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PURIFICATION AND PROPERTIES OF A RIBONUCLEASE FROM AVENA LEAF TISSUES

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SUMMARY

A ribonuclease was isolated and purified from Avena leaf tissues. The enzyme was shown to be an endonuclease which cleaves all the phosphodiester bonds forming nucleoside 2′,3′-cyclic phosphates. As shown by kinetic analysis, the ribonuclease has a preference for the secondary phosphate esters of 3′-GMP. The early release of 2′,3′-GMP was followed by the release of 2′,3′-AMP and 2′,3′-UMP, whereas a pronounced lag period preceded the release of 2′,3′-CMP. The purine/pyrimidine ratio in the mixture of nucleotides liberated during RNA hydrolysis was high at the beginning of the reaction and gradually decreased with longer incubation time. The relative purine specificity of the enzyme was also shown by using homopolymers as substrates. A mixture of poly A and poly U in the proportion 1:2, known to produce maximum secondary interaction, was not attacked. The enzyme hydrolyzed only the purine 2′,3′-cyclic phosphates to a considerable extent. The following reaction products inhibited the enzyme: 3′-AMP, 3′-GMP, 2′,3′-AMP and 2′,3′-GMP. 3′-CMP, 3′-UMP, 2′,3′-CMP and 2′,3′-UMP had no or little effect. The activity of the ribonuclease described above was found to increase rapidly in Avena leaf tissues in association with injury and/or senescence.

INTRODUCTION

Ribonuclease activity has been shown to increase rapidly in a number of plant tissues exposed to various stress conditions such as mechanical injury[1,2], osmotic shock[3,4], parasitic attack[1,5–7], water shortage[8], heat treatment[8] or leaf excision[9–13]. Preliminary evidence suggests that the stress-induced increase in ribonuclease level is dependent on new protein synthesis[3,14]. A prerequisite for the elucidation of the nature and significance of this phenomenon is a closer characterization of the enzymes affected. Preliminary evidence which shows that more than one nuclease is involved has recently been obtained[14]. In the present paper a detailed characterization of a

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ribonuclease, the activity of which is dramatically increased in excised Avena leaf tissues[14], is given.

MATERIALS AND METHODS

Preparation of crude extract. 120 g of 10-day-old *Avena sativa* L. leaf tissue were deep frozen (−15°) and homogenized in 360 ml of 0.05 M Tris–HCl buffer (pH 7.5) in a prechilled mortar with quartz sand. The resulting slurry was passed through 4 layers of cheesecloth and centrifuged at 16 000 × *g* for 30 min at 4°. The supernatant served as the crude extract.

Determination of enzyme activities. The determination of ribonuclease, phosphodiesterase and phosphomonoesterase activities was carried out as described earlier[14].

Purification of ribonuclease. The procedure of BARKER AND HOLLINSHEAD[15] was used with slight modifications as described previously[14].

Hydrolysis of RNA and identification of the digestion products. Highly polymerized yeast RNA was incubated with the enzyme in the presence of 0.005 M acetate buffer (pH 5.5). After incubation the reaction mixture was adjusted with Tris buffer to pH 7.8. The final concentration of the reaction mixture was 0.005 M with respect to Tris buffer. A DEAE-cellulose column (1 cm × 18 cm) was packed in 2 M $(NH_4)_2CO_3$ under slight pressure. Before applying the sample, the column was washed with distilled water until no CO_3^{2-} were detected in the effluent. An aliquot of the reaction mixture was then applied to the column which was subjected to a linear gradient of NH_4HCO_3, 0.0–0.4 M, (pH 8.6)[16]. The fractions were collected, and the absorbance of each fraction was measured at 260 mμ in a Beckman DU-2 spectrophotometer. The identity of the nucleotides present in the fractions belonging to various peaks was established by (a) ultraviolet spectra in acid and alkali, (b) paper chromatography and (c) high-voltage electrophoresis. Two-dimensional paper chromatography was carried out on Whatman No. 40 filter paper in the following systems: isobutyric acid–ammonia–water (66:1:33, by vol.) in the first dimension and isopropanol–HCl–water (170:44:36, by vol.) in the second dimension. A Shandon high-voltage electrophoresis apparatus was used for paper electrophoresis. The digestion products were separated either in 0.02 M formate buffer (pH 3.5), in 0.1 M borate buffer (pH 9.2) or in 0.1 M phosphate buffer (pH 7.5). The spots were marked under ultraviolet light and eluted with 0.1 M HCl or distilled water for 24 h at 4°. The nucleotides were identified on the basis of the spectral characteristics of the eluates, the R_F values or relative mobilities of the spots. Authentic samples of nucleotides served as controls.

