Structure of Amino Acyl Oligonucleotides from T_1 Ribonuclease Digests of Soluble Ribonucleic Acid Charged with Serine, Glycine, Threonine, and Alanine*

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ABSTRACT: Four kinds of amino acyl oligonucleotides, each labeled with a different radioactive amino acid, had previously been prepared in a purified state from T_1 ribonuclease digests of amino acyl ribonucleic acid. The structure of the oligonucleotide component of each substance was determined by degradation with alkali, venom phosphodiesterase, and pancreatic ribonuclease and by sequential degradation using periodate oxidation, amine-catalyzed cleavage, and phosphomonoesterase digestion.

Separate studies showed that the amino acids were attached to oligonucleotide by amino acyl linkage. From the results described, the terminal nucleotide sequences at the acceptor ends of the corresponding chains of yeast transfer ribonucleic acid were deduced. These were as follows: serine, . . .pGpCpCpA; glycine, . . . pGpCpApCpCpA; threonine, ...pGpCpApCpCpA; alanine, . . .pGpUpCpCpApCpCpA.

The previous paper (Smith *et al.*, 1966) described the isolation, in a purified state, of four kinds of amino acyl oligonucleotides from T_1 ribonuclease digests of yeast soluble ribonucleic acid (s-RNA). The present report is concerned with experiments which were carried out in order to characterize these derivatives of amino acyl RNA.

The structure of the oligonucleotide component of each of the four fragments was determined by the use of classical procedures adapted to a micro scale. The procedures employed made it possible to elucidate the complete structure of each oligonucleotide, leaving no assumptions about the order of bases. Separate experiments were performed in order to confirm the identity and mode of attachment of amino acids in the four fragments.

Experimental Procedures

High-voltage electrophoresis was carried out as described in the previous paper (Smith *et al.*, 1966). The solutions used for electrophoresis were as follows: pH 3.5, 5% acetic acid adjusted to pH 3.5 by addition of ammonia; pH 5.0, 1% acetic acid–0.002 M EDTA, adjusted to pH 5.0 by addition of ammonia; pH 9.3, 0.1 M potassium borate–0.002 M EDTA.

Storage of Purified Amino Acyl Oligonucleotides. After the final purification procedure described in the previous paper (Smith *et al.*, 1966) amino acyl oligonucleotides were freeze dried and ammonium formate or ammonium acetate was removed by means of an infrared lamp. The final products were stored in a desiccator at −20°.

Removal of Amino Acids from Amino Acyl Oligonucleotides by Hydrolysis in Mild Alkali. Between 2 and 4 OD_{260} units of each preparation was taken up in 100 μl of 0.2 M ammonium bicarbonate, pH 8.6, and incubated in an air-tight tube at 37° for 3 hr. The samples were diluted to 0.8 ml with distilled water and applied to small columns of DEAE-cellulose (Serva Entwicklungslabor, Heidelberg, Germany), 4 × 0.4 cm, which had been prepared in the cold room at 2°. Before use the columns were washed overnight with 1.5 M ammonium acetate, pH 6, followed by 0.015 M ammonium acetate, pH 5.5. The radioactivity due to free amino acids was washed through the columns in 0.015 M ammonium acetate, pH 5.5. The oligonucleotides were then eluted using 1.5 M ammonium acetate, pH 6.0. The amino acid and oligonucleotide fractions were finally freeze dried on parafilm in a vacuum desiccator which contained solid KOH and concentrated H_2SO_4.

Alkaline Hydrolysis of Oligonucleotides. A freeze-dried sample containing about 2 OD_{260} units of oligonucleotide after removal of amino acid was taken up in 40 μl of distilled water. One microliter of 10 N KOH was added giving pH 13–13.5. The sample was then sealed in a thin-walled glass capillary tube and heated at 80° for 1 hr. The tube was cooled and broken, and the contents were quantitatively transferred to a column

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[1] Abbreviations used in this paper: A, adenosine; G, guanosine; C, cytidine; U, uridine; Ap, Gp, Cp, and Up represent corresponding 2′- and/or 3′-nucleoside monophosphates; pA, pG, pC, and pU represent the 5′-nucleoside monophosphates; ApCp, adenylyl-(3′-5′)-cytidine (mixed 2′,3′)-phosphate, and other polynucleotides similarly.

of Dowex-1 resin for separation of the components of the digest.

Enzymes Used for Analytical Studies. Snake venom phosphodiesterase and pancreatic ribonuclease were purchased from Worthington Biochemical Corp., Freehold, N. J. Both enzymes were free from phosphomonoesterase activity. *Escherichia coli* alkaline phosphatase was a gift from Dr. Milton Schlesinger. The enzyme had been prepared by the method of Garen and Levinthal (1960) and was free from ribonuclease activity.

