Chemical Studies on Amino Acid Acceptor Ribonucleic Acids. III. The Degradation of Purified Alanine- and Valine-specific Yeast s-RNA's by Pancreatic Ribonuclease*

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Alanine- and valine-specific s-RNA's have been purified from yeast s-RNA by countercurrent distribution. Digestion with pancreatic ribonuclease followed by fingerprinting gave rise to a number of pure oligonucleotides, whose yield and structure are reported. The 5′-linked end group, which carries free 2′- and 3′-hydroxyl groups, was isolated from both s-RNA's and found to be cytidine (1 per 75 nucleotides). The localization of pseudouridylic acid, ribothymidylic acid, inosinic acid, N^2-dimethylguanylic acid, N^1-methylguanylic acid, and 5-methylcytidylic acid within specific sequences has been achieved, demonstrating that both similarities and differences exist between the two specific s-RNA molecules. The yields of oligonucleotides obtained from digests with pancreatic ribonuclease are sufficiently high to indicate that the starting material in each case is reasonably homogeneous with respect to nucleotide sequence. Individual oligonucleotides were identified by the qualitative and quantitative analysis of their degradation products.

A detailed understanding of the primary structure of amino acid–acceptor RNA (transfer RNA, s-RNA) would greatly increase our comprehension of the mechanism of protein synthesis (see review by Berg, 1961). Given the nucleotide sequences of a single s-RNA species, we could attempt, by model building, to deduce the important features of the secondary structure, such as the Watson-Crick base pairs, the "coding" site, and the portion of the molecule which interacts with the specific amino acid–activating enzymes. It is currently believed that for each amino acid there exists at least one specific s-RNA molecule, possibly several. The point has to be established, however, by showing that for each amino acid there are one or at most a very few specific nucleotide sequences. Highly purified transfer RNA specific for certain amino acids is now available, thanks largely to Holley's pioneering work (Holley and Merrill, 1959; Apgar *et al.*, 1962). Purifications of specific s-RNA's have been reported from many laboratories (Tanaka *et al.*, 1962, Zachau *et al.*, 1961; Doctor and Connelly, 1961; Stephenson and Zamecnik, 1961; Portatius *et al.*, 1961; Zubay, 1962), but in our experience Holley's countercurrent-distribution method has been the most reliable. The present paper is a progress report concerning our efforts to elucidate the nucleotide sequences in alanine- and valine-specific yeast transfer RNA's and describing the structure and yields of some of the oligonucleotides found. Portions of this work have been reported briefly (Ingram and Sjöquist, 1963).

Experimental

Purification of Acceptor RNA.—Yeast acceptor RNA was purchased from General Biochemicals (Chagrin Falls, Ohio), who apparently prepare it from pressed bakers' yeast (Fleischman's) by Holley's modification (Holley *et al.*, 1961) of the method of Monier *et al.* (1960). The material usually contained about 75% by weight of RNA; it was used directly.

Countercurrent distribution (Fig. 1) was carried out as described by Apgar *et al.* (1962) in a 100-tube automatic apparatus of the Craig-Post type in a room whose temperature was accurately controlled at 25°. In later experiments, a 200-tube automatic machine (E. C. Apparatus Co., Swarthmore, Pa.) was used in a laboratory which was efficiently air-conditioned at 25°. Batches of 1 g of the commercial RNA (=750 mg actual RNA) were distributed at a time. The procedure was easily reproducible, and results close to those of Holley were obtained; however, with the same solvent system, 400 transfers were required to obtain a distribution which Holley achieves in 200 transfers, presumably because of a slight difference in the operating temperature (2–3°). The partition coefficient of s-RNA in this system is exceedingly sensitive to changes in temperature.

The RNA was recovered from the grouped fractions (five tubes together) (Apgar *et al.*, 1962) and the relevant amino acid–acceptor activity determined by the DE-20 ion-exchange paper method previously described (Ingram and Pierce, 1962). The alanine-specific acceptor RNA (60 mg), the slowest-moving fraction, was redistributed in Holley's 1.9 M NaK phosphate buffer system for 844 transfers (Fig. 1). It formed now a rather narrow, almost symmetrical peak. The most active portions of this peak, recovered as above, were finally evaporated in a rotary evaporator and stored at −20°. Care was taken to avoid contamination with the so-called "finger nuclease."

The valine-specific acceptor RNA (98 mg) was redistributed (Fig. 2) as the alanine-specific RNA for 1015 transfers, when the peak showed signs of beginning to split into two. On one occasion two distinct peaks of valine acceptor activity were formed. Perhaps there are two valine-specific acceptor RNA molecules or perhaps the two fractions differ in some other way. This paper deals mainly with the slower, more active peak and only a few comments concerning the nucleoside end group of the second valine acceptor RNA peak will be presented.

The alanine acceptor activity of the twice-distributed

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material was usually 0.5–0.6 mole of alanine per mole of acceptor RNA (mw 25,000, Litt and Ingram, 1964). In the most recent experiments, using a new activating enzyme preparation, specific activities as high as 0.81 mole of alanine per mole of s-RNA were obtained. It is felt that these labeling figures represent minimum values, because the enzyme system used was a rather crude yeast extract and because in the assays only a very few micrograms of highly purified RNA are present and subject to degradation by traces of nucleases. There is also a considerable hydrolysis rate of the amino acyl s-RNA, so that a steady-state concentration will be reached below 100% amino acyl s-RNA formation. In addition, since both alanine and valine s-RNA terminate in cytidine, both would have to have the AMP moiety added before they could accept the amino acid from the activating enzyme (see Ingram and Sjöquist, 1963). Even if our incubation is optimal for this last enzyme, it may not be so for the terminal adding enzyme. More reliance will be placed in this and future papers on the chemical purity of our s-RNA preparations, as established by the ratios between the molar quantities of oligonucleotides found in fingerprints. By this criterion it is felt that the alanine-specific RNA may be better than 80% pure.

