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Heat shock proteins and thermoresistance in lizards

(lizards/hyperthermia/adaptation/heat shock proteins)

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ABSTRACT **The synthesis of heat shock proteins (hsps) at normal physiological and elevated temperatures has been correlated with the natural adaptation of an organism to heat in nine lizard species studied. These species differ drastically by their adaptation to elevated temperature and represent a spectrum of forms isolated from various geographical regions of the Union of Soviet Socialist Republics. The synthesis of hsps belonging to the hsp70 family and their correspondent mRNAs have been compared at different temperature regimes. This analysis has shown that lizards inhabiting the Middle Asia deserts are characterized by a higher content of hsp70-like proteins at normal physiological temperatures (2- to 5-fold differences) when compared with the forms from central and northern regions of the European part of the Union of Soviet Socialistic Republics. Analysis of hsp70 mRNA at different temperatures substantiated these observations, showing evident correlation between adaptation of a given form to hyperthermia and the quantity of hsp70 mRNA in the cells under non-heat-shock conditions. The results obtained with a wide spectrum of ecologically different lizard species, coupled with other relevant data, enable us to propose a general rule applicable to poikilothermic organisms. This rule postulates the direct correlation between the characteristic temperature of the ecological niche of a given species and the amount of hsp70-like proteins in the cells at normal temperature.**

Heat or other environmental stresses have been shown to induce the synthesis of a family of proteins, the so-called heat shock proteins (hsps), in a wide variety of cells from yeast to mammalian systems (for reviews, see refs. 1–3). Recent work has focused on the role of hsps in the assembly, folding, and transport of other cellular proteins under different conditions. The level of these special activities of hsps, often termed "chaperonins", depends on the state of the general translational machinery of the cell (4–6). One of the more interesting aspects of thermal biology in different systems is the interrelation between the induction of hsp synthesis and the development of thermoresistance. Hsps are thought to protect cells from the toxic effects of short-term environmental stress (1–3). However, although much information about the structure of hsps and their cellular function has been accumulated, almost all these data were obtained by studying culture cells or isolated organs and tissues. Only scattered facts concern the role of hsps in providing whole-body adaptation to the close species inhabiting ecological niches with strikingly different temperature regimes (7–10).

Our studies focus on the heat shock response in nine lizard species that inhabit different environments and exhibit various levels of thermoresistance. We found a direct correlation between the level of thermoresistance of a species and both the quantity of hsps belonging to the hsp70 family and the correspondent mRNA in the cells at normal physiological temperature.

MATERIALS AND METHODS

Lizards. Characterization of nine lizard species used in the study is given in Table 1.

Heat Shock and Labeling of Recently Synthesized Proteins. Adult lizards collected in different regions were acclimated to 25°C on a 12:12 photoperiod for at least 3 days before use in the experiments. To induce hsp synthesis, we used 1-hr exposures to different temperatures in special preheated chambers. After heat shock treatment, the animals were kept for 1 hr at 25°C. Recently synthesized proteins were visualized by injecting [^{35}S]methionine (50 μCi/g of body weight; 1 Ci = 37 GBq) i.p. After injection, the lizards were placed back at 25°C for a 1-hr incubation period. At the end of this period, the animals were decapitated, and the liver was removed over ice and homogenized in cold buffer containing 50 mM Tris·HCl, pH 6.8, and a protease inhibitor (1 mM phenylmethylsulfonyl fluoride). The homogenate was filtered and precipitated with 4 vol of ethanol. The pellet was washed twice by ethanol and stored in 70% ethanol at −20°C.

Protein Electrophoresis and Fluorography. Protein precipitates were dissolved in lysis buffer (16) and processed for two-dimensional electrophoresis by the method of O'Farrel *et al.* (17). Fluorography was done according to Bonner and Laski (18) with slight modifications. After electrophoresis, gels were soaked in acetic acid, followed by incubation in 15% diphenyloxazole in glacial acetic acid for 30 min. Diphenyloxazole was precipitated by washing the gels in distilled water with subsequent drying. Scanning of two-dimensional fluorograms and immunoblots was done by using densitometers XL Ultrascan and Opton. The incorporation of [^{35}S]methionine into hsps was determined by staining two-dimensional gels with Coomassie R-250, cutting out the corresponding spots that were dried on Whatman 3MM filter, and counting the radioactivity.