Digestion of Oligonucleotide with Snake Venom Phosphodiesterase. A sample containing about 2 OD_{260} units of oligonucleotide in freeze-dried form after removal of attached amino acid was taken up in 100 μl of 0.2 M ammonium bicarbonate, pH 8.6. A solution (5 μl) of snake venom phophodiesterase, 5 mg/ml, was added. The mixture was incubated for 35 min at 37° in an airtight tube. The sample was dried down with air flow, taken up in 50 μl of distilled water, and dried down a second time in order to remove ammonium bicarbonate. The contents of the tube were then taken up in 0.5 ml of distilled water and applied to a column of Dowex-1 resin for separation of the components of the digest.

Digestion of Amino Acyl Oligonucleotides with Pancreatic Ribonuclease. Between 2 and 4 OD_{260} units of amino acyl oligonucleotide in freeze-dried form was taken up in 40 μl of 0.02 M ammonium acetate, pH 5.5. A solution (1 μl) containing pancreatic ribonuclease, 10 mg/ml, was added and the mixture was maintained at 25° for 1 hr. The mixture was then applied to a column of Dowex-1 resin to fractionate the components of the digest. In some experiments the ribonuclease digest was fractionated directly by electrophoresis. For such studies the sample was taken up initially in 10–20 μl of 0.01 M ammonium acetate, pH 5.5, before addition of ribonuclease.

Analysis of Oligonucleotides for Minor Nucleosides. A freeze-dried sample containing about 2 OD_{260} units of oligonucleotide after removal of amino acid was taken up in 20 μl of distilled water and transferred to a 1-ml conical glass centrifuge tube. The sample was then treated with 2 μl of 2 M ammonium bicarbonate, pH 8.6; 5 μl of venom phospodiesterase, 5 mg/ml; and 5 μl of *E. coli* alkaline phosphatase, 100 μg/ml. The contents of the tube gave a pH of 8.5–8.6 after mixing. The tube was closed with a piece of parafilm and was immersed in a water bath at 37° for 2 hr. The contents of the tube were quantitatively transferred to parafilm. The sample was then freeze dried in a vacuum desiccator which contained solid KOH and concentrated H_2SO_4. After drying, the sample was redissolved in 5 μl of distilled water and two 2-μl aliquots were applied to two separate sheets of Whatman No. 1 paper. Control samples containing reference nucleosides were treated in the same way as the unknown samples and were applied to the paper at the same time. The two sheets of paper were then developed by descending chromatography at room temperature in different solvent systems: solvent A (Hall, 1965), 1-butanol–water–concentrated ammonium hydroxide (86:14:5); solvent B (Wyatt, 1951), 2-propanol–concentrated hydrochloric acid–water (680:170:144).

Quantitative Fractionation of RNA Digests on Microcolumns of Dowex-1 Formate. Dowex-AG-1-X10, 200–400 mesh, chloride form, was purchased from California Corp. for Biochemical Research, Los Angeles, Calif. The resin was converted to the formate form before use. In order to analyze digests containing 10–50 mμmoles of each RNA nucleotide, a procedure was developed using columns of resin 8 cm long and 0.2 cm in diameter. The columns were eluted according to the method described by Cohn (1955) except that Up was eluted with 0.05 M ammonium formate–0.05 M formic acid, pH 5.0. The columns were run under pressure equal to 40-cm head of water, and 0.5-ml fractions were collected. The chromatographic procedure was sufficiently sensitive to detect 20 mμmoles of an RNA nucleoside or of the nucleotides Cp, Ap, Up, pC, pA, and pU with an experimental error of $\pm 10\%$.

Sequential Degradation of Oligonucleotides Using Periodate Oxidation, Amine-Catalyzed Cleavage, and Phosphomonoesterase Digestion. A procedure was developed for determination of the terminal base in the unknown oligonucleotides after the attached amino acid had been removed. This was a modification of the technique described by Neu and Heppel (1964). The method could be used repeatedly on a given oligonucleotide sample to remove base residues from the 3′ terminus in a sequential manner. The sensitivity was sufficient to detect 10 mμmoles of released terminal base.

