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Selective Resistance to Desiccation of Nuclear Ribonucleic Acid Synthesis in Isolated Nuclei of *Artemia salina* Embryos during Pre-emergence Development

By DIDIER DE CHAFFOY and MASATOSHI KONDO
Departement Celbiologie, Universiteit Antwerpen, B-2610 Wilrijk, Belgium

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The developing gastrula embryos of *Artemia salina* are resistant to a complete redesiccation during a period of pre-emergence development. Isolated nuclei from these dehydrated embryos could retain a transcriptional activity *in vitro* comparable with that of non-desiccated controls. On the other hand, redesiccation of both prenauplii and nauplii completely destroys their viability as well as the nuclear transcriptional activity. However, those gastrula embryos that did not develop in a first incubation period could remain viable and develop with a considerable time-lag after a subsequent second incubation.

Dried encysted embryos of the brine shrimp, *Artemia salina*, exhibit remarkable biological properties. These dried gastrula embryos have been shown to be essentially dormant (Clegg, 1967, 1974) and to be able to resist a variety of severe conditions, such as extreme temperature (Hinton, 1954; Dutrieu & Chrestia-Blanchine, 1967), complete desiccation (Dutrieu, 1960; Clegg, 1967), various organic solvents (Morris, 1968; Iwanami, 1973) and even γ-irradiation (Bowen, 1963; Iwasaki, 1964*a*, 1965). Further, they could be reversibly hydrated and dehydrated (Morris, 1971).

In the present paper, we have examined effects of desiccation of the developing *Artemia* embryos on the nuclear transcriptional activity in isolated nuclei. We show here that developing gastrula embryos are resistant to a complete redesiccation, and isolated nuclei from these dehydrated embryos retain transcriptional activity comparable with that of non-desiccated controls. However, the resistance to redesiccation is limited only to a pre-emergence period, namely a period of development before emergence of prenauplii from the protective alveolar chorionic shell (Morris & Afzelius, 1967). It is suggested that glycerol accumulated in the encysted embryos during pre-emergence development (Clegg, 1964) might play an important role in protection of nuclear activity against dehydration.

Experimental

(*a*) *Growth and handling of Artemia embryos*

Encysted cryptobiotic embryos (Division of Sterno Industries, Harrison, NJ, U.S.A.; Utah Saltern species; 8 g dry wt.) were treated and grown essentially by the described method (Moens & Kondo, 1976). For RNA polymerase assay, nuclei were isolated from the animals grown under sterile conditions. The rates of emergence and hatching were estimated by observing a sample in an embryonic dish under a binocular microscope (Wild Heerbrugg M5) or by spreading the sample over a filter paper, followed by an enlarged projection on a screen. Desiccation of animals was done as described in the legend to Fig. 1. We define here operationally prenauplius and nauplius as emerged and hatched larvae, namely a prenauplius that has emerged by breaking the outer shell but still remains within the hatching membrane and a nauplius that has physically been out of the hatching membrane and swims away from the empty shell.

(*b*) *Isolation of nuclei*

The desiccated animals were hydrated at 0°C in a growth medium for 3 h (Iwasaki, 1964*b*) before they were homogenized with a small volume of buffer 1 (pH 7.5) containing 30 mM-Tris/HCl, 120 mM-KCl, 3 mM-magnesium acetate, 7 mM-2-mercaptoethanol and 250 mM-sucrose. The homogenate was transferred to a long narrow test tube, extra buffer 1 was added (to final volume 45 ml) and the tube was placed in ice/water for a few minutes to sediment unbroken materials as well as to float empty shells. Then the homogenate was carefully removed with a Pasteur pipette into a centrifuge tube, leaving behind any unwanted material, and was centrifuged at 4°C for 50 s at 1000 rev./min in a Beckman JA-20 rotor to eliminate the remaining large cell debris. The resulting supernatant was re-centrifuged in the same rotor for 2 min at 310*g* (2000 rev./min), and the nuclear pellet was carefully washed with buffer 2 (pH 7.6), containing 30 mM-Tris/HCl, 75 mM-KCl,

3mM-magnesium acetate, 2mM-2-mercaptoethanol and 250mM-sucrose. The nuclei were finally resuspended in 200–500 μl of buffer 2 and used immediately for RNA polymerase assay.