Partial Proteolytic Digestion of [^{35}S]Methionine-Labeled hsps. Enzyme digests using SV8 protease from *Staphylococcus aureus* (Sigma) were done according to the method of Cleaveland *et al.* (19). After two-dimensional electrophoresis the gels were briefly stained with Coomassie blue. The protein spots in question were cut out and soaked in 10 ml of 0.1% SDS/1 mM EDTA for 30 min at room temperature. These gel fragments were placed into the wells of concentrating gel for one-dimensional electrophoresis. In this instance, 10 μl of Laemmli lysis buffer (16) containing 20% glycerol and 10 μl of protease SV8 in the same buffer were subsequently loaded. SDS/PAGE was done until the leader dye reached the boundary between concentrating and separating gels. The current was switched off, and after 30 min of proteolytic digestion the electrophoresis was continued. The results were visualized by fluorography.

Immunoblotting. The proteins were transferred onto nitrocellulose paper by following the procedure of Towbin *et al.* (20). Rabbit polyclonal serum N7 raised against calf hsp73 and purified by affinity chromatography with ATP-agarose

Abbreviation: hsp, heat shock protein.

Table 1. Characteristics of inhabitance and temperature range of behavioral activity and hsp induction of nine lizard species

Species and faunistic group	Habitat	Temperature range of species activity, °C			Temperature range of induction and continuing synthesis of hsp68, °C	Refs.
		Minimal	Optimal	Maximal		
Turanian group						
Phrynocephalus interscapularis	Sand desert	15–17	20–34	37–42	39–50	11, 12
Phrynocephalus raddei	Sand and clay desert	11–14	17–30	38–40	39–47	11, 12
Phrynocephalus helioscopus	Clay desert	7–10	15–30	36–42	37–47	11, 12
Crossobamon eversmanni	Sand desert	10–13	16–23	24–29	37–45	11, 12
Teratoscincus scincus	Sand desert	8–10	16–22	26–30	36–43	11, 12
Iranian–Afghan						
Agama caucasica	Rocky slopes and canyons	8–10	20–26	28–38	37–47	12, 13
European group						
Lacerta agilis	Mountains, steppe, forests, and gardens	7–10	20–25	27–35	36–42	12, 14, 15
Lacerta vivipara	Deciduous and coniferous forests	12–15	18–25	27–32	35–42	12, 15
Caucasian–Asia Minor group						
Lacerta saxicola	Rocky mountains	7–10	19–24	28–32	36–42	12, 15

(21) was donated by B. A. Margulis (Institute of Cytology, Leningrad). Binding of the antiserum was detected with a horse radish peroxidase goat anti-rabbit conjugate (Sigma), followed by incubation with 3,3′-diaminobenzidine.

RNA Isolation and Dot Blot Analysis. RNA extraction, nick-translation of the probe, and hybridization of filters were done as described by Maniatis *et al.* (22). The total RNA isolated from lizard's liver was directly spotted onto nitrocellulose paper by using a Hybri-Dot apparatus and hybridized with a ^{32}P-labeled plasmid, pxL16P, containing the *hsp70* gene of *Xenopus laevis*. The hybridization was carried out in a buffer containing 50% formamide at 37°C for 16 hr. The pxL16P plasmid was given to us by M. Bienz (Medical Research Council Laboratory, Cambridge).

Determination of Protein Concentration and [^{35}S]Methionine Incorporation. Before the electrophoresis, aliquots of the protein samples were spotted onto GF/C glass filters and washed with 7% trichloroacetic acid and ethanol; radioactivity was then counted. To determine protein concentration, the proteins were precipitated with ice-cold 10% trichloroacetic acid, washed with ethanol, and dissolved in 0.1 M NaOH. Protein concentration was determined by the standard Lowry method (23).

