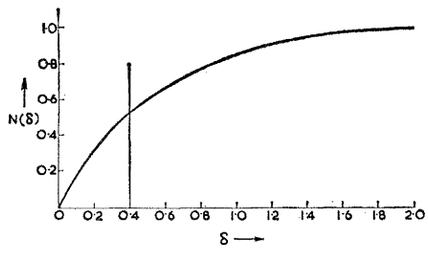

Fig. 3. Cumulative function $N(\delta)$ of the Bijvoet ratio δ (equal to $|\Delta I|/I$) for $\sigma_1^2 = 0.5$ and $k = 0.2$. The mean value is marked by a vertical line.

Table 2. Fractional number of reflections n_{90} with phase difference less than 90° from the heavy atom contribution, as a function of σ_1^2.

σ_1^2	P* equal to (%):			
	1	2	M.A.	M.C.
0.2	76.0	73.0	72.0	69.5
.4	88.5	82.3	81.5	77.5
.6	96.0	89.0	88.5	83.0
.8	99.8	93.5	94.5	89.0

* $P = 1$ for one heavy atom in the unit cell; $P = 2$ for two heavy atoms; P = M.A. for many heavy atoms with an acentric distribution; and, P = M.C. for many heavy atoms with a centric distribution.

Table 3. Mean value of the Bijvoet ratio δ as a function of σ_1^2. The value of k is taken to be 0.2. The other symbols are as in Table 2.

σ_1^2	P equal to			
	1	2	M.A.	M.C.
0.1	0.2796	0.2460	0.2400	0.2098
.2	.3831	.3289	.3200	.2738
.3	.4478	.3750	.3666	.3094
.4	.4824	.3951	.3919	.3290
.5	.4857	.3928	.4000	.3370
.6	.4529	.3704	.3919	.3354
.7	.3795	.3315	.3666	.3237
.8	.2720	.2800	.3200	.2993
.9	.1609	.2129	.2400	.2514

tion, is approximately equal to 0.28 for cobalt, 0.21 for iodine, 0.15 for platinum, and 0.17 for mercury. The anomalous effects are most prominent for $\sigma_1^2 = 0.5$; in this case, the mean value of δ (for $k = 0.2$) is as high as 40 percent, so that it should be possible to measure them quite accurately by means of counters. Although a theoretical formula is not available, the cumulative function $N(\delta)$ of δ for this example is shown in Fig. 3, as obtained from a practical case. Although the median value is about 40 percent, the number of reflections for which $\delta >$ 100 percent is as high as 15 percent (Fig. 3).

Thus, the anomalous dispersion method is one that deserves further consideration by protein crystallographers. In fact, as early as 1958, Blow (*10*) used this technique in conjunction with isomorphous replacement in his study of hemoglobin, and similar combined studies have since been made by others. Thus, provided that the errors of measurement of intensities can be reduced, the anomalous effects by themselves could be used for phase determination since the errors due to nonisomorphism are avoided.

Another difficulty with proteins, but often not with ordinary crystals, is that the reflections become very weak about 2 Å, resulting in poor data. With the large unit cells which occur in proteins, the foregoing distribution functions are valid, except for about a hundred reflections with small values of $(\sin\theta)/\lambda$. Thus, the smallness of the total number of reflections in relation to the number of atoms, which occurs with proteins, does not affect the phase-determining technique as such, but it certainly affects the clarity of the final diagram, even with correct phases.

Incidentally, even for finding out the positions of the heavy atoms in a protein structure, a combined application of the isomorphous crystal method and the anomalous dispersion method is the most fruitful one (*11*). This result was reached by application of the theoretical approaches to problems studied earlier (*12*). In fact, a combination of the isomorphous crystal and anomalous dispersion method has been used in the study of the structure of the protein lysozyme (*13*).

