The Oligonucleotide Nature and Synthesis of Acid-soluble Ribonucleic Acid in Amphibian Eggs and Embryos*  

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The total ribonucleic acid fraction of mature ovarian eggs of amphibia and fish is composed of two gross fractions, one soluble in cold 0.5 M perchloric acid and the other insoluble in the acid (1, 2). Furthermore, on prolonged treatment with mild alkali, about 80% of the acid is converted to mononucleotides, some 20% being recovered as oligonucleotide fragments, a few of which contain labile phosphorus (1).  

The presence of this peculiar ribonucleotide fraction in ovarian eggs and its virtual absence from somatic tissue suggests that it is unique to immature cells and that it may influence embryonic development. Consequently we have endeavored to (a) characterize further the chemical nature of acid-soluble RNA from ovarian eggs, and (b) study the metabolic activity of acid-soluble RNA and acid-insoluble RNA during amphibian embryonic development.  

EXPERIMENTAL PROCEDURE  

Ovarian Egg Studies  

Preparation of RNA—The total RNA from ovarian eggs of Rana catesbeiana was prepared by a phenol procedure and purified as described previously (1).  

Cellulose Column Chromatography—Either total RNA in 7 M urea or acid-soluble RNA in 7 M urea was applied to DEAE-cellulose columns (3), 1 X 20 cm, and eluted with a linear gradient from 7 M urea (2 liters) to 7 M urea + 0.3 M ammonium formate (2 liters) for separation of acid-soluble oligonucleotides, or from 7 M urea (4 liters) to 7 M urea + 0.8 M ammonium formate (4 liters) for separation of acid-soluble oligonucleotides from acid-insoluble RNA. The contents of the peak tubes were pooled and, to remove urea, each fraction was diluted 3-fold with distilled water and applied to small (1 X 2 cm) DEAE columns. The columns were washed with water, and the oligonucleotides were stripped from the DEAE with 50 bed volumes of 1 M NH₄HCO₃. The NH₄HCO₃ was removed by treatment of each sample with Dowex 50-H⁴+. After filtration the samples were neutralized with NH₄OH and were concentrated under reduced pressure without permitting the temperature to exceed 25°C. Aliquots of each sample were used for analysis of total phosphorus (4), terminal phosphorus, labile phosphorus, and nucleoside-nucleotide ratios.  

Determination of Terminal Phosphorus—To aliquots containing about 0.5 μmole of total phosphorus were added 0.5 mmole of NH₄Cl buffer, pH 9.0, and 10 μmoles of MgCl₂ (final volume, 3.0 ml). Then 0.05 ml of a bacterial alkaline phosphatase suspension (Worthington Biochemical Corporation, Freehold, N. J.) was added, and the samples were incubated for 30 minutes at 37°C. This represents an excess of enzyme since 5 μmoles each of CMP, CDP, and CTP are completely hydrolyzed to cytidine and P₁ in 30 minutes under these conditions. A blank for each sample was prepared and treated in exactly the same manner except without enzyme. At the end of the incubation period, the release of P₁ was measured (5) in both the blank and enzyme samples.  

Determination of Mononucleotide-Nucleoside Di- (Tri-)phosphate Ratio—Aliquots containing about 2.5 μmoles of total phosphorus were added 100 μmoles of NH₄Cl buffer, pH 9.0, and 5 μmoles of MgCl₂ (final volume, 1.2 ml). Then 0.05 ml of bacterial alkaline phosphatase was added, and the samples were incubated for 30 minutes at 37°C. At the end of the incubation period the samples were neutralized with HCl and were placed in a boiling water bath for 20 minutes to inactivate the alkaline phosphatase. After chilling, the preparations received 0.5 μmole of NH₄Cl buffer, pH 9.0, and 0.1 ml of snake venom phosphodiesterase (Worthington Biochemical Corporation, Freehold, N. J.). The samples were incubated at 37°C for 2 hours, diluted to 50 ml with distilled water, made 0.05 M with respect to NH₄OH, and percolated through Dowex 1-Cl columns, 1 X 2 cm. The nucleosides were eluted with 100 ml of 0.01 M NH₄Cl, and the nucleotides with 25 ml of 0.1 M HCl.  

Determination of Inorganic Pyrophosphate Contamination—To aliquots containing about 2.5 μmoles of total phosphorus were added 100 μmoles of Tris buffer, pH 7.4, and 10 μmoles of MgCl₂ (final volume, 3.0 ml). Then 0.01 ml of inorganic pyrophosphatase (Worthington) was added, and the samples were incubated for 40 minutes at 37°C. A blank for each sample was identically prepared and treated, except that enzyme was omitted. This represents an excess of enzyme since 5 μmoles of sodium pyrophosphate are completely converted to P₁ in 20 minutes under these conditions. At the end of the incubation period, the release of P₁ was measured (6) in both blank and enzyme samples.
Inorganic pyrophosphate contamination was subtracted from all other phosphorus values.

