Studies on *B. subtilis* Ribonuclease. I. Characterization of Enzymatic Specificity.

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B. subtilis ribonuclease was extensively purified from a bacterial medium with a yield of 11%. The enzyme was found to be free of contaminating ribonucleases and phosphatases. The course of enzymatic action was examined by characterization of different stages of digestion of high molecular weight RNA and various oligonucleotides. *B. subtilis* ribonuclease was thus found to hydrolyze —GpGp— and —GpAp— linkages about 100 times faster than other phosphodiester bonds, but did not have a sole specificity for purines as reported previously. At the last stage of digestion, only mono- and dinucleotides remained. Possible applications of the preferential specificity of the enzyme to the characterization of nucleotide sequences and preparation of large oligonucleotides are discussed.

B. subtilis produces an extracellular ribonuclease (Nishimura and Nomura, 1958, 1959) which was reported to hydrolyze only the secondary phosphate esters of the purine ribonucleoside 3′-phosphates of yeast ribonucleic acid (RNA). Thus at the end of digestion the mononucleotide fraction and the end groups of the isolated oligonucleotides consisted only of guanylic and adenylic acids. Among the mononucleotides produced, the ratio of guanylic to adenylic acid was about 3:1 (Nishimura, 1960). By comparison, pancreatic ribonuclease which is specific for the hydrolysis of phosphodiester bonds between pyrimidine riboside 3′-phosphates and adjacent purine or pyrimidine nucleotides (Schmidt *et al.*, 1951) releases cytidylic and uridylic acids (in yeast RNA) in a ratio of about 1:1 (Markham and Smith, 1952; Volkin and Cohn, 1953). Since a purine-specific enzyme would be very useful for the determination of nucleotide sequences in RNA, it was of interest to characterize further the specificity of *B. subtilis* ribonuclease as well as to explain the reason for the unexpected ratio of purine mononucleotides released in *B. subtilis* ribonuclease digests of RNA.

In this report we describe the extensive purification of *B. subtilis* ribonuclease. The second paper of this series (Hartley *et al.*, 1963) deals with the physical properties of this purified enzyme.

Using such enzyme preparations, digests of high molecular weight RNA as well as of various oligonucleotides of known base-sequences were examined. Contrary to the previously reported results of Nishimura and Nomura, our data show that while *B. subtilis* ribonuclease preferentially hydrolyzes -Gp/Ap- and -Gp/Gp-[1] linkages, other internucleotide bonds in RNA are hydrolyzed as well. Only mono- and dinucleotides are found in a complete digest.

MATERIALS AND METHODS

All spectrophotometric measurements were made with a Beckman DU spectrophotometer with silica cells of 1-cm light path and are expressed as absorbancy (A). A Leeds and Northrup meter equipped with microelectrodes was used for *p*H determinations. For paper chromatography with Whatman No. 3MM paper, two solvents were used, Solvent A (*n*-propanol-concentrated NH_4OH-H_2O, 55:10:35 by volume) and Solvent B (40 g of ammonium sulfate added to 100 m of 0.1 M sodium phosphate, *p*H 7.0).

Alkaline phosphatase[2] from *Escherichia coli* was used for removal of the terminal 3′-phosphate (Khorana and Vizsolyi, 1961). Oligonucleotides were hydrolyzed with snake venom diesterase[3] that contained insignificant amounts of 5′-nucleotidase (Koerner and Sinsheimer, 1957). Pancreatic ribonuclease[4] was also used for this purpose under the same conditions as described previously (Rushizky *et al.*, 1961).

RNA.—High molecular weight RNA was prepared from pressed cakes of bakers' yeast by treatment with detergent (Crestfield *et al.*, 1955). The fraction insoluble in M NaCl was then extracted with phenol. A 0.1% solution of this RNA in water at neutral *p*H was calculated to have an A_{260} of 24.3.

DNA.—Deoxyribonucleic acid from sperm was obtained from Nutritional Biochemical Corporation, Cleveland, Ohio.

Oligonucleotides.—Compounds terminating in guanosine 3′-phosphate were obtained from ribonuclease-T_1 digests (Rushizky and Sober, 1962) of yeast RNA. The other oligonucleotides were isolated from pancreatic ribonuclease digests of the same RNA (Rushizky and Knight, 1960).

