Finally, as will be detailed in the following paper, the specificity of the two enzymes appears to be the same.

ACKNOWLEDGMENTS

We would like to thank Dr. H. K. Schachman and Miss F. Putney, who performed and analyzed the analytical ultracentrifuge run, and Drs. F. Egami and H. Fraenkel-Conrat, who provided the sample of ribonuclease T_1 used here. We are also indebted to Dr. R. D. Cole for valuable discussion regarding the reaction of *p*-mercuribenzoate with proteins.

REFERENCES

Barrett, R. J., Friesen, H., and Astwood, E. B. (1962), *J. Biol. Chem. 237*, 432.
Bernfeld, P., Nisselbaum, J. S., Berkeley, B. J., and Hanson, R. W. (1960), *J. Biol. Chem. 235*, 2852.
Bonhoeffer, F., and Schachman, H. K. (1960), *Biochem. Biophys. Res. Commun. 2*, 366.
Boyer, P. D. (1954), *J. Am. Chem. Soc. 76*, 4331.
Doty, P., Boedtker, H., Fresco, J. R., Hall, B. D., and Haselkorn, R. (1959), *Ann. N.Y. Acad. Sci. 81*, 693.
Edsall, J. T. (1953), *Proteins 1B*, 634.
Fellig, J., and Wiley, C. E. (1959), *Arch. Biochem. Biophys. 85*, 313.
Garen, A., and Levinthal, C. (1960), *Biochim. Biophys. Acta 38*, 470.
Glitz, D. G., and Dekker, C. A. (1963), *Biochemistry 2*, 1185.
Glitz, D. G., and Dekker, C. A. (1964), *Biochemistry 3*, 1399 (this issue; following paper).
Gundlach, H. G., Stein, W. H., and Moore, S. (1959), *J. Biol. Chem. 234*, 1754.
Kalnitsky, G., Hummel, J. P., Resnick, H., Carter, J. R., Barnett, L. B., and Dierks, C. (1959), *Ann. N. Y. Acad. Sci. 81*, 542.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236*, 1372.
Michelson, A. M. (1959), *J. Chem. Soc.*, 3655.
Nishimura, S. (1960), *Biochim. Biophys. Acta 45*, 15.
Nishimura, S., and Novelli, G. D. (1963), *Biochem. Biophys. Res. Commun. 11*, 161.
Rushizky, G. W., Greco, A. E., Hartley, R. W., and Sober, H. A. (1963), *Biochem. Biophys. Res. Commun. 10*, 311.
Sato, K., and Egami, F. (1957), *J. Biochem. (Tokyo) 44*, 753.
Shapira, R. (1962), *Anal. Biochem. 3*, 308.
Spackman, D. H., Stein, W. H., and Moore, S. (1960), *J. Biol. Chem. 235*, 648.
Sueoka, N., and Cheng, T. Y. (1962), *J. Mol. Biol. 4*, 161.
Tabor, H., Tabor, C. W., and Rosenthal, S. M. (1961), *Ann. Rev. Biochem. 30*, 579.
Takahashi, K. (1961), *J. Biochem. (Tokyo) 49*, 1.
Tanford, C., and Hauenstein, J. D. (1956), *J. Am. Chem. Soc. 78*, 5287.
Ui, N., and Tarutani, O. (1961), *J. Biochem. (Tokyo) 49*, 9.
Yamagata, S., Takahashi, K., and Egami, F. (1962), *J. Biochem. (Tokyo) 52*, 272.

Studies on a Ribonuclease from *Ustilago sphaerogena*. II. Specificity of the Enzyme*

DOHN G. GLITZ† AND CHARLES A. DEKKER

From the Department of Biochemistry, University of California, Berkeley

Received September 24, 1963; revised July 7, 1964

Hydrolysis of RNA by the ribonuclease from *Ustilago sphaerogena* results in the formation of 3′-guanylic acid and oligonucleotides ending in guanosine-3′-phosphate. The enzyme acts first through the formation of guanosine-2′,3′-cyclicphosphate, and then more slowly hydrolyzes the cyclic ester to form the 3′-nucleotide. A copolymer of adenylic acid and guanylic acid is attacked at all guanylic acid residues, resulting in the formation of guanosine-2′,3′-cyclicphosphate, guanosine-3′-phosphate, and a series of oligonucleotides composed of adenylic acid but ending in a single guanylic acid residue. The enzyme is equally active on a number of RNA and polynucleotide substrates containing guanylic acid, and slightly less active on polymers containing inosinic acid. Polyadenylic acid is hydrolyzed at 0.002% the rate of RNA, while polyuridylic and polycytidylic acids, DNA, and bis-(*p*-nitrophenyl)phosphate are inert to the enzyme. Methylated albumin-kieselguhr chromatography has been used to examine partial degradation products of s-RNA during the digestion by a number of nucleases. The *Ustilago* enzyme initially produces large fragments from the RNA substrate, and then gradually reduces these fragments to small oligonucleotides.

In the preceding paper (Glitz and Dekker, 1964) the purification of an extracellular ribonuclease produced by the smut fungus *Ustilago sphaerogena* was described. The enzyme was obtained in a form which was judged to be highly purified, as indicated by electrophoretic homogeneity, the formation of a single sharp boundary in the ultracentrifuge, and a specific activity equal to or greater than those of other nucleases. In this paper the specificity and the mode of action of the nuclease will be considered.

* This work was supported in part by a grant (GB-882) from the National Science Foundation and in part by a U. S. Public Health Service Fellowship (GPM-18,787) from the National Institutes of Health.

† Submitted in partial fulfillment of the requirements for the Ph.D. degree in biochemistry at the University of California, Berkeley.

MATERIALS AND METHODS

Enzymes.—An extracellular ribonuclease from *U. sphaerogena* was prepared as described in the preceding paper (Glitz and Dekker, 1964). Unless otherwise stated, enzyme of specific activity 175,000 units/mg protein was used. Pancreatic ribonuclease A (Worthington Biochemical Co.) was rechromatographed by Dr. M. Irie of this laboratory and was a homogeneous preparation of specific activity 55,000 units/mg pro-

tein. The sample of micrococcal nuclease was purified by Miss G. Herold, using acetone and ammonium sulfate fractionation followed by chromatography on IRC-50 resin. The specific activity of this preparation was about 11,500 units/mg protein. All activities were measured in the assays described in the previous paper, except that in the case of micrococcal nuclease a *p*H of 8.6 was maintained using 0.04 M ammonium hydroxide–ammonium chloride buffer, and calcium chloride was present at a concentration of 0.005 M.

