

# THE EFFECT OF PROTEIN SYNTHESIS INHIBITION ON THE ENTRY OF MESSENGER RNA INTO THE CYTOPLASM OF SEA URCHIN EMBRYOS

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## ABSTRACT

Emetine is a potent inhibitor of protein synthesis in sea urchin embryos. At a concentration of the drug that rapidly inhibits protein synthesis in blastulae by 95%, uridine incorporation into RNA continues for more than 1 hr and presumptive histone messenger RNA is synthesized and transported into the cytoplasm where it is apparently associated with polyribosomes. Possible explanations of this result and its implications for the "informasome" theory of messenger transport in embryonic cells are discussed.

## INTRODUCTION

Prehatching sea urchin embryos do not produce detectable amounts of mature ribosomal RNA (1, 2) but do synthesize messenger RNA (mRNA)<sup>1</sup> which is translated on cytoplasmic polysomes (3-5), and high molecular weight, heterodisperse, rapidly labeled RNA, which seems to be confined to the nucleus (6).<sup>2</sup> Certain of the messenger RNA molecules synthesized and translated during early development have a well defined mobility on polyacrylamide gel electrophoresis and have been tentatively identified, on various grounds, as templates for histones (4). The embryos therefore present a favorable system for studying the transport of mRNA from the nucleus into the cyto-

plasm. It has been suggested that specific complexes between mRNA and newly synthesized protein ("informasomes") are involved in this process in loach and in sea urchin embryos (as well as other eukaryotic cells) (7). It was thus of interest to study the effect of protein synthesis inhibition on the synthesis and transport of definable mRNAs (histone mRNAs) destined for function in the cytoplasm.

## MATERIALS AND METHODS

### *Embryos*

Specimens of *Arbacia punctulata* were obtained from Florida and Woods Hole, Mass. Methods used to fertilize and demembranate eggs, and culture the embryos (18°-22°C) have been described in detail elsewhere (8).

### *Estimation of Protein Synthesis*

Embryos were incubated with leucine-<sup>14</sup>C (250 mCi/mmole) as indicated. They were collected by

<sup>1</sup> Abbreviations used in this paper: CTP, cytidine triphosphate; EDTA, ethylenediaminetetraacetate; mRNA, messenger RNA; POPOP, *p*-bis[2-(5-phenyloxazolyl)] benzene; PPO, 2,5-diphenyloxazole; SDS, sodium dodecylsulfate; TCA, trichloroacetic acid; tRNA, transport RNA; UTP, uridine triphosphate.

<sup>2</sup> Hogan, B., and P. R. Gross. 1971. Manuscript in preparation.

centrifugation, dissolved by vigorous agitation in 0.5 ml 0.5% sodium dodecylsulfate (SDS), and precipitated with 1.0 ml 10% trichloroacetic acid (TCA) containing 1 mg/ml cold leucine. Samples were filtered onto Millipore membranes, washed with 10% TCA, dried, and counted in a Beckman liquid scintillation counter with the use of a scintillant of toluene, *p*-bis[2-(5-phenyloxazolyl)]benzene (POPOP), and 2,5-diphenyloxazole (PPO).

### RNA Synthesis

Embryos were incubated with 5  $\mu\text{Ci/ml}$  uridine- $^3\text{H}$  (20 Ci/mole) for 15 min, collected by centrifugation, dissolved in 1.0 ml 0.5% SDS, and precipitated with 2.0 ml ice-cold 10% TCA. They were filtered, washed, dried, and counted as above.

### RNA and DNA Synthesis

Embryos that had been incubated with 10  $\mu\text{Ci/ml}$  uridine- $^3\text{H}$  were collected by centrifugation and dissolved in 0.6 ml 0.5% SDS. Half of the samples were assayed for radioactivity in DNA and RNA by making them 7% with cold TCA and collecting on membrane filters, which were then dissolved in methoxycellosolve and counted in a liquid scintillation system. The other half were assayed for radioactivity in DNA by making them 0.3 N with respect to NaOH and incubating at 37°C for 16 hr. They were then made 7% with TCA, filtered, and counted as above. Radioactivity in RNA was calculated by difference.

### Polysome Analysis

Embryos were washed twice with ice-cold calcium- and magnesium-free seawater and once with 0.02 M Tris pH 7.5, 0.2 M KCl, 0.005 M Mg acetate (Medium TKM). They were resuspended and homogenized in 3 volumes of the same buffer containing 0.1% Triton X100. The homogenate was centrifuged for 10 min at 10,000 *g* and the postmitochondrial supernatant was layered on top of a sucrose density gradient, appropriate details of which are given with each figure. Gradients were assayed for OD 260  $m\mu$  and acid-precipitable radioactivity as described (3).

