Chemical Studies on Amino Acid Acceptor Ribonucleic Acids. II. Attempts at Partial Digestion of Yeast Amino Acid Acceptor Ribonucleic Acid with Pancreatic Ribonuclease*

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Limited hydrolysis of mixed yeast amino acid acceptor RNA with pancreatic ribonuclease has been carried out under conditions which are aimed at stabilizing the secondary structure. Long oligonucleotides in the molecular weight range 11,000–16,000 were obtained, as well as a fraction of small oligonucleotides. Experiments with mixed and with purified alanine specific acceptor RNA indicate that only a few of the "additional" bases are liberated among the small oligonucleotides.

Complete digestion of amino acid acceptor RNA (s-RNA) with any of the presently available degradative enzymes produces a large number of small fragments. It would be of considerable use in nucleotide sequence studies if the action of such enzymes could be limited to a small number of specific sites, yielding large well-defined oligonucleotide fragments.

Previous work (Penniston, 1962; Penniston and Doty, 1963; Nishimura and Novelli, 1963; Zubay and Marciello, 1963) has established that Mg^{2+} stabilizes the secondary structure of acceptor RNA. These workers have also shown that Mg^{2+} inhibits the degradation of s-RNA by a number of ribonucleases and and phosphodiesterases.

We have examined the products formed when mixed yeast amino acid acceptor RNA is partially degraded by pancreatic ribonuclease at low temperatures in the presence of Mg^{2+}, and we have found that large fragments can be prepared from such digestion mixtures. The nucleotide compositions and approximate molecular weights of these fragments are reported, together with some preliminary studies on a similar partial digestion of purified alanine-specific acceptor RNA.

Materials and Methods

Acceptor RNA.—Yeast soluble RNA was obtained from General Biochemical Corp. (Chagrin Falls, Ohio), who make it essentially according to Holley *et al.* (1961). It was chromatographed on DEAE-cellulose (Ingram and Pierce, 1962). The material will be referred to as *mixed acceptor RNA*. In one experiment, alanine-specific acceptor RNA, purified from commercial yeast s-RNA by two countercurrent distributions (Apgar *et al.*, 1962; Ingram and Sjöquist, 1963), was used.

Ribonuclease.—Bovine pancreatic ribonuclease was obtained as a thrice-crystallized product from Worthington Biochemical Corp. (Freehold, N.J.).

Bentonite.—The commercial product was treated according to Fraenkel-Conrat *et al.* (1961).

Chelex-100.—Chelex-100, 50–100 mesh, was obtained from the California Corp. for Biochemical Research. It was washed with 2 N HCl, 2 N NH_4OH, and water, before use. It was shown to be capable of removing Mg^{2+} from RNA digests by testing such digests with Eriochrome Black-T before and after treatment with the resin (Laitinen, 1960).

Molecular Weight Determinations.—These were performed by short-column equilibrium ultracentrifugation, as described by Yphantis (1960). Runs were performed with a multichannel equilibrium centerpiece obtained from the Spinco division of Beckman Instrument Co. (Palo Alto, Calif.). The solvent used for the determinations was 0.2 M NaCl, 0.005 M potassium phosphate, *p*H 7.2, plus 0.5% formaldehyde. RNA in this solvent was allowed to stand overnight at room temperature before centrifugation. Determinations were made at 15,220 rpm for undegraded RNA and at 21,740 rpm for large fragments isolated from partial digests.

The partial specific volume of intact acceptor RNA was taken as 0.51 cm^3/g (Tissières, 1959). It was assumed that this value also applied to the large fragments isolated from partial digests.

Runs performed with 0.4 M NaCl instead of 0.2 M NaCl in the solvent gave essentially identical results. This indicated that errors due to charge effects (Johnson *et al.*, 1954) are probably not serious in this system.

Synthetic-boundary runs were performed in a Schachman-type cell (Schachman and Harrington, 1954). It was assumed that the relationship between the solute concentration and the area under the schlieren peak found for intact acceptor RNA was also valid for the large fragments produced by partial digestion. This assumption was justified by the fact that these fragments turned out to have nucleotide compositions (and thus presumably refractive index increments) similar to that of intact acceptor RNA.