Oligonucleotide (2–3 OD_{260} units) in freeze-dried form was taken up in 20 μl of distilled water and transferred to a 1-ml glass conical centrifuge tube. Two microliters of 0.2 M $NaIO_4$ was added, the contents of the tube were mixed, and after an interval of about 2 min 2 μl of 3.5 M cyclohexylammonium acetate, pH 10.5, was added. The contents of the tube were mixed again giving a final pH of about 10 (indicator paper). The tube was closed with a piece of parafilm and was immersed in a water bath at 45° for 90 min. The sample was then removed from the bath, treated with 2–5 μl of 1 M ethylene glycol, mixed, and allowed to stand 5 min at room temperature. At this point 5 μl of 2 M ammonium bicarbonate, pH 8.6, was added followed by 10 μl of a solution containing 100 μg/ml of *E. coli* alkaline phosphatase. The contents of the tube were mixed giving a final pH of 8.6–9. The tube was closed with a piece of parafilm and incubated as before for 2 hr at 45°.

After incubation the contents of the tube were diluted with 200 μl of ice-cold distilled water and applied to a column of DEAE-cellulose, 4 × 0.4 cm, in the cold room at 2°. The column had been washed overnight as described above. Following loading, the free base and alkaline phosphatase were washed through the column with 0.015 M ammonium acetate, pH 5.5. The oligonucleotide was then eluted using 1.5 M ammonium acetate, pH 6.0. After determination of spectral data, the oligonucleotide solution was dried down on parafilm as described above.

Estimation of Total Amino Acid per Mole of Terminal

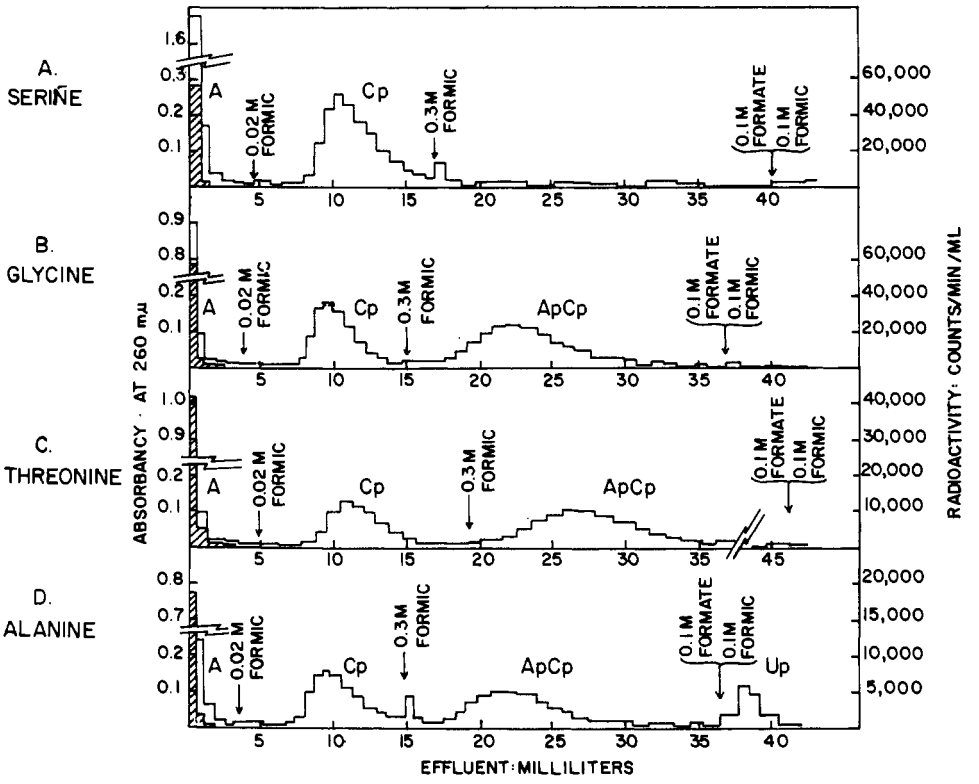

FIGURE 1: Chromatography of pancreatic ribonuclease digests of amino acyl oligonucleotides on small columns of Dowex-1-X10 formate, 200–400 mesh. The cross-hatched areas indicate radioactivity of column effluents. Before digestion, amino acyl oligonucleotides contained: (A) 74.3 × 10^3 cpm of [^{14}C]serine, 3.4 OD_{260} units of RNA; (B) 62.9 × 10^3 cpm of [^{3}H]glycine, 2.1 OD_{260} units of RNA; (C) 47.0 × 10^3 cpm of [^{3}H]threonine, 1.9 OD_{260} units of RNA; (D) 27.6 × 10^3 cpm of [^{14}C]alanine, 2.9 OD_{260} units of RNA. Ribonuclease digestion was carried out at pH 5.5 as described in the text. After loading of samples, the columns were washed with distilled water until the first change of solvent.

