tively unchanged, but the matrix in the nucleus has become lighter and more uniform, and the nuclear membrane is more sharply defined. With $\Delta E = 50$ ev (Fig. 1d) almost all the components of the cell have changed in contrast and now range from a very light gray to white. Notice that the lysosome-like bodies, and other smaller spheroid bodies, which were white at $\Delta E = 0$, are now black, and the other cellular components are white or gray, indicating that the energy loss spectrum is quite different at this 50-volt peak. The nuclear membrane is no longer distinguishable.

From this preliminary investigation of the cell by electron energy loss, one observes that various ultrastructures can be distinguished in the same cell through changes in image density. The change indicates that there is a substantial amount of information in the transmitted beam of electrons. To obtain this type of information with the conventional electron microscope would require different stains on several consecutive sections. It is hoped that further modification of the microscope, and proper selection of energy levels, may make it possible to localize selectively specific elements, such as metal ions (calcium, iron, magnesium, and others), on the basis of specific energy losses sustained by impinging electrons, and to improve resolution.

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Actinomycin D: Uptake by Sea Urchin Eggs and Embryos

Abstract. *Actinomycin D is excluded from unfertilized eggs and developing embryos of the sea urchin* Arbacia punctulata *until the blastula hatches. The rate of uptake of actinomycin D by embryos doubles as development progresses after hatching to the gastrula stage.*

Despite the wide use of actinomycin D in investigations of ribonucleic acid and protein synthesis, little is known about the ability of this drug to penetrate intact cells (*1*). This lack of information has resulted in the use of empirical dosage schedules in a variety of widely dissimilar experimental systems. Inhibition of RNA synthesis or protein synthesis after administration of the drug is considered proof of a direct effect of this antimetabolite on genetic transcription. However, the interpretation of experiments in which administration of the drug does not cause inhibition is difficult without evidence that the drug has penetrated to the site of its postulated action.

The marked increase in protein synthesis after fertilization of sea urchin eggs proceeds normally for several hours in the presence of actinomycin D (*2*). Current ideas about the fundamental events of early embryonic development are based, to a large degree, on this observation, particularly the concept that stable maternal messenger RNA's direct the production of early embryonic proteins (*3*).

Because the unfertilized sea urchin egg is almost completely impermeable to polypeptides and amino acids, we investigated the uptake and binding of actinomycin D-^{14}C (a polypeptide) in eggs and embryos of the sea urchin *Arbacia punctulata.*

Mature females were induced to spawn into seawater by electroshock (*4*). The eggs were washed in sterile seawater and counted in micropipettes.

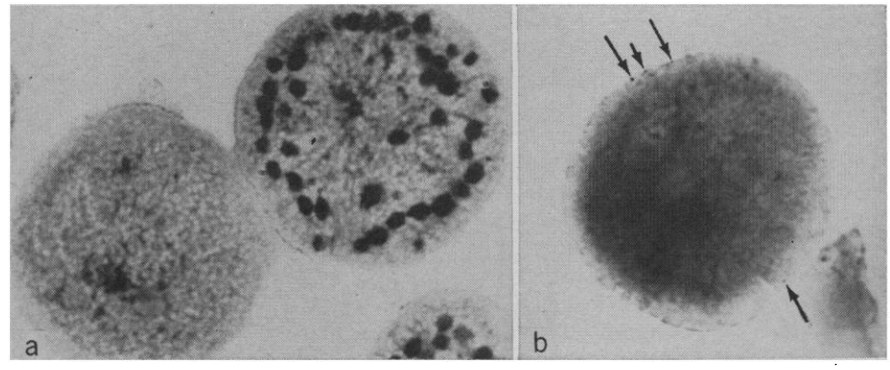

Fig. 1. (a) Control blastula in the upper right hand corner, control unfertilized egg at lower left. No artifactual grains are visible (× 750). (b) Unfertilized egg treated with actinomycin D-^{14}C for 4 hours. Grains are visible at periphery (upper left-hand corner), none over the egg itself (× 1013).