Digestion of Alanine- and Valine-specific RNA.—These were generally carried out 1 mg at a time in 20–50 μl of 0.2 M ammonium acetate, *p*H 7.6, containing 12.5 μg of pancreatic ribonuclease (Worthington Biochemical Corp., Freehold, N. J.). In some digests both pancreatic and takadiastase (T_1) ribonuclease were present simultaneously. The solution was incubated at 38° in a sealed glass capillary for 90–120 minutes. After evaporation on Parafilm in a vacuum desiccator, the residue was dissolved in 5 μl of water in a constriction pipet (Lang-Levy) and subjected to fingerprinting.

Fingerprinting Procedures.—Method B (Ingram and Pierce, 1962) was mostly used, but a substitute used as ascending solvent was a mixture of 1-butanol–pyridine–acetic acid–water in the volume proportions 50:100:15:60. Since pyridine absorbs ultraviolet light, the oligonucleotide spots were made visible by dipping the dry chromatogram in acetone containing 4% by volume of concentrated ammonia. After redrying, the spots were clearly visible. Although this method worked well in the humid summer months, it proved unreliable in the fall and winter; therefore the 1-propanol-ammonia solvent (Method C) is now preferred.

Method D. More recently, Method B has been modified for the use of Whatman 3MM paper which is 46 cm (18 in.) wide, with the double wicks tapered to 30 cm (12 in.). The cooled plate is now 51 × 61 cm (20 × 24 in.). For descending chromatography the dry paper was trimmed, and a 64-mm (2.5-in.) strip was sewn next to the row of oligonucleotides parallel to the direction of electrophoresis. The solvent was 1-propanol–concd NH_3–water in the volume proportions 55:10:35 (Lipsett *et al.*, 1961). In this solvent 2′,3′-cyclic phosphates run well ahead of the corresponding 2′- or 3′-phosphates. Separation of some methylated nucleotides is also very good. Very good fingerprints are obtained using Whatman 3HR paper sheets 46 × 57 cm (18.25 × 22.5 in.), with double wicks of the same paper dipping into the buffer vessels and overlapping the main sheet by 13 mm (0.5 in.). Background ultraviolet-absorbing material can be largely removed by previous washings of the paper with the solvent. Oligonucleotides containing the pGp... residue are strongly adsorbed unless the paper is first dipped into 0.05% EDTA, *p*H 7, and then washed chromatographically with water and dried.[1]

Characterization and Hydrolysis of Oligonucleotides.—The relevant areas of the chromatograms of fingerprints for which the pyridine solvent had been used were eluted with 0.5 N ammonia (100–200 μl/in.[2]). The extract was evaporated in a vacuum desiccator to remove remaining traces of pyridine before it was redissolved in 0.3 ml of either 0.5 or 1.0 N hydrochloric acid or in 0.2 M ammonium acetate, *p*H 7.6, for examination in the Cary 11M spectrophotometer with beam-focusing device. Only in the case of mononucleotides were the spectra used to identify the material in the particular spot. In all other cases the spectrum at this stage was used mainly to calculate the yield of that oligonucleotide, but the structure was established by the results of the acid or alkaline hydrolysis. 1-Propanol-ammonia chromatograms can be efficiently eluted with water only (100–200 μl/in.[2]).

Alkaline hydrolysis was mostly used, and this was carried out with 80 μl of 0.3 N KOH per fingerprint spot. Heating was done in a capillary at 37° for about 16 hours. The desalting procedure on CM 50 paper previously described (Ingram and Pierce, 1962) was used again with very good results. However, recent batches of this paper have had to be washed chromatographically with 1 N hydrochloric acid, and plenty of water, before the capacity of a 2 × 6–cm strip was high enough to neutralize 80 μl of 0.3 N KOH. After elution and evaporation on Parafilm, the residue in 5 μl of water was loaded onto 1.5 cm of wet Whatman 3MM paper. Electrophoresis was usually carried out on a cooled metal plate in 10% acetic acid brought to *p*H 2.7 with ammonia (see Method B, Ingram and Pierce, 1962). More recently, 20% acetic acid, adjusted to *p*H 2.7 or 3.0 with ammonia, has given superior separations on Whatman 3HR paper at lower voltage gradients. The mononucleotides were eluted, together with blank areas, and characterized by their spectra in acid as well as by their electrophoretic position. All the eluted papers were dried and weighed so that appropriate blank corrections could be applied to the optical-density readings.

Digestion of Oligonucleotides with Alkaline Phosphatase and Snake Venom Diesterase.—To the dried eluate of an oligonucleotide spot were added 20 μl of 0.2 M ammonium acetate, *p*H 7.6, and 2 μl of an *Escherichia coli* alkaline phosphatase solution (gift of Dr. C. Levinthal) which contained 16 mg/ml. Incubation was for 2 hours at 37° in a sealed glass capillary. The solution was evaporated to dryness before purification of the oligonucleotides by electrophoresis, as described for the alkaline hydrolysates. Alkaline phosphatase remained adsorbed at the starting line.

Relevant areas of the dry paper were eluted with 100 μl of 0.2 M ammonium carbonate, *p*H 8.5. Snake venom diesterase (Worthington Biochemicals, Freehold, N. J.) (5 μl containing 25 μg in the same *p*H 8.5 buffer) was added to the eluate. After incubation for 1 hour at 38°, and evaporation, the products of digestion were identified by electrophoresis as described for the alkaline hydrolysates. Further identification of nucleotides and nucleosides and their estimation was usually carried out by determining their spectra in 0.5 N HCl.

[1] Abbreviations used in this work: Ap, 2′- and/or 3′-phosphates of adenosine, ApGp, adenyl-(3′,5′)-guanosine phosphate; ψ-uridylic acid (ψUp), pseudouridine-(2′)-3′-phosphate; Tp, thymine ribosyl phosphate; MeAp, N^6-methyladenine ribosyl phosphate; 2-DMGp, N^2-dimethylguanylic acid; 1-MGp, N^1-methylguanylic acid; Ip, inosinic acid; pGp, guanosine-2′(3′),5′-diphosphate.

