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Inhibition of putrescine synthesis blocks development of the polychete *Ophryotrocha labronica* at gastrulation

[α-methylornithine/methylglyoxal bis(guanylhydrazone)/polyamines/embryogenesis]

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ABSTRACT **Developing eggs of the polychete *Ophryotrocha labronica* were analyzed for polyamines during the first 6 days after fertilization. The spermine content dominated initially, but gradually decreased. It was surpassed by putrescine, which rapidly increased to a maximum on the 3rd day, i.e., at the inception of gastrulation. The spermidine content was low during the entire period. Treatment of eggs with the putrescine synthesis inhibitor α-methylornithine from the onset of development led to developmental arrest at gastrulation and to an abnormally low content of putrescine in the treated embryos. Methylglyoxal bis(guanylhydrazone), an inhibitor of spermine and spermidine synthesis, had no visible effect on development. Our observations strongly suggest that putrescine synthesis is indispensable in early embryonic development of *Ophryotrocha*.**

The polyamines putrescine, spermidine, and spermine are essential components of prokaryotic and eukaryotic cells as well as of virus particles (1). When added to cell-free systems or cells in culture, the polyamines have been found to stimulate the synthesis of DNA, RNA, and protein. This multifaceted action may be due to interaction with nucleic acids or nucleic acid-containing structures, such as chromatin and ribosomes, for which the polyamines exhibit high affinity.

A finding of paramount importance has been that stimulation of cellular polyamine synthesis is associated with a shift from a quiescent to a proliferating state (1–3). In fact, the rate of polyamine synthesis has been shown to exhibit a high positive correlation with the rate of cell proliferation both *in vitro* (4) and *in vivo* (5). In continuously dividing cells, polyamines are synthesized in a biphasic pattern (6). The fact that polyamine synthesis increases prior to DNA synthesis as well as cell division suggests a possible involvement of the polyamines in the progression through these phases of the cell cycle.

Recent experiments with polyamine auxotrophs of *Escherichia coli* (7–9) and inhibitors of polyamine synthesis (10–14) emphasize the importance of the polyamines in cell growth and division, and lend further support for the contention that polyamines play an important role in nucleic acid and protein synthesis in the cell. The polyamine synthesis inhibitors used in the latter studies were α-methylornithine (Me-Orn), a competitive inhibitor of L-ornithine decarboxylase (putrescine synthesis), the initial enzyme in polyamine synthesis, and methylglyoxal bis(guanylhydrazone) (MeGAG), a potent inhibitor of *S*-adenosyl-L-methionine decarboxylase, an enzyme involved in spermidine and spermine synthesis. Both inhibitors caused a partial depletion of the cellular polyamine content, a condition that perturbed the traverse of the cell cycle (10–14).

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The metabolism of the polyamines during early embryonic development has been studied only in sea urchins (15) and amphibians (16, 17). These investigations suggest that the polyamines may participate actively in early embryonic development (e.g., by stimulating RNA synthesis). To date, however, this notion is based only on circumstantial evidence. In an attempt to analyze more directly the importance of the polyamines in early development we have subjected fertilized eggs of *Ophryotrocha labronica* to Me-Orn or MeGAG treatment and analyzed the embryos microscopically for possible interferences with normal development.

MATERIALS AND METHODS

Cultures of the marine polychete *Ophryotrocha labronica* LaGreca and Bacci from the Mediterranean (Naples) are kept in sea water at room temperature (18°–20°). The polychetes breed all through the year and provide a continuous supply of embryo material. Fertilized eggs are deposited cemented together as cylindrical egg packs, with each of the approximately 300 eggs provided with a separate, thin jelly capsule, permeable to nucleosides, amino acids, polyamines, etc. Development of the unpigmented eggs is easily followed in the dissecting microscope. Thanks to their smallness and transparency, the morphological differentiation of the eggs up to hatching can be satisfactorily followed and photographically recorded in hematoxylin-stained whole preparations without previous sectioning. Another great advantage is the strict synchronism in development that exists between all the eggs of an egg pack; part of the pack can accordingly serve as a very reliable control in experimental work.