### RNA Extraction and Polyacrylamide Gel Electrophoresis

Samples were dissolved in 0.5% SDS, 0.1 M NaCl, 0.001 M ethylenediaminetetraacetate (EDTA), 0.01 M Tris, pH 7.5, and shaken with an equal volume of phenol and of chloroform/1% isoamyl alcohol (v/v) at room temperature. The two phases were separated by centrifugation and the organic phase was removed. The deproteinization was repeated until no interphase

material remained and nucleic acid was precipitated from the aqueous phase with 2 volumes of ethanol at  $-20^\circ\text{C}$ . The precipitate was dissolved in 0.02 M Tris, pH 7.4, 0.005 M Mg acetate containing 100  $\mu\text{g/ml}$  DNase (Worthington Biochemical Corp., Freehold, N.J.), and incubated at 37°C for 7 min. After deproteinization the aqueous phase was made 0.1 M with NaCl, and RNA was precipitated with ethanol. Acrylamide gel electrophoresis was carried out as described (4).

Radioactive precursors were purchased from New England Nuclear Corp., Boston, Mass., and emetine hydrochloride from Mann Research Laboratories Inc., New York.

## RESULTS

### Effect of Emetine on Protein Synthesis

Fig. 1 shows that emetine is an effective inhibitor of protein synthesis in sea urchin embryos. At  $10^{-3}$  M it inhibited protein synthesis by 99%, while under similar conditions and concentrations acetylcycloheximide and cycloheximide inhibited by 62% and 37%, respectively, and streptovitacin A and sparsomycin were completely ineffective. Demembrated embryos were slightly more sensitive to emetine than embryos that had membranes, and unfertilized eggs were inhibited more slowly than embryos, where, as shown in Fig. 2, inhibition was complete within 4 min of adding the drug. The relative resistance of the

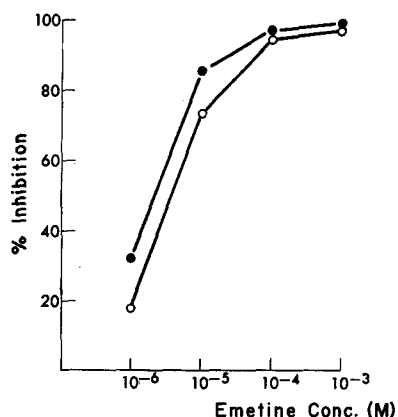


FIGURE 1 Effect of varying concentrations of emetine on protein synthesis in sea urchin embryos. Embryos at early cleavage ( $8 \times 10^8$  embryos/ml) with (open circles) or without (closed circles) fertilization membranes were incubated for 10 min with 0.25  $\mu\text{Ci/ml}$  leucine- $^{14}\text{C}$  and different concentrations of emetine. Protein synthesis was assayed as described in Methods.

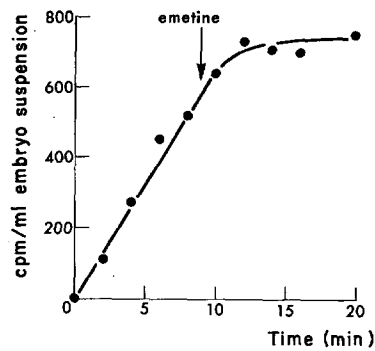


FIGURE 2 Time course of emetine inhibition of protein synthesis. Early gastrulae were incubated with  $0.2 \mu\text{Ci/ml}$  L-leucine- $^{14}\text{C}$  and  $5 \mu\text{g/ml}$  L-leucine. Samples were removed at various times for the assay of TCA-insoluble radioactivity. 9 min after the beginning of the incubation, emetine was added to a final concentration of  $10^{-4} \text{M}$ .

embryos to most protein synthesis inhibitors commonly used with eukaryotic cells is probably attributable to low permeability rather than to any peculiarity of the protein synthesizing system.

It was not possible to restore protein synthesis to the normal rate in embryos incubated for more than about 10 min with  $10^{-4} \text{M}$  emetine, though they were washed several times and reincubated in fresh seawater. Irreversibility of emetine action has been observed in HeLa cells (9) and is probably due to difficulty in washing the drug completely out of the cells.<sup>3</sup>

Polysomes from embryos incubated for 30 min with  $10^{-4} \text{M}$  emetine, and then pulse labeled with radioactive amino acids, were not very different in size from those present in control embryos but had little radioactivity incorporated into their nascent chains (Fig. 3). This result, like those obtained in HeLa cells (9), suggests that emetine acts by inhibiting the movement of ribosomes along the mRNA.

