EXTRACELLULAR RIBONUCLEASES FROM *BACILLUS SUBTILIS**

I. CRYSTALLIZATION AND SPECIFICITY

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SUMMARY

1. A method is described for large scale purification of two extracellular RNases obtained from *Bacillus subtilis*. They are here named RNase peak I and RNase peak II respectively.

2. RNase peak II was crystallised in the form of needles from homogeneous preparations obtained by the use of chromatography.

3. The specificity of the RNase peak I and peak II enzymes was quite different from that of bovine pancreatic RNase. They hydrolyzed the "core" remaining after treatment of the yeast RNA fraction with bovine pancreatic RNase. But they hydrolyzed only the secondary phosphate esters of the purine riboside 3'-phosphates in RNA and cyclic guanylic and adenylic acids were then isolated as intermediary products from the dialysate. The mononucleotides and end groups of the oligonucleotides produced by the enzymes were guanylic and adenylic acids.

4. The effects of metallic salts, polyvinyl sulfate and anti-RNase on the activity of RNase peak I and RNase peak II and bovine pancreatic RNase are described.

5. A protein, which has no RNase activity, was obtained in crystalline form by crystallisation of the fraction of peak I and the sedimentation coefficient of this inactive protein was about the same as that of active RNase peak II.

6. A latent RNase, which was activated by 4 *M* urea and was almost completely inhibited by anti-RNases, was detected in the bacterial cells.

INTRODUCTION

Bovine pancreatic RNase was first crystallized by KUNITZ[1] and since then RNases have been isolated from various mammalian organs[2–4], plants[5–8] and microorganisms[9, 10]. With the exception of pancreatic RNase and RNase T_1 from takadiastase[11], the RNases so far reported have not been examined physiochemically, because the amount of enzymes available has been small and it has not been possible to obtain them in a state pure enough for such experiments.

Abbreviations: RNA, ribonucleic acid; RNase, ribonuclease; EDTA, ethylenediaminetetraacetic acid; PCMB, *p*-chloromercuricbenzoate; CM-, carboxymethyl; BSER, *B. subtilisis* extracellular RNase.

* A preliminary note on this subject has already been published in the Proceedings of the Eighth Symposium on Nucleic Acid, Research Association of Nucleic Acid, Kyoto, 1959, p. 3.

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NISHIMURA AND NOMURA[12,13] reported that *B. subtilis* produces, as extracellular enzymes, a large quantity of RNases, that one of these enzymes, *i.e.* RNase peak II, could be highly purified by employing the carboxylic acid cation exchange resin IRC-50 (XE-64), and that the specific activity of this purified RNase peak II was comparable to that of crystalline bovine pancreatic RNase.

The present paper describes an improved method for large scale purification and crystallization of RNase peak II, and also the results of a study of the properties of both RNase peaks I and II, particularly their specificity and their immunological and physicochemical properties. The mode of action of these enzymes on RNA appears to be quite different from that of bovine pancreatic RNase. They produced, as terminal nucleotides, only guanylic and adenylic acids.

MATERIALS AND METHODS

The strain of *B. subtilis* used, the culture medium*, the assay procedure, activity unit and specific activity of the enzymes and substrate for RNases were described in previous reports[12,13]. The "core" produced by bovine pancreatic RNase was prepared by the method of TANAKA[14]. The protein content was assayed by the method of LOWRY *et al.*[15], and is expressed as tyrosine equivalent by using a standard tyrosine solution.

The crystalline bovine pancreatic RNase used was a commercial preparation supplied by the Sigma Chemical Company (Lot No. 38–087). Phosphomonoesterase was prepared from hypertrophic human prostate gland as described by MARKHAM AND SMITH[16]. The preparation had no RNase activity and therefore could not have affected the results of the end group determinations.

IRC-50 (XE-64) resin was obtained from the Rohm and Haas Co. and was prepared for use in batches and in columns by the procedure of HIRS[17].

Paper chromatographic solvents

For the separation of cyclic mononucleotides and nucleosides, Solvent I: isopropanol–water (70:30, v/v) with the addition of 0.35 ml of NH_3 solution (0.88 sp. g) for each liter of gas space in the tank, was used[18]. Solvent II: methanol–ethanol–water–conc. HCl (50:25:19:6, v/v/v/v) was used to separate mononucleotides from the enzymic hydrolysate of RNA[19]. For the purpose of identification of guanylic acid isomers Solvent III: satd.$(NH_4)_2SO_4$–isopropanol–1 *M* sodium acetate (80:2:20, v/v/v) was used[16,20]. In all cases ascending chromatography was carried out, using Toyoroshi No. 51 or No. 51 A paper.