Table 1. Radioactivity of unfertilized eggs plus incubation medium compared with that of egg homogenates and incubation medium alone. At time 0, 1 ml of seawater containing 73 μg of actinomycin D-^{14}C (specific activity, 11.4 mc/mM) and 127 μg of unlabeled actinomycin D was added with stirring to a suspension of 460,000 eggs in 9 ml of sterile seawater (final concentration of actinomycin D, 20 μg/ml). Portions (1 ml) of egg suspension were removed at the times indicated and homogenized directly ("egg-medium homogenate"). The eggs contained in other portions of the suspension were separated from the incubation medium and homogenized in 1 ml of distilled water as described in the text ("egg-water homogenate"). Portions (0.1 ml) of each type of homogenate and of the incubation medium alone were counted in 10 ml of Bray's solution in a liquid scintillation counter. Control cultures without actinomycin D were prepared with each experiment.

Incubation (min)	"Egg-medium homogenate"*	Radioactivity (count min^{-1} ml^{-1})	
		Incubation medium	"Egg-water homogenate"*
0	101,960	102,840	980
10		100,140	970
30	103,000	110,420	774
60	104,000	106,410	860
120			849
240	103,840	112,300	840

* Eggs occupied approximately 1 percent of the volume.

Eggs were fertilized by the addition of one drop of 1 percent fresh sperm suspension to every 10 ml of egg suspension (about 40,000 eggs per milliliter). Only cultures with 95 percent or better fertilization were used.

After incubation in seawater containing actinomycin D-^{14}C, the eggs or embryos were washed by repeated centrifugation through sterile seawater until successive washes contained a constant amount of radioactivity; they were then homogenized in distilled water, and the radioactivity was determined in Bray's solution in a liquid scintillation counter. In the case of the embryos a portion of the distilled water homogenate was treated with an equal volume of cold 10 percent trichloroacetic acid (TCA); the homogenate was then treated with cold 5 percent TCA, heated for 20 minutes at 90°C in 5 percent TCA, and extracted successively with ethanol, ethanol and ether, and ether. The ^{14}C content of the hot TCA supernatant ("nucleic acid fraction") and of the final precipitate ("protein fraction") was measured in a liquid scintillation counter.

Autoradiographic procedures were used to study the localization of bound actinomycin D-^{14}C. Cells exposed to the drug were washed in large volumes of sterile seawater and fixed in Carnoy's fixative; they were then dehydrated in a series of alcohols, cleared in xylene or propylene glycol, and embedded in paraffin or epoxy, respectively. Paraffin sections (5 μ thick) or plastic sections (1 μ thick) were coated with Kodak NTB-2 emulsion, exposed for various periods in the dark, and developed.

The amount of actinomycin-^{14}C which became attached to unfertilized eggs during the first 4 hours of contact with the drug did not increase after the first minute (Table 1) and never exceeded the concentration of actinomycin D-^{14}C present in an equivalent volume of incubation medium. This suggests the lack of facilitated transport into or extensive irreversible binding within the cells. The absence of significant amounts of bound actinomycin D-^{14}C was further demonstrated by the ability of eggs, first incubated for 4 hours in actinomycin D and then washed free of the drug and resuspended in seawater, to develop to fully differentiated and active plutei within 24 hours after fertilization.

Normally developing embryos exposed for short periods to actinomycin D-^{14}C at fertilization (0 minute), prior to hatching (360 minutes), at completion of hatching (480 minutes), or at early gastrulation (900 minutes) accumulated the drug at rates which increased rapidly after hatching (Table 2). Fractionation of egg homogenates into nucleic acid and protein fractions revealed that most of the radioactivity was associated with the protein fraction. There was an accumulation of radioactivity at the periphery of zygotes and unhatched blastulas, whereas no label was visible over the interior of cells. After hatching, radioactivity was evident over the interior of swimming blastulas (Figs. 1 and 2).

Table 2. Radioactivity in embryos developing in incubation medium containing seawater and actinomycin D. Eggs were fertilized as described in the text. At the times indicated, portions (1 ml) of embryo suspension were removed and added to 9 ml of actinomycin D-^{14}C (73 μg) and unlabeled actinomycin D (127 μg) in sterile seawater (final concentration of actinomycin D, 20 μg/ml). After 45 minutes the embryos treated with actinomycin were collected, washed, homogenized in 1 ml of distilled water, and counted as described in the text ("embryo-water homogenates"). The protein and nucleic acid fractions were prepared and their ^{14}C content measured as described in the text. The ^{14}C content of the incubation medium alone was determined as in Table 1. Control cultures without actinomycin D were prepared with each experiment. The majority of embryos hatched between 360 and 480 minutes.