To test the possibility that the drug was simply inhibiting amino acid uptake, two-cell embryos were incubated with  $0.2 \mu\text{Ci/ml}$  leucine- $^3\text{H}$  in the presence of  $10^{-4} \text{M}$  emetine. Samples were removed every minute for 10 min and the acid-soluble pool was extracted with 10% TCA. Emetine did not in any way alter the entry of the leucine- $^3\text{H}$  into this pool. Since it is unlikely that significant differences in the absolute pool sizes existed at the

<sup>3</sup> Grollman, A. P. 1969. Personal communication.

beginning of this experiment, emetine does, in fact, suppress translation.

### *Effect of Emetine on Cell and Embryo Morphology*

Eggs fertilized in  $10^{-4} \text{M}$  emetine produced fertilization membranes and the sperm and egg pronuclei fused, but the zygote never divided. Cells in older embryos appeared to undergo at most one division before mitotic arrest. These observations are consistent with a requirement for protein synthesis for entry of cells into mitosis (10, 11). Nuclei in embryos treated with  $10^{-4} \text{M}$  emetine for 30 min appeared larger and paler than those in normal embryos, but there was no other obvious cell damage.

### *Effect of Inhibition of Protein Synthesis on RNA Synthesis*

Fig. 4 shows the results of an experiment in which embryos were incubated with uridine- $^3\text{H}$  with and without emetine. Under these conditions normal embryos incorporate an increasing fraction (with time) of the labeled uridine into DNA, presumably as deoxycytidine. In the presence of emetine, DNA synthesis was inhibited, while uridine continued to be incorporated into RNA for 2 hr. There is an apparent stimulation of uridine incorporation after 60 min in the absence of protein synthesis. This may result from changes in the uridine nucleotide pool, although it is possible that RNA degradation is inhibited by inhibiting protein synthesis (see later).

When uridine was given as a 15 min pulse (Fig. 5), the results suggested that the rate of RNA synthesis declined slowly after the addition of emetine so that by 80 min the rate in inhibited embryos was 26% of the initial rate and 18% of the control. This conclusion is also subject to the qualification that the size of the endogenous uridine pool may have changed in the absence of protein synthesis.

### *Inhibition of Protein Synthesis and Entry of mRNA into the Cytoplasm*

When control embryos (blastulae) were incubated for 1 hr with uridine- $^3\text{H}$  and the cytoplasm was fractionated by sucrose gradient centrifugation, labeled RNA was found associated with the polysomes and postribosomal region of the gradient

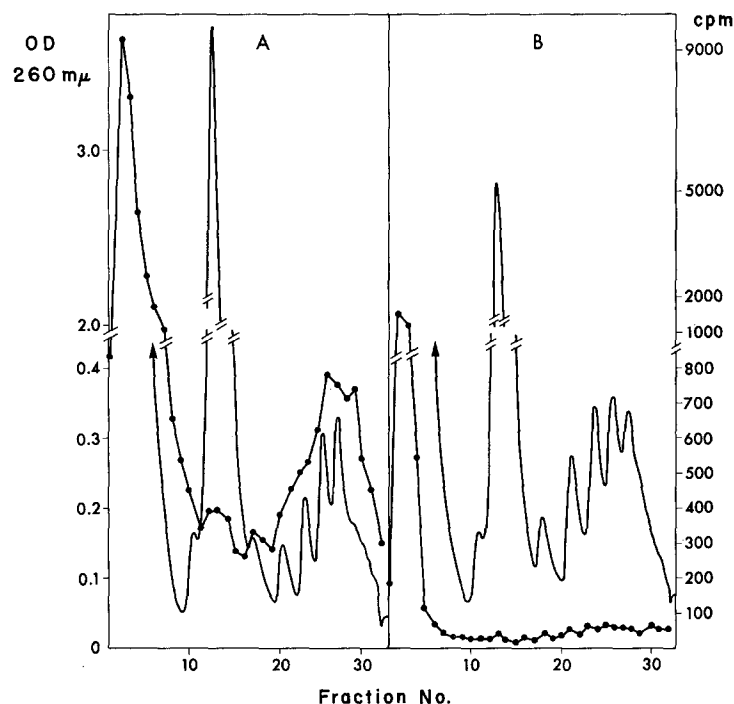


FIGURE 3 Labeling of nascent polypeptide chains in the presence of emetine. Embryos (early blastulae) were incubated for 30 min with  $10^{-4}$  M emetine and then labeled for 1 min with  $2 \mu\text{Ci/ml}$  amino acid- $^{14}\text{C}$  mixture. A postmitochondrial supernatant was prepared and layered on top of a 7–47% sucrose gradient in medium TKM. Centrifugation was carried out at 40,000 rpm for 75 min at  $4^\circ\text{C}$  in the SW 41 Beckman rotor. The gradients were assayed for OD 260  $m\mu$  (solid line) and TCA-insoluble radioactivity (closed circles) as described. (A) control. (B) incubated with emetine.