Preparation of anti-serum against the RNases of B. subtilis (*anti-BSER*)

Two rabbits were each injected intravenously with 1 ml of crude enzyme preparation fractionated with $(NH_4)_2SO_4$ (Table I, step 5, 1000 units/ml). A total of six injections was given, one injection being given every other day. Because the formation

* RNase activity in the culture medium at the maximum stage of production (about 70 h incubation) varied from 5 to 20 units/ml. The cause of this variation is unknown. Overheating of the medium during sterilization must be avoided. Sterilization of the medium was generally carried out at 110° for 15 min in an autoclave. The addition of trace elements (Fe^{++}, Mn^{++}, Mo^{++}, Cu^{++}, Zn^{++}, $B_4O_7^{--}$) reduced the production of both RNases. During cell culture strong aeration was necessary for RNase formation. 150 ml of culture medium was added to a flask (600 ml) and shaken at about 130 rev./min at 30° on a reciprocal shaker with a moving distance of 6 cm.

of antibody could not be detected by a precipitin test or by the inhibition of RNase activity, the Freund technique was applied. After homogenizing the enzyme fraction with Freund's adjuvant, about 20,000 enzyme units in a volume of 4.0 ml (Table I, step 6) were injected in four equal portions during 3 months into the subscapular region of each of two rabbits. The rabbits were bled 14 days after the last injection and their sera were separated. The same procedure was used for the preparation of antibody against bovine pancreatic RNase.

RESULTS

Large scale purification of the RNase

In order to treat large volumes of culture medium, the batch method, using IRC-50 (XE-64) resin, was applied instead of the ammonium sulfate fractionation previously described[11,12].

This procedure had the advantage that polysaccharide-like substances could be easily and completely removed.

The culture medium, including the cells, was immediately acidified to pH 2 with 4 *N* H_2SO_4. After standing overnight at 4°, the suspension was filtered with the aid of Hyflo Super Cel and the filter cake was washed once with water. The filtrate was adjusted to pH 4.2 with 10 *N* NaOH and to it was then added 0.02 of the filtrate weight of wet resin which had been previously treated with 0.01 *M* sodium phosphate buffer, pH 7.3. After the suspension had been again adjusted to pH 4.2, it was quickly filtered by suction. The filter cake thus obtained was dispersed in 0.2 *M* sodium phosphate buffer, pH 7.3, (0.05 of the volume of the initial culture medium); then 8% NH_3 solution was slowly added until the pH reached 7.3. The suspension was then filtered and the filter cake was washed with a small volume of the same buffer. The combined filtrates were then subjected to ammonium sulfate fractionation at pH 5.9. If adsorption and elution are not done within 15 min, the yield of the enzyme will be decreased.

Table I shows a typical purification and recovery chart obtained at the various steps of this procedure.

As Fig. 1 shows, it was possible to purify the enzyme, step 4 (Table I), by column chromatography using IRC-50 (XE-64) resin as described previously[12,13*]. Before the enzyme preparation was placed on a column, it was dialysed for about 6 h, adjusted to pH 3.5 and centrifuged to remove the precipitate which always formed.

Each enzyme fraction (peak I and peak II) was collected, dialysed against a hundred times its volume of deionized water at 4° for 4 h, lyophilized and rechromatographed. As Fig. 2 shows, rechromatography of each enzyme on separate columns yielded, in each instance, an single peak, a result which demonstrates that the multiple bands separated from the crude enzyme preparation by the columns of XE-64 could not be attributed to compounds arising from the main constituents by alteration on the column. When rechromatography was done, a load of 5 to 10 mg of enzyme dissolved in 2 to 5 ml of solution was employed, a column of 1.7 cm in diameter being used. The recovery of enzymes by a single passage on the chromatographic column ranged from 75 to 50%.

* Dr. T. TUVE pointed out that a CM-cellulose column could also be used for the purification of these bacterial enzymes[21].

TABLE I

PURIFICATION OF RNASE FROM *B. subtilis*

In case of step 1, 2 and 3, protein contents were determined with the dialysed samples.