TABLE I

ALANINE S-RNA: SOME OLIGONUCLEOTIDES FROM PANCREATIC RIBONUCLEASE DIGESTS[a]

Oligonucleotide	Spot No.	Yield (moles per mole cytidine)	Structure Determined by:	Product	Yield (mμ-moles)	Comment
ApCp	p2	1.1				Identified by characteristic position (Rushizky and Knight, 1960)
2-DMGpCp	p4	1.1	alk. phosphatase + SVD[b]	2-dimethyl-guanosine	12.7	Identified by electrophoretic position and spectrum
				pC	10.4	
			KOH	2-DMGp	10.7	HCl hydrol. → 2-DMGuanine, identified by chromatography in isopropyl alcohol-HCl, spectrum
				Cp	12.0	
GpCp	p5	2.6	alk. phosphatase + SVD	guanosine	18.1	
				pC	19.1	
ApGpCp	p6	0.9	T_1	ApGp	13.8	Identified by electrophoretic position and spectrum
				Cp	17.2	
ApUp	p9	0.4	KOH	Ap	15.9	
				Up	16.1	
GpUp	p14	3.8	alk. phosphatase + SVD	guanosine	42.8	
				pU	39.3	
1-MGpGpCp	p8	1.1	alk. phosphatase + SVD	1-methyl-guanosine	9.6	Identified by electrophoretic position and spectrum
				pG	10.6	
				pC	9.2	
			KOH	1-MGp	8.0	Identified by electrophoretic position and spectrum
				Gp	8.4	
				Cp	7.7	
ApGpUp	p10	1.9	T_1	ApGp		
				Gp		
				ApUp		
				Up		
			alk. phosphatase + SVD	adenosine	5.0	
				guanosine	6.2	
				pA	5.8	
				pG	5.5	
				pU	(6.1)	Low because of partial destruction by ultraviolet light on the electrophoresis paper
			KOH	Ap	12.5	
				Gp	13.5	
				Up	10.0	
IpGpCp	p11	1.0	alk. phosphatase + SVD	inosine	9.9	Identified by spectrum, electrophoresis, and chromatography in isopropyl alcohol–NH_3 as hypoxanthine after HCl hydrolysis
				pG	7.6	
				pC	12.8	
GpGpTp	p15	0.8	KOH	Gp	24.5	
				Tp	12.1	Identified by spectrum, electrophoresis at *p*H 3.0
GpGpApCp	p7	0.9	T_1	Gp		
				ApCp		Identified by electrophoretic position and spectrum
			KOH	Cp	6.7	
				Ap	9.7	
				Gp	15.7	

[a] Unless otherwise indicated, the products of degradation were identified by their electrophoretic position and by their spectra either in 0.5–1.0 N HCl or at *p*H 6.8. [b] SVD = snake venom diesterase.

RESULTS

Alanine-specific RNA: Pancreatic Ribonuclease Digestion.—Figure 2 shows the fingerprint of the alanine-specific acceptor RNA, digested with pancreatic ribonuclease (see also Table I). Not shown is the nucleoside end group which moves far to the cathode side. The only end group found was cytidine (see Ingram and Sjöquist, 1963), with about 8% of a slower-moving component, probably adenosine. The presence of cytidine as virtually the only end group with free 2′,3′-hydroxyl groups was confirmed by alkaline hydrolysis,

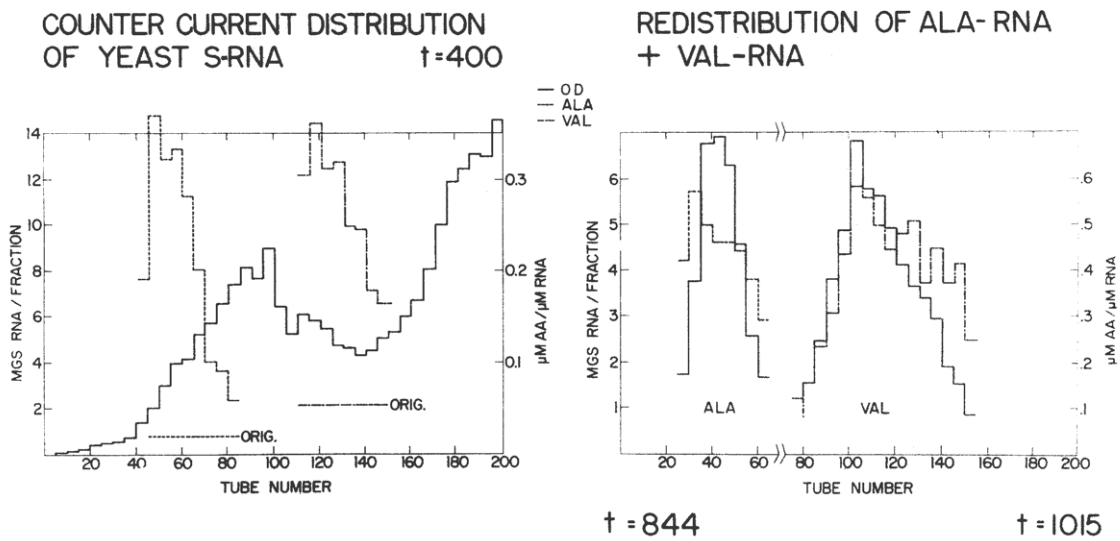


FIG. 1.—Countercurrent distribution of alanine- and valine-specific s-RNA (see text).

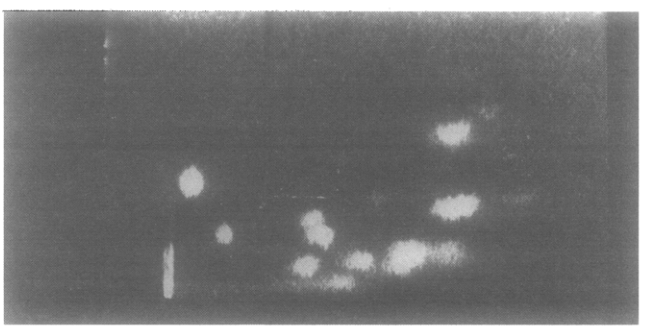
by the simultaneous digestion with both ribonucleases, and by the presence of a T_1 oligonucleotide which yielded no guanylic acid, but did yield one equivalent of cytidine on alkaline hydrolysis.

