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PROPERTIES AND SUBCELLULAR DISTRIBUTION OF RIBONUCLEASE ACTIVITY IN *CHLORELLA*

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Key Work Index—*Chlorella*; Chlorophyta; alga; enzymes; ribosomes; ribonuclease; RNA.

Abstract—RNase activity from *Chlorella* was partially purified. Two RNase activities were demonstrated, one soluble and the other ribosomal. The effects on ribonuclease activity of variations in pH and temperature, and of Mg^{2+}, Na^{+}, and mononucleotides were examined. The RNase activities (phosphodiesterases EC 3.1.4.23) were both endonucleolytic, releasing oligonucleotides, and cyclic nucleotide intermediates, but exhibited different specificities in releasing mononucleotides from RNA. The ribosomal activity released 3′-GMP, and after prolonged incubation 3′-UMP, but the soluble activity released 3′-GMP, 3′-AMP and 3′-UMP. Neither of the RNase preparations hydrolysed DNA, nor released 5′-nucleotides from RNA. Increased ribosomal RNase activity was related to dissociation of ribosomes, and latency of ribosomal RNase activity was demonstrated. The possible *in vivo* distribution of RNases is discussed.

INTRODUCTION

Intracellular RNases from a wide range of organisms have been previously investigated [1, 2] and many have been characterized in considerable detail, but investigation of RNases from plants has been concentrated on higher plant material [2, 3] of which the RNases of *Zea mays* have been investigated the most thoroughly [4–6].

Although RNase activity has been previously noted in, and extracted from, unicellular algae [7–9], no detailed examination of such activity has been previously reported. Amongst the aims of this investigation was examination of the sub-cellular distribution of the RNase activity of *Chlorella*, and examination of its properties as a basis for comparison of algal RNases with those from higher plants.

RESULTS

Extraction and purification of Chlorella *RNases*

The most effective method for extracting RNase from *Chlorella* was by means of the French cell. The 30000 *g* supernatant was centrifuged at 100000 *g* for 2 hr to produce a ribosomal pellet, and post-ribosomal supernatant. Soluble phase was produced from post-ribosomal supernatant as described in Experimental. Two RNase activities were detected, one associated with the soluble phase and the other with the washed ribosomal pellet. Preparation of acetone powders, and use of deoxycholate were less effective, as was also intracellular disruption by freezing and thawing.

Centrifugation of the 30000 *g* supernatant at 100000 *g* for 1 hr, produced a supernatant containing residual rRNA (100000 *g*/1 hr supernatant), and fractionation of this preparation on a column of Sephadex G-75 produced two peaks of RNase activity. One (RNase S1) had an apparent MW of (5×10^4) to 10^5, whereas that of the second (RNase S2) was *ca* 10^4. Data for a typical fractionation are given in Table 1.

Fractionation of the 100000 *g*/1 hr supernatant by chromatography on DEAE-cellulose, eluting with a 10–500 mM NaPi gradient (pH 6.4), also suggested the presence of two distinct RNase activities, RNase D1, and RNase D2, eluting at 150 and 250 mM Pi respectively.

Examination of the actions of RNases D1, D2, S1, and S2 on yeast RNA suggested that RNases D1, and S2

Table 1. Partial purification of 100000 *g*/1 hr supernatant from broken cells of *Chlorella* on Sephadex G-75

Fraction	Protein (mg/ml)	RNase units/ml*	RNase specific activity (units/μg protein)*	Relative specific activity
Broken cell preparation	111.3	50.1	0.45	
100000 *g*/1 hr supernatant	8.6	53.7	6.24	1.00
RNase S1	0.027	11.0	407.41	65.27
RNase S2	0.300	53.0	176.67	28.31