Adsorbents.—DEAE- and CM-cellulose (Peterson and Sober, 1956) (0.8 and 0.6 meq/g, respectively) were obtained from Brown Co., Berlin, N. H. Before use, fines were removed by repeated decantation, and the adsorbent was sieved and washed (Peterson and Sober, 1956). Columns were packed with increasing pressure to 15 psi using 100–230 mesh adsorbent equilibrated with the starting buffer (Table I) and washed until the conductivity and *p*H of the effluent solution were the same as those of the influent solution.

Assay for B. subtilis Ribonuclease.—To 0.8 ml of the assay solution (2.0 mg of yeast RNA per ml, and 0.125 M in Tris-Cl, *p*H 8.5) was added 0.2 ml of properly diluted enzyme solution; the mixture was held at 37° for 15 minutes. The reaction was stopped by the addition of 1 ml of 6% $HClO_4$. After 15 minutes at 0°, the precipitate was centrifuged out, 0.5 ml of the

[1] The abbreviations are those in use by the Journal of Biological Chemistry. A slanted line denotes hydrolysis of the internucleotide bond.

[2] We are grateful to Father Donald J. Plocke, Peter Bent Brigham Hospital, Boston, Massachusetts, for a gift of this enzyme preparation.

[3] Obtained from *Crotalus adamanteus* venom from Ross Allen's Reptile Institute, Silver Springs, Florida.

[4] Pancreatic ribonuclease (twice recrystallized) was obtained from Worthington Biochemical Corporation, Freehold, N. J.

TABLE I

CHROMATOGRAPHY OF *B. subtilis* RIBONUCLEASE SOLUTIONS

Cellulose Adsorbents, (g)	Column Dimensions (inside diameter × height in cm)	Starting Buffer	Limit Buffers[a]	Fraction of Limit Buffer[b] in Varigrad Chambers	Gradient Volume (ml)	Flow Rate (ml/hr)
DEAE, 100 g	4.4 × 55	0.01 M AmB, *p*H 8.6	0.05 M AmB, *p*H 8.6			100
CM(I), 95 g	4.4 × 55	0.001 M AmAc, *p*H 4.35	(A) 0.1 M AmAc, *p*H 4.35	3, 4 = 0.5 A 5 = A	2700	36
			(B) 0.1 M AmB, *p*H 7.6	6 = 0.1 B 7 = 0.5 B 8, 9 = B		
CM(II), 30 g	2.2 × 75	0.005 M AmAc, *p*H 4.35	(A) 0.05 M AmAc, *p*H 4.35	2 = 0.2 A 3 = A	2250	32
			(B) 0.2 M AmAc, *p*H 5.7	4 = 0.05 B 5, 6 = 0.25 B 7, 8 = 0.5 B 9 = B		
CM(III), 30 g	2.2 × 75	0.005 M NaAc, *p*H 4.4	(A) 0.1 M NaAc, *p*H 4.7	2 = 0.5 A 3 = A	2250	32
			(B) 0.2 M NaAc, *p*H 5.7 0.1 M NaCl	4, 5 = 0.25 B 6, 7 = 0.5 B 8, 9 = B		

[a] If not indicated in this column, the chambers of the Varigrad (Peterson and Rowland, 1961) contain starting buffers only. For example, in CM(I), chambers 1 and 2 contain the starting buffer only. Abbreviations used for designation of buffers: AmB, ammonium bicarbonate, AmAc, ammonium acetate, NaAc, sodium acetate. [b] Peterson and Sober (1959).

supernatant diluted with 4.5 ml of water, and the A_{260} determined against the proper blank. This assay was linear up to A_{260} values of about 0.7; there was no detectable lag period. An increase in A_{260} of 1.0 under these conditions was defined as 100 units of enzyme activity.