The number of nuclei was estimated by counting them under a Leitz Wetzler Orthoplan microscope with the aid of an Improved Neubauer (0.1mm × 0.0025mm^2). DNA content was determined as described by Sillero & Ochoa (1971) and Dische (1930), with calf thymus DNA (Sigma Chemical Co., St. Louis, MO, U.S.A.) as standard.

(c) *RNA polymerase assay in isolated nuclei*

Nuclei (final concn. 1×10^7–3×10^7/ml) were incubated at 30°C for 30min in a reaction mixture (50 μl) containing 30mM-Tris/HCl (pH7.6), 75mM-KCl, 2.4mM-magnesium acetate, 2mM-manganese acetate, 20mM-$(NH_4)_2SO_4$, 7mM-2-mercaptoethanol, 1mM-ATP, 0.25mM-CTP, 0.25mM-GTP, 0.05mM-UTP and 0.5 μCi of [^{3}H]UTP (The Radiochemical Centre, Amersham, Bucks., U.K.; 41Ci/mmol). Reaction was stopped by addition of 750 μl of 5% (w/v) trichloroacetic acid; the mixture was vortex-mixed well and placed in ice/water. After about 10min, acid-insoluble materials were collected on a glass-fibre disc (Whatman GF/C, 25mm) and washed carefully with 5% trichloroacetic acid. The radioactivity was determined as described elsewhere (Moens & Kondo, 1976).

RNA synthesis in isolated nuclei at concentrations of 1×10^7–10×10^7/ml was linear with concentration of nuclei, completely resistant to rifampicin (10 μg/ml; a gift from Professor Silvestri, Lepettit Co., Milano, Italy), indicating no mitochondrial contamination, and resulted in production of heterogeneous RNA (4–45S) (L. Swennen, L. Moens & M. Kondo, unpublished work).

Results and Discussion

(a) *Redesiccation of developing encysted embryos*

Desiccated dormant *Artemia* embryos can be fully hydrated by incubation in a growth medium (Moens & Kondo, 1976) at either 0° or 27°C for 3h (Iwasaki, 1964*b*), but a rapid resumption of metabolic activity in the embryos was observed only by incubation at higher temperatures (Muramatsu, 1960; Iwasaki, 1964*b*; Finamore & Clegg, 1969). The observed extent of water saturation in the hydrated embryos was about 60% (w/w) in our experiments after incubation at 30°C for several hours, in accordance with results obtained by others (Morris, 1971).

Dehydration of water-saturated embryos was achieved within 8h by desiccation *in vacuo* over $CaCl_2$ (Fig. 1; Clegg, 1967). Over 90% of the water in the embryos was eliminated in 5h under normal conditions, or after 1.3h when they had been previously placed in 5M-NaCl solution before desiccation (Fig. 1). These treatments of the hydrated embryos did not significantly alter their hatchability by a subsequent incubation at 30°C (see below).

(b) *Effect of redesiccation on embryonic development*

Under our standard growth conditions, the earliest emergence and earliest hatching were observed at about 6–7h and 10–11h respectively. A similar growth curve was reported by Ewing & Clegg (1969). We therefore estimate that the average durations required for emergence and hatching were approx. 6–7h and 4–5h respectively. As observed by Morris (1971), a proportion (30–40%) of the embryos was unable to respond to a first incubation, but only to a subsequent one that was preceded by desiccation.

Fig. 2 demonstrates the time required for the earliest hatching by a second incubation of the redesiccated embryos. When the first incubation was within a pre-emergence period (6–7h), an interruption of the embryonic development by dehydration appeared to have only a slight influence on the subsequent development initiated by a second incubation, as judged by a shortened time (8–9h) needed for the earliest hatching (Fig. 2).