* Units of RNase activity are defined in Experimental

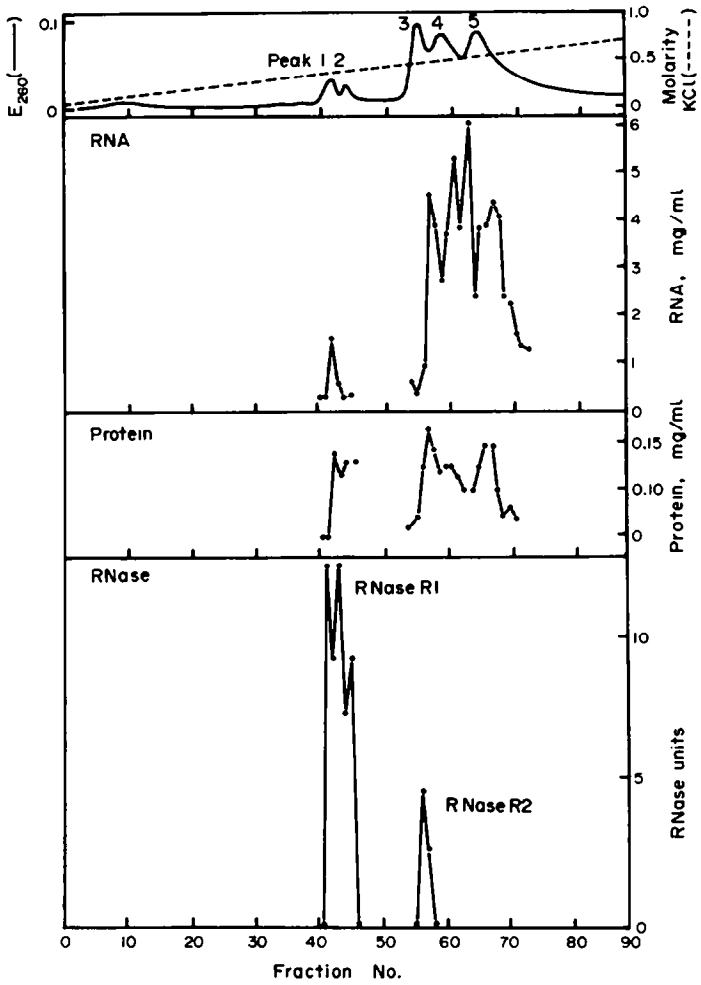

Fig. 1. Elution of RNase activity from *Chlorella* ribosomes bound to DEAE-cellulose using the method of Datta *et al.* [10] 5 ml ribosomal preparation (102 mg RNA/ml; 7 mg protein/ml) was applied to a DEAE-cellulose column (20 cm × 1.5 cm diam.). Elution was by a linear gradient of KCl (10 mM–1 M) at 4°; 5 ml fractions were collected.

were equivalent, and corresponded to the RNase activity of the soluble phase (see Experimental), and that RNases D2, and S1 were equivalent, and corresponded to the ribosomal RNase activity. Electrophoresis of the 100 000 *g*/1 hr supernatant on polyacrylamide, or starch gels also revealed two RNase activities.

Purification of RNases from Chlorella *ribosomes*

Preparations of ribosomes in 20 mM Tris–HCl buffer (pH 7.4), containing 10 mM Mg acetate, and 0.1 mM dithiothreitol, were bound to DEAE-cellulose as described by Datta *et al.* [10]. Ribosomal proteins were eluted from the bound ribosomes using a linear concentration gradient of KCl (10 mM–1 M) in the elution buffer. The A_{260} of the eluate was monitored, and its content of RNA, protein and RNase activity measured.

The fractionation (Fig. 1) produced two main groups of peaks absorbing UV at 260 nm, peaks 1–2, and peaks 3–5. Peaks 1–2 consisted mainly of protein, whereas most of the ribosomal RNA occurred in peaks 3–5. Estimation of S°_{20w} for peaks 4 and 5 indicated that they were composed mainly of ribosomal fragments (18S and 23S), with small amounts of RNA aggregation products (51S, 72S, 97S, 109S, and 122S) resulting from the relatively high concentrations of Mg^{2+} (10 mM) in the elution buffer.

Two peaks of RNase activity were detected. One of these (R1) was associated with the ribosomal protein peak, and the other (R2) with the first peak of rRNA. These RNase peaks showed identical specificity of nucleotide release from yeast RNA, and may represent the same enzyme bound with rRNA to differing extents.