Adenosine in Amino Acyl Oligonucleotides. Amino acyl oligonucleotide (2–4 OD_{260} units) was treated as described above to remove amino acid, except that the sample was taken up initially in 100 μl of 0.1 M ammonium bicarbonate, pH 8.6. The DEAE-cellulose column was washed as described above and then finally with distilled water. The sample, which had been incubated at 37°, pH 8.6, was diluted to 0.8 ml with distilled water and applied to the column at 2°. When the solution had passed into the column the free amino acid was washed through with distilled water. The oligonucleotide was then eluted from the column and its content of terminal adenosine was determined by digestion with pancreatic ribonuclease. The column fractions containing radioactivity were pooled and freeze dried. Ammonium bicarbonate was removed by heating the sample in an evacuated flask, using an infrared lamp. The sample was then taken up in a small volume of distilled water and dried with air flow on a sand bath at 80° overnight to ensure removal of any residual ammonium bicarbonate. Amino acids were estimated by the method of Moore and Stein (1948).

Determination of Ultraviolet Absorbancy was performed using a Zeiss PMQ spectrophotometer. For small volumes of solutions a microcell attachment was employed, using 0.3 ml of silica cuvets with a 1-cm light path. When it was required to determine absorption spectra for substances in solution, a Cary 15 recording spectrophotometer was used.

TABLE I: Electrophoretic Mobility[a] of Amino Acyl Oligonucleotides.

Amino Acid Label	Electrophoretic Mobility, pH 3.5		Electrophoretic Mobility, pH 5.0	
	Intact	pH 8.5 Treated	Intact	pH 8.5 Treated
Serine	−0.41	0	+0.32	+0.79
Glycine	−0.04	+0.23	+0.76	+1.06
Threonine	−0.03	+0.23	+0.75	+1.07
Alanine	+0.23	+0.46	+0.96	+1.18

[a] Electrophoretic mobility was measured relative to Up using U as a marker to correct for any flow of solvent.

TABLE II: Analyses of Oligonucleotides by Different Procedures.[a]

Amino Acid Label	Structure Found	Yields of Products (moles/mole of nucleoside)[b]		
		Alkali[c]	Venom Phosphodiesterase	Pancreatic Ribonuclease
Serine	CpCpA	A, 1.0 Cp, 2.0	C, 1.0 pC, 1.1 pA, 1.1	A-aa, 1.0 Cp, 2.1
Glycine	CpApCpCpA	A, 1.0 Cp, 3.1 Ap, 1.0	C, 1.0 pC, 1.9 pA, 2.0	A-aa, 1.0 Cp, 2.3 ApCp, 1.1
Threonine	CpApCpCpA	A, 1.0 Cp, 2.9 Ap, 1.2	C, 1.0 pC, 1.7 pA, 1.5	A-aa, 1.0 Cp, 2.2 ApCp, 1.1
Alanine	UpCpCpApCpCpA	A, 1.0 Cp, 4.4 Ap, 0.9 Up, 0.9	U, 1.0 pC, 3.6 pA, 1.9	A-aa, 1.0 Cp, 2.8 ApCp, 0.9 Up, 0.9

[a] Nucleosides and nucleotides were identified by position of elution on columns of Dowex-1 formate, spectra at pH 2 and pH 13, and, in some cases, by electrophoretic mobility at pH 3.5. Adenosine amino acid esters (abbreviated to A-aa above) were identified from spectral data and by experiments described in the text. ApCp was identified from its absorption spectrum and by the following experiment. The column fractions were freeze dried and treated with *E. coli* alkaline phosphatase at pH 8.6. Alkaline phosphatase was removed by chromatography on columns of Dowex-1 formate. Digestion of the nucleotide-containing material in 0.2 N KOH for 1 hr at 80° gave the products C and Ap in the molar ratio 1:1. [b] Yields of products were determined from ultraviolet absorbancy. The extinction coefficients of adenosine amino acid esters at 260 mμ (pH 2) were assumed to be the same as that for adenosine and the millimolar extinction coefficient for ApCp was taken as 18.9 at 260 mμ (pH 2). [c] The recoveries of products from alkaline hydrolysis were corrected for small losses due to deamination. Correction factors were found by treating known amounts of reference compounds in the same conditions.

Results

High-Voltage Electrophoresis. A summary of the electrophoretic mobilities of the oligonucleotides both before and after removal of attached amino acids is given in Table I. The fact that the intact fragments, with amino acid attached, were more basic than the corresponding oligonucleotide after release of amino acid, suggested that the amino groups of the amino acids were free in the original compounds. The alkaline lability of the amino acid–oligonucleotide linkage is consistent with the known properties of the amino acid ester bond of intact amino acyl RNA (Wolfenden, 1963).

