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CHARACTERIZATION OF A NUCLEASE FROM BARLEY SHOOTS

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; nuclease; ribonuclease; deoxyribonuclease; enzyme purification; enzyme characterization.

Abstract—A nuclease isolated from barley shoots is a nuclease I (EC 3.1.30.2) whose RNase to DNase activity ratio is 11 to 1. It appears to be an exonuclease for RNA since it can hydrolyse about 50% of the molecule with the liberation of 5'-AMP, 5'-GMP and 5'-UMP which appear in the ratio of 1:2.7:1.7. It cannot hydrolyse the cytidylyl 3'-bond. The M_r is 37000, the isoelectric pH is 4.4, and the energies of activation are for RNA 9.2 kcal/mole and for DNA 22 kcal/mole. The optimum pHs are 6.0 (RNase) and 6.5 (DNase). The enzyme is stable up to 60° and its DNase activity is more sensitive than its RNase activity to pHs above and below 6.5.

INTRODUCTION

A number of nucleases have been reported in microbial and plant tissues, but their individual functions *in vivo* are not well defined. The levels of nuclease activity have been viewed as high in comparison with the concentration of substrates [1]. These enzymes as well as other hydrolases are located in lytic compartments of cells where nucleic acids that are no longer functional are hydrolysed and the products are subsequently utilized in anabolic reactions [2]. For example, a number of hydrolases including RNase are in the vacuoles of yeast cells and of barley aleurone cells. In the yeast vacuole they represent individually up to 50% of the corresponding total activity of the lysate of the spheroplasts. This is likely a minimal value since destruction of some vacuoles and other lysosomal structures are lost during isolation.

Increased plant RNase, DNase, and other hydrolase activities occur in the lytic compartments of senescent leaves and in leaves damaged by stress. Tissue components such as nucleic acids and protein come under a controlled breakdown process whereby their degradation products become available for use elsewhere in the plant [3, 4]. Much remains to be learned about the control mechanisms.

In growth and differentiation, as in senescence, hormonal control can play a role in lytic activities. Phytochrome for example, has been reported to control ribosomal RNase in etiolated hypocotyls of *Lupinus albus* L. [5].

This report describes the characterization of a nuclease from barley shoots.

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RESULTS

Purification

The ammonium sulphate treatment of the crude shoot extract achieved a purification factor for RNase and DNase of 16 and 15 respectively as shown in Table 1. The very low activity precipitated by 40% saturation with ammonium sulphate was discarded. The Tris-acryl DEAE column increased the purification factor for the two activities to 170 and 136, respectively. The Tris-acryl DEAE product described in Table 1 is fractions 34 and 35 of Fig. 1. Most of the protein was removed from the column by the sodium chloride gradient before nuclease activity was eluted.

Purification was improved by chromatographing the Tris-acryl DEAE product with Sephadex G-75 (Fig. 2 and Table 1) whereby a purification factor at 1042 was achieved for RNase and 963 for DNase (fraction 33, Fig. 2) relative to the crude extract. The G-75 column separated the RNase activity into two parts, one with peak activity of fraction 33 and the other with peak activity at fraction 46. The latter enzyme is a major one (RNase I) that we have described in barley roots [6] but is only a minor RNase in shoots.

When fraction 33, Fig. 2 was chromatographed on the AMP-Sepharose column a single activity peak was obtained which contained a very low protein concentration. This peak was eluted when the sodium chloride in the eluant was 0.11–0.17 M. The fractions in this peak with the high activity (fractions 23 and 24 with combined volume 6 ml, Table 1) were eluted over the concentration range 0.13 M to 0.15 M sodium chloride. These combined fractions had less than 0.1 μg of protein per ml (Table 1). The disagreement between the purification factor for RNase and DNase may have been caused by errors in measuring the weak DNase activity relative to the RNase activity, and to the presence of RNase activity (e.g. RNase I) and perhaps DNase in the