Development (min)*	Radioactivity (count min^{-1} ml^{-1})			
	"Embryo-water homogenate"	Protein fraction	Nucleic acid fraction	Incubation medium
0	512	114	24	104,500
360	520	58	30	106,600
480	714	248	32	101.500
900	1064	334	53	103,400

* Time refers to duration of development before immersion for 45 minutes in the actinomycin D incubation medium.

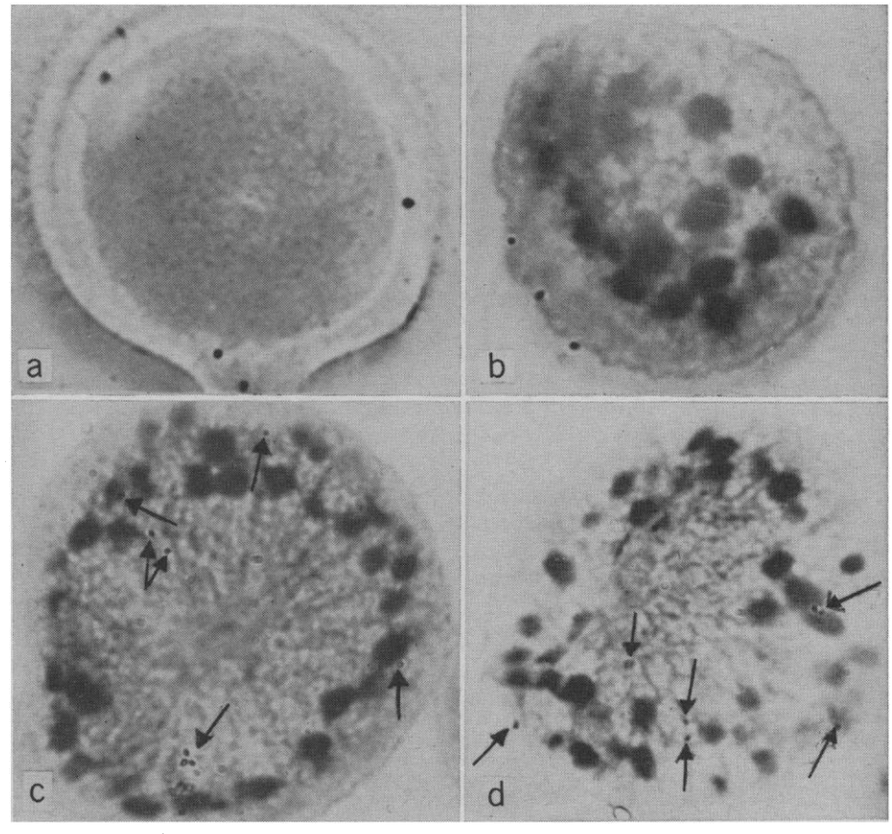

Fig. 2. (a) Appearance of egg 1 minute after fertilization. Actinomycin D-^{14}C is penetrating the fertilization membrane but appears to be excluded from the interior by the hyaline layer covering the egg (× 1350). (b) Unhatched blastula. Fertilization membrane still present. The actinomycin D-^{14}C appears trapped at the hyaline layer (× 1350). (c) Hatched blastula. Fertilization membrane is absent. Radioactivity is evident over the interior of the embryo (× 1350). (d) Early gastrula. Radioactivity is evident over the interior of the embryo (× 1350).

Calculations with Avogadro's number show that 6.7×10^{12} molecules of actinomycin D are bound to each sea urchin egg or unhatched embryo. Because this concentration is approximately equal to the concentration of actinomycin D in the volume of seawater displaced by an egg of *Arbacia punctulata* (*4*), no net accumulation within the space occupied by the eggs occurs. The drug is bound on first contact at a number of unknown sites on the outer surface of the egg or embryo. The fact that the concentration on the surface of cells is in equilibrium with the surrounding medium before hatching suggests that the binding is relatively weak. The autoradiographic studies show that no detectable radioactivity is trapped within cells before the membranes are removed at hatching. This conclusion is further strengthened by the inability of eggs to absorb sufficient actinomycin D after exposure for 4 hours before fertilization to prevent normal development to a highly differentiated, freely feeding stage.

