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Effects of Heat Treatment on the Symbiotic System of an Aphid Mycetocyte

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Abstract

The growth of the pea aphids that had been heat-treated at 37°C for 3 hr or longer at an early nymphal stage was severely retarded. These treated insects began viviposition significantly later than the control or did not produce progeny depending on the length of the heat treatment. The heat-treated insects did not synthesize symbionin, an endosymbiont-specific protein, and lost their pre-existing symbionin over the course of their post embryonic development. Electron microscopic observations suggested that abnormal embryos found in the heat-treated insects do not have normal endosymbionts in their mycetocytes.

Keywords: heat shock, aphid, endosymbiosis, symbionin, sterility, chaperonin, stress

1. Introduction

Intracellular symbiosis in the aphid mycetocyte is one of the best systems in which to study the interaction between two types of genomes in a single cell, the first of the eukaryotic host and the second of the prokaryotic symbionts. Also, this system might be useful in experimental search for the origin of cellular organelles (Margulis, 1970, 1981; Ishikawa, 1984a, 1989).

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Aphid endosymbionts *in vivo* are apparently regulated by the host so that they may produce only one protein, symbionin (Ishikawa, 1982). Since symbionin is not only abundant in the endosymbiont but also synthesized highly preferentially by the endosymbiont *in vivo* (Ishikawa, 1982), it may be a key protein in the maintenance of the symbiotic system. While on two-dimensional gel electrophoresis symbionin is an acidic protein with a molecular mass of 63,000, an electron micrograph of the negatively-stained molecules suggested that native symbionin is a 14 subunit homooligomer of 63,000 which is composed of two stacked rings of 7 subunits each (Hara and Ishikawa, 1990). Antigenicity and N-terminal amino acid sequence suggested that symbionin is highly homologous to the groEL protein, a heat shock protein of *E. coli* essential for cell viability and bacteriophage capsid assembly (Hendrix, 1979; Hara et al., 1990a,b, 1991). Symbionin is probably encoded by the endosymbiont's genome (Ohtaka and Ishikawa, unpublished), and synthesized by its ribosomes (Ishikawa, 1982; 1984b).

Under *in vitro* conditions and in the aged host, symbionts are able to produce numerous protein species other than symbionin (Ishikawa, 1984b,c). It seems that as the control of the host over the endosymbiont is somehow weakened, symbiont's other genes begin to be expressed (Ishikawa, 1984d). Of interest is how this control mechanism works.

To know this, a promising approach will be to see what entails the destruction of the symbiotic system. It has been reported that prokaryotic endosymbionts in aphids are successfully eliminated with various antibiotics (references cited in Houk and Griffiths, 1980; Fink, 1952; Brooks, 1963; Chang, 1974) and amoebae (Jeon and Ahn, 1978), it has been shown that heat treatment also brings about the destruction of their intracellular symbiotic systems.

In the present study we have demonstrated that brief heat treatment of newborn nymphs of the pea aphid causes elimination of the endosymbionts from the host insects.

2. Materials and Methods

Materials

A long-established parthenogenetic clone of pea aphics, *Acyrthosiphon pisum* (Harris) was maintained on young broad bean plants, *Vicia faba* (L.) at 15°C in a long-day regime with 18 hr light/6 hr dark.

Injection of antibiotics and radioactive precursor

Injections were done after anaesthetizing the insects in a stream of CO_2. In brief, this was performed by inserting an ultra-thin glass capillary tube into the second or third right leg. The capillary was connected to an air pump (Millipore) by a polyethylene tubing. A semi-quantitative injection was done by applying the air pressure on the solution in the capillary for a short, fixed length of time (Ishikawa, 1978). Each insect was injected with about 0.1 μl of L[^{35}S]methionine (1160 Ci/mmole, ICN) at 11.33 mCi/ml. The effect of cycloheximide was examined by injecting 0.1 μl of the drug at 200 μg/ml (about 10 ng/mg of body weight).

