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Thermotolerance and synthesis of heat shock proteins: These responses are present in *Hydra attenuata* but absent in *Hydra oligactis*

(stress proteins/ecology/evolution)

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ABSTRACT **Organisms respond to environmental stress by synthesizing a small number of highly conserved heat shock proteins. In organisms as diverse as bacteria, plants, invertebrates, and vertebrates, synthesis of these proteins is directly correlated with the acquisition of thermotolerance. While studying the freshwater coelenterate hydra, we observed that *Hydra oligactis* was extremely sensitive to thermal stress. In contrast, the related species *Hydra attenuata* survives short-term exposure to high temperatures. Furthermore, after incubation at an elevated but nonlethal temperature, *H. oligactis* did not become thermotolerant. *H. attenuata*, however, acquired thermotolerance after such a preincubation. In *H. attenuata* the major heat shock protein was found to be 60 kDa in size. *H. oligactis* did not synthesize detectable levels of this protein or any new species of proteins in response to stress. Several other species of hydra were found to behave like *H. oligactis* in response to stress. Thus, these findings provide direct support for the hypothesis that heat shock proteins are required for stress tolerance and that the major heat shock protein in hydra does not have any effects on normal growth or physiology. The findings also indicate that the presence of a heat shock response might be related to the natural environment in which an organism lives.**

Organisms as diverse as bacteria, plants, and mammals respond to environmental stress, such as exposure to heat or heavy metal ions, by the immediate synthesis of several heat shock proteins (hsps) and often the cessation of synthesis of most other proteins (1, 2). The major hsp in all organisms studied so far has a molecular mass of ≈70 kDa (hsp70). DNA sequence analysis of genes encoding hsp70 from a variety of organisms has shown them to be highly conserved in evolution (3–6). Because the hsp70 gene from *Drosophila* as well as hsp-encoding genes from *Dictyostelium* can be activated by heat shock in cells from highly divergent species (2), the induction mechanism itself also seems to be conserved. Recently, remarkable similarity of a heat shock transcription factor from organisms as diverse as *Drosophila* and yeast has been shown (7).

The apparent universality of the heat shock response and the high degree of conservation of the genes involved implies an important function for the proteins encoded by these genes. However, despite much information about the structure of the proteins and their genes, the cellular function of these proteins is unknown (2). The proteins are thought to protect cells from the toxic effects of short-term environmental stress (2). This conjecture is based on the observation that in many cell types and organisms induction of hsps correlates with an increase in the capacity for thermotolerance (8–10).

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In studying the heat shock response in the freshwater coelenterate hydra, we found a direct correlation between the ability to synthesize a hsp and survival of environmental stress.

MATERIALS AND METHODS

Hydra Culture. Animals were cultured at 17–18°C using standard methods (11).

Heat Treatment, Radioactive Labeling, Sample Preparation, and Gel Electrophoresis. Polyps were labeled by injecting 25 μCi of ^{35}S-labeled amino acids (Tran^{35}S-label, ICN, specific activity, 1100 Ci/mM; 1 Ci = 37 GBq) into the gastric cavity as described (12). For heat shock treatment polyps were transferred to prewarmed medium at the temperatures indicated, labeled with ^{35}S, incubated for 1 hr at the elevated temperature, and then washed in hydra medium (8). Thereafter, 10 polyps were homogenized in 50 μl of sodium dodecyl sulfate (SDS) sample buffer and boiled for 5 min. Protein extract from 10 polyps was loaded into each sample well of a 10% SDS/polyacrylamide gel. Fluorography was performed using EN^{3}HANCE (New England Nuclear). To label *Drosophila* proteins, cells of Schneider's cell line 3 (provided by S. Sharp, Department of Microbiology, University of California at Irvine) were incubated at 23°C or preincubated for 1 hr at 37°C and then incubated for 6 hr at 37°C after addition of [^{35}S]methionine (0.2 mCi/ml, Amersham, specific activity, 1200 Ci/mM) to the growth medium.

Measurement of Protein Synthesis Rates. Polyps were labeled at the experimental temperatures for 60 min as described above. After homogenization in SDS sample buffer, aliquots were taken and dried onto filter paper discs. The discs were washed with cold 10% trichloroacetic acid, 5% trichloroacetic acid, ethanol, and acetone, and the amount of radioactivity was determined with a liquid scintillation counter.