Purification of B. subtilis ribonuclease.—*B. subtilis* strain H (Nishimura and Nomura, 1958) was used to inoculate 150 ml of a medium containing 0.5% lactose, 1% neopeptone, 0.5% meat extract, 0.2% yeast extract, and 0.2% NaCl, at *p*H 7.2.[5] The inoculum was grown for 24 hours at 30° with aeration by shaking, assayed for *B. subtilis* ribonuclease activity (100–500 units per ml), and examined under the microscope for the characteristic rods of *B. subtilis*. Portions of 25 ml of inoculum were then transferred to each of six 4-liter Erlenmeyer flasks containing 1 liter of Nishimura's medium (Nishimura and Nomura, 1958) modified to contain 2% neopeptone in place of soybean extract. After shaking for 24 hours at 30°, the maximum amount of *B. subtilis* ribonuclease was obtained. The 6 liters of medium were pooled, the cells centrifuged out, and the supernatant solution (at 0°) brought to 0.4 M H_2SO_4 (*p*H 3.68 determined at a 1:1000 dilution) with concentrated H_2SO_4. After 16 hours at 5°, the *p*H of the solution was adjusted to 2.3 (undiluted) with conc. NH_4OH; 500 g of $(NH_4)_2SO_4$ per liter of solution was then added.

Due to the high density of this solution, the enzyme and other protein rose to the top upon standing for 24 hours at 5°. The clear yellow solution below the protein layer was drawn off; the residue was suspended in 1 liter of water and adjusted to *p*H 7 with NH_4OH. After extensive dialysis at 4° against three 10-liter volumes of water for 12–16 hours each, insoluble material was centrifuged out and the supernatant solution was then applied to a DEAE-cellulose column (Table I). The enzyme (at *p*H 8.6) passed through the column without being bound to the adsorbent; the last traces of *B. subtilis* ribonuclease activity were eluted with 0.05 M NH_4HCO_3, *p*H 8.6.

The crude *B. subtilis* ribonuclease solution (about 2 liters) was adjusted to *p*H 4.3 with 1 ml of glacial acetic acid and adsorbed on a CM-cellulose column previously equilibrated with 0.001 M ammonium acetate, *p*H 4.35. (CM [I], Table I). Two peaks with ribonuclease activity were thus isolated. Peak A was not bound to the adsorbent and passed through the column together with all the pigment which had accompanied the *B. subtilis* ribonuclease up to this point. Peak B was eluted after 81% of the total gradient volume had gone through the column. The fractions containing the highest specific activity were pooled, dialyzed, adjusted (by dilution) to the concentration of the starting buffer of the next column (no *p*H adjustments were necessary), and further purified by two chromatography steps on CM-cellulose (Table II). In both buffer systems the enzyme was eluted after 75–90% of the total gradient volume had passed through the column. After the third chromatography on CM-cellulose, the peak fractions were pooled and stored at 3° in the presence of chloroform at a concentration of about 1 mg/ml. *B. subtilis* ribonuclease does not lose activity under these conditions for several months.

Characterization of Ribonuclease Activity in Peak A.—An aliquot of about one-tenth of the pooled material in peak A was dialyzed and resubmitted to chromatography as described for CM-cellulose I (Table I). Other aliquots of peak A were compared with further purified *B. subtilis* ribonuclease from peak B with respect to *p*H optimum, effect of EDTA, $CuSO_4$, $MgSO_4$ (Table III), and enzymatic action on yeast RNA as described below.

Assay of B. subtilis Ribonuclease for Contaminating Enzymes.—Purified *B. subtilis* ribonuclease was tested for acid phosphatase and nonspecific phosphodiesterase (Koerner and Sinsheimer, 1957) as well as for deoxyribonuclease activity (Rushizky and Sober, 1962).

Mapping and Digestion of Yeast RNA and Oligonucleotides with B. subtilis Ribonuclease.—The mapping procedure has been described (Rushizky and Knight,

[5] We are grateful to Drs. S. Nishimura and H. Tizuka of the Institute of Applied Microbiology, University of Tokyo, for a gift of this culture.

TABLE II

PURIFICATION OF *B. subtilis* RIBONUCLEASE FROM 6 LITERS OF BACTERIAL MEDIUM

	Total Protein (as A_{280})	Enzyme Units ($\times 10^{-6}$)[a]	Specific Activity[b]	% Recovery
Original medium	460,000	49	106	100
After cell removal	375,000	56	150	114
$(NH_4)_2SO_4$ ppt	91,000	52	574	106
After dialysis	44,600	37	828	75
After DEAE-cellulose chromatography	12,000	33	2,740	66
After CM-cellulose chromatography (I)				
Peak A[c]	10,000	10	912	20
Peak B	1,000	17	16,100	35
After CM-cellulose chromatography (II)	122	13	105,300	26
After CM-cellulose chromatography (III)	12	5.4	446,000	11

[a] For a description of enzyme units and enzyme assay, see text. [b] Total units of enzyme/total A_{280}. [c] For a characterization of the enzymatic activity in these peaks, see text.