The *dinucleotide p4* gave 1.1 equivalent per 75 nucleotides of N^2-dimethyl-GpCp. The eluate from p4 was dephosphorylated and degraded with snake venom diesterase. Paper electrophoresis at *p*H 2.7 gave cytidine-5′-phosphate (and a little free cytidine due to 5′-nucleotidase contamination) and a nucleoside migrating to the cathode more rapidly than guanosine (ratio of mobility about 3 relative to uridine). The nucleoside had a spectrum in 1 N hydrochloric acid close to that published by Smith and Dunn (1959) for N^2-dimethylguanosine (λ_{max} 264 mμ; pronounced shoulder, ratio 280/260 mμ = 0.50). The amounts of cytidine-5′-phosphate (plus cytidine) and N^2-dimethylguanosine were 10.4 and 12.7 mμmoles, respectively.

The dinucleotide was found again in the digest of alanine s-RNA with both ribonucleases. It seems that under the conditions used, the bond next to N^2-dimethylguanosine-3′-phosphate is not hydrolyzed by ribonuclease T_1, although in other digests with this ribonuclease alone, splitting of this bond to give Cp2DMGp has been observed. Alkaline hydrolysis, followed by electrophoresis at *p*H 2.7, yielded cytidine-3′-phosphate (12.0 mμmoles) and N^2-dimethylguanosine-(2′)3′-phosphate (10.7 mμmoles); the nucleotides were identified by their electrophoretic position and by their spectra in 0.67 N HCl. The extinction coefficient of guanosine-3′-phosphate was used for the methylated nucleotide. N^2-Dimethylguanosine-3′-phosphate showed λ_{max} 266 mμ; ratio 280/260 mμ = 0.67. This nucleotide, after hydrolysis in 100 μl of 1 N HCl at 100° for 1 hour, gave N^2-dimethylguanine, characterized in chromatography in the isopropanol-HCl solvent; ratio: R_F-guanine/R_F-2-dimethylguanine = 1.7 which is close to the literature value (Smith and Dunn, 1959). The methylated base on the acid paper chromatogram had a characteristic brilliant light-blue fluorescence in ultraviolet light, easily distinguishable from the darker and less brilliant fluorescence of guanine.

The *trinucleotide p8* gave 1.0 equivalent per 75 nucleotides of N^1-methyl-GpGpCp. The structure was proved in the same way as dinucleotide p4. After dephosphorylation and degradation with snake venom diesterase, three major products were found on electrophoresis at *p*H 2.7: guanosine-5′-phosphate (and also a very small amount of guanosine), together with 10.6 mμmoles; cytidine-5′-phosphate, 9.2 mμmoles; and a nucleoside (9.6 mμmoles) whose mobility at *p*H 2.7 is intermediate between guanosine and N^2-dimethylguanosine (*vide supra*). This nucleoside had a spectrum in 1 N hydrochloric acid close to that given by Smith and Dunn (1959) for N^1-methylguanosine (λ_{max} 258 mμ; a weak shoulder; ratio 280/260 mμ = 0.71).

In another experiment, alkaline hydrolysis and electrophoresis at *p*H 2.7 gave cytidine-3′-phosphate (7.7 mμmoles), guanosine-(2′)3′-phosphate (8.4 mμmoles), and N^1-methylguanosine-(2′)3′-phosphate (8.0 mμmoles), identified by their spectra in 1 N HCl and by their position in electrophoresis. The N^1-methylguanosine-(2′)3′-phosphate moved a little more slowly than the unmethylated nucleotide and was clearly re-



260 Ala · s-RNA : pancr. RNAase

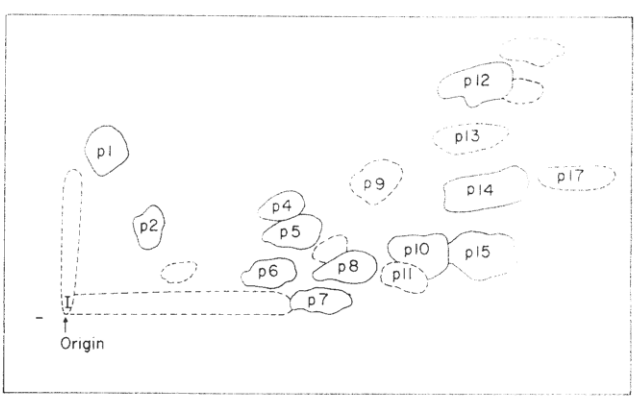


FIG. 2.—Alanine s-RNA. Fingerprint of pancreatic ribonuclease digest. Method B, modified for use with the pyridine-containing solvent; see Methods.