Embryo Studies

Twelve female frogs (Rana pipiens) each received intraperitoneal injections of 250 µC of 32P as orthophosphate per day for 3 days, followed by two daily injections of 1.0 mmole of unlabeled phosphate buffer, pH 6.8. On the last day of buffer injection, whole pituitaries were administered to induce ovulation (8). The following day the labeled eggs were fertilized and allowed to develop at 25°C. At the various stages of development, either 800 or 1000 embryos were manually stripped of jelly, and the RNA was extracted with the use of hot sodium chloride (9). The RNA was purified by repeated chloroform-octanol (5:1) extractions and by treatment with MgCl₂ (1). Acrid-soluble RNA was separated from acid-insoluble RNA by adjusting the total RNA fraction to 0.5 M with respect to perchloric acid, in the cold, followed by centrifugation at 30,000 × g. The acid-soluble RNA fraction was neutralized with KOH, and the insoluble potassium perchlorate was removed by centrifugation. Both acid-soluble and acid-insoluble RNA fractions were dialyzed against running distilled water for 3 hours. The acid-insoluble RNA preparations contained less than 2% DNA, as measured by the method of Ceriotti (10). Aliquots of each preparation were taken for ultraviolet absorbance measurements in a Zeiss spectrophotometer and for counting with a thin end window Geiger tube.

RESULTS

Ovarian Studies

Separation of Oligonucleotides in Total RNA—Fig. 1 shows a typical separation of total RNA in the presence of urea on DEAE-cellulose columns. Six acid-soluble peaks are separated from each other and from the larger acid-insoluble RNA. Recovery from the columns is usually about 95%. The double peak obtained in the acid-insoluble RNA region is characteristic of the ovarian egg RNA preparations.

The order of elution of the first six peaks suggests the presence of mononucleotides through hexanucleotides with each peak differing from its neighbor by one negative charge (3).

Analysis of Oligonucleotide Peaks—Table I shows a characterization of each peak by (a) ratio of terminal phosphorus to total phosphorus, (b) ratio of labile phosphorus to total phosphorus, (c) ratio of nucleoside from 5'-hydroxyl end or 3'-hydroxyl end to nucleotide residue, and (d) extent of hydrolysis and products obtained upon alkaline hydrolysis. The structures depicted for each peak are based on these values and are considered only as tentative.

One common feature of Peaks 2 to 6 is that no nucleosides are liberated upon alkaline hydrolysis. This finding suggests that no free 3'-hydroxyl end groups are present in any of these oligonucleotides.

It is quite clear that Peaks 1, 4, and 5 are mononucleotides, tetrnanucleotides, and pentanucleotides, respectively. Peak 6 shows a nucleotide to nucleoside ratio indicative of pentanucleotides, but the terminal phosphorus to total phosphorus ratio indicates extra terminal phosphorus. This interpretation is substantiated by analysis of alkaline hydrolyzates of the peak, and since no labile phosphorus is present, the pentanucleotides of Peak 6 contain most probably both 3'- and 5'-phosphoryl end groups.

Peaks 2 and 3, however, show a peculiarity that does not permit their ready identification: they each contain labile phosphorus. That these two peaks are not mixtures of nucleoside di- and triphosphates is shown by the fact that they are not converted to nucleosides on treatment with a bacterial alkaline phosphatase preparation known to contain not only phosphomonoesterase activity but di- and triphosphatase activity as well.

Upon alkaline hydrolysis, Peaks 2 and 3 are not quantitatively converted to mononucleotides. Only about 60% and 20%, respectively, of the total optical density units (at 260 µm) are recovered as mononucleotides from Dowex 1-chloride columns; the remaining optical density units of Peak 2 can be recovered from the columns with 0.01 M HCl, but stronger HCl solutions are necessary for recovery of the remaining units of Peak 3. These “alkali-resistant” fragments contain labile phosphorus, and all of the phosphorus of each fragment is converted to P₁ upon treatment with bacterial alkaline phosphatase. The “alkali-resistant” fragment of Peak 2 has a total phosphorus to labile phosphorus ratio of 2.1, whereas the corresponding fragment of Peak 3 shows a value of 1.6. The phosphorus analysis and the difference in elution pattern from Dowex 1-chloride columns indicate that the “alkali-resistant” fragment of Peak 2 is a nucleoside diphosphate, whereas the corresponding fragment of Peak 3 is a nucleoside triphosphate. Since no nucleosides are liberated upon

![Fig. 1. Separation of oligonucleotides from each other and from polynucleotides. A total of 250 mg of ovarian egg RNA was dissolved in about 10 ml of 7 M urea and applied to a DEAE column, 1 × 20 cm. The column was washed with 250 ml of 7 M urea, and the compounds were eluted with the use of linear gradients as indicated.](http://www.jbc.org/)

Geiger tube.