Identification of Radioactive Amino Acids Released by Mild Alkaline Treatment of the Labeled Fragments. The radioactive materials released by pH 8.5 treatment of the labeled fragments showed electrophoretic mobilities at pH 3.5, pH 5.0, and pH 9.3 which were identical with the mobilities of the expected free amino acids.

Determination of the Base Composition and Terminal Nucleotide Residues of Oligonucleotide Components. The purified oligonucleotides were subjected to hydrolysis with alkali, with snake venom phosphodiesterase, and with pancreatic ribonuclease. The products of each type of digestion were fractionated by anion-exchange chromatography using microcolumns of Dowex-1 formate (Figure 1).

Before digestion with alkali, the oligonucleotide fragments were treated to remove attached amino acids. The results of alkaline hydrolysis of the four oligonucleotides are given in Table II. The procedure caused small losses of products for which appropriate corrections were applied. All of the digests contained only one nucleoside, adenosine, indicating that in every case the residue at the 3′ end of the chain was pA.

Removal of attached amino acid, followed by digestion of the oligonucleotides with snake venom phosphodiesterase, gave results which are shown in Table II. The mild enzymic digestion procedure gave good recoveries of products. The data established that the serine, glycine, and threonine oligonucleotides terminated in a Cp residue while the alanine oligonucleotide possessed a Up residue at the 5′ end of the chain.

For hydrolysis of the oligonucleotides with pancreatic ribonuclease, two different kinds of approach were used. In the first kind of experiment, the amino acyl oligonucleotides were digested directly with pancreatic ribonuclease at pH 5.5. In the second kind of study the

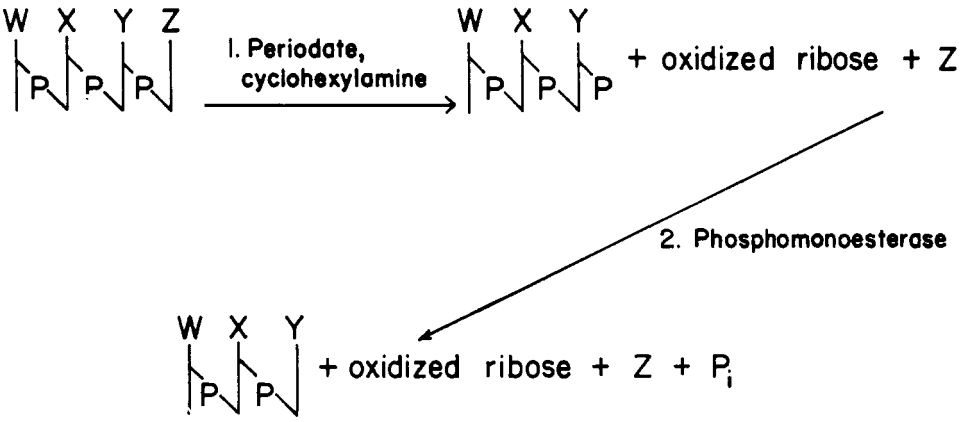

FIGURE 2: Sequential degradation of oligonucleotide. W, X, Y, and Z represent base residues in oligonucleotide chain.

amino acids were removed before digestion of the oligonucleotide at pH 7.5. The former type of experiment required a rather higher concentration of enzyme than the latter. We shall only consider the first type of experiment since this was found to give essentially the same data as the second and in addition provided information about the mode of attachment of amino acid to RNA.

Digestion of the amino acyl oligonucleotides with pancreatic ribonuclease at pH 5.5 yielded the results shown in Table II. The ribonuclease released adenosine amino acid ester from the 3′ end of each chain and produced the dinucleotide ApCp in addition to mononucleotides (Figure 1). The results obtained from the ribonuclease digestions were consistent with the composition data which had been derived from alkaline hydrolysis and venom phosphodiesterase digestion of the four oligonucleotides. Within the limits of the experimental error of the methods involved, all three procedures showed integral molar ratios of nucleosides and nucleotides.

Analysis of Oligonucleotide Components for Presence of Minor Nucleosides. From the results shown in Table II, it appeared that the four oligonucleotides did not contain any rare nucleosides (Hall, 1965). As a confirmatory test, each fragment was treated to remove attached amino acid and was then digested with a mixture of snake venom phosphodiesterase and *E. coli* alkaline phosphatase. This procedure caused complete degradation to nucleosides. The nucleoside mixture was then resolved by paper chromatography. The experiment showed that the serine, glycine, and threonine oligonucleotides contained only A and C while the alanine fragment contained A, C, and U. There was no evidence for the presence of G or any of the rare nucleosides of s-RNA.