Hydrolysis of pyrimidine and purine nucleoside cyclic phosphates. This was carried out as described earlier[14].

Hydrolysis of homopolymers and double-stranded structures. Hydrolysis of poly A, poly I, poly C, poly U and mixtures of poly A and poly U in a ratio of 1:2 was carried out as described by CHAKRABURTTY AND BURMA[17].

The use of gel filtration to distinguish between endonuclease and exonuclease action. The method of BIRNBOIM[18] developed for deoxyribonucleases was adapted to test the endo- or exonuclease activity of ribonuclease. The principles described were followed but Sephadex G-100 was replaced by Sephadex G-50 and 0.1 M acetate buffer (pH 5.5) was used for the elution of nucleotides and undigested RNA from a 0.8 cm × 15 cm column. Before the method was applied to Avena ribonuclease, its reliability was

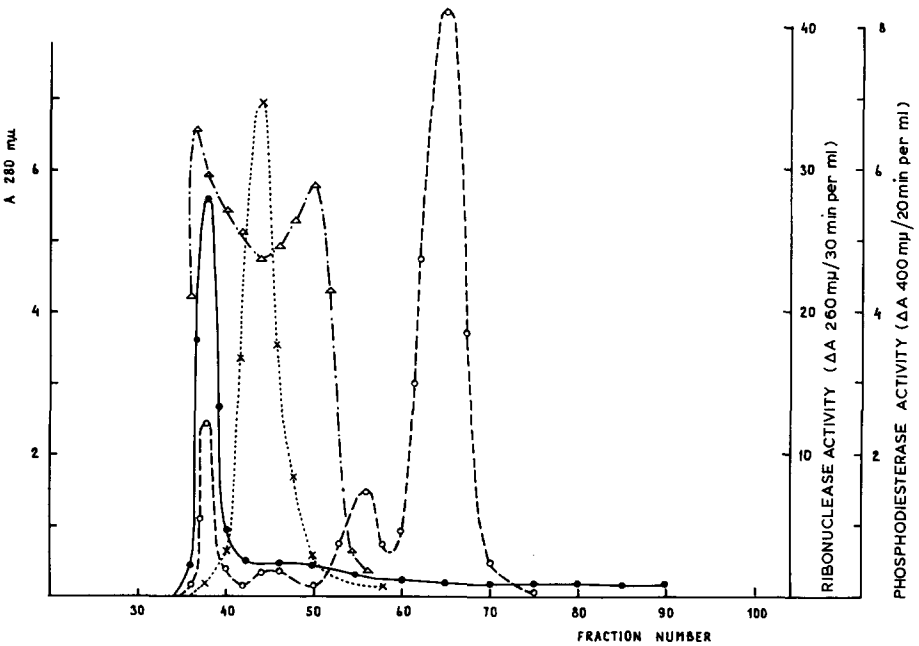

Fig. 1. Chromatography of ribonucleases and phosphodiesterases isolated from Avena leaves. Proteins from crude extracts centrifuged at 100 000 × *g* for 90 min were precipitated with $(NH_4)_2SO_4$ at 80% saturation, the precipitate was dissolved in 3 ml of 0.01 M Tris–HCl buffer (pH 7.5) and applied to a Sephadex G-75 column (2.4 cm × 100 cm) equilibrated with 0.01 M Tris buffer. Proteins were eluted with the same buffer and monitored at 280 mμ. The assay system for ribonuclease activity consisted of 1.5 mg yeast RNA, 100 μmoles of acetate buffer (pH 5.5) and 0.4 ml of effluent in a final volume of 2.0 ml. The incubation was carried out at 37° for 30 min. The increase in absorption at 260 mμ of acid soluble (0.3% $La(NO_3)_3$ in 2.5% trichloroacetic acid) digestion products was measured. The assay system for phosphodiesterase activity contained 1 μmole of bis-(*p*-nitrophenyl) phosphate, 100 μmoles of acetate buffer (pH 5.5) or 100 μmoles of Tris–HCl buffer (pH 8.8) and 0.3 ml of effluent in a final volume of 2.0 ml. After 20 min of incubation at 37°, the reaction was stopped with 1 ml of 0.3 M NaOH, and the increase in absorbance at 400 mμ due to the liberation of *p*-nitrophenol was measured. ●—●, protein; ○— —○, ribonuclease; × · · · · · ×, phosphodiesterase (pH 8.8); △—·—△, phosphodiesterase (pH 5.5).