RESULTS AND DISCUSSION

Differential Effect of Heat Shock on Morphology and Viability of *Hydra attenuata* and *Hydra oligactis*. Cultures of hydra are kept in the laboratory at 17–18°C. Temperatures above 24°C are generally found to be unfavorable, whereas temperatures of 37°C and higher cause immediate death of the polyps. To test the effect of thermal stress on hydra species from two different taxonomic groups (13), *H. attenuata* and *H. oligactis*, 30 polyps of each species were transferred to prewarmed (33°C) hydra medium and examined for viability. The results demonstrate that polyps of *H. attenuata* (Fig. 1

Abbreviation: hsp, heat shock protein.

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A–C) are not dramatically affected by exposure to 33°C for at least 90 min. Thirty minutes after exposure (Fig. 1*B*) the polyps are indistinguishable from polyps kept at the normal culture temperature of 17–18°C (Fig. 1*A*). Sixty minutes later (Fig. 1*C*) the somewhat contracted tentacles indicate that the polyps are in unfavorable conditions. However, all *H. attenuata* polyps examined at this time still exhibited normal behavior as indicated by tentacle movement (see polyp in center of Fig. 1*C*) and response to touch. Within 1–2 hr after being returned to 17–18°C, these polyps were again normal.

All *H. oligactis* polyps (Fig. 1 *D–F*), however, were dramatically affected by exposure to heat. After 30 min at 33°C (Fig. 1*E*) the tentacles and body column of the polyps were contracted and the upper region of the body column was conspicuously swollen. The polyps did not respond to stimuli and did not recover upon returning them to 17–18°C. Longer exposure (60 min) to this elevated temperature leads to the death of the animals and tissue disintegration (Fig. 1*F*).

Other forms of environmental stress (e.g., exposure to cadmium ions or sodium azide) had a similar differential effect. Exposure of *H. oligactis* to concentrations of these agents that are sublethal for *H. attenuata*, resulted in the same pattern of phenotypic changes and death seen with heat-shocked polyps (data not shown).

Thermotolerance Can Be Acquired by *H. attenuata*, But Not by *H. oligactis*. Short-term exposure of organisms to moderately elevated temperature generally provides some protection against the lethal effects of a subsequent shift to a higher temperature (2, 6). To determine if this acquired thermotolerance can be observed in hydra, polyps were either directly incubated at a high, lethal temperature (*H. attenuata*, 34°C; *H. oligactis*, 33°C) or preincubated for 2 hr at an elevated but nonlethal temperature (*H. attenuata*, 30°C; *H. oligactis*, 25°C) and subsequently exposed to the high temperature. The ability of the polyps to withstand the high temperature treatment was then tested. The results of this experiment are shown in Fig. 2. After pretreatment at 30°C, *H. attenuata* shows 100% survival at the high temperature for at least 4 days (Fig. 2*A*). We conclude that during the preincubation period at 30°C the polyps developed thermotolerance and are protected against the deleterious effects of the high temperature. In *H. oligactis*, however, preincubation at 25°C or 30°C did not lead to any protection. In contrast, preincubated animals were found to be more sensitive to the deleterious effects of the high temperature and died sooner than non-preincubated control animals (Fig. 2*B*). Because there is much evidence for the assumption that thermotolerance develops as a result of the heat shock response (2), the failure to induce thermotolerance in *H. oligactis* could be explained if this species does not express a heat shock response.

***H. attenuata* Synthesizes Heat Shock Proteins.** To test directly the hypothesis that the acquisition of thermotolerance is dependent on the synthesis of heat shock proteins, we further examined the heat shock response in *H. attenuata* and *H. oligactis*. When cultures of *H. attenuata* were shifted from 18°C to 24°C or higher temperatures, the pattern of protein synthesis changed rapidly and dramatically. The most obvious change was the appearance of a major polypeptide of apparent molecular mass 60 kDa (Fig. 3*A*). Although induction of this protein was detectable over a broad temperature range (22–23°C), the level of synthesis depended on the stress temperature, with the highest level at the highest tempera-