Table 1. Purification of the nuclease

Fraction parameters	Fractions				
	Crude extract	Precipitation from 40 to 80% saturation with $(NH_4)_2SO_4$	DEAE Tris–acryl column	Sephadex G-75 column	AMP Sepharose column
Volume (ml)	225	26.0	13.0	4.0	6.0
Total protein (mg)	3510	201	6.6	0.108	6×10^{-4}
Total RNase (U)	569	504	177	18	3.4
Recovery RNase (%)	100	89	31	3	0.6
Specific activity RNase (U/mg protein)	0.16	2.51	27.2	167	57×10^2
Purification factor, RNase	0	16	170	1042	3.3×10^4
Total DNase (U)	36	29	8.8	1.04	0.30
Recovery DNase (%)	100	81	24	3	0.83
Specific activity DNase (U/mg)	0.010	0.145	1.36	10	5×10^2
Purification factor, DNase	0	15	136	963	5×10^4
RNase: DNase ratio	16	17	19	17	11

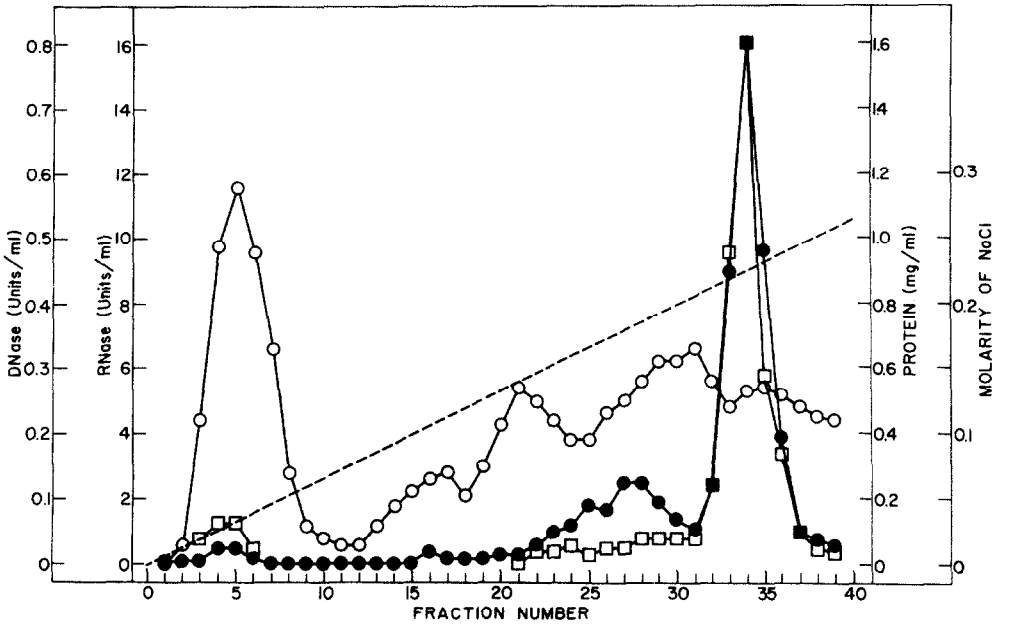

Fig. 1. DEAE Tris–acryl chromatogrpahy of the 40–80% $(NH_4)_2SO_4$ fraction. DNase activity □——□; RNase activity ●——●; protein ○——○; NaCl- - - - -.

crude extract which differed from the one that was isolated. The ratios of RNase to DNase activities vary from 11 to 19 depending on the purification step of Table 1.

Slab gel electrophoresis of the product from affinity chromatography showed only one band (R_f 0.4) when detected by the RNA-toluidine blue method and by the conventional silver stain method.

Enzyme parameters

The M_r by Sephadex G-75 gel filtration was 39 000, by the HPLC method 37 000, and by the nondenaturing electrophoretic procedure 32 000. The isoelectric pH was 4.4. For RNase activity the energy of activation ΔH_a was 9.2 kcal/mole, and the optimum pH was 6.0. For pH stability the activities as percent of control for the various pHs were: pH 5.5, 63%; pH 6.0, 89%; pH 6.5, 94%; pH 7.0, 100%; pH 7.5, 100%; and pH 8.0, 91%. The enzyme was thermally stable under the conditions of the experiment up to 60°; the 70° treatment lowered the activity to 47% of the control.

