The Mechanism of Protein Synthesis Activation After Fertilization of Sea Urchin Eggs

S. METAFORA, L. FELICETTI*, AND R. GAMBINO

Laboratory of Molecular Embryology, 80072 Arco Felice, Naples, and Laboratory of Cell Biology, Rome, Italy

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ABSTRACT Upon fertilization of Paracentrotus lividus eggs, the ability of their ribosomes to form a ternary complex with poly(U) and phenylalanyl-tRNA is enhanced. In addition, it is possible to extract from ribosomes of the unfertilized egg a protein factor that inhibits polyphenylalanine synthesis by decreasing the binding of mRNA and aminoacyl-tRNA to the ribosomes. The observed increase in the rate of protein synthesis that occurs at fertilization could be accounted for by the removal of this inhibitory protein from the ribosomes.

It is generally agreed upon that in the early development of the sea urchin egg, protein synthesis is largely directed by templates transcribed during oogenesis (1). However, the mechanism whereby the utilization of these templates is restricted in the unfertilized egg is still a matter of controversy. The suggestion of a translational inefficiency of the unfertilized-egg ribosomes (2-4) has been questioned on the basis of experiments that seem to indicate that these ribosomes are able to respond to poly(U) as efficiently as those of the fertilized egg (5-7). The experiments reported in this paper prove, however, that under appropriate experimental conditions, the ribosomes of the unfertilized egg are less efficient than those of the fertilized egg in supporting poly(U)-directed phenylalanine polymerization. Ribosomes of unfertilized eggs also display a lower ability to bind poly(U) and phenylalanyl-tRNA. We further show that a protein component of the unfertilized-egg ribosomes, which is released upon fertilization, is a powerful inhibitor of polypeptide synthesis.

MATERIALS AND METHODS

Chemicals

[14C]phenylalanine (513 Ci/mol) and [3H]poly(U) (7.76 Ci/mole) were obtained from Schwarz BioResearch, Escherichia coli stripped tRNA from General Biochemicals, poly(U) and GDPCP from Miles Laboratories, GTP from Sigma, dithiothreitol (DTT) from Calbiochem, Sephadex G-200 from Pharmacia, and trypsin and soybean trypsin inhibitor from Worthington.

Buffers

TKMD buffer: 50 mM Tris-HCl (pH 7.6)–0.4 M KCl–18 mM magnesium acetate–1 mM DTT; Modified Novelli’s Medium A: 50 mM Tris-HCl (pH 7.5)–25 mM KCl–4 mM magnesium acetate–0.3 M sucrose–1 mM DTT; TED buffer: 50 mM Tris-HCl (pH 7.5)–0.1 mM EDTA–1 mM DTT.

Fertilization and culture of sea urchin embryo

Eggs of Paracentrotus lividus were collected and handled as described (8).

Preparation of S-135 and native ribosomes from unfertilized eggs and embryos

All operations were performed at 4°C. Eggs and embryos were harvested by centrifugation, washed once with ice-cold calcium- and magnesium-free artificial sea water, and were finally suspended in 3 volumes of TKMD buffer. 1 mg of bentonite that had been centrifuged at low speed (9) was added per ml of solution, and the suspension was homogenized by five strokes of a hand-operated Dounce homogenizer with a tight-fitting pestle. The homogenate was centrifuged at 8000 × g for 10 min. The resulting supernatant was centrifuged twice at 15,000 × g for 15 min, then layered over 1 ml of 0.6 M sucrose in TKMD buffer, and was finally centrifuged in a Spinco model L ultracentrifuge at 135,000 × g for 90 min at 4°C. The ribosomal pellets were colorless and transparent. The red fluffy layer floating over the colorless pellet was discarded. Before use, the ribosomal pellets were suspended in modified Novelli’s Medium A; the 260/280 absorbance ratio of this suspension ranged between 1.80 and 1.95. The upper 1/4 of the supernatant from the 135,000 × g centrifugation was used as S-135. The protein concentration was estimated by the method of Lowry et al. (10).

Preparation of elongation factors, T1 and T2, from rat liver supernatant

This was as described by Felicetti and Lipmann (11).

Preparation of elongation factors, T1 and T2, from sea urchin supernatant

A partially purified preparation of T1 and T2 factors was obtained by ammonium sulfate fractionation and Sephadex G-200 chromatography of S-135 from 32 blastomere embryos. Details of the procedure will be described in a separate paper.