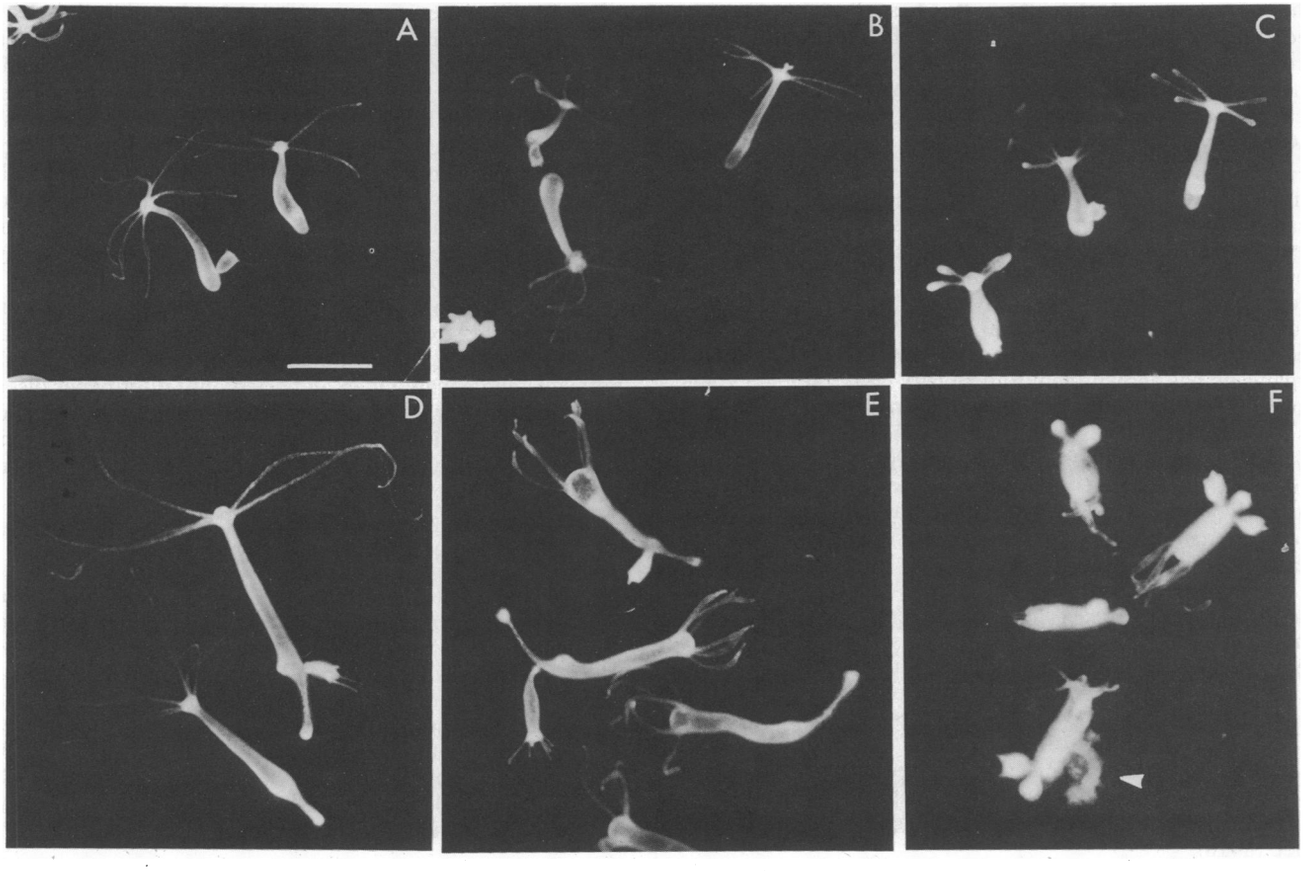

FIG. 1. Differential effect of heat shock on the morphology and viability of *H. attenuata* (*A–C*) and *H. oligactis* (*D–F*). Polyps were transferred to prewarmed (33°C) hydra medium and examined immediately (*A* and *D*), after 30 min (*B* and *E*), and after 90 min (*C* and *F*). Note the movement (slightly blurred tentacles) of *H. attenuata* polyps (*C*), indicating normal behavior. The arrow indicates disintegrating tissue. (Bar = 3 mm.)

