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PREPARATION AND PROPERTIES OF SEA-URCHIN RIBONUCLEASE

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SUMMARY

1. A method is described for the purification of a ribonuclease (polyribonucleotide 2-oligonucleotide transferase (cyclizing), EC 2.7.7.16) from mature, unfertilized eggs of the sea urchin *Psammechinus miliaris*. The enzyme was purified 19 000-fold with a yield of 48% with the aid of chloroform treatment, $(NH_4)_2SO_4$ fractionation, and by chromatography on Sephadex G-100, on SE-Sephadex C-50 and on CM-Sephadex C-50.

2. The enzyme had a pH-optimum at 5.3–5.5.

3. The enzyme was heat labile, loosing about 50% of its activity in 3 min at 60° and pH 5.3, 70% of its activity in 3 min at 60° and pH 2.1, and 80% of its activity in 3 min at 40° and pH 9.0.

4. The enzyme hydrolyzed yeast RNA completely to 2',3'-cyclic nucleotides, and finally hydrolyzed the purine cyclic nucleotides slowly to their corresponding 3'-isomers.

5. The enzyme hydrolyzed guanosine 2',3'-cyclic phosphate about three times faster than adenosine 2',3'-cyclic phosphate.

6. The purified enzyme was found to be free of deoxyribonuclease and non-specific phosphodiesterase activity.

7. A molecular weight of about 37 000 was estimated for the enzyme by comparative gel filtration experiments.

INTRODUCTION

During the last few years a large number of ribonucleases from various plant and animal sources have been investigated and more or less extensively purified (see refs. 1 and 2). Besides their major interest for comparative biochemistry, the investigations also have played an important role as aids in the structural analysis of the different RNA's involved in the biosynthesis of proteins. Very little is, however, known about the role the different ribonucleases play in the metabolism of the RNA, although it is well-known that processes such as maturation, fertilization and early differentiation of the cells greatly affect the state of the RNA (see refs. 3–5). Since sea-urchin

eggs long have served as an excellent material in studies of these early cell processes, an investigation of their ribonucleases was of great interest in this respect.

Our investigation has so far resulted in the isolation of a ribonuclease from mature, unfertilized eggs of the sea urchin *Psammechinus miliaris*[6]. The present report describes the purification procedure and some of the properties of the purified enzyme.

EXPERIMENTAL

Source of the enzyme

Mature, unfertilized eggs of the sea urchin *P. miliaris*, were collected at the Zoological station, Kristineberg. The ovaries were cut open and the outflowing eggs were collected in sea water and thereafter passed through bolting silk. The eggs were further washed by suspending them in sea water. After slow centrifugation the eggs were lyophilized.

Substrates

Yeast RNA (Schwartz BioResearch, N.Y.) was extensively dialyzed against distilled water before use. Highly polymerized DNA was obtained from N.B.C., Cleveland. Cyclic ribomononucleotides, ribonucleoside phosphates and ribonucleosides were obtained from Sigma, St. Louis and Schwartz BioResearch, N.Y. Cyclic mononucleotides were purified before use by paper electrophoresis in 0.05 M ammonium bicarbonate. Bis-*p*-nitrophenyl phosphate was a commercial product of Sigma, St. Louis.