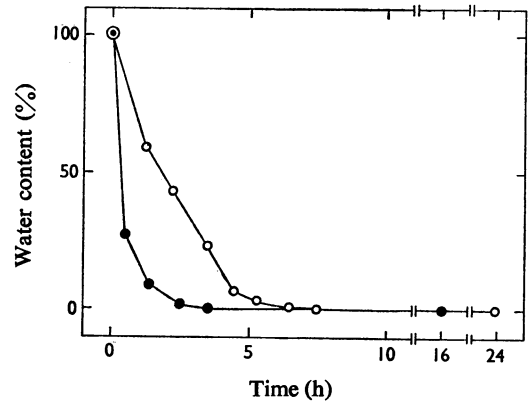

Fig. 1. *Dehydration of fully desiccated embryos*

Completely desiccated embryos (0.3–0.5g) were hydrated fully by incubation at 30°C for 5–8h. After hydration was completed, the encysted embryos were briefly washed with water, filtered on a filter paper to eliminate any attached water outside of the cysts and transferred to a plastic weighing dish. After the weighing, dishes were placed *in vacuo* over $CaCl_2$ and the weights were determined at the time-intervals indicated. ○, Control; ●, the encysted embryos were placed in 5M-NaCl solution for a few days between hydration and desiccation.

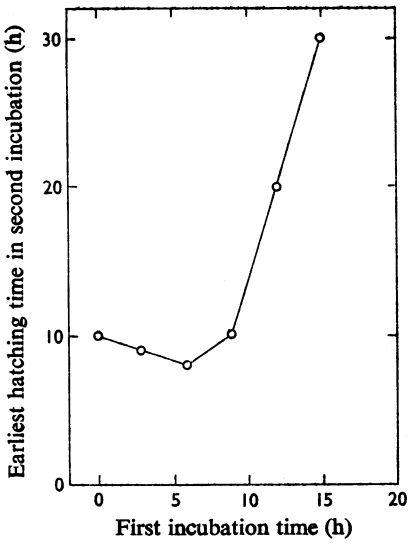

Fig. 2. *Time required for the earliest hatching in the second incubation*

The encysted embryos (8 g) were grown by a first incubation at 30°C with an optimal aeration for the times indicated in the Figure and then desiccated overnight as described in the legend to Fig. 1. Thus completely dehydrated encysted embryos were then grown for the second time by incubation at 30°C, and the time required for the earliest hatching of the swimming nauplii was determined by visual observation.

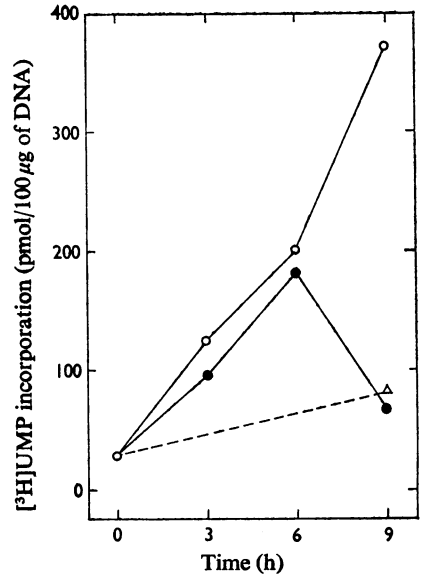

Fig. 3. *Effect of desiccation on RNA synthesis in isolated nuclei*

Nuclei were isolated from the embryos that had developed for the times indicated in the Figure, and their activity for RNA synthesis was assayed as described in the Experimental section. When embryos were desiccated, they were rehydrated at 0°C for 3 h before isolation of nuclei. The values were obtained mostly from duplicated sets of independent experiments, each experiment involving quadruplicate (or more) tubes. Nuclei were isolated from non-desiccated control (○), from desiccated (●) or from unemerged embryos at 9 h (△). The broken line indicates an expected increase in nuclear RNA synthesis by slowly developing embryos that may not hatch in the first incubation period.

