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[1] Talwar, G. P., N. C. Panda, G. S. Sareen, and A. J. Tolani, *Biochem. J.*, **82**, 173 (1962).

[2] Gupta, S. L., thesis, All India Institute of Medical Sciences, Department of Biochemistry.

[3] Sibatani, A., S. R. de Kloet, V. Allfrey, and A. E. Mirsky, these PROCEEDINGS, **48**, 471 (1962).

[4] Talwar, G. P., S. L. Gupta, and F. Gros, *Biochem. J.*, in press.

[5] Matthaei, J. H., and M. W. Nirenberg, these PROCEEDINGS, **47**, 1580 (1961).

[6] Hamilton, T. H., these PROCEEDINGS, **49**, 373 (1963).

[7] In contrast to many other strains of albino rats which begin testicular maturation at approximately one month of age, the males of the AIIMS colony do not show signs of spontaneous testicular maturation until they are near 45 days of age.

[8] Jervell, K. F., C. R. Diniz, and G. C. Mueller, *J. Biol. Chem.*, **231**, 959 (1958).

[9] Goldberg, I. M., and M. Rabinowitz, *Science*, **136**, 315 (1962).

[10] Reich, E., I. M. Goldberg, and M. Rabinowitz, *Nature*, **196**, 745 (1962).

[11] Hurwitz, J., J. J. Furth, M. Malamy, and M. Alexander, these PROCEEDINGS, **48**, 1222 (1962).

[12] Tata, J. R., *Nature*, **197**, 1167 (1963).

[13] Liao, S., and H. G. Williams-Ashman, these PROCEEDINGS, **48**, 1956 (1962).

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*OLD AND NEW RNA IN THE EMBRYOGENESIS OF THE PURPLE SEA URCHIN**

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The RNA involved in embryogenesis may be stored originally in the unfertilized egg as well as newly synthesized in the course of development. A major portion of the RNA reserve of the sea urchin egg is ribosomal. The ability of these ribosomes to participate in protein synthesis is limited by a lack of messenger RNA (mRNA);[1-3] but after fertilization the rate of ribosomal synthesis of protein increases, presumably because mRNA has been newly synthesized or in some way made available. Chemical measurements indicate that there is practically no change in the amount of RNA present through early development.[4, 5] Nevertheless, incorporation of various radioactive precursors into RNA of sea urchin embryos has been detected.[6, 7] If this incorporation indicates only turnover, then a substantial net synthesis of any one RNA component would have to come at the expense of another. An increase with development in the concentration of one such component, transfer RNA, was deduced previously[3] and has been corroborated by the data to be presented.

In the experiments reported here, the major components of RNA of the sea urchin egg have been characterized by sedimentation analysis, and their fate in the course of development has been examined together with the nature of the RNA newly synthesized at the various embryonic stages. The sedimentation pattern of incorporation following a short exposure (pulse) to radioactive precursor has been compared to that at the end of a subsequent incubation in the

presence of unlabeled precursor (chase). The portion of radioactive RNA of the pulse destined for stable components will be found associated with them following the chase. On the other hand, any rapidly degraded RNA will be detectable only after a pulse and not at the end of a chase. The course of embryogenesis of the sea urchin, it will be demonstrated, is marked by striking changes in the character of pulse and chase RNA (RNA_p and RNA_c, respectively).

Methods.—Batches of eggs from one to three females of the species *Strongylocentrotus purpuratus* (Pacific Bio-Marine Supply Company, Venice, California) were pooled. Fertilized eggs were allowed to develop[7] at 20°C in artificial sea water[8] in the presence of penicillin (30 mg/ml) and streptomycin (50 mg/ml). At the various developmental stages 1 ml of eggs or embryos were settled by light centrifugation and suspended to 5 ml with sea water for a period of 10 or 20 min, during which time they were exposed to a concentration of either 3.5 or 7 μM uridine-H^3 (2.77 mC/μmole; New England Nuclear Corporation). At the end of this pulse period the eggs were suspended in sea water containing unlabeled uridine (5 mM). Half of this suspension was centrifuged quickly. The embryos in the pellet were frozen immediately to −80°C and stored. The other half of the suspension was centrifuged lightly, then resuspended in 100 ml of sea water containing 0.5 mM uridine. These embryos continued developing for 4 hr. After this chase period, the embryos were centrifuged, and their pellet was frozen and stored as above.

