EFFECTS OF ACTINOMYCIN D ON MACROMOLECULE SYNTHESIS AND EARLY DEVELOPMENT IN SEA URCHIN EGGS*

Paul R. Gross and Gilles H. Cousineau

Department of Biology, Brown University, Providence 12, R. I.
and Marine Biological Laboratory, Woods Hole, Mass.

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Incorporation of exogenous amino acids into protein occurs in sea urchin eggs only after fertilization. The alteration in capacity for protein synthesis is a property of the ribosomes, since cell-free systems behave like the eggs in incorporation experiments. Nemer (1962) and Wilt and Hultin (1962) have demonstrated differential stimulation of phenylalanine incorporation by Poly-U in ribosomes from fertilized and unfertilized eggs. Since the particles from unfertilized eggs are stimulated to a greater extent than those from early developmental stages, it is proposed that functional messenger is presented only after fertilization.

There is no net synthesis of RNA in sea urchin embryos during early development (Schmidt, et al., 1948), so that any messenger RNA synthesis would have to be by turnover of pre-existing polymer. Alternatively, the egg might contain a store of messengers in "masked" form, which would become available at fertilization.

The present experiments had three purposes: (1) To determine whether RNA turnover does take place in Arbacia punctulata eggs, (2) to determine, if it does, to what extent Actinomycin D is inhibitory, and (3) to examine, if such inhibition occurs, concomitant effects on protein synthesis and differentiation in early development.

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Experiments

1. Inhibition of C^{14}-uracil uptake by Actinomycin D*; Concentration Dependence. Uracil-C^{14} is incorporated into RNA from fertilization on, and the rate increases during early development. Actinomycin D, when present from before fertilization, brings the incorporation to a stop at about four hours, and in concentrations above 20 µg/ml., allows no differentiation or orderly succession of cleavage planes. Division, however, continues even with more than 100 µg/ml. in the medium. The concentration dependence of the Actinomycin effect on uracil incorporation is summarized in Table I.

TABLE I

INHIBITION OF C^{14}-URACIL INCORPORATION IN ACTINOMYCIN-TREATED EMBRYOS

Conc. Act. D. µg/ml.	cpm/10^6 eggs	% inhibition	Development at 5-1/2 hrs.
0	840	0	normal early blastulae
1.4	304	64	normal early blastulae
5.7	197	77	very irregular blastulae, slight cleavage delay
24	54	94	"morulae" and multicellular masses; no normal forms
96	36	96	many cleavages, but no differentiation

Flasks all contained 5 ml. egg suspension, 1.86 x 10^4 eggs/ml., with Actinomycin D, 0-96 µg/ml. After 10 minutes pre-incubation, all suspensions were fertilized (0.05 ml. sperm suspension). 95+% membrane elevation in all flasks. Eggs were allowed to develop at 23.5°C for 5-1/2 hrs., then to each flask was added uracil-2-C^{14}, final concentration: 87.5 mµM/ml., 2.88 µc/ml. After 1 hr. incubation, 3 ml. samples were removed, washed rapidly with cold sea water, and suspended in cold 5% TCA with a 1000-fold excess of unlabeled uracil. After standing overnight, precipitates were collected on Millipore filters presoaked in TCA-cold uracil. Filters were washed through twice with 5% TCA and then with water, dried, and mounted on planchettes. Counting was in a guarded-detector gas-flow system with a background of 3 cpm. Efficiency was approximately 7% for this method.

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2. Uracil incorporation immediately following fertilization. Massive doses of Actinomycin D are required to reduce the already low normal incorporation during the first four hours of development. The most effective suppression was 79% over the first two hours, and this experiment is summarized in Table II. In other experiments, levels of 100 µg/ml. were ineffective in stopping incorporation during the first four hours; but always, uracil uptake ceased by 5 hours after fertilization. No subsequent decline in counts was observed.

