S_1 Nuclease Hydrolysis of Single-Stranded Nucleic Acids with Partial Double-Stranded Configuration†

G. W. Rushizky,* V. A. Shaternikov,‡ J. H. Mozejko, and H. A. Sober§

ABSTRACT: The single-strand specific nuclease S_1 from *Aspergillus oryzae* (EC 3.1.4.21) was purified 600-fold in 16% yield from dried mycelia. Determination of the isoelectric point of S_1 nuclease as 4.3–4.4 allowed adjustment of chromatographic conditions such that the enzyme was isolated free of contaminating ribonucleases T_1 and T_2. S_1 nuclease so purified was used for removal of single-stranded portions from the RNA of the *Escherichia coli* phage MS2, which has a helical content of about 65% in vitro. At 23°, increasing amounts of enzyme converted the RNA to mononucleotides in about equimolar base ratios. No small intermediates of chain length 2–8 were found. At 0°, MS2 RNA hydrolysis was slower and reached, in exhaustive digests, a plateau where 70% of the substrate RNA remained insoluble in 66% EtOH. With [^{32}P]MS2 RNA, strip chart counting of 6% acrylamide–6 *M* urea electrophoresis patterns of such digests gave recoveries of 80–91% in the form of defined oligomer bands. On 2.5% acrylamide–0.5% agarose gels, the molecular weights of the major oligomers were found to range from 25,000 to 41,000. Similar to purified $tRNA^{Arg}$ used as a control, these oligomers were not resistant to pancreatic RNase–RNase T_1 hydrolysis at 37°, and were not bound on hydroxylapatite at 50° in 0.14 *M* sodium phosphate (pH 6.8). Melting of the oligomers gave complex profiles without a clear T_m and showed an increase in A_{260} of 35% at 93° over that at 28°. Upon formaldehyde denaturation of MS2 RNA prior to S_1 nuclease hydrolysis, no resistant oligomers were found.

The single-strand specific nuclease S_1 of *Aspergillus oryzae* (Ando, 1966) has been purified and widely used for the characterization of nucleic acids (Schaller et al., 1969; Shichido and Ikeda, 1971; Sutton, 1971; Shichido and Ando, 1972; Beard et al., 1973; Vogt, 1973; Germond et al., 1974). At present, the products of S_1 hydrolysis of native single-stranded nucleic acids that contain, in part, double-stranded configurations (Boedtker, 1967) are not well characterized or known. In this study, S_1 nuclease was purified until free of contaminating nonspecific nucleases and RNases T_1 and T_2 and, then, RNA of the *Escherichia coli* phage MS2 was hydrolyzed with this S_1 nuclease under various conditions and the products were fractionated by polyacrylamide gel electrophoresis. The results show that all portions of MS2 RNA are not equally susceptible to hydrolysis by the enzyme.

Materials and Methods

Spectrophotometric measurements were made in cells with a 1-cm light path and are expressed as absorbancy (*A*). Two solvents were used for paper chromatography with Whatman No. 3MM paper: solvent A (1-propanol–concentrated NH_4OH–H_2O, 55:10:35, v/v) and solvent B (40 g of $(NH_4)_2SO_4$ added to 100 ml of 0.1 *M* Tris-HCl (pH 7.5)). This latter solvent was useful for base ratio determinations of DNA because it resolves pA, pG, pT, and pC in the order of increasing R_f values. Pancreatic RNase (Type II-A) and RNase T_1 were obtained from Sigma, St. Louis, Mo.

Nucleic Acids. RNA was obtained from MS2 essentially as described by Strauss and Sinsheimer (1963). ^{32}P-labeled MS2 RNA was isolated as reported (Glitz, 1968) and contained 0.5×10^6 cpm/μg of RNA, determinated by Cerenkov counting in H_2O at about 50% efficiency. Pure $tRNA^{Arg}$ (lot no. 15-141) was provided by Dr. A. D. Kelmers, Oak Ridge National Laboratories. Single-stranded DNA of the *E. coli* phage fd (Hoffmann-Berling et al., 1963) and calf thymus DNA (Colter et al., 1962) were prepared as described. Di- and trinucleotides used as substrates for S_1 nuclease were isolated from enzymatic digests of RNA (Rushizky and Sober, 1963) and DNA (Roberts et al., 1962).

Adsorbents. DEAE-cellulose (0.8 mequiv/g) and CM-cellulose (0.64 mequiv/g) were obtained from Schleicher & Schuell, Keene, N.H. Sephadex G-100 was obtained from Pharmacia. Columns were equilibrated until the effluent and influent pH and conductivity were the same.

