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PURIFICATION AND PROPERTIES OF BARLEY LEAF RIBONUCLEASE*

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Abstract—The principal ribonuclease from young barley plants was purified 29 200-fold by a six-step procedure. The enzyme showed a high specific activity (15 500 ΔA_{260} units/min/mg protein) and a molecular weight of about 25 000 was indicated by gel filtration and equilibrium sedimentation. Kinetic analysis of the cleavage of dinucleoside monophosphates and of yeast RNA indicated a base preference of Gua > Ade $\geq$ Ura $\gg$ Cyt, and was sensitive to the base located on either side of the phosphodiester bond. The enzyme resembles the Type I class of plant ribonucleases (E.C. 2.7.7.*x*).

INTRODUCTION

PARTIALLY purified RNase preparations have been obtained from various tissues of flowering plants, including roots, seeds, shoots and leaves,[1] but efforts to obtain RNases in pure form from plant tissues have been generally unsuccessful. RNase I obtained from corn endosperm by Wilson[2] appears to meet the general criteria of a pure plant RNase, but there are no reports of RNase preparations of equivalent purity and specific activity from the leaf tissue of any flowering plant. RNase preparations from aerial plant parts that have been studied in some detail show many common properties: they have acidic pH optima and readily degrade RNA to mononucleotides, with preferential release of purine nucleotides as the intermediate 2′,3′-cyclic phosphates[1] and subsequent hydrolysis of these cyclic phosphates to the corresponding 3′-phosphates.

Since bacterial, fungal and mammalian RNases show a wide range of specificities, it is surprising that the leaf RNases from a variety of higher plants should show a rather uniform mode of action. Studies of the specificity of RNases from the leaves of higher plants have all utilized enzyme preparations of seemingly low specific activity. It is possible that these RNase preparations contained more than a single enzyme capable of degrading RNA and the apparently uniform mode of action reported for leaf RNases may have been the result of two or more enzymes acting in concert, obscuring the unique specificity of each.

This paper is concerned with the preparation and partial characterization of an electrophoretically homogeneous RNase of high specific activity from young barley plants (*Hordeum vulgare* 'Larker'). Partial purification of this enzyme was described by Stelzig.[3]

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[1] BARNARD, E. A. (1969) *Ann. Rev. Biochem.* **38**, 677.
[2] WILSON, C. M. (1967) *J. Biol. Chem.* **242**, 2260.
[3] STELZIG, D. A. (1969) *Diss. Abs. B.* **29** (8), 2794.

RESULTS

Purification

RNase activity was extracted from macerated barley leaf tissue and found in the 100 000 *g* supernatant. The six-step purification procedure outlined in Table 1 resulted in a 29 200-fold purification of barley leaf RNase. The final product, Fraction VI, was obtained with 24% overall recovery of activity and showed a specific activity of 15 500 ΔA_{260} units/min/mg protein. The RNase activity was separated as a single peak in each of four successive fractionation steps which included gel filtration, chromatography on phosphonocellulose and on DEAE-cellulose and preparative polyacrylamide gel electrophoresis. Substantial enhancement of specific activity was obtained in each step. Fraction VI was free of DNase, nuclease and phosphomonoesterase activities and was used for subsequent characterization studies. Analytical gel electrophoresis of Fraction VI showed a single protein band over the range of 25–500 enzyme units/assay. Polyacrylamide gel electrophoresis of freshly prepared Fraction VI revealed a major band of RNase activity[4] which corresponded to the protein band. A very faint band of RNase activity was observed with slightly lower mobility than the major band. Multiple bands of RNase activity were noted by Wilson in corn endosperm RNase I.[4]

TABLE 1. PURIFICATION OF BARLEY LEAF RNase I

Fraction	Volume (ml)	Mg protein per ml	Enzyme* units	Specific activity†	Recovery (%)	Purification (fold)
I (Extraction) (4·5 kg tissue)	6900	19·8	73 200‡	0·53‡	100	1
II ($[NH_4]_2SO_4$)	640	15·4	60 000‡	6·1‡	82	11·4
III (Bio-Gel P30)	430	0·95	12 400	30	62	57
IV (Cellex-P)	1700	0·43	56 600	77	45	144
V (Cellex-D)	400	0·07	40 200	1400	32	2800
VI (Preparative electrophoresis	20	0·16	49 600	15 500	24	29 200

* An enzyme unit produces one A_{260} unit/min of acid-soluble nucleotides from yeast RNA at 37°.
† Enzyme units/mg protein.
‡ Corrected for higher molecular weight RNase activity separated in the first peak in the preparation of Fraction III.