For DNase activity ΔH_a was 22 kcal/mole and the optimum pH was 6.5. For pH stability the activities as percent of control for the various pHs were: pH 5.0, 20%; pH 5.5, 40%; pH 6.0, 80%; pH 6.5, 100%; pH 7.0, 79%;

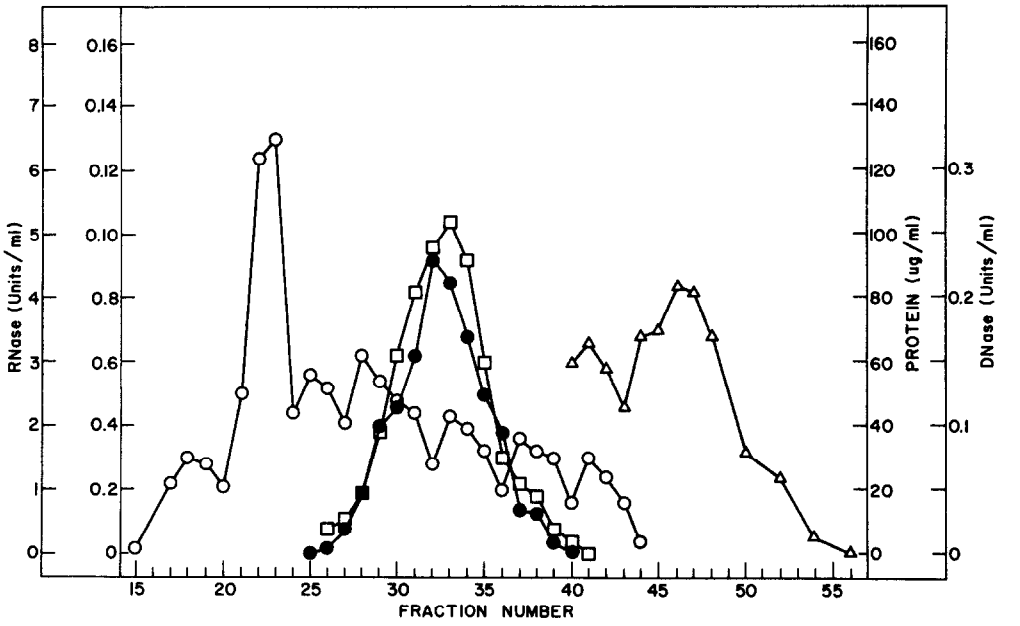

Fig. 2. Sephadex G-75 chromatography of the nuclease in fractions 34 and 35 from the DEAE Tris–acryl column. DNase activity □——□; RNase activity ●——● (high scale), △——△ (low scale); protein ○——○.

pH 7.5, 30%; and pH 8.0, 8%. The resistance to thermal inactivation was the same as for RNase activity.

Reaction with RNA

When RNA (Crestfield) was used at the 0.1 mg/ml level the main nucleotides formed were 5′-GMP and 5′-UMP in about equal proportions. Only a trace of 5′-AMP was present and essentially no 5′-CMP. The reaction was essentially complete within 15 min. The percentages of the RNA substrate represented by the two main products for various incubation times were: 15 min, 43%; 30 min, 47%; 60 min, 48%; 120 min, 50%; and 6 hr, 54%. No 3′-nucleotides or 2′,3′-cyclic nucleotides were formed.

When this RNA was used at 1 mg/ml level, 5′-AMP, 5′-GMP and 5′-UMP were produced in the ratio of 1:2.7:1.7, but again barely detectable amounts of 5′-CMP could be seen. The percentages of substrate represented by the sum of the products for various incubation times were: 2 min, 1.6%; 11 min, 3.7%; 20 min, 3.4%, 30 min, 3.4%; 120 min, 6%, 180 min, 7.9%; and 20 hr, 9.7%.