Properties of Chlorella *RNases*

The RNase activities of preparations of broken cells, of ribosomes, and of the soluble phase all showed optimal

Table 2. The effects of some mononucleotides on the RNase activity from the 100 000 *g*/1 hr supernatant of *Chlorella*

Nucleotide added	Relative RNase activity (% of control)	
	10^{-5} M nucleotide	10^{-3} M nucleotide
None	100.0	100.0
3'-AMP	25.9	21.0
3':5'-AMP	21.0	21.0
3'-UMP	18.9	23.1
3'-CMP	28.0	23.1
3'-GMP	93.0	93.0
ATP	25.9	0.4

activity at *ca* pH 5. At this pH, the RNase activity of broken cell preparations showed marked thermostability, with maximum activity at *ca* 90° during an incubation period of 10 min. $MgCl_2$ and NaCl at 0.01 mM and 1 mM did not affect the activities of the two RNase preparations (D1 and D2) previously resolved by chromatography on DEAE-cellulose. At 10 μM and 1 mM a number of mononucleotides markedly inhibited the RNase activity of the 100 000 *g*/1 hr supernatant (Table 2). An exception was 3'-GMP, which only gave a 7% inhibition.

To investigate the action of the RNases on RNA, RNase activities D1 and D2, obtained by fractionation of the post-ribosomal supernatant on DEAE-cellulose, were incubated with yeast RNA (10 mg/ml in 0.1 M Na citrate buffer, pH 5.2) at 37°. After 3 hr, portions of the incubate were examined by high voltage paper electrophoresis in one dimension, and PC, using solvent system 1, in the second. RNase D1 released 3'-GMP, 2':3'-GMP, 3'-UMP, and 3'-AMP, together with some oligonucleotides, indicating the action of a non-specific endonuclease. The occurrence of small amounts of nucleosides and bases in the incubate showed that some nucleotidase and nucleosidase activity was also present. RNase D2 released only 3'-GMP, and 2':3'-GMP, together with some oligonucleotides from RNA, but no nucleosides, or bases were detected. This is compatible with RNase D2 being an endonuclease, specific for the bonds between adjacent GMP residues in RNA and producing 2':3'-GMP as an intermediate.

Estimation was made of the individual nucleotide components of the commercial yeast RNA (BDH) used as substrate. Comparison of these figures with the relative amounts of the corresponding free nucleotides released from the RNA substrate by *Chlorella* RNases indicated a selective release of specific nucleotides (Table 3).

Preparations of ribosomes, soluble phase and 'pH 5 extract' (see Experimental) were incubated separately in 100 mM Na citrate buffer pH 5.2 at 37°, with added yeast RNA (10 mg/ml). After incubation, aliquots were cooled rapidly to 4°, and cleared by centrifugation at 15 000 *g* for 10 min. They were then fractionated on columns of Sephadex G-15, together with controls, and the A_{260} of the eluate monitored. Fractionation of all the incubates containing RNase showed the presence of undegraded RNA, oligonucleotides, and free mononucleotides; nucleosides were detected only in incubates containing soluble phase. Incubation of each preparation gave similar results with yeast RNA, or with endogenous *Chlorella* RNA, but the ribosomal preparation, soluble phase, and 'pH 5 extract' produced characteristic patterns of degradation products. Ribosomal, 'pH 5 extract', and soluble phase preparations all produced oligonucleotides as intermediates by endonucleolysis, and mononucleotides as end-products. The appearance of nucleosides in incubates indicated the presence of

Table 4. Nucleotides released from yeast RNA and *Chlorella* RNA by RNase preparations from *Chlorella*

Incubation	Incubation time (hr)	Nucleotides liberated	Ratio of nucleotide: 3'-GMP
Ribosomes	3	3'-GMP	—
+	6	3'-GMP	—
Chlorella RNA	9	3'-GMP	1.0
		3'-UMP	0.16
pH 5 extract	6	3'-GMP	1.0
+		3'-UMP	0.94
Chlorella RNA		3'-AMP	0.61
Soluble phase	9	3'-GMP	1.0
+		3'-UMP	0.61
Chlorella RNA		3'-AMP	4.5
		Adenosine	2.51
Soluble phase	6	3'-GMP	1.0
+		3'-UMP	0.79
yeast RNA		3'-AMP	1.46
		Adenosine	2.07