The gel filtration treatment (step 3) resulted in the separation of two peaks of RNase activity. The first peak to be eluted contained about 25% of the total RNase activity and had a MW in excess of 70 000 (excluded by Sephadex G75). This fraction contained also DNase, phosphomonoesterase and phosphodiesterase activities but was not studied further.

The second and major peak of RNase activity eluted during gel filtration was designated barley leaf RNase I, and was the basis of this study. This peak was relatively free of other nucleases and phosphoesterases. Wyen *et al.*[5] found a similar distribution of enzyme activities in extracts of oat leaf tissue (*Avena sativa*), using Sephadex G75 to effect separation. The RNase activities listed in Fractions I and II in Table 1 are corrected for the higher MW RNase activity which was separated from RNase I by gel filtration.

[4] WILSON, C. M. (1971) *Plant Physiol.* **48**, 64.
[5] WYEN, N. V., UDVARDY, J., SOLMOSY, F., MARRE, E. and FARKAS, G. L. (1969) *Biochim. Biophys. Acta* **191**, 588.

Enzyme Properties

The molecular weight of the barley leaf RNase I was estimated to be 25 000 ± 2000 by gel filtration with Sephadex G75. In the ultracentrifuge, the meniscus depletion sedimentation equilibrium procedure gave M_w $(1-\bar{V}\rho) = 7000$ at 20°. The plot of ln c vs. r^2 obtained from the equilibrium sedimentation photograph was linear indicating a monodisperse system. If one assumes a value of 0·27 for the term $(1-\bar{V}\rho)$, M_w is calculated to be 25 900 daltons.

The optimal pH of the enzyme was 5·5 in 0·1 M sodium acetate or sodium cacodylate buffers, and 4·9 in 0·1 M sodium citrate buffer. A pH shift of 0·5 units either side of the optimum reduced activity by about 20%.

TABLE 2. EFFECT OF INHIBITORS AND ACTIVATORS ON PURIFIED BARLEY LEAF RNase I

Reagent	Concentration (mM)	Relative activity* of enzyme (%)	Reagent	Concentration (mM)	Relative activity* of enzyme (%)
Cobalt chloride	3	41	*N*-acetyl imidazole	3	100
Cupric chloride	3	13	*N*-ethyl maleimide	3	95
Ferric chloride	3	16	Iodoacetic acid	3	90
Magnesium chloride	3	99	*N*-Bromosuccinimide	3	9
Manganese chloride	3	70	Dithioerythritol	3	99
Mercuric chloride	3	10	Sodium azide‡	1	96
Lead chloride	3	75		10	98
Zinc chloride	3	10		100	94
EDTA	3	94	Potassium chloride‡	10	99
Uranium acetate	3	11		100	97
PCMB	(0·5 sat.)	44	Control	0	100

* Fraction VI enzyme (2·6 units) was preincubated at room temp. with 8·4 μmol of compound in 1·8 ml of 0·1 M acetate buffer pH 5·5 containing 1·2 mg bovine serum albumin. After 30 min, 1 ml of 0·4% RNA substrate in the same buffer was added and the assay completed as described in Experimental.

† 1·4 ml of a saturated solution of PCMB was used for the preincubation mixture.

‡ No preincubation. Standard assay conditions in Experimental were followed except that NaN_3 or KCl was added to give final concentration shown.

The effects of a variety of small molecules and ions on barley leaf RNase I are shown in Table 2. Copper (II), iron (III), mercury (II), zinc (II) and uranium (III) all showed substantial inhibition. Since the more specific sulfhydryl inhibitors, iodoacetate and *N*-ethyl maleimide, produced little inhibition it may be concluded that this enzyme does not have an active-SH functional grouping. The inhibition by heavy metals was probably a non-specific effect.

Dithiothreitol, potassium, magnesium, EDTA, *N*-acetyl imidazole and azide had little or no effect. The latter was included because it was used to suppress microbial growth during the gel filtration step. Inhibition by *N*-bromosuccinimide could have resulted from thiol oxidation or interaction with tryptophan residues.