(Fig. 6 A). In embryos incubated with  $2 \times 10^{-5}$  M and  $1 \times 10^{-4}$  M emetine (Fig. 6, B and C) there was an increase in the number of polysomes (particularly dimers and trimers) and a significant decrease in the number of monomers in the cells. Labeled RNA was still found associated with polysomes, with a distribution similar to the control, although the specific radioactivity (cpm/OD 260  $m\mu$ ) was about twice as high as normal after treatment with  $1 \times 10^{-4}$  M emetine. Labeled RNA was also found in the subribosomal (20–60S) region of the gradient, but there was no accumulation there relative to the control, as might have been expected, were the RNA being transported to the cytoplasm as particles sedimenting somewhat more slowly than polysomes and monomeric ribosomes, and were the entry of the particles into polysomes being inhibited. In embryos incubated with the highest concentration of emetine,  $1 \times 10^{-3}$  M (Fig. 6 D), there was no decrease in the number of monomers in the cell but there was a pronounced increase in the number of dimers

and trimers, and labeled RNA was again localized in the polysome and postribosomal regions of the gradient.

To test whether the newly synthesized RNA sedimenting in the polysome region of the gradient was attached to ribosomes or sedimented there fortuitously as a result of association with protein, the experiment described in Fig. 7 was carried out. A postmitochondrial supernatant was prepared from embryos incubated for 1 hr with  $1 \times 10^{-4}$  M emetine and uridine- $^3\text{H}$ . Half of this supernatant was made 30 mM with EDTA before analysis by sucrose gradient centrifugation. This concentration of EDTA is sufficient to dissociate completely all the polysomes into subunits, and most of the labeled RNA was removed from the polysome region of the gradient, indicating that it was indeed attached to the ribosomes (13, 14). (This test does not show that the ribosomes attached to the RNA were in fact engaged in polypeptide chain elongation.)

When the RNA extracted from the polysomes

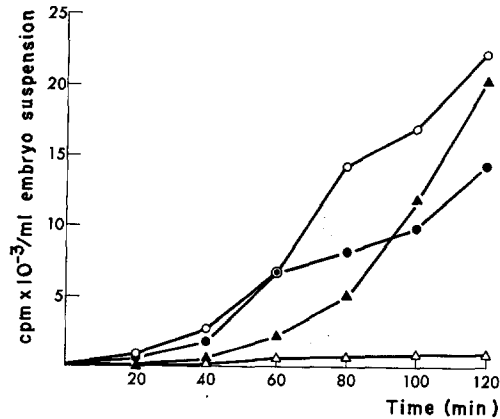


FIGURE 4 Effect of inhibition of protein synthesis on the incorporation of uridine into RNA and DNA. Demembrated embryos (late cleavage-early blastula) were incubated with  $10^{-4}$  M uridine- $^3$ H and samples were withdrawn at various times for assay of radioactivity in DNA and RNA as described in Methods. (Closed circles) control RNA; (open circles) RNA in embryos incubated with  $10^{-4}$  M emetine; (closed triangles) control DNA; (open triangles) DNA in embryos incubated with emetine.

of normal embryos and embryos incubated for 1 hr with  $1 \times 10^{-4}$  M emetine and uridine- $^3$ H was analyzed on polyacrylamide gels, the results shown in Fig. 8 were obtained. It is clear that no new 26, 18, or 5S ribosomal RNA entered the cytoplasm during this time. Most of the newly synthesized RNA was in three components migrating between 18 and 5S. These RNAs have been identified tentatively as mRNAs for histones, with a mean sedimentation velocity of 9S on sucrose density gradient analysis (4). Newly synthesized RNA was also found as a peak at approximately 20S and as heterogeneous molecules migrating more slowly than the 26S ribosomal RNA.

The total radioactivity associated with the three histone mRNA peaks of the inhibited embryos was about twice that found in the control, even though the amount of unlabeled (ribosomal) RNA entering the gels was the same in both cases. The radioactivity in the 4S and  $>26$ S regions was also higher in the gels of RNA from inhibited embryos than from the controls but little difference was observed in the 20S region.