Thus we are inclined to propose that these hatched swimming nauplii are produced from those embryos that had responded to the first incubation, but not from those showing delayed hatching (Morris, 1971). However, the total time needed for the earliest hatching of the embryos whose development was interrupted by desiccation became still longer than that for controls (Fig. 2). Nevertheless, we suggest that *Artemia* embryos during a pre-emergence period could be reversibly dehydrated without diminishing their viability.

On the other hand, when a first incubation was extended beyond the reversible period (i.e. the pre-emergence period), the earliest hatching was delayed by two- to three-fold (20–30 h) during a second incubation (Fig. 2). This seems to be because, once the embryos reach an emergence stage, they are obviously unable to remain viable during a subsequent desiccation. Hence only that delayed-hatching population that could not develop or still remained within a pre-emergence period during the first incubation was able to hatch during a second incubation. We note here that the delayed-hatching embryos require correspondingly a more prolonged second incubation time for the earliest hatching (Fig. 2). Although Morris (1971) observed no such delay for the time required for 50% hatching of these embryos, in contrast with our results, this discrepancy should be largely due to differences in growth conditions, e.g. temperature, oxygen supply etc.

(c) Effect of redesiccation on transcriptional activity

As reported previously (Swennen *et al.*, 1976), isolated nuclei of the cryptobiotic embryos exhibited very low activity for RNA synthesis *in vitro*. As shown in Fig. 3, the nuclear capacity for RNA synthesis increases progressively as the encysted gastrula embryos develop on incubation at 30°C. In agreement with the fact that the embryos in a pre-emergence period could be reversibly desiccated (see above), the nuclei isolated from these embryos retained their ability to incorporate [³H]UMP into RNA even after a complete desiccation, although

a slight decrease in RNA synthesis in these nuclei was observed compared with non-desiccated controls (Fig. 3). The observed nuclear resistance to desiccation should be one of the important factors that enable the desiccated embryos to be viable against dehydration.

On the other hand, nuclei derived from prenauplii or nauplii were completely inactivated for transcription *in vitro* when animals were previously desiccated (Fig. 3; D. de Chaffoy & M. Kondo, unpublished work). It should be noted here that a significant proportion (about 20%) of [^{3}H]UMP incorporation into RNA by isolated nuclei of the animals that had developed for 9h was due to the nuclear activity of the developing but still unemerged embryos, as indicated by the values obtained from non-desiccated samples in which prenauplii were killed by NaOCl treatment (Finamore & Clegg, 1969) before extraction of nuclei (△ in Fig. 3).

Desiccation of these unemerged embryos after 9h of incubation again diminished slightly nuclear activity for RNA synthesis, as was shown for the embryos of a pre-emergence period.

Although a precise mechanism that ensures the nuclear resistance to desiccation is not clearly known yet, we may offer one plausible explanation for it. Clegg (1964) observed that the encysted *Artemia* embryos accumulated a large amount of glycerol, which was then lost into the environment when prenauplii emerged. Thus it might be that the glycerol molecule replaces the water molecule in such a way that it can prevent biological macromolecules from being inactivated when the encysted embryos are dehydrated. In prenauplii or nauplii, however, glycerol protection against desiccation obviously could not operate. Possible functions of glycerol in *Artemia* were discussed by Clegg (1974).

Finally, the pre-emergence development in which a reversible desiccation is possible represents in the embryos a period of quite low metabolic activity. In this period, it has been reported that no DNA synthesis (Nakanishi *et al.*, 1962), no extensive RNA synthesis (McClean & Warner, 1971), except of some low-molecular-weight species (Susheela & Jayaraman, 1976), and no prominent protein synthesis *in vitro* on polyribosomal templates (Moens & Kondo, 1976) occur, but that a significant cell differentiation takes place in these embryos (Benesch, 1969).

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