Steps	*Volume (ml)*	*Total units*	*Total protein content*	*Specific activity (units/μg tyr.)*	*Yield (%)*
1. Culture medium	12,500	72,500	6,580	0.011	100
2. Supernatant after removal of precipitate formed by acidification	12,500	62,500	4,690	0.013	86
3. Eluate from IRC-50 resin (pH, 7.3)	800	33,600	312	0.11	46
4. Precipitate between 0.4 and 0.7 satn. of $(NH_4)_2SO_4$ (310–530 g/l)	50	22,000	125	0.18	30
5. Supernatant at pH 2	50	23,500	111	0.21	32
6. Precipitate between 0.48 and 0.62 satn. of $(NH_4)_2SO_4$ (370–470 g/l)	4	7,300	4.6	1.6	10

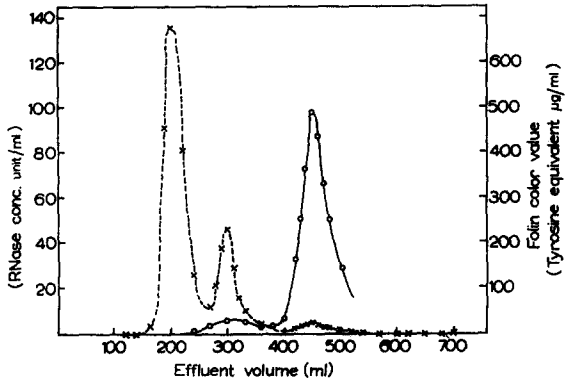

Fig. 1. Column chromatography of RNases from *B. subtilis*. Elution was performed with 0.2 *M* sodium phosphate buffer, pH 5.9 from a column (5 × 20 cm) of Amberlite IRC-50 (XE-64) equilibrated with the same buffer. ———, RNase activity; – – – –, protein content. The flow rate was about 1 ml/cm²/h.

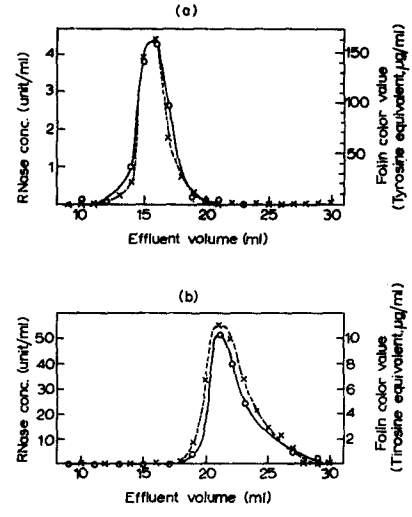

Fig. 2. Rechromatography of (a) RNase peak I and (b) peak II. Experimental conditions were the same as those described for Fig. 1, except that for this procedure a smaller column (0.9 × 20 cm) was used. ———, RNase activity; – – – –, protein content.

Crystallization of RNase peak II

The fraction, peak II, obtained by rechromatography was dialysed for 4 h and lyophilized. It was allowed to contain a small amount of residual sodium phosphate. The resulting wet paste was dissolved in deionized water at pH 5.9 (about 4 mg tyr. equivalent/ml). Saturated ammonium sulfate was then slowly added while the preparation was being stirred, until a very faint turbidity appeared. The suspension

was then left at 4° for 2 days. Needle-shaped crystals of RNase gradually formed and during the stirring typical "silkiness" was seen. When, during the next 2 days, saturated $(NH_4)_2SO_4$ was slowly added to about 0.7 saturation, two-thirds of the original protein crystallized out as needles (see Fig. 3). The specific activity (RNase unit/tyr. μg) of crystalline RNase and that of the RNase remaining in the mother liquid were almost the same (almost equal to that of crystalline bovine pancreatic RNase), a result which suggests that the original chromatographically homogeneous enzyme preparation was pure RNase. It was necessary to remove the red-brown colored material, and this was done by elution with the fast running protein peak near the solvent front in order to obtain crystals. Hence a small amount of this material was not completely removed from peak I and peak II by the initial column chromatography and rechromatography was employed to remove this impurity.

Inactive crystalline protein

Crystalline protein which did not possess RNase activity was obtained from the fraction of peak I by the procedure used for the crystallization of RNase peak II. The crystals of this inactive protein appeared in an ammonium sulfate solution more dilute than that required to produce crystals of RNase peak II. When about half of the protein was precipitated as needles (see Fig. 4), all the RNase activity in peak I remained in the mother liquid. The diffusion coefficient of the inactive protein measured in 0.2 *M* sodium phosphate buffer, pH 5.9, at 4°, was $85 \cdot 10^{-8}$ cm²/sec. This value was the same as that of bovine pancreatic RNase measured under the same conditions ($85 \cdot 10^{-8}$ cm²/sec). The sedimentation coefficient of this material was, as will be described below, almost the same as that of active RNase peak II.

Ultracentrifuge analysis

The ultracentrifuge studies were carried out with a Spinco Model E analytical ultracentrifuge. Analyses were performed in 0.2 *M* sodium phosphate buffer, pH 5.9.