1960). To check for the completion of digestion, equal amounts of RNA in 0.1 M Tris-Cl, *p*H 8.5, were hydrolyzed at increasing enzyme-substrate ratios (see legend to Table IV). Digestion was halted by treatment with phenol followed by extraction with ether. Recovery of nucleic acid material has been shown to be quantitative under these conditions (Rushizky *et al.*, 1961). A separate portion of the enzymatic digest was hydrolyzed with N KOH (24 hours at 23°) and assayed spectrophotometrically in order to measure the total amount of nucleotide material used for mapping. The mono- and oligonucleotides eluted from maps of the RNA digests were identified as previously described for the same compounds obtained after digestion with micrococcal nuclease (Rushizky *et al.*, 1962) and ribonuclease-T_1 (Rushizky and Sober, 1962). Compounds terminating in 2',3'-cyclic-terminal phosphates were separated from their corresponding 3'-phosphate forms by paper chromatography with solvent B.

TABLE III

EFFECT OF METALS AND EDTA ON ACTIVITY OF *B. subtilis* RIBONUCLEASE[a]

Compound Tested	M	Per Cent Activity	
		Peak A	Peak B
EDTA	0.001	− 3	−11
	0.01	+ 5	+ 5
	0.1	−13	− 8
$CuSO_4$	0.001	− 1	− 3
	0.01	−14	−41
	0.1	−98	−96
$MgSO_4$	0.001	−16	−28
	0.01	−37	−38
	0.1	−83	−81

[a] For a definition of peaks A and B, see Table II and the text.

Oligonucleotide digests were also freed of enzyme with phenol, and fractionated by paper chromatography with solvent A, B, or by mapping. Determination of the nucleotide composition of the oligonucleotide products in the hydrolyzates was facilitated by eluting the spots from the chromatograms for 3 hours with 0.01 M (0.1 M for papers developed with solvent B) ammonium acetate, *p*H 4.5, containing 0.02 units of ribonuclease T_2 per ml. Under these conditions oligonucleotides are completely hydrolyzed to nucleoside 3'-phosphates, which can be quantitatively determined by spectrophotometry at *p*H 7.0 (Rushizky and Sober, 1963).

RESULTS

B. subtilis ribonuclease was purified from 6 liters of bacterial medium by treatment with acid and ammonium sulfate, and by chromatography on DEAE- and CM-cellulose (Table I). The total purification was 4200-fold, and the yield 11% (Table II).

The *p*H optimum in 0.1 M Tris-Cl was at *p*H 8.5, with 50% of maximal activity remaining at *p*H 7.5 and 9.3. At *p*H 8.5, varying the substrate concentrations from 0.75 to 7.5 mg of RNA per ml had no effect on enzyme activity. EDTA (0.001–0.1 M) also did not affect the enzyme; $CuSO_4$ and $MgSO_4$ were inhibi-

TABLE IV

MONO- AND OLIGONUCLEOTIDES IDENTIFIED IN *B. subtilis* RIBONUCLEASE DIGESTS OF YEAST RNA

Stage 1[a]	Stage 2	Stage 3	Spot Number (Fig. 1)	Stage 4
G-cyclic-p	Gp,G-cyclic-p	Gp,G-cyclic-p	4, 5	Gp,G-cyclic-p
	Ap[b]	Ap,Cp	1	Ap,Cp
		Up	10	Up
	ApAp	CpAp,ApAp	2	CpAp,ApAp
ApGp	ApGp,CpGp	ApGp,CpGp	6	ApGp,CpGp
	UpGp	UpGp	11	UpGp
		ApUp,UpAp,UpCp,CpUp	8	ApUp,UpCp,CpUp
		UpUp	13	
		(ApUpUp),(UpUpCp)	14	
	(CpUp)Gp,(ApUp)Gp	CpUpGp,UpCpGp,ApUpGp	12	(CpUp)Gp
	(ApCp)Gp,ApApGp,CpCpGp	ApCpGp,CpApGp,CpCpGp	7	CpCpGp
		ApUpAp,(CpUpAp),UpCpCp	9	
		ApApCp,ApCpCp	3	ApCpCp
	(ApCpUp)Gp,(CpCpUp)Gp	(ApApUpUp),(CpCpUp)Gp[c]	15	