It is clear from the foregoing experiments that some 9S histone messenger RNA continues to be made and transported into the cytoplasm when 95% of the normal protein synthesis is inhibited in these embryos. It is not possible from the data

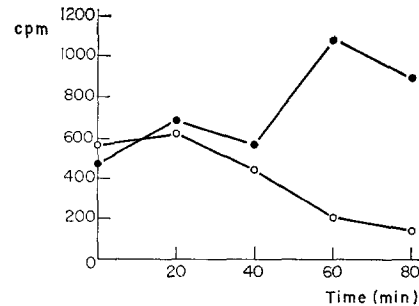


FIGURE 5 Effect of inhibition of protein synthesis on the rate of RNA synthesis. Embryos (morulae) were incubated with or without  $10^{-4}$  M emetine. At various times portions were removed and pulse labeled with 5  $\mu$ Ci/ml uridine- $^3$ H for 15 min. Uptake of radioactivity into TCA-insoluble material was assayed as described. (closed circles) control; (open circles) embryos incubated with  $10^{-4}$  M emetine.

presented to say *how much* histone messenger RNA is synthesized and accumulated in the cytoplasm under these conditions. For example, it is possible that when protein synthesis is inhibited the size of the intracellular uridine nucleotide pool is reduced, so that the specific radioactivity of the uridine triphosphate (UTP) is increased and a coincident inhibition of 9S mRNA synthesis and/or transport is hidden. Thus a 50% reduction in combined synthesis, transport, and attachment of ribosomes to histone messenger RNA, together with a fourfold increase in the UTP specific radioactivity, might produce the results obtained in Fig. 8. While this possibility is difficult to eliminate without measuring the specific activity of the nucleotide pools directly, an indirect estimate of changes in the pool were obtained in the experiment described below.

Evidence suggests (15) that a large part of the incorporation of radioactivity from uridine- $^3$ H and  $^{32}$ P into transfer RNA (tRNA) during the period of rapid cleavage in sea urchin embryos is into the pCpCpA terminal sequence. It has also been shown that in HeLa cells the *de novo* synthesis of tRNA is not affected by inhibiting protein synthesis.<sup>4</sup> Differences in the incorporation of cytidine- $^3$ H into tRNA of emetine-treated and normal blastulae during a 1 hr incubation were therefore taken as a measure of change in the specific radioactivity of the cytidine triphosphate (CTP) pool due to inhibition of protein synthesis. From the results shown in Fig. 9 it can be calcu-

<sup>4</sup> Penman, S. 1970. Personal communication.

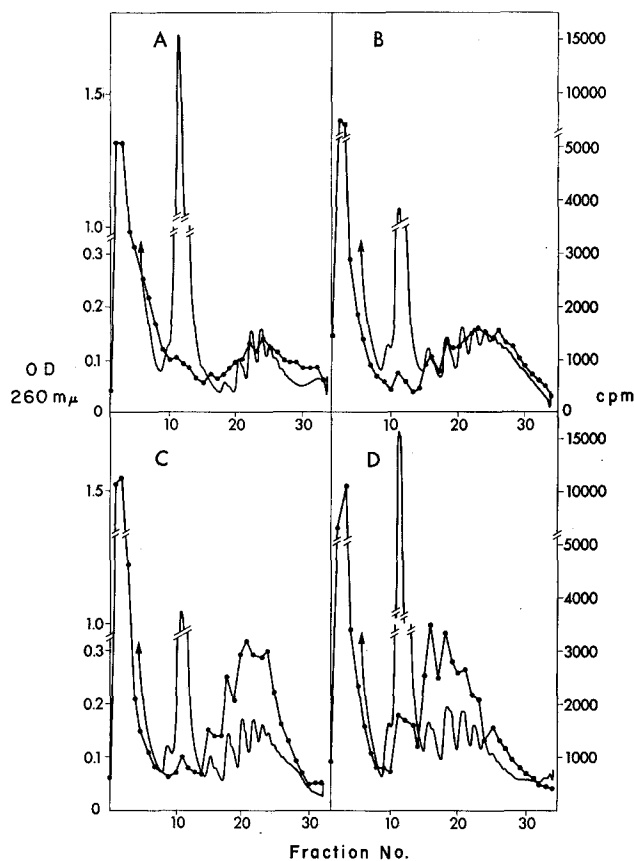


FIGURE 6 Effect of inhibition of protein synthesis on the entry of newly synthesized RNA into the cytoplasm. Early blastulae (with fertilization membranes) were incubated with  $20 \mu\text{Ci/ml}$  uridine- $^3\text{H}$  with or without various concentrations of emetine for 1 hr (label and drug were added simultaneously). Postmitochondrial supernatants were prepared and analyzed by sucrose density gradient centrifugation as described in Fig. 3. (solid line) OD  $260 \text{ m}\mu$ ; cpm. (A) control, (B) embryos incubated with  $2 \times 10^{-5} \text{ M}$  emetine, (C) with  $1 \times 10^{-4} \text{ M}$  emetine, and (D) with  $1 \times 10^{-3} \text{ M}$  emetine. Small polysome optical density peaks were resolved into two components. Similar structures in reticulocyte cell-free preparations of polysomes have been interpreted as polysomes containing an unpaired, small ribosomal subunit (12).