Assays for Nucleases. To 0.8 ml of assay solution (0.8 mg of MS2 RNA or fd DNA in either 0.1 *M* sodium acetate (pH 4.3)–0.001 *M* $ZnSO_4$ or 0.1 *M* Tris-Cl (pH 7.5)) was added 0.2 ml of diluted enzyme solution and the mixture held at 42° for 15 min. The reaction was stopped by the addition of 1 ml of 6% $HClO_4$ at 4°. After 15 min in ice, the precipitate was removed by centrifugation, 0.5 ml of the supernatant was diluted with 4.5 ml of water, and the A_{260} was measured against an appropriate blank. A range of dilutions was used in each assay. An increase in A_{260} of 1.0 was defined as 1 unit of enzyme activity. With the same nucleic acid solution, the reproducibility of this assay was better than ±6%.

Purification of S_1 Nuclease. Dried mycelia of *Aspergillus oryzae* (Takadiastase, Sankyo Co., Ltd., Tokyo, Japan), 20 g, were extracted, heated to 70°, and treated with $(NH_4)_2SO_4$ as described by Vogt (1973). All further steps were carried out at 4°. After centrifugation, the saturated ammonium sulfate supernatant was free of S_1 nuclease ac-

†From the Developmental Biochemistry Section, Laboratory of Nutrition and Endocrinology, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014. *Received March 4, 1975.*

‡State Scientific-Research Institute of Standardization and Control of Drugs, MS, Moscow, USSR.

§Deceased; November 26, 1974.

tivity. The precipitate was dialyzed for a total of 3 days vs. four 10-l. volumes of H_2O and then vs. 10 l. of 0.05 *M* sodium acetate (pH 5.0) plus 0.001 *M* $ZnSO_4$ (starting buffer, SB). The dialysate, 134 ml, was loaded on a 4 × 30 cm DEAE-cellulose column equilibrated with SB and eluted with a 5-l. linear gradient from SB to 0.7 *M* NaCl plus SB at a flow rate of 46 ml/hr. All fractions of the S_1 nuclease peak were pooled, dialyzed for 2 days vs. two 10-l. volumes of 0.02 *M* ammonium acetate (pH 4.4), and loaded on a 4 × 23 cm CM-cellulose column equilibrated with the same buffer at a flow rate of 30 ml/hr. The column was washed with 250 ml of 0.02 *M* ammonium acetate (pH 4.4) and the eluates were combined and dialyzed for three 8-hr changes vs. 10-l. volumes of H_2O. The crude S_1 preparation (about 700 ml) was precipitated with 2 volumes of acetone (Rushizky and Sober, 1962) dissolved in 15 ml of 2 *M* NaCl–0.001 *M* $ZnSO_4$–0.02 *M* Tris-HCl (pH 8.0) and further purified by passage through a 5 × 72 cm Sephadex G-100 column equilibrated with the latter buffer at a flow rate of 11 ml/hr. The enzyme emerged from the column before a wide A_{280} peak. Highly active S_1 nuclease fractions were pooled, dialyzed for at least 8 hr against two 10-l. volumes of 0.001 *M* NH_4EDTA (pH 6.8) and 5% glycerol, and stored at an A_{280} of 0.1 or more over $CHCl_3$ at 4°. The S_1 nuclease preparation was stable for at least 3 months under these conditions. All tests described below were conducted with suitable dilutions of such enzyme preparations.

Linearity of assay, pH optimum, EDTA inactivation, Zn^{2+} requirement, activity toward double-stranded nucleic acid (calf thymus DNA), and ratio of activities toward single-stranded RNA and DNA (MS2 RNA/fd DNA) were performed according to Vogt (1973). To test for contaminating phosphatases, MS2 RNA and fd DNA were hydrolyzed to 5′-mononucleotides and the digests examined by paper chromatography with solvent A which resolves nucleosides from nucleotides.

The possible presence of RNases such as T_1 and T_2 was checked by incubating S_1 nuclease in 0.4 *N* HCl for 24 hr at 0°, a treatment to which RNase T_1 and T_2, but not S_1 nuclease, are stable (Egami and Nakamura, 1969). After neutralization and salt removal by exhaustive dialysis, MS2 RNA digests prepared with treated S_1 nuclease were examined by polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis was performed in an EC 490 cell (E. C. Apparatus Corp., St. Petersburg, Fla.) using a gel slab (0.3 × 17 × 24 cm) and a 16-place Teflon slot former producing 1 mm × 1 cm sample wells. A volume of 25–50 μl/slot was used. Cyanogum 41 (Fisher) or acrylamide and bisacrylamide in the same weight ratio of 19:1 were used as obtained from Canalco, Rockville, Md. Electrophoresis with Peacock's buffer with 2.5% acrylamide–0.5% agarose composite gels, and staining with 0.005% "Stainsall" were performed as described (Dahlberg et al., 1969). Gel compositions in percent refer to total input concentration of monomer.