Assays

Ribonuclease activity was assayed essentially according to the procedure of DICKMAN, AROSKAR AND KROPF[7], using a 1% solution of RNA. Incubation was carried out at pH 5.0 and 25° for 25 min. After precipitation and centrifugation, 0.50 ml of the supernatant was diluted with 2.0 ml water. The amount of sea-urchin ribonuclease required to cause an increase in the 260-mμ absorbance of 0.10 was defined as one unit of ribonuclease activity. Cyclic-phosphatase activity was assayed according to the procedure of JOSEFSSON AND LAGERSTEDT[8] with the following modifications. Incubation (10 μl) was carried out in 0.05 M sodium acetate buffer (pH 5.0) and contained 0.10 μmole of 2',3'-cyclic ribonucleotide and about 2.5 units of ribonuclease activity. At zero time and after 60 and 120 min reaction time, 2-μl samples were withdrawn from the incubation mixture and applied to thin-layer plates. The thin-layer plates were developed by using isopropanol–ammonia–water (65:10:25, by vol., for adenosine and cytidine phosphates; 55:20:25, by vol., for guanosine phosphates; 60:10:30, by vol., for uridine phosphates) as solvent. The nucleoside 3'-phosphates were extracted with 0.2 ml of 0.01 M HCl. Adenosine 3'-phosphate was assayed at 258 mμ, cytidine 3'-phosphate at 278 mμ, guanosine 3'-phosphate at 256 mμ and uridine 3'-phosphate at 259 mμ. The amount of sea-urchin ribonuclease hydrolyzing 1 μmole of cyclic mononucleotide per min was defined as one unit of cyclic-phosphatase activity. Deoxyribonuclease activity was assayed according to the method of SCHNEIDER AND HOGEBOOM[9], the reaction mixture containing 0.05 M sodium acetate buffer (pH 5.0) and 0.01 M $MgSO_4$. Incubation was carried out for 60 min at 25°. Non-specific phosphodiesterase activity was assayed according to the procedure of IRIE AND

UKITA[10]. Incubation was made at 25° for 2 h in 0.10 M sodium acetate buffer (pH 5.0) and 0.10 M $MgSO_4$.

Identification of cyclic ribonucleotide digestion products

0.10 μmole of adenosine or guanosine 2′,3′-cyclic phosphate was incubated at 25° overnight in 0.10 M sodium acetate buffer (pH 5.30) with 2 units of ribonuclease activity. Identical incubations without enzyme served as controls. The incubation products were separated by chromatography on 20 cm × 20 cm thin layers of MN 300 cellulose (Fa. Macerey, Düren). Solvent: satd. $(NH_4)_2SO_4$–1 M sodium acetate (pH 6.0)–isopropanol (80:18:2, by vol.)[11]. The corresponding nucleosides, nucleoside 3′-phosphates, nucleoside 2′-phosphates and nucleoside 2′,3′-cyclic phosphates served as references.

Paper electrophoresis

Electrophoresis was carried out essentially according to Method B of INGRAM AND PIERCE[12] on a cooled metal plate in 20% acetic acid[13] adjusted to pH 3.0 with conc. ammonia and using Whatman 3 MC paper.

Estimation of purification

As an indication of enzyme purification, the ratio of units of activity per ml to 280-mμ absorbance was chosen.

Estimation of molecular size

The molecular size of the sea-urchin ribonuclease was determined with the aid of Sephadex G-100 (Pharmacia, Uppsala) according to the method of ANDREWS[14,15]. As reference substances, crystalline bovine pancreatic ribonuclease (Armour, Eastbourne), 2 × crystalline pepsin (Worthington Biochem., N.J.) and crystalline bovine serum albumin (Armour, Eastbourne) were used.

Isolation and purification of the enzyme

All operations were performed in a cold room at 4°, unless otherwise stated.

Preparation of homogenate. Lyophilized eggs of the sea urchin (4 g) were homogenized with simultaneous cooling in an ice-bath for 2 min (Potter–Elvehjem homogenizer, 2200 rev./min) in 4 equal batches with 0.10 M acetic acid (25 ml). The insoluble material was centrifuged off (27 000 × *g*, 15 min and 0°) and the slight yellow supernatants were collected. The sediments were washed with 0.10 M acetic acid (3 ml) and the centrifugation was repeated. The supernatants were combined (128 ml, Fraction 1, Table I) and the sediments were discarded.

Extraction with chloroform. The combined supernatants were shaken for 30 min with 1 vol. of cold chloroform (b.p. 61°) and 2 ml of 1-octanol (b.p. 195°). The phases were separated by centrifugation (7000 × *g*, 15 min and 0°). The clear yellow aqueous phase was collected, evaporated for 15 min *in vacuo* and then lyophilized. The red-yellow powder was dissolved in distilled water (10 ml). A small insoluble red-brown residue was centrifuged off (27 000 × *g*, 15 min and 0°) and the clear yellow supernatant was collected. The sediment was washed with a small volume of distilled water and the

centrifugation was repeated. The supernatants were combined (10.6 ml) and the sediment was discarded.