lated that the specific radioactivity of the tRNA (total cpm in the shaded area at 4S/total amount (measured in arbitrary units) of the 4S OD  $260 \text{ m}\mu$  peak) was in fact one and one-half times higher in the cytoplasm of the inhibited embryos than the control. The ratio of total counts in the 9S histone messenger complex in the inhibited embryos to the control was 2.0, and the corresponding ratio for the 20S peak was 1.2. There appeared to be little change in the total counts associated with cytoplasmic RNAs of slower mobilities than the 26S ribosomal RNA, or with heterogeneous, high molecular weight nuclear RNA (results not shown here) under the two conditions. These

results, while less than absolute measures of RNA synthesis, at least make very unlikely the possibility that during inhibition of protein synthesis there could be a large depression in 9S histone messenger RNA synthesis and transport coupled with a correspondingly large increase in the specific radioactivity of the precursor nucleotide pools.

#### DISCUSSION

It is clear from the data obtained in these experiments that when protein synthesis in sea urchin embryos is quickly reduced to 5% or less of the normal rate, the incorporation of uridine- $^3\text{H}$  into RNA continues for more than an hour (Fig. 4).

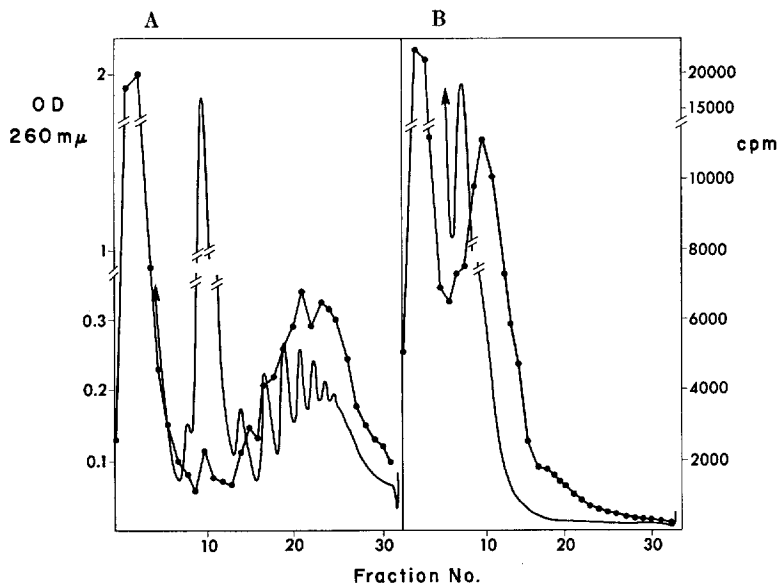


FIGURE 7 Effect of EDTA treatment on the sedimentation of RNA synthesized in the presence of emetine. Blastulae were incubated for one hour with  $20 \mu\text{Ci/ml}$  uridine- $^3\text{H}$  and  $1 \times 10^{-4} \text{ M}$  emetine, and a postmitochondrial supernatant was prepared. One-half of the supernatant was made  $30 \text{ mM}$  with EDTA before layering on a sucrose gradient for analysis as described in Fig. 3. (solid line) OD  $260 \text{ m}\mu$ ; (closed circles) cpm. (A) control, (B) after treatment with  $30 \text{ mM}$  EDTA.

Under these conditions several different kinds of RNA are produced. These include the three presumptive histone mRNA species, 20S polyribosomal RNA, heterogeneous cytoplasmic RNA of lower electrophoretic mobility than 26S ribosomal RNA, and tRNA (with the label probably as cytidine in the pCpCpA terminus). Heterogeneous, high molecular weight RNA of the nucleus is also synthesized. This result contrasts with the rapid inhibition of synthesis and processing of ribosomal RNA in HeLa cells treated with cycloheximide (16), although synthesis of high molecular weight, heterogeneous nucleoplasmic RNA is less sensitive in these cells to protein synthesis suppression (16), and synthesis of tRNA is unaffected.<sup>4</sup>

Lack of detailed information about the size of, and possible fluctuations in, the nucleoside triphosphate pools precludes making reliable estimates of how much of each kind of RNA is synthesized and accumulated in emetine-treated and normal embryos during a given incubation period. The results of the experiment represented by Fig. 9 make it rather unlikely, however, that the amount of any RNA species produced during the hour after exposure to emetine is markedly less than the normal. It is in fact possible that the total

production of cytoplasmic RNA during a 60 min incubation in emetine is greater than in controls. Incorporation data alone, for short pulses given during the incubation with emetine, suggest a slowly declining bulk rate of synthesis (Fig. 5), so that, all observations considered, there arises the possibility that inhibition of protein synthesis might inhibit RNA degradation. This is certainly a very speculative proposal at present, but it has been found in bacteria that decay of mRNA is suppressed by chloramphenicol (17), probably because the mRNA is protected from nucleases while stably complexed with ribosomes.