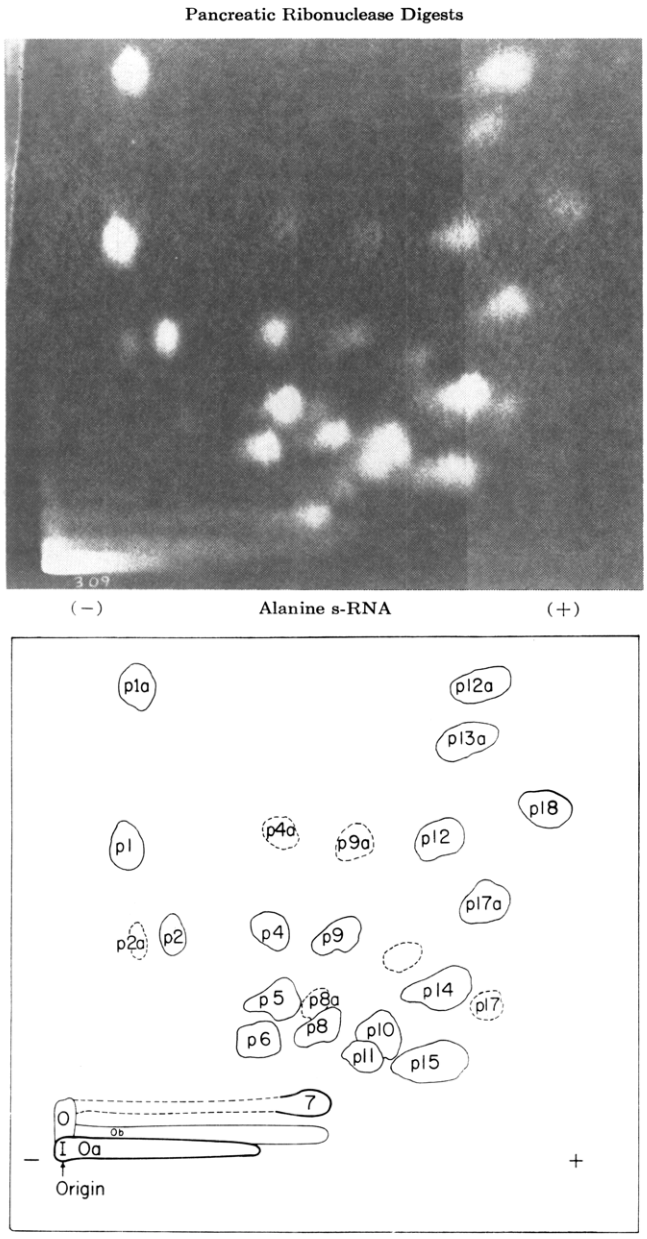


FIG. 3.—Alanine s-RNA. Fingerprint of pancreatic ribonuclease digest. Method D under Methods, using 20% acetic acid–NH_3, *p*H 2.7; Whatman 3HR paper.

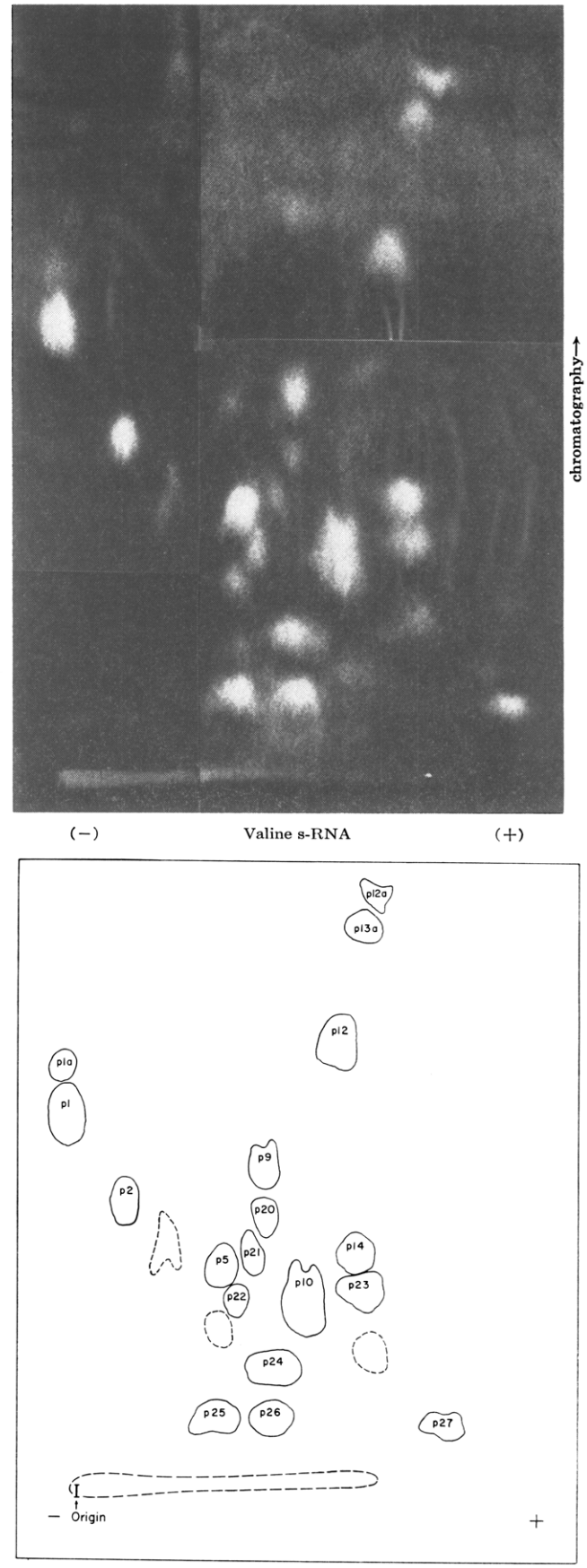


FIG. 4.—Valine s-RNA. Fingerprint of pancreatic ribonuclease digest. Method D under Methods, using Whatman 3MM paper dipped in 0.05% EDTA and dried, 10% acetic acid–NH_3, *p*H 2.7.

solved. The methyl derivative had λ_{max} at 260 mμ and a ratio 280/260 mμ of 0.70.

In the fingerprint of a digest of alanine s-RNA with both ribonucleases, a resistant dinucleotide was found (0.7 mole per mole of cytidine end group) which gave a spectrum in 0.1 M ammonium acetate at *p*H 6.8 closely resembling that of guanosine-3′-phosphate itself. Hydrolysis in 1 N HCl followed by chromatography in the isopropanol-HCl solvent showed only one spot, corresponding to guanine. Rechromatography of this material in isopropanol-NH_3 showed distinctly two bases, the slower of which corresponded to guanine. From the ratio of their R_F values (= 1.4) (Smith and Dunn, 1959) we can deduce that the faster-moving component is N^1-methylguanine, which would not be expected to separate from guanine in the acidic solvent. It seems that the bond next to N^1-methylguanosine-3′-phosphate is resistant to ribonuclease T_1 (McCully and Cantoni, 1951).

The combined action of both ribonucleases yields thymine ribotide (0.9 mole per mole cytidine), char-

acterized by its position on the fingerprint where it is slightly slower than Up in electrophoresis at *p*H 2.7, but faster than Up in chromatography. The spectrum of the isolated Tp showed λ_{max} = 265 mμ ratio 280/260 mμ = 0.67.