The purified enzyme preparation (Fraction VI) was stable at room temperature over a period of 24 hr. Very dilute solutions (1–5 enzyme units/ml) were less stable in the absence of substrate, but were readily stabilized by preparing the dilute enzyme solutions in buffer containing 1–2 mg of bovine serum albumin per ml of solution.

Digestion of Yeast RNA

Yeast RNA was rapidly degraded to a mixture of nucleotides by barley leaf RNase I. The distribution of products according to nucleotide chain length after various digestion periods is shown in Table 3. There was a rapid release of mononucleotides during the early stages of digestion, but over half of the products remained as oligonucleotides after digestion for 12 hr.

TABLE 3. CHAIN LENGTH DISTRIBUTION OF NUCLEOTIDE FRAGMENTS IN DIGESTS OF YEAST RNA BY BARLEY LEAF RNase I

Digestion time (hr)	Chain length distribution of nucleotide fragments (%)*			
	mono	di	tri	> tri
0·5	21	14	13	52
2	34	22	15	29†
12	44	23	8	25

Fraction VI enzyme (25 units) was incubated at 37° with 300 mg yeast RNA in 30 ml 0·1 M acetate buffer pH 5·5. Aliquots were withdrawn at the intervals indicated and the digestion products fractionated as in Experimental.

* Based on absorbance at 260 nm. † tetra, 7; > tetra, 22.

The base composition of the short chain fragments released during RNA digestion is shown in Table 4. The preference for cleavage next to a purine residue is shown by the high purine/pyrimidine ratio in the mononucleotide fractions. This ratio was 6·1 after 0·5 hr

TABLE 4. BASE COMPOSITION OF NUCLEOTIDE FRACTIONS FROM DEGRADATION OF YEAST RNA

Digestion time (hr)	Nucleotide chain length	Ade	Cyt	Gua	Ura	Pur/Pyr*
			(mol %)			
0·5	mono	21	0	65	14	6·1
2		17	trace	59	22	3·2
12		25	5	48	22	2·7
0·5	di	28	3	31	37	1·47
2		31	14	14	41	0·82
12		25	21	14	39	0·65
0·5	tri	26	20	21	33	0·89
2		23	29	11	37	0·78
12		19	40	9	31	0·39
2	tetra	18	37	9	35	0·37
	RNA substrate	23	20	27	29	1·0

The several nucleotide fractions were hydrolyzed to the free bases with $HClO_4$. The bases were separated by paper chromatography and eluted for quantitation.

* Ratio of purines to pyrimidines.

and 2·7 after 12 hr digestion. Guanine comprised 65% of the bases in the 0·5 hr mononucleotide fraction, followed by 21% adenine, 14% uracil and no cytosine. The overall order of base preference appeared to be Gua $\gg$ Ade $\geq$ Ura $\gg$ Cyt. The purine/pyrimidine ratio declined with time and increasing nucleotide chain length, with cytosine being the pre-

dominant base in the 12 hr trinucleotide and the 2 hr tetranucleotide fractions. The high degree of preference for cleavage next to a guanine residue, probably on both sides, may be concluded from the data in Table 5 which shows that 51% of the guanine in the RNA substrate was liberated as the mononucleotide during 0·5 hr digestion, increasing to 76% during 2 hr, with only a slight increase thereafter. The proportions of adenine and uracil liberated into this fraction were approximately equal to each other, but the rate of release was substantially less than that of guanine, and only 11% of the cytosine was released even after 12 hr digestion. The small increase in guanine mononucleotide as the digestion time was extended from 2 to 12 hr suggests that certain of the internucleotide phosphodiester bonds next to guanine residues are quite resistant to cleavage by barley leaf RNase I.

TABLE 5. LIBERATION OF MONONUCLEOTIDES FROM YEAST RNA BY BARLEY LEAF RNase I

Digestion time (hr)	Mononucleotides* A	C	G	U
0·5	19	0	51	10
2	25	trace	76	26
12	48	11	78	50

* Values indicate percentage liberation of the corresponding mononucleotide from substrate, and include combined total of 3′- and 2′,3′-monophosphates. Calculated from data in Tables 3 and 4.

