RIBONUCLEIC ACID METABOLISM FOLLOWING FERTILIZATION IN SEA URCHIN EGGS

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A fairly precise and complete picture of the mechanism of protein synthesis has now been achieved. Each amino acid is first attached to a specific transfer RNA (tRNA) and is then inserted into the proper position in the peptide chain as it is polymerized on the ribosome under the guidance of the messenger RNA (mRNA). In the act of transfer the tRNA is released and is then free to repeat the cycle it has just completed. Since it is obvious that the orderly process of cellular differentiation involves a pattern consisting of the initiation and termination of the synthesis of many different proteins, it is clearly of interest to see how various aspects of the scheme by which proteins are synthesized are used to control differentiation. Our concern in this paper is with the first step in the chain of development: fertilization.

The most dramatic event after fertilization is an increase in the incorporation of exogenously supplied amino acids into newly formed proteins.[1, 2] Hence, the first question to arise is: what change takes place upon fertilization that triggers the process of protein synthesis? It seems likely that a part of the answer will be found in the various RNA components which account for so much of the means by which protein synthesis occurs. It is with that we and other workers[10, 15] have examined the changes taking place in the several RNA components after fertilization. The technique employed here has been primarily that of adding P^{32}-labeled phosphate at the time of fertilization and following its incorporation into the RNA components. This provides a distinct advantage over labeled uridine[10, 15] since the composition of RNA being synthesized can be determined.

Experimental Details.—Materials: All experiments were carried out on eggs of *Lytechinus pictus*. These as well as the sperm were obtained by injection of 1 *M* KCl solution in the usual manner. Only batches of eggs that yielded greater than 95% fertilization were used.

RNA extraction: RNA was extracted from embryos, essentially as described by Scherrer and Darnell.[3] Centrifuged pellets containing about 4 million embryos were suspended in 5 ml of a hypotonic sodium acetate buffer (0.001 *M* NaAc, 0.01 *M* NaCl, pH 5). This produced instant lysis. Sodium dodecyl sulfate (20%) was added at once to a final concentration of 0.5 gm/100 ml. Five ml of freshly distilled phenol was added and the mixture shaken at 4° for 15 min. It was then centrifuged, the water layer removed, and the interphase resuspended in 5 ml of another acetate buffer (0.01 *M* NaAc, 0.1 *M* NaCl, 0.5% sodium dodecyl sulfate 0.001 *M* $MgCl_2$). A second phenol extraction was performed, and after centrifugation the separated water layer was combined with the first extract. The resultant was subjected to another phenol extraction. The separated water layer from this extraction was treated with 50 μg of deoxyribonuclease and still another phenol extraction performed. The RNA was precipitated from the final chilled aqueous layer by the addition of 2 vol of cold ethanol. The precipitate was dissolved in a sodium acetate buffer (0.01 *M* NaAc, 0.1 *M* NaCl, pH 5) supplemented by purified bentonite, 50 μg/ml. This mixture was dialyzed overnight against the same buffer. All RNA preparations had a 260–280 mμ extinction ratio of more than 2.

Sedimentation analysis: Sucrose (Merck) was dissolved in sodium acetate buffer (0.01 *M*, pH 5; 0.1 *M* NaCl; 0.001 *M* Versene). The gradient was constructed from the following amounts of such solutions expressed in weight percentage: 0.2 ml of 40%, 0.8 ml of 20%, 1 ml of 16%, 1 ml of 12%, 1 ml of 8%, and 0.4 ml of 4%. On top of this was layered 0.2 ml of the RNA

solution and it was then spun at 37,000 rpm in the Spinco model L ultracentrifuge at 4° for 4 hr. Fractions of 0.15 ml were collected, diluted with 0.1 ml of water, absorbance was read, and 100 μl transferred to planchettes, dried, and measured in a gas flow counter.