In the fingerprint of the pancreatic ribonuclease digest (Figs. 2, 3) of alanine s-RNA, Tp occurs as the *trinucleotide p15* with the structure GpGpTp (0.8 mole per mole cytidine). Alkaline hydrolysis and electrophoresis at *p*H 3.0 in 20% acetic acid–NH_3 gave Gp (24.5 mμmoles) and Tp (12.1 mμmoles). The latter was identified by its characteristic position in electrophoresis and by its spectrum (λ_{max} in 1 N HCl = 263 mμ).

The occurrence of *inosinic acid* (Ip) in s-RNA has been observed by D. B. Dunn (unpublished) and also by Holley *et al.* (1963). In the fingerprint of the pancreatic ribonuclease digest of alanine s-RNA it occurs as the *trinucleotide IpGpCp-(p11)*, an oligonucleotide just below the trinucleotides ApGpUp and GpApUp and not too well resolved from them. The yield is 1.0 mole per mole of cytidine. The structure was proved by dephosphorylation and degradation with snake venom diesterase. Only three products were found on electrophoresis at *p*H 3.0: cytidylic acid (presumably 5'-), 12.8 mμM; 5'-guanylic acid, 7.6 mμM; and inosine, 9.9 mμM. In view of the specificity of pancreatic ribonuclease, this proves the structure IpGpCp. The inosine was characterized by its electrophoretic position, by its spectrum in 1 N HCl (λ_{max} = 247 mμ), and by its hydrolysis with 1 N HCl at 100° to hypoxanthine which was identified chromatographically in the isopropanol-H_2O (NH_3) solvent.

Some larger oligonucleotides near the origin of the fingerprints will be the subject of a later paper.

Valine-specific RNA: Pancreatic Ribonuclease Digestion.—Figure 4 shows a fingerprint of the valine-specific acceptor RNA after digestion with pancreatic ribonuclease, e.g., of the slow valine s-RNA peak from the countercurrent distribution. The cathode side is not shown, but this yielded 12.8 mμmoles of cytidine and 5.2 mμmoles of adenosine. It also showed a minor amount of a component moving between adenosine and the application point. There was not enough material in this spot for further analysis.

The presence of cytidine as the main end group with free 2',3'-hydroxyl groups was confirmed by the alkaline hydrolysis of intact valine s-RNA. Cytidine was also found as the end group in one oligonucleotide obtained after digestion with ribonuclease T_1. All other oligonucleotides after T_1-digestion ended in (2')3'-guanylic acid.

It is of interest to mention here that preliminary results of the ribonuclease T_1 digest of the second valine s-RNA peak from the countercurrent distribution show two oligonucleotides that do not end in (2')3'-guanylic acid. One of these oligonucleotides ends in cytidine and seems to be identical with the one from the first valine-acceptor peak. It is present in a minor amount, however. The second oligonucleotide, not ending in guanylic acid, is the major one, and contains adenosine. In comparison with the first valine-acceptor peak, the ratio cytidine to adenosine is reversed.

Cytidine-3'-phosphate forms *spot p1*. Slightly above this spot in the fingerprint is 5-methylcytidine-3'-phosphate, accounting for 2.0 moles per 78 nucleotides. It has a cytidylic acid spectrum, but its maximum in 1 N HCl was at 284 mμ, and the ratio 280/260 was 3.1.

Spots p6 and *p8* contain uridine-3'-phosphate. Spot p6 is the cyclic form which was proved by its shift in R_F following chromatography of an alkaline hydrolysate. Together they account for 5.6 residues of uridylic acid per 78 nucleotides.

Pseudouridine-(2')3'-phosphate (ψUp) forms *spot p7*, accounting for 2.2 residues per 78 nucleotides. More ψUp (>0.5 residue) is found in a dinucleotide forming spot p20. Alkaline hydrolysis of p20 yields adenosine-(2')3'-phosphate (2.7 mμmoles) and (pseudouridine-(2')3'-phosphate (2.2 mμmoles), identified by their position in electrophoresis at *p*H 2.7 and by their spectra. In addition, ψUp was identified by its characteristic fluorescence in ultraviolet light after electrophoresis.

Thymine ribonucleotide was found in the trinucleotide *p21*, slightly below ApUp, and gave 0.5–0.6 residue per 78 nucleotides (see also Table II). From its position in the fingerprint we assume that the trinucleotide is in cyclic form. Alkaline hydrolysis of p11 followed by electrophoresis at *p*H 2.7 (20% acetic acid–NH_3) yields Ap (5.6 mμmoles), Gp (4.4 mμmoles) and Tp(6.5 mμmoles). The last was identified by its position in electrophoresis, where it moves slightly more slowly than Up, and by spectrum (λ_{max} in acid = 265 mμ, ratio 280/260 mμ = 0.70). Ribonuclease T_1-digestion of the trinucleotide yields free Tp (4.5 mμmoles) and the dinucleotide ApGp (5.0 mμmoles). Combined digestion of valine s-RNA with pancreatic and T_1 ribonucleases gave also free Tp but in a higher yield, corresponding to 1.0 mole per 78 nucleotides. It was identified by its position in the fingerprint (above Up) and by its spectrum. In addition, Tp is found in a tetranucleotide in the fingerprint from a ribonuclease T_1 digest (Ingram and Sjöquist, 1963).

Like alanine s-RNA, valine s-RNA also contains inosinic acid. It was found in the *trinucleotide p22* in the fingerprint, slightly below and to the right of GpCp (p5). The yield was 0.8 mole per 78 nucleotides, and its composition (IpApCp) was established following KOH hydrolysis and electrophoresis at *p*H 2.7; Ap (8.0 mμmoles), Cp (8.3 mμmoles), and Ip (7.9 mμmoles). Ip was characterized by its position in electrophoresis and chromatography in 1-propanol-NH_3 and by its spectrum (λ_{max} in 1 N HCl = 250 mμ). T_1 ribonuclease digestion of p22 yielded free Ip (5.5 mμmoles) and ApCp (4.9 mμmoles), which were identified after electrophoresis at *p*H 2.7.

Free inosinic acid was also found in a fingerprint obtained after the combined action of both ribonucleases. It was present in almost one residue per 78 nucleotides.