For t-RNA at the 1 mg/ml level the results were essentially the same. The ratios of the products were 1:2.4:1.6, and the percentages of the substrate represented by the sum of the products for the incubation times of 67, 150 and 360 min were 3.3%, 4.3% and 10.3%, respectively.

Slab gel electrophoresis of the reaction mixtures for both RNAs showed the unreacted RNA spot with R_f 0.30 which decreased in intensity with incubation time to yield a faint spot with R_f 0.4 and a major one with R_f 0.6.

Reaction with polynucleotides

The specific activities of the enzyme relative to polynucleotides were: poly A, 19.3; poly U, 1.0; poly C, 0.25; and poly G, 0. The specific activity relative to RNA was 9.5.

Reaction with dinucleoside monophosphates

The products in every case were the 5′-nucleoside phosphates and the separated nucleoside. The quantities of these compounds as percent of substrate are given in Table 2. UpC, GpC and ApC were hydrolysed to a greater extent than the other substrates. Replacing U, G or A on the 3′ end with C, i.e. CpC, CpG, CpU and CpA, resulted in complete resistance to hydrolysis.

Table 2. Hydrolysis of dinucleoside monophosphates by the purified nuclease*

Substrate	% of substrate hydrolysed by measurement of 5′-Nucleotide	Nucleoside
UpC	16	13
GpC	14	10
ApC	7	6
CpC	0	0
UpG	0.5	0.5
GpG	4	4
ApG	0.6	0.5
CpG	0	0
UpU	0.7	0.8
GpU	5	5
ApU	3	3
CpU	0	0
UpA	0.9	1
GpA	0.8	0.9
CpA	0	0
ApA	3	3

*Reactions: 50 μl of 10 mM substrate concentrations in 0.1 M NaOAc, pH 6, and 0.005 units RNase, 30°, 1 hr.

Reaction with p-nitrophenyl esters and nucleoside mono-, di- and triphosphates

The enzyme did not react with any of the *p*-nitrophenyl esters, the 5′-nucleotides, the 2′-nucleotides, or with 3′-TMP. The extent of hydrolysis of the 3′-nucleotides based on analyses for P was as follows: 3′-IMP, 0.26%; 3′-GMP, 0.31%; 3′-UMP, 0.32%; 3′-AMP, 0.23%; and 3′-CMP, 0.05%. The corresponding values based on analyses for the nucleoside product were: 3′-IMP, 0.28%; 3′-GMP, 0.40%; 3′-UMP, 0.35%; 3′-AMP, 0.38%; and 3′-CMP, 0.05%.

Reaction with inhibitors

Table 3 shows the concentrations of the inhibitors needed for 50% inhibition of the reaction rate. The compounds showed about the same inhibition of RNase as DNase, with the exception of dithiothreitol and the polyamines. RNase was not sensitive to dithiothreitol but was very sensitive to spermine. The Mn^{2+} ion stimulated DNase by about 50% and was used routinely in all DNase assays, whereas the same concentration was slightly inhibitory for RNase. Cu^{2+} ion exhibited its usual toxicity toward enzyme activity.

DISCUSSION

The nuclease appears to be a nuclease I (EC 3.1.30.2) according to Wilson's classification [1] since it hydrolyses both RNA and DNA at the 3′-positions to yield 5′-nucleotides. Its affinity for dinucleoside phosphates (Table 2) is much less than the affinity displayed for these substrates by the barley root RNase I described earlier [6] and which is also present in the shoots. The RNase I under the same conditions hydrolysed from 33 to 100% of each of the substrates. However, the nuclease I and the RNase I showed a similar preference for the polynucleotide substrates.

With RNA at the same concentration (0.1 mg/ml) their behaviour differed. The RNase I converted about 72% of the substrate to the characteristic 2′,3′-cyclic mononucleotides, but the nuclease I conversion was only about 50% to the 5′-nucleotides.