Preparation of the 1 M NH4Cl wash from ribosomes

Ten pellets of native ribosomes, derived from 40 g of eggs (fresh weight), were resuspended in 2 ml of TKMD buffer containing 1 M NH4Cl and left on ice for 8–12 hr. The suspension was clarified by centrifugation at 10,000 × g for 5 min and then centrifuged at 135,000 × g for 5 hr at 4°C in a Spinco
ultracentrifuge. The ribosomal pellets thus obtained are referred to as "washed ribosomes."

The upper 1/4 of the supernatant was dialyzed overnight against 2 liters of TED buffer. The heavy precipitate formed during dialysis was removed by centrifugation at 15,000 \( \times g \) for 10 min, the supernatant (hereafter called "ribosomal wash") was concentrated in a dialysis tube by treatment at 2°C with dry Sephadex G-200. The treatment with 1 M NH\(_4\)Cl for 12 hr extracted about 4% of the ribosomal proteins. The removal of NH\(_4\)Cl by dialysis resulted in the precipitation of about 70–80% of the proteins contained in the original wash.

**RESULTS**

Polymerizing activity of ribosomes from fertilized and unfertilized eggs

Preliminary experiments established that the optimum ionic concentrations for polyphenylalanine synthesis are 4–5 mM MgCl\(_2\) and 120 mM NH\(_4\)Cl for both unfertilized and fertilized egg ribosomes. As shown in Table 1, \( T_1 \) and \( T_2 \) factors are both needed for polyphenylalanine polymerization. Moreover, the ribosomes from fertilized eggs, in the presence of saturating amounts of \( T_1 \) and \( T_2 \), exhibit a polymerizing activity about 30% higher than the ribosomes from unfertilized eggs. This difference is more evident in kinetic experiments (Fig. 1).

**Enzymatic binding of phenylalanyl-tRNA to poly(U)-programmed ribosomes**

The enzymatic binding of aminoacyl-tRNA to ribosomes in the absence of polymerization was studied using a purified preparation of \( T_1 \) from rat liver (Table 2). In the absence of \( T_1 \) factor, ribosomes from both unfertilized and fertilized eggs bind very little \([^{14}C]Phe\)-tRNA. Addition of \( T_1 \) results in an about 10-fold stimulation. The binding activity of the ribosomes from fertilized eggs is about 40% higher than that of the ribosomes from unfertilized eggs. GTP is required for optimum binding, GDPPCP, a nonhydrolyzable analogue, can replace GTP with about the same efficiency. As expected, \( T_2 \) factor has no effect on the binding.

**TABLE 1. Stimulation of phenylalanine polymerization by rat liver elongation factors as measured with sea urchin ribosomes**

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>([^{14}C]Phe) polymerized (pmol)</th>
<th>Unfertilized</th>
<th>Fertilized*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosomes, no transfer factors</td>
<td>0.041</td>
<td>0.049</td>
<td></td>
</tr>
<tr>
<td>Ribosomes + ( T_1 )</td>
<td>0.038</td>
<td>0.319</td>
<td></td>
</tr>
<tr>
<td>Ribosomes + ( T_2 )</td>
<td>0.053</td>
<td>0.080</td>
<td></td>
</tr>
<tr>
<td>Ribosomes + ( T_1 ) + ( T_2 )</td>
<td>3.420</td>
<td>4.435</td>
<td></td>
</tr>
</tbody>
</table>

The incubation mixture contained, in a final volume of 0.25 ml, 12.5 \( \mu \)mol of Tris-HCl buffer (pH 7.4), 20 \( \mu \)mol of NH\(_4\)Cl, 1.25 \( \mu \)mol of MgCl\(_2\), 4 \( \mu \)mol of DTT, 25 \( \mu \)mol of poly(U), 0.25 \( \mu \)mol of GTP, 35 \( \mu \)mol of E. coli \([^{14}C]Phe\)-tRNA (12 \( \mu \)mol of \([^{14}C]Phe\))phenylalanine, prepared as described by Conway (15), 130 \( \mu \)mol of native (i.e. not extracted with NH\(_4\)Cl) ribosomes and, when indicated, 15 \( \mu \)mol of \( T_1 \) and/or 14 \( \mu \)mol of \( T_2 \) factors. The mixture was incubated at 37°C for 15 min and the reaction stopped with 3 ml of cold 5% trichloroacetic acid (TCA). The tubes were heated at 100°C for 10 min and the precipitate was collected on HA Millipore filters (0.45-\( \mu \)m pore size, 25-mm diameter), washed with four 3-ml portions of cold 5% TCA, and counted in a Nuclear Chicago liquid scintillation counter in Bray's solution (16).