The rate of protein synthesis during the first 4 hours of development in embryos treated with actinomycin was not significantly different from that in untreated controls (*2*); however, the effectiveness of the drug increased with subsequent development. Our results indicate that the lack of effect of actinomycin D on protein synthesis during early embryogenesis may be due to the inability of the drug to enter the cells. The arrest in development which occurs at the hatching stage in the presence of actinomycin D can best be explained by our observations that the embryo becomes vulnerable to actinomycin D only when the hatching process deprives it of its protective shell.

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Soluble Sulfatase in Growing Bone of Rats

Abstract. *Soluble sulfatase has been found in epiphyseal, articular, and rib cartilages and in metaphysis and bone marrow of the rat. The greatest activity in young rats is in the metaphysis. Young rats had higher levels of activity in epiphyseal and articular cartilage and in the metaphysis than did the older rats.*

There has been growing attention in the recent literature to degradative changes in proteinpolysaccharides in calicifying cartilage, after the demonstration of a cartilage protease (*1*). Since there is a rapid turnover of radioactive sulfate in epiphyseal cartilage, an attempt was made to demonstrate the presence of sulfatase in this tissue. Soluble sulfatase has been found in many tissues of different animal species and in *Proteus vulgaris* (*2*) but has not hitherto been reported in cartilage and bone. The method used was adapted from Dodgson *et al.* (*2*) and Roy (*3*). We did not attempt to differentiate between soluble arylsulfatase A and B.

Epiphyseal, articular, and rib cartilages, bone marrow, and metaphysis were dissected from freshly killed Wistar rats 28 to 116 days old. The tissues were ground up separately with a mortar and pestle (5 ml of distilled water was added to each), alternately frozen and thawed three times, and kept cold overnight. The suspension was made up to 12 ml with distilled water and was centrifuged at 20,000 rev/min for 30 minutes. The precipitate was washed and recentrifuged at 30,000 rev/min for 30 minutes. Four volumes of acetone at $-20°C$ were added to the combined supernatants to precipitate the enzyme. The mixture was centrifuged at 25,000 rev/min for 45 minutes, and the supernatant was discarded. The precipitate was dried in a vacuum desiccator after the residual acetone was removed with a stream of air.

The dried samples were dissolved in 0.6 ml of distilled water at 37°C for 1 hour, and 0.6 ml of 0.5*M* sodium acetate–acetic acid buffer (*p*H 5.3) was added. To 0.6 ml of this mixture was added 0.6 ml of 0.5*M* acetate buffer (*p*H 5.3) containing 21 mg of purified *p*-nitrocatechol sulfate to make a final substrate concentration of 0.063*M*. After incubation for 1 hour at 37°C, 3 ml of alkaline quinol was added to develop a red color (*3*). The absorbancy of the red solution was determined at 515 nm. The *p*-nitrocatechol present was read from a standard curve and reported in micrograms per milliliter. Blanks were run containing *p*-nitrocatechol sulfate, but no enzyme; control samples, containing enzyme that had been boiled for 3 minutes before addition of *p*-nitrocatechol sulfate, were also run. All boiled controls showed negligible activity. The activity of sulfatase in the metaphysis (showing the highest activity) was determined to be linear with time during the hour's incubation.

Soluble *p*-nitrocatechol sulfatase activity was found in all of the tissues tested (Table 1). There was considerable variation in the level from animal to animal, but nevertheless, in the case of epiphyseal and articular cartilage and of the metaphysis, the younger

Table 1. *p*-Nitrocatechol sulfatase activity in growing rat bone. Results are expressed as the mean ± standard deviation, in micrograms of nitrocatechol released per milligram of wet weight per hour. Figures in parentheses are the number of animals.

Tissue	Age of rats (days)		*P**
	28–35	76–111	
Epiphyseal cartilage	6.95 ± 3.90 (5)	1.78 ± 0.44 (7)	.009
Articular cartilage	4.22 ± 2.01 (4)	1.22 ± .28 (4)	.04
Costal cartilage	1.08 ± 0.29 (3)	0.76 ± .15 (4)	.17
Metaphysis	12.26 ± 4.25 (5)	6.23 ± 2.64 (6)	.03
Bone marrow	7.82 ± 3.16 (4)	9.25 ± 2.77 (4)	.58

* Statistical significance between young and old groups.