Isolation 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: Amino acyl ribonucleic acid (RNA) from yeast was digested with T_1 ribonuclease at pH 5.4. Amino acyl oligonucleotides were isolated directly from the digest by ion-exchange chromatography on columns of sulfonated polystyrene. The preparations were further purified by chromatography on columns of DEAE-cellulose and by high-voltage electrophoresis on paper. Analytical studies showed that the final products were homogeneous with respect to their oligonucleotide components. For each of the amino acids: serine, glycine, threonine, and alanine, only one amino acyl oligonucleotide could be detected. The methods described can be used for unfractionated or purified amino acid specific soluble ribonucleic acid.

When isolated from the cell, crude soluble ribonucleic acid (s-RNA) consists of a mixture of different polynucleotide molecules. This mixture appears to contain acceptor RNA's for each of the common protein amino acids. It is widely accepted that, in each case, the amino acid becomes attached to its specific RNA by means of an ester linkage to the 2′- or 3′-hydroxyl group of the terminal adenosine residue of the RNA (Zachau *et al.*, 1958; Preiss *et al.*, 1959; Feldman and Zachau, 1964; Wolfenden *et al.*, 1964; McLaughlin and Ingram, 1965a,b). Zachau *et al.* (1958) isolated adenosyl esters of amino acids from liver s-RNA after treating the amino acid–RNA complex with pancreatic ribonuclease.[1]

$$\ldots\text{pCpCpA-amino acid} \xrightarrow{\text{pancreatic ribonuclease}} \ldots\text{pCp} + \text{Cp} + \text{A-amino acid}$$

When T_1 ribonuclease (ribonucleate 3′-guanylohydrolase, EC 3.1.4.8) became available (Takahashi, 1961), it was of interest to see whether this enzyme would act in an analogous manner to pancreatic ribonuclease. From earlier work on the structure of s-RNA chains (Hecht *et al.*, 1958; Herbert and Wilson, 1962; Berg *et al.*, 1962), it would be expected that T_1 ribonuclease would release amino acyl oligonucleotides from s-RNA, *e.g.*

$$\ldots\text{pXpGpYpZpCpCpA-amino acid} \xrightarrow{T_1\text{ ribonuclease}} \ldots\text{pXpGp} + \text{YpZpCpCpA-amino acid}$$

where X, Y, and Z represent unknown bases. This expectation was confirmed by earlier studies in this laboratory (Smith *et al.*, 1964; Herbert *et al.*, 1964) which showed the presence of compounds having the properties of amino acyl oligonucleotides in pH 5.5 T_1 ribonuclease digests of amino acyl RNA.

The purpose of this paper is to describe the isolation in a purified state of four amino acyl oligonucleotides which have not previously been studied. The following paper (Smith and Herbert, 1966) describes the structure and properties of these compounds. The fragments were originally demonstrated by chromatography of pH 5.4, T_1 ribonuclease digests of amino acyl RNA on columns of DEAE-cellulose. When the properties of the amino acyl oligonucleotides were better understood, new isolation procedures were developed, using chromatography on columns of Dowex-50, DEAE-cellulose, and high-voltage electrophoresis. The new methods have allowed preparation of the amino acyl oligonucleotides with a high yield and purity.

Analytical studies (Smith and Herbert, 1966) have shown that each of the four terminal fragments isolated was homogeneous with respect to its oligonucleotide component.

Experimental Procedure

Preparation of Yeast s-RNA. The RNA was extracted from fresh bakers' yeast (Fleischmann's yeast, Standard Brands Inc., New York, N. Y.) according to the method of Holley (1963). The product was dialyzed against

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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; ATP, adenosine triphosphate; CTP, cytidine triphosphate.

distilled water, freeze dried, and stored in a desiccator at −20°. For the experiments described, it was assumed that an RNA solution in 0.05 M Tris hydrochloride, pH 7.5, 25°, having an optical density of 24 at 260 mμ, contained 1 mg of s-RNA/ml.

Preparation of Amino Acid Activation Enzymes. The procedure of Herbert *et al.* (1964) was modified for large-scale preparation of yeast extract. All operations were carried out in a cold room at 2°. Fresh bakers' yeast, 890 g, was crumbled into 400 ml of solution A (0.05 M Tris hydrochloride, 0.004 M $MgCl_2$, pH 7.5) at 2°. An even suspension of yeast was obtained by stirring with a glass rod. Glass beads, 1600 g of 0.2-mm diameter (Aloe Scientific, Seattle, Wash.), were added to the mixture. Portions of the suspension were then treated in a Waring Blendor at 90 v, allowing 20 min for each portion and making sure that the temperature of the extracts did not rise above 15°. After this treatment the glass beads were allowed to settle and the supernatant was decanted. The beads were washed with a further 250 ml of solution A at 2° and the washings combined with the main extract. The extracts were then treated as previously described (Herbert *et al.*, 1964) to remove cell debris, particles, and dialyzable material. The extracts were finally freeze dried and stored at −20° until required. Such preparations were active in catalyzing attachment of serine, glycine, threonine, alanine, phenylalanine, and tyrosine to s-RNA. After 6 months of storage there was a loss of activity toward alanine, phenylalanine, and tyrosine while for glycine, serine, threonine, and valine considerable activity remained.

Preparation of ribonuclease T_1 enzyme was carried out according to the method of Takahashi (1961). The properties and specificity of the preparation at pH 5.4 have been described in a previous paper (Herbert *et al.*, 1964).