Fractionation with $(NH_4)_2SO_4$. The combined supernatants were adjusted to pH 3.5 with formic acid (98–100%). Cold 0.10 M sodium formate buffer, pH 3.5 (8.45 ml), saturated with $(NH_4)_2SO_4$, was slowly added to the solution with continuous stirring and the solution was then left for 2 h with intermittent shaking. The precipitate which formed on standing was centrifuged off (12 000 × *g*, 20 min and 0°) and discarded. The clear yellow supernatant (17.8 ml) was collected and solid $(NH_4)_2SO_4$ (3.2 g) was slowly added to the solution. The solution was stirred continuously until all salt was

TABLE I

PURIFICATION OF RIBONUCLEASE FROM UNFERTILIZED EGGS OF THE SEA URCHIN *P. miliaris*

Figures are given for 4 g of lyophilized eggs.

Fraction	*Volume (ml)*	$A_{280\ m\mu}$	*Ribonuclease activity*		*Yield (%)*
			Units/ml	*Units/ml per* $A_{280\ m\mu}$	
1. Acid homogenate	128	10.5	9.20	0.876	100
2. $(NH_4)_2SO_4$ precipitate	1.7	10.9	617	56.6	89
3. Pooled active fractions of Sephadex G-100 effluent	22.5	0.053	43.5	821	83
4. Pooled active fractions of SE-Sephadex C-50 effluent	1.2	0.208	600	2 880	91
5. Pooled active fractions of CM-Sephadex C-50 effluent	3.4	0.010	165	16 500	48

dissolved and then left overnight. The precipitate which formed on standing was collected by centrifugation (12 000 × *g*, 20 min and 0°) and the supernatant was discarded. The precipitate was easily dissolved in distilled water to a slightly red solution (Fraction 2, Table I).

Chromatography on Sephadex G-100. The clear solution (1.7 ml) was applied to a 1.7 cm × 61 cm column of Sephadex G-100 (Pharmacia, Uppsala), prepared in and equilibrated with 0.047 M sodium acetate solution. The column was eluted with the same solvent at a constant flow rate of 20 ml per h. Fractions of 2 ml were collected and analyzed for 280-mμ absorbance and for ribonuclease activity. The active fractions were pooled (Fraction 3, Table I).

Concentration on SE-Sephadex C-50. The enzyme solution (22.5 ml) was adjusted to pH 4.5 with glacial acetic acid and was then applied to a 0.58 cm × 4 cm column of SE-Sephadex C-50 (Pharmacia, Uppsala). The column was prepared according to the directions of the manufacturer and equilibrated with 0.047 M sodium acetate buffer (pH 4.5). After the initial charge and a brief wash with the buffer, the column was washed with 0.10 M sodium acetate, which eluted the enzyme. Fractions of 0.4 ml were collected, assayed for ribonuclease activity, and those containing the activity were pooled (Fraction 4, Table I).

Chromatography on CM-Sephadex C-50. The enzyme solution (1.2 ml) was adjusted to pH 4.8 with glacial acetic acid and was then applied to a 0.5 cm × 35 cm column of CM-Sephadex C-50 (Pharmacia, Uppsala). The column was prepared in and equilibrated with 0.10 M sodium acetate buffer (pH 4.8). After an initial wash with the buffer the column was eluted at a constant flow rate of 5 ml per h, with an increasing NaCl gradient. The gradient was developed by pouring 1 M NaCl–0.10 M sodium acetate buffer into a constant volume mixing chamber containing 75 ml of the buffer. Fractions of 0.7 ml were collected by a drop counter. They were analyzed for 280-mμ absorbance and for ribonuclease activity and the active fractions were pooled (Fraction 5, Table I).