ascertained by using pancreatic ribonuclease A, an enzyme well known to be an endonuclease.

Determination of the base ratios. Acid hydrolysis was used for the determination of base ratios of the substrate as described by SMITH AND MARKHAM[19].

Materials

First leaves of 8–10-day-old *A. sativa* L. seedlings, grown under ordinary greenhouse conditions, were used for enzyme extraction.

All the mononucleotides were obtained from the Pabst Laboratories, Milwaukee, Wisc. *p*-Nitrophenylphosphate disodium salt, bis-(*p*-nitrophenyl) phosphate sodium salt and the homopolymers were products of Sigma Chemical Co., St. Louis, Mo. Sephadex G-50 and G-75 were purchased from Pharmacia Fine Chemicals, Uppsala. The other chemicals used were of analytical grade. Highly polymerized yeast RNA (lot No. 63411) was obtained from Calbiochem., Los Angeles, Calif.

RESULTS

Fractionation of enzymes in crude extracts

Chromatography of crude extracts on Sephadex G-75 revealed the presence of several nucleases and other phosphate-ester hydrolyzing enzymes. As shown by the chromatographic profile presented in Fig. 1, four well-defined peaks with RNA-splitting activity, one peak corresponding to alkaline phosphodiesterase and two peaks corresponding to acidic phosphodiesterases (as assayed by the use of bis-(*p*-nitrophenyl) phosphate), were revealed. The patterns obtained were highly reproducible, including the ratios of the various peaks. The ribonuclease corresponding to the main peak of ribonuclease activity was purified 150-fold, as described in a previous publication[14] and was analyzed for its mode of action.

Test of endo- or exonuclease activity

High-molecular-weight yeast RNA was digested with the purified enzyme for various intervals, and the digestion products were chromatographed on Sephadex