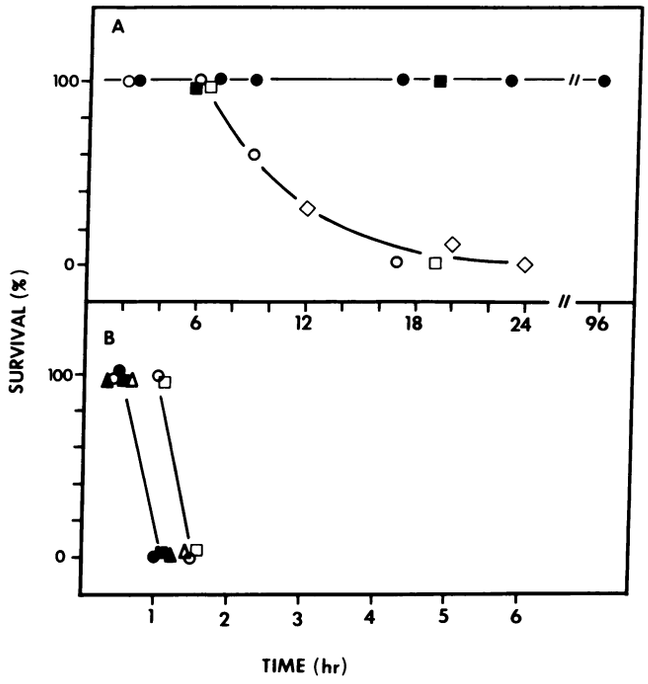

FIG. 2. Acquisition of thermotolerance in hydra. At $t = 0$ groups of 10 polyps were transferred from 18°C to either 25°C (▲) or 30°C (● and ■) and incubated for 2 hr. Such pretreated polyps were then subjected to a temperature of either 33°C (*H. oligactis*) or 34°C (*H. attenuata*). Controls consisted of groups of 10 polyps transferred directly from 18°C to 33°C or 34°C (△, ○, or □). (*A*) Results obtained with *H. attenuata*. (*B*) Results obtained with *H. oligactis*. At the indicated time the number of viable polyps was determined (different symbols represent different experiments). Note the differences between the time scales in *A* and *B*.

ture. We termed this major hsp hsp60. The observed changes in the pattern of protein synthesis occurred rapidly, requiring <1-hr exposure to the elevated temperature. In addition, another protein with a molecular mass of ≈80 kDa was synthesized in response to high-temperature exposure, although at levels much lower than the 60 kDa protein (Fig. 3*A*, ◁). Further analysis using SDS/12.5% polyacrylamide gels showed the additional presence of a 28-kDa hsp (data not shown). The hsps in the 80- to 90-kDa and 20- to 30-kDa size range are found in all eukaryotes (2), and the hydra proteins of this size may be members of this highly conserved group.

A characteristic feature of hsps is that their synthesis can be induced by a number of other stress treatments (2). To test whether hsp60 behaved in a similar way, we exposed *H. attenuata* to a variety of known inducers. We found that hsp60 was induced by cadmium chloride (25 μM) and sodium azide (0.5 mM) (data not shown). When hydra were exposed to both sodium azide and heat, the response is more dramatic than with either agent alone. Fig. 3*B* shows the protein synthesis pattern in polyps maintained at 17–18°C (lane 1) and after 1-hr exposure to a combination of 30°C and 0.5 mM azide (lane 2). Under these conditions only the characteristic hydra hsps, predominantly hsp60, were produced, whereas synthesis of most other proteins was repressed.

In all organisms studied so far the major heat shock gene encodes a 70-kDa protein (2). However, SDS gel electrophoresis of heat-shocked hydra proteins provides no evidence for the presence of an hsp of this molecular size. Fig. 3*C* shows a direct comparison between hsps found in *Drosophila* and those found in *H. attenuata*. Clearly, the hsp60 of hydra does not comigrate with any of the hsps of *Drosophila*. Because hsps can be subject to proteolysis during sample preparation (2), we examined the possibility that hsp60 might be a breakdown product of a 70-kDa protein. In one set of experiments the potent protease inhibitors phenylmethylsulfonyl fluoride (0.5 mM), sodium vanadate (0.2 mM), antipain (100 ng/ml), and leupeptin (100 μg/ml) were added to the SDS sample buffer immediately before homogenization. Upon SDS gel electrophoresis of samples treated in this way no change in the protein pattern was seen (data not shown). To