* 32 blastomeres.

**TABLE 2. Binding of \([^{14}C]phenylalanyl-tRNA\) to poly(U)-programmed ribosomes from sea urchin**

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>([^{14}C]Phe)-tRNA bound (pmol)</th>
<th>Unfertilized</th>
<th>Fertilized*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>1.800</td>
<td>2.550</td>
<td></td>
</tr>
<tr>
<td>– ( T_1 )</td>
<td>0.155</td>
<td>0.302</td>
<td></td>
</tr>
<tr>
<td>– GTP</td>
<td>0.709</td>
<td>0.604</td>
<td></td>
</tr>
<tr>
<td>– GTP + GDPPCP</td>
<td>2.050</td>
<td>2.750</td>
<td></td>
</tr>
<tr>
<td>– ( T_1 ) + ( T_2 )</td>
<td>0.150</td>
<td>0.293</td>
<td></td>
</tr>
</tbody>
</table>

The incubation mixture was the same as for the polymerization assay described in Table 1. The complete system contained, in a final volume of 0.25 ml, 130 \( \mu \)mol of native ribosomes and 15 \( \mu \)mol of \( T_1 \) factor from rat liver; 14 \( \mu \)mol of \( T_2 \) factor from rat liver; and 0.25 \( \mu \)mol of GDPPCP (when indicated). After incubation at 37°C for 15 min, the reaction was stopped with 3 ml of cold buffer containing 50 mM Tris-HCl (pH 7.4)–80 mM NH\(_4\)Cl–5 mM MgCl\(_2\). The diluted mixture was immediately filtered and the filters were washed with three 3-ml portions of cold washing buffer (the same that was used to stop the reaction). The filters were then dissolved in Bray's solution and counted in a Nuclear Chicago liquid scintillation counter.

* 32 blastomeres.

The ribosomes from unfertilized eggs are less active in binding aminoacyl-tRNA, even at saturating concentrations of ribosomes (Fig. 2).

**Binding of \([^{3}H]poly(U)\) to native ribosomes**

The different capacity to bind aminoacyl-tRNA of the ribosomes from unfertilized and fertilized eggs may be attributed to a fertilization-induced change in either the ribosomal sites for aminoacyl-tRNA binding or for mRNA binding. Therefore, the binding of \([^{3}H]poly(U)\) to ribosomes was studied by the Millipore filter technique of Moore (12). The binding of poly(U) to fertilized-egg ribosomes was 35% higher than to unfertilized-egg ribosomes.

**Effect of the ribosomal wash from unfertilized and fertilized eggs on polyphenylalanine synthesis and \([^{14}C]\)Phe-tRNA binding to ribosomes**

The difference in translational efficiency between unfertilized- and fertilized-egg ribosomes has been attributed to the presence of inhibitory factor(s) in the unfertilized-egg ribosomes (3). Ribosomes from unfertilized and fertilized eggs were extracted with 1 M NH\(_4\)Cl as described under Methods and the ribosomal wash was tested in a poly(U)-programmed cell-free system.

Addition of the ribosomal wash from unfertilized eggs to a poly(U)-programmed system containing ribosomes from fertilized eggs results in an up to 90% inhibition of phenylalanine polymerization (Figs. 3 and 4) and up to 75–80% inhibition of Phe-tRNA binding to ribosomes (Fig. 5). The experiments also show that 200 \( \mu \)g of ribosomal-wash proteins are required to obtain maximal inhibition of polymerization when the S-135 from fertilized eggs is used (Fig. 3), whereas the same extent of inhibition is obtained with about 50 \( \mu \)g of ribosomal-wash proteins when purified \( T_1 \) and \( T_2 \) factors (from either rat liver or sea urchin embryos) are used (Fig. 4).