RESULTS AND DISCUSSION

Features of Heat Shock Response in Lizard Species. Nine lizard species used in these experiments belong to six genus within three families (Agamidae, Gekkonidae, Lacertidae). According to their origin, they belong to four faunistic complexes (Table 1). The species were chosen for the analysis on the basis of differences in the temperature of their ecological niches. Experiments using one-dimensional SDS electrophoresis revealed the presence of 85-, 68-, and 25-kDa hsps after heat shock in the liver cells of all lizard species studied, hsps of 68 kDa and 85 kDa being the major components of heat shock response. A representative autoradiogram is shown in Fig. 1*a*. However, significant differences have been demonstrated regarding the temperature range necessary for hsp induction. It is evident from Table 1 that, in contrast to the representatives of the Lacertidae family, which can synthesize hsps within the 35–42°C range, the induction of hsps in all diurnal lizards of southern origin, such as *P. interscapularis* starts at temperatures at least 2–3°C higher and proceeds up to 47–50°C. Nocturnal species of southern origin (e.g., two gecko species: *T. scincus* and *C. everesmani*) occupy intermediate position in this respect (Table 1). Thus, there exists a positive correlation between the range of stress temperature and the average temperature of an ecological niche inhabited by a species. Similar data have been obtained by us when different species of silkworm and unicellular lizard parasites *Leishmania* were studied (7, 8) and by other authors investigating the heat shock response in different fish species (24, 25). It is noteworthy that two representatives of the Lacertidae family, *L. vivipara* and *L. saxicola*, practically never can be found in nature at the temperature that can induce hsps in their cells. In other words, such a temperature elevation really represents stress treatment for these lizards, and the hsps induced are necessary for the protection of an organism from the stress, thereby providing species survival. On the other hand, in thermoresistant and thermophylic species, such as *P. raddei*, *P. helioscopus*, and, in particular, *P. interscapularis*, which exhibit average body temperature during the summer period ranging from 41 to 44°C (11, 12), the induction and active synthesis of hsp68 occurs within a temperature interval normal for the species. However, in all lizards studied, temperature elevation induced the synthesis of major hsps, which reaches maximum at critical temperatures when hsps became the major proteins synthesized in the cell; concurrently the synthesis of normal cellular proteins drops. Further increase in the temperature caused lizards to go into a coma-like state from which the animal did not recover.

The detailed pattern of hsps expression in lizard species has been obtained by using two-dimensional electrophoresis. Besides the aforementioned hsp85, -68, and -25, in all species studied the presence of hsp46-48, -35, and other minor peptides has been demonstrated (data not shown). The analysis showed that in all species studied, hsp68, the major component of the heat shock response in lizards, is represented by two distinct isoforms, which we have named hsp68(+) and hsp68(−). Fig. 1 *b–e* represents fragments of a two-dimensional fluorogram of proteins synthesized in liver cells of *P. interscapularis* and *L. agilis* at 25°C and after heat shock, respectively. It is evident that the former species is characterized by a higher level of constitutive synthesis of hsp68 at the normal temperature. A similar pattern is characteristic for moderately thermophylic species (*P. raddei* and *P. helioscopus*). However, while in the cells of *P. interscapularis*, significant synthesis of the hsp68(−) isoform is evident at 25°C; in the other cases prominent synthesis of the hsp68(+) isoform is clearly seen (Fig. 1 *b* and *d*). Although the synthesis of both isoforms was increased after tempera-