The nuclease I was unable to hydrolyse dinucleotide monophosphates with a cytidylyl group at the 3′ end. It is likely that the cytidylyl location in the RNA rendered the enzyme incapable of further attack on the substrate. This is suggested also by the absence of 5′-CMP in the hydrolytic products from RNA and by the insignificant hydrolyses of 3′-CMP when this was used as substrate.

Liao [7] reported two forms of DNase in barley malt which appear to require a disulphide bridge for activity. The nuclease I may also have this requirement since it showed sensitivity to dithiothreitol for DNase activity. Liao's DNases had M_r 32 000 which is comparable to that of the nuclease I. However Liao's enzymes did not react with RNA, and had a pH optimum of 7.5, both in contrast to the properties of the nuclease I we describe.

The nuclease I we describe appears similar to the nuclease from barley seed described by Pietrzak *et al.* [8] with respect to M_r, optimum pH, and action on polynucleotides, but not with respect to the activity ratio of RNase and DNase (Table 1) which in their case was 3:1. No mention was made of DNase enhancement with Mn^{2+}.

Polyamines which are present in plant tissue reportedly retard senescence by decreasing protease and RNase activity [9–12]. Our nuclease I appears to be much more sensitive to these compounds than the RNase of rice [13] which showed only 24% inhibition at 1 mM spermine.

Table 3. Effect of inhibitors on the hydrolysis of RNA and DNA by the purified nuclease

Inhibitor	Concentration (mM) of inhibitors for 50% inhibition	
	RNase	DNase
Iodoacetate	5	5
Maleimide	8	5
Dithiothreitol	50	5
Ba^{2+}	8	7
Cu^{2+}	0.1	0.2
Zn^{2+}	3	7
Fe^{2+}	3	*
Mn^{2+}	4	†
Ca^{2+}	9	6
Mg^{2+}	5	5
Spermidine	7	1
Spermine	0.02	0.1
Putrescine	1	4

*Forms ppt in substrate Mn^{2+} solution.

†Present at 5 mM in all DNA substrate solutions.

EXPERIMENTAL

Preparation of tissue. Barley kernels (*Hordeum vulgare* L. variety Morex) were spread on blotting paper in pans (*ca* 30 cm × 30 cm), so that each kernel occupied about 1 cm^2 of space. Deionized H_2O was added so that the paper was completely satd. The trays were wrapped in plastic and placed in the dark at 15°. H_2O was added when necessary to keep the paper satd over a period of 6–7 days at which time the shoots (*ca* 2 cm) were removed with scissors as close to the axis as possible and frozen immediately. Accumulated stocks were freeze dried and stored at 4°.

Extraction. Dried shoots (20 g) and acid-washed sand (2 g) were ground with a mortar and pestle. The powdered shoots were stirred with 400 ml 0.1 M Tris chloride buffer pH 6.5 for 2 hr at 4°. The suspension was centrifuged at 4° for 30 min at 80 000 *g*. The supernatant was stored at 4° until used. The ppt was discarded.

Protein assays. Protein was assayed by the method of ref. [14], except for the enzyme solns from affinity chromatography which contained a very low protein conc. Protein in this soln was estimated by electrophoresing the soln by the side of 0.1–1 μg of bovine serum albumin and then staining the gel by the silver method described in the electrophoresis section. The quantity of protein in the sample was estimated from the intensity of the silver-protein band relative to an appropriate bovine serum band.

DNase and RNase assays. DNase was measured by method of ref. [7] with 5 mM Mn^{2+} in the substrate soln, and RNase by the method of ref. [15]. A unit of activity is a unit change in *A* at 260 nm per min. Specific activity is the units per mg protein.

$(NH_4)_2SO_4$ *precipitation.* The shoot extract was brought to 40% satn with $(NH_4)_2SO_4$ at 4° and stirred for 2 hr. The suspension was centrifuged at 80 000 *g* for 30 min, after which the supernatant was removed and the ppt suspended in 0.1 M Tris chloride buffer, pH 7. The supernatant was made 80% satd with

$(NH_4)_2SO_4$ with stirring as before. The suspension was centrifuged as before and the supernatant was discarded. The pellet was suspended in the 0.1 M Tris chloride buffer. The two solns of dissolved pellets were dialysed exhaustively against the Tris chloride buffer.