RESULTS

Purification

The results of a typical purification experiment are given in Table I. The high increase of the specific activity when expressed as units per ml per $A_{280\ m\mu}$, almost 19 000-fold, was not always obtained, but varied from 10 000-fold to 20 000-fold in several experiments owing at least partly to variations in the original content of activity of the various egg batches used.

The ribonuclease activity was obtained as a well-defined peak after chromato-

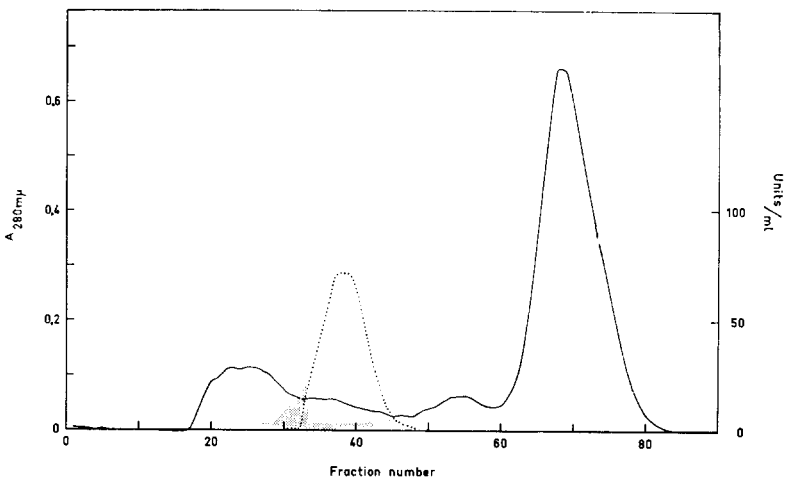

Fig. 1. Chromatography of sea-urchin ribonuclease (...) on Sephadex G-100. 1.7 ml of an $(NH_4)_2SO_4$-precipitated enzyme solution, equivalent to 4 g of lyophilized sea-urchin eggs, were applied in the cold room to an 1.7 cm × 61 cm column, equilibrated and eluted with 0.047 M sodium acetate. Flow rate, 20 ml/h. Effluent collected in 2-ml fractions. $A_{280\ m\mu}$ (———).

graphy on Sephadex G-100, and in high yields. A representative elution pattern is shown in Fig. 1. The very minute amounts of enzyme available and the low stability of its activity necessitated the concentration procedure on SE-Sephadex, although this purification step involved a relatively great loss of activity. In the final purification step on CM-Sephadex, the enzyme was eluted in a single peak, as may be seen from a typical elution pattern given in Fig. 2. Although extensively purified, the en-

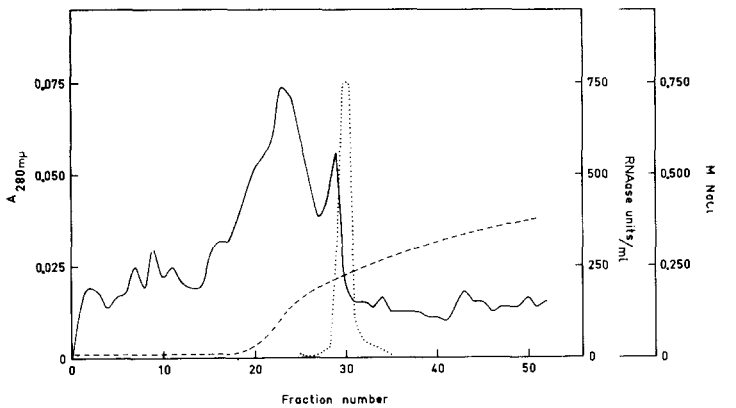

Fig. 2. Chromatography of sea-urchin ribonuclease (. . .) on CM-Sephadex C-50. 1.2 ml of an enzyme solution, concentrated on SE-Sephadex and equivalent to 4 g of lyophilized sea-urchin eggs, was applied in the cold room to an 0.5 cm × 35 cm column. The column was equilibrated with 0.10 M sodium acetate buffer (pH 4.8), and eluted by a gradient developed by using 1 M NaCl (- - - -). Flow rate, 5 ml/h. Effluent collected in 0.7-ml fractions. $A_{280\ m\mu}$ (———).

zyme certainly was still not pure, because no corresponding peak in the absorbance at 280 mμ was obtained.