A photograph of the schlieren patterns produced during a typical run with intact crude acceptor RNA is given in Figure 1a. Figure 1b shows the concentration dependence of the apparent weight-average molecular weight of acceptor RNA. Since this concentration dependence was quite small, ultracentrifuge measurements on large oligonucleotide fragments were carried out at single concentrations below 5 mg/ml, and errors due to concentration dependence were assumed negligible.

Concentrations of RNA stock solutions were estimated from the ultraviolet absorption at 260 mμ. The absorbance of a 0.10% solution of s-RNA in 0.01 M phosphate, *p*H 7.2, in a 1-cm cell at 25° was taken as 21.4 (Stephenson and Zamecnik, 1961). Extinction coefficients at 260 mμ of RNA fragments were determined experimentally by measuring the absorbance at 260 mμ of solutions before and after

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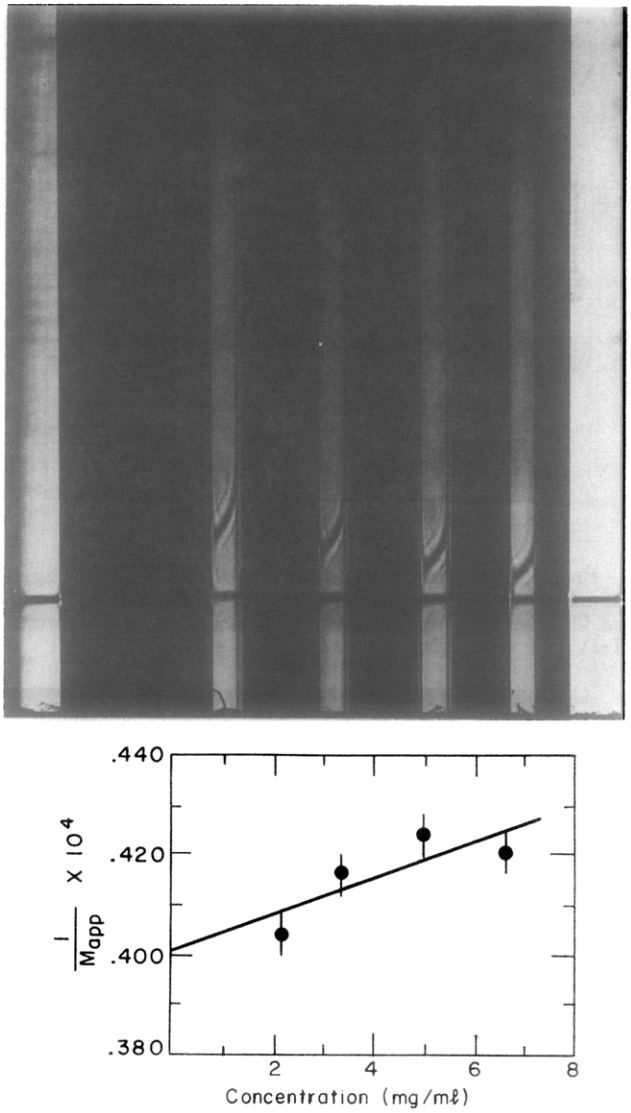

FIG. 1.—Schlieren photograph of short-column equilibrium ultracentrifuge run on mixed acceptor RNA (a, upper); and concentration dependence of the apparent molecular weight of mixed acceptor RNA (b, lower). (a) Speed of rotation, 15,220 rpm; bar angle, 75°. Concentrations of solute are (left to right) 6.65, 5.00, 3.32, and 2.22 mg/ml. Photograph taken 2 hours 10 minutes after attainment of speed. (b) Ordinate, reciprocal of the apparent molecular weight $\times 10^4$; abscissa, concentration in mg/ml. Points calculated from photograph in (a).