DEAE-Trisacryl M chromatography. A column (2.5 cm × 30 cm) of DEAE-Trisacryl M was prepared in equilibrium with 0.01 M Tris chloride buffer, pH 7 in accordance with the instructions of the manufacturer, LKB Instruments Inc., Gathersburg, MD. A 26 ml aliquot of the soln containing the enzyme which had been precipitated by adjusting the $(NH_4)_2SO_4$ concn from 40 to 80% satn was applied to the column at 4° by upward flow of 28 ml per hr. The column was washed with 56 ml of the buffer at this flow rate. The column was then developed with a linear NaCl gradient. Buffer (150 ml) containing 0.3 M NaCl was pumped into 150 ml of the buffer with stirring as the latter was pumped on the column at 28 ml per hr, and four fractions/hr were collected.

Gel filtration. A column (2.6 cm × 92 cm) of Sephadex G-75 (Pharmacia) was equilibrated in accordance with the manufacturer's instructions with 0.025 M NaOAc buffer, pH 6.5, which was also 0.12 M NaCl. Enzyme soln (13 ml), which contained 6.6 mg of protein from the DEAE Trisacryl M column (fractions 34 and 35), was equilibrated with the above buffer by dialysis and was then pumped by upward flow onto the column at 25 ml/hr. The void vol. of the column was 130 ml. The column was eluted with the acetate–NaCl buffer at 25 ml/hr. The effluent was monitored for A at 280 nm and six fractions/hr were collected. All operations were done at 4°.

Electrofocusing. This was performed with the product from Sephadex G-75 chromatography (Table 1) as described in ref. [6].

Affinity chromatography. The ligand was 5'-AMP which was coupled with AH-Sepharose 4B (Pharmacia) by the method of ref. [16]. The column (0.9 cm × 20 cm) was prepared as described in ref. [6]. The enzyme soln from the G-75 gel filtration (4 ml containing 0.11 mg protein) was dialysed against 0.005 M NH_4OAc, pH 6 and applied to the top of the column at 4° which had been equilibrated with this buffer. A linear NaCl gradient was applied by pumping 60 g of 0.015 M NH_4OAc, pH 6 in 0.3 M NaCl into an equal wt of the 0.015 M NH_4OAc which in turn was pumped on the column by downward flow at 12 ml/hr and four fractions/hr were collected.

Slab gel electrophoresis. The equipment was the 16 cm BioRad Protean cell. The electrophoresis of the non-denatured enzyme was done by the procedure of Sigma (Bulletin MKR-137) with the 9% polyacrylamide level. Slabs were stained by the silver stain method (BioRad, Bulletin 1089) and by the method of ref. [17] in which electrophoresed slabs are incubated in RNA soln and then stained with toluidine blue. The RNase locations where the RNA had been hydrolysed appeared white on the blue RNA-toluidine blue background.

The electrophoresis of RNA in reaction solns was done with the above equipment and with 10% polyacrylamide gels prepared in formamide [18]. RNA was located by the toluidine blue procedure in which blue RNA spots were obtained on a clear background.

Determination of M_r *by gel filtration.* The Sephadex G-75 column described in the section on gel filtration was equilibrated with 0.2 M NaOAc, pH 6.5 which was 0.003 M NaN_3. The standard proteins (Sigma) were ribonuclease A, chymotrypsinogen A, ovalbumin and bovine serum albumin with M_rs of 13 700, 25 000, 45 000 and 66 000, respectively. Two mg quantities of each were chromatographed at a flow rate of 25.7 ml/hr and the effluent was monitored at 280 nm. The enzyme (194 units RNase, 28 units DNase) in 8.1 ml of solvent was chromatographed separately. The M_r of the enzyme was determined from a standard curve of log M_r plotted against K_{av} for the standard proteins, where $K_{av} = \frac{V_E - V_o}{V_t - V_o}$ and V_o = void vol.; V_t = total column vol.; V_E = elution vol.