Base composition: The base composition of the P^{32}-labeled RNA was determined by pooling the desired fractions, adding carrier yeast RNA, and dialyzing the solution against distilled water. The solution was then lyophilized and hydrolyzed with 0.3 *M* KOH at 37° for 18 hr. The hydrolysate was acidified with perchloric acid and centrifuged to remove any remaining acid-precipitable material as well as $KClO_4$. After neutralizing the supernatant solution with KOH and removing the resultant $KClO_4$, the solution was placed on a Whatman no. 3 filter paper previously immersed in a 0.05 *M* formate buffer, pH 3.2. The sample was then subjected to electrophoresis in the manner described by Smith.[4] The nucleotides were located by their ultraviolet absorption and radioactivity. The radioactive spots were cut out and counted in a Tricarb scintillation counter.

Bacterial contamination: Early in our work it became evident that the contamination of either the eggs or sperm by bacteria greatly falsified the results. Even though collections were made in sterilized sea water the contamination was heavy, particularly in the sperm. The most frequently occurring contaminant appearing on beef heart infusion agar plates was *Escherichia coli*. Thus, separating the eggs from sea water by centrifugation did not remove the contamination. Proceeding in this way, one would expect that incubation of freshly fertilized eggs in P^{32} medium would yield primarily bacterial RNA. The results of this procedure are shown in Figure 1 where the pattern of counts observed in the sucrose gradient fractionation of RNA extracted after 3 hr incubation is presented. The three-peaked distribution has the characteristic shape of bacterial RNA. The base composition of the fractions from the 16 and 23 S peaks was determined and is listed in Table 1 together with the composition of ribosomal RNA from *E. coli* and

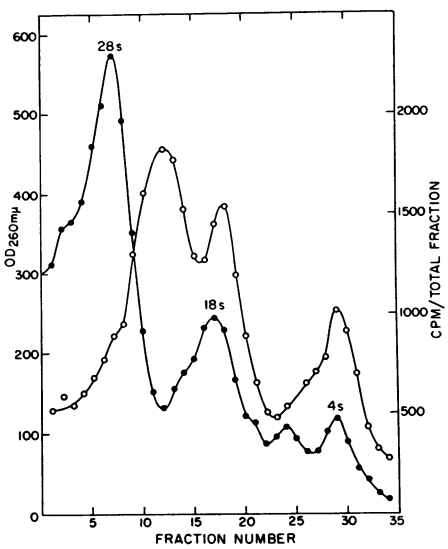

FIG. 1.—Distribution of radioactivity in RNA from fertilized eggs, incubated in sterile sea water for 3 hr in presence of radioactive P^{32}. No attempt was made to remove bacterial contamination. Filled circles: optical density. Open circles: counts/min/fraction. On the basis of previous experience and marker location we assign sedimentation constants of 28S, 18S, and 4S to the first, second, and third peaks, respectively. The radioactive peaks in fractions 12, 18, and 29 then correspond to 23S, 16S, and 4S, respectively.

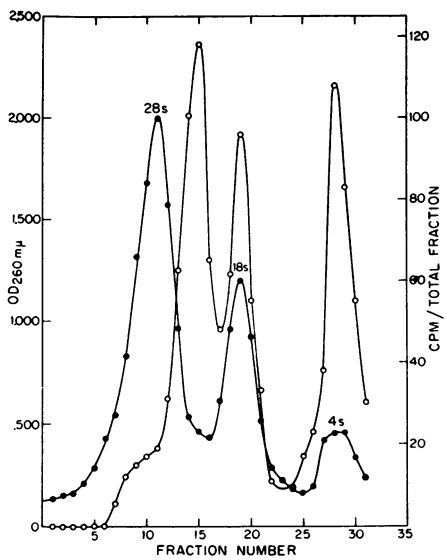

FIG. 2.—Distribution of radioactivity in RNA from unfertilized eggs, incubated in sterile sea water supplemented by 50 γ/ml of streptomycin and 300 units/ml of penicillin for 3 hr in presence of P^{32}. The radioactive peaks at fractions 15, 19, and 29 are assigned as 23S, 18S, and 4S, respectively.