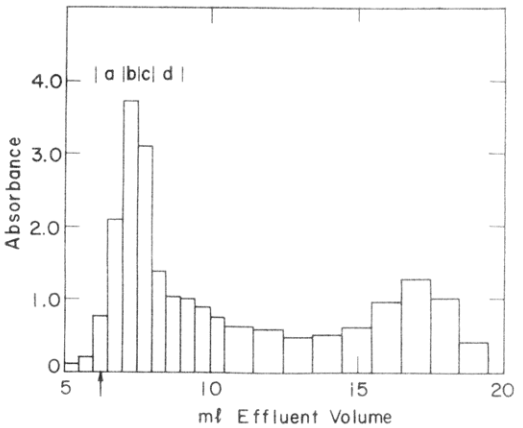

FIG. 2.—Elution profile of a partial ribonuclease digest of mixed acceptor RNA. A 0.9 × 22-cm column of Sephadex G-75 fine was equilibrated with 0.1 M triethylammonium acetate. The sample (1.1 mg/0.4 ml) was applied and the column eluted with 0.1 M triethylammonium acetate at a flow rate of 10 ml/hr. Intact acceptor RNA gave a single peak at 6.0–6.5 ml (marked with an arrow) when passed through this column. Fractions a–d were used for molecular weight studies (see Table I). See text for digestion conditions.

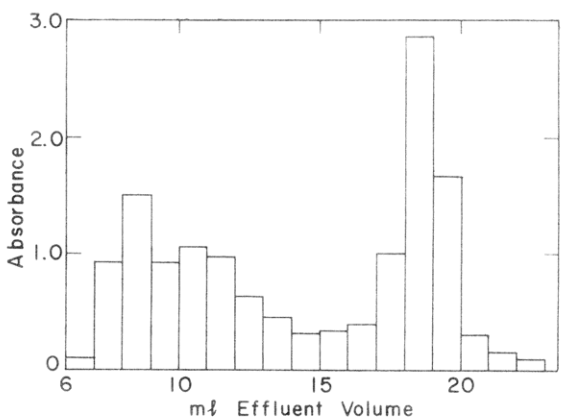

FIG. 3.—Sephadex G-75 elution profile of a 1-hour digest of mixed acceptor RNA (0.6 mg) with pancreatic ribonuclease in 0.2 M ammonium acetate, 0.01 M EDTA, *p*H 7.5 0°. The details of the chromatography are the same as for Fig. 2, except that 0.05 M ammonium formate was used as the eluent.

alkaline hydrolysis. The absorbance of intact acceptor RNA increased 34% on hydrolysis in 0.3 N KOH for 16 hours at 37°; the large fragments showed an increase of 26% after similar treatment. (Absorbances of alkaline hydrolysates were measured directly in 0.3 M KOH.)

Partial Enzymatic Digestions.—The digestion mixtures (total volumes 1–2 ml) contained, per ml final volume, 100 μmoles Tris-acetate, *p*H 7.5; 10 μmoles magnesium acetate, and 2.0 mg RNA. Solutions were chilled to 0° and 20 μg pancreatic ribonuclease per ml was added. The incubation at 0° was terminated after 1 hour by addition of 2–3 mg bentonite per ml digest (Fraenkel-Conrat *et al.*, 1961). The *p*H was adjusted in the cold to 5.5–6.0 with acetic acid. The bentonite and adsorbed ribonuclease were removed by brief centrifugation.

The *p*H was adjusted to 7.5–8.0 with NH_4OH, and the solution was passed through a 0.9 × 3-cm column of Chelex-100 (NH_4^+ form). Digests were stored frozen until used.

The bentonite treatment was shown to produce essentially total inhibition of ribonuclease under conditions much more favorable to ribonuclease action than those used in the partial digestions. No acid-soluble nucleotides were produced when acceptor RNA, bentonite, and ribonuclease, at concentrations similar to those used in the partial digestions, were incubated at 37° for 1 hour in 0.15 M potassium acetate, *p*H 6.

Fractionation of Digests on Sephadex G-75.—The digests (1–4 mg) were concentrated in a rotary evaporator to a volume of about 0.5 ml. They were then applied to 0.9 × 22-cm columns of Sephadex G-75-fine (Pharmacia). Elution was carried out with 0.05 M ammonium formate or 0.1 M triethylammonium acetate, *p*H 7, at a flow rate of 4–8 ml/hour.