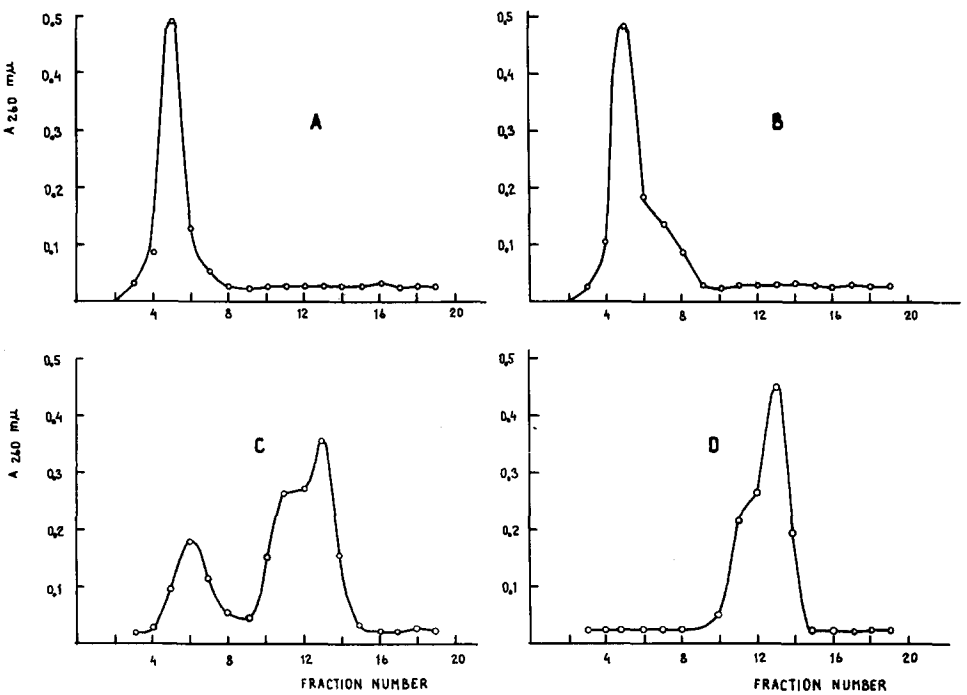

Fig. 2. Gel filtration of digestion products obtained after incubation of RNA with Avena ribonuclease. 2 mg of highly polymerized yeast RNA dissolved in 1 ml of 0.1 M acetate buffer (pH 5.5) was incubated with 100 μg of lyophilized Avena ribonuclease preparation. 0.1 ml of the incubation mixture was withdrawn at 0-time as well as after various incubation periods. The samples were thoroughly mixed and shaken with 3 μl of diethylpyrocarbonate to stop nuclease activity[20]. 0.4 ml of 0.1 M acetate buffer (pH 5.5) was added to each sample, and the samples were applied to Sephadex G-50 columns (0.8 cm × 20 cm) equilibrated with acetate buffer. The nucleic acid and the breakdown products were eluted with 0.1 M acetate buffer (pH 5.5). 2-ml fractions were collected, and the absorbance of the fractions was measured at 260 mμ. A, 0-time; B, 30-min incubation at 0°; C, 12-h incubation at 37°; D, 48-h incubation at 37°.

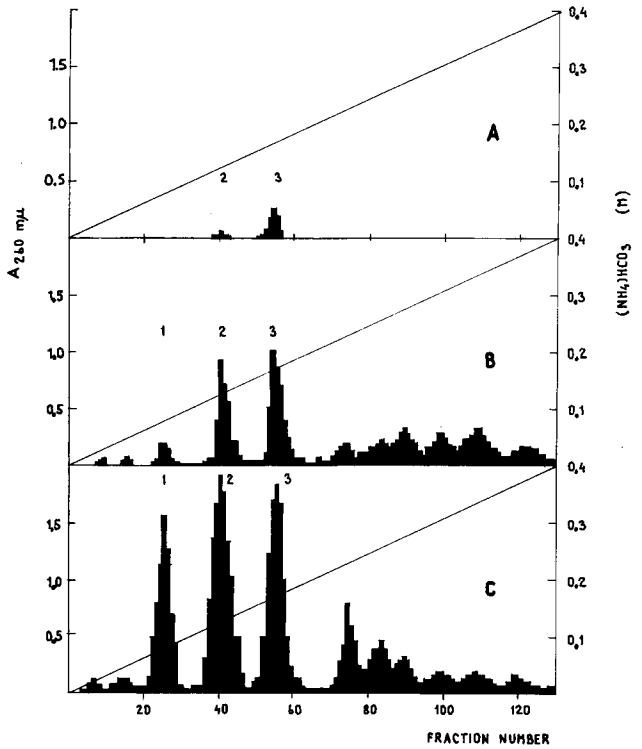

Fig. 3. Ion-exchange chromatography of the digestion products of yeast RNA hydrolyzed with Avena ribonuclease. 15 mg of high-molecular-weight yeast RNA was incubated at 37° with 3 mg of lyophilized Avena ribonuclease preparation in 3 ml of 0.005 M acetate buffer (pH 5.5) and 0.001 M $MgCl_2$. 1-ml samples were withdrawn at various intervals (A, 15 min; B, 6 h; C, 24 h), adjusted to pH 7.8 with Tris–HCl buffer (final Tris buffer concentration 0.005 M), and applied to a DEAE cellulose column (1 cm × 18 cm). The nucleotides were eluted with NH_4HCO_3. 3-ml fractions were collected.