Sequential Degradation of Oligonucleotide Components. In order to find the complete nucleotide sequences of the glycine, threonine, and alanine fragments, further analytical information was required. This was obtained by sequential degradation of the oligonucleotides using periodate oxidation, amine-catalyzed cleavage, and removal of 3′-terminal phosphate with *E. coli* phosphomonoesterase as illustrated in Figure 2. The method used was similar to that described by Neu and Heppel (1964) except that smaller quantities of oligonucleotide were employed.

The yield of oligonucleotide fragment produced by the degradation was usually 70–80% of the theoretical value. For this reason it was not found practicable to carry out more than three sequential degradations on a given sample of starting material. Following the degradations, the residual oligonucleotide fragment was hydrolyzed with alkali in order to determine its composition and 3′-terminal nucleotide residue.

The results of the sequential degradation studies are shown in Table III. From these data and the analyses of the serine fragment described above, it is clear that the oligonucleotide components of all four amino acyl oligonucleotides contained the expected common terminal sequence . . .pCpCpA.

Nucleotide Sequences of Oligonucleotide Components and Corresponding Chains of Transfer Ribonucleic Acid (t-RNA). The results described above established that the four preparations were homogeneous with respect to their oligonucleotide components. The analytical data made it possible to deduce the complete nucleotide sequence of each of the amino acid labeled oligonucleotides (Table II).

From previous studies (Herbert *et al.*, 1964), it was known that for every 100 phosphodiester linkages hydrolyzed by the T_1 ribonuclease used to prepare the fragments at pH 5.4, at least 93 were adjacent to Gp residues. It was therefore concluded that the terminal nucleotide sequences of the acceptor RNA from which the four amino acid labeled oligonucleotides had been derived were as follows: serine, . . .GpCpCpA; glycine, . . .GpCpApCpCpA; threonine, . . .GpCpApCpCpA; alanine . . .GpUpCpCpApCpCpA.

Evidence Concerning Mode of Attachment of Amino Acid to Oligonucleotide. The separation of the components of pH 5.5, pancreatic ribonuclease digests of amino acyl oligonucleotides on columns of Dowex-1 (Figure 1) gave a distinct peak containing adenosine

TABLE III: Sequential Degradation of Oligonucleotides.

Amino Acid Label	No. of Degradations Performed	Bases Released by Degradations[a]			3′-Terminal Nucleoside of Residual Fragment[b]
		1st	2nd	3rd	
Serine	1	Adenine			
Glycine	2	Adenine	Cytosine		C
Threonine	2	Adenine	Cytosine		C
Alanine	3	Adenine	Cytosine	Cytosine	A

[a] The bases released by degradations were identified by means of absorption spectra at pH 2, pH 13.5, and by electrophoretic mobility. [b] The 3′-terminal nucleosides of the residual oligonucleotide fragments were identified by alkaline hydrolysis followed by separation of the products on columns of Dowex-1 formate.

together with amino acid radioactivity at the commencement of each run. Electrophoresis at pH 3.5 showed that the radioactivity was associated with compounds which were more basic than adenosine itself. Exposure to mild alkaline conditions caused degradation of these compounds to adenosine and free amino acid. The presence of the amino acids attached to adenosine was found to protect the adenosine against periodate oxidation. The results obtained suggested that the amino acids were originally attached to the oligonucleotide fragments by acyl ester linkage to the 2′- or 3′-hydroxyl groups of the ribose of the terminal adenosine residue. This result would be expected on the basis of earlier studies in other laboratories in which adenosine amino acid esters were isolated directly from amino acyl RNA (Zachau *et al.*, 1958; Preiss *et al.*, 1959; Marcker and Sanger, 1964).

Quantitative Estimation of Amino Acid per Mole of Terminal Adenosine. Using the ninhydrin procedure of Moore and Stein (1948), it was shown that, within the limits of experimental error, each fragment contained 1 mole of amino acid/mole of terminal adenosine. However, when the amount of amino acid present was calculated on the basis of radioactive label, there was usually less than 1 mole/mole of terminal adenosine. Table IV illustrates the results of this experiment. It appeared that this result was due to dilution of radioactive amino acids used to label s-RNA in the yeast cell-free system (Smith *et al.*, 1966).

TABLE IV: Amino Acids Recovered from Amino Acyl Oligonucleotides.