[a] Digestion conditions: 3.5 mg of RNA in 0.5 ml of 0.1 M Tris-Cl, *p*H 8.5, were digested for 6 hours at 37° with 1.75, 195, and 3850 units of *B. subtilis* ribonuclease added in 0.1 ml of water. This corresponds to stages 1–3, respectively. Stage 4: 3.5 mg of RNA in 0.4 ml of buffer as above were digested for 8 hours at 37° with four equal portions (total units: 77,000) of enzyme added at 2-hour intervals. For a definition of enzyme units, see text. [b] Except for the case of Gp, compounds terminating in 2',3'-cyclic-terminal phosphate and 3'-phosphate are listed together. [c] Stages 1–2 contained core material, while in Stage 3 only tetra- and in Stage 4 only tri- and smaller oligonucleotides were found.

TABLE V

HYDROLYSIS OF TRINUCLEOTIDES BY *B. subtilis* RIBONUCLEASE[a]

Compound	Units of Enzyme per mμmole of Compound	Compounds Isolated	Compounds Deduced	mμMoles of Compounds Isolated after Varying Times of Digestion (hours) at 37° 0	1	2	4
(CpUp)Gp	2.9	(CpUp)Gp		740	745		743
		% hydrolysis			0		0
ApUpGp	1.9	Ap,A-cyclic-p			24		57
		UpGp			25		58
		ApUpGp		841	820		786
			Ap/UpGp		25		58
		% hydrolysis			3%		7%
ApApCp	1.6	Ap,A-cyclic-p[b]			87	110	
		Cp			50	63	
		ApAp			51	60	
		ApCp			91	112	
		ApApCp		900	745	710	
			ApAp/Cp		51	62	
			Ap/ApCp		89	112	
		% hydrolysis			16%	20%	
ApApUp	1.6	Ap,A-cyclic-p			47	69	
		ApUp			46	65	
		Up			29	30	
		ApAp			29	29	
		ApApUp		460	379	357	
			ApAp/Up		29	29	
			Ap/ApUp		46	68	
		% hydrolysis			17%	21%	
ApGpUp	1.75	Ap,A-cyclic-p			25	57	
		GpUp			23	55	
		Up			90	153	
		ApGp			89	157	
		ApGpUp		690	570	476	
			Ap/GpUp		24	55	
			ApGp/Up		90	155	
		% hydrolysis			16%	30%	
ApGpCp	2.2	Ap,A-cyclic-p			9	17	
		GpCp			9	16	
		ApGp			55	78	
		Cp			57	79	
		ApGpCp		590	523	490	
			ApGp/Cp		56	79	
			Ap/GpCp		9	17	
		% hydrolysis			11%	16%	
ApGpCp	0.3	ApGpCp		75		78	
		% hydrolysis				0	
UpApGp	2.9	Up,U-cyclic-p			239		500
		ApGp			236		486
		UpApGp		728	486		240
			Up/ApGp		238		493
		% hydrolysis			33%		67%
ApApGp	2.0	Ap,A-cyclic-p			227		447
		ApGp			230		440
		ApApGp		630	400		191
			Ap/ApGp		230		440
		% hydrolysis			37%		70%
GpGpCp	0.05	G-cyclic-p			77		
		GpCp			78		
		GpGpCp		79	0		
			Gp/GpCp				
		% hydrolysis			100%		
GpApCp	0.03	G-cyclic-p,Gp			78		
		ApCp			80		
		GpApCp		80	0		
			Gp/ApCp				
		% hydrolysis			100%		

TABLE V (continued)

Compound	Units of Enzyme per mμmole of Compound	Compounds Isolated	Compounds Deduced	mμMoles of Compounds Isolated after Varying Times of Digestion (hours) at 37° 0	1	2	4
GpApUp	0.01	G-cyclic-p			193	310	
		ApUp			199	307	
		GpApUp			304	185	
			Gp/ApUp	493	196	309	
		% hydrolysis			40%	63%	
GpGpUp	0.01	G-cyclic-p			247		
		GpUp			241		
		GpGpUp		797	553		
			Gp/GpUp		244		
		% hydrolysis			31%		