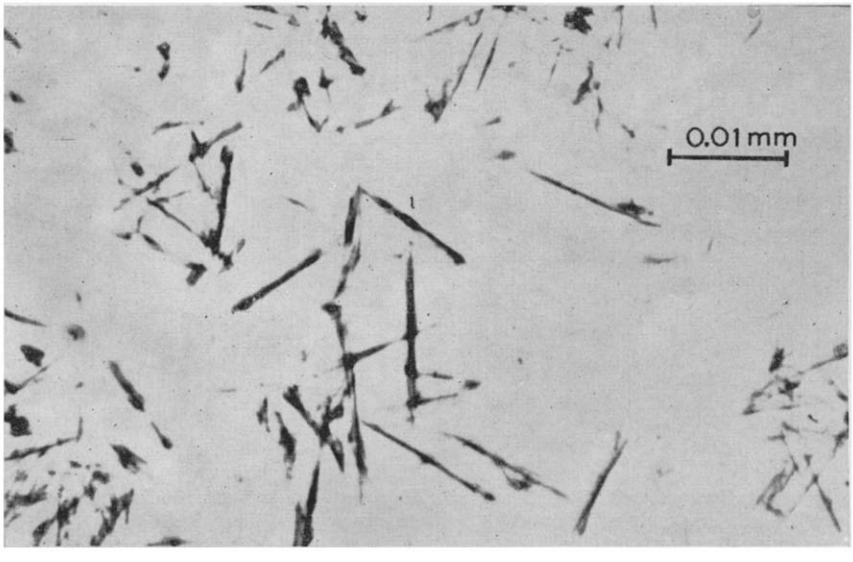

Fig. 3. Crystals of RNase, peak II.

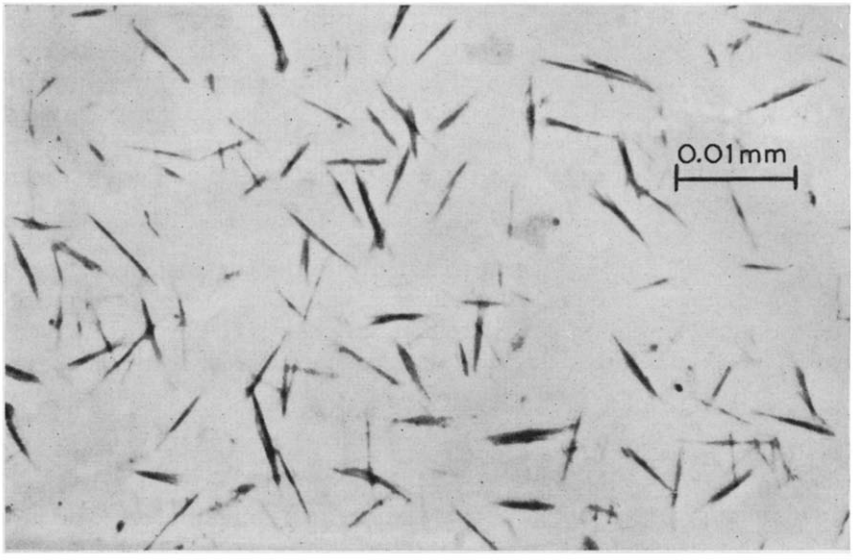

Fig. 4. Crystals of inactive protein from the fraction of peak I.

Fig. 5 shows typical ultracentrifuge pictures with a synthetic boundary cell. Sedimentation coefficients of each sample were determined; (s_{20} was 1.4 for RNase peak II (the rechromatographed preparation), 1.2 for inactive crystalline protein and 1.6 for bovine pancreatic RNase. These data showed that RNase peak II was ultracentrifugically homogeneous and the difference in s_{20} between RNase peak II and pancreatic RNase could scarcely be observed.

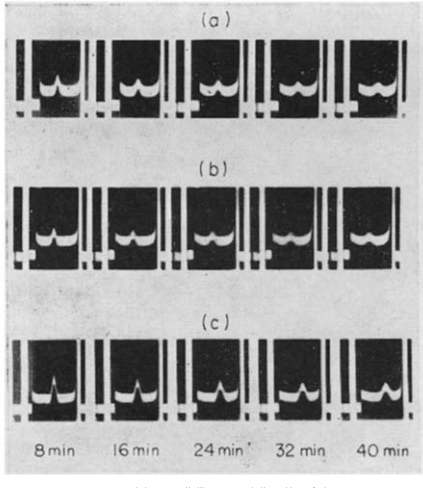

Fig. 5. Sedimentation patterns of (a) RNase peak II (about 3 mg/ml) after sedimentation of 8, 16, 24, 32 and 40 min at 57,000 rev./min at 19°, (b) inactive crystalline protein (about 3 mg/ml) at 57,000 rev./min at 17° and (c) bovine pancreatic RNase (5 mg/ml) at 57,100 rev./min at 28°; solvent 0.2 *M* sodium phosphate buffer, pH 5.9; bar angle 50°.