Amino Acid Label	Moles of Amino Acid Recovered from Purified Fragment/Mole of Terminal Adenosine	
	Total Amino Acid	Labeled Amino Acid
Serine	0.9	0.6
Glycine	1.0	0.2
Threonine	1.0	0.2
Alanine	0.8	0.6

The dilution of amino acid label was most marked with the glycine- and threonine-labeled oligonucleotides. These fragments had been found to give unexpectedly high yields of ultraviolet-absorbing material during their purification (Smith *et al.*, 1966) and showed two separate amino acyl adenosine spots, one radioactive and the other nonradioactive, on electrophoresis after digestion with pancreatic ribonuclease. The evidence suggested that, although the preparations were homogeneous with respect to their oligonucleotide components, a considerable proportion of the amino acid attached to oligonucleotide was chemically different from the amino acid carrying radioactive label. This inhomogeneity apparently arose from the occurrence of the terminal sequence . . .GpCpApCpCpA in a number of different acceptor RNA's and the inability of the purification procedures to resolve the resultant amino acyl oligonucleotides which differed only in the neutral amino acid esterified to RNA.

Discussion

The isolation and determination of structure of amino acyl oligonucleotides provide a direct and relatively rapid procedure for finding the terminal nucleotide sequences of individual amino acid acceptor RNA's. The methods described here and in the previous paper (Smith *et al.*, 1966) are quite simple and have the advantage of being generally applicable to any amino acid acceptor RNA, in unfractionated or purified form.

Since the present study has involved a quite different approach from that used in other laboratories, it is interesting to compare the information it has provided on nucleotide sequences of individual acceptor RNA's of yeast with the data so far available from other methods. The terminal nucleotide sequence . . .GpUpCpCpApCpCpA determined by the present procedure for alanine-acceptor RNA is in accord with the terminal

sequence found by Holley *et al.* (1965) in two different species of purified alanine RNA. There is some disagreement in the literature concerning the terminal nucleotide sequence of serine-acceptor RNA from yeast. By the method described here, using unfractionated s-RNA, it has only been possible to detect one terminal sequence, . . .GpCpCpA. Karau and Zachau (1964) isolated two different species of serine RNA in pure form from yeast, using countercurrent distribution. Both types of RNA were found to possess the terminal sequence . . .GpCpCpA (Dütting and Zachau, 1964; Melchers *et al.*, 1965; Dütting *et al.*, 1965). This is consistent with the results of the present study but differs from the earlier report of Cantoni *et al.* (1963), who isolated one type of serine RNA and reported the terminal sequence to be . . .GpCpApApCpCpA. More recently, Bergquist and Robertson (1965) obtained five different fractions of yeast serine-acceptor RNA in partially purified form using partition chromatography on columns. Bergquist (1965) concluded that the terminal sequence of all these chains was . . .Gp(ApCp)-CpCpA. The reason for the discrepancy between the results of different laboratories concerning the terminal sequence of serine RNA is difficult to understand; it might possibly be explained by the use of different strains of yeast.

In addition to the acceptor RNA's for the amino acids it has recently been found that there are RNA molecules which carry derivatives of amino acids. Pearlman and Block (1963) reported evidence for the occurrence of *N*-acetyltyrosyl-s-RNA in rat liver, Marcker and Sanger (1964) have identified *N*-formylmethionyl-s-RNA from *E. coli*, and Hall and Chheda (1965) have isolated N^6-(*N*-formyl-α-aminoacyl)adenosines from yeast s-RNA. The experiments of Carlsen *et al.* (1964) suggest that in chicken liver, there may be a polynucleotide acceptor for phosphoserine. It is of interest, therefore, that for the amino acids used in the present study there was no evidence for attachment of derivatives of amino acids to RNA in the conditions used. If oligonucleotide complexes carrying derivatives of the labeled amino acids had been released by T_1 ribonuclease digestion of RNA, these substances should have been easily detected since they would be expected to have different chromatographic and electrophoretic properties from the corresponding amino acyl oligonucleotides. However, the present experiments were not designed to detect such compounds and the fact that they were not found does not preclude the possibility that intact yeast cells might contain acceptor RNA's for derivatives of the amino acids used.