G-50. The chromatographic profiles are shown in Fig. 2. At zero time, a single peak representing the undigested RNA was obtained (Fig. 2A). At short incubation times, a small amount of digested material appeared as a shoulder of the main peak (Fig. 2B). After longer incubation, a peak corresponding to the position of the first peak was shifted towards higher elution volumes corresponding to a decrease in average molecular weight (Fig. 2C). After extensive digestion, the hydrolysis products yielded upon chromatography a single peak corresponding to mononucleotides. The shoulder probably represents low-molecular-weight oligonucleotides (Fig. 2D). The results clearly show that the ribonuclease investigated is an endonuclease.

Identification of breakdown products

The progress of RNA hydrolysis was followed by column chromatography of the breakdown products on DEAE-cellulose. As may be seen in Fig. 3, three main peaks were obtained the first of which (in the order of elution sequence) appeared significantly later than the other two. With digests of longer incubation periods, profiles were obtained in which the three main peaks were followed by a number of smaller peaks representing oligonucleotides[16].

TABLE I

NUCLEOTIDE COMPOSITION OF THE PEAKS OBTAINED BY COLUMN CHROMATOGRAPHY ON DEAE-CELLULOSE OF ENZYMATIC DIGESTS OF RNA

Fractions corresponding to Peaks 1, 2 and 3 (*cf.* Fig. 3) were pooled, evaporated to dryness under vacuum at 40° and taken up in small amounts of water. Aliquots were subjected to high-voltage electrophoresis in 0.1 M phosphate buffer (pH 7.5). The spots marked under ultraviolet light were eluted with water for quantitative spectrophotometric estimation. The total amount of nucleotides present in Peaks No. 1, 2 and 3 is taken as 100%.

Peak No.	*Time of enzymatic digestion (h)*	*Composition (%)*							
		3'-AMP	*2',3'-AMP*	*3'-GMP*	*2',3'-GMP*	*3'-UMP*	*2',3'-UMP*	*3'-CMP*	*2',3'-CMP*
1	6	—	—	—	—	—	—	—	7
2	6	4.2	21.8	—	—	—	16	—	—
3	6	—	—	9.7	41.3	—	—	—	—
1	48	—	—	—	—	—	—	0.9	16.1
2	48	4.8	22.2	—	—	1.5	17.5	—	—
3	48	—	—	7.4	29.6	—	—	—	—

The nature of the three main peaks eluted in the region of mononucleotides was investigated by spectral analysis, paper chromatography and high-voltage paper electrophoresis. Peak 1 was identified as 2',3'-CMP *plus* minute amounts of 3'-CMP, Peak 2 as a mixture of 2',3'-AMP and 2',3'-UMP containing small amounts of 3'-AMP and 3'-UMP as well. Peak 3 contained 2',3'-GMP and 3'-GMP. Quantitative data on the composition of the peaks are given in Table I.

It may be concluded from the results presented in Fig. 3, and Table I that the ribonuclease cleaves all the phosphodiester bonds with an early release of 2',3'-GMP, followed by the release of 2',3'-AMP and 2',3'-UMP. 2',3'-CMP is released after a considerable lag period only. Upon longer incubation 3'-GMP, 3'-AMP and traces of 3'-UMP also appear in the incubation mixture.

TABLE II

PURINE/PYRIMIDINE RATIOS IN THE MIXTURE OF MONONUCLEOTIDES LIBERATED DURING HYDROLYSIS OF RNA BY AVENA RIBONUCLEASE

Incubation time (h)	*Purine/pyrimidine ratio*
6	3.35
24	1.78

As shown in Table II the enzyme has a pronounced relative purine specificity in the earlier stages of enzyme action. There is a relative shift in favor of the pyrimidine nucleotides as the enzymatic breakdown progresses.

No 5'-nucleotides occurred among the breakdown products as shown by high-voltage electrophoresis in 0.1 M borate buffer (pH 9.2).