Gel electrophoresis of protein

Whole bodies of insects were homogenized in lysis buffer (0.06 M Tris-HCl, 10% (v/v) glycerol, 2% (w/v) sodium lauryl sulfate, 5% (v/v) β-mercaptoethanol, pH 6.8), and heated in boiling water for 5 min. Insoluble materials were removed by centrifuging at 5,000 rpm at room temperature.

Polyacrylamide gel electrophoresis of protein samples was performed as described by Laemmli (1970) using a 3–10% tandem slab gel ($120 \times 120 \times 1$ mm). Proteins in lysis buffer were electrophoresed at 30 mA constant current at room temperature until the bromophenol blue tracking dye migrated to the end of the gel (about 2.5 hr). The slab gel was stained for 18 hr with Coomassie blue in a solution containing 50% (v/v) methanol, 7.5% (v/v) acetic acid and 1% (w/v) trichloroacetic acid and destained by shaking in 7.5% (v/v) acetic acid containing 5% (v/v) methanol. Fluorography (Bonner and Laskey, 1974) of the gel was performed using Enlightening (NEN) and Fuji X-ray film (RX) at -80°C. Dried gel slabs were exposed to film for about 20 hr.

Electron microscopy

Embryos were dissected from adult insects, fixed overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C, washed twice with 0.1 M cacodylate buffer (pH 7.4) and subjected to the second fixation in 1% OsO_4 containing 0.1 M cacodylate buffer (pH 7.4) for 2 hr on ice (Karnovsky, 1965). The fixed tissues were stained *en bloc* in 1% uranyl acetate for 40 min at room temperature, dehydrated in a graded ethanol series and embedded in a mixture of EM-Spurr set (NSA, DER-736, ERL-4206, S-1) (Nissin EM). Thin sections were stained with lead citrate and observed in a JEM 100CX electron microscope.

3. Results

Survival rate

Nymphs obtained within 12 hr of viviposition were divided into groups of 20 individuals, and kept at 15°C, 37°C or 42°C in a breeding container, in which young broad bean plants were grown. Temporal survival rates were determined at these temperatures (Fig. 1). No mortality was observed in the

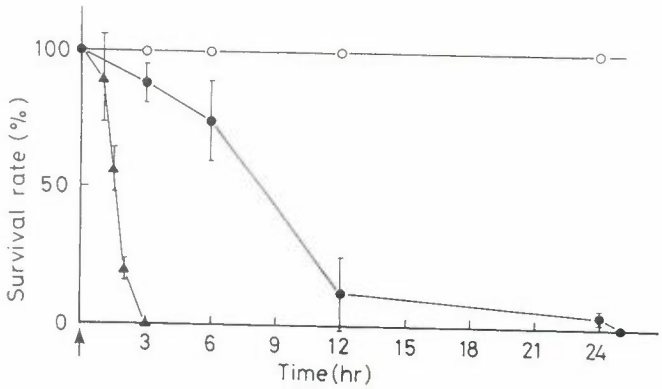

Figure 1. Survival rate of heat-treated insects. Newborn nymphs were divided into groups of 20 individuals, and kept at 15°C (○), 37°C (●), or 42°C (▲) in breeding containers in which young broad bean plants were grown. Individuals that survived were counted at intervals after the beginning of heat treatments. Arrow indicates the beginning of heat treatments. Vertical bars represent standard deviations (n=3).

control group (15°C), while 100% mortality occurred at 37°C after 25 hr and at 42°C after 3 hr.

Growth rate

Newborn nymphs, exposed to 37°C for 6 hr or 12 hr or unexposed, were maintained at 15°C as described above. Growth rates were determined by weighing them in groups at different time intervals. The growth of heat-treated insects was severely retarded (Fig. 2). Diminished body weight was directly related to length of treatment at 37°C. In addition, while the control insects began to produce offspring at about the 13th day of birth, the heat-treated insects began viviposition significantly later or did not produce offspring at all throughout their entire life.