Alkaline phosphatase from *Escherichia coli* was purchased from Worthington. The preparation of snake venom phosphodiesterase was a gift of Dr. M. Laskowski and was believed to be quite free of endonuclease activity (see Georgatsos and Laskowski, 1962). Polynucleotide phosphorylase was prepared from cells of *Micrococcus lysodeikticus* (Miles Chemical Co., Clifton, N. J.) through the second ammonium sulfate step of the method of Singer and Guss (1962).

Nucleic Acids and Polynucleotides.—Wheat germ s-RNA and high-molecular-weight RNA were prepared as previously described (Glitz and Dekker, 1963). Ribosomal RNA from *E. coli* was a gift of Dr. B. Lane, while yeast RNA was a commercial product (Schwarz) which had been subjected to repeated acid and alcohol precipitation (Frisch-Niggemeyer and Reddi, 1957). RNA from tobacco mosaic virus (TMV-RNA) was a gift of Dr. H. Fraenkel-Conrat. Salmon sperm DNA was a high-molecular-weight product purchased from Calbiochem.

Biosynthetic polynucleotides were obtained from a number of sources. Polyadenylic acid (poly-A) and polyuridylic acid (poly-U), synthesized using polynucleotide phosphorylase, were purchased from the Miles Chemical Co. Polycytidylic acid (poly-C) was a gift of Dr. S. Ochoa. A mixed polymer of adenylic and guanylic acids (poly-AG)[1] was synthesized according to the methods outlined in Steiner and Beers (1961), using polynucleotide phosphorylase and the corresponding nucleoside diphosphates (Sigma). The product contained 40% guanylic acid, as determined by spectral data and analysis of alkaline hydrolysates. A mixed polymer of adenylic and inosinic acids (poly-AI) was prepared by deamination of poly-A, using the conditions described by Wyatt (1951) but with reduction of the time of treatment to 2 hours. Analysis of the poly-AI sample indicated about 25% inosinic acid. Chemically synthesized oligonucleotides containing guanylic acid (oligo-G) and mixed adenylic and guanylic acids (oligo-AG) in both $2' \rightarrow 5'$ and $3' \rightarrow 5'$ linkages were gifts of Dr. A. M. Michelson, and were prepared as described by him (Michelson, 1959a).

Other Materials.—Nucleoside-2',3'-cyclicphosphates were prepared from the corresponding nucleotides using the method of Michelson (1959b), and purified by paper chromatography using Whatman 3MM paper and a 2-propanol-ammonia-water (7:1:2) solvent system. Buffers of Tris (tris[hydroxymethyl]aminomethane, Sigma Chemical Co., Sigma 121) and imidazole (Aldrich Chemical Co., Milwaukee) were adjusted with HCl to the desired *p*H. All chemicals not otherwise specified were of reagent grade.

Methods.—The nature of the base in the mononucleotides and at the 3-phosphorylated end of the oligonucleotides formed by *Ustilago* nuclease action on RNA was established by spectral analysis of the nucleosides produced by sequential hydrolysis with phosphomonoesterase and alkali. Details are given in the legend to Figure 1.

DEAE-cellulose chromatography with 7 M urea in the elution buffers was performed in a manner similar to that described by Tomlinson and Tener (1962). Specific conditions can be found in the legends to Figures 1 to 3. Concentrated urea solution was deionized by passage through a column of mixed-bed resin (Bio-Rad AG 501) before incorporation into chromatographic buffers.

Mono- and oligonucleotides eluted from DEAE-cellulose columns were desalted using the method of Rushizky and Sober (1962), sometimes employing a linear gradient of ammonium carbonate to obtain fractionation of components. In one case salts were removed by dialysis against distilled water. Nucleosides and nucleotides were identified by spectral data and chromatographic and electrophoretic behavior. Paper chromatography was performed using two solvent systems: 2-propanol-ammonia-water (7:1:2), descending; and *p*H 7.1 sodium phosphate buffer, 0.1 M, containing ammonium sulfate at a concentration of 40 g/100 ml, ascending. Alkaline hydrolysates were spotted directly, unneutralized, for chromatography in the latter system. Paper electrophoresis was carried out at *p*H 3.5 using 0.05 or 0.1 M ammonium formate buffer.

Methylated albumin-kieselguhr chromatography was conducted as outlined in the previous paper (Glitz and Dekker, 1964). In a few instances the sodium chloride gradient was extended to 1.25 M, in which case the volume of eluting buffer was also increased such that in all elution patterns a given tube number represents the same salt concentration. Prior to chromatography wheat germ s-RNA was incubated with nucleases as described in the legends to Figures 7 to 10.

Results

End-Group Analysis of Nuclease-Digestion Products.—Preliminary experiments, in which RNA was digested with the *Ustilago* enzyme and then spotted directly on paper for either chromatography or electrophoresis, indicated that the only noncyclic mononucleotide formed by the action of the enzyme was guanosine-3'-phosphate. Upon alkaline hydrolysis of the digest little nucleoside was detected, further indicating that the enzyme produced 3'- rather than 5'-phosphorylated endings from RNA. The results of the experiment designed to establish the nature of the base at the newly formed 3'-phosphorylated ends are summarized in Table I. It is seen that only guanylic acid ends are formed in appreciable quantities as nuclease digestion proceeds.

Composition of Nuclease Digests of Polynucleotides.—Chromatography of *Ustilago* nuclease digests of RNA using DEAE-cellulose with 7 M urea present in the elution buffers resulted in patterns such as that illustrated in Figure 1. Very similar patterns were obtained using both sodium chloride and sodium acetate gradients in the elution scheme, with a variety of column dimensions and quantities of digest ranging from 5 to 100 mg. Digests of wheat germ s-RNA, high-molecular-weight RNA, and TMV-RNA were all similar, although the latter two sources generally gave a slightly better separation of peaks.

The components of the peaks of Figure 1 have been identified as follows: Peak A contained mostly protein, although some nucleoside and free base were also present. Peak A was quantitatively less important in

[1] Abbreviations used in this work: poly-AG, a mixed polymer of adenylic and guanylic acids; poly-AI, a mixed polymer of adenylic and inosinic acids; oligo-G, chemically synthesized oligonucleotides containing guanylic acid; oligo-AG, chemically synthesized oligonucleotides containing mixed adenylic and guanylic acids.