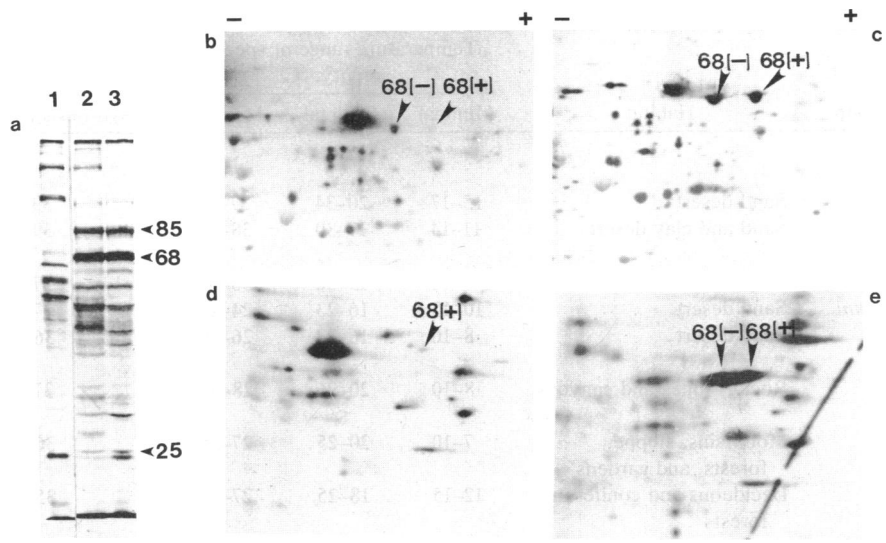

FIG. 1. Analysis of heat-shock proteins synthesized in liver cells of lizards at different temperatures. (*a*) Autoradiogram of 10% SDS/PAGE gel of [^{35}S]methionine-labeled liver proteins of *A. caucasica* (lane 1, 25°C; lane 2, 43°C; and lane 3, 45°C). Positions and sizes of major lizard hsps in kDa are indicated at right. Fluorograms of two-dimensional electrophoresis of liver proteins, isolated from *P. interscapularis* (*b* and *c*) and *L. agilis* (*d* and *e*) at normal temperature of 25°C (*b* and *d*) or after heat shock treatment of 42°C (*e*) or 45°C (*c*). (+) and (−) isoforms of hsp68 are indicated by arrowheads.

ture elevation, at critical temperatures the synthesis of hsp68(+) dropped virtually to zero level, while synthesis of hsp68(−) reached maximal value.

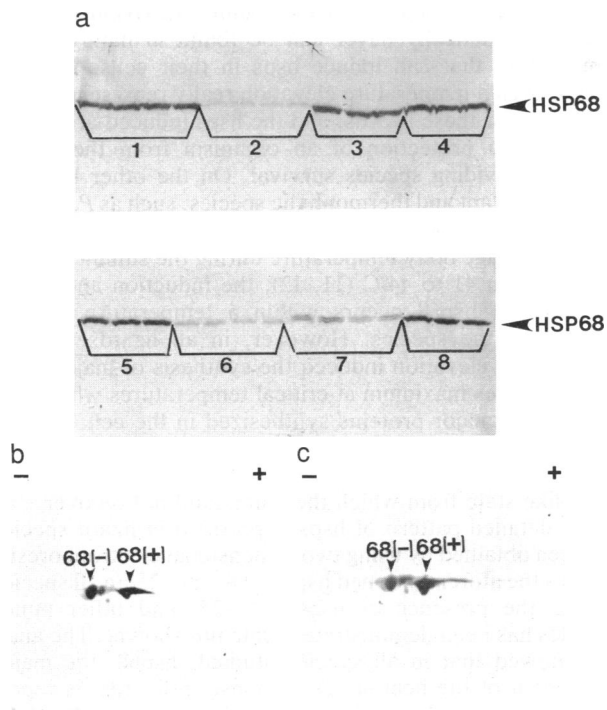

FIG. 2. Immunoblots of hsps isolated from different lizard species. (*a*) Proteins (5 μg of protein per lane) were separated on SDS/PAGE and transferred to nitrocellulose. Proteins were detected by using the polyclonal antibodies N7 raised against calf hsp73. Lanes: 1, *P. interscapularis* (100%); 2, *L. vivipara* (17%, 5%); 3, *P. raddei* (78%); 4, *T. scincus* (72%); 5, *A. caucasica* (91%); 6, *L. saxicola* (37%); 7, *L. agilis* (46%); and 8, *C. eversmanni* (82%). Each lane represents protein isolated from one individual. Relative hsp68 contents in % (in parentheses) are based on scanning the corresponding bands of immunoprecipitation. (*b* and *c*) Immunoblot analysis of *P. interscapularis* proteins separated by two-dimensional electrophoresis by using the same antibodies as in *a* at 25°C (*b*) and 45°C (*c*) (150 μg of protein per lane).

Structural-Immunological Characteristic of hsp70-Like Proteins and Pattern of Their Synthesis at Elevated Temperature. Fig. 2*a* represents one-dimensional immunoblotting of proteins isolated from different lizard species treated with polyclonal antibodies raised against calf hsp73. The only band of precipitation clearly seen in all species indicates high conservation of these proteins in evolution. Two-dimensional immunophoregrams representing liver proteins of *P. interscapularis* (Fig. 2 *b* and *c*) clearly illustrate that hsp68(−) and hsp68(+) belong to the same hsp70 family. Comparison of these immunoblots with correspondent autoradiograms (Fig. 1 *b* and *c*) indicates complete coincidence of sites of immunoprecipitation with correspondent spots on the autoradiogram. It is necessary to emphasize that both constitutively synthesized and heat-inducible hsp70-like proteins are present in the liver cells of this species at 25°C (Fig. 2*b*), whereas temperature elevation leads to different changes in the expression of both isoforms (Fig. 1 and Fig. 2*c*).