Table 3. Comparison of the base composition of RNA hydrolysate obtained by alkaline hydrolysis, with that from the action of *Chlorella* RNases

Base	Alkaline hydrolysis of commercial yeast RNA (bases/100 bases)	Enzymic hydrolysis of RNA (bases/100 bases)	
		RNase D1	RNase D2
A	35.2	58	0
U	29.6	11	0
G	22.0	31	100
C	13.0	0	0

RNase D1 and D2 were obtained from fractionation of preparations on DEAE-cellulose.

nucleotidase activity in the soluble phase. The mononucleotides produced in preparations of 'pH 5 extract' were similar to those obtained with soluble phase preparations, but quantitatively and qualitatively different from those produced by the ribosomal preparations (Table 4).

Results showed that the RNase activity of the soluble phase and of the 'pH 5 extract' released 3'-GMP, 3'-UMP, and 3'-AMP from RNA. After 3 hr and 6 hr of incubation, ribosomal preparations had released only 3'-GMP from RNA but after 9 hr, low concentrations of 3'-UMP were also detected. The absence of nucleosides in ribosomal incubates, and their presence in incubates of soluble phase suggested that nucleotidases were confined to the soluble phase. No 5'-nucleotides were detected in any of the incubates. Prolonged incubation times were used since mononucleotides were final end-products produced at low concentrations by *Chlorella* endonucleases.

RNase activities R1 and R2, extracted from ribosomal preparations bound to DEAE-cellulose (Fig. 1) were dialysed against 3 changes of 20 mM Tris–HCl buffer pH 7.4, at 4° for 24 hr to remove any nucleotide contaminants. Yeast RNA (10 mg/ml in 0.1 M Na acetate buffer, pH 5), previously freed from nucleotide contaminants, was incubated with RNase activities R1 and R2 for 10 hr together with controls, and the hydrolysate fractionated on a column of Sephadex G-15. PC of the pooled mononucleotide fractions using solvent system 3 showed that both 3'-GMP and 3'-UMP were produced, and their relative molarities were estimated to be 4.5:1. The release of 3'-UMP from RNA by RNase activities R1 and R2 contrasted with earlier results in which 3'-GMP had been the major product with only very low levels of 3'-UMP (Table 4).

Products of the incubation of preparations of ribosomes, and soluble phase with 2':3'-AMP (0.25 mg/ml of 100 mM Na citrate buffer pH 5.2) together with control incubations, were fractionated on Sephadex G15, and by TLC on cellulose using Solvent 2. Results showed that, compared with soluble phase preparations, ribosomal preparations caused little hydrolysis of added 2':3'-AMP. In ribosomal incubates some 3'-AMP was observed together with very low levels of adenosine and adenine. Thus, although ribosomal preparations do not release either 2':3'-AMP or 3'-AMP from yeast RNA, they appear to catalyse limited hydrolysis of these nucleotides. Both 2':3'-AMP, and 3'-AMP were almost completely hydrolysed to adenosine, and adenine by soluble phase incubates.

Action of RNase activity S2 on 2':3'-cyclic nucleotides

RNase activity S2, obtained by fractionation of the 100 000 *g*/1 hr supernatant of *Chlorella* cells on Sephadex G-75 (see Table 1), was incubated separately with each of a number of 2':3'-cyclic nucleotides, and also with yeast RNA at 37°. After 2 hr, degradation products from RNase, and control incubates were fractionated using TLC. Conversion of 2':3'-AMP to 3'-AMP, and adenosine was pronounced, as was the conversion of 2':3'-UMP to 3'-UMP, and uridine. However, hydrolysis of 2':3'-GMP to 3'-GMP, and hydrolysis of 2':3'-CMP to 3'-CMP and cytidine was slight.

Subcellular distribution of RNases in Chlorella

Suspensions of broken cells, obtained using a French pressure cell at 24 MPa, were subjected to centrifugal fractionation. The homogeneity of the various organelle fractions obtained was estimated as described in Experimental, and each sub-cellular fraction was examined for RNase activity. RNase activity was consistently associated with the washed ribosomal pellet, and the soluble phase. The results of a typical estimation of RNase activity in the ribosomal preparation and post-ribosomal supernatant are shown in Table 5.