TABLE III

HYDROLYSIS OF NUCLEOSIDE CYCLIC PHOSPHATES BY AVENA RIBONUCLEASE

The reaction mixture contained 500 μg substrate, 30 μmoles of sodium acetate buffer (pH 5.5) and 100 μg of lyophilized ribonuclease preparation in a final volume of 0.3 ml. After incubation of the reaction mixture at 37° for 24 or 48 h the samples were evaporated to a suitable volume and subjected to high-voltage electrophoresis in 0.1 M phosphate buffer (pH 7.5). The spots marked under ultraviolet light were eluted with 0.1 M HCl for 24 h for quantitative spectrophotometric determination.

Substrate	*Percentage of cyclic phosphate hydrolyzed*	
	24 h	*48 h*
2′,3′-AMP	40.5	65.0
2′,3′-GMP	55.6	74.0
2′,3′-UMP	6.7	13.5
2′,3′-CMP	—	1.4

Hydrolysis of nucleoside cyclic phosphates

As shown in Table III the enzyme cleaved only the purine nucleoside cyclic phosphates to any considerable extent.

Effect of end-products on RNA hydrolysis

Data summarized in Table IV show that the purine cyclic nucleotides and the purine nucleoside 2′(3′)-phosphates inhibited the ribonuclease considerably. The pyrimidine nucleotides, whether cyclic or noncyclic, had no effect. In contrast to the nucleoside 2′(3′)-phosphates, the nucleoside 5′-phosphates did not inhibit the enzyme. 3′,5′-AMP also proved to be inhibitory.

Effect of Avena ribonuclease on homopolymers and double-stranded structures

As shown in Table V Avena ribonuclease splits all 4 homopolymers in the follow-

TABLE IV

INHIBITION OF AVENA RIBONUCLEASE ACTIVITY BY END-PRODUCTS

Ribonuclease activity was determined as described in the legend to Fig. 1 in the absence and presence of various end-products, respectively. I and II: results of separate experiments.

Substance added to the reaction mixture	*Inhibition of ribonuclease activity (%)*	
	I	*II*
2′(3′)-AMP	64	61
2′(3′)-GMP	33	43
2′(3′)-UMP	6	0
2′(3′)-CMP	6	3
2′,3′-AMP	48	45
2′,3′-GMP	22	32
2′,3′-UMP	0	0
2′,3′-CMP	0	0
3′,5′-AMP	47	56

TABLE V

EFFECT OF AVENA RIBONUCLEASE ON HOMOPOLYMERS

The reaction mixture contained 1 μmole of substrate (expressed in terms of nucleotide residues), 180 μmoles of sodium acetate buffer (pH 5.5), 13.5 μmoles of $MgCl_2$, 100 μg of lyophilized ribonuclease in a total volume of 2.2 ml. Incubation was carried out at 37° for 30 min and 1 h, respectively. After incubation 0.5 ml of a 0.25% uranyl acetate solution in 2.5% trichloroacetic acids was added. The mixture was allowed to stand overnight at 4° and then centrifuged at 12 000 × *g* for 10 min at 4°. The absorbance of the supernatant fluid was measured at 260 mμ against a zero-time blank. The results are expressed as the percentage of the homopolymer converted to acid-soluble material.

Homopolymer	*Percentage of degradation after hydrolysis with Avena ribonuclease for*	
	30 min	*1 h*
Poly A	29.2	41.5
Poly I	49.1	54.6
Poly U	16.5	31.2
Poly C	21.5	30.0

ing order poly I > poly A > poly U > poly C. A mixture of poly A and poly U in the proportion 1:2 was not attacked by the enzyme.

DISCUSSION

The ribonucleases of higher plants have been investigated less systematically than the ribonucleases of microorganisms and animal tissues. No fully specific ribonuclease has been isolated so far from higher plants. Ribonucleases have been isolated and purified from 8 higher plant species[21–30]. It is not easy to compare the properties of these proteins as the enzymes were characterized by a variety of experimental approaches. A general property of higher plant ribonucleases seems to be that they are endoenzymes which cleave all the phosphodiester bonds of the RNA chain without any strict base specificity. These properties are shared by the present enzyme. The Avena ribonuclease has a relative purine specificity, with a preferential release of 2′,3′-GMP in the early phases of enzymatic digestion. The marked lag period in the release of 2′,3′-CMP is also very characteristic of the mode of action of Avena ribonuclease. Although the substrate used in the present experiments was comparatively rich in purines (G 26.1, A 28.4, C 20.6 and U 24.9%) as shown by the analysis of acid hydrolysates, this can hardly account for the observed relative purine specificity of the enzyme studied. There is some evidence that the relative guanine specificity in the early phase of the reaction and the resistance of bonds involving 3′-CMP residues may be common characteristics of higher plant ribonucleases[23–25]. This feature of relative specificity would deserve more detailed, comparative studies on several higher plant ribonucleases.