Insects treated at 37°C for 12 hr not only did not produce progeny but their body color was unusually bright yellow (Fig. 3). On the other hand, longevity of these sterile insects was equal to, or even longer than, that of the control (data not shown).

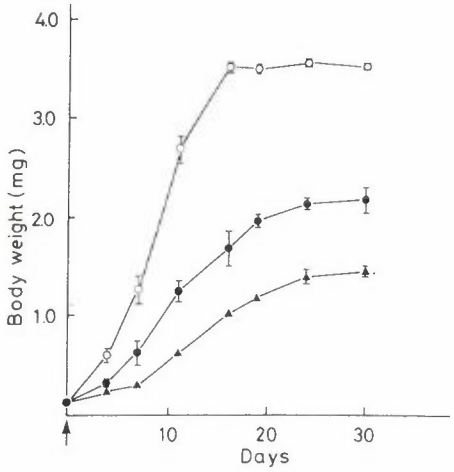

Figure 2. Post-embryonic growth of heat-treated insects. Newborn nymphs were treated at 37°C for 6 hr (•) or 12 hr (▲), and raised afterward at 15°C in a long-day regime. The growth was determined by weighing them in groups at intervals. The control insects were kept throughout at 15°C (○). Arrow indicates the day of birth. Vertical bars represent standard deviations (n=3).

Protein synthesis in heat-treated insects

To examine biochemical changes in the heat-treated insects which are liable to sterility and growth retardation, protein synthesis in the whole tissue of insects was studied. Twenty day old, apterous insects, which were exposed to 37°C at an early nymphal stage for 3 to 12 hr, were injected with [^{35}S]methionine and the proteins were extracted 3 hr later. When necessary,

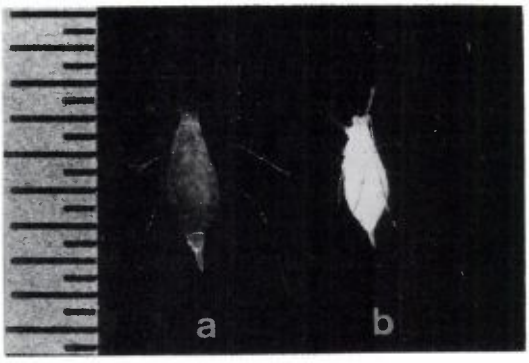

Figure 3. Twenty-day apterous insects. (a) Control; (b) treated at 37°C for 12 hr at the newborn stage. Scale represents 0.5 mm.

the insects were injected with cycloheximide 3 hr before the administration of the radioactive precursor. It has been known that under these conditions the only protein synthesized by unheated insects is symbionin (Ishikawa, 1982; 1984b). Protein samples were subjected to separation on one-dimensional gel electrophoresis. When the gel was stained with Coomassie brilliant blue (CBB) (Fig. 4a), it was noted that one protein band indicated by an arrow was missing