This result suggests that the crude S-135 might contain some factor capable of interfering with the action of the
inhibitor. In contrast, as shown in Table 3, the ribosomal wash from fertilized eggs results in a slight (but reproducible) stimulation of polyphenylalanine synthesis. When the unfertilized-egg ribosomal wash was tested in a system containing unfertilized-egg ribosomes, a 10-13% inhibition was observed.

A different result was obtained when ribosomes extracted with 1 M NH₄Cl were used in the cell-free system (Table 3). In this case, the ribosomal wash from unfertilized eggs had no effect on polyphenylalanine synthesis; the ribosomal wash of fertilized-egg ribosomes caused a slight stimulation of the activity of ribosomes from both unfertilized and fertilized eggs.

These data indicate that the ribosomal wash of unfertilized eggs contains a factor capable of inhibiting polypeptide synthesis in a poly(U)-directed cell-free system and that this factor is no longer present in the ribosomes of fertilized eggs. It is also clear that after NH₄Cl extraction, the ribosomes lose their ability to respond to the inhibitory factor(s) present in the extract.

**Time course of inhibition of polyphenylalanine synthesis**

Maximal inhibition of polyphenylalanine synthesis can be detected as early as 2 min after addition of the ribosomal wash to a cell-free system containing native ribosomes and S-135 from fertilized eggs (32 blastomeres) (Fig. 6). These results suggest that the inhibitory substance quickly combines with some component of the cell-free system.

**Effect of ribosomal wash from unfertilized eggs on [³H]poly(U) binding to ribosomes**

The inhibition of [¹⁴C]Phe-tRNA binding by the ribosomal wash could be due either to a modification of the ribosomal sites for aminoacyl-tRNA binding, or of those involved in mRNA binding. In order to discriminate between these two alternatives, [³H]poly(U) was incubated for 5 min at 37°C with purified native monosomes from fertilized eggs in the absence or in the presence of different amounts of ribosomal wash solution and the incubation mixture was analyzed on sucrose gradients. 30 μg of ribosomal wash causes 75% inhibition of...
poly(U) binding (Fig. 7B), whereas 60 µg causes nearly 100% inhibition (Fig. 7C).

**Preliminary characterization of the inhibitory factor**

As shown in Fig. 8, the ribosomal wash loses 60% of its inhibitory activity after incubation for 4 min at 50°C. 1 µg/ml of trypsin completely destroys the inhibitory factor in 20 min at 30°C. Filtration of a concentrated preparation of ribosomal wash (about 5 mg of protein) through a Sephadex G-200 column shows that the inhibitory activity is eluted as a symmetrical peak with the bulk of the ribosomal proteins. These observations suggest that the inhibitory factor is a protein with a molecular weight of less than 200,000.

**Effect of ribosomal wash from unfertilized eggs on polyphenylalanine synthesis**

The data reported in the previous sections indicate that the factor present in the ribosomal wash inhibits polypeptide synthesis by preventing the formation of the ternary complex of mRNA-aminocacyl-tRNA-ribosomes. The next question is whether ribosomes from different sources are inhibited by this factor, and to what extent. Therefore, 80S and 70S ribosomes were used; the results are shown in Table 4.

The inhibition ranges from 65% for rabbit reticulocytes ribosomes to 88% for rat liver ribosomes. Ribosomes from E. coli K12 are inhibited to about the same degree as the ribosomes from eukaryotic organisms.

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![Fig. 1. Kinetics of phenylalanine incorporation with native ribosomes from unfertilized and fertilized eggs. Incubation conditions and assay as in Table 1, except that 175 µg of [14C]Phe-tRNA (60 pmol of [14C]phenylalanine) per tube were used.](image)

![Fig. 2. Effect of increasing amounts of ribosomes, from fertilized and unfertilized eggs, on phenylalanyl-tRNA binding. The incubation mixture contained, in a final volume of 0.25 ml, ribosomes from unfertilized and fertilized (32 blastomeres) eggs and 15 µg of T1 from rat liver. The assay for enzymatic binding is described in Table 2.](image)

![Fig. 3. Inhibition of polyphenylalanine synthesis by the ribosomal wash from unfertilized eggs in the presence of S-135. The incubation mixture contained, in a total volume of 0.25 ml, 1.7 mg of S-135, 100 µg of native ribosomes from sea urchin embryos at the blastula stage and increasing amounts of ribosomal wash; the assay was performed as described in Table 1.](image)