Developing eggs were collected daily for polyamine analysis during the first 5 days after fertilization. Five egg packs (about 1500 eggs) were sufficient for the quantitative analysis of the polyamines, because a very sensitive analytical technique was employed. The method is based on dansylation of the polyamines, thin-layer chromatographic separation, and fluorescence intensity analysis of their derivatives (18). The developing eggs were disrupted by sonication in 200 μl of 0.2 M $HClO_4$ at 0–5°. After 1 hr in the cold, the homogenate was centrifuged at 1000 × *g* for 15 min and the entire cell extract was dansylated according to the method of Seiler (18). The dansylated derivatives were dissolved in toluene and concentrated, so that the entire amount extracted from 1500 developing eggs was applied on the chromatography plate. The details of the method as applied have been described elsewhere (19).

Me-Orn was kindly donated by Merrell International Research Center, Strasbourg, France, and MeGAG was purchased

Abbreviations: Me-Orn, DL-α-methylornithine; MeGAG, methylglyoxal bis(guanylhydrazone).

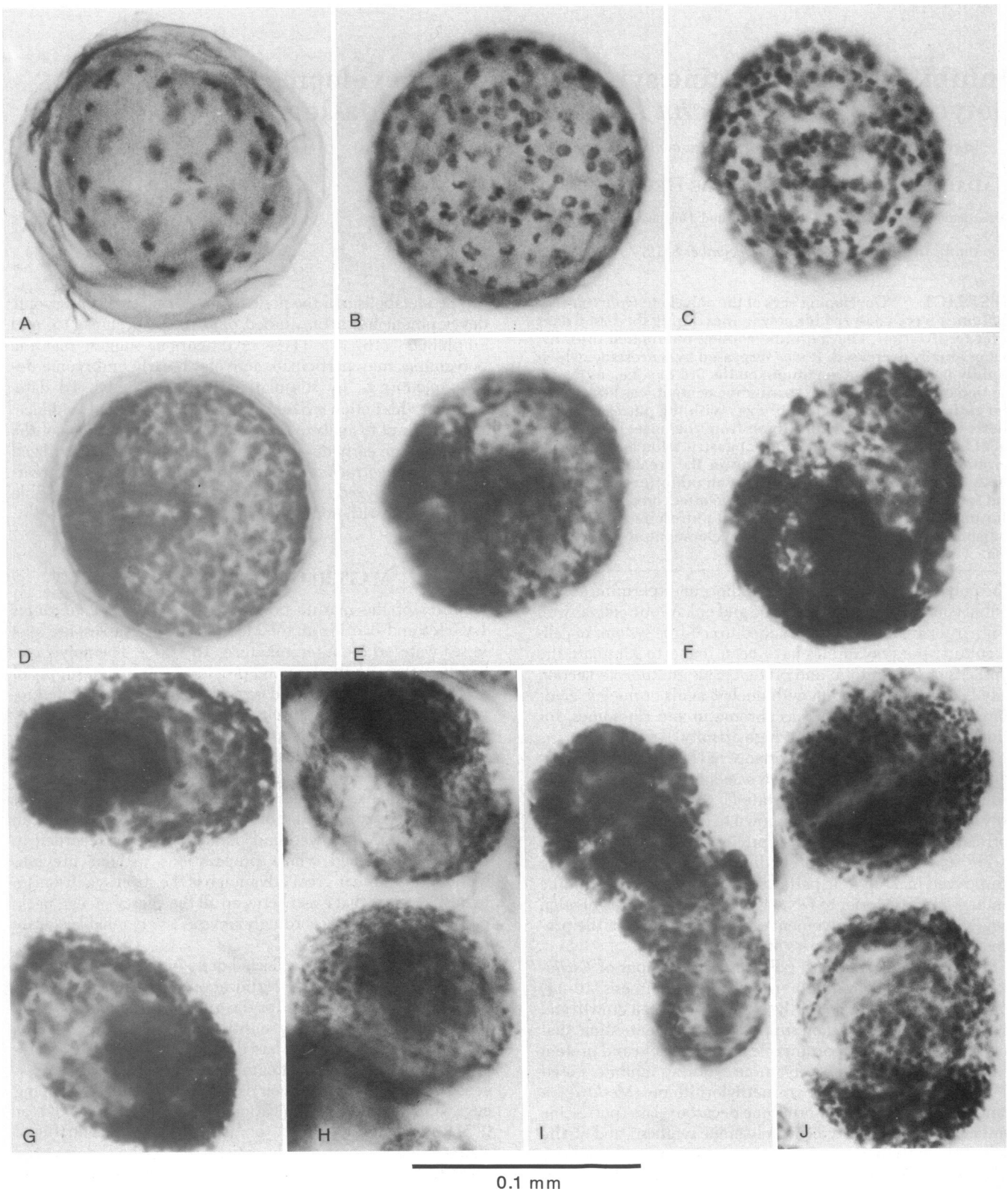

FIG. 1. (*A–F*) Normal developmental changes in embryos of *Ophryotrocha labronica* 1–6 days after fertilization. On the 3rd day (*C*) gastrulation is under way; it is completed on the 4th day (*D*). (*H*) *Ophryotrocha* embryos treated for 6 days with Me-Orn (10 mM). Notice that the embryos, which in development are far behind the DL-ornithine-treated controls (*G*), have not yet completed gastrulation. (*J*) *Ophryotrocha* embryos treated for 8 days with Me-Orn (10 mM). No developmental progress has been made compared with embryos treated for 6 days (*H*). Controls (*I*) are hatched on the 8th day.

from Aldrich Chemical Co., Milwaukee, WI. They were dissolved in sea water at 10 and 0.1 mM concentrations, which have been found to effectively inhibit cell proliferation *in vitro* (10, 11, 13).

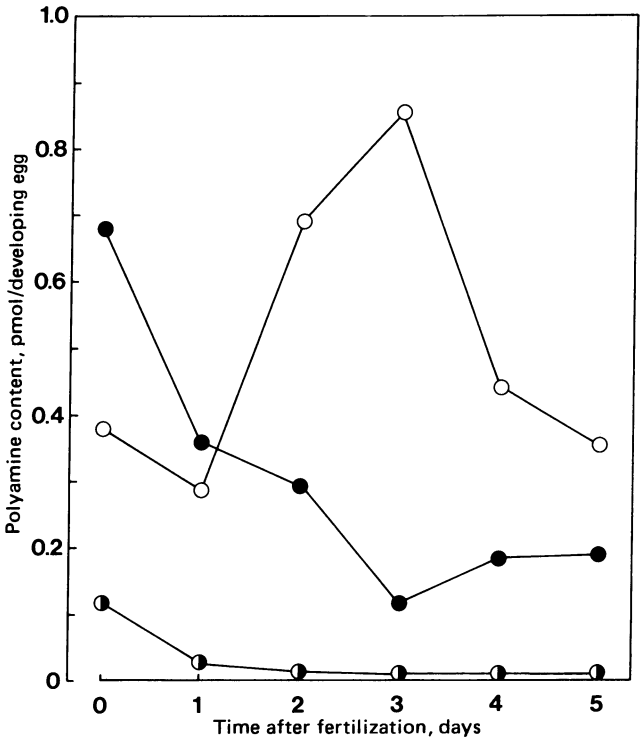

FIG. 2. Polyamine content of developing eggs of *Ophryotrocha labronica*. ○, Putrescine; ◑, spermidine; and ●, spermine. Each point represents 1500 developing eggs.