The *trinucleotide p23* yielded 0.7 mole per 78 nucleotides. In the fingerprint this spot is located slightly below GpUp. Alkaline hydrolysis followed by electrophoresis at *p*H 2.7 gave guanylic acid (14.4 mμmoles) and uridylic acid (6.7 mμmoles). However, acid hydrolysis of the Gp spot in 1 N HCl for 1 hour, followed by chromatography in isopropanol-H_2O (NH_3 in vapor phase), yielded two spots, both of which gave a blue fluorescence in ultraviolet light when exposed to HCl vapor. The slower-moving spot corresponded to guanine. From the ratio of their R_F values (= 1.45) (Smith and Dunn, 1959) we can deduce that the faster-moving component is N^1-methylguanine. It gave a spectrum in 1 N HCl similar to the one published by Smith and Dunn (1959) with a maximum at 258 mμ and a weak shoulder.

Digestion of p23 with ribonuclease T_1 followed by electrophoresis at *p*H 2.7 yielded two spots. One corresponded to Up, the other migrated a little further than Gp. Following HCl hydrolysis, the composition of the latter was proved by chromatography in isopropanol-H_2O (NH_3 in vapor phase) and spectral analysis. The result showed guanine and N^1-methyl-

TABLE II

VALINE s-RNA: SOME OLIGONUCLEOTIDES FROM PANCREATIC RIBONUCLEASE DIGESTS[a]

Oligonucleotide	Spot No.	Yield (moles per 78 nucleotides)	Structure Determined by:	Product	Yield (mμmoles)	Comment
ApCp	p2	1.9	KOH	Ap	18.6	
				Cp	17.8	
ApUp	p9	1.0	KOH	Ap	11.6	
				Up	11.2	
ApψUp	p20	0.5	KOH	Ap	2.7	Identified by electrophoretic position and spectrum, and ψUp; also by fluorescence in ultraviolet light
				ψUp	2.2	
ApGpT	p21	0.5	KOH	Ap	5.6	See text
				Gp	4.4	
				Tp	6.5	
			T_1	ApGp	5.0	Identified by electrophoretic position and spectrum. KOH-hydrolysis of ApGp → Ap, Gp
				Tp	4.5	
GpCp	p5	3.5	KOH	Cp	31.0	
				Gp	29.5	
IpApCp	p22	0.8	KOH	Ap	8.0	
				Cp	8.3	
				Ip	7.9	See text
			T_1	ApCp	4.9	Identified by electrophoretic position and spectrum. KOH-hydrolysis of ApCp → Ap, Cp
				Ip	5.5	
ApGpUp GpApUp	p10	2.9	KOH	Ap	15.0	
				Gp	15.6	
				Up	15.0	
			T_1	ApGp	20.7	After HCl-hydrolysis, Gp + ApUp separated into guanine, adenine, and Up in isopropyl alcohol–H_2O (NH_3 in vapor phase)
				Up	19.5	
				Gp + ApUp	10.3	
GpGpCp	p24	1.1	KOH	Cp	14.4	
				Gp	27.8	
			alk. phosphatase + SVD[b]	guanosine	7.8	Identified by electrophoretic position (20% HOAc-NH_3, *p*H 2.7) and spectrum
				pC	8.2	
				pG	8.0	
ApGpApUp(?)	p26	0.8	KOH	Ap	22.0	
				Gp	13.8	
				Up	9.0	
pGpGpUp	p27	0.6	KOH	pGp	5.6	Identified by electrophoretic position and spectrum
				Gp	5.4	
				Up	5.7	
GpApApCp	p25	0.9	KOH	Ap	17.1	
				Cp	11.0	
				Gp	10.0	
			T_1	ApApCp	9.6	Identified by electrophoretic position and spectrum. KOH-hydrolysis of ApApCp → 2Ap, C*p*
				Gp	9.9	
GpUp	p14	1.1	KOH	Gp	19.1	
				Up	17.2	
1-MGpGpUp	p23	0.7	KOH	Gp + 1-MGp	14.4	After acid hydrolysis, 1-MGuanine and guanine were separated by chromatography in isopropyl alcohol–H_2O (NH_3 in vapor phase)
				Up	6.7	
			T_1	1-MGpGp	5.0	1-MGpGp separated into 1-MGuanine and guanine in chromatography in isopropyl alcohol–H_2O (NH_3 in vapor phase) subsequent to HCl-hydrolysis
				Up	4.8	

[a] Unless otherwise indicated, the products of degradation were identified by their electrophoretic positions and by their spectra in either 0.5–1.0 N HCl or at *p*H 6.8. [b] SVD = snake venom diesterase.

guanine. Thus the two spots in electrophoresis after ribonuclease T_2 digestion of p23 account for 1-MGpGp (5.0 mμmoles) and Up (4.8 mμmoles).

As mentioned before in connection with alanine s-RNA, the phosphodiester linkage between N^1-methylguanosine and guanosine is resistant to ribonuclease T_1. This was also confirmed in another experiment where valine s-RNA was digested with both pancreatic and

T_1-ribonuclease. In a fingerprint from this digest 1-MGpGp was found, accounting for 0.8 mole per 78 nucleotides. In addition, 1-MGp was found in a trinucleotide, Up1-MGpGp following ribonuclease T_1 digestion of valine s-RNA (Ingram and Sjöquist, 1963). Again the phosphodiester bond between 1-MGp and Gp proved resistant toward ribonuclease T_1.

The *trinucleotide p27* yielded after alkaline hydrolysis pGp (5.6 mμmoles), Gp (5.4 mμmoles), and Up (5.7 mμmoles), accounting for 0.6 mole per 78 nucleotides. The identification of pGp was accomplished by electrophoresis at *p*H 2.7, in which pGp moves further than Up, and by its spectrum.

