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Quantitative Analysis of Metamorphosis Induced by L-Glutamine in Embryos of the Sea Urchin, *Hemicentrotus pulcherrimus*

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ABSTRACT—Metamorphosis of the sea urchin, *Hemicentrotus pulcherrimus*, can be induced by L-glutamine as reported previously for *Pseudocentrotus depressus* [15]. To analyze more precisely the process of metamorphosis induced by L-glutamine, the development of the echinus rudiment (ER) was classified into six stages. The stage at which the larvae underwent the normal metamorphosis by glutamine treatment was confirmed. The time of the glutamine treatment required for metamorphosis (the eversion of ER) was over 10 hr but treatment for more than 25 hr tended to decrease the number of metamorphosed larvae, although the larval arms had mostly been resorbed. The time of glutamine treatment to induce the metamorphosis, depended on the development of ER; more time was required for the younger larvae and less time for the older. The high mitotic activities observed in the cells of the ciliary bands were markedly decreased in the glutamine-treated larvae to metamorphose. These findings suggested that a degenerative process of metamorphosis including cell death is induced by L-glutamine.

INTRODUCTION

Larvae of marine invertebrates undergo metamorphosis in response to enviromental cues [2]. In echinoderms, bacterial and algal films are effective in inducing metamorphosis of sea star [7, 14] and sea urchin [5, 9, 16] larvae. As the active components of the natural metamorphosis-inducing substances, free fatty acid components have been extracted from red algae [6] and a pheromonal peptide has been isolated from the sand in the adult habitat [1], which were effective in inducing metamorphosis of sea urchin and sand dollar, respectively. However, the induction mechanisms of the metamorphosis are scarcely known.

L-Glutamine induced metamorphosis in the larvae of sea urchins, *Strongylocentrotus intermedius* [10] and *Pseudocentrotus depressus* [15]. In *P. depressus*, the effective duration of the glutamine-treatment for metamorphosis was 4–8 hr, when the eversion of the echinus rudiment (ER) was scored as a criterion of metamorphosis, while on the other hand, a 2-hr treatment was sufficient for causing the early changes such as the cessation of larval swimming and the beginning of the arm-shortening. Therefore, the early changes induced by short glutamine treatment were not sufficient to evoke the later change, the ER-eversion [15]. Herein, metamorphosis was induced in another sea urchin, *Hemicentrotus pulcherrimus* by L-glutamine, and the relationship between the concentration or duration of treatment and the rate of metamorphosis was analyzed. Also the mitotic activity of larvae was determined by BrdU incorporation. The findings demonstrated that a degenerative process is included in the induction of metamorphosis.

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MATERIALS AND METHODS

Larvae derived from one pair of a male and a female of *H. pulcherrimus* were reared by the method of Noguchi [13] with slight modifications as described previously [15]. Embryos were cultured in beakers stirred with a paddle connected to a motor (30 or 72 rpm) at 18–20°C and were fed *Chaetoceros gracilis* every one or two days. Larvae competent for metamorphosis were obtained over a period of 47 days (from 4th May to 19th June), beginning from the 40th day after insemination.

In each experiment, 50–100 larvae were selected from the culture beakers and transferred into artificial sea water (ASW: 420 mM NaCl, 9.0 mM KCl, 10 mM $CaCl_2 \cdot 2H_2O$, 24.5 mM $MgCl_2 \cdot 6H_2O$, 25.5 mM $MgSO_4 \cdot 7H_2O$, 2.15 mM $NaHCO_3$). After rinsing twice in ASW, every 10 larvae were randomly apportioned into each well of a 12-well plastic plate previously filled with 2.5 ml ASW or L-glutamine-containing ASW (adjusted with NaOH at pH 7.8–8.0). Stages of larvae in the experiments were selected according to purposes of the experiments and most of the larvae were finally squashed on a glass slide to check the developmental stage of the echinus rudiment (ER).

To see the effects of L-glutamine on the mitotic activity, the larvae were labelled with 4 μM 5-bromodeoxyuridine (BrdU) (Sigma Chemical Co.) for 4 hr (24–28 hr or 42–46 hr after starting the glutamine treatment), fixed with 10% formalin and stored in 70% ethanol at 4°C. Fixed larvae were placed on a poly-L-lysine-coated glass slide and were dried out. After dipping in 2N HCl for 30 min, BrdU in larvae was detected with a Cell Proliferation Kit (Amersham) using 0.01% Triton X to permeate antibodies.

