Autoradiogram of an SDS-polyacrylamide (10 to 20% gradient) gel of proteins from seedlings preincubated at the specified temperature for 1 hr then labeled with [^{35}S] methionine for 1 hr at the same temperature. Extracts of tissues containing equal cpm were loaded in the lanes with the exception of lane 8. Lane 1, 25 C; lane 2, 28 C; lane 3, 31 C; lane 4, 34 C; lane 5, 37 C; lane 6, 40 C; lane 7, 43 C; lane 8, 45 C.

material decreases rapidly during the first 3 hr at 40 C and then stays at a low level for the next 2 hr (data not presented).

The heat shock proteins synthesized in cultured cells and seedlings are very similar (Fig. 3). Slightly different approximate molecular weights were reported earlier for the HSP's of soybean cells [2]. The proteins have now been more accurately sized by the use of additional molecular weight markers and the 103 K, 85.5 K, 82 K, 80 K, 14 K and 11 K bands reported earlier [2] are now designated 94 K, 80 K, 75 K, 72 K, 19 K and 16 K, respectively.

Out of general interest to see how similar the HSP's in a dicot and a monocot might be, the HSP's from soybean and corn (*Zea mays*) seedlings were compared (Fig. 4). Although not identical there is a surprising similarity in heat shock bands between the two widely separate species.

The storage proteins are synthesized on mRNAs that are present in a very large number of copies per cell [7, 8], unlike the situation with the

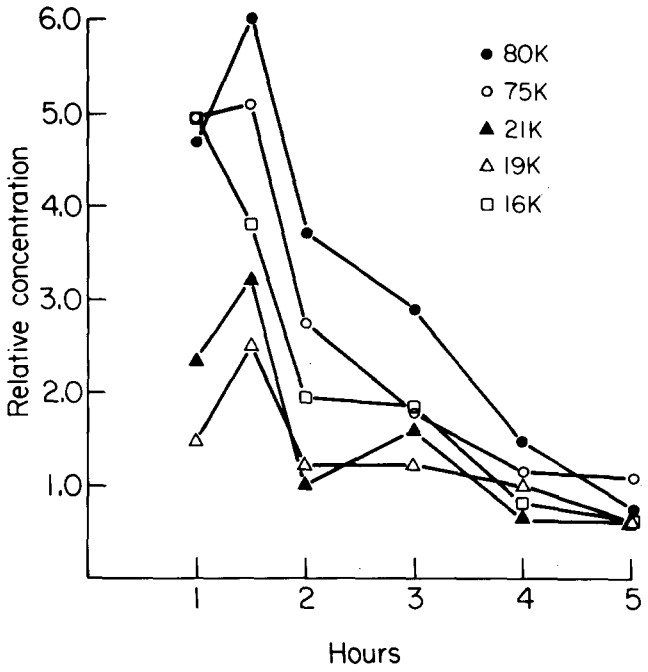

Figure 2. Effect of a continuous heat shock on the synthesis of specific heat shock proteins in soybean seedlings. Seedlings were incubated at 40 C for the times indicated followed by a 1 hr labeling with [^{35}S]-methionine at 40 C. Individual heat shock bands (80 K, 75 K, 21 K, 19 K and 16 K) on autoradiograms after gel electrophoresis were scanned in a densitometer.

majority of mRNAs in the seedling. Accordingly it was of interest to determine whether storage protein synthesis is under the same type of control after a heat shock as are most proteins in the seedling. Young seeds actively synthesizing storage proteins were incubated at 25 C or 40 C for various periods and labeled with [^{35}S] methionine. The proteins were extracted and analyzed on polyacrylamide gels (Fig. 5). Surprisingly, in addition to the synthesis of normal HSPs the synthesis of the storage proteins seems to increase during a heat shock of 40 C for 1 hr (lanes 1, 2). The storage proteins are relatively poor in methionine [4]. Had a label other than methionine been used, even darker bands of the storage proteins would undoubtedly have been seen in the autoradiograms. Lane 5 which shows the HSPs in seedlings enables one to see more clearly which bands correspond to the HSPs and which to the storage proteins. It does appear that the higher molecular weight HSPs are decreased relative to the smaller HSPs indicating a possible competition between the storage protein mRNAs and those of the larger HSPs during translation. In addition to the storage proteins there are several other unidentified non-HSP bands whose synthesis appears to be increased during a heat shock.