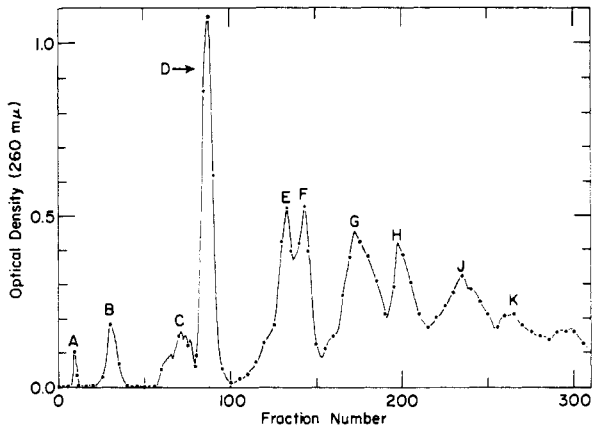

FIG. 1.—DEAE-cellulose chromatography of a *Ustilago* nuclease digest of s-RNA in the presence of urea. Twenty-five mg of wheat germ s-RNA was hydrolyzed for 24 hours at 28° using 55 μg of a *Ustilago* nuclease preparation of specific activity 160,000 units/mg protein. The digest was then chromatographed using a column of DEAE-cellulose of dimensions 2.3 cm diameter by 21 cm length and an elution buffer containing 7 M urea and a linear gradient of sodium acetate ranging from 0.0 to 0.4 M. A total elution volume of 3 liters was used. Fractions of 10 ml were collected using a flow rate of 0.5 ml/min. Partial identification of the components of peaks A-K is presented under Results.

TABLE I

3′-PHOSPHATE END-GROUP DETERMINATION OF *Ustilago* NUCLEASE DIGESTION PRODUCTS[a]

Sample	Nucleoside Content (OD units)		Nucleotide Content (OD units)	Per Cent Nucleoside
	Guanosine	Others		
Control				
0 hours	0.9	4.7	240	2.3
4 hours	0.8	2.8	206	1.7
24 hours	2.6	4.1	280	2.4
Experimental				
5 minutes	6.4	3.6	233	4.1
15 minutes	6.5	2.7	185	4.9
4 hours	7.0	2.6	180	5.1
24 hours	19.4	5.0	160	13.2

[a] Sixty mg of wheat germ s-RNA was digested with 60 μg of *Ustilago* nuclease in a total volume of 12 ml of imidazole-albumin buffer, *p*H 7.0. At the time periods indicated 10-mg aliquots of RNA were removed from the reaction mixture, treated with 60 mg of bentonite to remove the nuclease, adjusted to *p*H 8, and incubated for 4 hours at 28° with 25 μg of *E. coli* phosphomonoesterase. The aliquot was then hydrolyzed in 1 M KOH for 24 hours at 30°, neutralized with Dowex-50 (H^+), and chromatographed on a column of Dowex-1 formate of dimensions 1.5 cm diameter by 13 cm length. Nucleosides were fractionated using 0.005 M ammonium formate buffer, *p*H 3.7, and nucleotides eluted using 2.0 M ammonium formate buffer. Control samples were treated identically, except that the *Ustilago* nuclease was left out of the incubation mixture. All quantities are expressed in terms of optical density units measured at 260 mμ.

digests of TMV-RNA and wheat germ high-molecular-weight RNA. Only guanosine-2′,3′-cyclicphosphate (G-cyclic-p) was found in peak B. Three components were isolated from peak C using chromatography on DEAE-cellulose with an ammonium carbonate gradient. Each component produced guanylic acid and one other nucleotide (cytidylic, uridylic, or adenylic acids) upon alkaline hydrolysis. Incubation of the

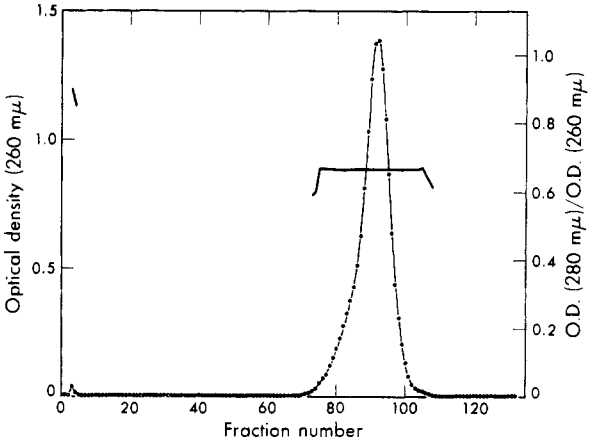

FIG. 2.—Rechromatography of mononucleotide from a *Ustilago* nuclease digest. Fifty mg of wheat germ s-RNA was hydrolyzed and chromatographed as in Fig. 1, except that a column 45 cm long was used with 4 liters of elution buffer containing a sodium chloride gradient of 0.0–0.4 M in 7 M urea. The mononucleotide fraction, peak D of Fig. 1, was then diluted to 1 liter, the *p*H was adjusted to 9 with ammonium hydroxide, and the fraction was adsorbed on a column of Dowex-1 (formate) of dimensions 1.3 cm diameter by 7 cm length and then eluted using a parabolic gradient of ammonium formate buffer, *p*H 3.7, ranging from 0.02 to 1.0 M. Ten-ml fractions were collected using a flow rate of 0.5 ml/min.

components containing cytidylic or uridylic acid with pancreatic ribonuclease produced 3′-cytidylic acid or 3′-uridylic acid plus G-cyclic-p, indicating that the original sequences were CpG-cyclic-p and UpG-cyclic-p. Peak D contained only a single mononucleotide, guanosine-3′-phosphate. Rechromatography of peak D using Dowex-1 is shown in Figure 2. Peaks E and F were fractionated into three major components by DEAE-cellulose chromatography using an ammonium carbonate gradient. Each of these components contained guanylic acid plus one of the other common nucleotides. Treatment with pancreatic ribonuclease resulted in the hydrolysis of the compounds containing cytidylic or uridylic acids as one nucleotide, giving 3′-guanylic acid plus the appropriate pyrimidine-3′-nucleotide. Alkaline hydrolysis of the third fraction, most of which was found in peak F, gave both the 2′ and 3′ isomers of adenylic acid but only the 3′ isomer of guanylic acid. The original sequences were thus identified as CpGp, UpGp, and ApGp. Peaks G to K were assumed to be oligomers of greater chain length.