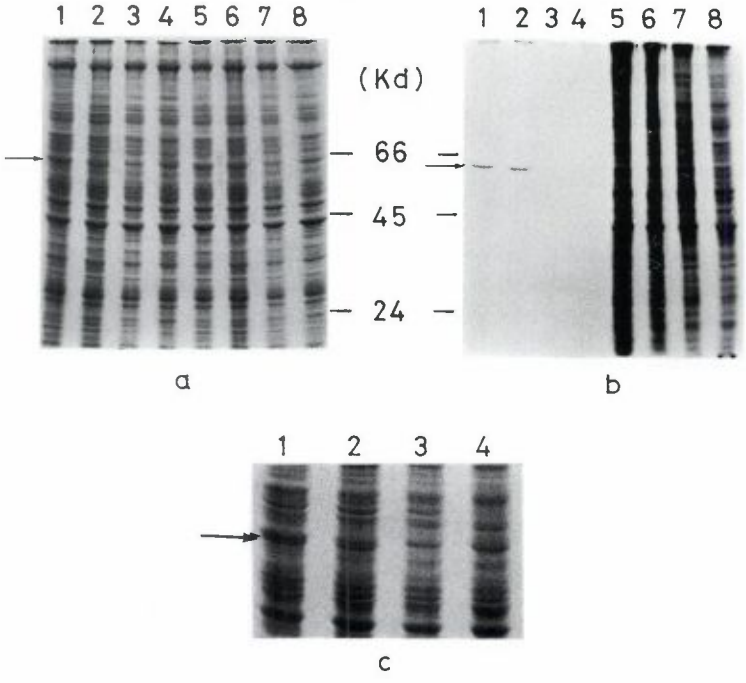

Figure 4. SDS-polyacrylamide gel electrophoresis of proteins in heat-treated insects. Twenty-day insects were injected with L[^{35}S]methionine and the proteins extracted 1 hr later. Groups of insects were treated at 37°C for various lengths of period at their newborn stage. The proteins were resolved on polyacrylamide gel electrophoresis as described by Laemmli (1970). In several experiments, cycloheximide was injected into the insects 3 hr prior to the injection of the radioactive precursor. (a) Stained with Coomassie blue; (b) fluorographed; (c) part of (a), enlarged. 1, 5 Untreated insects; 2, 6 treated at 37°C for 3 hr; 3, 7, treated at 37°C for 6 hr; 4, 8, treated at 37°C for 12 hr; 1–4, injected with cycloheximide. Arrows indicate the position of symbionin.

from the samples extracted from the insects treated at 37°C for 6 hr (lanes 3 and 7, see also lane 3, Fig. 4c) and 12 hr (lanes 4 and 8, see also lane 4,

Fig. 4c). No other difference between the proteins from the heat-treated and control insects was apparent.

A fluorogram of the same gel is shown in Fig. 4b. An arrow in the figure indicates the band due to symbionin (Ishikawa and Yamaji, 1985), the only protein that was synthesized by the control insects under the influence of cycloheximide (lane 1). Upon comparing Fig. 4b with 4a, it was indicated that the protein missing from the heat-treated insects (lanes 3, 4, 7 and 8, Fig. 4a; lanes 3 and 4, Fig. 4c) is symbionin. While the 3 hr-treated insects were able to synthesize symbionin (lane 2, Fig. 4b), those treated for 6 hr or longer did not synthesize the protein (lanes 3 and 4, Fig. 4b). The same is true for the insects which did not receive cycloheximide (lanes 5–8, Fig. 4b). In insects treated at 37°C for 6 hr (lane 7) and 12 hr (lane 8), the band due to symbionin was selectively missing.

Diminution of symbionin during post-embryonic development of the heat-treated insects

While the amount of symbionin in normal insects remained almost unchanged during post-embryonic development (Ishikawa et al., 1985), Fig. 4 clearly indicates that the insects heat-treated at the newborn stage neither contain nor synthesize symbionin when grown up. To estimate changes in the amount of symbionin during the course of post-embryonic development of the heat-treated insects, proteins of the whole insects at three stages of development were resolved on polyacrylamide gel.

Insects that had been treated at 37°C for 12 hr at the newborn stage were raised at 15°C under a long-day regime. Shown in lane 1 of Fig. 5 is a resolution of proteins from untreated insects at the newborn stage as a control. Comparison of thickness of the bands due to symbionin suggested that the symbionin content in the heat-treated insects decreases during post-embryonic development from a normal level at the newborn stage (lane 2, Fig. 5) to none at 10 days after birth (lane 4, Fig. 5).