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Messenger RNA in Early Sea-Urchin Embryos: Cytoplasmic Particles

Abstract. *Three structures containing messenger RNA can be demonstrated in the cytoplasm of early sea-urchin embryos: (i) particles that sediment more slowly than ribosomes and contain newly synthesized DNA-like RNA, (ii) light polyribosomes, which also contain this newly synthesized RNA, and (iii) heavy polyribosomes, which seemingly contain only already existing or "maternal" messenger RNA and account for the bulk of the synthesis of protein.*

A newly described class of ribonucleoprotein (RNP) particles, which sediment more slowly than ribosomes, occurs in fish embryos (*1*, *2*). Because they have a unique buoyant density, about 1.42 to 1.45 g/cm^3, as determined with CsCl gradients (*2*, *3*), these particles are distinct from ribosomal subunits, which have a buoyant density of 1.52 g/cm^3. Evidence for the existence of messenger RNA (mRNA) in these particles was drawn from their apparent ability to stimulate incorporation into protein in a cell-free ribosomal preparation (*2*). We report here evidence for the existence of similar particles in early sea-urchin embryos and demonstrate that their RNA is mRNA because it forms hybrids with DNA in high proportion.

The sea-urchin embryo synthesizes new mRNA before the eight-cell stage (*4*, *5*). We show now that this new RNA appears in the cytoplasm, not only in the above-mentioned RNP particles, but also as a complex with ribosomes, in the form of polyribosomal structures. However, the significance of the synthesis of cytoplasmic mRNA by the cleavage-stage embryo is not immediately apparent, since there is varied evidence that such a synthesis need not contribute to embryonic development

at this time. Experiments both with parthenogenetic merogones (*6*) and parthenogenetically activated enucleated eggs (*7*), and treatment with radiation (*8*) and actinomycin (*9*), exclude the cell nucleus and nuclear, or DNA-dependent, synthesis of RNA from a necessary involvement either in much of early morphogenesis or in the activation of early protein synthesis. Although in none of these cases has development been described as normal, a substantial amount of "development" does appear to occur, and a normal rate of protein synthesis, regulated by the activity of mRNA (*10*), is sustained through the cleavage stages solely by supposed "maternal" mRNA, which had been stored in the unfertilized egg, awaiting activation (*9*).

Thus a paradoxical situation arises in which new mRNA appears, but seemingly without function in the events of protein synthesis and development of the early embryo. Toward a resolution of this paradox we have examined what now seem to be three classes of cytoplasmic structures containing mRNA: (i) light RNP particles, containing new RNA, (ii) slowly sedimenting or light polyribosomes, also containing new RNA, and (iii) rapidly sedimenting or heavy polyribosomes, which most likely contain already existing or maternal mRNA and account for the bulk of the synthesis of protein. We present evidence here that these three structures exist, and suggest that it is through their functions and interrelationships that the resolution of the paradox may be attained.

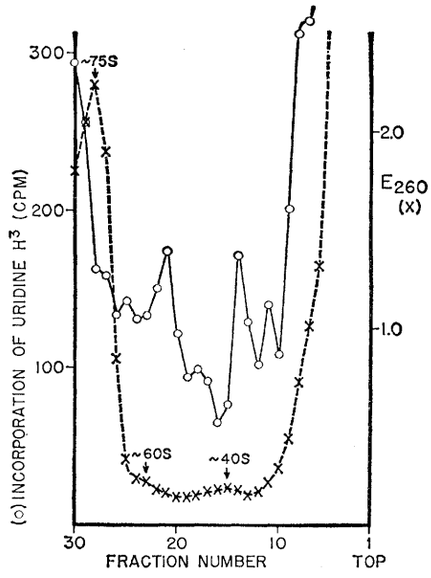

Fig. 1. Incorporation of uridine-H^3 in slowly sedimenting particles of cleavage-stage embryos. Embryos (1 ml) at the four-cell stage were incubated at 19°C in 10 ml of sea water for 2 hours with uridine-H^3 (10 μc/ml; 3.84 c/mmole). Embryos were homogenized, and the 12,000*g* supernatant was analyzed by sedimentation. Centrifugation was for 12 hours in the Spinco SW25.1 rotor at 24,000 rev/min.