A biosynthetic polymer consisting only of adenylic and guanylic acids (poly-AG) was digested using the *Ustilago* enzyme and then chromatographed in the DEAE-cellulose system with 7 M urea buffers. The elution pattern is shown in Figure 3. The individual peaks have been identified on the basis of elution position and optical-density ratio (plotted in the heavy line in Fig. 3). Peak A is primarily G-cyclic-p, while peak B is guanylic acid (Gp). Peak C is the dinucleotide of adenylic and guanylic acids, ApGp, and peak D is the trinucleotide ApApGp. Peaks E through M are believed to be oligomers of increasing chain length of adenylic acid ending in a single guanylic acid residue (i.e., ApApApGp, ApApApApGp, etc.). The sequences postulated above are confirmed by the data obtained in the end-group determination, illustrated in Figure 4. The sole nucleoside found is guanosine (peak 1). Both the 2′ and 3′ isomers of adenylic acid (peak 2) are found, indicating that this material arose from alkaline hydrolysis of internal portions of the

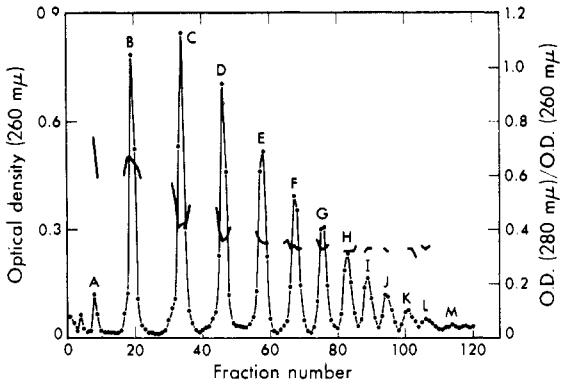

FIG. 3.—Fractionation of a *Ustilago* nuclease digest of poly-AG. Five mg of poly-AG was incubated at 28° for 24 hours with 75 μg of *Ustilago* nuclease, specific activity 175,000 units/mg protein, and adsorbed on a column of DEAE-cellulose of dimensions 1.4 cm diameter by 17 cm length. Chromatography was conducted using 1 liter of 0.02 M Tris hydrochloride, *p*H 7.5, and 7 M urea, employing a linear gradient of 0.0–0.4 M NaCl. Fractions of 8 ml were collected using a flow rate of 0.5 ml/min. Peaks A through M have been identified as G-cyclic-p, Gp, ApGp, ApApGp, etc.

TABLE II

ACTIVITY OF *Ustilago* NUCLEASE WITH VARIOUS POTENTIAL SUBSTRATES[a]

Substrate	Method of Assay Used in Comparison: RNAase Assay	*p*H-stat	Chromatography	Relative Activity
s-RNA, wheat germ	+	+	+	100
High-mw RNA, wheat germ	+	+	+	100
Ribosomal RNA, *E. coli*	+	+		100
Yeast RNA, commercial product	+	+		100
Poly-AG			+	*ca.* 100
Oligo-G			+	*ca.* 100
Oligo-AG			+	*ca.* 100
Poly-AI	+		+	68
Poly-A	+		+	0.002
Poly-U	+		+	0.001
Poly-C			+	0.001
DNA, salmon sperm	+		+	0.001
bis-(*p*-Nitrophenyl)-phosphate	+			0.001
U-cyclic-p			+	0.001
C-cyclic-p			+	0.001
A-cyclic-p			+	0.001
G-cyclic-p		+	+	2.5

[a] Activities were measured in the usual quantitative ribonuclease assay, in the *p*H-stat, or by comparison of the appearance of product as isolated by paper chromatography or DEAE-cellulose-column chromatography. In the case of bis-(*p*-nitrophenyl)phosphate, activity was measured in a phosphatase assay similar to that of Garen and Levinthal (1960).

chain, but no guanylic acid is apparent. Peak 3 appears to be unhydrolyzed ApG.

Mixed polymer of adenylic and inosinic acids has been hydrolyzed by the *Ustilago* enzyme and chromatographed on DEAE-cellulose as shown in Figure 5. The pattern is quite complex in comparison to that of Figure 3, but a tentative identification of peaks has been made on the basis of elution position and spectral data. Peaks B, D, F, and H show the gradually decreasing optical density ratio (250 mμ/260 mμ at *p*H 2)

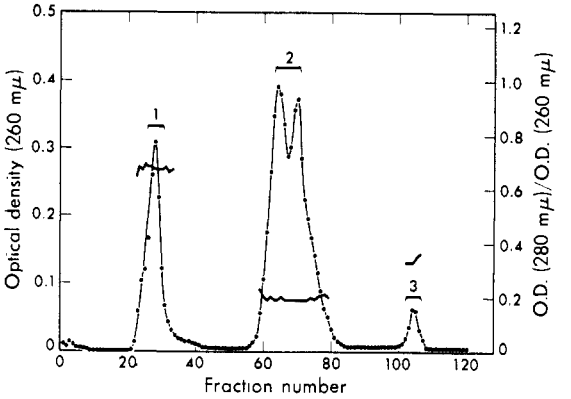

FIG. 4.—End-group determination of the poly-AG digest. The materials of Fig. 3 were pooled, dialyzed for 8 hours against distilled water to remove urea, treated with 125 μg of *E. coli* alkaline phosphatase for 4 hours at 28°, and hydrolyzed for 24 hours at 30° using 1 M KOH. The hydrolysate was then neutralized and adsorbed on a column of Dowex-1 formate of dimensions 1.3 cm diameter by 7 cm length. Elution was carried out using a parabolic gradient of water to 1.0 M ammonium formate, *p*H 3.7. Fractions of 8 ml were collected using a flow rate of 0.5 ml/min. Peak 1 is guanosine, peaks 2 are the 2′ and 3′ isomers of adenylic acid, and peak 3 is the dinucleoside phosphate ApG.

expected of the series Ip, ApIp, ApApIp, and ApApApIp, while the constant ratio of peaks C, E, G, and the early part of peak I indicate that these are the mono-, di-, tri-, and tetranucleotides of adenylic acid, respectively.

Chemically synthesized oligomers of guanylic acid (oligo-G) and adenylic and guanylic acids (oligo-AG) were treated with nuclease from *U. sphaerogena* and the digests were spotted on paper for chromatography in the 2-propanol-ammonia–water system. In both cases the only mononucleotide formed in quantities sufficient to be measured was guanylic acid. In early stages of the digestion up to 50% of the mononucleotide was found as the 2′,3′-cyclic phosphate, while at later stages the greater part was found as the 3′-nucleotide. More than 50% of the material remained unhydrolyzed in each case.

The relative rates of hydrolysis of the four common nucleoside-2′,3′-cyclicphosphates have been measured using the *Ustilago* enzyme. Only G-cyclic-p was hydrolyzed at a rate which could be measured. Quantitative comparison of the relative rates of hydrolysis of the cyclic phosphates as well as a number of other actual and potential substrates may be found in Table II.

Mode of Enzyme Attack.—Methylated albumin-kieselguhr chromatography of a number of samples of RNA and RNA components is illustrated in Figure 6. Not shown are adenylic acid, eluted almost entirely in the first two fractions, and wheat germ high-molecular-weight RNA, eluted in fractions 55 to 75. Fraction K was isolated by DEAE-cellulose chromatography as in Figure 1, and was composed of oligonucleotides of chain length of about 6. The differences seen in the elution profiles made it seem likely that this method of examination of RNA could be used to follow, in a semiquantitative fashion, the course of degradation of an RNA sample by a ribonuclease.