The enzyme preparation gave no significant increase in absorbance when tested for deoxyribonuclease activity, with 12 units of ribonuclease activity added to the

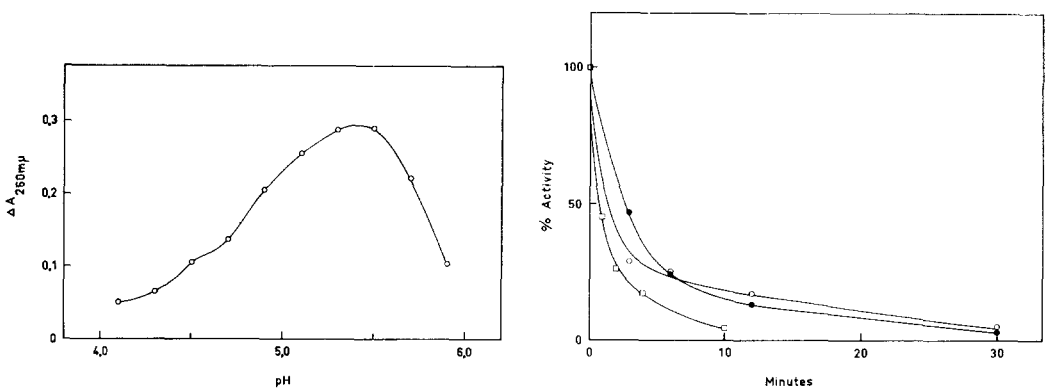

Fig. 3. Influence of pH on sea-urchin ribonuclease activity. Incubations (1 ml) were carried out in 0.10 M sodium acetate buffers (ionic strength was adjusted to 0.10 with NaCl) and contained each, 1.1% yeast RNA and 2.3 units of ribonuclease. Digestion at 25° for 25 min.

Fig. 4. Stability of sea-urchin ribonuclease activity. Each test solution (1.1 ml) contained 70.3 units of ribonuclease. Samples of 0.1 ml were withdrawn for assays as indicated. ○, 0.01 M HCl (pH 2.1 and 60°); ●, 0.05 M sodium acetate buffer (pH 5.3 and 60°); □, 0.05 M sodium borate buffer (pH 9.0 and 40°). All test solutions were adjusted to an ionic strength of 0.05 with NaCl.

incubation, or for non-specific phosphodiesterase activity, with 20 units of ribonuclease activity added to the incubation mixture, according to the assay procedures described in EXPERIMENTAL.

Effect of various enzyme concentrations

An investigation of the dependence of $\Delta A_{260\ m\mu}$ on enzyme concentration showed a linear correlation to a maximum of 0.5 for $\Delta A_{260\ m\mu}$.

Effect of pH

The sea-urchin ribonuclease showed a rather sharp maximum activity at pH 5.3 to 5.5, when the effect of pH was investigated in 0.10 M sodium acetate buffers (Fig. 3).

Stability

The stability of sea-urchin ribonuclease was determined at pH 2.1, 5.3 and 9.0 at 40° and 60° (Fig. 4). The enzyme appeared to be rather heat labile in comparison with other ribonucleases. Solutions of the purified enzyme were unstable when stored at 4°. Frozen preparations, however, retained their activity for more then 2 years.