Electron microscopic observation of the mycetocytes of the heat-treated insects

To observe the morphological changes accompanying the biochemical events observed, the mycetocytes in the embryos of normal and heat-treated insects were examined in an electron microscope (Fig. 6). Figure 6a shows part of a normal embryonic mycetocyte containing numerous endosymbionts. When sections of the embryo taken from a parent treated at 37°C for 12 hr at its newborn stage were examined, no symbiont such as in Fig. 6a was observed in

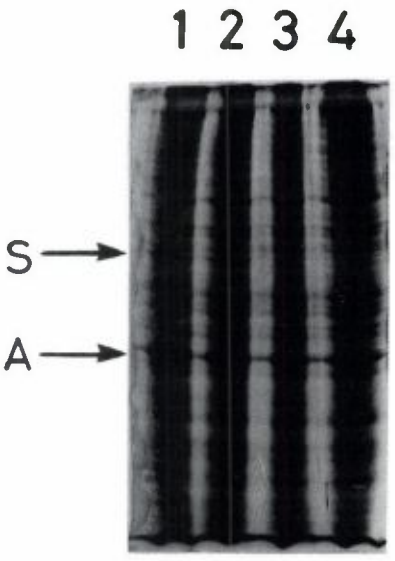

Figure 5. Diminution of symbionin in heat-treated insects. Insects that had been treated at 37°C for 12 hr at their newborn stage were raised at 15°C under a long-day regime. Proteins were extracted from these insects at various stages and resolved on polyacrylamide gel. 1, Newborn nymphs, untreated; 2, newborn nymphs immediately after the treatment; 3, insects 6 days after the treatment; 4, insects 10 days after the treatment. S, Symbionin; A, actin.

the mycetocyte. Instead, there were many vacuole-like structures that might otherwise be occupied by the symbionts (Fig. 6b). In particular, it is noteworthy that nucleus and mitochondria remained intact where all the symbionts were lost from the mycetocyte (Fig. 6b).

4. Discussion

Previous studies have demonstrated that injection of rifampicin into aphids leads to a production of undersized and sterile progeny. Biochemical and morphological studies have indicated that such a change in progeny is closely related to disappearance of endosymbionts (Ishikawa and Yamaji, 1985).

The present paper showed that a brief heat-treatment of newborn nymphs results in growth retardation and sterility of these insects. The result may account for the fact that most aphid species are habitants of the temperate region and that even in that region their multiplication is not conspicuous in summer. Like the effect of injected antibiotic, the effect of heat-treatment was accompanied with loss of endosymbionts, the fact which is evidenced by

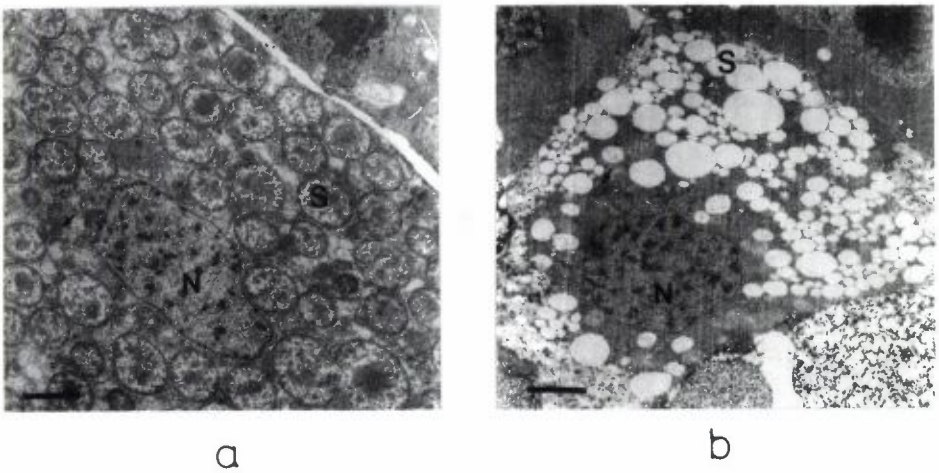

Figure 6. Electron micrographs of part of embryonic mycetocytes in untreated and heat-treated insects. Embryos were taken out of 25 day-old insects. (a) Untreated insects; (b) treated at 37°C for 12 hr at the newborn stage. N, Nucleus of the mycetocyte; S, endosymbiont or vacuole-like structure. Arrows represent mitochondria in the mycetocyte. Bars represent 2 μm.