RESULTS

Developmental stages of the echinus rudiment in 8-armed plutei of H. pulcherrimus

I classified 8-armed plutei having an echinus rudiment (ER) into six stages, as shown in Fig. 1, according to the outer appearance (a-g) and the skeletal figures of larvae

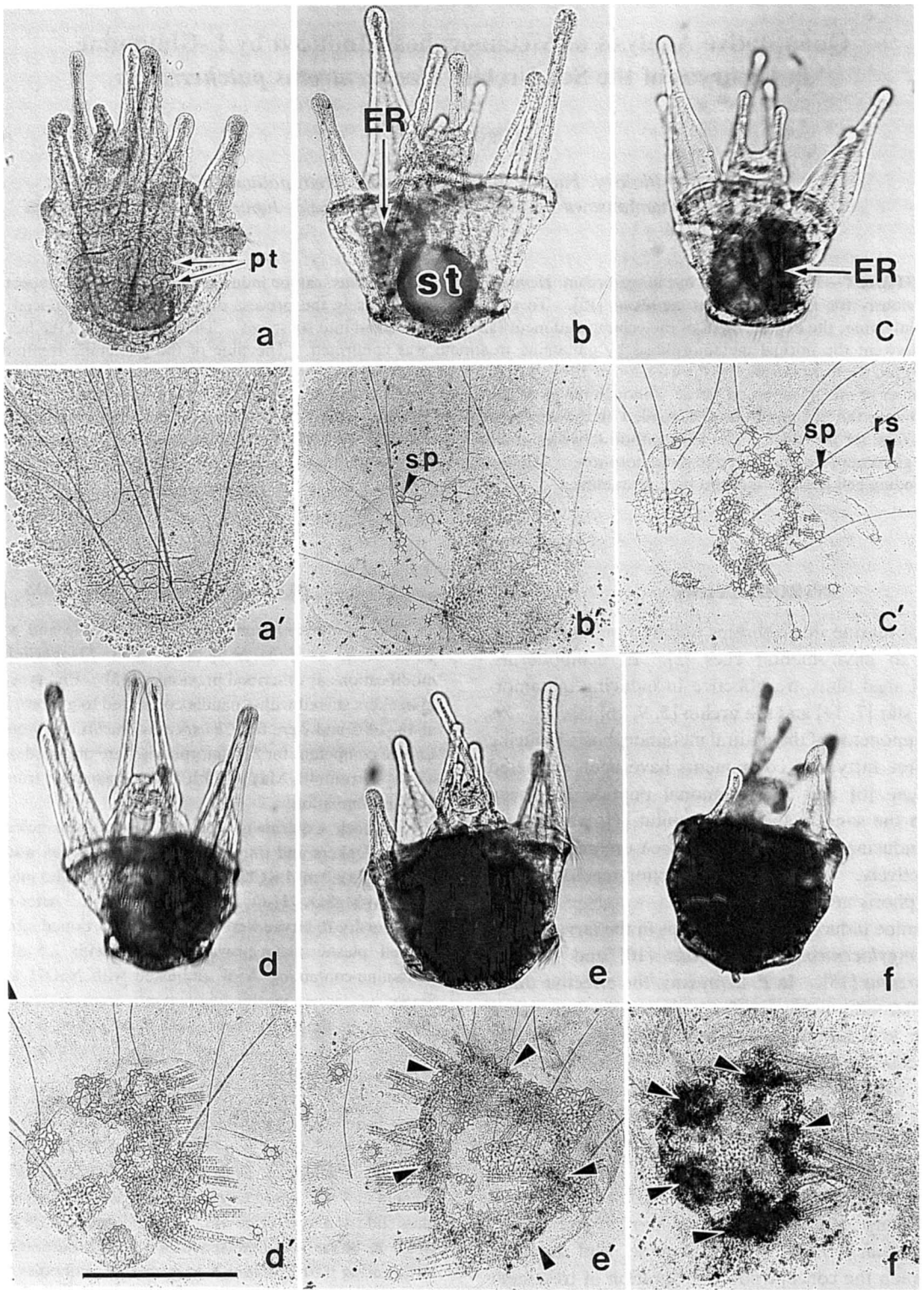

FIG. 1. Stages of the echinus rudiment in the 8-armed plutei of *H. pulcherrimus*. Development of the echinus rudiment (ER) was classified into six stages. a-f are micrographs of larvae. a'-f' are micrographs of the squashed larvae to see the skeletal structures of a-f, respectively. a, a'; Stage 1, primary tube feet (pt) are moving within the larval body. b, b'; Stage 2, Spines are formed (from rudimental spines to 4 segmented ones). A rudiment of spine (sp) is recognized. c, c'; Stage 3, ER became untransparent. Spines have 5 to 10 segments. Rossete-like skeletons (rs) are observable at the top of the tube feet. d, d'; Stage 4,: Spines have 11–15 segments. e, e'; Stage 5, ER fully grows. Spines have 15–20 segments. Dark materials (pointed with arrowheads) are seen among the network skeleton of ER. f, f'; Stage 6, Arms become short. Spines have over 20 segments and much more dark materials are formed in ER. Larvae were cultured at 18–20°C and reached stage 1 by about 30 days, to stage 6 for further additional 10 days. pt, primary tube feet. st, stomach. sp, skeleton of spine. rs, rosset (skeleton of tube foot). Bar, 500 μm.