[a] For a description of the isolation procedure by paper chromatography and mapping, see text. The hydrolysis of a phosphodiester bond is indicated by a slanted line, /. [b] The progress of hydrolysis of the trinucleotides was deduced as follows: In the case of ApApCp (1 hour digestion in 0.1 M Tris-Cl, pH 8.5), 51 millimicromoles (mμmoles) of ApAp and 50 mμmoles of Cp were found, accounting for 51 mμmoles of the original substrate hydrolyzed as ApAp/Cp. Similarly, 87 mμmoles of Ap plus A-cyclic-p were correlated with 91 mμmoles of ApCp, giving 89 mμmoles of Ap/ApCp. In all cases, per cent hydrolysis is expressed in terms of the fraction of original trinucleotides split.

tory, but only at rather high concentrations (Table III). The enzyme did not lose activity upon treatment with 0.4 N H_2SO_4 for 18 hours at 4°.

B. subtilis ribonuclease was inactive toward *p*-nitrophenylphosphate and bis(*p*-nitrophenyl)phosphate. When DNA was incubated for 8 hours at 37° with 22,000 units of enzyme per mg substrate in 0.1 M Tris-Cl, *p*H 8.5, no mono- or oligonucleotides were released as determined by paper chromatography using solvent B.

After the first column chromatography on CM-cellulose, two forms of *B. subtilis* ribonuclease were isolated. One of these, (peak A, Table II) was not bound to the adsorbent at *p*H 4.3 and had a considerably lower specific activity than peak B. Even small amounts of peak A material were not bound to CM-cellulose under identical conditions. However, the two forms of *B. subtilis* ribonuclease did not differ from each other with respect to *p*H optimum, effect of EDTA, $CuSO_4$, $MgSO_4$, and enzymatic action on yeast RNA. When *B. subtilis* was grown as described but in a 300-liter batch with aeration by forced air rather than shaking, no peak A ribonuclease was obtained by the same purification procedure.

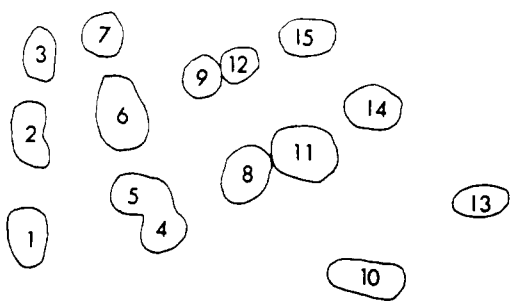

FIG. 1.—Drawing of a map showing the fractionation on Whatman No. 3 paper of 3.5 mg of RNA digested by *B. subtilis* ribonuclease (stage 3, Table IV). First dimension: electrophoresis (left to right) in 0.02 M ammonium formate, *p*H 2.7, for 17 hours at room temperature and 6 v/cm. Second dimension: paper chromatography in descent, with 55:45 (v/v) tertiary butanol: 0.02 M ammonium formate, *p*H 3.8. The paper was serrated so that the solvent could run off the paper without edge effects. Running time was 36–40 hours at room temperature. For an identification of the compounds, see Table 4.

The course of enzymatic action on RNA was followed by examining RNA digests prepared with 0.5, 55, 1100, and 22,000 units of *B. subtilis* ribonuclease per mg of substrate (stages 1–4, Table IV). The first mono- and oligonucleotides released were G-cyclic-p and ApG-cyclic-p (stage 1). At the next stage compounds terminating in Gp predominated with traces of Ap and ApAp present as well. Further enzymatic action (stage 3) resulted in the liberation of Up and Cp and significant amounts of oligonucleotides free of guanylic acid, in addition to the compounds already found in stage 2. "Core" material (large oligonucleotides that did not move during paper electrophoresis and chromatography) was evident until stage 3, but absent from stage 4 digests. At this last stage, mono- and dinucleotides and only traces of trinucleotides were found.

In order to compare our results with those of Nishimura (1960), the ratio of Gp to Ap (found in mononucleotide form) was determined. At stage 3 the ratio was 2.9, while at stage 4 it was 1.6.

The compounds listed in Table 4 were found in both the 2′,3′-cyclic-terminal phosphate and 3′-phosphate form; the proportion of these was not obtained for stages 1–3. In stage 4 the per cent (of the total amount) of a compound isolated in the cyclic-terminal phosphate form were as follows: Gp, 41: Ap, Cp, Up, 90–95; CpAp, ApAp, UpAp, UpCp, and CpUp, 75–85; ApGp, CpGp, and UpGp, 5–10.