Nucleotide Composition of Fractions.—To determine the nucleotide compositions of the fractions, the digestion was carried out as above, except that the

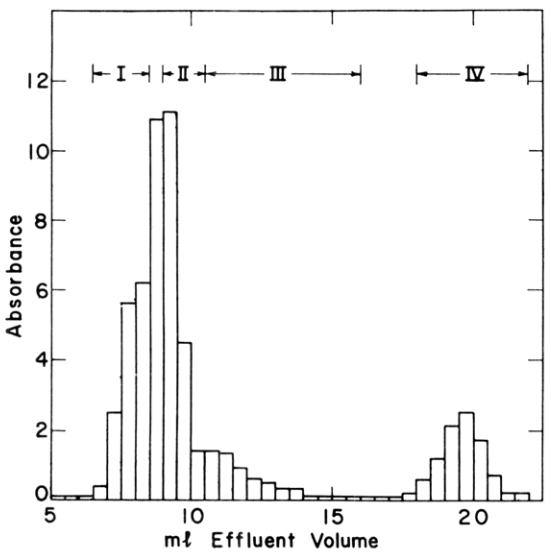

FIG. 4.—Elution profile of a partial ribonuclease digest of mixed acceptor RNA (1.3 mg). Details are the same as for Fig. 2, except that digestion was carried out in 0.2 M ammonium acetate, 0.01 M magnesium acetate, 0.002 M Tris-acetate, *p*H 7.5, and elution was with 0.05 M ammonium formate. Fractions were combined into groups I–IV and used for nucleotide composition studies (see Table II).

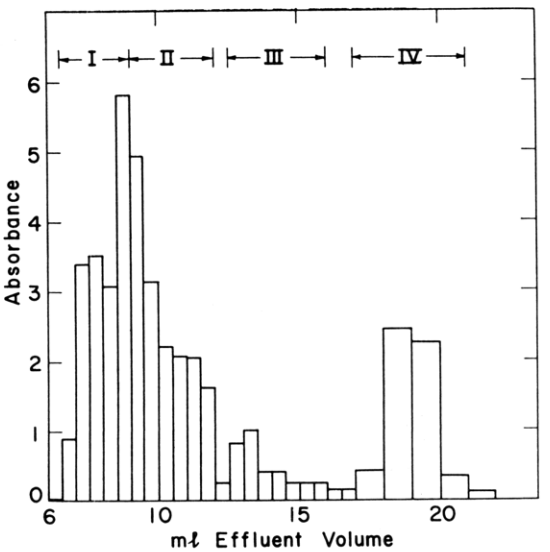

FIG. 5.—Elution profile of a partial ribonuclease digest of alanine specific RNA (1.5 mg). Details are the same as for Fig. 4. Fractions were combined into four groups, I–IV, for the nucleotide composition studies.

quantity of Tris-acetate was decreased to 2 μmoles/ml and 200 μmoles ammonium acetate per ml final volume was added. In this way the accumulation of salt in the fraction of small oligonucleotides was avoided.

Appropriate fractions from Sephadex columns were evaporated in a rotary evaporator to remove volatile salts. The samples were taken up in 75 μl 0.3 N KOH and hydrolyzed at 37° for 14–18 hours in sealed capillaries. K^+ ions were removed by eluting the hydrolysates from 2 × 6-cm strips of CM-50 cation exchange paper with 100–200 μl H_2O (Ingram and Pierce, 1962). The strips were further eluted with 0.5 N NH_4OH to remove any nucleosides (especially cytidine) which were present.

The separation and identification of the nucleotides was carried out by a modification of the two-dimensional mapping procedure (method C) of Ingram and Pierce (1962). Electrophoresis was performed on Whatman 3HR paper at 20 v/cm for 3.5–4 hours in 20% acetic acid adjusted to *p*H 3.0 with concentrated ammonium hydroxide. This was followed by ascending chromatography in the system of Markham and Smith (1952) which contains isopropanol-water (70:30 v/v) plus ammonia in the vapor phase (0.35 ml concentrated NH_4OH in a beaker per liter of gas space). The eluates from the spots were made 0.1 M in HCl and the spectra were recorded with a Cary Model 11 M Spectrophotometer.