![Fig. 4. Inhibition of polyphenylalanine synthesis by the ribosomal wash from unfertilized eggs in the presence of purified T1 and T2 factors. The incubation mixture contained, in a total volume of 0.25 ml, 15 µg of T1, 14 µg of T2, 115 µg of native ribosomes from embryos at the blastula stage, and increasing amounts of ribosomal wash. The assay was performed as in Table 1.](image)

![Fig. 5. Inhibition of [14C]phenylalanyl-tRNA binding to ribosomes by the ribosomal wash from unfertilized eggs in the presence of purified T1 factor. The incubation mixture was the same as for the polymerization assay (see Table 1) except that 0.25 µmol of GDPCP was substituted for GTP to avoid any polymerization in the course of the reaction. It contained, in a total volume of 0.25 ml, 115 µg of native ribosomes from embryos at the blastula stage, 15 µg of T1 factor, and ribosomal wash protein. Incubation, Millipore filtration, and counting are described in Table 2.](image)

![Fig. 6. Time course of inhibition of polyphenylalanine synthesis. The incubation mixture contained, in a total volume of 0.25 ml, 115 µg of native ribosomes, 1.5 mg of S-135 from fertilized eggs (32 blastomere stage), and 30 µg of ribosomal wash (where indicated). The tubes were incubated at 37°C for different lengths of time; the assay for phenylalanine polymerization was performed as in Table 1.](image)

![Fig. 7. Effect of the ribosomal wash from unfertilized eggs on [3H]poly(U) binding to ribosomes. 1 ml of S-135 prepared from a homogenate of four blastomere-stage embryos was layered on a 26-ml 17–50% sucrose gradient in TKM (with 1 ml of 60% sucrose buffer at the bottom). The gradient was centrifuged for 210 min at 24,000 rpm in a SW 25.1 Spinco rotor at 4°C; the monosome peak was pooled and pelleted by centrifugation at 135,000 × g for 6 hr. Two 4-mg units of monosomes, in 0.1 ml of a medium containing 70 mM Tris·HCl (pH 7.5)–80 mM KCl–10 mM MgCl2–1 mM DTT, were incubated for 5 min at 37°C with [3H]poly(U) (30,000 cpm, 15% efficiency). The reaction mixture was quickly layered on a 4.83-ml 10–30% sucrose gradient in the incubation buffer. The gradient was centrifuged for 95 min at 38,000 rpm in a SW-39 Spinco rotor at 24°C. 2-drop fractions were collected from the bottom of the tube. Each fraction was monitored for absorbancy at 260 nm and for radioactivity after dilution with 0.4 ml of water. Radioactivity was measured by dissolving an aliquot of each sample in 5 ml of Bray's solution and counting in a Nuclear Chicago Spectrometer. A, control; B, +30 µg of ribosomal wash; C, +60 µg of ribosomal wash.](image)

![Fig. 8. Heat inactivation of the inhibitory activity of the ribosomal wash from unfertilized eggs. 260 µg of ribosomal wash protein in TED buffer was heated for 4 min at different temperatures. The residual inhibitory activity was tested in a poly(U)-directed cell-free system containing 150 µg of native ribosomes from embryos at the blastula stage, 15 µg of T1, and 14 µg of T2 elongation factors from rat liver (see assay for phenylalanine polymerization in Table 1). The inhibition obtained at 30°C is assumed to be 100%.](image)

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**TABLE 3. Effect of ribosomal wash on a poly(U)-directed cell-free system from unfertilized or fertilized sea urchin eggs**

<table>
<thead>
<tr>
<th>[3H]phenylalanine polymerized (pmol/assay)</th>
<th>Ribosomal wash</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native</td>
</tr>
<tr>
<td>(A)</td>
<td></td>
</tr>
<tr>
<td>Unfertilized</td>
<td>4.600</td>
</tr>
<tr>
<td>Fertilized</td>
<td>7.487</td>
</tr>
<tr>
<td>(B) NH4Cl-washed</td>
<td></td>
</tr>
<tr>
<td>Unfertilized</td>
<td>1.223</td>
</tr>
<tr>
<td>Fertilized</td>
<td>4.110</td>
</tr>
</tbody>
</table>

The incubation mixture for phenylalanine polymerization contained, in a final volume of 0.25 ml, 115 µg of native or NH4Cl-washed ribosomes, 1.29 mg of homologous S-135 from unfertilized or fertilized eggs, and 30 µg of 1 M NH4Cl ribosomal wash, where indicated. The assay was performed as described in Table 1.