RESULTS

Under present culture conditions the first 6 days of embryonic development of *Ophryotrocha* proceed as shown in Fig. 1. After this period the larvae begin to leave the egg pack and by 8 days after fertilization (Fig. 1*I*) most of them are free swimming. The photographs demonstrate that gastrulation is under way on the 3rd day and is completed on the 4th day. On the 6th day the mouth region of the larvae is cleary defined and jaws are present.

Fig. 2 shows a representative set of data for the polyamine content during early embryonic development. Similar results were obtained at a different time of the year, thus indicating that polyamine changes are not subject to seasonal changes in this animal. At the onset of development spermine is the major polyamine in the *Ophryotrocha* embryo (Fig. 2). Already on the 2nd day it is surpassed by putrescine, which increases steeply and holds a strikingly dominant position for the rest of the investigated period, with a maximum on the 3rd day. Spermine shows a decreasing trend over the first half of the period, but during the second half the curve is leveling out. Spermidine is all the time on a low level, with the highest value at the beginning of development.

When relating the polyamine content (Fig. 2) to the embryonic development (Fig. 1), one finds that the decrease in spermine content characterizes the whole cleavage period, the part of early development that shows the highest frequency of cell division. The subsequent stabilization in spermine content sets in during gastrulation and is maintained during early larval development. The rapid increase of putrescine immediately precedes gastrulation, with the maximum attained at the onset of this process. To test the functional significance of this increase of putrescine, *Ophryotrocha* embroys were treated with the polyamine synthesis inhibitors Me-Orn or MeGAG from the start of development. Me-Orn (10 mM) was found to arrest the embryos at gastrulation and further development was lacking even at the time of hatching of the controls (Fig. 1). An equimolar concentration of DL-ornithine had no demonstrable effect. Nor did the other inhibitor, MeGAG, interfere with normal embryogenesis. As was to be expected, embryos treated with Me-Orn contained markedly less putrescine on the 3rd day than controls (Table 1).

Table 1. Effect of Me-Orn on the polyamine content of developing eggs of *Ophryotrocha labronica*

Me-Orn, mM	Content, pmol/developing egg		
	Putrescine	Spermidine	Spermine
0	0.752	0.011	0.105
10	0.166	0.012	0.097

Me-Orn was added on day 0. Polyamines were assayed on day 3.

DISCUSSION

The curves in Fig. 2 show a manifest increase in putrescine synthesis in the *Ophrytrocha* embryos just before gastrulation. On the other hand, they give no indications of any significant increase in spermidine and/or spermine synthesis during the investigated period. This impression gains support from our observation that MeGAG had no visible effect on early embryonic development. The general decrease in polyamines during the first 2 days of development suggests that the embryos then are compelled to utilize an existing supply, which is gradually reduced. However, our unpublished experiments have shown that [^{3}H]ornithine is converted into spermidine and spermine at a low but significant rate and suggest that polyamine synthesis is maintained despite the fact that the spermidine and spermine contents decrease. These data suggest a conversion of spermidine and spermine into other substances, e.g., by conjugation with acetyl groups or by enzymic oxidations. The decline in spermine content during the cleavage period fairly well reflects the frequency of cell division in the embryos, which is highest on the first day and gradually falls off for the rest of the period. The fact that the highest spermidine and spermine content in *Ophryotrocha* is observed at the time when cell multiplication is predominant, is a feature in accordance with observations on mitotically active mammalian cells (4). However, the new and interesting finding is that in *Ophryotrocha* spermine is surpassed by the rapidly increasing putrescine just before gastrulation, i.e., at the crucial stage that marks the beginning of embryonic differentiation. Furthermore, putrescine appears to be directly involved in these forthcoming events, because embryos deprived of putrescine synthesis by Me-Orn were unable to gastrulate and develop further, whereas treatment with the spermidine and spermine synthesis inhibitor MeGAG had no visible effects.