Enzymatic Hydrolysis of Nucleic Acids and Oligonucleotides. Diluted aliquots of the purified enzyme were held with 0.1–3.0 mg of substrate for 3 hr at 0, 23, or 42° in either 0.1 *M* sodium acetate (pH 4.3)–0.002 *M* $ZnSO_4$ (low salt buffer) or in 0.02 *M* sodium acetate (pH 4.3)–0.25 *M* NaCl–0.002 *M* $ZnSO_4$ (high salt buffer). Low salt buffer was used for base ratio determinations, and high salt buffer for all other S_1 nuclease digestions. The volume of the digests varied from 0.1–0.5 ml. After hydrolysis S_1 nuclease was removed, at 23°, by two extractions with an equal volume of phenol saturated with 0.1 *M* sodium acetate (pH 4.3). Traces of phenol were removed either with ether followed by aeration with N_2, or by precipitation of nucleic acid with 2 volumes of EtOH.

To compare enzymatic digests prepared with varying S_1 nuclease/substrate ratios, the amount of enzyme required to hydrolyze fd DNA or MS2 RNA to mononucleotides as above in low salt buffer was defined as *E*.[1]

Strip Chart Counting (SCC) of ^{32}P in Polyacrylamide Gel Strips. After electrophoresis, strips were cut out using the sample well and Bromophenol Blue marker position as guides, and sealed in a single thickness of plastic film (Saran wrap). The gels were counted in an Actigraph III thin-layer plate conveyor system with a micromil window (Model 1006) connected to a Model 8703 decade scaler and printer (all from Nuclear Chicago, Des Plaines, Ill.). The scaler was operated at 1050 V, with a time constant of 10 sec and a chart speed of 60 cm/hr. Total counts were obtained by summation of scaler counts set to a 0.5-min interval. The background correction (blank gels) was 6 cpm. Compared to scintillation counting of excised gel slices, the recovery of ^{32}P counts by SCC ranged from 0.6 to 0.8% (Rushizky and Mozejko, 1974).

Isoelectric Focusing (Vesterberg, 1970). All equipment and reagents were from LKB Producter, Stockholm. A 110-ml column (Model No. 8101) was employed with 1% ampholytes of pH range 3–10 or 4–6. Gradients were prepared with the LKB No. 8121 mixer and pumped into the column with 10–40 ml of protein (total A_{280} = 7–28) solution at 4 ml/min. Constant current levels were obtained after 24–48 hr at 4° and 500 V. The column was emptied at a flow rate of 2 ml/min, yielding 60 fractions of 1.8 ml.

Formaldehyde Denaturation of MS2 RNA (Boedtker, 1967). RNA at 1 mg/ml was held for 15 min at 80° in 2% formaldehyde–0.05 *M* sodium borate (pH 8.5) and quick-cooled in ice.

Hydroxylapatite Chromatography. Hydroxylapatite (Main et al., 1959) was employed as described by Kohne and Britten (1971) at 50°. Calf thymus DNA, MS2 RNA, $tRNA^{Arg}$, or large oligonucleotides prepared by S_1 nuclease hydrolysis of MS2 RNA were used. About 0.2 mg of nucleic acid material/ml of packed bed volume was loaded in 0.14 *M* sodium phosphate (pH 6.8) and washed, and the bound fraction (double-stranded nucleic acid) was eluted with 0.5 *M* sodium phosphate (pH 6.8). Recoveries of uv-absorbing material were better than 96%. Loading and elution of samples were at flow rates of 0.5 ml/min, and new adsorbent was used for each experiment.

Heat denaturation of nucleic acid samples was carried out in an ACTA III spectrophotometer (Beckman Instruments, Fullerton, Calif.) and jacketed cells, in 0.2 *M* NaCl–0.01 *M* sodium phosphate (pH 6.8)–0.001 *M* NH_4EDTA (pH 6.8) at a temperature rise of 20°/hr.

Resistance of Nucleic Acid Material to RNase Digestion (Gillespie and Spiegelman, 1965; Monckton and Naora, 1974). As a test for double-stranded RNA, samples were held at 0 or 37° in 0.02 *M* Tris-HCl (pH 7.5), 0.002 *M* $ZnSO_4$, and 0.25 *M* NaCl for 14–18 hr with pancreatic RNase and RNase T_1 each at enzyme/substrate ratios of 1:20. Fractionation of digests was by paper chromatography with solvent A. The material left at the origin ("core") and

[1] Abbreviations used are: *E*, the amount of S_1 nuclease required to hydrolyze 1 mg of nucleic acid to mononucleotides; SCC, strip chart counting of ^{32}P-labeled oligonucleotides in polyacrylamide gel strips.