Examination of the binding of RNase to ribosomes

Three ribosomal fractions were prepared in fractionation medium containing either low (0.06 M), intermediate (0.2 M), or high (0.4 M) concentrations of KCl. The sp. act. of the RNase in each ribosomal pellet preparation, and soluble phase was assayed (Table 6).

If reversible binding of soluble RNases to ribosomes had occurred during sub-cellular fractionation, increasing concentrations of monovalent cations in the extraction medium might be expected to alter the binding by ionic competition with the RNase for binding sites on the ribosome [12, 13]. Such altered binding would be reflected in the changed sp. act. of the RNase. However, the slight decrease in sp. act. obtained (Table 6) was similar in both the ribosomal preparation and the soluble phase, indicating that the effect is more likely to be a general inhibitory effect of KCl on RNase.

During elution of RNase activity from *Chlorella* ribosomes bound to DEAE-cellulose [10] (Fig. 1), loosely-bound RNase, which would elute at low concentrations of KCl, was not detected. However, between 0.3 M and 0.4 M KCl, a major peak of RNase activity was eluted, and between 0.5 M and 0.6 M KCl, a minor peak

Table 5. RNase activity of the ribosomal preparation and soluble phase from *Chlorella*

Fraction	Protein (mg/ml)	RNA (mg/ml)	RNase specific activity	
			RNase units/mg protein*	RNase units/mg RNA*
Ribosomes	2.0	2.5	2.96	2.37
Soluble phase	2.1	0.4	7.94	23.1

* Units of RNase activity are defined in Experimental

Table 6. Effect of KCl on the RNase activity of ribosomes and soluble phase

	Specific activity of RNase			
	Ribosomal preparation		Soluble phase	
Concentration of KCl (mM)	Specific activity (units*/mg protein)	Relative specific activity	Specific activity (units*/mg protein)	Relative specific activity
60	0.727	1.0	20.0	1.0
200	0.555	0.76	18.57	0.93
400	0.625	0.86	16.79	0.84

* Units of RNase activity are defined in Experimental.

was eluted. The necessity of relatively high concentrations of KCl to elute RNase activity indicated that the RNases were strongly bound to the ribosomes *in vitro*. Furthermore, the properties of these RNases with respect to the nucleotides they release from RNA, were dissimilar to those of the activity in the post-ribosomal supernatants.

Latency of ribosomal RNase from Chlorella

At low temperatures, in buffers of ionic strength 0.1 to 0.2 M, and pH 7, or with concentrations of Mg^{2+} greater than 10 mM, ribosomes show little or no RNase activity. Under these conditions ribosomes are structurally intact [20–22, 26, 28]. However, changes in these stabilizing conditions affect ribosomal structure, and previously latent RNase activity becomes detectable. A preliminary examination of *Chlorella* ribosomes was made in this respect by exposing them to either 25 mM EDTA (pH 7) at 37°, or 4 M urea at 37°, and with one set of controls in the fractionation buffer at 4° and another at 37°. RNase activity was estimated by following release of acid-soluble nucleotides from the endogenous RNA in the ribosomal preparations. From the results (Fig. 2) it can be seen that treatment of ribosomes with EDTA or urea induced RNase activity, whereas such activity was virtually absent from ribosomes previously incubated at 37° and 4° without additions.

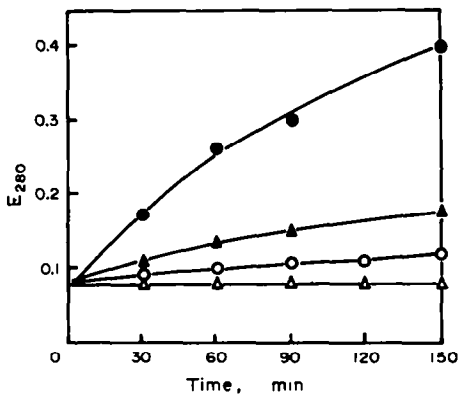

Fig. 2. Release of acid-soluble nucleotides from *Chlorella* ribosomes during incubation with EDTA, and urea. Ribosomal preparations in 0.1 M Na citrate buffer (pH 6) were kept at 37° in the presence of 25 mM Na–EDTA (●), or of 4 M urea (▲). Controls contained only the buffered ribosomal preparation and were maintained at 37° (○) or 4° (△).