Attachment of Labeled Amino Acids to s-RNA. Nucleoside triphosphates were obtained from California Corp. for Biochemical Research, Los Angeles, Calif. The radioactive-labeled amino acids [U-^{14}C]serine, [U-^{3}H]-serine, [U-^{14}C]alanine, [2-^{3}H]glycine, [U-^{14}C]threonine, and [U-^{3}H]threonine were purchased from Nuclear Chicago Corp., Des Plaines, Ill. The specific radioactivity of ^{14}C-labeled amino acids was 1–2 c/mole and of ^{3}H-labeled amino acids 30–40 c/mole. Labeled amino acids were attached to RNA as previously described (Smith *et al.*, 1964). The incubation mixtures containing labeled RNA were cooled on ice and treated with an equal volume of 90% phenol in water together with one-tenth volume of 1 M potassium acetate, pH 5. The aqueous layer was removed and the phenol was extracted a second time with 0.1 M potassium acetate, pH 5. The combined aqueous extracts were treated with 2.5 volumes of ethanol at −20° and allowed to stand at −20° overnight. The RNA was sedimented in a Sorvall centrifuge at 13,200*g* for 30 min at 0°. The precipitate was then taken up in 10 mM potassium acetate, pH 5.4, giving a concentration of 2–5 mg/ml, and was dialyzed for 24 hr against 50 volumes of 10 mM potassium acetate, pH 5.4, at 0–4° with two changes of buffer. Preparations of amino acyl RNA which were not used immediately were frozen and stored at −20° If the RNA was to be stored for more than a week, the solutions were freeze dried before storage at −20°.

Digestion with T_1 Ribonuclease. The final digestion mixture (pH 5.4) contained 3–4 mg/ml of labeled RNA, 10 mM potassium acetate, 2 mM EDTA, and 0.044 OD_{280} unit (220 activity units of Takahashi) of T_1 ribonuclease/ml (for experiments where DEAE-cellulose chromatography was to be used) or 0.015 OD_{280} unit (75 activity units) of T_1 ribonuclease/ml (for experiments where Dowex-50 chromatography was to be used). The mixture was made up on ice and immediately after addition of the enzyme was transferred to an incubation bath at 37°. Incubation continued for 90 min for experiments where DEAE-cellulose chromatography was used and for 35 min for experiments where Dowex-50 chromatography was employed.

DEAE-Cellulose Chromatography. DEAE-cellulose was purchased from Serva Entwicklungslabor, Heidelberg, Germany, and was treated by the procedure of Staehelin (1961) before use. For elution of columns, a stock solution of 10 M ammonium formate was prepared which, when diluted ten times, gave pH 5.2 at 20°. This buffer was diluted as required to give solutions varying from 0.01 to 1.0 M. The pH of such solutions fell within the range 5.1–5.2 at 20°.

Dowex-50 Chromatography. Dowex-AG-50W-X2, 200–400 mesh, was purchased from California Corp. for Biochemical Research, Los Angeles, Calif. Before use the resin was washed at 2° with 1 N HCl and then with 0.2 M ammonium formate, pH 2.7. The buffers used for eluting the columns were adjusted to give the pH indicated at 20°.

Determination of Radioactivity. Samples of 0.2 ml or less were added to glass vials, treated with 20 ml of the scintillation mixture previously described (Herbert *et al.*, 1964), and counted in a Nuclear Chicago liquid scintillation counter.

High-voltage electrophoresis was performed on a cooled metal plate 61 × 36 cm as described by Ingram and Pierce (1962, method B). Whatman 3MM paper was washed before use. In each run one sheet of paper 61 × 30.5 cm was used for separation of samples and two sheets of paper 45.8 × 30.5 cm were used as wicks. Sufficient paper for 12 runs was prepared and washed with about 8 l. of 1 N acetic acid. The paper was then washed with 8–12 l. of distilled water and finally allowed to dry at 25° for several days. The washing procedure was found to reduce the background ultraviolet adsorption due to interfering substances in the paper.

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.

Before the commencement of a run, the electrical conductivity of unknown samples was determined in order to be sure that salt had been removed (Radiometer conductivity meter). The paper was wet loaded, samples being applied as spots in volumes of 1–25 μl. Runs were performed for 3–4 hr at 2000–3000 v. After a run the paper was allowed to dry at room temperature and the