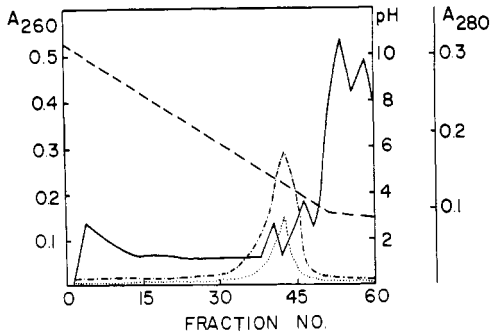

FIGURE 1: Isoelectric focusing of S_1 nuclease after DEAE-cellulose chromatography at pH 5.0 as described in the text. The fractions were assayed for RNase and DNase activity after 1:1 dilution with water since 50% sucrose did not affect S_1 nuclease activity; pH (- - -), A_{280} (—). Enzymatic activity with fd DNA (—·—·) or MS2 RNA (. . .) was assayed as described in the text and is presented as acid-soluble A_{260}.

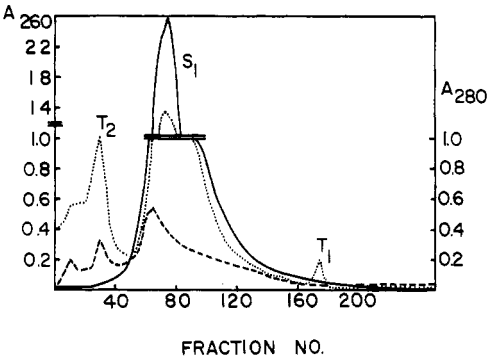

FIGURE 2: Chromatography of crude S_1 nuclease preparations on DEAE-cellulose at pH 5.0. Contaminating enzyme activities were identified as described in the text; A_{280} (- - -). Enzymatic activity with fd DNA (—) or MS2 RNA (. . .) was assayed as given in the text and is presented as acid-soluble A_{260}.

Table I: Purification of S_1 Nuclease from 20 g of Sankyo Takadiastase.[a]

	Total Protein as A_{280}	Enzyme Units with DNA	Specific Activity DNase/ A_{280}	DNase/ RNase	% Recovery
Crude extract	16,000	8,200	0.51	0.18	100
After heat step	14,300	5,700	0.40	1.3	70
After $(NH_4)_2SO_4$ precipitation	615	5,300	8.7	1.5	65
After DEAE-cellulose at pH 5.0	150	4,800	32	1.9	58
After CM-cellulose at pH 4.4	112	4,600	41	2.0	56
After Sephadex G-100	4.3	1,310	306	2.0	16

[a] For a description of enzyme units and enzyme assay, see text. Specific activity is defined as total units of enzyme/total A_{280}.

Table II: Release of Mononucleotides in Partial S_1 Nuclease Digests in Low Salt Buffer of MS2 RNA or fd DNA.[a]

Substrate	Units of Enzyme per mg per substrate	% Mononucleotide Released C	A	G	U (or t)	Comments
MS2 RNA	0.08		Trace		Trace	
	0.14		21		15	
	0.55	31	41	29	41	
	1.70		Set to 100%			Molar ratios C/A/G/U = 25:23:27:24
	1.70		Trace		Trace	No Zn^{2+} present
fd DNA	0.075		Trace		Trace	
	0.37	11	14	11	10	
	0.55	34	44	32	36	
	1.10	51	58	56	48	
	3.30		Set to 100%			Molar ratios C/A/G/T = 20:25:20:35
	3.30		Trace		Trace	No Zn^{2+} present

[a] 1.5–2.0 mg of substrate and diluted enzyme were held in 0.5 ml of 0.1 *M* sodium acetate (pH 4.3) and 2 m*M* $ZnSO_4$ for 3 hr at 23° and fractionated by paper electrophoresis and paper chromatography (Rushizky and Sober, 1963). The results shown are the average of triplicate analyses used for each stage of hydrolysis. "Trace" indicates that the spots were seen in the relevant areas but were not present in amount sufficient for spectrophotometry, i.e., less than a total A_{260} of 0.15.

mononucleotides were eluted with 0.03 *M* NaOH at 37° for 14–18 hours, and recoveries of A_{260} were compared to those eluted from blank areas.