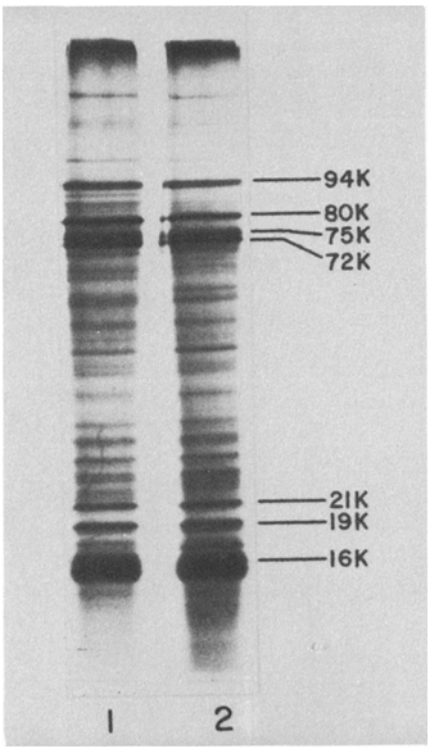

Figure 3. Comparison of heat shock proteins from cultured cells and seedlings of soybean. Autoradiogram of an SDS-polyacrylamide gel of proteins from seedlings (lane 1) and cells (lane 2) preincubated at 40 C for 1 hr, followed by a 1 hr label with [^{35}S] methionine.

That the 11S proteins continue to be synthesized during a heat shock is shown more clearly by using an antiserum produced against the 11S storage proteins to immunoprecipitate the newly synthesized 11S proteins from other proteins in the developing seed extract. The data in Fig. 6 confirm the results of Fig. 5 that a heat shock does not cause an inhibition of synthesis of the 11S storage proteins, but on the contrary the synthesis of 11S proteins may be increased.

Soybean plants do reach heat shock temperatures in the field especially under non-irrigated conditions [9]. A rapid increase in temperature such as has been used by us experimentally, is unlikely in nature. It was, therefore, of interest to determine whether a gradual increase in temperature such as might occur in the field would alter the nature of the heat sock response. Accordingly, the incubation temperature of seedlings was gradually raised 3 °/hr from 25 to 52 C and the seedlings were labeled during the terminal hour. The incorporation of label into hot TCA insoluble material both after a sudden heat shock and after a gradual increase in temperature are