For further processing, the eggs or embryos were homogenized in a solution of 0.5% sodium dodecyl sulfate (SDS), 1 mM $MgCl_2$, 0.1 *M* NaCl in 0.01 *M* acetate buffer pH 6.0. Samples were immediately submitted to phenol extraction at 60°.[9] Purified RNA[10] in the above solution less SDS was applied to a 5–20% linear sucrose gradient,[11] containing 100 μg/ml bentonite. Samples were centrifuged for 11 hr at 25,000 rpm in the Spinco head SW 25.1. Equal fractions were collected. Optical densities at 260 mμ were determined, and the radioactivities of aliquots of the fractions in scintillation fluid[12] were measured in a scintillation counter. Duplicate pulse-chase experiments with different batches of eggs were performed for every embryonic stage represented. All figures have been normalized to a total of 10 $O.D._{260}$ units of RNA per gradient.

The sedimentation constants of the various RNA peaks were estimated by adding to the sucrose gradients a trace of P^{32}-labeled RNA, prepared from L-cell fibroblasts (gift of R. Perry), containing components of known sedimentation constants.[13] Using the 18S component of the P^{32}-marker RNA as a standard, the approximate S values of the other peaks were derived by the method of Martin and Ames.[14]

Major RNA components of eggs and embryos: Sedimentation analysis of the RNA of the eggs of *S. purpuratus* performed after extraction at 60° in the presence of SDS revealed four major components as determined by absorbancy at 260 mμ. The sedimentation constants of the four peaks of egg RNA from the bottom to the top of the gradient (Fig. 1) were estimated to be 25–28S, 18S, 13S, and 4S, respectively. Phenol extraction at room temperature in the absence of SDS produced only 28S, 18S, and 4S RNA. The first two of these components correspond to the known ribosomal RNA's;[15] the last, to transfer RNA. Phenol extraction of egg ribosomes with SDS at 60° gave the 13S RNA as well as the 28S and 18S material. This RNA component was apparently derived from ribosomal RNA as a breakdown product. The RNA of two other species, *Arbacia punctulata* and *Lytechinus pictus*, were not susceptible in this way to these conditions of extraction.[16] The amount of extracted RNA sedimenting at 13S diminishes considerably in the course of development of *S. purpuratus*, from about 30% in the egg to less than 5% in the pluteus.

Approximately 6% of the total RNA of the egg is 4S. This value rises by a factor of 2 and 3 when the blastula and gastrula stages, respectively, are reached (Fig. 2). A net increase in the concentration of transfer RNA during development had been previously[3] deduced from measurements of polypeptide synthesis by cell-free ribosomal preparations from other species of sea urchin.

Pulse and chase RNA: (a) *The unfertilized egg:* Nucleosides are utilized to a lesser degree before than after fertilization;[7] nevertheless, their incorporation into the RNA of the unfertilized egg is detectable. The characteristics of this incorporation were studied by sedimentation analysis of RNA from eggs that had been incubated with H^3-uridine for a 20-min pulse and those that had been subsequently incubated in unlabeled uridine for a 4-hr chase, following the 20-min pulse. After the pulse, radioactivity was distributed through the gradient into peaks of approximately 4,

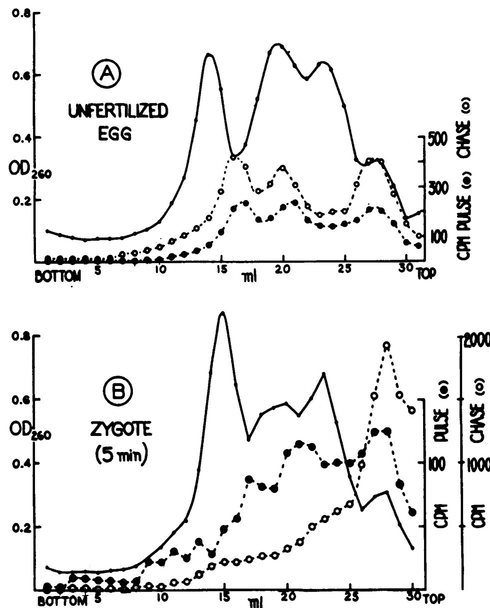

FIG. 1.—Sedimentation diagrams of RNA from (*A*) unfertilized and (*B*) just-fertilized eggs. Eggs were pulsed for 20 min with H^3-uridine (7 μM) and chased for 4 hr in the presence of 0.5 mM unlabeled uridine. Solid curve represents absorbancy at 260 mμ; dotted lines represent radioactivities of RNA_p (●) and RNA_c (○).