(a'-g').

Stage 1: The larva has ER with five primary tube feet which are moving within the larval body. The larva presented in Fig. 1a was slightly compressed to show well the primary tube feet. No skeleton was observable except for the skeletons of larval arms (a'). Stage 2: Beginning of spine-formation to four segmented spines. The larva becomes bigger than Stage 1. In ER, many triangle or ring shaped skeletons are found. Among the skeletons, a rudiment of spine is discernible (sp in b'). Stage 3: ER becomes untransparent (c) and has small spines with 5–10 segments. The primary tube feet have a rossete-like skeleton (rs) on their top, and network-like skeletons are formed (c'). Stage 4: ER becomes bigger and the spines have 11–15 segments. A complicate network of skeletons is formed which binds to the root of each spine (d, d'). Most of the larvae swim in the middle of the culture beaker until stage 4. Stage 5: ER is fully grown in the larval body (e). There are 15–20 segments in the spine and some dark materials are found at their root (e'). The rossetes of the tube feet become complicate (e'). When stirring of sea water is stopped, larvae at this stage tend to sediment. Stage 6: Arms become slightly narrow in width and short. There are over 20 segments in the spine. In some larvae the spine is extruded through the vestibules of ER. Larvae sink to the bottom of the beaker if stirring of sea water is stopped and swim in that area. The ER is filled with dark materials (f, f').

In our culture system, these stages were all found in the same beaker from the day of 40th after insemination and time of each stage was not identified, although the formation of ER began at the 27–30th days and the primary tube feet were found in larvae at first at the 34th day after fertilization.

Effective concentration of L-glutamine for induction of metamorphosis

About 300 larvae at stage 3–5 were collected from the middle layer of the culture beakers, and 10 larvae were randomly apportioned into each well of 12-well plastic plates filled with ASW containing various concentrations (10^{-7} M–10^{-2} M) of L-glutamine and were left for 24 hr at 20°C. Thirty larvae were examined for each concentration. After 24 hr, the larvae were washed twice and reared in ASW. They were checked at 24 hr and 48 hr after the start of treatment for metamorphosis-specific characters such as the cessation of swimming, the start of arm-retraction, the extruding of primary podia and the eversion of ER. At 24 hr, no metamorphosed larvae (ER everted larvae) were found (Fig. 2), although the cessation of swimming was observed in larvae treated with higher concentrations than 10^{-6} M. At 48 hr, metamorphosis had occurred in 53% of the larvae treated with 10^{-4} M glutamine and in 76% of the larvae treated with 10^{-3} M glutamine (Fig. 2). Arms had shortened in all larvae treated with 10^{-3} M and 80%, 72% and 50% in the larvae treated with 10^{-4} M, 10^{-5} M and 10^{-6} M glutamine, respectively. Larvae treated with 10^{-7} M glutamine and control larvae did not show any shortening of the arms. In 10^{-2} M glutamine, the larvae degenerated instantly. The percentage of metamorphosed larvae increased slightly on the next day (at 60 hr) (Fig. 2). Treatment of larvae with GABA at 10^{-6} to 10^{-4} M for 24 hr could not induce any metamorphosis for up to 100 hr. At 60 mM, KCl was also ineffective in inducing metamorphosis of *H. pulcherrimus* larvae.

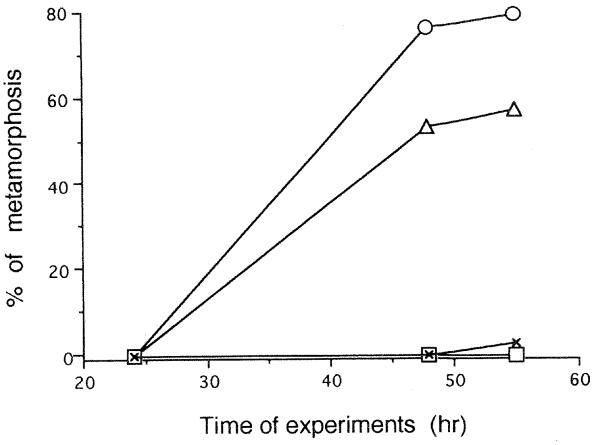

FIG. 2. Effective concentration of L-glutamine needed to induce metamorphosis. Each 30 larvae at stage 3–5 were treated with L-glutamine in ASW for 24 hr with concentrations ranging from 10^{-7} M to 10^{-2} M. 10^{-3} M(○), 10^{-4} M(△) and 10^{-5} M(□) to 10^{-7} M(□) glutamine. Control(×). Metamorphoses had occurred in the groups of larvae treated with 10^{-4} M and 10^{-3} M glutamine by 48 hr after the start of treatment.