The M_r was determined similarly by HPLC with a I-125 protein column (Waters Associates). The enzyme (200 μg, 0.7 units) was applied and the column was developed at 0.5 ml/min with 0.1 M NaOAc, pH 5.85. Fractions were collected every min for 20 min and assayed for activity. The M_r standards (Sigma) were ovalbumin, myoglobin and cytochrome c with M_rs of 45 000, 17 000 and 13 500, respectively.

M_r *by slab gel electrophoresis.* The procedure was that described in the Sigma Bulletin MKR-137. The standards used were α-lactalbumin, carbonic anhydrase, egg albumin and bovine serum albumin with M_rs of 14 200, 29 000, 45 000 and 66 000, respectively. These standards and the enzyme were electrophoresed on gels of 6, 7, 8 and 9% acrylamide. From 1 to 4 μg of the standards and from 0.1 to 0.4 μg of the enzyme were electrophoresed. The four standards were run in duplicate and then silver stained. The enzyme was also run in duplicate on separate gels and stained by a toluidine blue method [17].

Reaction with RNA. Yeast RNA prepared by the method of ref. [19] was obtained from the Sigma. This preparation is a representative distribution of yeast RNAs and represents 60% to 70% of the RNA in yeast. Except for the work with t-RNA, this RNA was used for the substrate. It was dissolved (0.1 mg/ml) in 0.1 M NaOAc, pH 6. To 2.8 ml of the substrate, 20 μl of enzyme soln containing 0.65 unit RNase was added and the reaction was incubated at 30° for up to 6 hr. At 15, 30, 60, 120 and 180 min and 6 hr, 500 μl aliquots were removed and frozen immediately. These were examined chromatographically for nucleotides produced [20] with a scanning spectrophotometer. Products were identified by elution times and by absorbance ratios at selected wavelengths from 230 to 290 nm which were compared with the corresponding data for reference compounds.

The Crestfield RNA and t-RNA (Type X-S, Sigma) were prepared at 1 mg/ml 0.005 M NaOAc, pH 6. To 1 ml of each substrate 25 μl of enzyme soln containing 0.1 unit RNase was added and the solns were incubated at 30° with aliquots removed periodically from 2 min to 16 hr. Nucleotide products were identified chromatographically as before. In addition, the reactions were examined electrophoretically for RNA.

Reaction with polynucleotides. Poly A, poly U, poly G and poly C (Sigma) were prepared at 0.1 mg/ml in 0.1 M NaOAc, pH 6. To 1 ml of poly A, 10 μl of enzyme soln (0.005 unit RNase) was added; to 1 ml of poly U and poly G, 0.05 unit of the enzyme was used; and for 1 ml poly C, 0.1 unit of the enzyme was used. The increase in A at 260 nm was followed as for the RNase assay.

Reaction with dinucleoside phosphates. Dinucleoside phosphate solns (50 μl of 10 mM) (Sigma) in 0.1 M NaOAc, pH 6, were incubated with 0.005 unit RNase, at 30° for 1 hr, after which the solns were frozen. When needed, the solns were thawed and 10 μl of each was chromatographed for nucleoside and nucleotide [20]. The dinucleoside phosphates examined were: uridylyl(3' → 5')cytidine (UpC), guanylyl(3' → 5')cytidine (GpC), adenylyl(3' 5')cytidine (ApC), cytidylyl(3' → 5')cytidine (CpC), uridylyl(3' 5')guanosine, (UpG), guanylyl(3' → 5')guanosine, (GpG), adenylyl(3' → 5')guanosine (ApG), cytidylyl(3' → 5')guanosine (CpG), uridylyl(3' → 5')uridine (UpU), guanylyl(3' → 5')uridine (GpU), adenylyl(3' → 5')uridine (ApU), cytidylyl(3' → 5')uridine (CpU), uridylyl(3' → 5')adenosine (UpA), guanidylyl(3' → 5')adenosine (GpA), cytidylyl(3' → 5')adenosine (CpA) and adenylyl(3' → 5')adenosine (ApA).