TABLE 1

BASE COMPOSITIONS OF RIBOSOMAL RNA

Base	Sea urchin	*E. coli*	Observed
Adenine	22.5	27.0	27.0
Guanine	29.4	27.6	28.2
Uracil	20.7	21.9	21.9
Cytosine	27.4	22.5	22.9

sea urchins.[5] The good agreement between the observed composition and *E. coli* suggests that the RNA was of bacterial origin.

An attempt was made to suppress bacterial growth by adding 50 μg/ml of streptomycin and 300 units/ml of penicillin to the sterilized sea water. The RNA produced under these conditions was greatly reduced, the specific activity being about 30 times less, as can be seen by a careful comparison of Figure 2, representing this experiment, and Figure 1. The number of colony-forming bacteria also showed a sharp decrease. But on both these counts it was evident that the addition of antibiotics had not completely repressed bacterial contamination.

We then turned to filtration in an attempt to remove the bacteria. Since the sperms as well as the bacteria easily pass through ordinary filter paper, whereas the embryos do not, it was possible to wash the former away from the latter at the end of the incubation period. In general 600 ml of sterile sea water was sufficient to wash free of bacteria and sperm 400 ml of an egg suspension containing about 10^4 eggs per ml. The following routine was adopted. Eggs were suspended at a concentration of 10^4/ml in sterilized, artificial sea water supplemented with 50 μg/ml and 300 units/ml of penicillin. Radioactive phosphate was then added in the amount of 0.5 μC/ml and, after a 3-hr incubation to permit equilibration of the label, fertilization was carried out by adding 4 ml of sperm suspension (absorbance $\sim$1.2 at 650 mμ). The embryos were agitated by bubbling sterile air. At the end of the desired incubation period 0.1 ml of 0.1 *N* HCl per 100 ml of suspension was added to break the membranes.[6] The embryos were then collected on filter paper and washed with about 600 ml of sterile sea water. The embryos were then washed off the filter paper, centrifuged, and used for RNA extraction.

Results.—The incubation of unfertilized eggs in the presence of P^{32} provides the necessary control for our other experiments. The results of fractionating the RNA isolated after a 5-hr incubation in the presence of P^{32} in the manner described in the previous section is shown in Figure 3. The distribution of material according to the optical density at 260 mμ is the usual one, but no counts have entered this material as the absence of counts along the baseline shows. Thus the background for the other experiments is indeed zero.

In the next experiment the eggs were incubated for 3 hr in P^{32}, then fertilized, and incubated another 90 min. This brought them to the 4-cell stage of development. The isolated RNA produced the sucrose gradient pattern shown in Figure 4. No radioactivity can be found in the first 20 fractions, but thereafter in the 4S region there is a substantial rise in counts.

Since this behavior proved to be reproducible, we investigated the RNA produced by pooling the fractions in the 4S region and determining the base composition in the manner described above. The activity of the four components were: cytidylic acid 2012, adenylic acid 648, guanylic acid 243, and uridylic acid 213 cpm. This distribution is strikingly different from the composition of tRNA. Its explanation seems possible only by assuming that the addition of the pCpCpA sequence to the end of already existing tRNA is taking place. In such an addition the radioactive phosphorus of cytidine and adenosine triphosphate would reappear in the 3′ position of the neighboring nucleotide upon hydrolysis. Thus the fourth nucleotide from the end will be labeled. Now it has been shown that about 60 per cent of all fourth positions in tRNA are occupied by adenylic acid, and 20 per cent each by guanylic

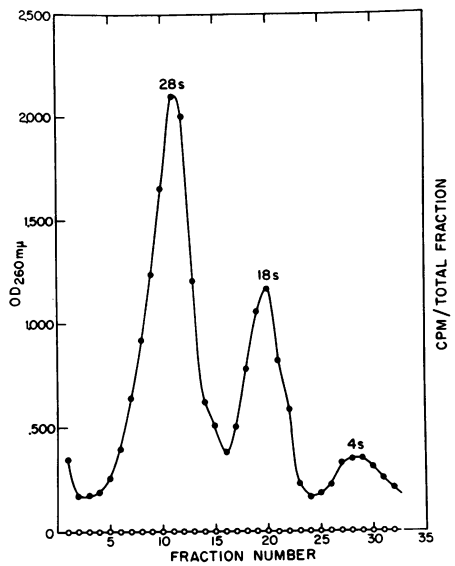

FIG. 3.—Distribution of radioactivity in RNA from unfertilized eggs, suspended in sterile artificial sea water supplemented by antibiotics for 3 hr. The eggs were then incubated for 5 hr in the presence of P^{32} and washed free of contamination by selective filtration.

