ELECTROPHORETIC EXAMINATION OF SOLUBLE PROTEINS SYNTHESIZED IN EARLY SEA URCHIN DEVELOPMENT[1]

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It has been demonstrated that fertilization in the sea urchin greatly stimulates protein synthesis in the egg (Nakano and Monroy, 1958; Hultin and Bergstrand, 1960). Recent evidence indicates that most, if not all, of the messenger (m)RNA required for this synthesis is present in the unfertilized egg in an inactive or "masked" form (Tyler, 1965). For example, the parthenogenetically activated non-nucleate fragment incorporates amino acids into protein to the same extent as does the nucleate fragment or even the fertilized whole egg (Tyler, 1963; Denny and Tyler, 1964) when tested either as intact cells or homogenates. This observation is also supported by radioautographic studies of amino acid incorporation by egg fragments (Brachet, Ficq, and Tencer, 1963). Additional evidence is provided by experiments with actinomycin D which inhibits RNA synthesis but permits protein synthesis and early development in sea urchin eggs (Gross and Cousineau, 1963; Gross, 1964). There is, apparently, some new mRNA synthesized during early development (Nemer, 1963, Wilt, 1963, 1964; Gross, Malkin, and Moyer, 1964; Gross, Kraemer, and Malkin, 1956; Baltus

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et al., 1965). The evidence, however, indicates that this RNA is initially inactive and is unmasked at later stages (Spirin, Belitsina, and Aitkhozhin, 1964; Tyler, 1963, 1965). These considerations lead to the expectation that the proteins synthesized during early development may be primarily those for which the mRNA has been stored in the unfertilized egg.

In the experiments reported here disc electrophoresis was employed for preliminary identification of the kinds of soluble new proteins formed by the developing sea urchin egg in the presence and absence of actinomycin D. The results support the aforementioned masked mRNA-concept in that no significant qualitative changes were found in the new proteins formed during early development.

Materials and Methods. Artificial sea water prepared by the formula of Tyler (1953) was used throughout the study. Gametes of the sea urchin, Strongylocentrotus purpuratus (Stimpson), were obtained by 0.55 M KCl injection. The eggs were exposed briefly to pH 4.1 sea water, to remove the jelly coat, washed several times in pH 8 sea water, inseminated with a small amount (ca. 0.1 ml per 100 ml of suspension) of a 1% sperm suspension in 10^{-3} M EDTA-sea water, and cultured at 16.5°C in 92 mm diameter petri dishes containing 2.5×10^5 eggs/8 ml sea water until the desired developmental stage was reached. Actinomycin D[4] treatment (20 µg/ml sea water) was started 30 minutes before insemination and the culturing continued in the same solution. At various stages of development, 0.05-0.10 ml of C^{14}-L-valine (spec. act. 200 C/mole, 2.5 µc/ml sea water) were added to the cultures and 1 hour later, 8 ml of 0.1 M C^{12}-L-valine was added for a 20 minute "chase." Embryos were then collected by centrifugation at 1,000 x g, washed 2 times with ice-cold sea water and homogenized in 0.58 M sucrose (1 part embryo:2 parts sucrose) in a Teflon-glass homogenizer. The homogenate was frozen-thawed twice in an

[4] Courtesy of Dr. H. B. Woodruff, Merck & Co., Inc.

ethanol-dry ice mixture and rehomogenized. The homogenate was then centrifuged at 100,000 x g for 2 hours in the cold and the supernatant removed and stored in the deep freeze until used for electrophoresis.

Disc electrophoresis was carried out using 7.5% acrylamide gels and buffers prepared according to the formulae described by Canalco Industrial Corp., Baltimore, Maryland. Glass tubing, O.D. 7 mm x 63 mm, was used with the heights of the upper and lower gels 6 mm x 44 mm respectively. The embryo-supernatant (0.025 ml) was layered over the upper gel and electrophoresis carried out at room temperature at 4 ma constant current/tube. After the bromphenol blue tracking dye (added to the upper buffer vessel until a blue tinge was noted) had migrated 35 mm, the electrophoresis was discontinued, the gel removed and cut into 1.3 mm slices with a razor blade in a plastic jig. Each slice was placed in a separate test tube and the protein eluted from the gel with 0.5 ml of diluted lower buffer (1 part buffer:9 parts distilled water) for a minimum of 14 hours, with agitation, in the cold. Early experiments indicated that no additional protein was eluted after 14 hours, and this time was used throughout the study. At least one gel for each extract was left intact and stained for protein with Naphthol Blue Black. The eluate from each slice was dried on filter paper and processed by Tyler's (1963) modification of the Mans and Novelli technique (1961) in order to determine the radioactivity of trichloracetic acid-insoluble protein. Radioactivity was determined by a Packard Tri-Carb liquid scintillation spectrometer with a counting efficiency of 50%.