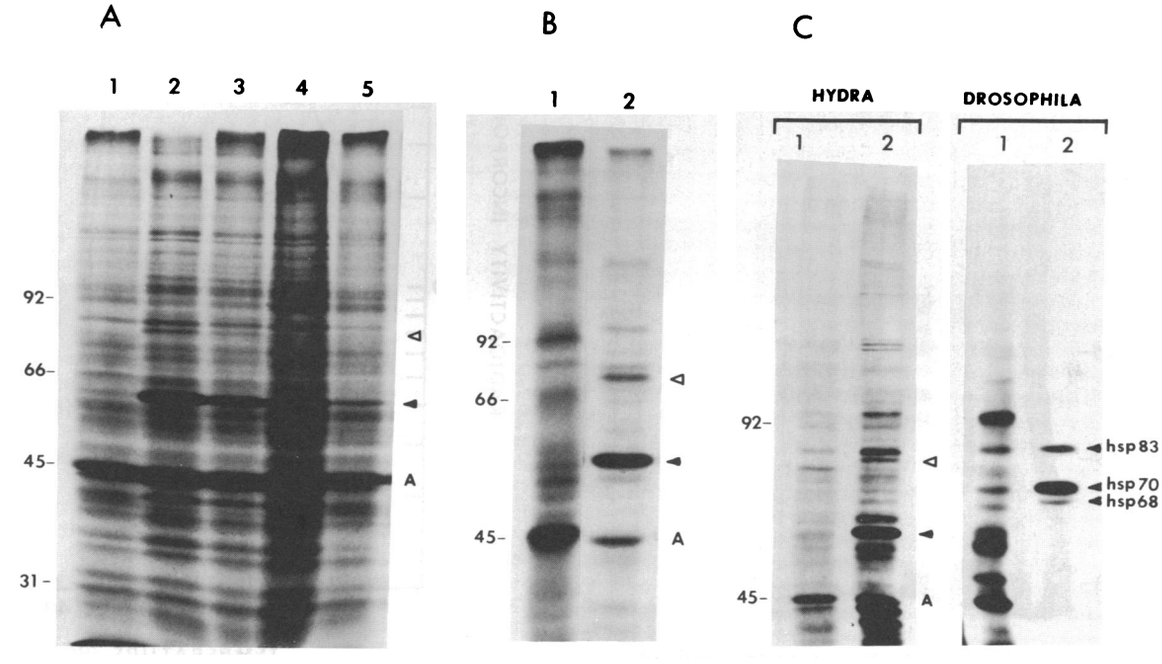

FIG. 3. Protein synthesis in heat-shocked polyps of *H. attenuata*. (*A*) Patterns of protein synthesis at 17–18°C (lane 1), 33°C (lane 2), 30°C (lane 3), 26°C (lane 4), and 24°C (lane 5). Protein extract from 10 polyps was loaded into each sample well of a 10% SDS/polyacrylamide gel. (*B*) Effect of sodium azide on heat-shocked polyps. Polyps were incubated for 1 hr at 30°C in medium containing 0.5 mM sodium azide (lane 2). Control polyps (lane 1) were maintained at 17–18°C. (*C*) Comparison of hsps in *H. attenuata* (lane 1 at 17–18°C; lane 2 at 30°C) and *Drosophila* (lane 1 at 23°C; lane 2 at 37°C). Hydra and *Drosophila* proteins were labeled as described in *Materials and Methods*. ◀, hsp60; ◁, 80-kDa hsp; and A, a protein that migrates to the expected position for actin.

further check for proteolytic activity in the hydra sample, *Drosophila* cells that had been labeled [^{35}S]methionine and heat-shocked were mixed with unlabeled heat-shocked hydra before homogenization. After SDS gel electrophoresis no degradation of the *Drosophila* hsps could be detected (data not shown). Finally, because hsp70 from *Drosophila* has been shown to have autoprotease activity (14) it is important to note that this activity is completely absent under our conditions (Fig. 1*C*). Thus, there is no evidence that hsp60 is the result of proteolytic cleavage of a larger protein.