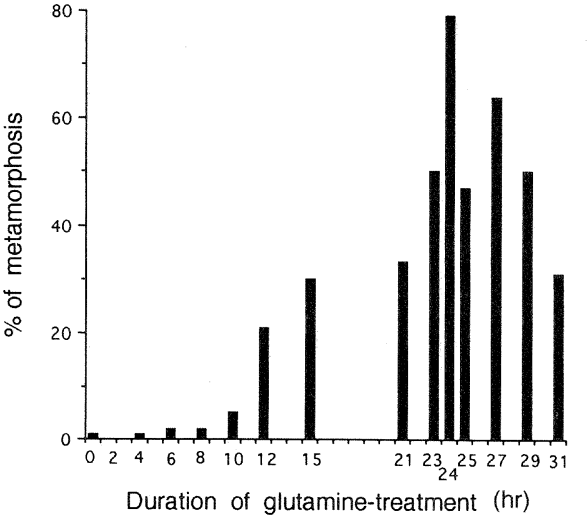

FIG. 3. Induction of metamorphosis by various times of glutamine treatments. Each 15–50 larvae (stage 3–5) were put into 10^{-4} M L-glutamine containing ASW and then transferred into glutamine-free ASW after 2 hr, 4 hr, 6 hr—31 hr. Control larvae (0 hr) were reared throughout the experiments in glutamine-free ASW. Metamorphosed larvae were counted at 55 hr after the start of the experiments. Metamorphosis was scarecely induced by 2-hr to 8-hr treatments. Metamorphosed larvae increased by the treatment for more than 12 hr, and reached the maximum after the 24-hr treatment. Treatment longer than 25 hr decreased the percentage of metamorphosis.