Results and Discussion. Development of the embryos in the presence of actinomycin D was delayed and eventually they died at the blastula stage without hatching. Typically, at 4 hours of development when the control embryos had reached the 16-cell stage, the treated embryos were in the 8-cell stage. The delay was more pronounced in advanced embryos.

Thus control embryos were late blastulae at 23 hours of development and had begun to gastrulate an hour later while the experimentals remained as unhatched immotile blastulae.

A group of embryos was labeled during the period 3-4 hours after fertilization (Figure 1). The C^{14}-labeled proteins at this stage were resolved into seven components. Despite the fact that the gels were unstained when sliced, the slices were sufficiently accurate so that any given peak appeared, in different gels, with a range of 2-3 slices.

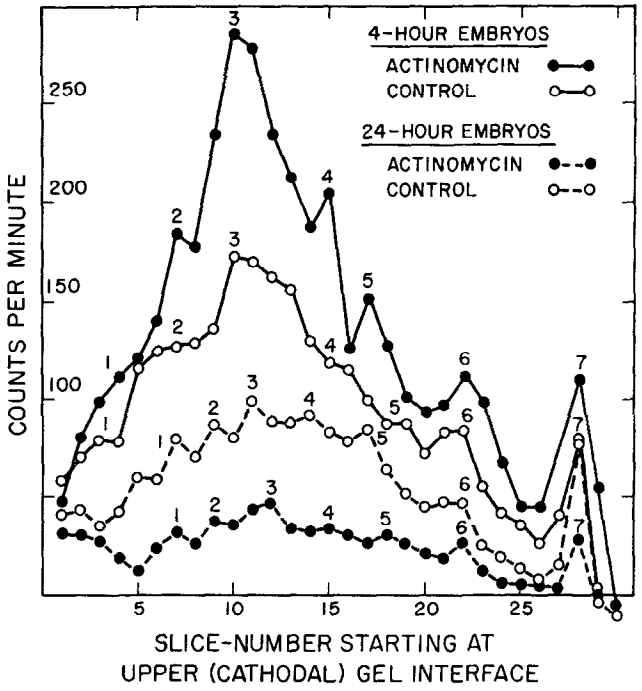

Figure 1. Electrophoretic (acrylamide gel) profiles of radioactivity of proteins extracted from S. purpuratus embryos after incorporation of C^{14}-L-valine at (I) 3-4 hours and at (II) 23-24 hours after fertilization, cultured with (IA and IIA) and without (IC and IIC) actinomycin D (20 μg/ml); protein content per 0.25 ml sample for IA, IC, IIA and IIC = 0.40, 0.35, 0.26, and 0.46 mg respectively; total radioactivity in protein per applied sample for IA, IC, IIA and IIC = 39,800, 38,200, 22,275 and 15,900 counts per minute respectively. Numbers (1-7) indicate repeatedly identifiable "peaks."

The peaks labeled 1-7 were readily reproducible. The region between the upper-lower gel interface and peak 1, however, was difficult to resolve and any peaks detected in this region were considered artifacts.

The pattern of labeled proteins of the actinomycin D treated embryos labeled 3-4 hours after fertilization was not significantly different from the controls; the same seven peaks were detected. Similar experiments were performed during the period of 23-24 hours of development when actinomycin D had considerably reduced the rate of protein synthesis (Malkin et al., 1964). The results (Figure 1) showed that the pattern of labeled proteins not only resembled that of the younger stage but also that there was no significant qualitative difference between the experimental and control embryos. In addition, the Naphthol Blue Black stained gels which distinguished 16 clearly separable components were identical in pattern for the two stages and for the experimental and control embryos. A similar experiment carried out on normal prism stage embryos revealed no significant qualitative differences when compared with the earlier stages.

These results are consistent with the above hypothesis that protein synthesis during early development is primarily dependent on the "masked" mRNA's present in the unfertilized egg. They are also consistent with the findings of Monroy et al. (1961) that there is no qualitative change, at least to the late gastrula stage, in electrophoretic patterns of proteins. In contrast, however, our methods which deal with acid insoluble proteins did not detect a reported (Gross, 1964) difference in proteins between actinomycin-treated and controls at the blastula stage as resolved by sucrose density gradient centrifugation.

Our methods are subject to several limitations. There is the possibility that new kinds of proteins are synthesized during early development but that they are obscured by other labeled proteins. Resolution is obviously limited by the number of slices. Furthermore, the present results apply to those proteins that are soluble in the solutions employed

for extraction, electrophoresis and elution, and insoluble in 5% TCA. This method does, however, lead to highly reproducible results and allows one readily to compare the results with those obtained by previous investigators.

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