Thus, the synthesis of a polyamine has been proved indispensable in early embryonic development. The developmental arrest observed in the absence of putrescine synthesis is difficult to assess at the present time, and only tentative suggestions can be made about the underlying mechanisms. Experiences from other invertebrate embryos, above all sea urchin embryos (20), show that blocking of gastrulation is particularly found in connection with blocking of RNA synthesis. Inhibitors of RNA synthesis do not prevent embryos from passing through cleavage. Their arrest of gastrulation is primarily conceived as due to interference with the synthesis of rRNA for the embryo's own production of ribosomes (20), which starts at this stage. However, also tRNAs and new mRNAs appear in the embryos at this time. Taken together, these events indicate that a reorganization

and a general activation of the genome is occurring in the embryos at gastrulation. How this change is attained is still completely unknown. In the search for possible models for gene activation much work has been done to find cellular substances that are likely to interact with the genome both at the DNA level and at the chromosomal protein level. Among possible candidates emerging are also the polyamines, and some evidence has even been produced in support of the idea that polyamines are gene activators (21–23). With regard to this and in view of the nature of the disturbances induced by Me-Orn in the *Ophryotrocha* embryos, we find it justified to suggest that putrescine is actively involved in gene activation in early embryonic development.

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1. Bachrach, U. (1973) *Function of Naturally Occurring Polyamines* (Academic Press, New York).
2. Raina, A. & Jänne, J. (1975) *Med. Biol.* **53**, 121–147.
3. Morris, D. R. & Fillingame, R. H. (1974) *Annu. Rev. Biochem.* **43**, 303–325.
4. Heby, O., Marton, L. J., Wilson, C. B. & Martinez, H. M. (1975) *J. Cell. Physiol.* **86**, 511–522.
5. Andersson, G. & Heby, O. (1977) *Cancer Res.*, **37**, 4361–4366.
6. Heby, O., Gray, J. W., Lindl, P. A., Marton, L. J. & Wilson, C. B. (1976) *Biochem. Biophys. Res. Commun.* **71**, 99–105.
7. Dion, A. S. & Cohen, S. S. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 213–217.
8. Morris, D. R. (1973) in *Polyamines in Normal and Neoplastic Growth*, ed. Russell, D. H. (Raven Press, New York), pp. 111–122.
9. Srinivasan, P. R., Young, D. V. & Maas, W. (1973) *J. Bacteriol.* **116**, 648–655.
10. Fillingame, R. H., Jorstad, C. M. & Morris, D. R. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 4042–4045.
11. Mamont, P. S., Böhlen, P., McCann, P. P., Bey, P., Schuber, F. & Tardif, C. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1626–1630.
12. Williams-Ashman, H. G., Corti, A. & Tadolini, B. (1976) *Ital. J. Biochem.* **25**, 5–32.
13. Heby, O., Marton, L. J., Wilson, C. B. & Gray, J. W. (1977) *Eur. J. Cancer* **13**, 1009–1017.
14. Andersson, G. & Heby, O. (1977) *Cancer Lett.* **3**, 59–63.
15. Manen, C.-A. & Russell, D. H. (1973) *J. Embryol. Exp. Morphol.* **29**, 331–345.
16. Caldarera, C. M. & Moruzzi, G. (1970) *Ann. N.Y. Acad. Sci.* **171**, 709–722.
17. Russell, D. H. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 523–527.
18. Seiler, N. (1970) *Methods Biochem. Anal.* **18**, 259–337.
19. Heby, O., Sarna, G. P., Marton, L. J., Omine, M., Perry, S. & Russell, D. H. (1973) *Cancer Res.* **33**, 2959–2964.
20. Stearns, L. W. (1974) *Sea Urchin Development: Cellular and Molecular Aspects* (Dowden, Hutchinsson & Ross, Stroudsburg, PA).
21. Dion, A. S. & Herbst, E. J. (1967) *Proc. Natl. Acad. Sci. USA* **58**, 2367–2371.
22. Barros, C. & Giudice, G. (1968) *Exp. Cell Res.* **50**, 671–674.
23. Fischer, H. A., Ahrens, P., Hähle, K. & Thiele, H. (1974) *Histochemistry* **38**, 223–228.