Embryos of the sea urchin *Lytechinus pictus* were allowed to develop in the presence of penicillin (10 mg/ml) and streptomycin (20 mg/ml) (*10*). Two hours after fertilization (four-cell stage), the embryos were incubated with 5 μM uridine-H^3 (4.0 mc/μmole; New England Nuclear Corp.) for 2 hours, until the 16-cell stage was reached. During the last 2 minutes of this period L-leucine-C^{14} (0.2 μc/ml; 111 μc/μmole; Schwarz Bioresearch) was present. The embryos were washed thoroughly with cold sea water and then with homogenization medium consisting of the following: sucrose, 0.25*M*; ammonium chloride, 0.24*M*; 0.01*M* $MgCl_2$; 0.01*M* tris-HCl, *p*H 7.8. In the experiments reported here a gentle homogenization was effected by hand with a Teflon pestle for 30 seconds. Later experiments indicated that two strokes with a Dounce homogenizer was more suitable. Nuclei and mitochondria were removed by centrifugation of the homogenate at 12,000*g* for 30 minutes. The supernatant fluid was layered on a linear sucrose gradient (15 to 30 percent by weight in homogenization medium). The gradients were centrifuged in a Spinco SW25.1 rotor at 24,000 rev/min for either 2, 3, or 12 hours. Approximately 30 equal fractions were collected. Absorption at 260 mμ (E_{260}) was measured, and then carrier albumin (200 μg) was added to each sample. Cold 5-percent trichloroacetic acid was added, and the resulting precipitates were plated on glass-fiber filter discs and dried. The radioactivity was measured by liquid scintillation spectrometry.

The embryonic preparations (with leucine excluded) were sedimented for 12 hours at 24,000 rev/min (Fig. 1). The ribosomal component (75*S*), as shown by its absorption at 260 mμ, traveled to within five fractions of the bottom of the gradient. The bulk of the soluble proteins and free nucleic acids remained near the top (not included), and accounted for about half of the total absorbing material in the gradient. Between these extremes there was a region of relatively little absorption. However, substantial incorporation of uridine-H^3 occurred in this region, with peaks of radioactivity being evident. These peaks do not correspond to the small ribosomal components, which display absorbance at approximately 38 to 40*S* and 60*S*. In 12 such experiments with uridine-H^3 and five others with leucine-C^{14}, six peaks of radioactivity were observed repeatedly with substantial incorporation of H^3 (up to 5000 count/min). The approximate sedimentation constants of the peaks of labeled RNA and labeled protein were calculated with the 75*S* ribosome as a reference. The high degree of reproducibility is shown in the average values and standard deviations as follows. Labeled RNA sedimented at 66±2, 57±2, 49±2, 40±2, 31±1, and 20±1*S*. Labeled protein showed similar *S* values: 65±1, 57±7, 50±1, 39±1, 31±1, and 22±1.

The 20 to 65*S* region (fractions 10 to 25) of the gradient of Fig. 1 was extracted directly with an equal volume of phenol, and the RNA in the aqueous phase was precipitated with ethanol.

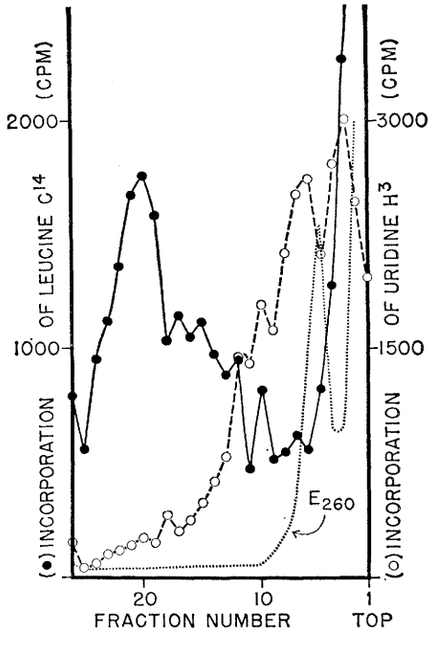

Fig. 2. Incorporation of uridine-H^3 and leucine-C^{14} in the polyribosomes of cleavage-stage embryos. Embryos (1 ml) at the 4-cell stage were incubated at 19°C in 10 ml of sea water for 2 hours with uridine-H^3 (10 μc/ml; 4.63 c/mmole). At the end of this period L-leucine-C^{14} (111 c/mole) was added at 0.2 μc/ml for 2 minutes. Embryos were homogenized, and the 12,000*g* supernatant was analyzed by sedimentation. Centrifugation for 2 hours in the Spinco SW25.1 rotor 24,000 rev/min.