Increase in RNase activity with increased dissociation of ribosomes

Ribosomes were prepared in fractionation medium (see Experimental) containing either 0.1 mM or 10 mM Mg acetate, and incubated in this medium at 37°. Aliquots were removed at 30 min, 60 min, and 6 hr intervals, and centrifuged at 10 000 *g* for 20 min to remove precipitated RNA. Fractionation on Sephadex G-15 at 4° indicated that each incubate contained low MW oligonucleotides, and mononucleotides. Increases in the concentrations of these components with time indicated that at both concentrations of Mg^{2+} ribosomes showed RNase activity, but that at the lower concentration (0.1 mM) where ribosomes were highly dissociated, the RNase activity was more pronounced.

DISCUSSION

Comparison of the properties of *Chlorella* RNases with those reported for RNases from a variety of sources, shows that the *Chlorella* enzymes have close similarity with those from the tissues of higher plants [2]. The RNases of *Chlorella*, like most other plant RNases, have activity optima at pH 5 (dependent on buffer composition). In higher plants, the pH optimum of RNase I (classification of Reddi [3]), which is found in the soluble phase of tissue homogenates, occurs at 5, and RNase II activity, from ribosomal preparations, has a higher optimum, between 6 and 7 [4–6]. The pH optima of most prokaryote RNases occur between 7 and 8 [1].

Chlorella RNases show marked thermostability [24, 25] and in common with RNases from other sources, as assessed by gel filtration, a relatively low MW of about 10^4. RNase N_1 from *Neurospora crassa* [14] has a MW of 10^4, RNase from *Chalaropsis* [15] a MW of 1.2×10^4, and RNases I and II from *Zea mays* [4] MWs of 2.3×10^4, and 1.7×10^4 respectively.

Partially purified RNases from *Chlorella* are unaffected by Mg^{2+} and Na^+. However, as has been reported for RNases from other sources, Mg^{2+} and K^+ do alter the activity of the ribosomally-bound RNases of *Chlorella* by affecting the degree of association with the ribosome [10, 16–20]. Also, like other nucleases, *Chlorella* RNases are inhibited by free nucleotides [1, 21, 26].

The enzymic action of *Chlorella* RNases as indicated

by the pattern of nucleotides liberated from RNA, is similar to that shown by most RNases from higher plant sources [2–3]. Plant RNases I and II exhibit specificity for the release of purine nucleotides from RNA. Often 3′-GMP is released in highest concentration, with that of 3′-AMP next and other nucleotides present in substantially lower concentrations, or even absent [22–26]. Like most higher plant RNases, the RNases of *Chlorella*, which liberate 2′:3′-cyclic nucleotides, are endonucleases [4, 22–26]. Although both the ribosomal and soluble RNases from *Chlorella* release principally purine nucleotides from RNA (Tables 3 and 4), they produce different hydrolysis patterns. The ribosomal RNase liberates mainly 3′-GMP with, after prolonged incubation, low concentrations of 3′-UMP. If the ribosomal location of this enzyme is not artefactual [20], these properties suggest a specific function at the ribosome, which might be to produce specific cleavages during activation or deactivation of RNA. In *E. coli*, ribosomal RNase has been implicated in mRNA metabolism [27, 28] and in the processing of precursors of rRNA [29, 30, 33, 41], mRNA [31–33] and tRNA [34]. Soluble RNase activity in *Chlorella* liberates 3′-GMP, 3′-AMP, and 3′-UMP from RNA (Tables 3 and 4) suggesting a function, less specific than that of the ribosomal RNase, such as catabolism of 'redundant' RNA. If the soluble RNase from *Chlorella* was not located in the cytosol *in vivo*, it might derive from vacuolar, or lysosomal structures destroyed during sub-cellular fractionation [2, 35, 42, 56–57]. In common with other plant RNases, RNases from *Chlorella* show differing abilities to hydrolyse cyclic nucleotides [3, 23–24].