These results are in line with the preferential hydrolysis of phosphodiester bonds between guanosine 3′-phosphate and other bases observed in RNA (stages 1–2, Table IV). Thus, among the mononucleotides, the per cent of G-cyclic-p is lowest; similarly, dinucleotides terminating in Gp such as ApG-cyclic-p are hydrolyzed at a faster rate than those without Gp, i.e., ApA-cyclic-p.

The determination of enzymatic specificity of *B. subtilis* ribonuclease also involved the comparison of rates of hydrolysis of trinucleotides (Table V). (CpUp)Gp was hardly split under the conditions leading to partial cleavage of ApUpGp, UpApGp, ApGpUp, ApGpCp, ApApCp, and ApApUp. By comparison, the -GpGp- and -GpAp- sequence in GpGpCp, GpApCp, GpGpUp, and GpApUp was split about 100 times faster than the six trinucleotides. The preferential hydrolysis of these two sequences is in agreement

TABLE VI

HYDROLYSIS OF DINUCLEOTIDES BY *B. subtilis* RIBONUCLEASE[a]

Compound	Units of Enzyme per mμmole of Compound	Compounds Isolated	Compounds Deduced	mμMoles of Compounds Isolated after Varying Times of Digestion (hours) at 37° 0	1	4	5
ApGp	730	ApGp		730			720
		% hydrolysis		0%			0%
ApCp	68	ApCp		445			450
		% hydrolysis		0%			0%
GpCp	256	Gp,G-cyclic-p					478
		Cp					490
		GpCp		481			0
			Gp/Cp				485
		% hydrolysis		0%			100%
GpCp	1	GpCp		210	211	213	
		% hydrolysis		0%	0	0	
CpGp	150	CpGp		1740			1700
		% hydrolysis		0%			0%
UpGp	1110	UpGp		350			349
		% hydrolysis		0%			0%
GpUp	285	Gp,G-cyclic-p					925
		Up					912
		GpUp		910			0
			Gp/Up				918
		% hydrolysis		0%			100%
ApUp	70	ApUp		370			371
		% hydrolysis		0%			0%

[a] For a description of the procedure see legend to Table V and text.

with the results obtained at the first stage of digestion of RNA. Thus, the first mono- and small oligonucleotides that appear are Gp and ApGp (stage 1, Table IV).

The rate of hydrolysis of several dinucleotides by *B. subtilis* ribonuclease was also investigated (Table VI). This showed that GpCp and GpUp were split at enzyme-substrate ratios at which CpGp, UpGp, ApGp, ApUp, and ApCp were resistant to cleavage.

The hydrolysis of GpCp and GpUp is in agreement with the preferential splitting of phosphodiester bonds between guanosine 3′-phosphate and the 5′-hydroxyl terminus of other bases noted above in *B. subtilis* ribonuclease digests of RNA. As a corollary, the bond between Gp and the neighboring base in Gp-terminal oligonucleotides was particularly resistant, since at all stages of digestion (Table IV) oligonucleotides were either free of Gp or contained Gp at the 3′-phosphate end as in ApGp.

When a mixture of Gp-terminal (tetra- and larger) oligonucleotides obtained from ribonuclease-T_1 digests was incubated for 2 hours at 37° with 33,000 units of *B. subtilis* ribonuclease per mg, no tri- or larger oligonucleotides remained. ApGp, CpGp, UpGp, and some dinucleotides not containing Gp were isolated. Smaller amounts of Ap and Up, but no Gp were found. The absence of free Gp thus confirms the resistance postulated for XpGp sequences (where Xp = Ap, Up, or Cp) in Gp-terminal oligonucleotides noted above.

DISCUSSION

The *B. subtilis* ribonuclease described here is comparable to that of Nishimura (1960) with respect to the strain of *B. subtilis* used as well as to the composition of the growth medium. Our substitution of neopeptone for soybean extract reduced the amount of polysaccharide material but did not noticeably affect the yield or enzymatic properties of the enzyme. The extracellular *B. subtilis* ribonuclease is bound on CM-cellulose at acid *p*H, is stable to acid at *p*H 2.0, is not activated by EDTA, and is not significantly affected by $CuSO_4$ and $MgSO_4$ except at high concentrations of these metals. Hydrolysis of RNA with *B. subtilis* ribonuclease involves a cyclic-terminal phosphate intermediate form. At an intermediate stage of digestion (stage 3, Table IV) we obtain a Gp–Ap ratio of 2.9. Furthermore, our *B. subtilis* ribonuclease has an enzymatic activity (on a weight basis) equal to that of pancreatic ribonuclease. The above results agree with those reported by Nishimura. However, the *p*H optimum of our enzyme is at *p*H 8.5 in Tris-Cl and it is inhibited to about 50% by 0.1 M sodium phosphate *p*H 8.5. This is in contrast with the previously reported *p*H optimum of 7.5 (0.1 M Tris-Cl) and lack of phosphate inhibition.