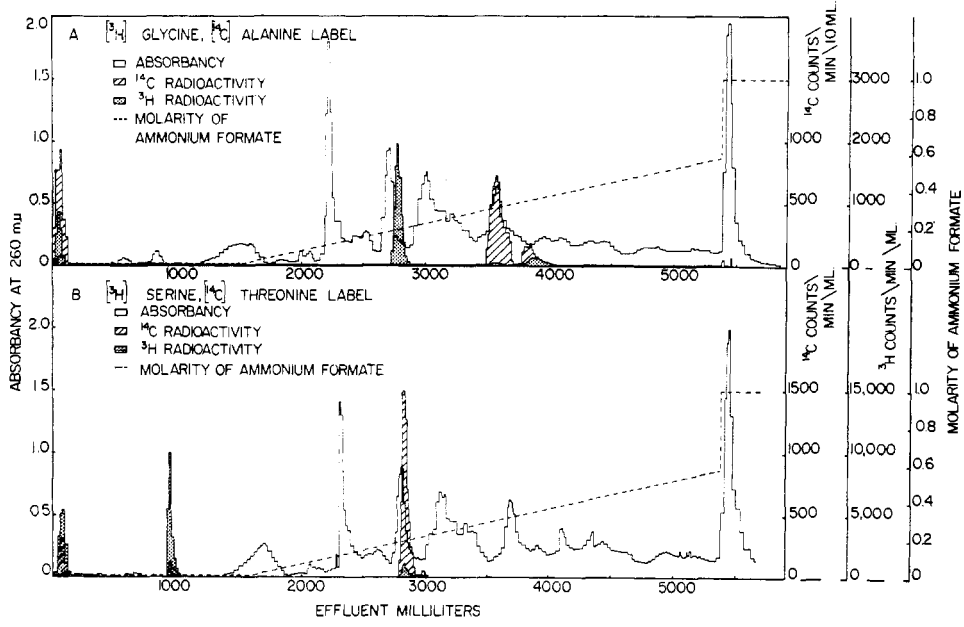

FIGURE 1: DEAE-cellulose chromatography of RNA which had been incubated for 90 min with T_1 ribonuclease at 37°, pH 5.4. The samples which were digested had contained initially: (A) 42.2 mg of RNA labeled with 0.065 μmole of [^{3}H]glycine (4786 × 10^3 cpm) and 0.018 μmole of [^{14}C]alanine (18.8 × 10^3 cpm); (B) 4.58 mg of RNA labeled with 0.085 μmole of [^{3}H]serine (1269 × 10^3 cpm) and 0.029 μmole of [^{14}C]threonine (101 × 10^3 cpm). In each case the digested RNA was chilled on ice, diluted with an equal volume of distilled water at 2°, and applied to a column of DEAE-cellulose 13.5 × 2.3 cm in the cold room. The column was eluted at 2° using 1.6 l. of 0.01 M ammonium formate, pH 5.2, followed by a linear concentration gradient of ammonium formate, pH 5.2, starting with 2 l. of 0.01 M ammonium formate in the mixer and 2 l. of 0.6 M ammonium formate in the reservoir. A flow rate of about 100 ml/hr was maintained by use of a Sigmamotor Fingerpump. The gradient-making device was disconnected after 3.7 l. of buffer had flowed from it and the column was finally eluted with 1 M ammonium formate, pH 5.2.

position of RNA components was determined by means of an ultraviolet lamp. In order to find the location of radioactive areas, the paper was cut into strips which were then scanned using a Vanguard Instruments radioactivity scanner.

Results

Chromatography of Total RNA Digests on Columns of DEAE-Cellulose. s-RNA was charged simultaneously with a ^{14}C- and a ^{3}H-labeled amino acid. The labeled RNA was then incubated with T_1 ribonuclease at pH 5.4 and the resulting digest was chromatographed on a column of DEAE-cellulose at 2°. The column was eluted with increasing concentrations of ammonium formate, pH 5.2.

Figure 1 shows the chromatograms obtained in two separate experiments where the RNA had initially been labeled with (A) [^{3}H]glycine and [^{14}C]alanine, (B) [^{3}H]serine and [^{14}C]threonine. For each of the amino acids used to label the RNA, there was one peak of radioactivity at the commencement of the run due to free amino acid, and another peak later in the elution profile, in the region where oligonucleotides were eluted. In addition, in the chromatogram illustrated by Figure 1A, there was a minor peak of radioactivity eluted from the column at a concentration of 0.35 M ammonium formate. This small peak was not found in every experiment performed and may have been due to complex formation between oligonucleotides. Table I shows the recoveries of radioactivity in the main labeled peaks for each of the amino acids used to label the RNA. It should be noted that the radioactivity recovered in these peaks did not account for more than 70% of the total

TABLE I: Recovery of Radioactivity from DEAE-Cellulose Columns.[a]

Radioactive Amino Acid	% Recovered as Free Amino Acid	% Recovered as Amino Acyl Oligonucleotide
Serine	36	30
Glycine	14	43
Threonine	10	53
Alanine	30	36

[a] Recoveries are expressed as per cent of total radioactivity of particular amino acid in RNA digest applied to DEAE-cellulose column.

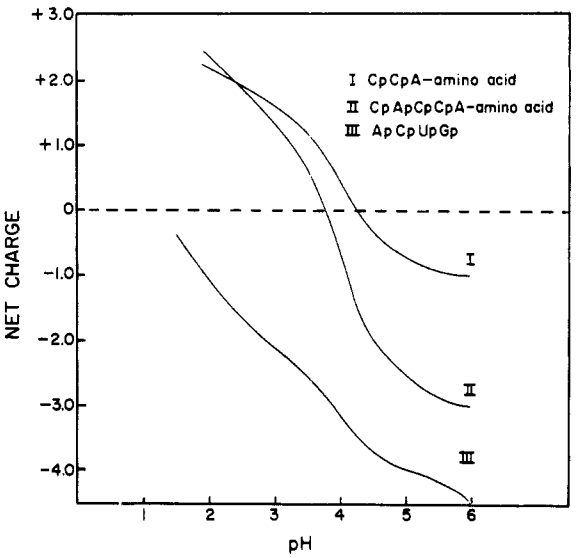

FIGURE 2: Net charge per molecule of amino acyl oligonucleotides (I and II) and of a possible oligonucleotide product of T_1 ribonuclease digestion of RNA (III), plotted as a function of pH. The net charges were calculated from the data of Smith (1955).

radioactivity originally present in the RNA digest. Similar recoveries were observed in earlier studies (Herbert *et al.*, 1964).

The ultraviolet absorbancy patterns of the column effluents in the two chromatograms shown in Figure 1 appeared to be very similar. In each case the first sharp peak of absorbancy at 260 mμ was eluted at a concentration of 0.1 M ammonium formate. On the basis of the ratios of absorbancy at 260 and 280 mμ, it seems likely that the major component of this peak was guanosine monophosphate. For both columns, a considerable amount of ultraviolet-absorbing material was eluted when the column was washed finally with 1 M ammonium formate. This suggested that the digests under study contained some large RNA fragments. These fragments may have been the result of incomplete digestion of the RNA. However, the amount of radioactivity present in such material was extremely small. Separate experiments showed that during 37° digestion of amino acyl RNA at pH 5.4 with limiting amounts of T_1 ribonuclease, terminal amino acyl oligonucleotides could be released almost quantitatively while the total RNA remained only partially digested.

Preliminary Conclusions Concerning the Properties of Amino Acyl Oligonucleotides. The results described above suggested that an amino acid–oligonucleotide fragment was released from each of the amino acyl RNA's under investigation and that these fragments could be arranged in order of increasing chain length as follows: serine < glycine = threonine < alanine. The data suggested that the glycine and threonine fragments might be of similar size. Paper electrophoresis of the labeled fragments after DEAE-cellulose chromatography showed that, at pH 3.5, the serine, glycine, and threonine components were positively charged while the alanine component possessed a negative charge.