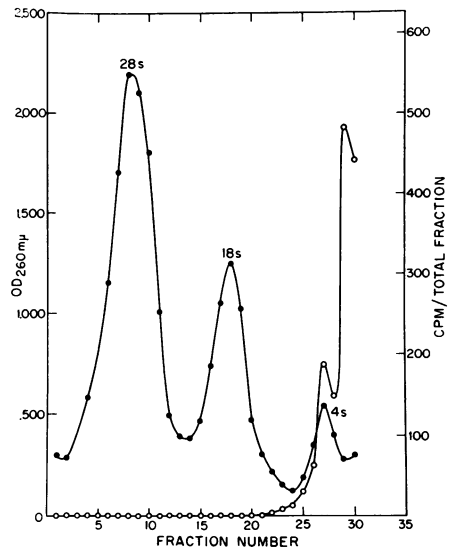

FIG. 4.—Distribution of radioactivity in RNA from fertilized eggs suspended in sterile artificial sea water together with radioactive P^{32}. Following a 3-hr preincubation period in presence of the isotope, the eggs were fertilized, and incubation was continued up to the 4-cell stage ($1^1/_2$ hr).

and uridylic acid.[7, 8] On this basis the label in the hydrolysate should be in the ratio of 10:3:1:1 for C:A:G:U, respectively. Our observed result is 10:3.1:1.2:1.1. The agreement is excellent. We conclude that the only metabolic event at the RNA level from the moment of fertilization to the four-cell stage is the addition or turnover of the pCpCpA terminal grouping of transfer RNA.

A similar experiment was then performed at the 32-cell stage. The resulting sucrose gradient pattern is shown in Figure 5. Here it is seen that radioactivity appears throughout the gradient but the concentration in the 4S region remains distinctive. The newly formed high-molecular-weight RNA is most likely to be messenger RNA. A composition similar to that of the DNA would support this. Base analysis of the pooled fractions 1–20 showed the composition to be in the mole ratio of 19.0:21.3:31.0:28.7 for G:C:A:U, respectively. This checks with the base composition of sea urchin DNA found to be 40.0 per cent G + C from apparent buoyant density in the CsCl gradient.[9] Analysis of the pooled fractions in the 4S region showed a similar result to that obtained before: 10:3.7:1.3:1.0 for C:A:G:U, respectively.

In a final experiment, development was carried forward to the blastula stage, that is, for 8 hr. The results are shown in Figure 6. It is seen that both the messenger and transfer RNA components have increased further. The base compositions were found to be much the same as in the previous experiment. For the pooled 1–20 fractions the mole ratios of 20.5:18.9:29.8:30.8 were found for G:C:A:U, and for the pooled fractions in the 4S region the ratios 10:3.9:1.3:1.2 were found.

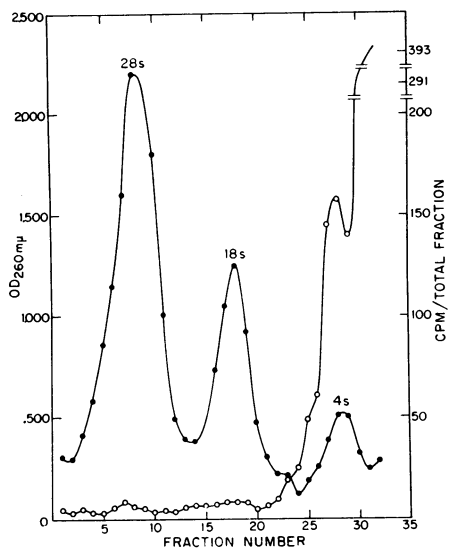

FIG. 5.—Distribution of radioactivity in RNA from fertilized eggs incubated with P^{32} until the 32-cell stage.