17, and 22S (Fig. 1*A*). Following the chase, the same distribution persisted, but with a slight increase in specific activity. The RNA represented by these peaks, apparently, is of a relatively high degree of stability. Since it does not contribute appreciably to the pattern of optical absorbancy, it probably comprises a very small fraction of the total RNA. In both the pulse and chase the 4S RNA was the only major component labeled. The additional incorporation during the chase may have come from nonexchangeable precursor pools that had accumulated during the pulse.[7, 10] A much longer chase would be needed to determine the extent of turnover of the pulse-labeled material.

(b) *The just-fertilized egg:* Exposure of eggs 5 min after fertilization to a 20-min pulse of H^3-uridine produced a pattern of incorporation similar to that of the unfertilized egg (Fig. 1*B*). Although qualitatively the same, the incorporation in the RNA_p of the fertilized egg was greater than that of the unfertilized egg. Furthermore, in contrast to the behavior of the unfertilized egg, a 4-hr chase of the fertilized egg resulted in some incorporation into the major non4S RNA components together with a predominant amount in the 4S RNA (Fig. 1*B*). The increase in specific activity during the chase was too great to decide whether or not the RNA_p peaks at 17 and 22S disappeared or were obscured by the pattern of the chase.

(c) *Pregastrula stages:* At the 8- to 16-cell cleavage stage, the radioactive RNA of a 10-min pulse was spread through the sucrose density gradient but with a peak displayed at approximately 10S (Fig. 2*B*). Incorporation during the chase was mainly into the 4S RNA with appreciable labeling of the 13S RNA and smaller activity in the 28 and 18S regions. The same pattern was displayed up to the 24-hr mesenchyme blastula stage (Fig. 2*C*). During development to this stage, the pulse-labeled peak at 10S became progressively broadened toward the heavy region of the gradient, and the degree of utilization of labeled uridine increased. Figure 2*A* represents a 10-min pulse and 4-hr chase following the first division ($1^1/_2$ hr). The character of the RNA_p is intermediate between that of the just-fertilized egg and those in the cleavage and blastula stages; that is, it is apparently a composite containing the 17 and 22S peak as well as the 10S peak.

(d) *Postgastrula stages:* A pronounced change in the characteristics of the RNA_p occurred after gastrulation. In the pulse of the 45-hr gastrula (Fig. 2*D*), the 10S region no longer predominated in amount of incorporation. Instead, the radioactivity was dispersed throughout the gradient with substantial amounts present in regions of greater than 30S. The corresponding RNA_c was also much different. A large amount of incorporation was found for the first time in the 28 and 18S RNA. At this stage the optical absorbancy of the 13S component was reduced from a peak to a small shoulder, and the radioactivity associated with it also described a shoulder. In the prism (60 hr) and in the 72-hr pluteus (Fig. 2*E*) the pattern of incorporation of RNA_p was similar to that seen in the gastrula. After chasing, a large amount of activity was found in the major RNA components.

Developmental relationships involving 4S and non4S RNA: The 4S and non4S RNA can be viewed separately by graphically subtracting the activity (the product of the specific activity and total optical density units) of the 4S RNA from that of the total RNA. The resultant non4S

FIG. 2.—Sedimentation diagrams of RNA from embryos at various stages. Embryos were pulsed for 10 min with H^3-uridine (3.5 μM) and chased 4 hr in the presence of 0.5 mM unlabeled uridine. Representations are the same as for Fig. 1: RNA_p (●), RNA_c (O).

RNA's of various stages have been represented on a single scale in Figure 3 against the optical density pattern of the gastrula stage. It is apparent that with development the degree of labeling increases as markedly as the pattern of sedimentation behavior changes. The shift to the heavier region of the gradient is coincident with the pronounced rise in incorporation in ribosomal RNA at gastrulation.