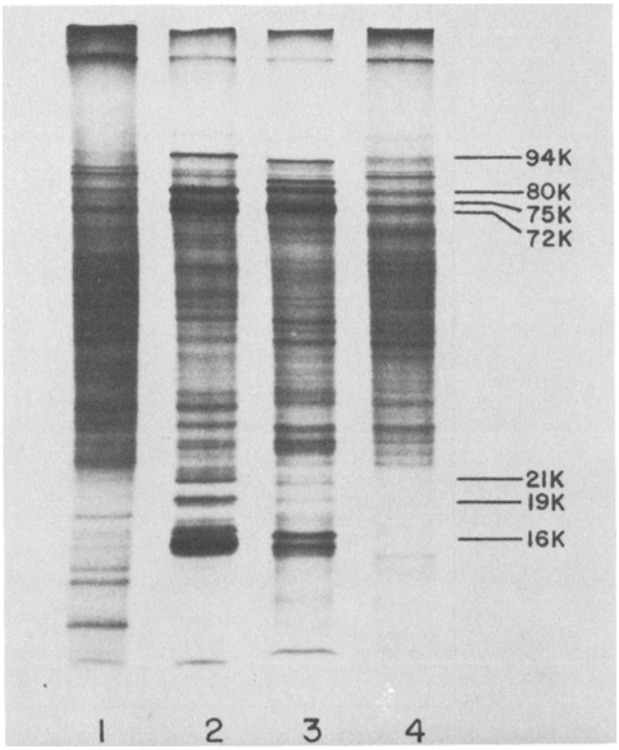

Figure 4. Comparison of HSPs from soybean and corn seedlings. Autoradiogram of an SDS-polyacrylamide gel of proteins from soybean and corn seedlngs preincubated at 40 C for 30 min, followed by a 1 hr label with [^{35}S] methionine. Controls were labeled at 25 C for 1 hr. Lanes 1 and 2, soybean, 25 C and 40 C respectively; lanes 3 and 4, corn, 40 C and 25 C respectively.

shown in Fig. 7. The incorporation of label into protein in seedlings rapidly transferred from 25 C to the heat shock temperature reaches a maximum at 37 C and rapidly decreases thereafter, and by 45 C, incorporation is barely detectable. In contrast, however, seedlings exposed to a gradual increase in temperature show a dramatic shift in the temperature (43 C) at which maximum protein synthesis occurs and substantial protein synthesis continues as high as 49 C.

Extracts of the seedlngs exposed to a gradual increase in temperature were analyzed by electrophoresis (Fig. 8). Heat shock proteins continue to be synthesized in large amounts up to 46 C and can even be detected at 49 C. Unlike the situation where normal protein synthesis is greatly reduced when the temperature is suddenly increased (Fig. 1), the synthesis of non-HSPs does not seem to decrease at temperatures even as high as 46 C, when the temperature is gradually increased.

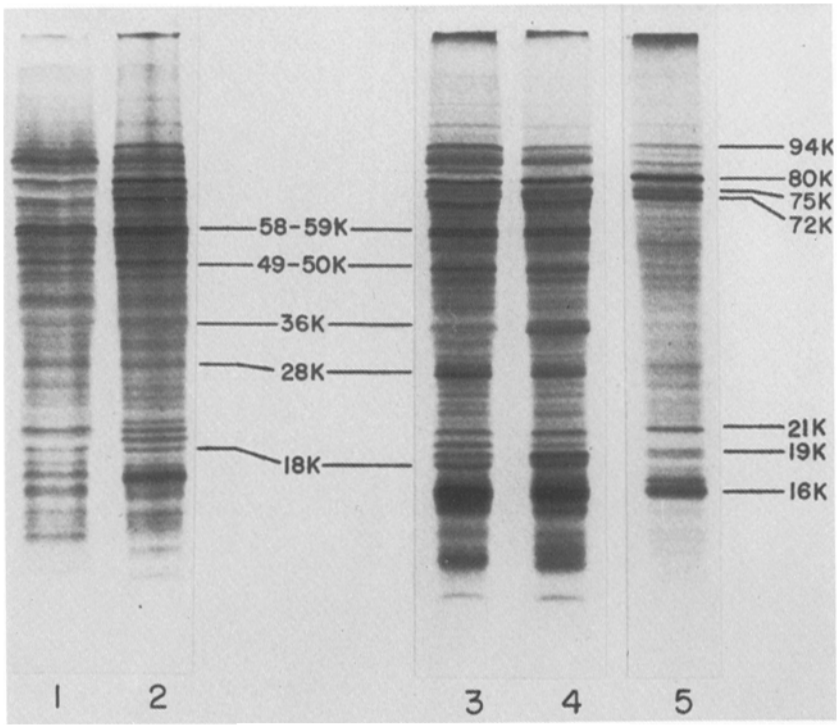

Figure 5. Synthesis of proteins by developing cotyledons of soybean exposed to a heat shock of 40 C and labeled for 1 or 2.5 hr at 40 C with [^{35}S] methionine. Lane 1, 1 hr at 25 C; lanes 2 and 3, 1 hr at 40 C; lane 4, 2.5 hr at 40 C; lane 5, soybean seedling, 1 hr at 40 C. The band at 58–59 K daltons has by comparison with the data of Sengupta et al [17] been tentatively identified as the 11S protein precursor and the bands at 36–38 and 18–20 kd are the mature 11S proteins. Similarly the bands at 68–76 kd [3] are the precursors of the α, α' (76–83 kd) mature proteins, and the 49–50 kd band is the precursor to the β subunit (53 kd).