Limitations of DEAE-Cellulose Chromatography for the Present Study. While direct chromatography of RNA digests on DEAE-cellulose was useful for preliminary fractionation studies, this technique was found to have some disadvantages when larger quantities of amino acyl oligonucleotides were needed for structural determinations. The requirement for greater quantities of RNA made it necessary to use larger columns of DEAE-cellulose. Such columns take longer to operate than small columns and losses of amino acyl oligonucleotides due to hydrolysis of the amino acyl ester bond was greatly increased. Experience with DEAE-cellulose chromatography suggested that the position of elution of amino acyl oligonucleotides was influenced by the presence of other oligonucleotides in the digestion mixtures. This difficulty did not appear to be serious with terminal fragments up to five nucleotides in length but became more important with longer terminal oligonucleotides. The problem may be overcome by the use of 7 M urea in eluting buffers (Penswick and Holley, 1965). However, in the present experiments it was found more convenient to substitute an alternative method to direct DEAE-cellulose chromatography.

The Basis of a New Method for Separation of Amino Acyl Oligonucleotides from the Other Components of a Ribonuclease Digest. It was known from previous studies (Herbert and Wilson, 1962) that for unfractionated yeast s-RNA, the predominant bases in the first five positions from the monoesterified ends of the chains are cytosine and adenine. From the behavior of the amino acyl oligonucleotides of serine glycine, threonine, and alanine during electrophoresis, it was apparent that three of the four components examined were slightly basic at pH 3.5. This property appeared to be consistent with the expectation that the compounds contained no uracil residues.

Amino acyl oligonucleotides might have structures similar to I and II represented conventionally in the upper part of Figure 2. A neutral amino acid esterified to the end of an oligonucleotide chain would be expected to contribute unit positive charge to the molecule, below pH 6, due to ionization of its amino group. The p*K* values associated with the amino groups of cytosine and adenine, bound in the polynucleotide chain, probably lie within the pH range 3–5. Below pH 4.5, therefore, each adenine and cytosine residue would contribute increasing positive charge to the molecule. In addition to these cationic groups, the primary phosphate groups associated with the phosphodiester bridges of the RNA chain would be fully ionized above pH 2.5, each providing one negative charge.

In Figure 2 are shown the curves of net charge *vs.* pH for the molecules depicted in the same figure. The curves were calculated using the dissociation curves of the ionizing groups of adenylic and cytidylic acids given by Smith (1955). It should be emphasized that the calculations were based upon assumed values for the dissociation constants of the various groups in the

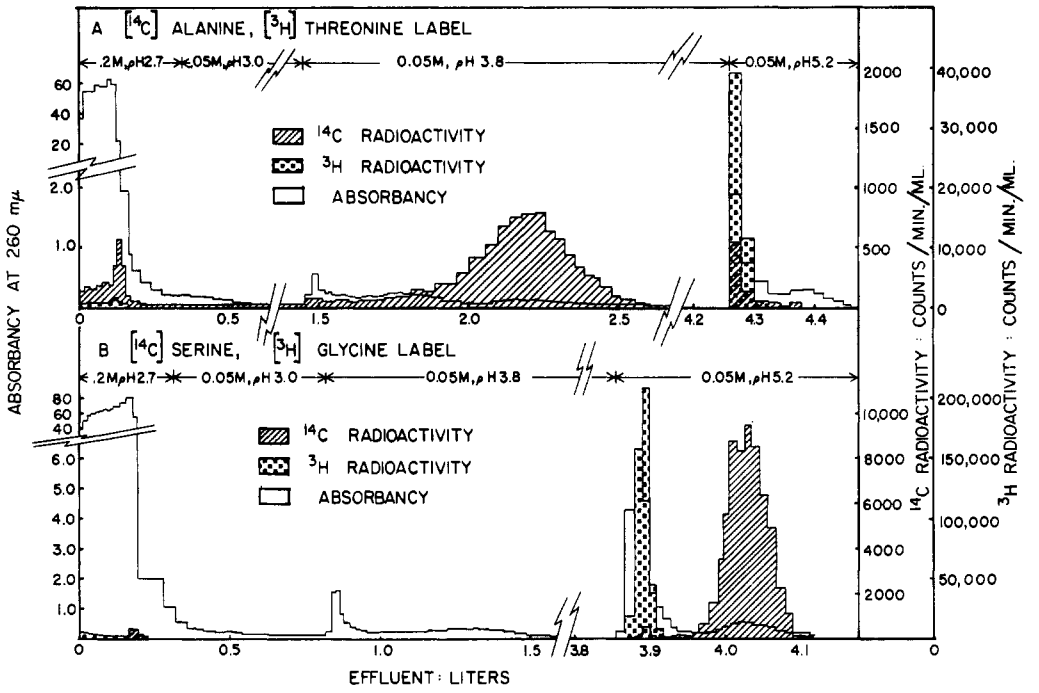

FIGURE 3: Chromatography on columns of Dowex-50-X2 of RNA which had been incubated for 35 min with T_1 ribonuclease at 37°, pH 5.4. The samples which were digested had contained initially: (A) 315 mg of RNA labeled with 0.405 μmole of [^{14}C]alanine (445 × 10^3 cpm) and 0.348 μmole of [^{3}H]threonine (1544 × 10^3 cpm); (B) 531 mg of RNA labeled with 0.614 μmole of [^{14}C]serine (673 × 10^3 cpm) and 1.3 μmoles of [^{3}H]glycine (5997 × 10^3 cpm). For both experiments the RNA digest was chilled on ice, adjusted to pH 2.5 by addition of 2 N formic acid, and applied to columns of Dowex-50-X2, 200–400 mesh, 10.0 × 1.0 cm, at 2°. The samples took 2–3 hr to load at a flow rate of about 60 ml/hr. The columns were then eluted with ammonium formate buffers of the concentration and pH indicated, with flow rates of about 100 ml/hr at 2°.

polymeric structure under study. In addition, the effects upon net charge of any possible interactions with other ions in solution have not been considered.