In *H. oligactis* No New Proteins Are Induced by Heat Shock. Upon exposure of *H. oligactis* to elevated but nonlethal temperatures, we were unable to detect any qualitative changes in the protein synthesis pattern. As shown in Fig. 4, the patterns of protein synthesis for heat-shocked *H. oligactis* polyps (lanes 4 and 5) and control animals maintained at 17–18°C (lane 3) are identical. For comparison, lanes 1 and 2 show the effect of heat shock on *H. attenuata*. Fig. 4 also demonstrates that the combination of sodium azide and heat has a much more dramatic effect on *H. oligactis* than on *H. attenuata*. Whereas in *H. attenuata* (lane 2) this combination results in the production of the characteristic set of hsps and the decline of most other protein synthesis, in *H. oligactis* the same conditions result in the cessation of protein synthesis (lanes 6 and 7), suggesting a much higher sensitivity of *H. oligactis* polyps to this stress treatment. Absence of induction of hsps was seen in several strains of *H. oligactis* (data not shown). Because these strains have been isolated from different geographic locations (Switzerland, Holland, United States) this lack of response would appear to be a characteristic feature of this species and not simply a strain-specific trait.

Because the maximum induction temperature for hsps is known to vary (2), the lack of response in *H. oligactis* could be due to suboptimal test temperatures. Since a decrease in total protein synthesis is considered to be a constant and highly reproducible phenomenon occurring under severe heat shock conditions (1, 2), we examined the rate of protein synthesis in *H. oligactis* at various temperatures. Fig. 5 demonstrates that increasing the temperature to 26°C resulted in an increased incorporation of radioactive amino acids into total protein. However, when animals were incubated at temperatures >26°C, a rapid decrease in protein synthesis was seen. This is consistent with the finding that animals exposed to temperatures >33°C die within minutes (cf. Fig. 1 D–F). The findings indicate that in *H. oligactis* temperatures >26°C must be considered as heat shock conditions. Fig. 4 (lanes 3–5) shows that when exposed to these temperatures, *H. oligactis* polyps do not synthesize any heat shock proteins. Correspondingly, after preincubation at either 25°C or 30°C (Fig. 2) the polyps do not develop thermotolerance.

Correlation Between Presence of hsp60 and Thermotolerance in Other Hydra Species. Upon examining the heat shock response in different species of hydra in addition to *H. attenuata* and *H. oligactis*, we found that hsp60 is also found in *Hydra magnipapillata* (strain sf-1) (ref. 15; data not shown). As summarized in Table 1, of five additional species examined, all failed to synthesize any of the hsps expressed by *H. attenuata* or any other new species of proteins in response to the treatment. The absence of a heat shock response was correlated in all cases with a low level of resistance to environmental stress of the sort seen in *H. oligactis*.

Correlation with Ecological Data. Due to the absence of induction of hsp synthesis and the reduced tolerance to stress, we would expect to find species such as *H. oligactis* in a more restricted range of habitats than species such as *H.*

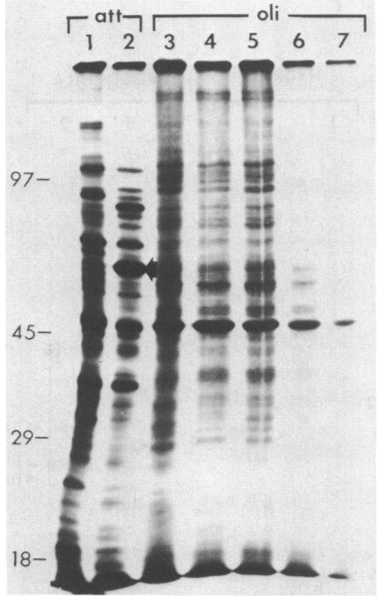

FIG. 4. Differential effect of heat shock on protein synthesis in *H. attenuata* (att) and *H. oligactis* (oli). *H. attenuata* at 16°C (lane 1) and at 30°C in medium containing 0.5 mM sodium azide (lane 2). *H. oligactis* at 16°C (lane 3), 26°C (lane 4), 30°C (lane 5), at 26°C in medium containing 0.5 mM azide (lane 6), and at 30°C in medium containing 0.5 mM sodium azide (lane 7). Proteins were analyzed using a 12.5% SDS/polyacrylamide gel system. Arrow, 60-kDa hsp.