One posssible effect of the heat shock treatment is a protection from death when cells are subsequently exposed to potentially lethal temperatures. To determine whether this is true, soybean seedlings were preincubated for 1 hr at either 25 C, 34 C or exposed to a gradual increase in temperature of 3 °/hr from 25 to 40 or 43 C, before being exposed to a killing temperature of 52 C for 1 hr. At the end of the 52 C treatment hypocotyl sections were stained with Evans blue to determine the extent of cell death. The data in Fig. 9 show that when seedlings are suddenly transferred from 25 to 52 C, almost all the cells are killed. If the seedlings are, however, preincubated at a moderate heat shock temperature of 34 C prior to exposure to 52 C about half the cells are protected from cell death. A gradual increase of 3 C per hr from 25 to 40 or 43 C prior to exposure to 52 C, completely prevents cell death.

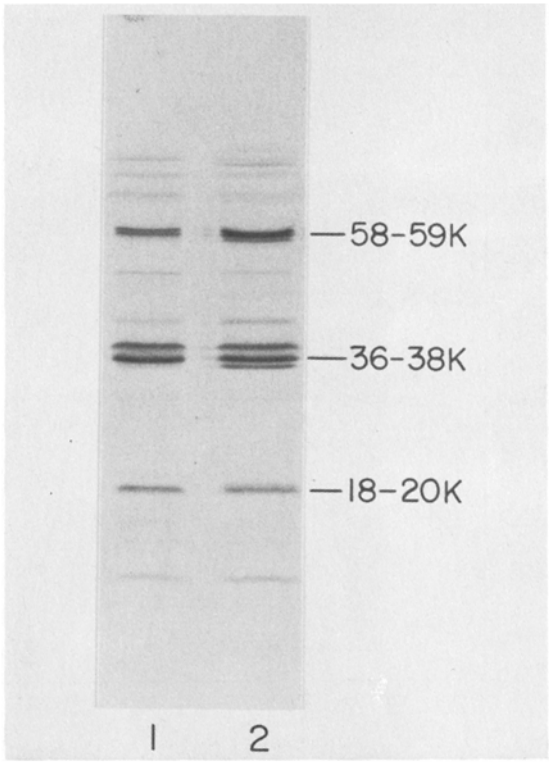

Figure 6. Analysis of proteins synthesized *in vivo* by developing soybean cotyledons after immunoprecipitation with antiserum to 11S protein. Developing seeds (about 200 mg in weight) were incubated at 25 (lane 1) and 40 C (lane 2) for 2.5 hr with [^{35}S] methionine. They were then extracted and the 11S proteins immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. A 2.5 hr incubation was used to allow processing of the precursor. The 58–59 K bands are the precursors of the acidic (36–38 K) and basic (18–20 K) subunits of the 11S protein.

Discussion

The number and size of heat shock proteins in soybean seedlings is identical to that shown by cultured cells of soybean [2]. There is not as complete an inhibition of normal protein synthesis after a heat shock in soybean or corn as compared to *Drosophila*. The temperature at which maximum synthesis of HSPs is induced in soybean seedlings is the same as that for soybean cells in culture [2]. During the preparation of our results for publication results similar to some of those presented here describing the heat shock phenomenon in isolated hypocotyl sections of soybean seedlings have been reported [12]. Exposure of soybean seedlings continuously to a heat shock temperature of 40 C, results in the initial increase in the relative amounts