From the curves shown in Figure 2, it can be seen that the isoelectric points of the hypothetical compounds I and II lie between pH 3.5 and pH 4.5. Below pH 3 the molecules are basic and should be bound by cation-exchange resins. At this pH most of the oligonucleotides of a ribonuclease digest will be acidic (*e.g.*, Figure 2, structure III) and should not be retained by such resins.

This suggests a relatively simple method which would make use of differences in net charge for separation of amino acyl oligonucleotides from the other components of a ribonuclease digest. However, it should be noted that this method depends upon the natural predominance of cytosine and adenine residues at the acceptor end of the s-RNA chain. If three of the base residues shown in the structures I and II in Figure 2 were replaced by uracil, then the molecules would have a net negative charge over the pH range 2–6 and could not be distinguished from other oligonucleotides on the basis of net charge.

Chromatography of RNA Digests on Columns of Dowex-50. In these experiments s-RNA was again labeled with the four amino acids serine, glycine, threonine, and alanine. The amino acyl RNA was treated with T_1 ribonuclease at pH 5.4, and the resulting digests were fractionated on columns of sulfonated polystyrene (Dowex-50-X2) at 2°.

The separation shown in Figure 3A was carried out on a digest of RNA which had initially been labeled with [^{14}C]alanine and [^{3}H]threonine while that illustrated in Figure 3B was performed with a digest of RNA having [^{14}C]serine and [^{3}H]glycine label. For both RNA digests over 95% of the total ultraviolet-absorbing material applied to the Dowex-50 column was immediately washed through in 0.2 M ammonium formate, pH 2.7, or 0.05 M ammonium formate, pH 3.0. During this washing period about 10% of the radioactivity present in the digests also passed through the column, presumably in the form of free amino acids. Subsequent elution of the columns with buffers of increasing pH released a separate peak of radioactivity associated with ultraviolet-absorbing material for each of the amino acids used to label the RNA. The radioactivity recovered in each of these peaks accounted for about 70% of the radioactivity of the corresponding labeled amino acid present in the RNA digest applied to the Dowex-50 column.

Final Purification of Amino Acyl Oligonucleotides. On the basis of the amount of ultraviolet-absorbing ma-

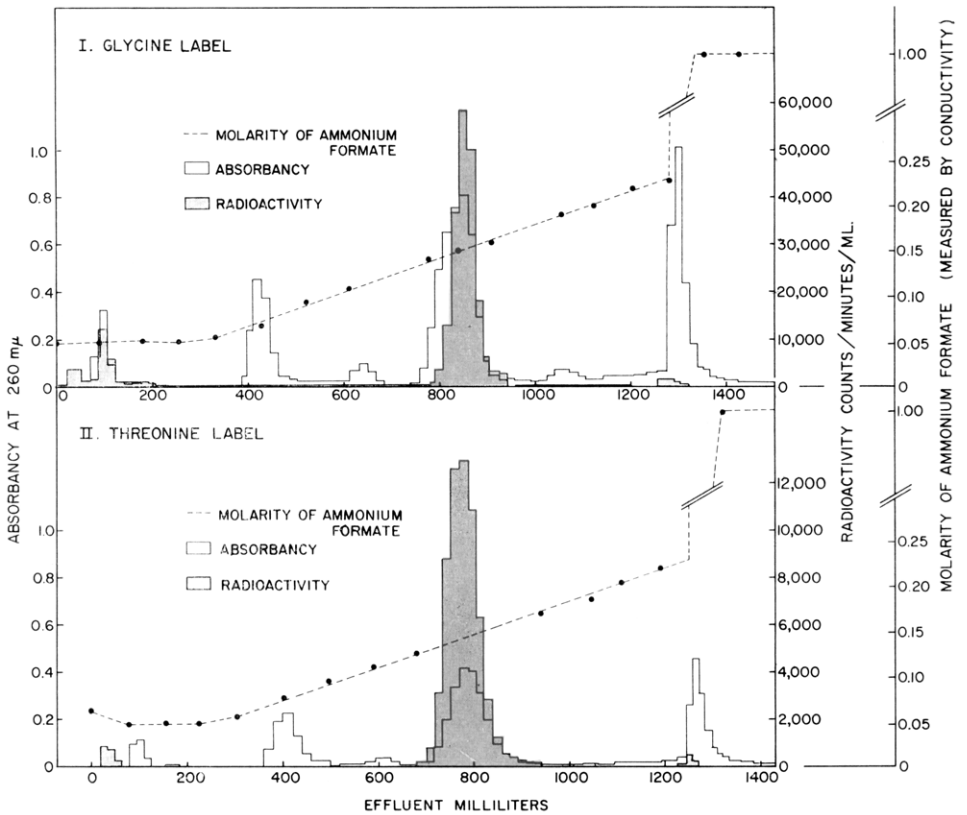

FIGURE 4: Chromatography of partially purified glycine and threonine oligonucleotides on columns of DEAE-cellulose. Peaks of amino acyl oligonucleotide material from previous chromatography on Dowex-50 resin columns were freeze dried and, after removal of salt, were taken up in a small volume of distilled water and applied to columns of DEAE-cellulose, 6 × 2.3 cm, which had been preequilibrated with solutions of ammonium formate, pH 5.2, at 2°. The material applied to column I contained 173.2 OD_{260} units of oligonucleotide and 3829 × 10^3 cpm equivalent to 0.715 mole of [^{3}H]glycine and that applied to column II contained 97.5 OD_{260} units of oligonucleotide and 1305 × 10^3 cpm corresponding to 0.253 μmole of [^{3}H]threonine. Both columns were eluted at 2° with 300 ml of 0.05 M ammonium formate, pH 5.2, followed by a linear concentration gradient of ammonium formate pH 5.2 starting with 500 ml of 0.05 M ammonium formate in the mixer and 500 ml of 0.25 M ammonium formate in the reservoir.

terial present in each of the four peaks of radioactivity recovered from Dowex-50 columns, the amino acyl oligonucleotides had been purified 50–100-fold from the T_1 ribonuclease digest. In order to purify these components further, the fractions from each peak were pooled and freeze dried, and ammonium formate was removed by sublimation. The four samples were then chromatographed on columns of DEAE-cellulose as shown in Figures 4 and 5. The technique used for these fractionations was similar to that employed for complete RNA digests (Figure 1) except that the linear concentration gradients of eluting solvents were less steep than in the previous experiments. It is of interest to note that the concentrations of ammonium formate required to elute the purified amino acyl oligonucleotides from DEAE-cellulose (Figures 4 and 5) were generally lower than those required to elute the same components before purification (Figure 1).