Table 1. Formation of hybrids between DNA and labeled RNA from slowly sedimenting particles. The procedure and its details have been described (*4*). The various labeled RNA preparations were obtained as follows: (a) Ribosomal and 4S RNA was extracted from gastrulae incubated with uridine-H^3 overnight. (b) RNA of light particles was obtained by extraction with an equal volume of phenol of the material in the sucrose gradient corresponding to the region from fractions 8 to 25 in Fig. 1. (c) RNA from hybrids was the purified DNA-like RNA (*4*). The values are averages of duplicate determinations.

H^3RNA		Hybrid (%)
Input (count/min)	Hybrid (count/min)	
a. *Ribosomal + 4S*		
665	1	0.2
584	0	0
570	1	0.2
b. *RNA of light particles*		
380	159	41.9
412	183	44.4
380	160	42.0
c. *RNA from hybrid*		
166	87	52.4
438	231	52.7

The purified RNA was analyzed (*4*) for its ability to form DNA-RNA hybrids with the DNA prepared from the sperm of *L. pictus* (Table 1). Hybrid formation of the RNA from the 20 to 65*S* region was compared with that of ribosomal RNA. Hybrids with labeled ribosomal RNA cannot be detected in the presence of unlabeled RNA. On the other hand, a high proportion of the RNA of the 20 to 65*S* region forms DNA-RNA hybrids. Approximately 43 percent of the input material was recovered as hybrids. Since the apparent efficiency of the method for different ratios of RNA to DNA was 52 percent, we can account for 83 percent of the labeled RNA obtained from the components sedimenting between 20 and 65*S* as mRNA at this early embryonic stage.

Sedimentation for 2 hours revealed that the incorporation of leucine in nascent protein, after pulse labeling, took place predominantly in very heavy polyribosomes, in agreement with the observation of Stafford *et al.* (*12*) and consistent with the observations of Monroy and Tyler (*13*). The bulk of labeled nascent protein was around fraction 20 (Fig. 2). Centrifugation for an additional hour resulted in all of the components' traveling further (Fig. 3), with the ribosome peak clearly removed from the soluble proteins at the top of the gradient. Figure 3, *a* and *b*, shows analyses of the same 12,000*g* supernatant; however, Fig. 3*b* represents a portion treated with ribonuclease (10 μg/ml) for 1 minute at 25°C before sedimentation. The ribonuclease treatment completely destroyed the polyribosomes, the labeled protein being shifted into the ribosome peak, which was not affected by the treatment. This labeled protein was nascent since it disappeared from the polyribosomes and ribosomes on further incubation of the embryos with unlabeled leucine. Thus these heavy structures are indeed polyribosomes since their integrity depends on RNA strands, presumably mRNA, susceptible to attack by ribonuclease. Furthermore, hardly any newly synthesized RNA is associated with these active, heavy polyribosomes. Therefore, the synthesis of protein may be attributed for the most part to unlabeled or already existing mRNA. This assignment agrees with the results of experiments by Denny and Tyler (*7*), Gross *et al.* (*9*), and Nemer and Bard (*10*), and it suggests that the regulation of the rate of protein synthesis after fertilization is governed by the activity of "maternal" mRNA stored in the cytoplasm of the unfertilized egg.