The course of digestion of a sample of wheat germ s-RNA by pancreatic ribonuclease is shown in Figure 7. After only a few minutes of digestion a measurable quantity of material is eluted in an intermediate region between the small oligonucleotide region and the

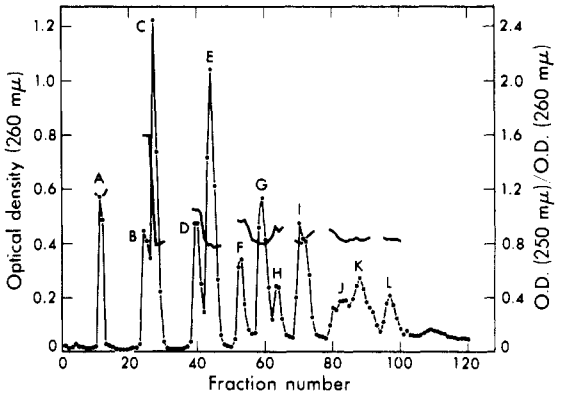

FIG. 5.—Fractionation of a *Ustilago* nuclease digest of poly-AI. Five mg of poly-AI was digested and chromatographed as in Fig. 3. The optical densities were measured after adjustment of the *p*H to about 2. The peaks have been tentatively identified as follows: peaks B, D, F, and H are probably composed of the series Ip, ApIp, ApApIp, and ApApApIp. Peaks C, E, G, and the early part of peak I appear to consist of the di-, tri-, tetra-, and pentanucleotides of adenylic acid.

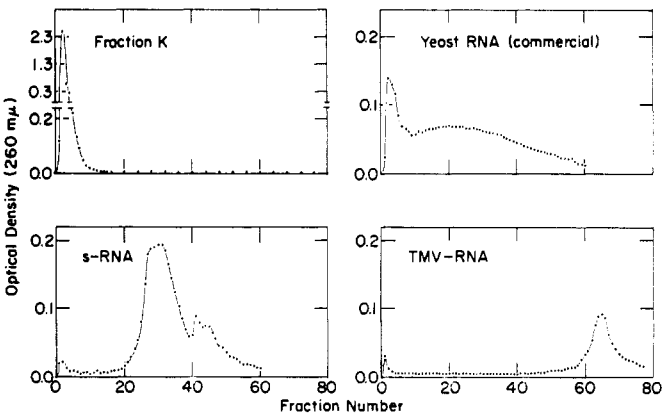

FIG. 6.—Methylated albumin-kieselguhr chromatography of RNA. Samples (1.5 mg) of nucleic acid (*ca.* 1 mg in the case of TMV-RNA) were adsorbed on a column of methylated albumin-kieselguhr of dimensions 2.3 cm diameter by 2.8 cm length and chromatographed using 800 ml of 0.05 M NaH_2PO_4 buffer employing a linear gradient of 0.05–1.25 M NaCl. Fraction K was obtained by DEAE-cellulose chromatography of a *Ustilago* nuclease digest of s-RNA, as illustrated in Fig. 1. Fractions of 10 ml were collected using a flow rate of 2.5 ml/min.

initial position of elution of the s-RNA peak. After 1 hour of digestion most of the s-RNA has been altered, but still only a small percentage of the ultraviolet-absorbing material is eluted in the very early fractions. At 2 hours very little of the material is eluted in the original position; more extensive digestion (e.g., 24 hours, not shown) results in elution of almost all the degraded RNA in the first few fractions.

A very different kind of degradation pattern is found if s-RNA is digested with snake venom phosphodiesterase, as shown in Figure 8. In this case material does not appear in the intermediate area between the elution positions of small oligonucleotides and the original s-RNA peak. Instead the quantity of material eluted in the initial s-RNA peak gradually decreases, while the materials eluted in the first few fractions show a corresponding increase in amount. Paper chromatography was used to fractionate the low-molecular-weight components formed by venom diesterase action on s-RNA. At all stages of digestion examined, ranging from 10 to 50% hydrolysis of the substrate, only mononucleotides were found in this fraction, and its composition corresponded approximately to the composition of wheat germ s-RNA substrate.

The degradation pattern obtained using wheat germ s-RNA and micrococcal nuclease is presented in Figure 9. The result appears to have characteristics of both the pancreatic RNAase and snake venom–phosphodiesterase digestions already shown. Some material appears rapidly in the intermediate area, between the first fractions and the initial elution position of the s-RNA peak, but quantitatively this intermediate fraction remains rather small. Material eluted in the first few fractions appears rapidly also, and continues to increase in quantity as the digestion period proceeds and the height of the initial s-RNA peak decreases.

The action of the *Ustilago* enzyme on wheat germ s-RNA is illustrated in Figure 10. In this experiment the molar ratio of substrate to enzyme was about 10^5. After only a few minutes of digestion material appears in the intermediate area of the elution diagram, while after 2 hours a large part of the original s-RNA peak is altered in its position of elution. Rapidly eluted material is not found in short term digests but appears at a later stage. Very similar degradation patterns were obtained using the *Ustilago* enzyme with s-RNA at digestion temperatures of 13–50°, or with high-molecular-weight RNA in place of the s-RNA substrate.

DISCUSSION

Specificity of the Enzyme.—Chromatography of *Ustilago* nuclease digests of wheat germ RNA using DEAE-cellulose and including urea in the elution buffers (see Fig. 1) should result in the separation of mono- and oligonucleotides on the basis of net negative charge according to Tomlinson and Tener (1962). However, in agreement with Bartos *et al.* (1963), it was found that oligonucleotides of low purine-pyrimidine ratio were eluted slightly ahead of those of high ratio when the net charge was the same. In spite of a less complete fractionation of components compared with that achieved by the aforementioned workers, a good deal of information has been obtained from this initial fractionation. Most important, only guanylic acid endings have been found in the digest. This is illustrated strikingly in Figure 2, which shows no trace of any mononucleotide component other than guanosine-3′-phosphate. If the *Ustilago* enzyme catalyzed the hydrolysis of any bond other than that at the 3′-phosphate of guanylic acid, other mononucleotides would be expected in the digest. Similarly, the dinucleotide fraction (peaks E and F of Fig. 1) of the digest contains measurable quantities of only the three dinucleotides consisting of cytidylic, uridylic, or adenylic acid linked through the 3′-phosphate group to the 5′-hydroxyl of guanylic acid (i.e., CpGp, UpGp, and ApGp). The mono- and dinucleotide fractions ending in a 2′,3′-cyclicphosphate show the same composition as the corresponding 3′-phosphate-ended fractions.