The rapidly synthesized non4S RNA consists of a component (m) that is rapidly degraded, concordant with its function as messenger, and a component (pr) that is precursor to stable or ribosomal RNA (s).[10] The fraction, $pr/(m + pr)$, of the rapidly synthesized RNA ($m + pr$), converted to s, can be

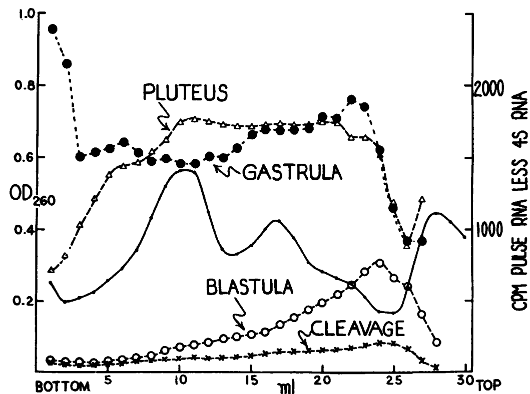

FIG. 3.—A comparison of the sedimentation characteristics on non4S RNA_p at various embryonic stages obtained by graphically subtracting the radioactivity of 4S RNA from the RNA_p of Fig. 2.

estimated for an interval long enough for essentially all of pr to be converted to s. An adequate chase represents such an interval. Thus, if the first RNA products (4S RNA + m + pr) in a given interval are derived from the same precursor pool, P, then

$$P_c / P_p = (m + pr)_c / (m + pr)_p = 4S\ RNA_c / 4S\ RNA_p,$$

$$\text{or } (m + pr)_c = (m + pr)_p\ (4S\ RNA_c / 4S\ RNA_p). \quad (1)$$

If $s = pr$, then

$$pr / (m + pr)_c = s / (m + pr)_p\ (4S\ RNA_c / 4S\ RNA_p)$$
$$= non4S\ RNA_c / (non4S\ RNA_p)\ (4S\ RNA_c / 4S\ RNA_p). \quad (2)$$

Calculations of the fraction of the total radioactivity (cpm) of the rapidly synthesized non4S RNA, as represented in Figure 3, converted to stable non4S RNA yield the values given in Table 1. In the pregastrula stages this fraction was approximately 0.31. The postgastrula stages retained much more of the rapidly synthesized RNA in the ribosomal constituents, about 0.52 in the 45-hr gastrula and 0.59 in the 72-hr pluteus.

While the ratio $s/(m + pr)$ and thus s/m apparently increase in the course of development, the ratio s/4S RNA_c also increases (Table 1). This ratio rises from a value of 0.5 in the early cleavage stages to 2.2 in the 72-hr pluteus. Therefore, during development the synthesis of ribosomal RNA increases relative to both messenger and transfer RNA.

Discussion.—The development of the sea urchin embryo proceeds in distinct phases, each with a characteristic pattern of RNA synthesis.

Before and shortly after fertilization: The RNA_p of unfertilized and just-fertilized eggs has two peaks of approximately 17 and 22S; however, incorporation is somewhat higher after fertilization. In the unfertilized egg the pattern persists through a 4-hr chase indicating a relatively high degree of stability, whereas in a chase of the fertilized eggs incorporation in stable RNA of different sedimentation behavior obscures the pulse pattern, indicating either that the RNA_p has been metabolically degraded or that the synthesis of this type of RNA has diminished. Irrespective of its degree of stability, this RNA_p may be the first messenger, serving to inaugurate the events of embryogenesis. The synthesis of such mRNA could account for the very low but detectable protein synthesis in the unfertilized egg.[2] Its continued production and use after fertilization may satisfy the need for protein synthesis in cell division[17] and account for the rise in this synthesis after fertilization.[1–3, 18]

Pregastrula stages: During the early cleavage stages, the nature of the RNA_p experiences a transition (Fig. 2*A*) to a pattern with a predominant peak of approximately 10S (Fig. 2*B*). This may be a transition between the synthesis of messengers patterned by the maternal genome for use before and immediately after fertilization and that of messengers to be involved in the developmental process. This pattern of RNA_p appearing in all of the pregastrula stages exhibits a progressive developmental change, whereby the peak broadens toward the heavier region of the gradient. This peak may comprise a group of mRNA's whose average size increases during this period of development (as the embryo learns longer words?). These messengers may herald differentiation.