Results

S_1 nuclease of *Aspergillus oryzae* was purified by the procedure of Vogt (1973), modified to detect and remove RNase T_1 and T_2 produced by the same mold. To facilitate purification by ion-exchange chromatography, isoelectric focusing over two pH ranges, 3–10 (Figure 1) and 4–6, was performed to locate the isoelectric point of S_1 nuclease, which was found to be 4.3–4.4. Since the isoelectric points of RNase T_1 and T_2 are at pH 2.9 and 5.0, respectively (Egami and Nakamura, 1969), DEAE-cellulose chromatography of S_1 nuclease was performed at pH 5.0 rather than at pH 7.5 as described by Vogt (1973). RNase T_1, T_2, and S_1 were thus separated from each other (Figure 2). The two RNases were identified by their lack of DNase activity, by their enzymatic activity ratios at pH 4.5/7.5 of about 20 for RNase T_2 and about 0.5 for RNase T_1, by their stability to 0.4 *N* HCl at 4° for 24 hr, and by persistence of enzymatic activity in 0.05 *M* EDTA (Egami and Nakamura, 1969); as well as by patterns of mono- and oligonucleotides obtained by mapping of their enzymatic digests of MS2 RNA (Rushizky and Sober, 1962, 1963).

Further purification of S_1 nuclease was achieved by chromatography on CM-cellulose at pH 4.4 under conditions where RNase T_2 was bound to the absorbent while S_1 nuclease was not. After concentration and exclusion chromatography on Sephadex G-100, purification was 600-fold and the yield 16% (Table I). The amount of enzyme so obtained from 20 g of Takadiastase was sufficient to hydrolyze about 0.75 g of MS2 RNA or 0.4 g of fd DNA to mononucleotides in low salt buffer at 23° (Table II). S_1 nuclease activity toward DNA was examined because of the characteristic property of the enzyme to hydrolyze both DNA and RNA. However, the difference in activity toward both substrates may be not significant or misleading since the more sensitive acid solubility assay (Table I) was found to depend on the previous history of the substrate. Thus, fd DNA was hydrolyzed twice as fast as heat-denatured (Roberts et al., 1962) calf thymus DNA, and heat denaturation rendered MS2 RNA a better substrate as well. The ratio found here for DNase/RNase activity of our S_1 nuclease was 2 (Table I) rather than 6–7 as observed by Vogt (1973). The S_1 nu-

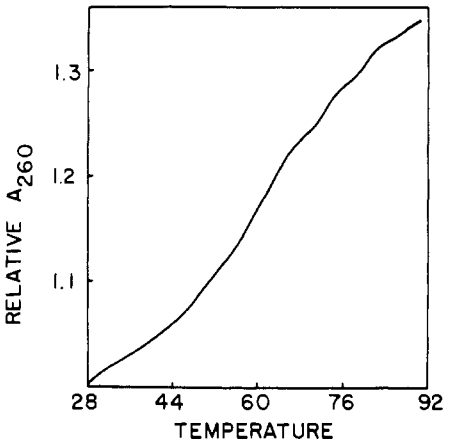

FIGURE 3: Heat denaturation of the resistant oligomer fraction (prepared with S_1 nuclease at 0°, E = 18 in high salt buffer) in 0.2 M NaCl-0.01 M sodium phosphate (pH 6.8)-0.001 M NH_4EDTA (pH 6.8) at a temperature rise of 20°/hr.

clease prepared here also differed from that of Vogt with respect to activity in 0.3% sodium dodecyl sulfate, 1 M urea, or 5% formamide. DNA/RNA hybridizations are often carried out in the presence of these denaturing agents. At 23° in low salt buffer, and at E = 18, the rates of hydrolysis of fd DNA or MS2 RNA to acid-soluble products were reduced to 12, 21, and 23% and 15, 24, and 11% respectively, of those of controls. Vogt (1973) observed no decrease in S_1 nuclease activity in the presence of any one of the three reagents. On the other side, our S_1 nuclease exhibited properties similar to the enzyme isolated by Vogt. Thus, the pH optimum, Zn^{2+} requirement, and inability of both enzymes to hydrolyze native calf thymus DNA were alike.

As shown in Table II, S_1 nuclease digests of MS2 RNA or fd DNA showed no marked preference of the enzyme for hydrolysis next to a particular base. Complete digests of the nucleic acids to nucleotide 5-monophosphates gave correct base ratios (Strauss and Sinsheimer, 1963; Hoffmann-Berling et al., 1963) in better than 97% yields.

Table II also shows that S_1 nuclease, dialyzed for 24 hr against 0.05 M NaCl-0.001 M EDTA-0.05 M sodium acetate (pH 4.3), was inactive toward MS2 RNA or fd DNA. Treatment of S_1 nuclease with 0.4 N HCl for 24 hr at 0° followed by neutralization and salt removal by dialysis destroyed all enzymatic activity vs. MS2 RNA or fd DNA as verified by polyacrylamide gel electrophoresis with 6% gels. S_1 nuclease hydrolysis of short oligonucleotides derived from DNA (TpCp, ApCp, ApGpGp, TpTpGp, TpGpGp) or RNA (CpGp, ApGp, CpCpGp, ApApCp, ApCpCp) to mononucleotides required amounts of enzyme similar to those required for the hydrolysis of nucleic acids.