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### Table I

**Analysis of oligonucleotides obtained from acid-soluble RNA fraction**

<table>
<thead>
<tr>
<th>Peak</th>
<th>Terminal phosphorus*</th>
<th>Total phosphorus*</th>
<th>Ep</th>
<th>5'-Nucleotide**</th>
<th>Alkaline hydrolysis†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.91, 1.11</td>
<td>0.36, 0.33, 0.25</td>
<td>15,000</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>0.77, 0.71, 0.63</td>
<td>0.36, 0.33, 0.25</td>
<td>7,200</td>
<td>33</td>
<td>2.94</td>
</tr>
<tr>
<td>3</td>
<td>0.83, 0.83, 0.83</td>
<td>0.50, 0.56, 0.44</td>
<td>4,900</td>
<td>25</td>
<td>3.70</td>
</tr>
<tr>
<td>4</td>
<td>0.31, 0.31, 0.32</td>
<td>0</td>
<td>10,600</td>
<td>92</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0.22, 0.20, 0.22</td>
<td>0</td>
<td>9,400</td>
<td>85</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0.32, 0.31, 0.28</td>
<td>0</td>
<td>7,500</td>
<td>77</td>
<td>0</td>
</tr>
<tr>
<td>Acid-insoluble RNA</td>
<td>0.025</td>
<td></td>
<td>7,900</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Includes monoesterified phosphorus and pyrophosphate.
† Individual chromatographic separations and determinations.
‡ Adjusted to 1 M with respect to H₂SO₄ and heated at 100° for 10 minutes.
§ Ep, Optical density at 290 μμ per mole of phosphorus.

**Snake venom phosphodiesterase treatment after removal of terminal phosphorus by bacterial alkaline phosphatase.
† No nucleosides found in any of the hydrolysates.
‡ Includes monoester 2'- and 3'-phosphate.
§ Includes nucleoside 3',5'-diphosphate as in Peak 6 and monoesterified phosphorus and pyrophosphate as in Peaks 2 and 3.

### Table II

**Amount of acid-soluble RNA in embryos**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Total RNA µg/10 embryos</th>
<th>Acid-soluble RNA % total RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two-cell</td>
<td>8.1</td>
<td>89</td>
</tr>
<tr>
<td>Gastrula</td>
<td>8.8</td>
<td>89</td>
</tr>
<tr>
<td>Early neurula</td>
<td>8.4</td>
<td>82</td>
</tr>
<tr>
<td>Late neurula</td>
<td>11.4</td>
<td>84</td>
</tr>
<tr>
<td>Hatching</td>
<td>15.9</td>
<td>79</td>
</tr>
<tr>
<td>Larva I</td>
<td>24.3</td>
<td>48</td>
</tr>
<tr>
<td>Larva II</td>
<td>65.0</td>
<td>37</td>
</tr>
</tbody>
</table>

![Fig. 2](http://www.jbc.org/)

**Fig. 2.** Synthesis of total RNA during development. ●, Micrograms of total RNA per 10 embryos (bases on optical density at 290 μμ). □, Total counts per minute in total RNA.
DEAE-cellulose columns. This value indicates the average molecular weight of acid-insoluble RNA to be about 14,000.

**Embryo Studies**

Occurrence of Acid-soluble RNA in Developing Embryos—Table II shows the quantity of total RNA recovered from 10 embryos at the various stages of development and also the percentage of the total RNA that is soluble in 0.5 M perchloric acid. Early embryos contain large quantities of acid-soluble RNA, the amount of which decreases markedly after hatching. This decrease apparently takes place at a time when the total quantity of RNA is increasing rapidly in the embryos.

$^{32}$P Incorporation into Acid-soluble and Acid-insoluble RNA in Developing Embryos—Fig. 2 shows the relation between the $^{32}$P counts in total RNA and the net increase in total RNA, measured spectrophotometrically, at the various stages of development. It is apparent that the increase in $^{32}$P counts in the total RNA fraction is related directly to the net synthesis of the total RNA during development. However, there is a marked difference in the incorporation of $^{32}$P into acid-soluble RNA and acid-insoluble RNA (Fig. 3). Between gastrulation and hatching, acid-soluble RNA is synthesized at a rapid rate, whereas the synthesis of acid-insoluble RNA is sluggish during this time. However, from hatching through larva stage II, the rate of synthesis of acid-insoluble RNA increases while the synthesis of acid-soluble RNA diminishes.