Molecular size

An investigation of the molecular size of the sea-urchin ribonuclease was made by comparative experiments with proteins of known molecular weight utilizing gel

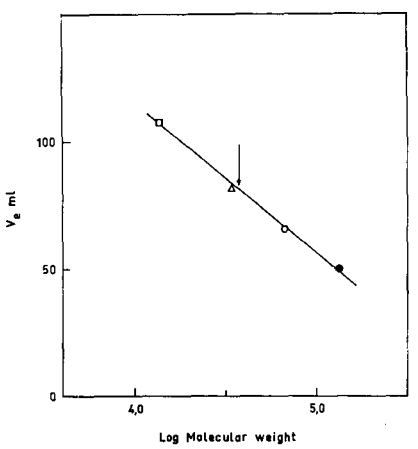

Fig. 5. Plot of elution volume, V_e, against log (mol. wt.) for proteins on a Sephadex G-100 column (1.7 cm × 59 cm). The column was equilibrated and eluted at room temperature with 0.5 M ammonium acetate at a rate of 10 ml/h with the aid of a constant pump device. Either 10 mg each of serum albumin, pepsin and pancreatic ribonuclease or 10 mg each of serum albumin, and pepsin and 117 units of sea-urchin ribonuclease activity were applied in 1.0 ml of 0.50 M ammonium acetate to the column. Effluent was collected in 1.0-ml fractions, which were read at 280 mμ and assayed for ribonuclease activity. Values are averaged for 2 separate experiments of the two kinds of protein mixtures, which were run successively on the same column. Arrow indicates value for sea-urchin ribonuclease. ●, bovine serum albumin dimer; ○, bovine serum albumin; △, pepsin; □, bovine pancreatic ribonuclease.

filtration on Sephadex G-100. With the assumption that the same relation between molecular weight and V_e, as found for the reference proteins, is valid for this ribonuclease the V_e obtained gave an estimated molecular weight of about 37 000 for the purified enzyme (Fig. 5).

Specificity

Products of total digestion of RNA. Incubations of the purified sea-urchin ribonuclease with yeast RNA were analyzed by paper electrophoresis and thin-layer chromatography after various incubation periods at 25°. After shorter incubation times oligonucleotides were identified as early products *plus* 2′,3′-cyclic nucleotides

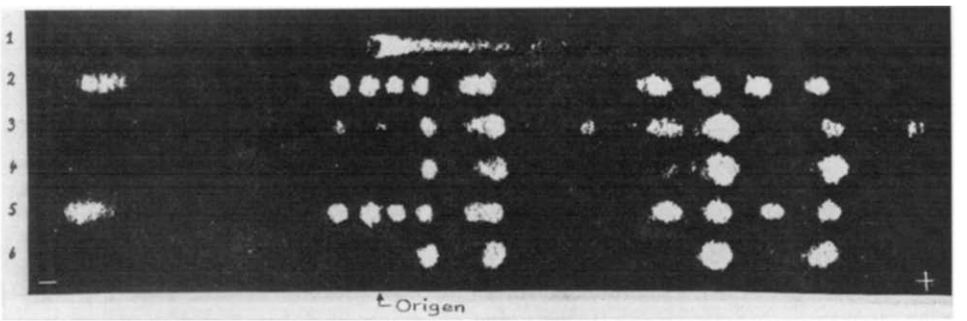

Fig. 6. One-dimensional electrophoresis pattern. Whatman 3 MC paper, 20% acetic acid–conc. NH_3, (pH 3.0). Electrophoresis took 3 h at 2500 V, applied to 57 cm × 15 cm paper according to Method B of INGRAM AND PIERCE[12]. 1, 200 μg yeast RNA kept in 25 μl of 0.02 M sodium acetate buffer (pH 5.3), for 24 h and 25°. 2, references (from right to left): U-2′,3′-*P*; U-3′-*P*; G-2′,3′-*P*; G-3′-*P*; A-2′,3′-*P*; A-3′-*P*; C-2′,3′-*P*; C-3′-*P*; C; G; and A. 3, 200 μg yeast RNA and 5.7 units of sea-urchin ribonuclease kept in 25 μl of 0.02 M sodium acetate buffer (pH 5.3), for 24 h and 25°. Identified products (from right to left): U-2′,3′-*P*; G-2′,3′-*P*; G-3′-*P*; A-2′,3′-*P*; A-3′-*P*; C-2′,3′-*P* and G (traces). Spots of unidentified products represent traces of oligonucleotides. 4, 0.1 μmole of each of the 2′,3′-cyclic ribonucleotides and 5.7 units of sea-urchin ribonuclease kept in 25 μl of 0.02 M sodium acetate buffer (pH 5.3), for 24 h and 25°. Identified products (from right to left): U-2′,3′-*P*; G-2′,3′-*P*; G-3′-*P* (traces); A-2′,3′-*P*; A-3′-*P* (traces); and C-2′,3′-*P*. 5, references as (2). 6, 0.1 μmole of each of the 2′,3′-cyclic ribonucleotides kept in 25 μl of 0.02 M sodium acetate buffer (pH 5.3), for 24 h and 25°.