Barley leaf RNase I probably functions as a phosphotransferase enzyme. The composition of the 0·5 hr mononucleotide fraction shown in Table 6 reveals the presence of purine 3′-phosphates, and 2′,3′-cyclic phosphates. Uracil was present only as the nucleoside 2′,3′-cyclic phosphate, and cytosine nucleotides were absent, as indicated previously. Qualitative examination of the 12 hr mononucleotide fraction showed the same general distribution of products, except that cytidine-2′,3′-cyclic phosphate was now present in small amounts.

TABLE 6. COMPOSITION OF THE MONONUCLEOTIDE FRACTION FROM DIGESTION (0·5 hr) OF YEAST RNA BY BARLEY LEAF RNase I

Nucleotide	Mol (%)	Nucleotide	Mol (%)
Ado-2′,3′-P	12	Guo-2′,3′-P	44
Ado-3′-P	9	Guo-3′-P	21
Cyd-2′,3′-P	0	Urd-2′,3′-P	14
Cyd-3′-P	0	Urd-3′-P	0

The mononucleotide fraction from the 0·5 hr digest was separated into components by TLC. The appropriate areas were scraped from the plates, eluted with 0·01 M HCl and the absorption spectra measured.

Some further information about the base specificity of barley leaf RNase I was obtained from the distribution of base residues in the dinucleotide fractions as shown in Table 7. The Pur/Pyr ratios of the ω-fractions are consistently higher than those of the α-fractions;

both ratios decrease as digestion proceeds. The high incidence of the NpGp type (or NpG > p) in the early stages of digestion probably results from preferential cleavage of internucleotide bonds having guanine at the 3′-position of the phosphodiester bond. The proportion of the NpGp type drops rapidly as the digestion proceeds and becomes the least common type after 12 hr, suggesting that cleavage occurs readily on either side of a guanine residue. UpNp and NpUp types predominate after 12 hr and represent the accumulation of resistant types. In all probability, phosphodiester bonds between pyrimidine nucleosides are very resistant to cleavage by this enzyme. The high cytosine content of the 2 hr tetranucleotide fraction and of the 12 hr trinucleotide fraction probably results from a higher incidence of cytosine in the interior positions.

TABLE 7. DISTRIBUTION OF BASE RESIDUES IN THE DINUCLEOTIDE FRACTIONS FROM DIGESTION OF YEAST RNA BY BARLEY LEAF RNase I

Digestion time (hr)	α-Terminus* Ade	Gua	Cyt	Ura	Pur/Pyr†	ω-Terminus* Ade	Gua	Cyt	Ura	Pur/Pyr†
	(Mol %)					(Mol %)				
0·5	29	23	6	42	1·08	28	37	0	35	1·80
2	30	13	13	44	0·76	33	14	15	38	0·89
12	20	12	21	47	0·47	29	17	21	33	0·85

* α-Terminus = 5′-terminus, ω = 3′-terminus; see Ref. 6.
† Ratio of purines to pyridmidines.

The composition of the short chain fragments of RNA digestion suggests a complex type of base specificity in which the rate of cleavage of the phosphodiester bonds is influenced by the nature of the bases present on both sides of the phosphodiester linkage. Cleavage is most rapid on either side of a guanine residue, and least rapid on either side of a cytosine residue.

TABLE 8. CLEAVAGE OF DINUCLEOSIDE MONOPHOSPHATES BY BARLEY LEAF RNase I

Substrate	Enzyme units used*	Relative cleavage rate†	Substrate	Enzyme units used*	Relative cleavage rate†
GpA	0·125	0·31	CpA	2·5	0·02
GpC	0·125	0·09	CpC	500	< < 0·001
GpG	0·025	1·00	CpG	0·625	0·08
GpU	0·125	0·34	CpU	500	< < 0·001
ApA	2·5	0·02	UpA	2·5	0·02
ApC	2·5	0·006	UpC	50	< 0·001
ApG	0·25	0·18	UpG	0·25	0·18
ApU	2·5	0·02	UpU	50	< 0·001
GpG + UpU	0·025	1·00	GpG + CpC	0·025	1·00

The assay consisted of 25 nmol of each substrate and the indicated amount of Fraction VI enzyme in 0·5 ml 0·1 M acetate buffer. The substrate and enzyme were each brought to 25°, mixed rapidly and the change in absorbance observed at the appropriate wavelength for at least 10 min.

* The enzyme unit is defined in Table 1.

† The values listed are relative to the cleavage rate of GpG. The absolute rate of cleavage of GpG was 23·3 nmol/min/RNase unit.