The sub-cellular distribution of RNase (Table 5), with most activity in the post-ribosomal supernatant and relatively low activity in the ribosomes, is similar to that described for much higher plant material [2]. In diverse higher plant tissues including those from pea [44], *Zea mays* [4–6], sugar cane [24], and wheat [43] most of the RNase activity is located in the soluble fraction. Ribosomal RNase has been demonstrated in a variety of higher plant material including pea [11], *Phaseolus* [22], *Avena* [26], *Zea mays* [4–6, 18–19], apple leaf [37], sugar cane leaf [24], and *Lupinus albus* [38]. In the present work, however, no RNase activity was detected in mitochondrial or plastid preparations from *Chlorella* [17, 37].

The latent activity exhibited by the ribosomal RNase of *Chlorella* (Fig. 2) is similar to that observed with *E. coli* [43], *Salmonella typhimurium* [55], and also with some higher plant material [17]. However, Hsiao [19] has presented evidence that in some higher plants, ribosomal RNase may be active when the ribosome is intact. The effect of decreasing the concentration of Mg^{2+} in the medium is to cause dissociation of ribosomes to subunits, then further dissociation to component RNA macromolecules and proteins [12, 16, 18, 43–44]. In *Chlorella*, heterogeneity of rRNA at low concentrations of Mg^{2+} (0.1 mM) confirms that disintegration of the ribosome occurs, but the appearance of RNase activity cannot be equated at present with the release or disruption of subunits.

Although RNase activity in *Chlorella* appeared to be associated with ribosomes, and was not easily removed by low (<0.4 M) concentrations of KCl (Table 6 and Fig. 1), it must be pointed out that the validity of such *in vivo* association has been questioned for a number of other organisms [19–20, 39–40, 42]. Payne and Dyer [42] have demonstrated with *Vicia faba* that RNase activity observed *in vitro* is derived from 'soluble'/lysosomal RNase by non-specific binding, however, since forms of RNase differing in electrophoretic mobility [4–6], and in apparent enzymic activity and properties [30, 45] have been detected in plant ribosomes, this observation may not be applicable to all plant tissue.

EXPERIMENTAL

Algal cultures. Cultures of *Chlorella saccharophila* var *saccharophila* strain 211/9a were obtained from the Purchasing Centre for Algae and Protozoa, Cambridge, U.K. Algae were maintained at 22° on algar slopes (2% Oxoid agar no. 3 in a *Chlorella* growth medium [45] containing 1% glucose) illuminated at 2.5 klx and subcultured every 10 weeks. Experimental cultures were grown autotrophically in a medium [45] sparged with filtered air enriched with 5% CO_2 at a rate of 1 l. per l. of medium per min. Cultures were maintained at 22° and 12 klx in an illuminated H_2O bath. The inoculum was a 10 ml culture started by transfer from an agar slope, and grown in light for 2 days at 22°. Cells were harvested from batch culture after 7–14 days' growth.

Estimation of RNase activity. Samples (0.5 ml) were incubated with 0.5 ml of 1% (w/v) yeast RNA (BDH) in 100 mM Na citrate buffer, pH 5.2. The RNA was previously freed from contaminating nucleotides on a column of Sephadex G-15, and the high MW RNA fraction was diluted to a concn of 1% (w/v) and stored at −20° until required. After the enzymic reaction, the incubate was cooled in ice for 3 min, and 0.2 ml of 2.5 M $HClO_4$ at 2° was added to stop the reaction. After standing at 2° for 10 min, the ppt was removed by centrifuging at 6000 g for 10 min at 4°. After 15× dilution of the supernatant, acid-soluble nucleotides were estimated from A at 260 nm. The unit of RNase activity used is defined as that activity increasing the A_{260} of incubates by 0.001 per min under the described assay conditions.

Extraction of RNase. Washed cells were suspended in fractionation medium (2 mM Na–EDTA, 10 mM Mg acetate, 60 mM KCl, and 1 mM dithiothreitol in 50 mM Tris–HCl buffer, pH 7.6) for preparation of ribosomes and soluble phase, and broken using a French pressure cell, pre-cooled to 2°. Whereas pressure of 24 MPa was used for attempted recovery of intact organelles, 137 MPa was used for routine isolation of ribosomes and post-ribosomal supernatant.