which increased in amount as the digestion time was lengthened. During extensive digestion periods the purine cyclic nucleotides slowly underwent hydrolysis to their 3′-nucleotides, while no parallel hydrolysis of the pyrimidine cyclic nucleotides was observed (Fig. 6). Small amounts of guanosine were also identified among the products indicating 3′-nucleotidase activity (Fig. 6). After prolonged incubations the traces of oligonucleotides disappeared completely.

Hydrolysis of 2′, 3′-cyclic nucleotides. Investigation of the cyclic-phosphatase activity of the purified sea-urchin ribonuclease on synthetic substrates confirmed that

TABLE II

CYCLIC-PHOSPHATASE ACTIVITIES OF PURIFIED SEA-URCHIN RIBONUCLEASE

Substrate	*Cyclic-phosphatase activity (munits/units of ribonuclease activity)*
Adenosine 2′,3′-cyclic phosphate	0.04
Guanosine 2′,3′-cyclic phosphate	0.13
Cytidine 2′,3′-cyclic phosphate	0.00
Uridine 2′,3′-cyclic phosphate	0.00

the enzyme was capable of splitting only the purine 2',3'-cyclic nucleotides and had no action on the pyrimidine 2',3'-cyclic nucleotides under the same conditions (Fig. 6). Quantitative analysis of the hydrolysis revealed that guanosine 2',3'-cyclic phosphate was hydrolyzed about 3 times faster than the corresponding adenosine derivative (Table II). Chromatographic analysis also proved the 3'-isomers to be the product of the hydrolysis of the purine cyclic nucleotides.

According to the specificity analysis sea-urchin ribonuclease thus digests yeast RNA completely to 2',3'-cyclic nucleotides and then hydrolyzes the purine cyclic nucleotides at a slower rate to their corresponding 3'-isomers.

DISCUSSION

The very minute amounts of enzyme present in the egg material as well as difficulties in collecting large batches of egg material limit the possibilities of purifying the sea-urchin ribonuclease in sufficient amounts for its chemical investigation. Knowledge of the sea-urchin ribonuclease is, however, of importance in work attempting to determine its role in the cell processes connected with the early development of the sea urchin.

Considering the properties of the sea-urchin ribonuclease, its optimal pH of 5.3–5.5 compares with the values of 5.3 for the spleen-acid ribonuclease[16], 5.1 for tobacco-leaf ribonuclease[17], 5.5 for pea-leaf ribonuclease[18], 5–6 for spinach ribonuclease[19] and 5.5 for ryegrass ribonuclease[20]. The heat-labile character of the sea-urchin ribonuclease is unusual among the ribonucleases and can be compared only with the stability observed for the spleen-acid ribonuclease[16] and for the pea-leaf ribonuclease[18].

Similar to the ribonucleases obtained in a purified state from plants and in contrast to pancreatic ribonuclease and other animal ribonucleases, the sea-urchin ribonuclease completely hydrolyzes yeast RNA to 2',3'-cyclic nucleotides. In addition, the specificity of the sea-urchin ribonuclease to hydrolyze only the purine 2',3'-cyclic nucleotides to their 3'-isomers and not the corresponding pyrimidine nucleotides, is parallelled only by the ribonucleases of tobacco leaf[21] and ryegrass[20,22].

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