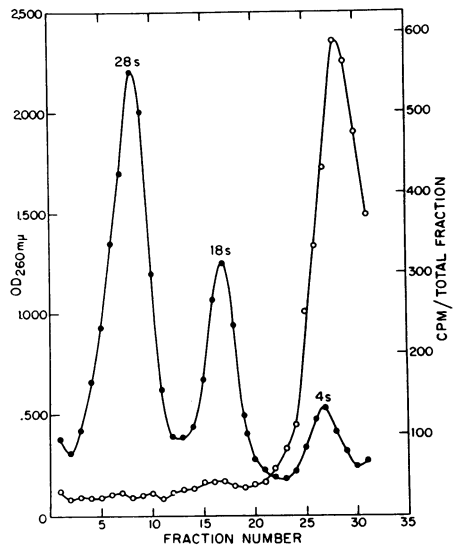

FIG. 6.—Distribution of radioactivity in RNA fertilized eggs incubated with P^{32} until the blastula stage.

Discussion.—The conclusions to be drawn from these experiments are straightforward. As expected, unfertilized eggs do not show any significant metabolic activity of RNA. Growth from fertilization to the four-cell stage is accompanied by uptake of radioactive phosphorus in the transfer RNA only. Base analysis shows this to be consistent with the addition of pCpCpA to the end of the tRNA molecules. Continued growth to the 32-cell stage and the blastula stage involves the production messenger RNA as well as further end additions to transfer RNA. Thus, within this limited view, the addition of the pCpCpA segment to the transfer RNA,[10] either *de novo* or by turnover, is the first event to occur with respect to the RNA components following fertilization. At the same time this might be a part of a mechanism for triggering protein synthesis upon fertilization.

The concept of triggering implies that all the other components needed for protein synthesis are present at the moment of fertilization. This appears to be the case. Maggio[11] has shown that RNA from sea urchin eggs stimulates protein synthesis. The same results have been observed in this laboratory for RNA from frog eggs.[12] Thus mRNA appears to be present. Moreover, it has been shown that the amount of tRNA as well as the amount of ribosomal RNA per embryo does not increase until the gastrula stage.[13] Thus the other RNA components also seem to be present. Hence the initiation of protein synthesis which is an immediate consequence of fertilization can be triggered by a single event. The addition of pCpCpA to the tRNA could be that triggering event in view of the work presented here.

It is important to point out that an alternate proposal has been made by Hultin[1] to account for the onset of protein synthesis following fertilization. His observations, which have been independently confirmed,[14] are that ribosomes from unfertilized eggs are inactive in cell-free protein-synthesizing systems in contrast to

those from fertilized eggs. This would suggest that some inhibitor masks the active sites on ribosomes. Whether the completed tRNA molecules, perhaps with their amino acids attached, might play a role in releasing the inhibitor and activating the ribosome remains to be explored. At present, the extent to which these two proposals overlap or are indeed mutually exclusive is unclear.

The onset of mRNA synthesis somewhere between the 4-cell stage and 32-cell stage indicates that the increased anabolic activity of RNA after fertilization takes place in two steps: first, an activating process (tRNA) using preprogrammed mRNA independent of parallel gene activation. The second step involves a gradual synthesis of mRNA which is directly gene-dependent and directs the development and differentiation. The molecular weight distribution of mRNA through all the sucrose gradient without any distinguishing peaks indicates a great variety of mRNA molecules, capable of synthesizing a great variety of proteins.

Summary.—Unfertilized eggs do not show any significant metabolic activity of RNA. Growth from fertilization to the four-cell stage is accompanied by uptake of radioactive phosphorus in the pCpCpA end of tRNA. Continued growth to blastula stage involves the production of mRNA as well as further end addition of the pCpCpA segment to the tRNA. Possible triggering mechanisms for protein synthesis upon fertilization are discussed.

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