Fractionation of partial digests of MS2 RNA or fd DNA by two-dimensional electrophoresis and chromatography or by paper chromatography with solvent A revealed no oligomers of chain lengths 2–8; instead, only mononucleotides and material larger than N = 8, i.e., that did not move away from the origin (core), were found.

The hydrolysis of MS2 RNA in high salt buffer by S_1 nuclease at 0°, E = 2–18 for 17 hr was also characterized by polyacrylamide gel electrophoresis. MS2 RNA yielded a series of distinct oligomers on 6% gels containing 6 M urea. There were little if any changes between the distribution and stain uptake of the oligomers prepared at E = 2 or E = 18. After phenol extraction of the digests to remove the enzyme, preparative isolation of the oligomers was carried out by precipitation with 2 volumes of EtOH. After centrifugation, the precipitate was dissolved in 0.1 M Tris-HCl (pH 7.5) and the EtOH step repeated. The residue, dissolved in H_2O, contained 70% of the A_{260} of the MS2 RNA used.

To characterize the material in this "resistant fraction" aliquots were used for various tests.

Paper chromatography with solvent A revealed no mono- or oligonucleotides of chain length less than 8, and acrylamide electrophoresis through 4% gels containing 6 M urea indicated the absence of MS2 RNA.

On hydroxylapatite under conditions where all but 2–4% of calf thymus DNA was bound in 0.14 M sodium phosphate at 50°, only 18–21% of the A_{260} of the "resistant fraction" were bound in 0.14 M and eluted by 0.5 M sodium phosphate. As controls, 27% of purified $tRNA^{Arg}$ and 62% of MS2 RNA were similarly found in the 0.5 M sodium phosphate eluent. Since recoveries of all nucleic acid samples were better than 96%, these results show low but similar amounts of double-stranded segments in both the resistant fraction and $tRNA^{Arg}$.

The resistant fraction was also checked for double-stranded RNA by resistance to pancreatic RNase-RNase T_1 in 0.25 M NaCl-0.002 M $ZnSO_4$-0.02 M Tris-HCl (pH 7.5) at 37 or 0°. The percent of double-stranded RNA was determined by the A_{260} recovered in the core fraction after paper chromatography with solvent A. RNase treatment of the resistant fraction at 37 or 0° reduced the percent of A_{260} in the cores to 2 and 5%, respectively. As a control purified $tRNA^{Arg}$ was treated with both RNases at 37 or 0° under the same conditions as the resistant fraction, and the amount of A_{260} in the cores found to be less than 5% at both digestion temperatures. Furthermore, a second treatment of the resistant fraction with S_1 nuclease in high salt buffer at E = 18 and 37° reduced the A_{260} core fraction to 8%, while at 0° more than 92% of the total A_{260} were recovered as core material. Stability of the resistant fraction to nuclease treatment was thus only obtained with the S_1 enzyme at 0°.

This stability was lost when MS2 RNA was treated with formaldehyde at 80° before hydrolysis by S_1 nuclease at E = 18 at 0° and in high salt buffer. Denaturation of the MS2 RNA rendered it susceptible to the enzyme since 95% of the A_{260} was converted to mononucleotides and no core material was found as determined by paper chromatography with solvent A. Similarly, on 6% acrylamide gels, no material was visible after staining with Stains-all.

In the presence of 0.3% sodium dodecyl sulfate, 1 M urea, or 5% formamide, S_1 nuclease at E = 2, at 0 or 23°, and in high or low salt buffer produced no large oligomers from MS2 RNA. However, this was not due to instability of the resistant fraction, but because the RNA was not hydrolyzed at all as visualized by electrophoresis of such digests on 6% acrylamide-6 M urea gels.

Thermal denaturation of the resistant fraction in 0.2 M NaCl showed a compound transition with no clear melting point, and an increase in A_{260} of 35% at 93° over that at 28° (Figure 3). Thus, melting of the resistant fraction resembled that of tRNAs (Bina-Stein and Crothers, 1974).