[6] RICHARDS, D. and LASKOWSKI, M., SR. (1969) *Biochemistry* **8**, 1786.

Action on Dinucleoside Monophosphates

The complex pattern of base specificity indicated for barley leaf RNase from studies of RNA digestion was generally confirmed by observing the rates of cleavage of the dinucleoside monophosphates. The cleavage rates of these substrates relative to that of GpG at the 0·05 mM concentration are shown in Table 8. In all four series, the cleavage rate was fastest when guanine was present in either position, with the fastest rate being observed with GpG. Conversely, the presence of cytosine in either position led to lowered rates and when two pyrimidines were present, no cleavage could be observed, even though the enzyme concentration was increased greatly. The exceedingly low rate of cleavage of YpY substrates (where Y is any pyrimidine nucleoside) was not the result of enzyme inhibition since the cleavage of GpG proceeded normally in the presence of CpC or UpU.

TABLE 9. K_m VALUES FOR CLEAVAGE OF DINUCLEOSIDE MONOPHOSPHATES BY BARLEY LEAF RNase I

Substrate	K_m ($M \times 10^4$)	V_m (μmol substrate cleaved/min/RNase unit)
ApA	0·50	2·62
ApG	0·79	23·0
ApC	1·26	1·06
GpG	0·21	77·4
CpA	0·47	1·40

K_m and V_m values obtained from double reciprocal plots using five dinucleoside monophosphate substrates are shown in Table 9. The values found are of the same order of magnitude as obtained for RNases M and RNase T_2[7] using similar substrates. Although only a limited number of substrates was tested, the results show the high preference for cleavage next to a guanine residue, and reduced rate of cleavage next to a cytosine. Useful kinetic data could not be obtained for YpY types because of the extremely low rate of cleavage.

DISCUSSION

Barley leaf RNase I shows several of the properties reported for other plant RNase preparations:[1] acidic pH optimum (5·5); preference for purines, especially guanine, at the point of cleavage; and the formation of 2′,3′-cyclic phosphates (phosphotransferase activity), followed by cleavage of the purine nucleotide cyclic phosphates to the corresponding 3′-phosphates. Reddi[8] has proposed the Type I designation for plant RNases with these properties. The Enzyme Commission designation E.C. 2.7.7.*x* is appropriate for barley leaf RNase I. The specific activity of the Fraction VI preparation (15 500 ΔA_{244} units/min/mg protein) is several times that reported for other leaf RNases. The only other plant RNase preparation reported to have comparable specific activity is corn endosperm RNase I (17 850 ΔA_{260} units/min/mg protein, recalculated from data of Wilson[2]). The high specific activity, high fold-purification (29 200, 24% recovery), elution as a single peak during

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[8] REDDI, K. K. (1966) in *Procedures in Nucleic Acid Research* (edited by CANTONI, G. L. and DAVIES, D. R.), p. 71, Harper & Row, New York.

each of the chromatography steps and during preparative gel electrophoresis, and behavior during equilibrium sedimentation suggest that Fraction VI is a homogeneous enzyme preparation.

The preference of barley leaf RNase for cleavage of yeast RNA next to guanine residues is shown in the composition of the mononucleotide fraction of the 0·5 hr RNA hydrolysate and the base distribution in the dinucleotide fractions. The pattern of action of barley leaf RNase I on the dinucleoside monophosphate substrates provides direct evidence that the enzyme is sensitive to the base residues on both sides of the phosphodiester bond. The more rapid cleavage of substrates of the types GpN and NpG (where N is A, C, G or U) and the relatively slow cleavage of NpC and CpN (where N is A, C or U) correlates well with the composition of the mono- and oligonucleotide fragments from RNA digestion. In all probability, oligonucleotides containing only pyrimidines are resistant to further cleavage under the conditions employed. After 12 hr, the mono-, di- and tri-nucleotide fragments contained nearly all of the guanine (>95%), 80% of the adenine, 72% of the uracil and 50% of the cytidine present in the original RNA. Oligonucleotides with chain length greater than 3 which remained in the 12 hr digest (25% of the RNA substrate) must have consisted primarily of blocks of pyrimidines, especially cytosine, with adenine residues interspersed along the chains.