The specificities of the enzymes

The following experiments indicated that the specificities of RNase peak I and peak II were quite different from that of bovine pancreatic RNase. The RNase hydrolyzed only secondary phosphate esters of purine riboside 3'-phosphates. No difference has so far been found between the enzyme specificity of RNase peak I and that of peak II.

TABLE II

HYDROLYSIS OF THE "CORE" BY RNASE PEAK I AND PEAK II

The incubation mixtures contained 5 mg of the "core" and indicated amounts of RNase in 0.5 ml 0.067 *M* phosphate buffer, pH 7.3. After 15 min incubation at 30°, the reaction was stopped by adding 0.2 ml of 2 *M* HCl. The precipitate formed was removed by centrifugation and 0.1 ml of the supernatant was added to 10 ml of water. The absorbance at 260 mμ was measured in a Beckman-type spectrophotometer. Complete solubilization of the "core" would give an increase of 0.7 O.D. units.

RNase	*Units of added RNase*	*Absorbance at 260 mμ*	*Increase of absorbance*
Untreated	—	0.47	—
RNase peak II	0.025	0.74	0.27
RNase peak II	0.1	0.73	0.26
RNase peak I	0.025	0.81	0.34
RNase peak I	0.1	0.76	0.30
Bovine pancreatic RNase	0.025	0.45	—0.02
Bovine pancreatic RNase	0.1	0.45	—0.02

Hydrolysis of the "core": The yeast RNA fraction resistant to bovine pancreatic RNase (the "core") was incubated with pancreatic RNase, RNase peak I and peak II respectively. As Table II shows, an increase in the acid soluble component was produced only by RNase peak I and peak II.

Formation of cyclic purine mononucleotides as intermediary products: This was studied by the method of MARKHAM AND SMITH[18]. About 5 units of each RNase were added to 1.0 ml 5 % RNA solution at pH 7.5 in cellulose dialysis tubing suspended in 30 ml of water. After 4.5 h of incubation at 30°, 0.4 of the original RNA became dialysable. The dialysate was collected and lyophilized. Lyophilized samples were dissolved in a minimum volume of water and were then spotted on a filter paper for chromatography. The chromatogram was developed with Solvent I for 16 h at room temperature. The fast running spot (which corresponds to band 6 of MARKHAM AND SMITH and contains all the cyclic nucleotides except that of guanine) was eluted, kept overnight in 0.1 *N* HCl at 4° to split cyclic phosphates to 2'- or 3'-phosphates, and then rechromatographed with Solvent II. The R_F value and absorption spectrum, then showed that, when RNase peak I and peak II were used, only adenylic acid could be detected, although cytidylic and uridylic acids appeared from the spot formed by the control experiment in which pancreatic RNase was used. When, however, the more slowly moving spot (which corresponds to band 5 of MARKHAM AND SMITH) was judged by the R_F value, typical absorption spectrum and fluorescence by u.v. irradiation after treatment with HCl gas, cyclic guanylic acid was detected. The ratio of amounts of cyclic adenylic to cyclic guanylic acids was about 1:6.

Liberation of 3'-guanylic acid from cyclic guanylic acid: Cyclic guanylic acid was

incubated with RNase peak II. The chromatogram was developed with Solvent III for 16 h at room temperature. Primarily 3'-guanylic acid was detected in the hydrolysate.

The determination of end groups in RNA hydrolysate: Mononucleotides and terminal groups of oligonucleotides produced by RNase peak II were identified by the method of MARKHAM AND SMITH[22]. 36 mg of yeast RNA was incubated at 30° for 1 h with 100 units RNase peak II in 1.0 ml 0.025 *M* phosphate buffer, pH 7.5. After this exhaustive digestion, the hydrolysate was kept overnight in 0.1 *M* HCl at 4° and then incubated with prostate phosphomonoesterase at 30°, pH 5.3, for 1 h. To make sure that enough of the enzyme was added to liberate completely the terminal phosphoryl groups, the rate of dephosphorylation of 2'- and 3'-guanylic acids was estimated and 10 times the amount of enzyme required for complete digestion was used. 10 *M* KOH were then added to give a final concentration of 1 *M* and the digest was incubated at 37° for 20 h, so that all the nucleoside derived from the dephosphorylated terminal groups was liberated. KOH in the digest was neutralized by the addition of 60% $HClO_4$. After excess $HClO_4$ had been neutralized by NH_3 solution, the precipitate was removed by centrifugation. The nucleosides were then isolated from the supernatant by chromatographing them with Solvent I as shown in Fig. 6. They were eluated with water and estimated spectrophotometrically; 6.8 μmoles guanos-