In the experiments reported here, we exploited the fact that early sea urchin embryos synthesize three species of polyribosomal RNA that have been identified by several independent tests as mRNA for histones. They are certainly being translated on cytoplasmic polyribosomes and they have well defined electrophoretic mobilities (4). These properties make them useful for a study of the relation between protein synthesis and mRNA synthesis and transport. The data (Figs. 8 and 9) show that both of the latter continue in the presence of emetine, for newly synthesized histone mRNA can be isolated from polyribosomes.

Rapidly labeled RNA sedimenting in the poly-

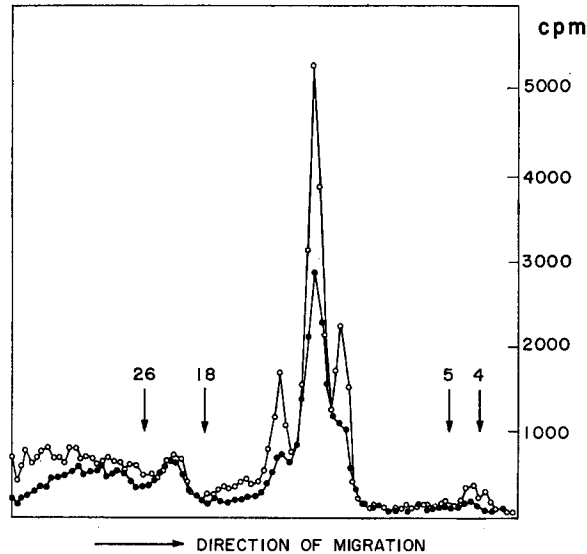


FIGURE 8 Analysis by polyacrylamide gel electrophoresis of newly synthesized RNA from polysomes of control and inhibited embryos. Blastulae were incubated for 1 hr with 20  $\mu\text{Ci/ml}$  uridine- $^3\text{H}$  with or without  $1 \times 10^{-4}$  M emetine. A postmitochondrial supernatant was prepared and analyzed by sucrose density gradient centrifugation (17–50% sucrose in TKM centrifuged in the SW 27 rotor at 25,000 rpm for 3.5 hr at 4°C). Material sedimenting faster than the 80S monomer was pooled and precipitated with 2 volumes of absolute ethanol at  $-20^\circ\text{C}$ . The precipitate was collected by centrifugation and RNA was extracted with phenol and chloroform/isoamyl alcohol at room temperature as described. The RNA was analyzed on 10 cm polyacrylamide gels at 5 mA/gel for 2.5 hr. The gels were cut into 1 mm slices which were dissolved in 0.5 ml  $\text{NH}_4\text{OH}$ , and the hydrolyzed RNA was counted in a liquid scintillator system. Details of these procedures are given in reference 4. The OD 260  $\mu\text{m}$  tracings of the two gels were identical and the positions of the 26, 18, 5, and 4S RNA peaks are indicated by arrows. (closed circles) cpm control; (open circles) inhibited embryos.

some region in sucrose gradients is largely removed from this region by treatment with EDTA (Fig. 7), indicating that the labeled RNA is actually attached to ribosomes rather than a protein-RNA complex produced artifactually and with sedimentation properties like those of polyribosomes.

The finding that mRNA transport into the cytoplasm continues during inhibition of protein synthesis for a significant interval (i.e., an hour at least—greater than the intermitotic interval for cleavage stages) has several consequences. It suggests, first, that messenger transport is not coupled in any obligatory way with *ongoing* protein synthesis. Secondly, it means either that mRNA transport requires no proteins, or that if special transport proteins are required (e.g., for packaging of mRNA into particles) there must be a pool of these in the cell. In terms of the time needed for a cell to traverse a cleavage cycle, the pool must be large. It has been reported that newly synthesized, nonribosomal RNA continues to enter the poly-

somes of HeLa cells when protein synthesis is inhibited by puromycin (18) or by cycloheximide (19), but in neither of the latter cases is there detailed information about the identity of the RNA message.