In the second paper of this series, devoted to the study of the physical properties of the enzyme (Hartley *et al.*, 1963) the *B. subtilis* ribonuclease was further purified (2-fold) by exclusion chromatography on Sephadex G-75. Electrophoresis on polyacrylamide at *p*H 5 revealed the presence of about 15% of contaminating protein without ribonuclease activity. The *p*H optimum and enzymatic action on RNA of this more highly purified enzyme did not differ from that of the preparations used in this study, nor from that of peak A or B (Table II). Since the character of the enzymatic action on RNA remained unchanged but the specific activity was increased at various stages of the purification process, the presence of two or more enzymes in our *B. subtilis* ribonuclease may be ruled out.

Kickhoefen and Buerger (1962) reported that pancreatic ribonuclease is stable in phenol. With both crude and highly purified *B. subtilis* ribonuclease, we found that enzyme preparations could be quantita-

tively desalted and concentrated (10-fold) by phenol extraction (Rushizky *et al.*, 1963). This procedure should reduce the loss of enzyme in the dialysis step (Table II) and facilitate a further purification of peak A ribonuclease.

In our hands, *B. subtilis* ribonuclease does not show the "simple" purine specificity reported by Nishimura (1960). Instead, the enzyme hydrolyzed -GpGp- and -GpAp- linkages in trinucleotides about 100 times faster than any other sequence. If this preference is maintained in RNA, then the enzyme may become a valuable tool (Dekker, 1960) for the isolation and characterization of large oligonucleotides. Given a polynucleotide of chain length 100 and an equal proportion of all four bases, a partial hydrolysis would thus yield (25) (0.5) = 12–13 oligonucleotides which, if present in sufficient quantities, could be isolated and identified with existing methods.

Another example of a preferential specificity for neighboring bases (Ap and Tp) was reported for micrococcal nuclease digests of thymus deoxyribonucleic acid (Roberts *et al.*, 1962). The same enzyme preferentially hydrolyzed bonds next to Ap and Up in RNA from tobacco mosaic virus, and was especially effective in attacking those regions of an RNA molecule which contained several adjacent Ap and/or Up residues. Thus, Ap and Up were the first compounds that could be identified in such digests (Rushizky *et al.*, 1962). The action of the enzyme on nucleic acids appears to be similar to its action on polyadenylic acid (Alexander *et al.*, 1961). In this case, the substrate is first hydrolyzed into large fragments which decreased in size as the reaction progressed; analogously, the hexamer of pA was preferentially split in the middle giving pApApA and ApApA.

There is another application of *B. subtilis* ribonuclease to the characterization of nucleotide sequences. This involves the resistance of Gp-terminal dinucleotides to hydrolysis. Compounds such as ApGp, CpGp, and UpGp are not split under conditions where other dinucleotides (either free of Gp, or containing Gp but not at the 3′-phosphate end, e.g., GpCp and GpUp) are hydrolyzed. The base next to Gp in Gp-terminal oligonucleotides may thus be identified as shown for the case of a mixture of two pentanucleotides.

$$\begin{array}{ll} x\ \text{ApApCpUpGp} & \xrightarrow{\textit{B. subtilis}} \\ y\ \text{ApApUpCpGp} & \xrightarrow{\text{Ribonuclease}} \end{array} \begin{array}{l} x\ \text{UpGp} \\ \text{CpGp, mono-, and dinucleotides} \end{array}$$

This procedure is applicable to Gp-terminal compounds, but not to oligonucleotides containing Gp at a position other than the 3′-phosphate terminus (such as ApGpUp) derived from RNA digests prepared with other enzymes. *B. subtilis* ribonuclease may thus hydrolyze ApGpUp to Ap and GpUp (Table V).

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