The DEAE-cellulose chromatography yielded the serine-labeled component in a homogeneous state as judged by high-voltage electrophoresis. However, the glycine-, threonine-, and alanine-labeled components were still not completely homogeneous. Further purification of the alanine fragment was accomplished by rechromatography on DEAE-cellulose while contaminants were removed from the glycine and threonine fragments by high-voltage electrophoresis at pH 3.5.

Homogeneity of Final Products during High-Voltage Electrophoresis. In order to test the homogeneity of the final preparations, the amino acyl oligonucleotides were subjected to high-voltage electrophoresis at pH 3.5. Each preparation was run in its original state and after treatment at pH 8.6 in order to remove the attached amino acid. The result of such an experiment is shown in Figure 6.

Each of the four components gave one major ultraviolet-absorbing spot, both in the intact condition and after removal of the attached amino acid. This was found to be true whether electrophoresis was carried out at pH 3.5 or pH 5.0. In every case, treatment at pH

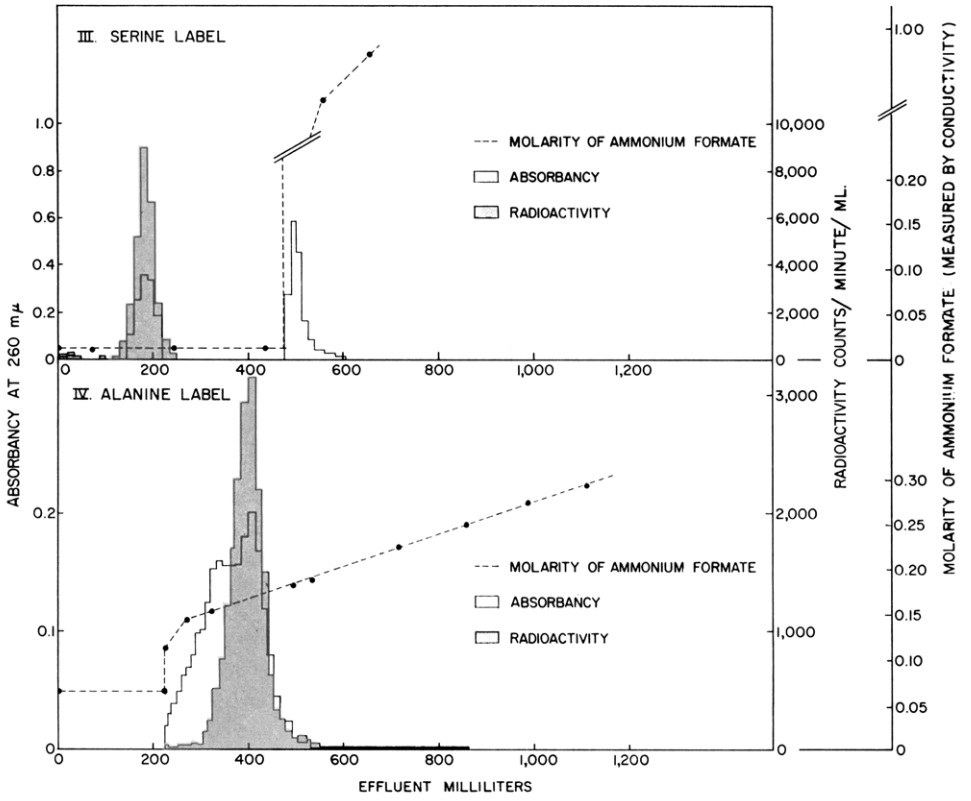

FIGURE 5: Chromatography of partially purified serine and alanine oligonucleotides on columns of DEAE-cellulose. Peaks of amino acyl oligonucleotide material from previous chromatography on Dowex-50 resin columns were concentrated and applied to columns as described under Figure 4. The material applied to column III contained 43.96 OD_{260} units of oligonucleotide and 353 $\times$ 10^3 cpm corresponding to 0.318 μmole of [^{14}C]serine. Column III, 4.0 $\times$ 2.3 cm, was eluted with 0.01 M ammonium formate, pH 5.2, at 2°. The material applied to column IV contained 31.3 OD_{260} units of oligonucleotide and 278 $\times$ 10^3 cpm corresponding to 0.125 μmole of [^{3}H]alanine. Column IV, 3.0 $\times$ 1.0 cm, was eluted at 2° with 200 ml 0.05 M ammonium formate, pH 5.2, followed by a linear concentration gradient of ammonium formate, pH 5.2, starting with 500 ml of 0.15 M ammonium formate in the mixer and 500 ml of 0.35 M ammonium formate in the reservoir.

8.6 caused almost complete disappearance of the original ultraviolet-absorbing spot and the appearance of a new spot, closer to the anode than the original spot. For the original material, the ultraviolet-absorbing spot was found to contain radioactivity, whereas after pH 8.6 treatment, the new ultraviolet-absorbing region contained no radioactivity; the radioactivity was now located in the area where free amino acid would be expected to be found.