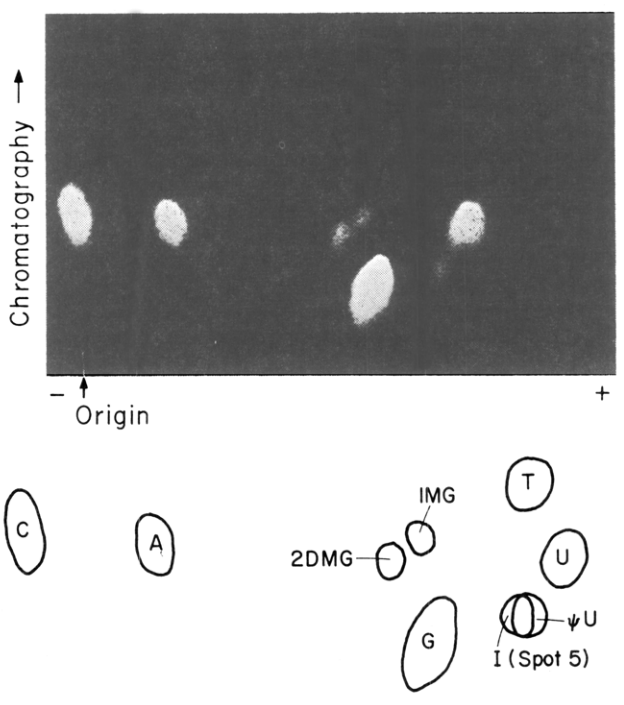

FIG. 6.—Two-dimensional map of an alkaline hydrolysate of fraction II, Fig. 5. (a, upper) Contact print of map. Approximately 0.2 mg of hydrolyzed RNA was "wet loaded" on a half-inch line, 11 inches from the short edge of an 18 × 22-1/2 inch sheet of Whatman 3HR paper. The paper had previously been washed with 1-propanol–water–concd NH_4OH (55:35:10, v/v) to reduce the background of ultraviolet-absorbing impurities. Electrophoresis was in 20% (v/v) acetic acid, adjusted to *p*H 3.0 with concd NH_4OH. For chromatography the isopropanol-H_2O (70:30) system, with NH_3 in the vapor phase, was used (see text). (b, lower) Tracing of two-dimensional map (a).

RESULTS

A typical Sephadex G-75 elution profile of a 1 hour digest of *mixed acceptor RNA* is shown in Figure 2. Similar elution profiles were obtained when partial digests were first heated 15 minutes at 65° in 1% formaldehyde and then chromatographed on G-75 columns with 0.1 M triethylamine acetate plus 1% formaldehyde as eluent.

If the digestion was carried out for 1 hour at 0° in the presence of 0.01 M EDTA instead of 0.01 M Mg^{2+}, the elution profile shown in Figure 3 was obtained. It is evident that the replacement of Mg^{2+} by EDTA accelerates the degradation considerably.

The molecular weights observed for some of the G-75 column fractions of Figure 2 and for intact mixed acceptor RNA are given in Table I. Similar results were obtained with two other fractionated digestion mixtures. The errors given reflect the estimated

precision of the determinations, not the absolute accuracy. The molecular weights obtained for column fractions were unaffected when the solutions made up for the ultracentrifuge runs (containing 0.5% formaldehyde) were heated at 55° for 30 minutes prior to ultracentrifugation, a treatment which is known to break up any secondary structure (Haselkorn and Doty, 1961). This establishes that the milder formaldehyde treatment usually used was probably effective in breaking up any hydrogen-bonded aggregates which might have been present.

TABLE I

MOLECULAR WEIGHTS OF INTACT MIXED ACCEPTOR RNA AND OF FRACTIONS FROM SEPHADEX COLUMN (FIG. 2)

Sample	Molecular Weight
Fraction a	15,900 ± 800
Fraction b	14,400 ± 700
Fraction c	13,200 ± 700
Fraction d	10,800 ± 500
Intact acceptor RNA	25,000 ± 500

with maximum near 260 mμ. However these spectra displayed more absorption at 250 mμ than could be accounted for by ψUp alone, indicating some contamination by Ip. Spot 5 from fraction IV showed essentially no fluorescence and had a spectrum (in 0.1 M ammonium acetate, *p*H 7) with a peak at 250 mμ, characteristic of inosinic acid.