The high level of homology between hsp68(−) and hsp68(+) has been further substantiated by using limited proteolysis of these proteins by protease SV8 (Fig. 3). However, when comparing both isoforms besides several

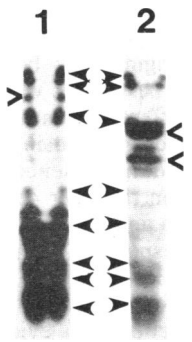

FIG. 3. Fluorogram resulting from proteolytic digestion of [^{35}S]methionine-labeled and SDS/PAGE-purified hsp68(−) and hsp68(+) of *P. interscapularis*. These proteins were labeled for 3 hr *in vivo* at 25°C after heat shock treatment (45°C for 1 hr). Enzyme digestion by SV8 protease from *S. aureus* was done as described (21). Homologous (►) and nonhomologous (>) products were derived from partial proteolytic digestion.

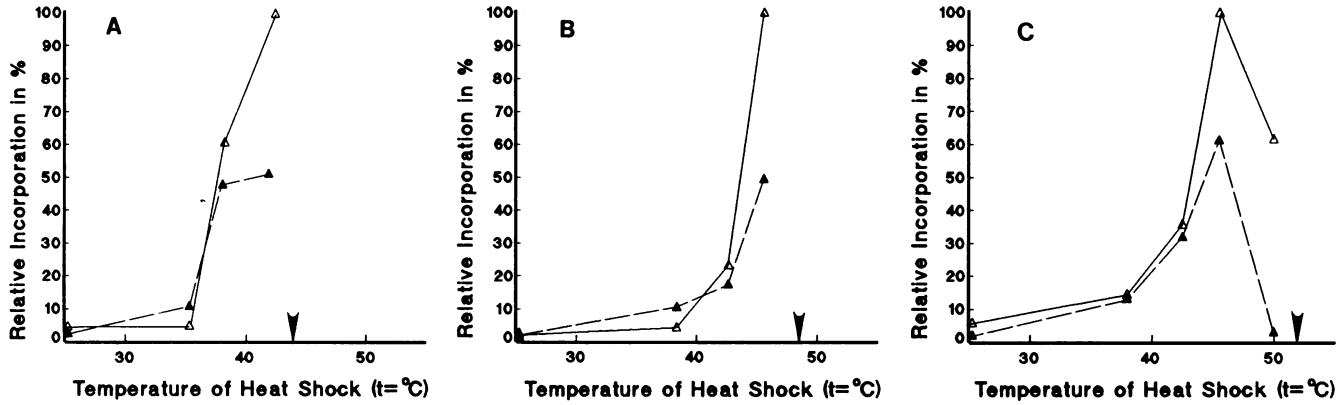

FIG. 4. Comparison of hsp68(−) and hsp68(+) synthesis in different desert species of lizards. (*A*) *T. scincus*. (*B*) *P. raddei*. (*C*) *P. interscapularis*. △, hsp68(−); ▲, hsp68(+). The temperatures that caused lizards to go into a coma-like state are indicated by arrows. Maximal incorporation of [^{35}S]methionine into hsp68(−) after heat shock treatment was arbitrarily taken as 100%; all other measurements are expressed relative to this value. All determinations represent the means of triplicate measurements.

methionine-containing peptides with identical molecular masses, one can see a few nonhomologous peptides. These data indicate that hsp68 isoforms in lizards are encoded for by two related but different genes. The fluorogram also illustrates higher rate of hsp68(−) synthesis, thus confirming the results of other above-mentioned experiments. Moreover, differences in the independent expression of these two proteins at elevated temperature were also detected. Thus, for *T. scincus* and *P. raddei*, hsp68(+) represents the earliest inducible protein synthesized at 36° and 38°C, respectively. Further temperature elevation results in approximately the same level of synthesis of both isoforms, whereas at critical temperature clear-cut dominance of hsp68(−) is evident. This conclusion is also true for another thermophylic species, *P. interscapularis*. However, in this case, the rate of hsp68(−) synthesis exceeds that of hsp68(+) at all temperatures tested (Fig. 4*a–c*).