The pattern of incorporation of uridine-H^3 in RNA is distinctly different from that of the nascent protein. The labeled RNA (Fig. 2) sediments predominantly in the region two to eight fractions ahead of the ribosomal component, as indicated by its absorption at 260 mμ. Very little of the newly synthesized RNA is associated with the heavy polyribosomes, which are actively engaged in protein synthesis. In Fig. 3*b* all of the labeled RNA was destroyed, and none of it remained associated with the ribosome peak after digestion with ribonuclease. Thus no newly synthesized ribosomes are detectable in the cytoplasm at this early stage. The digestion of any nonribosomal RNA that might have stuck to the ribosomes was complete or else the amount of such RNA was too little for detection. The sedimentation characteristics of the material heavier than ribosomes (approximately 95 to 170*S*) and its destruction by the enzyme, in a manner analogous to the disruption of aggregates of ribosomes held together by mRNA, indicate that the labeled RNA belongs to light polyribosomal structures. The newly synthesized RNA of the polyribosomes of these early embryos is messenger according to the criteria of its sedimentation characteristics and its ability to form hybrids with DNA in high proportion (*4*).

Apparently (*6–9*) the early embryo can sustain a normal rate of protein synthesis that does not depend on newly synthesized RNA, and yet new messenger RNA is synthesized. The bulk

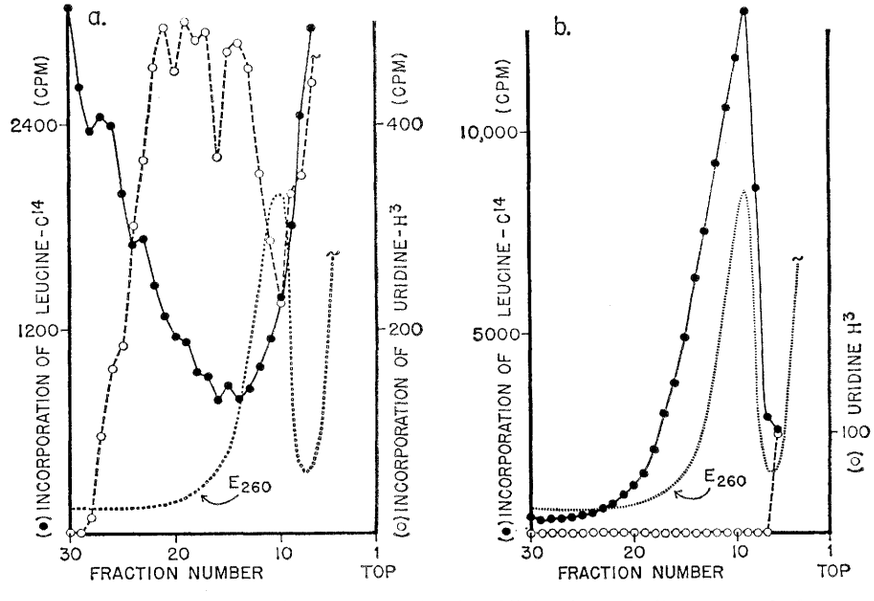

Fig. 3. Incorporation of uridine-H^3 and leucine-C^{14} in the polyribosomes of cleavage-stage embryos. The conditions were the same as for Fig. 2, except the uridine was at 3.84 c/mmole, and the centrifugation was for 3 hours: (*a*) control; (*b*) the 12,000*g* supernatant was treated with ribonuclease (10 μg/ml) for 1 minute at 25°C before analysis.

of protein synthesis occurs in heavy polyribosomes, whereas most of the newly synthesized cytoplasmic mRNA is found in structures separate from these, in the light polyribosomes and in RNP particles. The name "informosome" has been proposed (*2*) for these RNP particles, since they are believed to bear genetic information or mRNA. That these RNP particles are separate entities and not simply complexes of mRNA with ribosomal fragments might be concluded from the evidence of their buoyant density (*2*, *3*).