Some earlier investigations were concerned with the determination of terminal nucleotide sequences of mixed populations of s-RNA. Lagerkvist and Berg (1962) labeled the acceptor ends of s-RNA from *E. coli* with [^{32}P]nucleotides and used this technique in determining the structure of twelve different types of terminal sequence from T_1 ribonuclease digests of the RNA. Herbert and Wilson (1962) carried out somewhat similar studies with s-RNA from yeast and rat liver and concluded that the proportions of different types of terminal sequences were similar in yeast, rat liver, and *E. coli*. In addition to these investigations, Berg *et al.* (1962) were able to isolate and deduce the structure of oligonucleotides from the acceptor ends of leucine- and isoleucine-specific s-RNA of *E. coli*. The experimental approach used by these workers depended upon differential ^{32}P labeling of the acceptor chains under study. It is interesting to note that the most common type of terminal sequence found by Lagerkvist and Berg (1962) in s-RNA of *E. coli* was . . .GpCpApC-pCpA and that this same sequence has been detected in *E. coli* leucine-specific s-RNA by Berg *et al.* (1962) and in yeast glycine-, threonine- and phenylalanine-specific s-RNA's in this laboratory (Smith and Herbert, 1965).

As more structural information becomes available on t-RNA, considerable interest centers on the chemical nature of the sites which determine the different types of specificity exhibited by this RNA. While it seems probable that the coding recognition triplet for transfer of amino acids into protein will be found deep in the polynucleotide chain (Holley *et al.*, 1965) there is, as yet, little direct information on the position of this site or of the regions of the molecule which might be expected to be concerned in attachment of t-RNA to the ribosome and in recognition of the activated amino acid. Since all t-RNA's perform the same function for different amino acids, it seems likely that a comparative study of the structure of different types of acceptor chains will reveal systematic similarities and differences in structure which can be related to known differences in specificity. The techniques described here and in the previous paper (Smith *et al.*, 1966) may prove to be of value for this kind of study.

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Enzymatic Elimination from a Substituted Four-Carbon Amino Acid Coupled to Michael Addition of a β-Carbon to an Electrophilic Double Bond. Structure of the Reaction Product*

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ABSTRACT: When *N*-ethylmaleimide was added to reaction mixtures containing β- or γ-substituted four-carbon amino acids and pyridoxal phosphate enzymes normally catalyzing elimination, ammonia and liberated substituent were formed as expected, but α-ketobutyrate did not appear. In its place a compound accumulated which contained the elements of *N*-ethylmaleimide and the four-carbon chain of the substrate. The portion of this reaction product corresponding to the first two carbons of the amino acid was shown to be present as an α-keto acid function. The rate of decomposition of the product obtained after oxidative decarboxylation was similar to the rate of hydrolysis of succinimide in dilute alkali. The two degradation products isolated after strong acid hydrolysis were shown to be identical with synthetic preparations of the diastereoisomers of α-methyl-β-carboxyglutaric acid. The original enzyme reaction product was also present as the corresponding two diastereoisomers, but it is not known whether these arose from spontaneous racemization. These results indicate that the structure of the enzyme reaction product was α-keto-3-(3′-(*N*′-ethyl-2′,5′-dioxopyrrolidyl))-butyric acid.

This structure reflects a new kind of pyridoxal phosphate enzymatic reaction: the elimination of an electronegative substituent from a four-carbon amino acid, coupled to Michael addition of the third carbon to the electrophilic double bond of maleimide. Mechanisms were considered for the formation of the new reaction product.

In pyridoxal-P potentiated enzymatic reactions of the type termed "γ elimination" (reaction 1), it was found that, when *N*-ethylmaleimide (EM[1]) (or another maleimide derivative) was added to the reaction mixture (reaction 2), ammonia and the electronegative substituent "A" were liberated in expected amounts, but a large part of the α-ketobutyrate failed to appear (Flavin

$$ACH_2CH_2CH(NH_2)COOH + H_2O \longrightarrow AH + NH_3 + CH_3CH_2COCOOH \quad (1)$$

$$ACH_2CH_2CH(NH_2)COOH + H_2O + EM \longrightarrow AH + NH_3 + KEDB \quad (2)$$

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[1] Abbreviations used in this work: KEDB, α-keto-3-(3′-(*N*′-ethyl-2′,5′-dioxopyrrolidyl)) butyric acid, structure VII, formerly designated XMal; EDP, 2-(3′-(*N*′-ethyl-2′,5′-dioxopyrrolidyl))-propionic acid, VIII, formerly YMal; HEDB, α-hydroxy-3-(3′-(*N*′-ethyl-2′,5′-dioxopyrrolidyl))butyric acid, XI, formerly ZMal; KDB, α-keto-3-(3′-(2′,5′-dioxopyrrolidyl))butyric acid; KPDB, α-keto-3-(3′-(*N*′-phenyl-2′,5′-dioxopyrrolidyl))butyric acid; EM, *N*-ethylmaleimide. For KEDB and EDP, the subscripts 1 and 2 refer, respectively, to the electrophoretically slower and faster moving diastereoisomers.