Previously, Lindquist and Didomenico (26) described asynchronous syntheses of hsps belonging to different families in *Drosophila*. Independently regulated synthesis of two proteins belonging to the hsp70 family in lizards enables one to propose a different role for these proteins in the heat shock response. In other words, hsp68(+), which is synthesized over a broad range of optimal and moderately high temperatures, resembles in this respect cognate proteins of mammals, *Drosophila*, and yeast (2, 3). On the other hand, hsp68(−), which is usually actively synthesized only at critical and subcritical temperatures (*P. intrascapularis* being an exception), probably represents the major inducible protein and plays a pivotal role in protecting cells from thermal damage under conditions of severe temperature stress.

The Content of hsp68 Proteins and the Rate of Their Synthesis in Lizards from Contrasting Temperature Niches. The results of immunoblot analysis shown in Fig. 2*a* clearly demonstrate a strong positive correlation between the level of hsp68 in the cells of a lizard species at normal physiological temperature and the average temperature of an ecological niche inhabited by that species (Table 1). In general, thermophylic species have a higher content of hsp68 in their cells. This relationship is especially evident when species from mostly contrasting environments are compared. Thus, 5 to 6-fold differences in the hsp68 content under non-heat-shock conditions have been revealed when the most thermophylic species *P. interscapularis* is compared with *L. vivipara*, which inhabits the northern regions of the European part of the Union of the Soviet Socialistic Republics. A linear relationship is apparent between the increase in the average temperature of the ecological niche of a species and the enhanced content of hsp68 in the cells. It is noteworthy that while a high hsp68 content is characteristic for all species of the southern origin, certain differences may be also seen within this group between diurnal and nocturnal species. Thus the hsp68 content in two nocturnal species, *T. scincus* and *C. eversmanni* was at least 20% lower when compared with a typical diurnal species such as *P. interscapularis* (Fig. 2*a*). A similar correlation is evident when comparing species differing by the altitude of their ecological niche. Thus, the cells of *L. saxicola*, which inhabits humid Caucasus canyons and may be found at altitudes up to 3500 m, contain less hsp68 by a factor of 2.5 in comparison with *A. caucasica*, which inhabits the low hills of Kopet-Dug, an area characterized by a hot and dry climate (Fig. 2*a*).

High Level of hsp68 mRNA Transcripts in Lizard Species of Southern Origin. The high level of hsp70 group proteins found in the cells of the thermophylic lizards may be from a high constitutive expression of the corresponding genes at a normal physiological temperature. Hybridization of dot blots containing RNA from different lizard species with the hsp70 clone of *X. laevis* clearly indicates drastic differences in mRNA content of the species studied. A relatively high level of hsp68 mRNA at 25°C has been demonstrated in all thermophylic species and to a significantly less degree in *T. scincus*, a typical nocturnal form. In contrast, only very faint hybridization was demonstrated in *L. agilis* (see Fig. 5 and Table 1). Another feature characteristic of thermophylic species is the ability to increase the intensity of hsp68 transcription up to subcritical temperatures, whereas in *L. agilis* this response is not so dramatic. These results suggest that the heat shock response is a physiologically relevant phenomenon in intact organs of the adult lizards and that *in vivo* induction of the major heat shock genes is at the level of transcription.

Interestingly, high levels of hsp70 mRNA synthesis at 45° and 49°C have been previously demonstrated by us in a

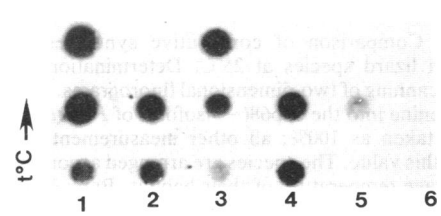

FIG. 5. Dot blot analysis of 5 μg of total RNA isolated from lizard liver and probed with a plasmid containing *X. laevis hsp70* gene. Columns: 1, *P. interscapularis* (25°C, 42°C, and 45°C); 2, *P. raddei* (25°C and 43°C); 3, *T. scincus* (25°C, 37°C, and 41°C); 4, *P. heliscopus* (25°C and 43°C); 5, *L. agilis* (25°C and 42°C); 6, yeast tRNA.