If any new mRNA does not participate in protein synthesis (*6–9*), we should expect that a means for regulating its activity is available in the cytoplasm. The existence of such mRNA-protein complexes as informosomes would allow us to formulate at least two hypothetical ways in which the activity of mRNA could be programmed, or protein synthesis regulated before or during translation: (i) The fate of particular messenger RNA's might hinge on that of the informosomes to which they belong. This fate may be investigated from the standpoint of the metabolic stability of the RNA or its ability to form polyribosomes through interaction with ribosomes. Such an interaction has been indicated already (*2*) in the fish embryo. (ii) Another method for regulation is offered if the mRNA remains complexed with protein during polyribosome formation. Such a sustained complex might allow the protein of the informosome to exert an influence on template function. Consistent with the idea of such an inactive polyribosome would be the interpretation of Figs. 2 and 3*a*, that the light polyribosomes containing newly synthesized messenger RNA are for the most part not engaged in protein synthesis. That they are incompletely loaded with ribosomes is indicated by their range of sedimentation constants and the range of sizes of their mRNA deducible from sedimentation behavior (*4*).

A solely cytoplasmic regulation of protein synthesis appears to be operative immediately after fertilization (*6–9*). Such a regulation may certainly influence embryogenesis and cellular differentiation.

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Messenger RNA in Early Sea-Urchin Embryos: Size Classes

Abstract. *Rapidly labeled RNA from four-cell embryos and blastulae of sea urchins was analyzed by sedimentation and for ability to form DNA-RNA hybrids. The RNA was derived from polyribosomes and from the "gel interphase," an extraction compartment resulting from treatment of whole embryos with phenol and known to be enriched with nuclei. The RNA from both sources displayed a high degree of structural complementarity to DNA. This DNA-like RNA of the polyribosomes sedimented in discrete classes, rather than in the sedimentation continuum demonstrable for the labeled RNA of the gel interphase. Thus messenger RNA appears to emerge in the cytoplasm in discrete size classes.*

The information for embryonic development resides ultimately in the messenger RNA's (*1*) which direct the synthesis of specific proteins. We have attempted to characterize, particularly through sedimentation analysis, the messenger RNA (mRNA) synthesized by early sea-urchin embryos. We now describe the peculiarities of the rapidly labeled RNA derived both from its nuclear site of origin and from its ultimate site of functionality in protein synthesis, the polyribosome (*2*).

In higher organisms rapidly labeled RNA is located predominantly in the nucleus of the cell (*3*). However, only a portion of the nuclear RNA may be messenger, as judged by the criteria of being either DNA-like (D-RNA) (*4*, *5*) or being able to stimulate incorporation into protein (*6*). In the rapidly growing cells usually studied a large proportion of the labeled nuclear RNA is precursor to ribosomal RNA (*7*, *8*). However, in the early embryo of the sea urchin *Lytechinus pictus*, the labeled RNA from a cellular partition that may be equivalent to the nucleus is predominantly D-RNA. The relative proportion of D-RNA in this compartment decreases with development of the embryo.

Labeled RNA that can be considered predominantly messenger, according to its DNA-like base composition, has been extracted from polyribosomes of tissue culture cells and analyzed by ultracentrifugation (*9*). The rapidly labeled RNA derived from polyribosomes (*9*, *10*) and that of total or nuclear pulse-labeled RNA (*4*, *7*, *11*, *12*) both display a polydisperse continuum upon sedimentation. Such patterns support the concept that mRNA synthesized by the cell consists of hundreds of molecular species in a continuous array of sizes; this array would account for the spectrum of known sizes of proteins. In our study with homogenates from early embryos we have uncovered a situation in which mRNA, derived from polyribosomes, appears to have been produced in discrete size classes.

All of the rapidly synthesized RNA of the cell can be obtained through extraction with 0.5 percent sodium dodecyl sulfate (SDS) and phenol at 60°C (*11*). All the pulse-labeled RNA was extracted by this procedure from embryos at the 4-hour cleavage stage, which had been incubated with uridine-H^3 for 15 minutes, and from 10-hour blastulae, incubated for 10 minutes. The purified RNA (*13*) was then submitted to sedimentation analysis. The sedimentation diagram (Fig. 1) indicated a polydisperse continuum throughout the gradient with a peak at approximately 10*S*. The labeled RNA of the blastula was also polydisperse,