Another common property of higher plant ribonucleases, except for those releasing nucleoside 5′-phosphates[27–29], is the production of nucleoside 2′,3′-cyclic phosphates. It was clearly indicated in the present study that nucleoside 2′,3′-cyclic phosphates accumulate as first products during enzymatic digestion. It is customary to classify plant ribonucleases according to their ability to convert the pyrimidine

nucleoside 2′,3′-cyclic phosphates into the corresponding nucleoside 3′-phosphates. According to the terminology of REDDI[31], the enzymes which hydrolyze only the purine cyclic phosphates belong to group "plant ribonuclease I" and those hydrolyzing both the purine and pyrimidine cyclic phosphates belong to group "plant ribonuclease II". Since, however, even in those cases in which the enzyme hydrolyzed all four nucleoside cyclic phosphates, the rate of hydrolysis of the pyrimidine cyclic nucleotides was generally low[25–27] and because the pyrimidine cyclic nucleotides are hydrolyzed nonenzymatically even in neutral solutions[32], the validity of the above distinction has been questioned[28]. None of the nucleases from higher plants have been tested systematically in enzyme assays directly on all four nucleoside 2′,3′-cyclic phosphates as substrates. Thus, proper controls (complete assay system minus enzyme) which make the calculation of the amount of nonenzymatic breakdown possible, have not been used in most cases. In the present work we carried out direct assays to check the ability of Avena ribonuclease to hydrolyze the nucleoside 2′,3′-cyclic phosphates. It was found that only the purine cyclic phosphates had been attacked to any considerable extent (Table III). Similar studies on plant ribonucleases which have been claimed to hydrolyze also pyrimidine cyclic phosphates are warranted.

An important feature of the Avena ribonuclease is the inhibition of enzyme activity by end-products, a property not reported so far for plant ribonucleases. The relative purine specificity of the enzyme is in line with the observation that only purine derivatives (both 2′,3′-cyclic phosphates and nucleoside 3′-phosphates) were found to be effective inhibitors of enzyme activity.

It is an open question in how far the relative specificity of a ribonuclease established on the basis of experiments carried out on natural substrates agrees with results obtained by using synthetic homopolymers. Only some of the plant nucleases have been studied from this point of view and none of the investigations were very extensive. REDDI[33] claims to have been able to confirm the relative specificity of tobacco ribonuclease also by using homopolymers; TUVE AND ANFINSEN[25] noted the low speed of the degradation by spinach ribonuclease of poly U as compared to poly A and poly C, an observation which is in fairly good agreement with the data obtained with natural substrates. Other workers failed to observe such a relationship[24]. According to KADO[30], the ribonuclease of cucumber does not hydrolyze poly A although the 3′-AMP linkages of RNA are attacked. In our experiments the relative purine specificity of the enzyme was equally demonstrated by using both RNA and synthetic homopolymers as substrates.

The activity of the Avena ribonuclease described in the present paper was found to increase dramatically in excised leaf tissues[14]. The mechanism of this increase and that of other rapid changes in ribonuclease level in "stressed" plant tissues[1–11] are an intriguing problem of metabolic regulation. Our previous paper[14] and a recent report by SAHAI SRIVASTAVA[13] on the increase in chromatin-associated nuclease activity in excised barley leaves suggest that the various nucleases of the plant cell are affected by leaf excision to different extents. The ribonuclease described in the present paper is only one of the nucleases the activity of which is increased upon leaf excision. A closer characterization of other nucleolytic enzymes affected by leaf excision or other stress conditions is in progress with the aim to throw more light on the mechanism and role of rapid changes in nuclease levels in plant tissues.