The hydrolysis of G-cyclic-p to form 3′-guanylic acid occurs at a slow rate in comparison to the hydrolysis of RNA (see Table II), while no other cyclicphosphate tested appears to be hydrolyzed to a significant extent. The hydrolysis of cyclic-phosphate-ended mono- or oligonucleotides to the corresponding 3′-phosphate-ended compound is probably size dependent, as judged by results obtained using the constant-*p*H automatic titrator (*p*H-stat). In the *p*H-stat the reaction was carried out at *p*H 7.0, thus the second dissociable hy-

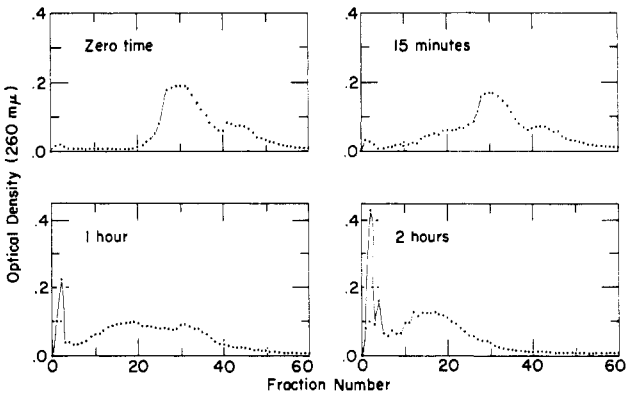

FIG. 7.—Digestion of s-RNA by pancreatic ribonuclease. s-RNA (7.5 mg) in a volume of 5.0 ml was incubated at 28° in the presence of 0.038 μg of pancreatic ribonuclease A. At the time intervals indicated samples of 1.0 ml were withdrawn and chromatographed as in Fig. 6, except that only 600 ml of elution buffer was employed with a salt gradient of 0.05–0.95 M.

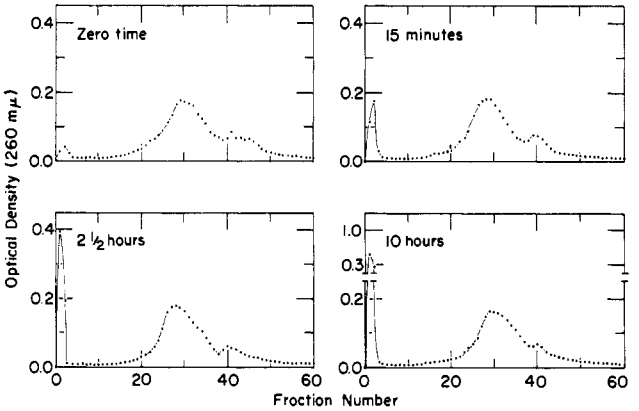

FIG. 8.—Digestion of s-RNA by snake venom phosphodiesterase. Digestion and chromatography were carried out as in Fig. 7, using *ca.* 7.5 μg of snake venom phosphodiesterase.

drogen of phosphoric acid (pK_a = 6.7) was titrated. This dissociation can occur from free or monoesterified phosphate groups, but not from phosphodiesters. Thus the formation of cyclicphosphate ends by cleavage of a linear polynucleotide would not be measured in the *p*H-stat, while the opening of the cyclicphosphate would permit dissociation and thus titration. The initial rate of RNA hydrolysis is about twenty-five times as great as the rate of G-cyclic-p opening, when both reactions are measured using the *p*H-stat (see Table II). This indicates that the rate of hydrolysis of the cyclicphosphate ends formed by hydrolysis of RNA and presumably present as oligonucleotide is more rapid than the hydrolysis of cyclic mononucleotide. Further support for this conclusion arises from the presence of small cyclicphosphate-ended digestion products (mono- and dinucleotides) in relatively large quantities, as seen in Figure 1. At the trinucleotide level and above, discrete cyclic nucleotide peaks are no longer apparent and cyclic-ended materials appear only as slight shoulders to the main peaks representing the noncyclic components of the elution diagram.

Rechromatography of the mononucleotide peak obtained by DEAE-cellulose chromatography in the presence of urea shows that only 3′-guanylic acid is present in this fraction of the digest (see Fig. 2). The substrate wheat germ s-RNA contained measurable quantities of methylated guanylic acids (see Glitz and Dekker, 1963), and the chromatographic technique employed in Figure 2 would have separated at least some isomers of these components had they been present in the digest. While the absence of any peak which could be identified as a methylated derivative of guanylic acid suggests that the *Ustilago* enzyme may not act on such nucleotides, this aspect of the specificity of the enzyme should be further investigated using a more sensitive assay procedure. McCully and Cantoni (1961) have reported that ribonuclease T_1, which is specific in the hydrolysis of RNA at guanylic acid residues, does not hydrolyze RNA at residues of 1-methylguanylic acid and N^2-dimethylguanylic acid.

The digestion of poly-AG by the *Ustilago* enzyme provides confirmatory evidence for the proposed guanylic acid specificity. Oligomers ending in guanylic acid with chain lengths up to about eleven nucleotides are found in the digest (see Fig. 3) in rather complete separation.

Analysis of the components of the digest of poly-AI indicates that cleavage is occurring adjacent to both inosinic acid and adenylic acid residues. The latter reaction is surprising since poly-A was shown to be very resistant to the *Ustilago* nuclease, being slowly hydrolyzed only at very high enzyme concentrations (see Table II). Two possibilities are suggested: (a) the Ap-X bond in poly-AI is more susceptible to attack than the Ap-A bond in poly-A, or (b) secondary reactions of the nitrous acid have modified the original poly-A substrate creating susceptible bonds of unknown nature. Cleavage of the poly-AI at adenylic acid residues does not appear to have occurred nonenzymatically since control experiments (no enzyme added) showed no production or prior existence of acid-soluble nucleotides upon incubation of the poly-AI under the usual assay conditions (imidazole buffer, *p*H 7.0, 28°, for 15 minutes). Sato-Asano and Fujii (1960) have reported that RNAase T_1 hydrolyzes deaminated RNA at inosinic acid and, less rapidly, at xanthylic acid residues.

The quantitative data of Table II show all natural RNA samples tested to be equivalent as substrates for the *Ustilago* enzyme. To the degree of accuracy used in the measurements, guanylic acid–containing synthetic polymers were also hydrolyzed at about the same rate, while the mixed polymer of adenylic and inosinic acids was about two-thirds as good a substrate. The hydrolysis of poly-A at a very slow rate appears to be real: the small quantity of acid-soluble materials obtained had spectral properties similar to those of adenylic acid. Consideration was given to the possibility that this slow hydrolysis is owing to a very minor impurity in the poly-A preparation, perhaps inosinic acid or some other susceptible derivative; however, alkaline hydrolysis followed by paper chromatography showed no nucleotide other than adenylic acid present in the polymer. No other homopolymer appears to be hydrolyzed at a significant rate. Similarly, DNA does not appear to be hydrolyzed, and no activity is apparent using a nonnucleotide phosphodiester, bis-(*p*-nitrophenyl)phosphate. The hydrolysis of G-cyclic-p to form only the 3′ isomer, and the incomplete hydrolysis of oligo-G and oligo-AG, which contained both 2′ → 5′ and 3′ → 5′ linkages (see Michelson, 1959a) indicate that 2′-linked esters of guanylic acid are not hydrolyzed by the enzyme. Thus it seems reasonable to conclude that in a natural RNA substrate guanylic acid is the only nucleotide attacked, and that reaction occurs first through the formation of a 2′,3′-cyclicphosphate, followed by opening of this ring to form the 3′ isomer of guanylic acid.