Reaction with nucleoside mono-, di- and triphosphates; p-nitrophenyl phosphate; bis-p-nitrophenyl phosphate; thymidine

3'-monophosphate p-*nitrophenyl ester; and thymidine 5'-monophosphate* p-*nitrophenyl ester.* The nucleoside phosphates (Sigma) were 5'-ATP, 5'-GTP, 5'-CTP, 5'-UTP, 5'-ADP, 5'-UDP, 5'-CDP, 5'-GDP, 5'-TMP, 5'-IMP, 5'-CMP, 5'-UMP, 3'-TMP, 3'-IMP, 3'-CMP, 3'-AMP, 3'-UMP, 3'-GMP, 2'-CMP, 2'-AMP, 2'-UMP and 2'-GMP. These substrates (2 mmol) were dissolved in 5 ml 0.1 M NaOAc, pH 6. Each substrate soln (0.5 ml) was incubated with 20 μl of enzyme soln (0.065 unit RNase) at 30° for 2 hr. From each reaction and each substrate blank 0.1 ml was removed and assayed for inorganic phosphorus [21] and another 0.1 ml was assayed for nucleoside by HPLC [20].

The reactions with *p*-nitrophenyl phosphate substrates were performed as described in ref. [18] with the quantity of enzyme mentioned above.

Optimum temperature. The RNase and DNase assays were done as described in the section on RNase and DNase assay procedure except that the reactions were carried out at 30°, 35°, 43°, 49°, 53° and 63°. In each case for RNase 5 μl of enzyme soln was used which contained 0.004 unit. For DNase 0.14 unit was used for the reaction at 30° and 35°, 0.07 unit for the reaction at 43°, and 0.03 unit for the reaction at 53° and 63°. The energy of activation was determined in the usual way by means of the Arrhenius equation.

Thermal stability. For the thermal stability of RNase 0.004 unit in 50 μl 0.1 M NaOAc, pH 6, was maintained in spectrophotometer cuvettes for 1 hr at 4°, 30°, 40°, 50°, 60° and 70°, following which 0.95 ml of substrate was added and the activity was determined at 30° as described in the section for RNase assay. Similarly, for DNase 0.045 unit DNase in 50 μl 0.1 M NaOAc, pH 6.5, were kept at 4°, 30°, 40° and 50° for 1 hr prior to determining the activity. Also, 0.093 unit of the enzyme was maintained at 60° and 70°C for 1 hr prior to measuring activity.

pH optimum. RNA and DNA were dissolved at 0.1 mg/ml in 0.1 M NaOAc buffer at pH 4.6, 5.3, 5.5, 6.0 and 6.5 or in 0.1 M Tris chloride buffers at pH 6.5, 7.0, 7.5, 8.0 and 8.5. For RNase 0.01 unit and for DNase 0.03 unit were used.

pH stability. For DNase, 60 μl of enzyme soln containing 0.06 unit was added to 60 μl of each of 0.2 M NaOAc buffers pH 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5, and to each of 0.2 M Tris chloride buffers pH 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. Solns were incubated at 4° overnight. Duplicate portions (50 μl) of each soln were assayed for DNase activity. Similarly for RNase 20 μl of enzyme soln containing 0.015 unit was added to 20 μl of each of the above buffers. After incubation, duplicate 25 μl portions of each soln were assayed for RNase activity. Control enzyme solns were not incubated but were kept frozen until all solns were assayed.

Effect of enzyme-inhibiting substances on RNase and DNase activity. The compounds used are shown in Table 3. The anion of the metals was Cl^-. The inhibitors were dissolved at appropriate concns in the substrate solns. For RNase 5 μl of enzyme (0.02 unit) was added to 1 ml of the substrate or substrate plus inhibitor. Activity was determined as for the standard RNase assay. The effect of the compounds on DNase was examined similarly except that in each case 25 μl of the enzyme (0.04 unit) was used and the DNA substrate soln in all cases was 5 mM with respect to Mn^{2+}, which stimulates DNase activity by *ca* 50%.

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