Although the major ultraviolet-absorbing component in every run migrated as a single spot, many samples showed a very faint second spot. This was due to the fact that preparations of untreated amino acyl oligonucleotides frequently contained small amounts of free oligonucleotide and amino acid due to hydrolysis during storage, while pH 8.6 treated material showed traces of intact amino acyl oligonucleotide, resulting from incomplete hydrolysis.

The evidence obtained from electrophoresis thus suggested that the four substances were homogeneous. Structural studies described in the following paper (Smith and Herbert, 1966) established that the fragments isolated were amino acyl oligonucleotides.

Progress of Purification Yields of Products. The data shown in Table II summarize the progress of the purification of the four amino acyl oligonucleotides. It is clear that the greatest purification was achieved in the initial chromatography of the digests on columns of Dowex-50. The later procedures served to remove oligonucleotides of short chain length such as CpCpA and CpC which behaved similarly to the amino acyl oligonucleotides on the cation-exchange columns.

The yields of the glycine- and threonine-labeled oligonucleotides in terms of ultraviolet-absorbing material were higher than the corresponding values for the serine and alanine fragments and appeared to be higher than the yields that would have been expected from single species of amino acid acceptor RNA. Subsequent studies showed that the oligonucleotide components of the glycine and threonine compounds both possessed the structure CpApCpCpA which appears to be a frequent terminal sequence in s-RNA. This

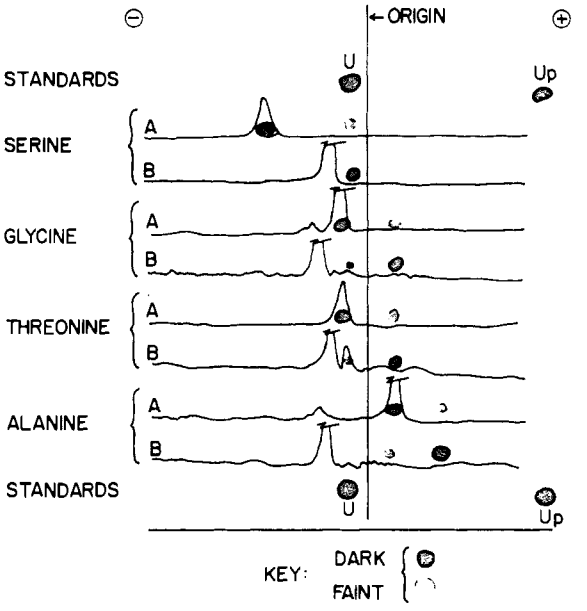

FIGURE 6: High-voltage electrophoresis of purified amino acyl oligonucleotides at pH 3.5. The shaded areas represent traces of ultraviolet-absorbing spots detected on the paper. Superimposed on each tracing is the record obtained from the radioactivity scanner. For every amino acid indicated, the record A resulted from electrophoresis of untreated amino acyl oligonucleotide and the record B from the same material after pH 8.5 treatment. Reference samples of U and Up were used as markers. In each case 2 OD_{260} units of fragment dissolved in 20 μl of distilled water was divided into two equal aliquots. One sample was kept on ice while the other was treated with ammonium bicarbonate to give a final concentration of 0.1 M (pH 8.6) and maintained at 37° for 2 hr. The ammonium bicarbonate was then removed by repeated drying down with a flow of compressed air at room temperature. The sample was finally redissolved in 10 μl of distilled water and both pH 8.6 treated and untreated samples were applied to paper for electrophoresis.

evidence, together with further structural data presented in the following paper (Smith and Herbert, 1966), showed that the fragments containing [^{3}H]glycine and [^{3}H]threonine, although homogeneous with respect to their oligonucleotide components, contained appreciable amounts of nonradioactive amino acids other than the original labeled amino acid attached to RNA.

From these observations it was clear that increase in specific radioactivity (Table II) was not, by itself, a reliable index of extent of purification of the amino acyl oligonucleotides. Consequently, the compounds were purified until they appeared to be homogeneous as judged by a number of different criteria including tests of physical properties and chemical analyses.

Discussion

For each of the four amino acids attached to RNA in the present experiments, only one labeled amino acyl oligonucleotide appeared to be produced. This result was demonstrated by both anion-exchange chromatography on DEAE-cellulose and cation-exchange chromatography on Dowex-50. However, the best recoveries of radioactivity in amino acyl oligonucleotides from the amino acid radioactivity originally bound to RNA were about 70%. It is possible that if 10% or less of the acceptor RNA for a certain amino acid had given rise to a small amount of a second type of oligonucleotide ester, this might not have been detected in the conditions used. However, the experiments described were repeated many times and apart from the very slight cross-contamination effect described below, no evidence was found for more than one oligonucleotide ester for serine, glycine, threonine, or alanine. It seems reasonable to conclude that if the acceptor RNA for any one of the four amino acids studied consists of more than one molecular species, then each type of molecule must have the same terminal nucleotide sequence. For the alanine and serine acceptors, independent evidence is available which is in agreement with this conclusion. In determining the nucleotide sequence of alanine RNA from yeast, Holley and co-workers found two species of acceptor chain which differed in one nucleotide position, one molecule containing a dihydrouridylic acid residue and the other a uridylic acid residue at this position (Holley *et al.*, 1965). For serine RNA from yeast, Zachau and co-workers have found several different acceptor RNA's which appear to possess a common terminal sequence (Melchers *et al.*, 1965; Dutting *et al.*, 1965).