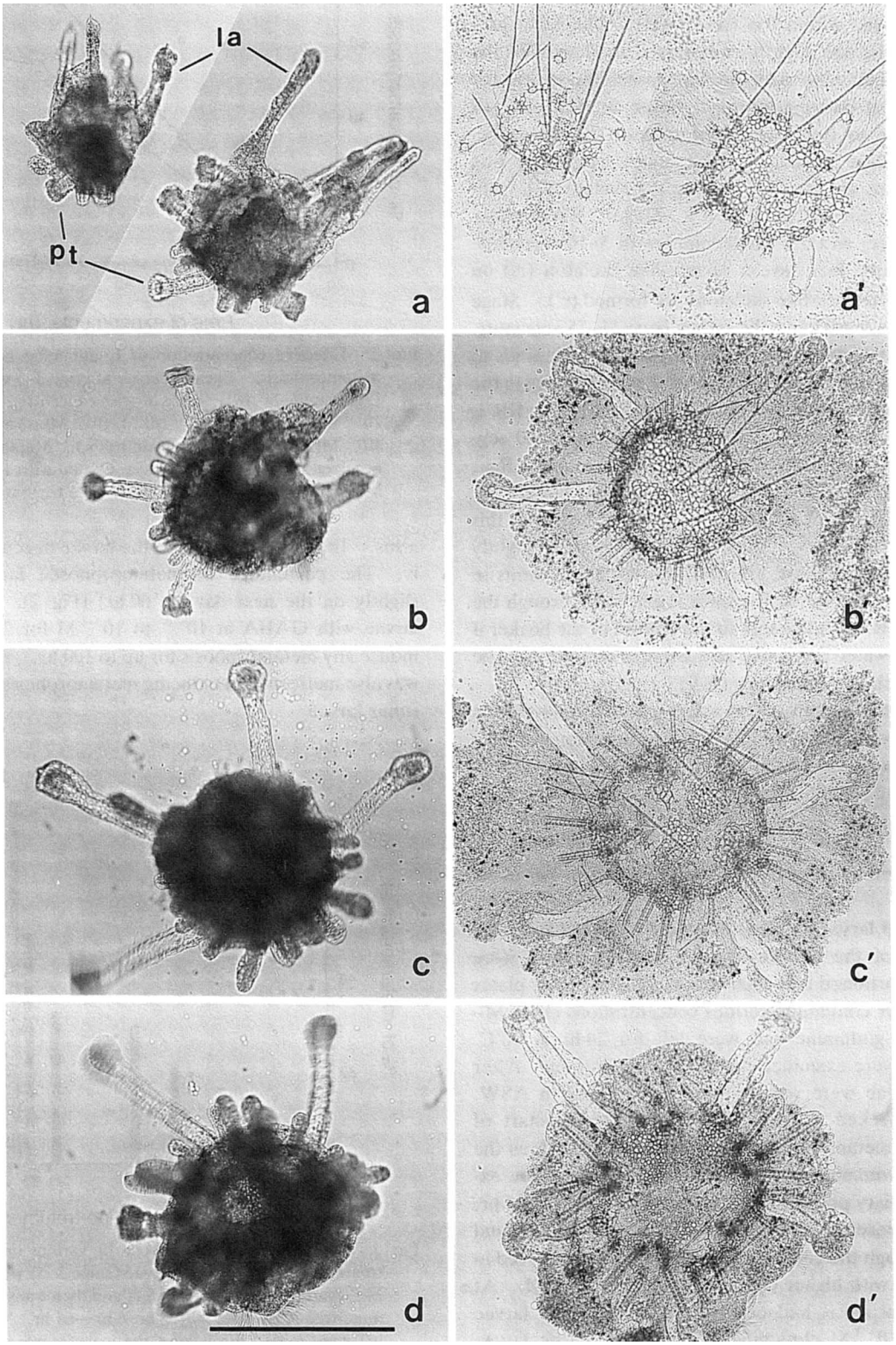

FIG. 4. Developmental stage of larvae competent to metamorphose. Larvae were treated with 10^{-4} M glutamine for 24 hr, and at 48 hr the larvae or juveniles (a-d) were squashed to check the developmental stage of ER (a'-d'). Left larva in a, a'; the youngest metamorphosed larva at stage 2. Right larva in a, a'; stage 3. b, b'; stage 4. c, c'; early stage 5. d, d'; late stage 5. la, larval arm. pt, primary tube foot. Bar, 500 μm.

Effective time of glutamine treatment

Fifteen to 50 larvae each at stage 3 to 5 were exposed to 10^{-4} M glutamine for various durations and the metamorphosed larvae were counted at 55 hr after the beginning of the experiment. Treatments for 2 to 8 hr were not effective in inducing metamorphosis (Fig. 3). The percentage of metamorphosis increased by the treatment for over 10 hr, and reached a maximum by the treatment for 24 hr. Further increase in the treatment time from 25 hr to 31 hr caused a gradually decrease in the number of metamorphosed larvae (Fig. 3). With the increase in the duration of glutamine treatment, the larval arms became shorter. The larvae exposed to glutamine for longer than 25 hr tended to take the shape of black balls, in which the arms were resorbed but the larvae could not evert ER.

Larval stage competent for metamorphosis induced by L-glutamine

To know the larval stage competent to react to the glutamine treatment, 260 larvae were subjected to 10^{-4} M glutamine and 58 control larvae (cultured in ASW without glutamine) presented in Figs. 2, 3 and 6 were examined for metamorphosis at two or three days after the treatment and every larval stage were confirmed by squashing (Fig. 4). In the control series, none of the larvae at stages 1 to 6 metamorphosed. Out of 111 stage 1 or 2 larvae treated with glutamine for 20–24 hr, only one stage-2 larva metamorphosed (Fig. 4a, 4a', left). Thereafter, the percentage of metamorphosed larvae increased as the developmental stage proceeded: 61% at stage 3 (Fig. 4a, 4a', right), 75% at stage 4 (Fig. 4b, 4b') and 100% at stage 5 (Fig. 4c, 4c' and 4d, 4d'). However, at stage 6, half of the larvae could not metamorphose (Fig. 5a and 5b). The youngest larva that metamorphosed had spines with one or two segments (Fig. 4a', left). Out of 14 larvae with 5-segmented spines, six larvae metamorphosed (43%); this is in sharp contrast to those having 3- or 4-segmented spines which underwent no metamorphosis. Eleven out of 14 (79%) larvae with 7- to 8-segmented spines metamorphosed. The prolongation of the glutamine treatment up to 31 hr could not induce metamorphosis in larvae

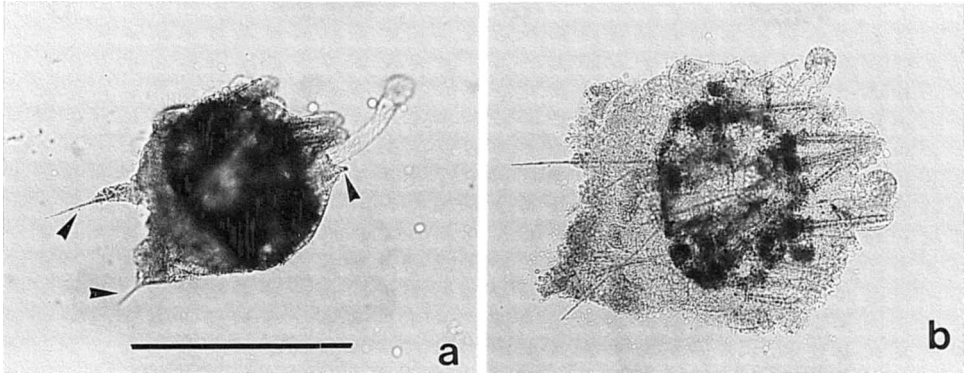

FIG. 5. Unmetamorphosed larva of stage 6. The larva treated with 10^{-4}M L-glutamine for 24 hr was observed on the third day. Light microgrphs of incompletely metamorphosed larva at stage 6 (a) and of a squashed figure (b) of a. Arrowheads show degenerated larval arms. Bar, 500 μm.