The lag in release of cytosine-containing mononucleotides from the degradation of yeast RNA appears to be a property common to plant RNases,[1,2,5,9] although the degree and nature of this lag have not been well documented. The cleavage rate of GpG relative to the YpY types at 0·05 mM was at least 1×10^3 and may have been 1×10^6 for the CpY types. The apparent tendency to cleave the phosphodiester bond on either side of a guanine residue and lack of cleavage between pyrimidine residues, especially cytosine, suggest that barley leaf RNase I may be useful for detecting blocks of pyrimidines in structural studies of RNA.

EXPERIMENTAL

Extraction and purification procedure. Barley (*Hordeum vulgare* 'Larker') plants were grown in the field and the tops excised at the soil level at the 4–5 leaf stage. The fresh tissue (4·5 kg) was macerated in a food chopper and homogenized at 4° in 4 l. of 0·02 M sodium acetate buffer, pH 5·5 (buffer A). After standing 2–3 hr at 4°, the homogenate was squeezed through cheesecloth and clarified by centrifuging at 4000 *g* for 15 min to give Fraction I. The plant tissue could be frozen and stored at −20°, either whole or macerated, for several months without loss of activity. The clarified supernatant (Fraction I) was treated with solid $(NH_4)_2SO_4$ at 4° and the material precipitating at 30% saturation was discarded. The supernatant was adjusted to 80% saturation and the precipitate collected by centrifugation. The active pellet was suspended in 300 ml of buffer A. The suspension was stored at −10° and was stable over several months. The suspension was thawed and dialyzed against distilled H_2O. Insoluble material was removed by centrifuging and the active supernatant constituted Fraction II.

Chromatography and preparative electrophoresis. Chromatography and preparative electrophoresis separations were conducted at 0–4°. Column effluents were monitored at 260 nm for protein and the eluates assayed for RNase activity. Fraction II was applied in 150 ml portions (3 g total protein) to a 8 × 55 cm column of Bio-Gel P30 (100–200 mesh) and eluted with buffer A, using a flow rate of 140 ml/hr. Sodium azide (0·02%) was added to the buffer to retard microbial growth and the columns were recycled repeatedly. The first peak of RNase activity eluted from the Bio-Gel P30 column contained 20–25% of the RNase applied, plus DNase and other phosphoesterases. The second peak of RNase activity contained the balance of the RNase activity (75–80% of the amount applied) with little of the other phosphoesterases found in the first peak. Several batches of the eluate containing the major peak of activity were pooled to give 3 l. of solution (Fraction III) which contained 1·8 g of protein. Fraction III was applied to a 5·2 × 48 cm column of Cellex-P (Bio-Rad phosphonocellulose) that had been equilibrated with buffer A. The sample was followed by more buffer A until a large inactive protein peak was eluted at which time a NaCl gradient was applied. The elution gradient was obtained with 4 l. buffer A and 4 l. buffer A containing 1 M NaCl and a flow rate

[9] TANG, W. J. and MARETZKE, A. (1970) *Biochim. Biophys. Acta* **212**, 300.

of 2 ml/min. RNase activity was eluted in the range of 0·2–0·4 M NaCl. The active fraction was dialyzed against distilled water to give Fraction IV (1700 ml, 0·73 g protein). Fraction IV was applied to a 2·5 × 45 cm column of DEAE-cellulose equilibrated with buffer A. The column was eluted with 15 vol. of the same buffer followed by a linear NaCl gradient at a flow rate of 2 ml/min. The gradient consisted of 2 l. of buffer A and 2 l. of buffer A containing 0·3 M NaCl. A single band of RNase activity was eluted in the range 0·12–0·14 M NaCl. The active fraction was dialyzed against distilled H_2O and lyophilized to give Fraction V. About 20% of the activity eluted from the column was lost during dialysis and lyophilization. A portion of Fraction V containing 20–40 mg protein was dissolved in 3 ml of 0·05 M Tris–borate buffer, pH 8·1, containing 1·5 mM EDTA and subjected to electrophoresis in a Buchler Poly-Prep apparatus using the general procedure of Kiesiel and Graf.[10] A single band of RNase activity was obtained (Fraction VI) and stored at −20°. Characterization studies were performed on this electrophoretically homogeneous sample. Protein determinations were made by the method of Lowry *et al.*[11] For solutions of very low protein concentrations the method was modified by using more concentrated reagents to permit estimations down to 0·6 μg protein/ml.