DISCUSSION

Our results confirm the observation of others that Mg^{2+} inhibits the action of pancreatic ribonuclease on acceptor RNA. In the presence of Mg^{2+} large fragments accumulate in the digestion mixtures which account for about 65% of the total material in the digest, both for alanine-specific and mixed acceptor RNA. These fragments are heterogeneous; in the case of the mixed acceptor RNA, their molecular weights range from about 11,000 to 16,000. Although no direct molecular weight measurements were made in the case of partial digests of alanine-specific acceptor RNA, behavior of the digests on Sephadex columns suggests a similar molecular weight range for the large fragments.

About 20% of the material in the partial digests of both alanine-specific and mixed acceptor RNA is accounted for by low molecular weight mono- and oligonucleotides that are completely included in Sephadex G-75.

It seems that Mg^{2+} ions stabilize the secondary structure of acceptor RNA and thus protect those ribonuclease sensitive sites which are present in helical regions. Sensitive sites present in nonhelical regions of the molecule are preferentially attacked. By virtue of its strong binding to the phosphate groups (Penniston, 1962), Mg^{2+} neutralizes the negative charge and thus decreases the tendency toward strand separation. The secondary structure remains intact, even in molecules which have suffered a few enzymatic cleavages.

In the absence of Mg^{2+} we may speculate that sites in nonhelical regions are still attacked preferentially, at first. However, these initial breaks might well introduce enough instability in the secondary structure to unmask other susceptible bonds. Thus a more rapid and more extensive hydrolysis would ensue.

TABLE II

NUCLEOTIDE COMPOSITION OF MIXED ACCEPTOR RNA AND OF FRACTIONS OBTAINED BY CHROMATOGRAPHY ON SEPHADEX (FIG. 4) OF A PARTIAL RIBONUCLEASE DIGEST OF RNA
(moles/100 moles of nucleotides and nucleosides)

Fractions[a] (Fig. 4)	Cp	Ap	Gp	Up	ψUp+Ip	MeAp	MeGp	Tp	pGp	C+A	Total Micromoles per Fraction
Acceptor[b] RNA	28	22	25	18	3.2	0.5	1.8	1.1	0.6	1.3	
I	29	21	32	10	3.0	1.0	1.7	1.5	1.0	none	1.9
II	30	19	32	11	2.6	0.9	2.4	1.5	0.9	none	2.1
III[c]	25	19	33	22					none	none	0.65
IV	36	14	18	21	5.5	none	trace	none	none	5.5[d]	1.3
Total[e]	30	19	29	14	3.1	0.4	1.4	1.0	0.6	1.2[d]	6.0

[a] Symbols: ψUp represents the 3′(2′)-monophosphate of uracil-5-ribose; Ip represents 3′(2′)-monophosphate of inosine; MeAp and MeGp represent the mixed methylated adenylic and guanylic acids. [b] Data of Ingram and Pierce (1962) for yeast acceptor RNA. [c] This analysis was done using electrophoresis only; therefore the additional components were not resolved. Thus Ap includes MeAp, Gp includes MeGp, and Up includes ψUp, Ip, and Tp. [d] After this hydrolysate had been eluted from CM-50 paper with water, the paper was further eluted with 200 μl 1 N NH_4OH. This alkaline eluate gave a spectrum characteristic of free cytidine. As no cytidine was observed on the two-dimensional map of the water eluate, it was assumed that the alkaline eluate contained all of this component present in fraction IV. Adenosine was not observed in this fraction. [e] Total by adding the composition of each of the four fractions and including a correction for the percentage of the total material in each fraction.

The elution profile for another partial digest of *mixed acceptor RNA* is shown in Figure 4. Nucleotide compositions found for intact mixed acceptor RNA and for the various fractions from this column are given in Table II.