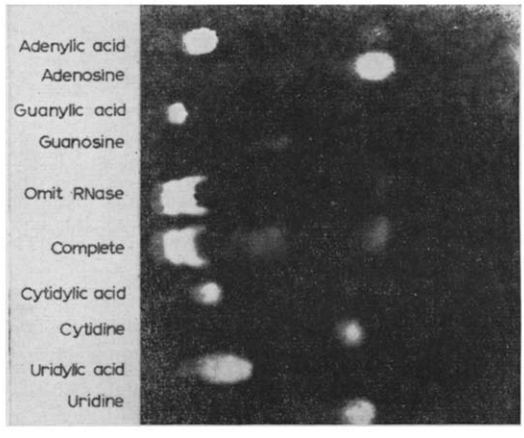

Fig. 6. Demonstration of the presence of end groups in RNA hydrolysate by phosphomonoesterase treatment.

ine, 1.9 μmoles adenosine and about 0.4 μmoles of the mixture of cytidine and uridine were produced from 36 mg yeast RNA which consisted of 20.6 μmoles guanylic acid, 19.8 μmoles adenylic acid, 14.0 μmoles cytidylic acid and 19.7 μmoles uridylic acid. Intact RNA treated in the same manner in the absence of RNase gave only a small amount of nucleosides (see Fig. 6). In the same procedure, the formation of end groups in the RNA by the action of RNase peak II was plotted as a function of time as shown in Fig. 7.

To confirm further whether or not the hydrolysis of secondary phosphate esters of guanosine and adenosine riboside 3'-phosphate was catalysed by a single enzyme, the ratio of guanylic to adenylic acid liberated by the RNase in various purification stages was measured. It was found that the enzyme in the supernatant fraction after

the addition of H_2SO_4 and that in the eluate from the XE-64 resin obtained by the batch method both showed the same specificity. 100 units/ml of RNase peak II were respectively heated to 100° for 10 min at pH 4.0 in 0.025 *M* acetate buffer; pH 6.0, in phosphate buffer; and at pH 8.3, in tris(hydroxymethyl)aminomethane buffer. The activity was lost, by about 45 % in the first of these buffers, by 48 % in the second and by 38 % in the third. This partially inactivated enzyme also had the same specificity. Identification of nucleotides was made by ascending chromatography using Solvent II. Zone electrophoresis in starch for 5.5 h at 7 V/cm in tris(hydroxymethyl)-aminomethane buffer, pH 8.3, was carried out. It was found that the enzyme then moved towards the cathode as a single peak. The activity shown as a broad band by electrophoresis in acetate buffer, pH 4.0 migrated towards the cathode. Enzyme aliquots from the fast and slow moving portions of the broad band had the same specificity.

TABLE III

EFFECT OF METALLIC SALTS AND OTHER SUBSTANCES ON THE ACTIVITY OF RNASE

The substance to be tested was dissolved in 0.067 *M* tris(hydroxymethyl)aminomethane buffer, pH 7.3, in case where a metallic salt was added to incubation mixture.

		Percent of activity		
Inhibitor	*Concentration*	*Bovine pancreatic RNase*	*RNase peak I*	*RNase peak II*
PCMB	$1.0 \cdot 10^{-2}$ *M*	100	89	100
Pb	$1.0 \cdot 10^{-2}$ *M*	45	70	91
Mg	$1.0 \cdot 10^{-2}$ *M*	97	73	91
Fe^{++}	$1.0 \cdot 10^{-2}$ *M*	35	43	77
Fe^{+++}	$1.0 \cdot 10^{-2}$ *M*	32	60	65
Ca	$1.0 \cdot 10^{-2}$ *M*	87	—	85
Mn	$1.0 \cdot 10^{-2}$ *M*	84	69	85
Zn	$1.0 \cdot 10^{-2}$ *M*	4.8	35	65
Cu^{++}	$1.0 \cdot 10^{-2}$ *M*	19	59	71
Co	$1.0 \cdot 10^{-2}$ *M*	71	—	61
Hg	$1.0 \cdot 10^{-2}$ *M*	19	15	14
EDTA	$1.7 \cdot 10^{-2}$ *M*	—	—	100
EDTA	$1.7 \cdot 10^{-3}$ *M*	—	100	100
Polyvinyl sulfate	500 μg/ml	28	103	114

The effects of metallic salts and other substances on the activity of the three RNases

The effects of metals on RNase peak I and peak II were almost the same as the effects of metals on bovine pancreatic RNase, although, as Table III shows, Zn^{++} and Cu^{++} had lower inhibitory effects on RNase peak I and peak II than on pancreatic RNase. A notable difference was, however, observed between the effects of polyvinyl sulfate on the bacterial and pancreatic RNases. Although bovine pancreatic RNase was, as other workers[23–25] have also shown, almost completely inhibited by polyvinyl sulfate, RNase peak I and peak II were not inhibited at all at the same concentrations of this substance.