REFERENCES

1 T. O. DIENER, *Virology*, 14 (1961) 177.
2 B. G. SHINDE, B. K. CHANDRASEKHAR AND V. SANTILLI, *Phytopathology*, 54 (1964) 908.
3 G. BAGI AND G. L. FARKAS, *Phytochemistry*, 6 (1967) 161.
4 G. BAGI AND G. L. FARKAS, *Experientia*, 24 (1968) 397.
5 R. ROHRINGER, D. J. SAMBORSKI AND C. O. PERSON, *Can. J. Botany*, 39 (1961) 775.
6 L. LOVREKOVICH, Z. KLEMENT AND G. L. FARKAS, *Science*, 145 (1964) 165.
7 G. L. FARKAS AND L. LOVREKOVICH, *Phytopathology*, 55 (1965) 519.
8 L. D. DOVE, *Plant Physiol.*, 42 (1967) 1176.
9 K. KISBÁN, M. HORVÁTH, L. DÉZSI, J. UDVARDY AND G. L. FARKAS, *Acta Botan. Acad. Sci. Hung.*, 10 (1964) 275.
10 J. UDVARDY, G. L. FARKAS, G. FORTI AND E. MARRÈ, *Giorn. Botan. Ital.*, 73 (1966) 318.
11 H. P. BALZ, *Planta*, 70 (1966) 207.
12 J. UDVARDY, G. L. FARKAS, E. MARRÈ AND G. FORTI, *Physiol. Plantarum*, 20 (1967) 781.
13 B. I. SAHAI SRIVASTAVA, *Biochem. Biophys. Res. Commun.*, 32 (1968) 533.
14 J. UDVARDY, G. L. FARKAS AND E. MARRÈ, *Plant Cell Physiol. Tokyo*, 10 (1969) 257.
15 G. R. BARKER AND J. A. HOLLINSHEAD, *Biochem. J.*, 103 (1968) 230.
16 M. STAEHELIN, *Biochim. Biophys. Acta*, 49 (1961) 11.
17 K. CHAKRABURTTY AND D. P. BURMA, *J. Biol. Chem.*, 243 (1968) 1133.
18 H. BIRNBOIM, *Biochim. Biophys. Acta*, 119 (1966) 198.
19 J. D. SMITH AND R. MARKHAM, *Biochem. J.*, 46 (1950) 509.
20 F. SØLYMOSY, I. FEDORCSÁK, A. GULYÁS, G. L. FARKAS AND L. EHRENBERG, *European J. Biochem.*, 5 (1968) 520.
21 M. HOLDEN AND N. W. PIRIE, *Biochem. J.*, 60 (1955) 39.
22 R. MARKHAM AND J. L. STROMINGER, *Biochem. J.*, 64 (1956) 46P.
23 K. K. REDDI, *Biochim. Biophys. Acta*, 28 (1958) 386.
24 L. SCHUSTER, *Biochim. Biophys. Acta*, 33 (1959) 452.
25 T. W. TUVE AND C. B. ANFINSEN, *J. Biol. Chem.*, 235 (1960) 3437.
26 A. J. MEROLA AND F. F. DAVIS, *Biochim. Biophys. Acta*, 55 (1962) 431.
27 C. M. WILSON, *Biochim. Biophys. Acta*, 76 (1963) 324.
28 T. L. WALTERS AND H. S. LORING, *J. Biol. Chem.*, 241 (1966) 2870.
29 T. L. WALTERS AND H. S. LORING, *J. Biol. Chem.*, 241 (1966) 2881.
30 C. J. KADO, *Arch. Biochem. Biophys.*, 125 (1968) 86.
31 K. K. REDDI, in G. L. CANTONI AND D. R. DAVIES, *Procedures in Nucleic Acid Research*, Harper and Row Publ., New York–London, 1966, p. 71.
32 W. SZER AND D. SHUGAR, *Biochem. Prep.*, 10 (1963) 142.
33 K. K. REDDI, *Biochim. Biophys. Acta*, 30 (1958) 638.