TABLE II

EFFECT OF ACTINOMYCIN ON UPTAKE OF C^{14}-URACIL AND VALINE IN EARLY CLEAVAGE

Observation	cpm/10^6 eggs		% inhibition by Act. D
	control	Act. D	
1st hr. incorporation, uracil	2.13×10^3	1.23×10^3	42
end of 2nd hour	7.58×10^3	1.57×10^3	79
1st hr. incorporation, valine	7.13×10^4	7.16×10^4	-0.4
end of 2nd hour	20.4×10^4	19.5×10^4	4.4

Cell density: 1.18×10^4/ml. L-valine-1-C^{14}; 41 mµM/ml., 0.24 µc/ml., Uracil-2-C^{14}; 21 mµM/ml., 0.66 µc/ml. Actinomycin D, 65 µg/ml., added 30 min. before fertilization.

Autoradiograms show that the uracil label is (1) intracellular, (2) nuclear and cytoplasmic, mainly the latter after 3 hrs., and (3) RNA'ase digestible. Since it is RNA that uracil labels, the failure of Actinomycin to stop incorporation immediately after fertilization probably reflects a very low permeability of the cell to this substance. The concentration dependence data are consistent with such a conclusion.

3. Protein synthesis. The data in Table II show that even with a significant depression of early RNA turnover by Actinomycin D, there is no appreciable effect on protein synthesis. (Hultin, 1961, has shown that

exogenous valine labels protein in sea urchin eggs, and that both the incorporation and mitosis are blocked by Puromycin.) The data in Table III show that incorporation of C^{14}-valine continues for at least seven hours after RNA turnover has stopped in Actinomycin-treated cells, and the rate does not differ significantly from that in untreated embryos.

TABLE III

UPTAKE OF C^{14}-VALINE IN NORMAL AND ACTINOMYCIN-TREATED EMBRYOS

Time post fertilization (hrs.)	dpm/10^6 embryos X 10^{-5}		Developmental Stage	
	Control	Act. D.	Control	Act. D.
1	1.19	0.96	2-cell	2-cell
3	3.00	2.98	16, 32 cell	irregular 8, 16-cell
5	3.99	4.13	early blastula	32 cells and up, irregular
12	5.06	6.06	gastrula	undifferentiated multicellular mass

Cell density: 3.46 X 10^4/ml. U-C^{14}-L valine, 0.53 mμM/ml., 0.11 μc/ml.; Actinomycin D, 115 μg/ml., added 20 minutes before fertilization.

Discussion

The present results confirm scattered earlier studies (e.g., Markman, 1961) in the observation of significant metabolic activity of RNA during early cleavage. This is in contrast to the notion that such differentiation as occurs in early development depends upon purely cytoplasmic processes. The suppression of uracil uptake by Actinomycin D, an inhibitor of RNA polymerase (Hurwitz, et al., 1962) suggests that an important part of the turnover represents synthesis of messenger RNA. This favors the idea that ribosomes receive newly-synthesized messengers after fertilization, which event would serve, among other things, to turn on RNA polymerase.

But the continued synthesis of protein long after RNA turnover has stopped, and at the same rate as in controls, offers a difficulty, for it

means that either all the information necessary for such synthesis is already present in the egg at fertilization, or that the small amount of new RNA made before the Actinomycin block becomes effective is adequate to support a maximum level of protein synthesis for the whole of early development.

Persistence of cell division, in the absence of differentiation, when RNA synthesis has stopped, leads to a possible explanation.

Continued cell division is surprising, in view of the antimitotic effect of Actinomycin in cell cultures (Reich, et al., 1962). But it must be recalled that in the embryos, cleavage continues for many hours even in the absence of a nucleus (Harvey, 1956). This, with Hultin's finding (1961) that some protein synthesis is needed for each division, suggests that an important fraction of the protein synthesis during early development is associated with mitotic activity. The (stable) messages for this class of protein would appear then to be stored in the cytoplasm; they might associate functionally with ribosomes only after fertilization. The initially low turnover of RNA would then be concerned only with production of messengers for the first steps in differentiation. They do come early, possibly as early as the 4th cleavage in this egg. The absolute amounts of such "differentiation" proteins might not be very great; but any interference with their production would prove ultimately lethal, because it would upset the balanced interrelation between mitosis and cellular differentiation, without which normal development cannot proceed.

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