Mode of Enzyme Attack.—Methylated albumin-

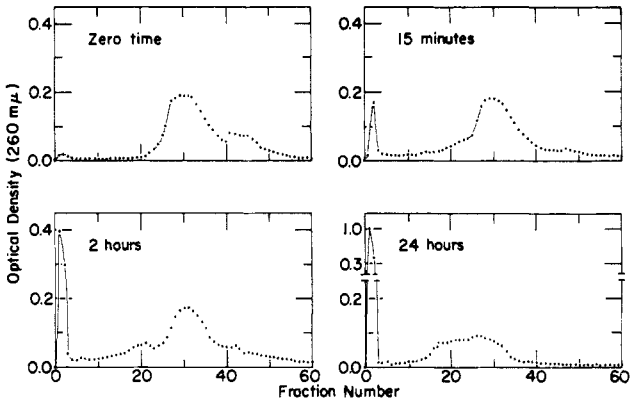

FIG. 9.—Digestion of s-RNA by micrococcal nuclease. Digestion and chromatography were carried out as in Fig. 7, using 0.12 μg of micrococcal nuclease.

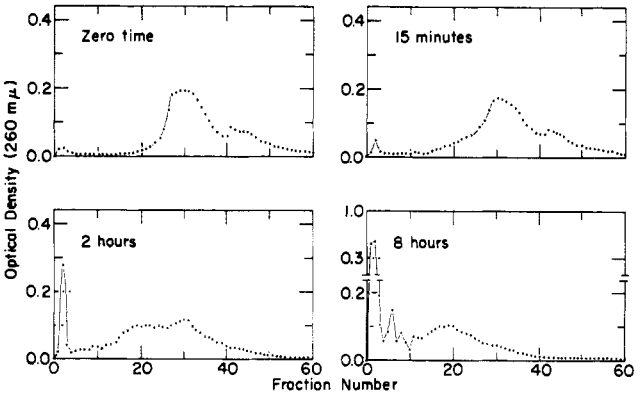

FIG. 10.—Digestion of s-RNA by *Ustilago* nuclease. Digestion and chromatography were carried out as outlined in Fig. 7, using 0.015 μg of *Ustilago* nuclease.

kieselguhr chromatography provides a very rapid and relatively simple method for the semiquantitative estimation of oligo- and polynucleotides of varying chain length. Assuming the fractionation obtained to be primarily a function of chain length, the time course of the degradation of RNA by nuclease can be followed using the position of elution and magnitude of the peak of ultraviolet-absorbing materials to approximate the size and quantity of different degradation products. Some idea of the size of material eluted at a given position can be obtained by comparison with the position of elution of known polymers as shown in Figure 6. This technique should provide a very sensitive measure of the breakage of a few bonds in a polynucleotide molecule, since the fragments of a large molecule might be expected to be eluted considerably earlier than the intact polymer.

The action of the *Ustilago* enzyme, like that of pancreatic ribonuclease (see Figs. 7 and 10), appears to be highly destructive of polynucleotide chains. Although the substrate RNA is present in a very large molar excess, almost all of the RNA is affected by the digestion process well before the hydrolysis nears completion. The simplest interpretation of the data of Figure 10 is that the enzyme molecule does not become firmly attached to any single molecule of substrate, but instead passes from substrate molecule to substrate molecule, breaking one or more bonds in each case but not fully degrading any single molecule attacked. Eventually, as judged by elution diagrams of complete digests, all of the substrate is reduced to material which is eluted from the column in the area of small oligonucleotides. The similarity of the patterns obtained from *Ustilago*-nuclease and pancreatic-ribonuclease digestion of s-RNA indicates that these two enzymes act in a generally similar manner.

The pattern obtained in the digestion of wheat germ s-RNA by snake venom phosphodiesterase is very different from that found in the previous two cases (see Fig. 8). The snake venom enzyme has been called an exopolynucleotidase, since it acts by stepwise removal of 5′-mononucleotides starting at the 3′-hydroxyl end of a polynucleotide chain (see Razzell and Khorana, 1959). The methylated albumin-kieselguhr patterns obtained using this enzyme with an s-RNA subtrate indicate that only two classes of material are present in the partial digests: the initial peak of what appears to be unaltered RNA, and a peak eluted in the first two or three fractions, chromatographically identified as mononucleotides. Examination of the composition of the mononucleotides released showed no variation from the composition of the substrate s-RNA, suggesting that hydrolysis occurred by the random and total degradation of substrate molecules. However, the additional hydrolysis from each molecule of substrate of a few terminal nucleotides, such as those at the acceptor end of the s-RNA (see Preiss *et al.*, 1961), probably would not have been detected. A further test of the suggested random and total hydrolysis mechanism could be made using some better criterion of the degree of intactness of the apparently unaltered s-RNA peak, perhaps amino acid–acceptor ability.

The patterns obtained by chromatography of micrococcal nuclease digests of wheat germ s-RNA are in many respects intermediate between those of the *Ustilago* or pancreatic enzymes and those of the snake venom phosphodiesterase. The immediate appearance of material eluted in the intermediate range of the elution diagram (see Fig. 9) has thus far been interpreted as an endonucleolytic action resulting in the formation of what appears to be large fragments of the s-RNA substrate. Equally rapid is the appearance of small materials which, by analogy with the snake venom case, can be most simply interpreted as resulting from an exonucleolytic action. Such a combined endo- and exonuclease activity has been proposed for this enzyme in the reaction with a DNA substrate (Dirksen and Dekker, 1960).

General Conclusions

The work reported in this paper shows that the specificity of the *Ustilago sphaerogena* ribonuclease is quite similar to that of the T_1 ribonuclease of Sato and Egami (1957), a second enzyme of fungal origin. Each nuclease is highly specific in the hydrolysis of guanylic acid esters to form 3′-guanylic acid–ended degradation products. The previous paper pointed out certain similarities in the chemical and physical characteristics of the two enzymes, as well as a few differences. In view of this partial resemblance, further comparison of the T_1 and *Ustilago* nucleases should be of interest with regard to both their mechanism of action and their properties as proteins.