Postgastrula stages: The final form taken by RNA_p in this progression occurs in all of the postgastrula stages. Its sedimentation pattern is polydisperse with a large proportion of radioactivity of greater than 30S. It is a pattern that has been demonstrated in highly developed growing cells.[9, 10, 19] The sharp rise in ribosomal RNA synthesis, seen in the chase experiments with gastrulae, coupled

with the appearance of substantial radioactivity in RNA_p greater than 30S, agrees with the hypothesis of Scherrer, Latham, and Darnell[19] that this part of the rapidly synthesized RNA is precursor to ribosomal RNA. The calculated proportion of non4S RNA_p that is converted to 28 and 18S RNA rises from less than 30 per cent in the early cleavage stages to greater than 60 per cent in the late pluteus. Thus, the ratio of synthesis of labile or messenger RNA to ribosomal RNA is greater in the pregastrula than in the postgastrula stages. The reservoir of functional ribosomes in the unfertilized egg[1-3] may largely serve the needs of the embryo through the early stages. During this period a greater emphasis may be placed on the synthesis of mRNA needed to program the functional ribosomes already present than on the construction of new ribosomes.

Summary.—Distinctly different sedimentation patterns are displayed by the rapidly synthesized RNA of three phases in development: (1) the patterns of the unfertilized and just-fertilized egg have characteristically two peaks (approximately 17 and 22S); (2) a peak of approximately 10S dominates in the pregastrula stages; and (3) the postgastrula pattern is polydisperse. The last is coincident with a markedly enhanced synthesis of ribosomal RNA, as detected by incorporation in chase experiments.

TABLE 1

DEVELOPMENTAL CHANGES IN THE RELATIVE SYNTHESES OF VARIOUS CLASSES OF RNA

		Ratio of incorporation (range)	
Stage	Hr	$s/(m + pr)$*	s/4S RNA_c
Cleavage	1–10	0.31 (0.30–0.33)	0.54 (0.53–0.56)
Blastula	20–28	0.34 (0.32–0.38)	0.87 (0.65–1.10)
Gastrula	45–49	0.52	1.14
Pluteus	72–76	0.59 (0.55–0.62)	2.20 (1.9–2.50)

* Fraction of rapidly synthesized non4S RNA ($m + pr$) converted to stable or ribosomal RNA (s = non4S RNA_c).

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[1] Nemer, M., *Biochem. Biophys. Res. Comm.*, **8**, 511 (1962).
[2] Wilt, F., and T. Hultin, *Biochem. Biophys. Res. Comm.*, **9**, 313 (1962).
[3] Nemer, M., and S. G. Bard, *Science*, **140**, 664 (1963).
[4] Schmidt, G., L. Hecht, and S. J. Thannhauser, *J. Gen. Physiol.*, **31**, 203 (1948).
[5] Elson, D., T. Gustafson, and E. Chargaff, *J. Biol. Chem.*, **209**, 285 (1954).
[6] Villee, C. A., M. Lowens, M. Gordon, E. Leonard, and A. Rich, *J. Cell. Comp. Physiol.*, **33**, 93 (1949); Abrams, R., *Exptl. Cell Research*, **2**, 235 (1951); Scarano, E., and H. M. Kalckar, *Publ. Staz. Zool. Napoli*, **24**, 188 (1953).
[7] Nemer, M., *J. Biol. Chem.*, **237**, 143 (1962).
[8] Tyler, A., *Biol. Bull.*, **104**, 224 (1953).
[9] Scherrer, K., and J. E. Darnell, *Biochem. Biophys. Res. Comm.*, **7**, 486 (1962).
[10] Perry, R., these PROCEEDINGS, **48**, 2179 (1962).
[11] Britten, R. J., and R. B. Roberts, *Science*, **131**, 32 (1960).
[12] Bray, G. A., *Anal. Biochem.*, **1**, 279 (1960).
[13] Homma, M., and A. S. Graham, *Biochim. Biophys. Acta*, **61**, 642 (1962).
[14] Martin, R. G., and B. N. Ames, *J. Biol. Chem.*, **236**, 1372 (1961).
[15] Hall, B. D., and P. Doty, *J. Mol. Biol.*, **1**, 111 (1959).
[16] Unpublished data.
[17] Hultin, T., *Experientia*, **16**, 410 (1961).
[18] Hultin, T., *Exptl. Cell Research*, **25**, 405 (1961).
[19] Scherrer, K., H. Latham, and J. E. Darnell, these PROCEEDINGS, **49**, 240 (1963).