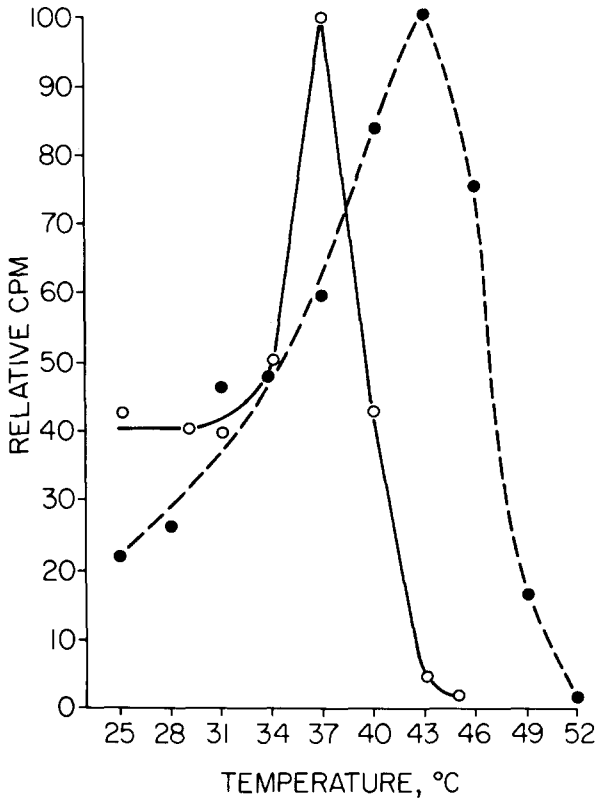

Figure 7. Comparison of the incorporation of label into hot TCA-precipitable radioactivity, between seedlings transferred suddenly from 25 C to the heat shock temperature (o—o) or subjected to a gradual increase of 3 C per hour from 25 C to the various final temperatures (o--o). All seedlings were labeled with [^{35}S] methionine during the terminal hour of the temperature treatment. For the gradual temperature increase, seedlings were maintained at 25 C prior to the temperature increase so that the total time of incubation for all treatments prior to the label was the same.

of the major HSPs followed by a decrease in their synthesis at times longer than 2.5 hr. A very similar situation was found with tobacco cells in culture [2].

The storage proteins in the developing soybean embryos appear to be under a different control during a heat shock than are most other normally synthesized proteins. Their synthesis is not reduced like most proteins but on the contrary may be increased. This is true for several other abundant proteins in the developing embryo. The storage proteins in soybean are synthesized as precursors which require 1 hr or longer to be processed into the mature forms which can be either larger or smaller than the original precursor [3, 18]. It would be of interest to determine what structural properties the mRNAs of the storage proteins share with those of the HSPs

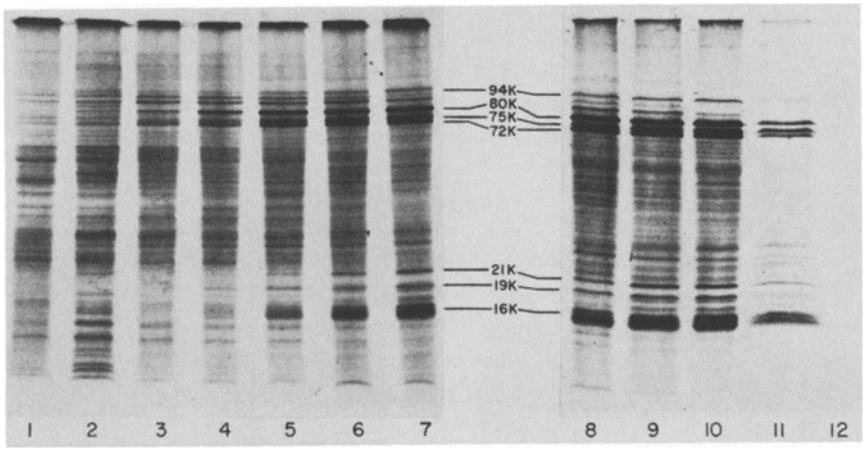

Figure 8. Effect of a gradual increase in temperature of 3°/hr on protein synthesis in soybean seedlings: The cells were labeled during the terminal hour of incubation at the temperature shown below with [^{35}S] methionine as in the legend to Fig. 7. Lane 1, 25 C; lane 2, 28 C; lane 3, 31 C; lane 4, 34 C; lane 5, 37 C; lanes 6 and 8, 40 C; lanes 7 and 9, 43 C; lane 10, 46 C; lane 11, 49 C; and lane 12, 52 C. Equal counts were loaded in all lanes with the excpetion of lanes 11 and 12 where the counts were too low to do this.

that enable their translation at higher temperatures. The storage protein mRNAs are very abundant being present in several thousand copies per cell [7]. Not all abundant mRNAs behave similarly to the storage protein mRNAs, as can be seen for example in seedlings where the synthesis of several prominent protein bands seen at the control temperature are inhibited at heat shock temperature (Fig. 1).