Amino acyl adenosines, derived from s-RNA, have been known for some time (Zachau *et al.*, 1958; Marcker and Sanger, 1964; Feldman and Zachau, 1964; Wolfenden *et al.*, 1964; McLaughlin and Ingram, 1965a,b). However, technical difficulties have delayed the isolation of purified amino acyl oligonucleotides. Although it was found that digestion of amino acyl RNA at pH 5.4 yielded substances having the expected properties of amino acyl oligonucleotides (Smith and Herbert, 1963; Herbert *et al.*, 1964), it proved difficult to purify sufficient quantities of these compounds for structural analysis. The observation that these substances became attached to strong acid cation-exchange resins in acid solution provided a method of purification which overcame many earlier difficulties. The strong binding of the amino acyl oligonucleotides to sulfonated polystyrene at pH 3.0 makes it possible, by exhaustive washing, to remove contaminating oligonucleotides which in other conditions form weak complexes with the amino acyl oligonucleotides and are very troublesome to remove. This washing procedure allows higher recoveries of the amino acid esters than are usually obtained after direct chromatography on DEAE-cellulose.

The main basis for fractionation of the RNA digests on columns of Dowex-50 resin appears to be the net charge on the oligonucleotides. However, once at-

TABLE II: Progress of Purification of Amino Acyl Oligonucleotides.

Amino Acid	Stage of Prepn	Total OD_{260} Units of Oligonucleotide[a]	mμmoles of Labeled Amino Acid/OD_{260} Unit of Oligonucleotide	% Recovery of Radioactivity Originally Attached to RNA
Serine	Total RNA digest	12,780.0	0.048	100
	After Dowex-50	44.0	11.5	73
	After DEAE-cellulose	20.3	19.6	58
Glycine	Total RNA digest	12,780.0	0.102	100
	After Dowex-50	173.2	4.80	72
	After DEAE-cellulose	71.6	5.45	59
	After electrophoresis	45.0	5.96	35
Threonine	Total RNA digest	7,600.0	0.046	100
	After Dowex-50	97.5	3.14	58
	After DEAE-cellulose	38.1	4.21	48
	After electrophoresis	27.9	5.01	34
Alanine	Total RNA digest	7,600.0	0.053	100
	After Dowex-50	34.9	7.14	72
	After DEAE-cellulose	12.6	8.75	50

[a] Optical density at 260 mμ was determined in 0.05 M ammonium formate, pH 5.2.

tached to the resin, amino acyl oligonucleotides do not behave as would be expected from considerations of charge alone. The glycine and threonine oligonucleotides have been shown to possess the structure II shown in Figure 2 (Smith and Herbert, 1966) and would be expected to have isoelectric points in the region of pH 3.8. However, it is clear from Figure 3 that extensive washing of the Dowex-50 columns at 2° with pH 3.8 buffer failed to elute these fragments. A possible explanation for this observation is that nonpolar interactions may cause retention of the oligonucleotide on the columns at 2°.

In several experiments where an amino acid with a given isotopic label was used, small amounts of this isotope were detected in chromatogram peaks in which the predominant label was due to another isotope, associated with a different amino acid. For example, in Figure 1B a very small amount of ^{14}C radioactivity, originally associated with [^{14}C]threonine, was found in the peak of ^{3}H label which had originated from [^{3}H]-serine and *vice versa*. The effect occurred mainly with the amino acids serine, threonine, and glycine and varied from one batch of radioactive amino acid to another. The most probable explanation is that it was due to traces of contaminating radioactive amino acids present in the labeled amino acid originally attached to RNA, although interconversion of labeled amino acids in the cell-free system used to label the RNA could not be ruled out. The effect was not investigated further since the labeled amino acid component of each peak of amino acyl oligonucleotide was subsequently isolated and characterized by means of its electrophoretic mobility (Smith and Herbert, 1966).

As already mentioned, a difficulty encountered in preparation of amino acyl oligonucleotides was dilution of radioactive amino acids used to label the RNA. This apparently occurred during attachment of amino acids to RNA using the yeast cell-free system. It did not seem likely that the RNA preparations added to the cell-free system contained unlabeled amino acids bound by amino acyl linkage, since separate experiments showed that the percentage of RNA chains accepting labeled amino acids was not significantly altered by preincubation of the RNA at pH 10 followed by separation on columns of Sephadex G-25.

For the purposes of isolation of a single amino acyl oligonucleotide in the present study, two types of isotope dilution effects can be distinguished: (1) simple dilution of a radioactive-labeled amino acid with unlabeled amino acid of the same species; (2) dilution of the radioactive amino acid by unlabeled amino acids which are themselves chemically different but which become attached to RNA chains having the same terminal nucleotide sequence as the acceptor RNA for the labeled amino acid. This effect arises from the inability of the purification procedure to resolve amino acyl oligonucleotides which differ only in the neutral amino acid esterified to RNA.

The results shown in Table II combined with data presented in the following paper (Smith and Herbert, 1966) show that dilution of the labeled serine and alanine was relatively small and could be accounted for by the mechanism (1) given above. However, the glycine- and threonine-labeled oligonucleotides contained consider-

able amounts of nonradioactive amino acids, and it seemed likely that in this case the dilution was due to a combination of effects 1 and 2.

Ishida and Miura (1965) recently reported the results of fractionation of T_1 ribonuclease digests of amino acid labeled RNA on columns of DEAE-cellulose. These workers have studied species differences in various amino acid specific s-RNA's as shown by different elution profiles obtained on columns of DEAE-cellulose. Although they did not study any of the amino acyl oligonucleotides described here, the results reported are broadly in agreement with observations in this laboratory.

The procedures which have been described may be of value for comparative study of transfer RNA's from different organisms. It seems important to determine whether such RNA's show differences in nucleotide sequence since differences in chromatographic properties and species specificity toward amino acid activating enzymes have already been demonstrated (Doctor and Mudd, 1963; Sueoka and Yamane, 1962; Ishida and Miura, 1965). Another possible application of the methods reported is in the partial ribonuclease digestion of amino acid specific s-RNA. Since most of the amino acyl oligonucleotides so far isolated do not contain uridylic acid residues, it seems reasonable to expect that some longer amino acyl oligonucleotide fragments, obtained from partial ribonuclease digests, would have a net positive charge at pH 2.5 and might thus be isolated on cation-exchange columns. This would allow the determination of sequences deeper in the RNA chain without the need to fractionate the RNA.

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