estimating the symbionin content and observing the mycetocyte ultrastructure. The result is consistent with our previous assumption that the endosymbionts are essential to growth and reproduction of the host insect. It is not excluded that the heat-treatment brings about the loss of endosymbiont indirectly via physiological changes in the treated insect. However, the selective inhibition of the symbionin synthesis by the treatment (lanes 3 and 4, Fig. 4c) suggests that the endosymbiont is, at least, one of the most sensitive targets.

In telescoping of generations of parthenogenetic aphids, even ovarioles of newly born nymphs contain embryos at early stages. Mature aphids that had been heat-treated at a newborn stage, though not producing progeny, contained abnormal embryos in the ovariole (data not shown). In the mycetocytes of these abnormal embryos were many vacuole-like structures instead of endosymbionts (Fig. 6). These vacuoles might represent spaces that were filled by endosymbionts. If correct, this suggests that the heat-treatment disrupted pre-existing symbionts in the embryo rather than prevented symbionts from infecting the early embryo. In this context, the symbiotic system of XD-amoeba with X-bacteria may be suggestive. In the latter system, the symbiont has plasmids that code for polypeptides essential to maintain the symbiosis harmonious. Heat treatment tends to eliminate these plasmids leading to disruption of the symbiotic system (Jeon, 1983). It is possible that in the symbiotic

system of the aphid mycetocyte similar proteins are also involved in order to keep its endosymbionts.

One of these proteins could be symbionin because the synthesis of symbionin was stopped not only in the aphid that had been heat-treated at a newborn stage (Fig. 4) but also in the insect heat-treated in an adult stage where numerous endosymbionts existed in the mycetocyte. In the latter case, the stop of the symbionin synthesis seemed to be followed by disintegration of the endosymbiont structure (data not shown). This assumption is also consistent with our recent finding that symbionin is a member of "chaperonin" (Hemmingsen et al., 1988), an assembly factor required for oligomeric proteins in eukaryotic cell organelles (Hara et al., 1990a, 1990b; 1991).

Although its biochemical mechanisms are not clear, similar change in body color due to heat-treatment (Fig. 3) has been also noted in cockroaches (Brooks, 1963).

According to the Serial Endosymbiosis Theory (SET), DNA-containing organelles of eukaryotic cells are evolutionary descendants of intracellular symbionts (Margulis, 1970). In fact, the mycetocyte symbiont of aphids could be looked upon as an organelle because of its properties as follows: (1) it is transmitted through host's generations, (2) it is essential for the host's multiplication, (3) it is so specialized as to synthesize *in vivo* only one protein, and (4) it does not multiply itself *in vitro* (Ishikawa, 1989). However, an important difference lying between the true organelles and intracellular symbionts is how the hosts respond to an environmental stress. When the heat shock is imposed on an eukaryotic cell, it manages to survive the stress as a whole. As a rule, it does not eliminate either mitochondria or chloroplast under such an environmental stress (Nover and Neumann, 1984). It is likely that the true organelles, according to the SET, are so closely integrated into the cell's entity, and that the "symbiosis" is kept stable and harmonious under a wide range of environmental conditions (Margulis, 1981). By contrast, intracellular symbioses such as those in the aphid mycetocyte and amoeba are extremely vulnerable. A brief heat-treatment easily disrupts these symbiotic systems, which suggests that these systems are stable only under very limited conditions. It is conceivable that evolutionarily the intracellular symbiosis will become adapted to a wider range of conditions because its vulnerableness cannot be profitable for either the host or symbiont in order to survive environmental stresses.

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