References

Bartos, E. M., Rushizky, G. W., and Sober, H. A. (1963), *Biochemistry 2*, 1179.

Dirksen, M. L., and Dekker, C. A. (1960), *Biochem. Biophys. Res. Commun. 2*, 147.

Frisch-Niggemeyer, W., and Reddi, K. K. (1957), *Biochim. Biophys. Acta 26*, 40.

Garen, A., and Levinthal, C. (1960), *Biochim. Biophys. Acta 38*, 470.

Georgatsos, J. G., and Laskowski, M., Sr. (1962), *Biochemistry 1*, 288.

Glitz, D. G., and Dekker, C. A. (1963), *Biochemistry 2*, 1185.
Glitz, D. G., and Dekker, C. A. (1964), *Biochemistry 3*, 1391 (this issue; preceding paper).
McCully, K. S., and Cantoni, G. L. (1961), *Biochim. Biophys. Acta 51*, 190.
Michelson, A. M. (1959a), *J. Chem. Soc.*, 1371.
Michelson, A. M. (1959b), *J. Chem. Soc.*, 3655.
Preiss, J., Dieckmann, M., and Berg, P. (1961), *J. Biol. Chem. 236*, 1748.
Razzell, W. E., and Khorana, H. G. (1959), *J. Biol. Chem. 234*, 2105.
Rushizky, G. W., and Sober, H. A. (1962), *Biochim. Biophys. Acta 55*, 217.
Sato, K., and Egami, F. (1957), *J. Biochem. (Tokyo) 44*, 753.
Sato-Asano, K., and Fujii, Y. (1960), *J. Biochem. (Tokyo) 47*, 608.
Singer, M., and Guss, J. (1962), *J. Biol. Chem. 237*, 182.
Steiner, R. F., and Beers, R. (1961), Polynucleotides, New York, Elsevier, p. 377.
Tomlinson, R. V., and Tener, G. M. (1962), *J. Am. Chem. Soc. 84*, 2644.
Wyatt, G. R. (1951), *Biochem. J. 48*, 581.

Differences in the Methylated Base Composition of Valyl and Leucyl Soluble Ribonucleic Acids of *Escherichia coli*

ARTHUR BANK, SUZANNE GEE, ALAN MEHLER, AND ALAN PETERKOFSKY

From the Laboratory of Biochemistry, National Institute of Dental Research, National Institutes of Health, Bethesda, Md.

Received May 18, 1964

The base compositions of soluble ribonucleic acids (s-RNA) of *Escherichia coli* that accept valine and leucine have been examined. To label specifically the methylated bases, the s-RNA was isolated from a methionine auxotroph cultured on methionine with radioactive tritium in the methyl group. The s-RNA was enzymatically charged with either valine or leucine and treated with an amino acid *N*-carboxyanhydride, which preferentially reacts with the charged species. The s-RNA precipitated with the resulting insoluble polypeptide was analyzed for major and minor bases. Although no interpretable differences were found in the contents of major bases, different patterns of minor bases were associated with the two species of s-RNA. Thymine, 6-methylaminopurine, 6-dimethylaminopurine, and possibly 2-*N*,*N*-dimethyl-6-hydroxypurine were found in the valine-specific s-RNA; thymine, 1-methylamino-6-hydroxypurine, and a dimethylpurine were present in the leucine-specific s-RNA.

The structures of s-RNA's have been the subject of intensive investigations since their discovery as intermediates in protein synthesis (Hoagland *et al.*, 1957; Hoagland *et al.*, 1958). Detailed structural analyses of purified s-RNA's are required for an understanding of how each species of s-RNA accepts a specific activated amino acid and transfers it to a ribosome-bound peptide. Differences either in the over-all base composition or in sequences of nucleotides could identify the sites on s-RNA molecules responsible for their specific enzymatic reactivities. Important contributions have been made by several authors who have reported many differences between highly purified species of yeast s-RNA (Stephenson and Zamecnik, 1961; Holley *et al.*, 1961, 1963; Zamecnik, 1962; Madison *et al.*, 1963; Cantoni *et al.*, 1963; Ingram and Sjöquist, 1963; Doctor *et al.*, 1963), as well as certain common sequences (Zamir *et al.*, 1964). Berg *et al.* (1962) have also reported differences in the terminal nucleotide sequences of leucine- and isoleucine-specific s-RNA's isolated from *Escherichia coli*. This paper documents significant differences in the methylated base composition of valyl and leucyl s-RNA's isolated from *E. coli*.

Several methods have been reported for the isolation of specific amino acid–acceptor s-RNA's using techniques such as countercurrent distribution (Zachau *et al.*, 1961; Apgar *et al.*, 1962), column chromatography (Ofengand *et al.*, 1961; Hartmann and Coy, 1961; Nishiyama *et al.*, 1961; Sueoka and Yamane, 1962; Tanaka *et al.*, 1962), and periodate treatment (Zamecnik *et al.*, 1960; Von Portatius *et al.*, 1961). A method for the isolation of specific s-RNA's using amino acid *N*-carboxyanhydrides has been described previously (Zachau, 1962; Simon *et al.*, 1962, 1963, 1964; Mehler and Bank, 1963). In this method, a mixture of s-RNA's is charged enzymatically with the amino acid of the s-RNA to be isolated; the free amino group of this amino acid serves as the initiator for the polymerization of an *N*-carboxyanhydride resulting in the preferential precipitation of the charged species of s-RNA as an ester of an insoluble polypeptide.

In the experiments described here specific *E. coli* s-RNA's were precipitated as γ-benzyl-L-glutamate polymers. These precipitates were then hydrolyzed with alkali and the resulting nucleotides were separated by column chromatography. For the detection of the methylated bases known to be present in s-RNA (Davis and Allen, 1957; Littlefield and Dunn, 1958; Smith and Dunn, 1959) we specifically labeled these bases in a methionine auxotroph grown on tritiated methionine.

Experimental

Materials.—s-RNA from *E. coli*, strain B, was obtained commercially from General Biochemicals. Its amino acid acceptance was 0.340 for valine and 0.388 for leucine measured as mμmoles of amino acids per μmole of nucleotide (Berg *et al.*, 1961). Amino acids were measured using commercial radioactive compounds with specific activities of 1–200 μcuries per μmole; nucleotide concentrations were determined as optical density at 260 mμ in a Cary spectrophotometer. Tritiated s-RNA was prepared from the *E. coli* K_{12}-W6 mutant as described previously by Peterkofsky *et al.* (1964) using commercial methyl-labeled tritiated methionine with a specific activity of 27 μc per μmole. Its amino acid acceptance was comparable to that of