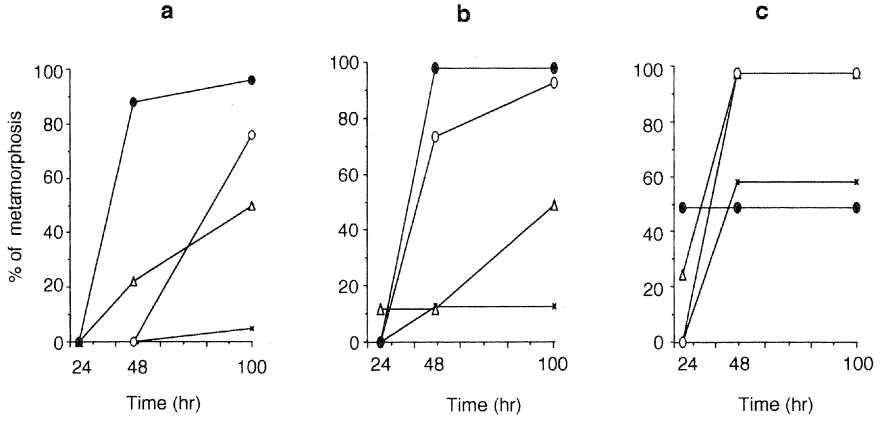

FIG. 6. Effects of glutamine on the various developmental stages of larvae. Three groups of larvae in different developmental stages (a; stage 3–5, b; early stage 6, c; late stage 6) were exposed to 10^{-4} M glutamine for 15 hr(△), 20 hr(○) and 24 hr(●), and were checked for their metamorphosis at 24 hr, 48 hr and 100 hr after the start of glutamine treatment. Control groups (×) were kept in ASW without glutamine. Among larvae in stage 6, the larvae with spines extruded from vestibules of ER were identified as late stage 6. The younger larvae required a much longer treatment and more time to metamorphose.

younger than stage 3.

The reactivity of larvae to glutamine was further studied in relation to the development and duration of treatment (Fig. 6). Three groups of larvae, stage 3–5 and early and late stage 6, were subjected to 15 hr-, 20 hr- and 24 hr-glutamine treatments. In the 1st group (stage 3–5), larvae were swimming in the middle layer of the culture beaker and did not sediment when stirring was stopped. These larvae subjected to the 24-hr treatment did not metamorphose at 24 hr but 92% of them metamorphosed by 48 hr, while the metamorphosis of larvae treated for 20 hr was observed about one day later, at 100 hr (Fig. 6a). Most of the larvae at the early stage 6 (larvae having fully grown ER and sedimented) treated for 20 hr or 24 hr, were induced to metamorphose by