Preparation of ribosomes and soluble phase. A suspension of cells in fractionation medium (2 mM EDTA, 10 mM Mg acetate, 60 mM KCl, and 1 mM dithiothreitol in 50 mM Tris–HCl buffer, pH 7.6) was passed through a French pressure cell at 137 MPa, and 4°. This preparation was centrifuged at 30 000 *g* for 30 min and the pellet discarded. The supernatant (A) was centrifuged at 100 000 *g* for 90 min to produce pellet B (ribosomes) and supernatant B. The pH of supernatant B was adjusted to 5 with HOAc and the pellet C ('pH 5 extract'), obtained after centrifugation at 20 000 *g* for 10 min, was resuspended in 0.1 M Na citrate buffer (pH 5.2). The remaining supernatant was the soluble phase. Pellet B (ribosomes) was washed twice, and resuspended in fractionation medium.

Preparation of subcellular organelles. To ensure max yield of intact organelles, a sporopollenin-less strain (211/9a) of *Chlorella* was chosen, and cells fragmented at 2° in a French pressure cell at 24 MPa [46–47]. Fractional centrifugation was effected using the medium of ref [48]. Coarse debris and intact cells were removed by centrifuging at 200 *g* (2 min); nuclei with some chloroplast contamination were obtained at 500 *g* (3 min); chloroplasts sedimented at 2000 *g* (10 min); mitochondria at 20 000 *g* (25 min); and ribosomes at 100 000 *g* (180 min). Each pellet was resuspended in H_2O at 2°, and made 0.01% (w/v) with respect to Na deoxycholate. After homogenizing in a Potter–Elvejhem homogenizer, preparations were assessed for homogeneity using organelle-specific assays. Mitochondrial preparations were checked using cytochrome oxidase activity.

Peroxysome preparations were examined for glycollic oxidase activity. Nuclear preparations were checked using DNA as a marker. Chloroplast preparations were examined using chlorophyll as a marker. Ribosomal preparations were checked by measuring their NADH–cytochrome *c* reductase activity.

PC and TLC of nucleotides. The solvent systems used were: (1) 2-methylpropan-2-ol (55 ml), and 20 mM HCO_2NH_4 soln (45 ml) previously adjusted to pH 3.7 with HCO_2H; (2) *iso*PrOH (140 ml), 18 M ammonia, (30 ml), and H_2O (30 ml); (3) *iso*-butyric acid (66 ml), 18 M ammonia, (1 ml), and H_2O (33 ml). TLC was on cellulose (MN cellulose 300 G) using solvent systems 2 or 3.

High voltage electrophoresis. On Whatman 3 MM paper strips (8 × 57 cm) or on sheets (46 × 57 cm). For some separations, Whatman No. 1 papers were run in one dimension using HOAc–HCO_2H buffer (pH 2), and in the second dimension using PC.

Soluble proteins were separated using electrophoresis on polyacrylamide gels [49, 50]. For examination of RNase activity in gels, the gel was pre-run at 4° for 30 min to remove ammonium persulphate. It was then sliced longitudinally, and half was used for RNase estimation in serial slices. The other half was stained for protein or RNA.

During starch gel electrophoresis [51] of RNases, a running buffer of pH 6 was used to maximize the RNase activity. To detect RNase, gels were incubated for 20 min with 0.5% RNA in 0.1 M Na citrate buffer (pH 5.2), washed twice with buffer and then incubated in the same buffer for 30 min. The gels were stained with 0.2% (w/v) toluidine blue in 0.5% (v/v) HOAc. RNase activity appeared as colourless areas against a blue background.

Estimation of protein and RNA. Protein was estimated using the method of ref. [52]; the method ref. [53] was used to estimate RNA.

Identification of nucleotide components of RNA. RNA samples were hydrolysed at 37° in 150 mM KOH for 10 hr. After neutralizing with $HClO_4$, $KClO_4$ was removed by centrifuging and the supernatant evaporated to dryness. The residue was redissolved in 50 mM HCl, and fractionated on a column of Dowex-50 (Cl^- form) [54].

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