The decrease in amount of acid-soluble RNA, the concomitant increase in $^{32}$P incorporation into acid-insoluble RNA, and the increase in total RNA per embryo indicate that after hatching the embryo is synthesizing primarily acid-insoluble RNA.

Fig. 4 shows the specific activities of the two RNA fractions during development. The specific activity of acid-soluble RNA exceeds several fold the specific activity of acid-insoluble RNA from the two-cell stage through hatching. However, at some time after hatching, the specific activities of the two RNA fractions become equal and remain so through the larva stages.

Mononucleotide Composition of RNAs during Development—To determine if the acid-soluble RNA fraction from embryos is similar to that found in ovarian eggs (1), $^{32}$P-labeled larva preparations were mixed with unlabeled calf liver RNA and hydrolyzed in mild alkali. The resulting mononucleotides were separated by column chromatography (6, 7) and counted. Table III compares the mononucleotide composition, based on $^{32}$P counts, of acid-soluble and acid-insoluble RNA fractions from the larvae with that of mature ovarian eggs. No major differences are apparent in the larva and ovarian egg fractions, indicating that the same acid-soluble RNA fraction is present in both ovarian eggs and developing embryos.

**DISCUSSION**

In our earlier studies of the acid-soluble RNA of amphibian eggs (1), we reported that only about 80% of this RNA fraction was hydrolyzed to mononucleotides by mild alkaline hydrolysis and that the remaining 20% could be recovered as so-called "alkali-resistant" compounds. Furthermore, we reported the presence of labile phosphorus in some of the nonmononucleotide peaks. The present results corroborate these initial observations and indicate that the "alkali-resistant" compounds are primarily nucleoside di- and triphosphates containing pyrophosphate linkages. The acid-soluble RNA fraction itself is actually a mixture of oligoribonucleotides varying in chain length from mononucleotides through pentanucleotides. However, it must be restated that structures depicted in Table I are only tentative; further analyses are required before definite structures can be assigned to each peak. No base analyses of the oligonucleotides were attempted because each DEAE peak probably contains mixtures of compounds of similar chain length.

Developing amphibian embryos, particularly at the early
stages, contain large quantities of acid-soluble oligonucleotides. Over 80% of the total RNA fraction, which contains less than 2% DNA, is composed of oligonucleotides, whereas from 40 to 60% of ovarian egg RNA was reported to resemble oligonucleotides (1). This difference can be explained by the fact that ovarian egg RNA was prepared from whole mature amphibian ovaries that contained not only eggs but also follicle cells and connective tissue. Most probably the follicle cells and connective tissue contributed acid-insoluble RNA to these preparations without adding significant amounts of oligonucleotide material.

The developing embryo shows two major stages in the synthesis of RNA-like substances. Immediately after gastrulation but before hatching, it is engaged principally in the synthesis of oligonucleotides, but after hatching it produces polynucleotides primarily. No simple precursor-product relationship between the oligonucleotide and polynucleotide fractions is apparent. Indeed the total radioactivity of the free acid-soluble nucleotide pool exceeds several fold the radioactivity in either the oligonucleotide fraction or the polynucleotide fraction at all stages of development and could easily account for the observed incorporation into the polynucleotides as well as the oligonucleotides. However, the possibility still remains and is being explored that oligonucleotides are converted directly to polynucleotides during amphibian embryonic development.

**SUMMARY**

Amphibian embryos in the early stages of development contain large quantities of acid-soluble oligonucleotides, the amount of which decreases markedly after hatching at a time when the total quantity of ribonucleic acid is increasing rapidly in the embryos. This acid-soluble ribonucleic acid is composed of a mixture of oligoribonucleotides that can be separated by diethylaminoethyl cellulose chromatography into six discrete peaks with components that vary in chain length from mononucleotides through pentanucleotides.

Between gastrulation and hatching, acid-soluble oligonucleotides incorporate $^{32}$P rapidly, whereas the simultaneous synthesis of acid-insoluble ribonucleic acid is slow. From hatching through the larva stages, the rate of synthesis of acid-insoluble ribonucleic acid increases while the synthesis of oligonucleotides diminishes. However, the turnover (measured by the specific activity of incorporated $^{32}$P) of acid-soluble oligonucleotides exceeds several fold that of acid-insoluble ribonucleic acid from the two-cell stage through hatching. At some time after hatching, the specific activities of the oligonucleotide and polynucleotide fractions become equal and remain so through the larva stages.

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**REFERENCES**

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