It should be pointed out that blocking DNA synthesis with hydroxyurea in these embryos causes a selective decay of the 9S histone mRNA complex in polyribosomes (4). The finding that emetine inhibits DNA synthesis (as traced in a uridine incorporation study) while permitting entry of 9S RNA into the polyribosomes appears to be a contradiction. This vanishes, however, when DNA synthesis in the presence of emetine is studied directly and in detail by thymidine- $^3\text{H}$  incorporation (results not shown here). It is found, in essence, that while emetine does bring DNA synthesis to a halt, this requires about an hour, during which time 9S RNA can be produced.

A somewhat puzzling result is the observation that new mRNA in the cytoplasm of embryos whose amino acid incorporation had been reduced



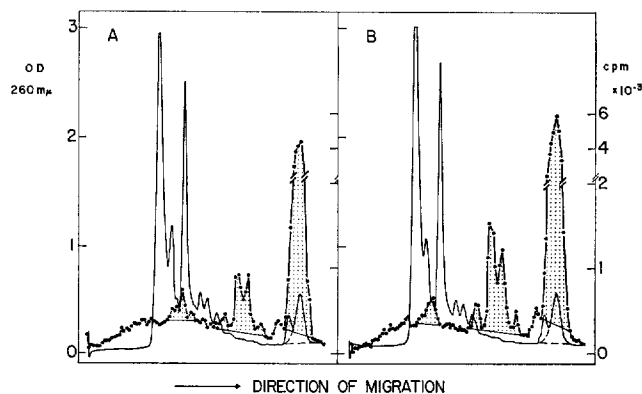


FIGURE 9 Total cytoplasmic RNA synthesized in the presence and absence of  $10^{-4}$  M emetine. Blastulae were incubated for 1 hr with  $10 \mu\text{Ci/ml}$  cytidine- $^3\text{H}$  ( $26.4 \text{ Ci/mmole}$ ) with (B) or without (A)  $1 \times 10^{-4}$  M emetine. They were then harvested, washed, and homogenized in TKM containing 0.1% Triton X100. The homogenate was centrifuged for 10 min at  $10,000 g$  and the supernatant ("cytoplasm") was precipitated with 2 volumes of ethanol at  $-20^\circ\text{C}$ . RNA was extracted as described in Methods, and after DNase treatment equal OD  $260 \text{ m}\mu$  amounts were analyzed on 10 cm 2.6% polyacrylamide gels at 10 v/cm for 2.75 hr. (solid line) OD  $260 \text{ m}\mu$ , (closed circles) cpm per slice. The specific radioactivity of the tRNA in A and B was calculated by dividing the total cpm in the shaded area at 4S by the total amount (measured in arbitrary units) of the 4S OD  $260 \text{ m}\mu$  peak (indicated by dashed lines). The areas used for summing the total cpm in the three presumptive histone mRNA peaks and 20S RNA are also shaded.

to 5% of the control was loaded with ribosomes in a roughly normal way (as judged by the *relative* distribution of radioactivity and OD at  $260 \text{ m}\mu$ ). The mRNA did not, furthermore, accumulate in small polyribosomes, in monomers, or in the post-ribosomal fractions. When, on the other hand, incorporation was reduced still further, mRNA *did* accumulate significantly in small polyribosomes (Fig. 6 C, 6 D).

Emetine is structurally similar to cycloheximide (9, 20) and is thought to inhibit protein synthesis in the same way, i.e., by inhibiting the translocase function (transferase II), slowing the movement of ribosomes along the message (21, 22). Cycloheximide and, by inference, emetine may also have a separate effect upon initiation (21, 23). The mode of action of emetine can be used to account for the behavior of ribosomes and unlabeled mRNA (i.e., preexisting) in these experiments. There is some difficulty, however, in accounting for the apparently normal loading of *new* mRNA when translation is reduced to 5% of the control (assuming that the incorporation reflects the actual translation rate).

With 5% the normal rate of protein synthesis and a normal mean chain completion time of 2 min (24, 25), it should follow that a ribosome requires about 40 min in the inhibited system for

transit of an average mRNA. The time available for loading is less than the 60 min labeling period, because labeled RNA appears in polyribosomes only after a 15 min delay (which eliminates nuclear leakage as a source of the RNA, since the nuclei become radioactive at once). Hence, only the first labeled mRNA to enter the cytoplasm after emetine treatment should be fully loaded, and labeled mRNA should accumulate in small polyribosomes. The expected effect was marginal or absent at 95% inhibition, but it did appear with larger doses of the drug and more severe inhibition. The simplest model thus leads to a prediction that is not unequivocally satisfied.

Whatever the eventual explanation for these particular observations, the fact remains that a defined mRNA can be synthesized and transported into polyribosomes when 95–97% of the protein synthesis has been eliminated, and that the mRNA so transported does not accumulate detectably in postribosomal ribonucleoprotein particles. Proposals on the mechanism of mRNA transport will have to account for these findings.

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