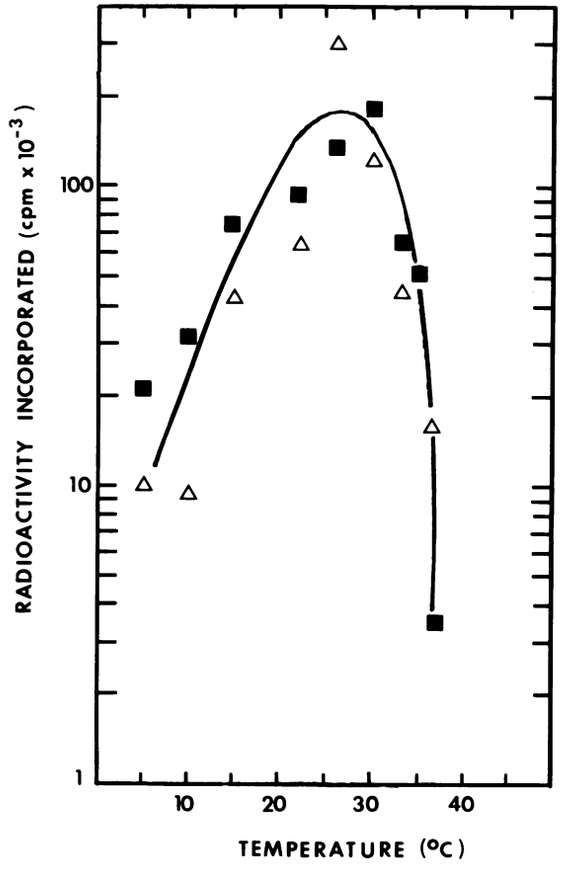

FIG. 5. Rate of protein synthesis in *H. oligactis* at various temperatures. The ordinate shows the incorporation of Tran^{35}S-label (ICN) into acid-insoluble material. The abscissa shows the test temperatures used. The two symbols represent two independent experiments.

Table 1. Correlation between presence of hsp60 and thermotolerance in several species of hydra

Species	hsp60	Thermotolerance
Hydra attenuata	+	+
Hydra magnipapillata (sf-1)	+	+
Hydra oligactis		
Swiss strain	−	−
United States strain	−	−
Dutch strain	−	−
Pelmatohydra robusta	−	−
Hydra carnea	−	−
Hydra utahensis	−	−
Hydra hymenae	−	−
Hydra circumcincta	−	−

The thermotolerance and the presence of hsp 60 were assayed for all species as described for *H. attenuata* in the text.

attenuata. Such habitats would be characterized by stable temperatures and absence of such materials as heavy metal ions. In support of our hypothesis are field observations (16) showing a direct correlation between an increase in temperature and the disappearance of *H. oligactis* from surface waters. Furthermore, consistent with our findings are previous studies on cultured polyps of *H. oligactis* (17) showing a low upper-lethal temperature. Thus, low upper-lethal temperatures appear to exclude *H. oligactis* from shallow pond habitats and also may contribute to their observed (18) strong seasonal fluctuation and rapid population decline after an increase of surface water temperature.

Summary. In examining the response of various species of hydra to heat and other forms of stress, we obtained unexpected results. We found species of hydra that are both unable to acquire thermotolerance and unable to synthesize a major hsp in response to stress. This finding was unexpected in light of the fact that organisms that evolved both before or after hydra synthesize hsp70 upon exposure to elevated temperature (2). In examining a total of eight different hydra species, we found a strong correlation between the presence or absence of hsp60 synthesis and the presence or absence of the ability to survive exposure to stress (Table 1).

The findings obtained in hydra appear to indicate that the heat shock response is not as universal as previously assumed. It might well be that selection to retain a strong heat shock response has been applied only to organisms living in relatively unstable habitats. The fact that, as far as we know, all of the organisms so far studied have been found in habitats characterized by changing temperatures and other forms of stress may explain why absence of hsp synthesis and inability to survive short-term exposure to stress has not been previously observed.

Finally, an unexpected result was the finding that the major hsp of hydra is not the virtually ubiquitous hsp70, but rather a 60-kDa protein. The relationship between hsp60 and hsp70 will be clarified when we have obtained sequence data from the gene encoding hsp60. Preliminary data show that an anti-hsp70 monoclonal antibody (provided by S. Lindquist, Department of Molecular Genetics and Cell Biology, University of Chicago), which reacts with 70-kDa proteins in a variety of different species, does not bind to hydra hsp60 (data not shown). Thus, hsp60 might be a truncated version of hsp70 lacking the domain that is recognized by the monoclonal antibody. An alternative and interesting possibility is that the hydra species synthesizing hsp60 may have recruited a protein evolutionarily unrelated to hsp70 to serve as their major hsp.

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