Analytical electrophoresis. Analytical polyacrylamide gel disc electrophoresis was performed with a Buchler Poly-Analyst apparatus according to the procedure of Ornstein and Davis.[12] Gels were stained for protein with Amido Schwartz and for RNase activity by the method of Wilson.[4] Aliquots of Fraction VI containing 20–80 μg protein and 1–20 units of enzyme were used for protein and RNase detection, respectively.

MW estimation. Meniscus depletion sedimentation equilibrium experiments were made with a Spinco Model E ultracentrifuge according to the method of Yphantis.[13] The sample was prepared in 0·2 M Tris–borate, pH 8·1 containing 3·0 mM EDTA and the equilibrium speed was 27 690 rpm. The plot of ln c vs. r^2 gave a straight line and was used for the calculation of $M_w(1-\bar{V}\rho)$. The MW was also estimated by gel filtration on Sephadex G75 following the procedure of Andrews.[14] Protein standards of MWs ranging from 12 400 to 45 000 were used to obtain a plot of V_e/V_0 vs. log MW.

RNase assay. RNase activity was measured by the method of Tuve and Anfinson[15] using commercial yeast RNA in 0·1 M sodium acetate buffer at pH 5·5. One unit of RNase activity is defined as the amount of enzyme which produces one A_{260} unit/min of acid-soluble nucleotides from yeast RNA at 37°. This is approximately equivalent to the release of 0·1 μmol of acid–soluble nucleotide/min. A variety of commercial RNA samples were used with equivalent results.

Action of RNase on yeast RNA. Commercial yeast RNA was fractionated with EtOH–H_2O and a fraction having $S_{20,w} = 3{\cdot}8S$ was used for degradation studies. The base composition was 23% Ade, 20% Cyt, 27% Gua and 29% Ura, with only trace amounts of minor bases, suggesting that the sample was derived from ribosomal RNA. Barley RNase I (25 units) was added to 300 mg of the above RNA dissolved in 30 ml 0·1 M sodium acetate buffer, pH 5·5 at 37°. Aliquots were withdrawn at intervals of 0·5, 2 and 12 hr. The reaction was terminated by flash heating to 100° and the pH adjusted to 7·8. The digests were fractionated by chromatography on DEAE-cellulose according to the method of Tener[16] and the chain-lengths of the fractions estimated by the procedure of Seaman.[17] The α- and ω-terminal nucleosides in the dinucleoside fractions were identified by the method described by Uchida *et al.*[18] The nucleosides and mononucleotides obtained by these procedures were characterized by use of one and two dimensional chromatography on cellulose and cellulose–DEAE-cellulose (19:1) thin layers and a variety of developing solvents.[18,19] Identifications were made by inspection under UV. The base composition of the original RNA and of the various mono- and oligonucleotide fractions was determined by PC after hydrolysis with perchloric acid.[20] Quantitative estimations of the components in the mononucleotide and dinucleotide fractions were made by scraping appropriate UV-absorbing or fluorescing areas from the plates and eluting with 1 or 2 ml of 0·01 M HCl (48 hr) with occasional shaking. The concentration of each component was determined by measuring the absorption at appropriate wavelengths. Absorption spectra were used to confirm identification of the components.

Cleavage of dinucleoside monophosphates. The rate of cleavage of the dinucleoside monophosphates was determined at pH 5·5 and 25° according to the spectrophotometric method of Imazawa *et al.*[6] Appropriate aliquots of the substrate, enzyme and 0·1 M acetate buffer, pH 5·5, were selected to give a reaction vol.

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[20] BENDICH, A. (1957) *Methods Enzymol.* **3**, 715.

of 0·50 ml, a substrate concentration of 0·05 mM, and a rate of cleavage corresponding to 0·001–0·003 ΔA/min. Enzyme dilutions of 10-, 100- and 1000-fold were made from stock solution (2480 U/ml) with the above acetate buffer containing 2 mg bovine serum/ml.

The K_m and V_m values were obtained from double reciprocal plots. The reaction mixture consisted of 0·5 ml substrate in 0·1 M acetate buffer, pH 5·5, and 10 μl of enzyme in the same buffer containing 1 mg bovine serum albumin/ml. Substrate concentrations ranged from 0·02 to 0·1 mM and the progress of the reaction was followed by the spectrophotometric method of Imazawa *et al.*[6]

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