The *trinucleotide p10* (ApGp)Up is located between UpApCp (p22) and 1-MGpGpCp (p23). It accounts for 2.9 moles of oligonucleotide per 78 nucleotides. Its composition, Ap (15.0 mμmoles), Gp (15.6 mμmoles), and Up (15.0 mμmoles), was established by alkaline hydrolysis followed by electrophoresis at *p*H 2.7. In another experiment, p10 was digested with ribonuclease T_1 followed by electrophoresis at *p*H 2.7. Three main spots were obtained: ApGp (20.7 mμmoles), Gp + ApUp (10.3 mμmoles), and Up (19.5 mμmoles). Acid hydrolysis of Gp + ApUp followed by chromatography in isopropanol-NH_3 yielded almost molar ratios of guanine, adenine, and uridylic acid. The trinucleotide p18 was also treated with alkaline phosphatase. Rechromatography in 1-propanol-NH_3 and digestion with snake venom diesterase was followed by electrophoresis at *p*H 2.7 (20% acetic acid–NH_3). Adenosine and guanosine were found as nucleosides in the ratio 2:1. Hence the composition of p10 was established to be ApGpUp (2 moles) and GpApUp (1 mole).

In the pancreatic ribonuclease fingerprint two *tetranucleotides* occur (*p25* and *p26*). Alkaline hydrolysis of p25 yielded Ap (17.1 mμmoles), Cp (11.0 mμmoles), and Gp (10.0 mμmoles). T_1 digestion of p25 gave ApApCp (9.7 mμmoles) and Gp (9.9 mμmoles). ApApCp is also found as one residue per valine s-RNA in a fingerprint resulting from digestion with the two ribonucleases. We can therefore deduce that p25 is a tetranucleotide with the sequence GpApApCp.

The second tetranucleotide, p26, moves a little further than GpApApCp in electrophoresis, *p*H 2.7, but with the same R_F in chromatography. Alkaline hydrolysis followed by electrophoresis yields Ap (22.0 mμmoles), Gp (13.8 mμmoles), and Up (9.0 mμmoles).

Digestion of p26 with ribonuclease T_1 gave no clear proof of its nucleotide sequence. ApGp and ApUp were present, but so were Gp and some other minor spots. Probably p26 is contaminated with other nucleotide material. Its position in the fingerprint and its base composition indicate, however, that it might be a tetranucleotide. This is in agreement with Holley *et al.* (1963) who finds a tetranucleotide with this composition in a pancreatic ribonuclease digest of valine s-RNA from yeast.

The finding of ApGp and ApUp after ribonuclease T_1 digestion of p26 suggests that the sequence is ApGp-ApUp. This is supported by the result of a fingerprint after digestion with both ribonucleases in which neither ApApGp nor ApApUp could be found, which would be the case if the structure of the tetranucleotide was either ApApGpUp or GpApApUp.

Base Compositions of Alanine and Valine Transfer RNA's.—In Table III are shown the base compositions of alanine and valine RNA's based mainly on the analyses of mono- and oligonucleotides derived from digests with pancreatic ribonuclease. The data are supported by the results of total KOH hydrolysis and of simultaneous digestion with the two ribonucleases. In the pancreatic ribonuclease digest it is easy to note and discard those trace amounts of contaminating oligonucleotides which still exist. In a total KOH hydrolysate several contaminants would give rise to appreciable amounts of the four main nucleotides. For this reason the data from pancreatic ribonuclease digestion are considered to be more reliable.

TABLE III

COMPOSITION OF ALANINE AND VALINE S-RNA[a]

	Alanine	Valine
Ap	8 ⎫ 8	13 ⎫ 13
MeAp	0 ⎭	0 ⎭
Up	13 ⎫	14 ⎫
ψUp	2 ⎬ 16	3 ⎬ 18
Tp	1 ⎭	1 ⎭
1-MGp	1	1
2-DMGp	1	0
Gp	23 ⎫	17 ⎫
pGp	1 ⎬ 25	1 ⎬ 19
Ip	1 ⎭	1 ⎭
Cp	23 ⎫	24–25 ⎫
5-MCp	⎬ 24	1–2 ⎬ 26–28
Cytidine	1 ⎭	1 ⎭
	75	77–79
ApCp	2	3
ApGp	4	4
ApUp	2	3
ApψUp		1
ApApCp		1

[a] The composition is based mainly on the analyses of mono- and oligonucleotides from digests with pancreatic ribonuclease (see text). The bases are grouped according to their expected base pairing character. A minimum of 11 and 13 bases in alanine and valine s-RNA, respectively, cannot find partners for base pairing of the Watson-Crick type.

The differences between the two RNA species are very clear, particularly in the "additional" bases and in the contents of adenylic acid. In the two RNA's it is apparent that a minimum of 11 and 13 bases, respectively, cannot find partners for base pairing. The methylated guanylic acids are placed in a class by themselves because they probably cannot form base pairs of the Watson-Crick type at all.

DISCUSSION

The results indicate clearly the differences which exist between alanine- and valine-specific s-RNA from yeast. Particularly striking are the differences and the similarities in the sequences around the additional bases, such as those summarized in Table IV. No obvious reason emerges for the formation of the additional bases in the sequences where they occur.

The methods employed in this work are classical now and they are adequate up to the level of tetra- or even pentanucleotides. For oligonucleotides which are con-

TABLE IV

ALANINE AND VALINE S-RNA: COMPARISON OF SEQUENCES

Alanine s-RNA	Valine s-RNA
GpGpTp	ApGpTp
IpGpCp	IpApCp
1-MGpGpCp	1-MGpGpUp
2-DMGpCp	
	ApψUp
	(pyrimidine)p5-MCp

siderably longer than five, new methods of separation and for sequencing will be needed.

In the present work, emphasis was placed on obtaining quantitative results, and in many cases duplicate or triplicate analyses. In particular, this applies to the analysis of the oligonucleotides and to the yields of the oligonucleotides themselves. The high yields obtained for all oligonucleotides except ApUp in alanine s-RNA and ApGpTp in valine s-RNA lead us to believe that the starting preparations are reasonably homogeneous with respect to nucleotide sequences. It is particularly reassuring to note that our results are, on the whole, in very good agreement, both qualitatively and quantitatively, with those of Holley *et al.* (1963) and of Doctor *et al.* (1963) for similar preparations of specific s-RNA's. The few minor differences which do exist will presumably be eventually resolved, particularly as new unusual bases are being discovered.

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