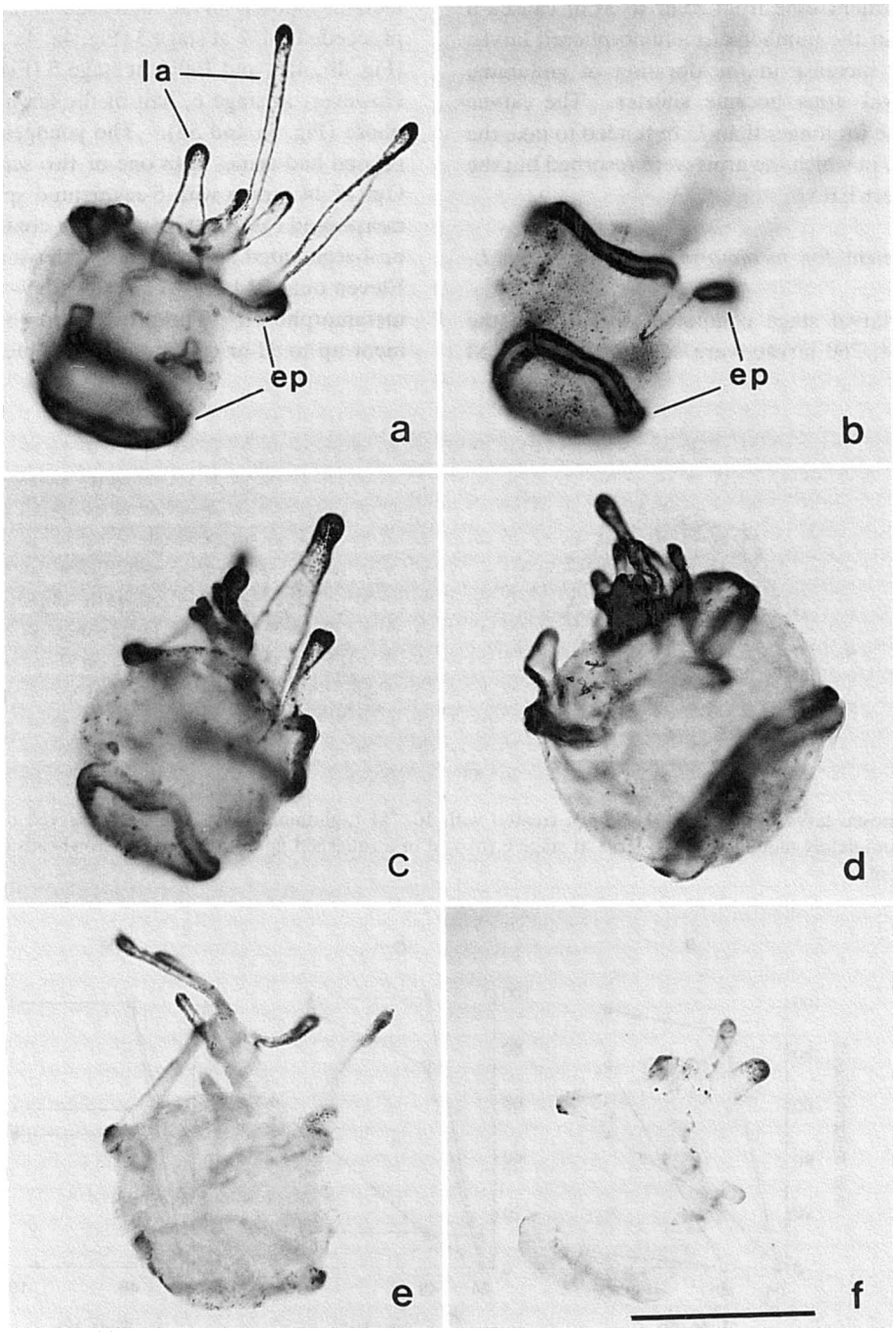

FIG. 7. Suppression of mitotic activity by the treatment with L-glutamine. Larvae at stage 3–5 were treated with 10^{-4}M glutamine for 6 hr (c, d) and 24 hr (e, f), and were labeled with 4 μM BrdU for 4 hr, at 24–28 hr (c, e) and 42–46 hr (d, f) after the start of treatment. Control larvae, without glutamine treatment, were labeled for 4 hr with the same concentration of BrdU (a, b; a control larva in different optical sections). BrdU incorporated in the epithelial cells of ciliary bands at arms (la) and epaulettes (ep) of larva (a, b) markedly decreased in larvae treated with glutamine for 24 hr (e, f) but did not decrease in the larvae treated for 6 hr (c, d). la, larval arm. ep, epaulette. Bar, 500 μm.

48 hr (Fig. 6b), while for larvae at late stage 6 (sedimented larvae whose spine had extruded out from the vestibule of ER), the 15-hr and 20-hr treatments were sufficient for the induction of metamorphosis in 100% of them by 48 hr. The 24-hr treatment rather inhibited the metamorphosis of about 50% of larvae (Fig. 6c). The larval arms had resorbed but could not evert ER. Autonomous metamorphosis occurred at a high rate (60%) in the control larvae of the late stage 6 (Fig. 6c).

In conclusion, the time of glutamine treatment necessary to induce metamorphosis was longer for the younger larvae and shorter for the older ones.

Suppression of mitotic activity by the treatment with L-glutamine

Competent larvae (stage 3–5) were incubated with 4 μm BrdU for 4 hr and labelled cells were detected immunocytochemically. Mitotic activity was high in the epithelial cells of ciliary bands of arms and epaulets (Fig. 7a,b). Two groups of larvae treated with 10^{-4} M glutamine for different times (6 hr and 24 hr), were labelled with 4 μM BrdU for 4 hr between 24 and 28 hr or 42 and 46 hr after the start of the glutamine treatment. At the 24th hr of the 24-hr treatment, every larval arms (26 larvae) had begun shortening and at the 46th hr, a half of the larvae had been induced to metamorphose. Meanwhile, in larvae treated with glutamine for 6 hr, larval arms had scarcely become short at the 24 hr after the start of the 6-hr treatment, although during the next 4 hr of BrdU treating three out of 11 larvae had begun to shorten their arms, and at the 46th hr the arms of three larvae out of 13 had become short but no larva had metamorphosed. BrdU labelling was found at both 24–28 hr (Fig. 7c) and 42–46 hr (Fig. 7d) after the start of treatment in the larvae treated with glutamine for 6 hr similar to the control (Fig. 7, a,b). By contrast, in the larvae treated with glutamine for 24 hr, labelling of BrdU (24–28 hr) markedly decreased from the epaulets and most of the arms (Fig. 7e). Fig. 7f shows one of the unmetamorphosed larvae treated with glutamine for 24 hr and labelled with BrdU for 4 hr (42–46 hr). Except for the arm tips, there was almost no labelling of BrdU. In the metamorphosed one, no labelling was found (the micrograph not shown).