southern species of silkworm (7). In this case, the increase in hsp synthesis at temperatures as high as 45–49°C was unexpected because all other insect species studied in this respect, such as *Drosophila*, could not synthesize hsps at temperatures >40°C (26). Therefore, the analysis of hsp70 mRNA at standard (25°C) and elevated temperatures in the nine lizard species studied correlates well with the hsp content at corresponding temperatures. A similar relationship was revealed in the experiments evaluating the rate of constitutive synthesis of both hsp68 isoforms. Fig. 6 clearly illustrates that the decrease in the rate of constitutive synthesis of these proteins correlates well with the decrease in the average temperature characteristic for a species-specific ecological niche.

The data presented here suggest that in parallel with behavioral adaptation to heat characteristic for all animals of deserts (14), some molecular mechanisms conferring heat resistance to an organism at the cellular level have also been fixed during evolution. The higher content of hsp70-like proteins found in the cells of thermophylic species at normal temperature probably represents one of such adaptive mechanisms.

Previously, *Xenopus* hsp70 genes were shown to be highly and constitutively active during oogenesis and to become heat-inducible in early embryos (27). The data of Bienz (28) suggested that unshocked oocytes might contain a low level of active heat shock transcription factor, which would, under normal conditions, activate heat-shock gene promoters. Such a model could also explain our results, if one assumes that during evolution natural selection favors different levels of heat shock transcription factor in related forms (e.g., lizard species) depending on the average temperature of their inhabitance. A higher concentration of hsps in the species of southern habitats in comparison with northern habitats seen under normal non-heat-shock conditions apparently reflects the readiness of such forms to react to the abrupt changes in the environmental temperature. Under desert field conditions, the temperature of sand surface and an animal's burrow may differ by 15–20°C (11–15). In other words, the diurnal animals may encounter severe heat stress when hunting or escaping from a predator. The data reported here coupled with the results of our previous studies on this subject with other model systems (different species of silkworm and *Leishmania*) enable us to postulate a general rule. This rule holds, at least, for poikilothermic organisms and states that there is a strong positive correlation between the content of hsp70-like proteins in the cells of a given animal form under normal non-heat-shock conditions and the average temperature of that animal's ecological niche. Therefore, the increased constitutive expression of heat-shock genes belonging to the hsp70 family apparently represents a molecular mechanism providing a species survival under high-temperature conditions typical for deserts of Middle Asia. These data favor the idea of the importance of heat-shock proteins in the induction of whole-animal thermoresistance.

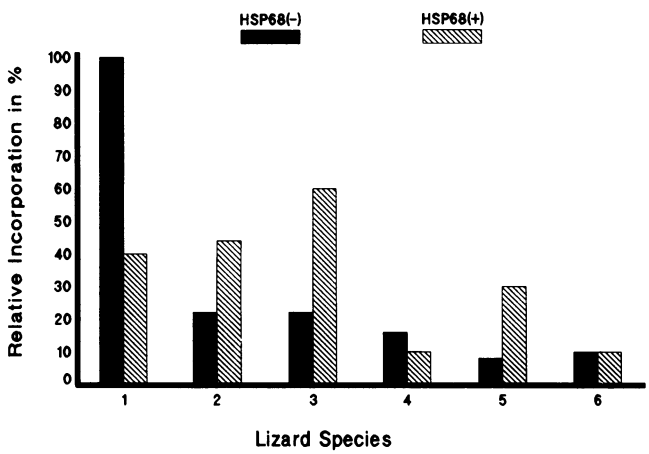

FIG. 6. Comparison of constitutive syntheses of two hsp68 isoforms in lizard species at 25°C. Determinations represent the results of scanning of two-dimensional fluorograms. Incorporation of [^{35}S]methionine into the hsp68(−) isoform of *P. interscapularis* was arbitrarily taken as 100%; all other measurements are expressed relative to this value. The species are arranged according to decrease in the average temperature of their habitat. Bars: 1, *P. interscapularis*; 2, *P. raddei*; 3, *P. helioscopus*; 4, *T. scincus*; 5, *L. agilis*; and 6, *L. saxicola*.

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