A single partial digestion was carried out on *alanine-specific acceptor RNA*. The digest was freed of Mg^{2+} and chromatographed on Sephadex G-75 as described above. The elution profile is shown in Figure 5, and the nucleotide composition of the fraction is discussed in the text. A two-dimensional map of the alkaline hydrolysate of fraction II is shown in Figure 6.

The two-dimensional mapping procedure gives only a partial separation of pseudouridylic and inosinic acids. Therefore these were eluted and measured together. However, some discrimination between them was possible from the spectrum of the eluted spots and also from the degree of fluorescence observed when the spots on the paper were exposed to ultraviolet light for 3–5 minutes. Thus spot 5 (Figure 6b) from fractions I and II of the alanine RNA digest had the brilliant blue-white fluorescence characteristics of ψUp, and had a spectrum (in 0.1 M ammonium acetate, *p*H 7)

The degree of specificity inherent in these limited digestions has not been established. However, the distribution of the "additional bases" among the fragments produced by partial digestion of alanine-specific acceptor RNA suggests that some degree of specificity exists. Thus inosinic acid is found predominantly in the peak of small oligonucleotides (IV) in Figure 6; N^2-dimethylguanine, on the other hand, occurs only in the large fragments (I and II), which also contain most of the pseudouridylic acid. The "additional" base N^1-methylguanine appears in both the large and the small fragments, as if incomplete cleavage had occurred at a relatively insensitive internucleotide linkage. However, since only one residue of each of the "additional" nucleotides N^1-methylguanylic acid, N^2-dimethylguanylic, ribothymidylic acid, and inosinic acid is present per molecule of intact alanine-specific acceptor RNA (Ingram and Sjöquist, 1963), a completely specific cleavage should lead to the appearance of each of these components in only one chromatographic peak. With the exception of N^1-methylguanylic acid, this is the case. It may be possible to improve the degree of specificity by modifying the conditions of digestion and to obtain better separation of the large pieces.

Finally, it should be pointed out that these results do not really support the model of acceptor RNA recently proposed by McCully and Cantoni (1962). If the "additional bases" were all concentrated in a nonhelical loop, one would expect, with both mixed and alanine-specific RNA, that the content of such bases would be reduced in the large fragments and enriched in the small fragments resulting from partial ribonuclease digestion. For the most part, our findings do not agree with this prediction.

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Purification and Properties of a Polygalacturonic Acid-*trans*-eliminase Produced by *Clostridium multifermentans**

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A strain of *Clostridium multifermentans*, although unable to ferment D-galacturonic acid, fermented polygalacturonic acid vigorously with the production of an extracellular pectic enzyme. With polygalacturonic acid as the substrate, this enzyme, with an optimal *p*H of 8.5, produced reaction products which strongly absorbed light at 235 mμ, indicating a *trans*-elimination mechanism. The clostridial enzyme was partially purified and was freed of contaminating pectinesterase. Based on the relationship between increase in absorbancy at 235 mμ and increase in reducing group formation, polygalacturonase appeared to be absent in the crude culture fluid. Activity of the enzyme was dependent on critical levels of certain divalent cations. Calcium, strontium, and manganese showed the greatest stimulation. The major end product from polygalacturonic acid was *O*-(4-deoxy-β-L-*threo*-hexopyranos-4-enyluronic acid)-(1 → 4)-D-galacturonic acid. Purified enzyme showed no activity on pectin of high methoxyl content (96% esterified) but crude enzyme preparations were able to attack this substrate since they also contained pectinesterase.

A most significant contribution to our knowledge of the enzymic degradation of pectic substances was the discovery by Albersheim, *et al.* (1960) of an enzyme capable of splitting the α-1,4-glycosidic bonds of pectin by a *trans*-elimination mechanism. Prior to this it had been universally assumed that pectinolytic enzymes break these bonds only by hydrolysis. The cleavage of the glycosidic bond of pectin by *trans*-elimination results in the loss of the proton from carbon atom 5 of a methyl galacturonate residue forming an unsaturated bond between carbon atoms 4 and 5. The products absorb light at approximately 235 mμ. There was no evidence of *trans*-elimination when the enzyme was incubated with polygalacturonic acid. This indicated a specific requirement for the presence of carbomethoxy groups rather than free carboxyl

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