For quantitation of the resistant fraction, [^{32}P]MS2 RNA hydrolysates were analyzed by SCC of 6% gels containing 6 M urea. As shown in Table III, 87–91 and 78–84% of the total ^{32}P were so recovered in the large oligomer region of the gels at E ratios of 2 and 18, respectively. The

Table III: Strip Chart Counting of ^{32}P in S_1 Nuclease Digests in High Salt Buffer of [^{32}P]MS2 RNA Fractionated by Polyacrylamide Gel Electrophoresis.[a]

	Total Cpm of ^{32}P Found in Areas of		
E Ratio	MS2 RNA	Large Oligomers	
E = 0	4300	0	See Figure 3A
at 0°	4501	0	
	4400	0	
	4414	0	
E = 2	310	4031	
at 0°	141	4150	
	185	3841	
	0	3980	
	93	3879	
E = 18	0	3470	
at 0°	0	3781	
	0	3600	
	0	3512	
	0	3523	See Figure 3B
	0	3634	
E = 18	0	1830	See Figure 3C
at 23°	0	1717	
	0	1200	

[a] [^{32}P]MS2 RNA was hydrolyzed for 3 hr at 0 or 23° in 0.02 *M* sodium acetate (pH 4.3)–0.25 *M* NaCl–0.002 *M* $ZnSO_4$ with varying amounts of S_1 nuclease. The digests (600,000 cpm/gel strip) were fractionated in 6% acrylamide–6 *M* urea gels and recovery of ^{32}P activity was determined by strip chart counting (see text). Because of the low efficiency of SCC, the amount of ^{32}P so accounted for ranged from 4300 to 4500 cpm measured with gels containing unhydrolyzed MS2 RNA. Lower amounts of ^{32}P in 3B and 3C are due to migration out of the gel of larger amounts of mononucleotides. *E* = 1 is defined as the amount of S_1 nuclease (1.7 units, see Table II) required to hydrolyze 1 mg of MS2 RNA to mononucleotides upon digestion for 3 hr at 23° in 0.1 *M* sodium acetate (pH 4.3)–0.002 *M* $ZnSO_4$.

mol wt of the major oligomer peaks, determined by electrophoresis through 2.5% acrylamide–0.5% agarose, ranged from 25,000 to 41,000 (Figure 4B). At 23°, resistance of large oligomers to high amounts of S_1 nuclease was considerably reduced compared to that at 0°, both in quantity (^{32}P counts) and size (oligonucleotide band distribution). Thus, about 3520/4401 cpm × 100 = 80% of the total cpm of [^{32}P]MS2 RNA fractionated per gel was recovered in the large oligomer region (Figure 4B) with digests prepared at *E* = 18 and 0°. By contrast, hydrolysis of [^{32}P]MS2 RNA under identical conditions but at 23° instead of 0° produced few if any oligomers of mol wt 25,000 or larger, and the yield of total cpm per gel decreased to 1830/4401 cpm × 100 = 41%. This showed that about half of the material in Figure 4B was reduced in size to such an extent that it moved out of the gel during electrophoresis.

Discussion

At present, the mechanism leading to the single-stranded specificity of S_1 nuclease is not understood. It is clear, however, that double-stranded DNA molecules are attacked by the enzyme as well if they are either nicked by physical means or strained by superhelix formation and that this action depends more on salt and temperature conditions than the hydrolysis of single-stranded DNA (Beard et al., 1973; Méchali et al., 1973; Germond et al., 1974). S_1 nuclease was also reported to hydrolyze duplex DNA, although at a much slower rate than single-stranded DNA (Godson, 1973; Johnson and St. John, 1974), but this has been questioned (Beard et al., 1973). The latter disagreement may be due to the use of impure S_1 preparations since *Aspergillus oryzae* also produces one or more endonucleases that hydrolyze double-stranded DNA (Ando, 1966). We considered the possible presence of such DNases an indication of RNase contamination as well, even though electrophoresis through polyacrylamide gels in dodecyl sulfate buffers revealed no extraneous protein (Godson, 1973; Vogt, 1973; Johnston and St. John, 1974). The use of S_1 nuclease for RNA/DNA hybridizations would not reveal RNases since these are routinely added for the removal of nonhybridized RNA (Gillespie and Spiegelman, 1965).

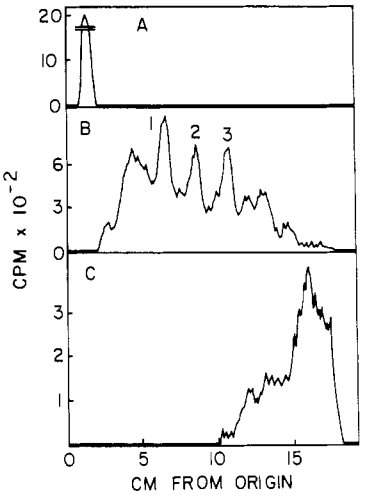

FIGURE 4: Strip chart counting of ^{32}P in S_1 nuclease digests of ^{32}P-labeled MS2 RNA fractionated by polyacrylamide gel electrophoresis as described in Table III. (A) Unhydrolyzed MS2 RNA, total cpm 4300, counted on 0–5000-cpm range. (B) MS2 RNA, hydrolyzed at *E* = 18 and 0°, total cpm 3523, counted on 0–1500-cpm range. Molecular weights of peaks 1 = 41,000, 2 = 34,000, 3 = 25,000. (C) MS2 RNA hydrolyzed at *E* = 18 as in (B) but at 23°. Total cpm 1830 counted on 0–500-cpm range. Electrophoresis is from left to right.