When the temperature is gradually increased at the rate of 3°/hr the seedlings respond differently to the temperature increase than do seedlings given a sudden heat shock (Fig. 7, 8). The temperature at which maximum protein synthesis occurs is shifted several degrees higher and in addition there appears to be a protection of normal protein synthesis from heat shock inhibition. A gradual increase in temperature is more representative of conditions that plants are exposed to in the field.

An additional effect of the heat shock treatment may be the protection of plant cells from death that might be caused by extreme heat stress. A prior mild heat shock or a gradual increase in temperature into the heat shock range (40 to 43 C) enables cells to survive when subsequently exposed to a potentially lethal temperature (Fig. 9). A very similar situation has been reported for *Drosophila* [16, 17], and for yeast [15].

The high temperatures that we have studied do occur in plants under field conditions. Leaf temperatures of several plants have been measured. In full sunlight it is not uncommon for leaf temperatures to be 10 to 15 C

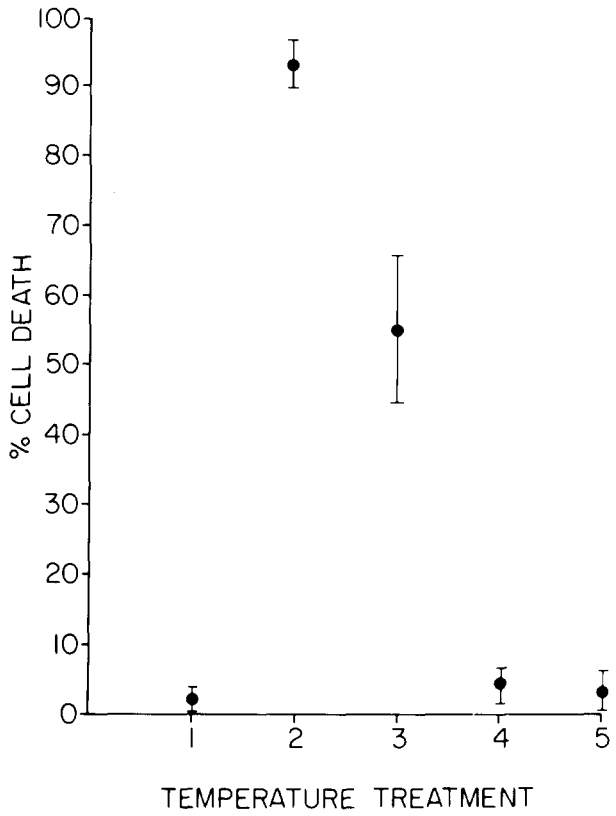

Figure 9. Protection from cell death in seedlings by prior heat shock treatments. Seedlings were exposed to different temperature treatments prior to incubation at 52 C for 1 hr. Cell death in hypocotyl sections was determined by Evans blue staining.
Treatment 1, 25 C for 1 hr followed by 25 C for 1 hr
Treatment 2, 25 C for 1 hr followed by 52 C for 1 hr
Treatment 3, 34 C for 1 hr followed by 52 C for 1 hr
Treatment 4, increase of 3 °/hr from 25 to 40 C, followed by 52 C for 1 hr
Treatment 5, increase of 3 °/hr from 25 to 43 C, followed by 52 C for 1 hr.

above air temperatures and to exceed 40 C [5, 6, 14]. The heat shock phenomenon thus appears to be not just an artificial response induced only under special laboratory conditions, but seems to have relevance to plants in nature in their ability to cope with stress induced by high temperatures.

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