Anti-B.subtilis *extracellular RNase*

As Fig. 8 shows, anti-BSER and anti-bovine pancreatic RNase inhibited the enzyme activity of their own antigen at a very low concentration. Anti-BSER did not

inhibit pancreatic RNase activity at all. A precipitation reaction between the antiserum and pancreatic RNase could not be detected. The reaction between anti-pancreatic RNase and RNases from *B. subtilis* was also negative. The addition of inactive crystalline protein had no effect on the reaction of anti-BSER with RNase peak II. This was shown by incubation of 0.01 ml antiserum with 10 μg tyr. equivalent, inactive crystalline protein for 20 min before the addition of 0.01 units of RNase peak II.

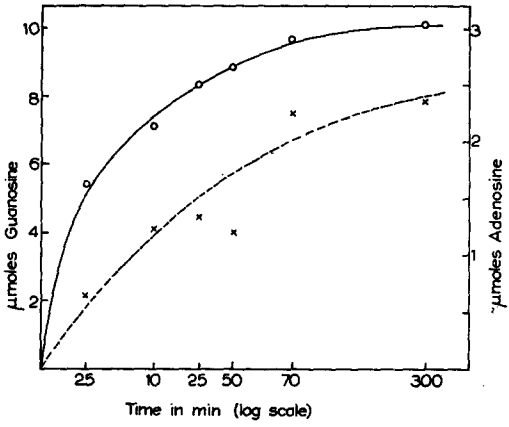

Fig. 7. Formation of end groups during the digestion of RNA by RNase peak II as a function of time. The reaction mixture contained 80 mg yeast RNA (consisting of 45 μmoles guanylic acid, 44 μmoles adenylic acid, 31 μmoles cytidylic acid and 43 μmoles uridylic acid) and 10 units RNase peak II in 1.0 ml 0.033 *M* phosphate buffer, pH 7.3. O—O, guanosine; ×—×, adenosine.

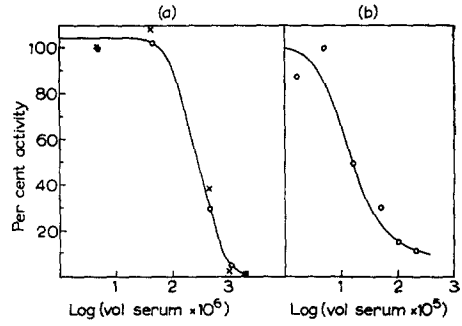

Fig. 8. Neutralization curve of RNase with anti-RNase (a) the reaction of anti-BSER (from rabbit 890) with ×—×, RNase peak I; and O—O, RNase peak II. (b) The reaction of anti-pancreatic RNase (from rabbit 888) with O—O, pancreatic RNase. 0.02 units of each RNase was incubated with antiserum as indicated, in 0.25 ml 0.067 *M* phosphate buffer, pH 7.3 at 4° for 10 min, and 0.25 ml 1% RNA in phosphate buffer was added for the assay of enzyme activity. It was previously confirmed that enzyme-antibody reaction was completed within 5 min.

Activation of latent RNase by urea treatment

NISHIMURA AND MARUO[26] have reported the presence in *B. subtilis* of an intracellular RNase which had properties quite different from those of the extracellular RNases and was completely inhibited by the addition of EDTA. They also found that the cell lysate showed a low degree of alkaline RNase activity, which was inhibited by the addition of anti-BSER. When, however, the lysate was treated with 4 *M* urea, the alkaline RNase activity was increased about 3 times and also, as Table IV shows, the RNase thus activated was completely inhibited by the antiserum. These RNase activities were retained in the supernatant fraction after ultracentrifugation at 100,000 × *g* for 2 h. By this treatment about half of the RNA in the original supernatant was precipitated. Activation by urea of purified RNase peak I and peak II and intracellular acid RNase did not occur.

DISCUSSION

Extracellular RNase peak II from *B. subtilis* is a crystalline RNase and is the first RNase to be crystallized from a source other than mammalian pancreas. Its high specific activity, chromatographic pattern and sedimentation analysis strongly suggests that it is a homogeneous protein.