S_1 nuclease preparations purified through the Sephadex G-100 step of Vogt's (1973) procedure were found to contain RNase T_2, regardless of whether crude amylase (Sigma) or Takadiastase (Sankyo) were used as starting material. Heating crude S_1 nuclease to 70° resulted in inactivation of contaminating nucleases (Vogt, 1973), which we confirm (Table I). However, the heat step is more efficient for the removal of RNase T_1 than RNase T_2. Thus in Takadiastase the ratio of RNase T_1/RNase T_2 activity is about 10 (Egami and Nakamura, 1969) while after heating this ratio is about 0.1 (Figure 2). Sigma amylase gave similar heat inactivation results as Sankyo Takadiastase.

Assay of S_1 nuclease in the mere presence of EDTA was not a suitable test for RNase contamination. While RNases T_1 and T_2 are fully active in 0.001 *M* NH_4EDTA (pH 6.8), so was S_1 nuclease. In fact, Sigma crude amylase appeared to contain amounts of S_1 nuclease that varied 2–5-fold between different lots, a discrepancy that could be abolished by 0.001 *M* EDTA at neutral pH.

Even in 0.05 *M* EDTA (Germond et al., 1974) S_1 nuclease inactivation was incomplete when checked by polyacrylamide gel electrophoresis of MS2 RNA digests. To demonstrate EDTA inactivation of the enzyme, extensive dialysis against 0.001 *M* EDTA in 0.05 *M* NaCl at pH 4.3 was required (Vogt, 1973).

In the absence of RNase contamination, S_1 nuclease was assumed to hydrolyze MS2 RNA in single-stranded portions at a much faster rate than in double-stranded portions.

If this assumption was correct, then MS2 RNA hydrolysis should be temperature and salt dependent by analogy to results obtained with superhelical SV40 DNA, where only one group of breaks was obtained in 0.25 *M* NaCl, but two groups of breaks were observed in 0.075 *M* NaCl, and where the rate of S_1 cleavage decreased fivefold in going from 0.01 to 0.25 *M* NaCl, as expected from the increase in the fraction of helical DNA with increasing ionic strength (Beard et al., 1973). Furthermore, after removal of single-stranded RNA, the remaining double-stranded portions of MS2 RNA should exist as oligomers resistant to further S_1 treatment. This differs from results obtained with enzymes such as RNase T_1 with which RNA hydrolysis at 0° in 0.02 *M* Mg^{2+} and 0.2 *M* NaCl continues upon addition of further enzyme so that there is a progressive increase in the amounts of smaller fragments produced (Holley, 1968; Adams et al., 1969). As shown by Min Jou et al. (1972), the segments of MS2 RNA for which the nucleotide sequence is known consist of a series of loops similar to those in the cloverleaf model of tRNAs (Nishimura, 1974). The loops may be folded over each other but do not have the double-stranded character of, for example, calf thymus DNA. On acrylamide gel electrophoresis in 6 *M* urea, such loops should open and the constituent fragments appear in distinct bands. Such bands were found in the molecular weight range of 25,000–41,000. For characterization on hydroxylapatite or by RNase resistance, the resistant oligomers were therefore compared with purified $tRNA^{Arg}$. Similar results but no evidence for helical content were obtained. In this respect, more information was deduced from the effect of formaldehyde denaturation of MS2 RNA prior to S_1 hydrolysis and from the melting behavior of the resistant oligomer fraction. Our data thus indicate that to S_1 nuclease, the tRNA-type loops in MS2 RNA appear similar to double-stranded nucleic acid portions. This facilitates their isolation in better yields than may be obtained with other nucleases lacking such a single-strand specificity. Nevertheless, definitive proof of the specific S_1 nuclease hydrolysis would require isolation and sequence determination of the postulated matching oligomers. In the case of MS2 RNA, this would amount to nucleotide sequence determination of about two-thirds of the molecule. Short of this, S_1 nuclease hydrolysis of single-stranded nucleic acids with partial double-stranded configurations should be of use for the isolation and characterization of large oligomers from such compounds.

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