**Control of RNase contamination in the ribosomal wash**

It was important to rule out the possibility that the ribosomal wash was contaminated with ribonucleases. Although the thermolability of the inhibitory factor speaks against such a possibility, more compelling indirect evidence is provided by the following observations: (a) If significant amounts of
Table 4. Effect of ribosomal wash from unfertilized eggs on polyphenylalanine synthesis with ribosomes from different sources

<table>
<thead>
<tr>
<th>Ribosomes</th>
<th>[14C]Phe polymerized (pmol/assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No addition</td>
</tr>
<tr>
<td>(A) 80S-type</td>
<td></td>
</tr>
<tr>
<td>Sea urchin</td>
<td>3.424</td>
</tr>
<tr>
<td>Rabbit reticulocyte</td>
<td>2.430</td>
</tr>
<tr>
<td>Rat liver</td>
<td>3.275</td>
</tr>
<tr>
<td>Pea germ</td>
<td>2.378</td>
</tr>
<tr>
<td>(B) 70S-type</td>
<td></td>
</tr>
<tr>
<td>E. coli K12</td>
<td>3.004</td>
</tr>
</tbody>
</table>

The incubation mixture (see Table 1) contained, in a final volume of 0.25 ml, 200 μg of ribosomes from different sources, 15 μg of T, and 14 μg of T, factors from rat liver for 80S ribosomes, 300 μg of an ammonium sulfate preparation (40-70% of saturation) from an S-135 prepared from E. coli K12, and 150 μg of ribosomal wash, where indicated.

RNase were present in the ribosomal wash from unfertilized eggs, a cell-free system containing either native or NH4Cl-washed ribosomes should be inhibited to the same degree; this is not the case (Table 3). (b) When [14C]phenylalanyl-tRNA was incubated with the ribosomal wash at 37°C for different lengths of time, the decrease in trichloroacetic acid-insoluble radioactivity was the same in the presence or in the absence of the ribosomal wash. (c) Finally, the percentage inhibition of polyphenylalanine synthesis was not changed when a 5-fold excess of poly(U) or [14C]phenylalanyl-tRNA was used in the incubation mixture.

CONCLUSIONS

The results described in this paper indicate that, upon fertilization, changes occur in the ribosomes (or at least in a certain percentage of the ribosomal population) whereby their ability to interact with mRNA and aminoacyl-tRNA is enhanced. In addition, we were able to show that a specific protein component, capable of inhibiting polyphenylalanine synthesis by preventing the formation of the ternary complex (ribosome-mRNA-aminocyl-tRNA), is part of the unfertilized-egg ribosomes. It is still not clear whether this protein functions by altering the binding sites specific for mRNA and aminoacyl-tRNA or by causing a distortion of the ribosomal structure as a whole.

Release of this factor probably occurs soon after fertilization. The release may be mediated by proteases; a transient activation of proteases after fertilization has been described (18). We further suggest that after its release, the inhibitory factor is inactivated by some component present in the cell sap. Indeed, we have observed that the amount of inhibitor required to obtain 90% inhibition of polyphenylalanine synthesis is about 4-fold greater with crude S-135 than with purified elongation factors.

We have also found that ribosome preparations from unfertilized eggs are only slightly sensitive (about 13% inhibition) to the inhibitor (Table 1). This could be explained by the presence in the unfertilized eggs of a heterogeneous population of ribosomes, only part of which is active in protein synthesis. It indeed appears that, in the unfertilized egg, only about 10% of the total ribosomal population is active in protein synthesis (14).

We wish to express our gratitude to Prof. Alberto Monroy for his encouragement and stimulating discussions in the course of this work, and to thank Prof. J. Brachet and Prof. H. Chantrenne for their critical review of the manuscript. We are indebted to Mr. A. Del Rio for his skillful technical assistance. Some of these experiments were carried out at the Zoological Station, Naples.