TABLE IV

ACTIVATION OF LATENT RNASE BY UREA TREATMENT

20 g wet cells which were harvested after 70 h cultivation and washed once with water and twice with 0.01 *M* $MgCl_2$ were ground with 60 g of powdered quartz for about 20 min, suspended in 100 ml 0.01 *M* $MgCl_2$ and centrifuged for 40 min at 10,000 × *g*. The supernatant solution was mixed with an equal volume of 8 *M* urea (dissolved in 0.1 *M* phosphate buffer, at pH 6.0 containing 0.02 *M* NaCl) for 2 h at 4°. The untreated sample was only mixed with the above buffer. After the treatment, samples were diluted 5 times with 0.067 *M* pH 7.3 phosphate buffer, for the assay of alkalline RNase, and 10 times with 0.067 *M* pH 5.9 phosphate buffer, for the assay of acid RNase. Incubations with RNA were made for 4 h and 2 h respectively.

Treatment	*RNase activity (increase of absorbance at 260 mμ)*	
	Activity at pH 7.3 in the presence of $0.7 \cdot 10^{-4}$ M EDTA (Alkaline RNase)	*Activity at pH 5.9 (Acid RNase)*
Untreated	0.043	0.223
Untreated + $1.7 \cdot 10^{-2}$ *M* EDTA	—	0.023
Untreated + antiserum	0.000	—
Treated with 4 *M* urea	0.143	0.217
Treated + $1.7 \cdot 10^{-2}$ *M* EDTA	—	0.014
Treated + antiserum	0.020	—

The enzymes, *i.e.*, peak I and peak II, were stable and soluble under acidic conditions. The optimum pH for the action of both RNases was 7.5. The chromatographic behavior of the enzymes was very similar to that of bovine pancreatic RNase although the bacterial enzymes were slightly more acidic than pancreatic RNase. Further, the various metals used had the same effect on both the bacterial and pancreatic enzymes. The sedimentation coefficient of RNase peak II was about the same as that of pancreatic RNase. It was extremely interesting to find that, although the enzymes RNase peak I and II were quite similar in respect of the properties just mentioned, they were, when they were compared with pancreatic RNase, quite different in their specificity. Preliminary evidence[27] suggested that the amino acid composition of RNase peak II was different from that of bovine pancreatic RNase. Studies of comparative biochemistry and relation between enzymic activity and protein structure will be helped by the isolation of this new RNase.

SATO-ASANO[28] has shown that RNase T_1 in takadiastase obtained from *Aspergillus oryzae* by EGAMI and his collaborators specifically hydrolyzes secondary phosphate esters of purine riboside 3'-phosphate. Her experiments showed, however, that this action of RNase T_1 formed only guanylic acid as the digestion product of RNA; it did not form adenylic acid. RNase T_1, compared with pancreatic RNase, appeared to be a very acidic protein. Anti-BSER did not react with RNase T_1. It could therefore be said that the extracellular RNases from *B. subtilis* were also different in many respects from RNase T_1.

It is possible to consider that RNase peak II consists of two enzymes, one of which liberates guanylic acid from RNA, while the other liberates adenylic acid. Yet the enzyme, at various stages of the purification, and also the partially heat-inactivated enzyme produced guanylic and adenylic acids from RNA at constant rates. Crystalline RNase moreover, had the same specific activity as the enzyme

remaining in the mother liquid. In zone electrophoresis in starch, RNase peak II migrated as a single peak. It is therefore felt that a single enzyme is responsible for the liberation of both guanylic and adenylic acids from RNA.

Crystalline protein, which did not possess RNase activity, was obtained from the fraction of peak I. The physicochemical properties of this protein were very similar to those of RNase peak II. The biological interpretation of this fact is at present unknown, but the possibility may be considered that this crystalline protein is a precursor of RNase or a degraded product of it produced by the action of the exoprotease of this bacterium formed during its growth.

Recently DICKMAN *et al.*[29] announced the presence of latent RNase in mouse pancreas, and ELSON[9] reported that ribonucleoprotein in *Escherichia coli* contained latent RNase which was activated by urea treatment. The properties of this enzyme resembled those of pancreatic RNase. As Table IV shows, the experiments here described indicate that latent RNase activated by urea is present in cells of *B. subtilis*. This RNase might be the same as extracellular RNase, because its activity was inhibited by anti-BSER. This enzyme did not attach to the microsomal fraction, but was in contrast with the results obtained by ELSON with *E. coli*, found in the supernatant fraction. ROTH[30] described an RNase inhibitor which was found in the supernatant fraction prepared from rat liver homogenate. It is therefore possible that such an inhibitor is present in the cells of *B. subtilis* and is combined with RNase before the excretion of this enzyme. Investigation of this possibility has not been completed.

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