DISCUSSION

Sea urchin embryos transform through metamorphosis from the larvae swimming by ciliary movements to the benthic juveniles moving with the spines and the tube feet. When larval metamorphosis is induced by L-glutamine, a series of changes are observable; 1) the cessation of swimming, 2) the beginning of arm-shortening, 3) the extrusion of tube feet out of the vestibule of ER and 4) the eversion of the ER [15]. The early changes 1)–3) were supposed not to be linked to the ER-eversion, because the time of glutamine treatment to induce the early changes of metamorphosis was too short to induce the ER-eversion [15]. Accordingly, in this paper ER-eversion was used for a criterion of metamorphosis.

Development of ER had been described by MacBride [8] from the beginning of the ER formation; an apposition of the amniotic cavity and the hydrocoele. Herein, the classification of the ER stages began after most of the fundamental structures of ER had been formed, and succeeded the period of growth, and finally ended to the over growing stage of ER (late stage 6). The formation of the ER progresses by an accumulation of thyroid hormones which may be derived from algae [4]. L-glutamine did not have any effect on young larvae undergoing the ER formation, the period of which was sensitive to thyroid hormones, but induced metamorphosis within two days at the various stages of larvae whose ER was growing. During the experiments, control larvae, cultured in ASW without glutamine over two days, showed their arms shortened but remained thick in the wide of arms, although the arms of the glutamine-treated or metamorphosing larvae were shortened with thinning. The shortening of larval arms in the control was probably caused by the active swimming of larvae that made chances to dash the arms against the wall of the culture plate, because when larvae were treated with glutamine for too short a time or too low a concentration to induce metamorphosis but enough to induce the cessation of swimming, the larval arms remained intact.

The youngest larva which was induced to metamorphose by L-glutamine had spines with two or three segments (stage 2), although the older larvae having five segmented spines (stage 3) metamorphosed more frequently (Fig. 4a). These stages are far younger than those usually expected to be competent for metamorphosis (stage 5-6; larvae with fully grown ER) [4, 6]. During metamorphosis, larval arms are resorbed and become short. In younger larvae, the larval arms remained longer than those of the older metamorphosed larvae (Fig. 4). This also implies that the arm-shortening is essentially not linked to the ER-eversion.

Progressing the developmental stage, the time of the glutamine treatment required for the induction of metamorphosis became shorter (Fig. 6). Fu-shiang Chia [3] proposed a possible mechanism of metamorphosis in marine invertebrate larvae that a competent larva acquires a chemical substance which he called a metamorphic factor. He maintained that the factor is masked until the larva receives an environmental cue which produces a catalytic factor. To date, there is no information about either metamorphic factor or catlytic factor above described. The metabolism of glutamine relating to metamorphosis should be studied.

Glutamine showed an inhibitory effect on the mitotic activity of the epithelial cells of ciliary bands when the glutamine treatment was long enough to make metamorphosed larvae (Fig. 7). Uptake of BrdU into the epithelial cells had markedly decreased for 4 hr after the treatment with glutamine for 24 hr, while in larvae treated for 6 hr with glutamine, the uptake of BrdU did not show any decreases. Since larval arms began to shorten, but no metamorphosis

occurred during these four hours, the suppression of mitotic activity may be an event belonging to catalytic one in metamorphosis.

In the anuran tadpole, thyroid hormone, a metamorphosis-inducing hormone, decreases the mitotic activity of epithelial cells of tail skin and induces cell death which occurs during metamorphosis [11, 12]. It is unknown whether the thyroid hormone which accumulated in 8-armed plutei [4] decreases the mitotic activity in sea urchin larvae or not. The glutamine-induced mitotic inhibition observed in sea urchin larvae (Fig. 7) suggests that the cell death occurs also in the epithelial cells of ciliary bands preceeding metamorphosis. Further studies are required to elucidate the relationship between the inhibition of mitotic activity and ER-eversion, and the effects of thyroid hormones accumulated in larva and those of L-glutamine given as a metamorphosis inducer.

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