The Separation of Soluble Ribonucleic Acids on Benzoylated Diethylaminoethylcellulose*

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ABSTRACT: Substitution of the hydroxyl groups of DEAE-cellulose by aromatic acids increases the nonionic attractions between it and polynucleotides. Benzoylated DEAE-cellulose (BD-cellulose) and a mixed benzoylated–naphthoylated DEAE-cellulose (BND-cellulose) have physical properties suitable for column chromatography. The constituents of yeast soluble ribonucleic acid can be fractionated by elution from such columns with a positive salt gradient. More strongly bound polynucleotides require the addition of alcohols or urea to release them. The strength of the binding of transfer ribonucleic acids (tRNAs) to these materials is dependent upon physical factors (temperature, concentration of divalent cation, and pH) which affect their secondary structures as well as upon their primary structures. These effects can be used in the chromatographic purification of tRNAs and are illustrated for those of brewer's yeast (*Saccharomyces cerevisiae*). Location and relative quantitation of acceptor activities for 20 amino acids in the eluate from such a column run at neutral pH are described. The $tRNA^{Phe}$ is more strongly held to BD-cellulose than any other and is quantitatively removed from all but traces of other acceptor activities in a single step. Further fractionation of $tRNA^{Asp}$ and $tRNAs^{Gly}$ was achieved by rechromatography under acidic conditions.

Interest in the chemistry and biology of tRNA has led to the development of several methods for the preparative and analytical separation of individual species of tRNA from the complex mixture present in crude sRNA. Methods used include those based on distribution between two liquid phases either in the countercurrent distribution apparatus (Apgar *et al.*, 1962; Karau and Zachau, 1964) or on chromatographic columns holding one phase stationary (Tanaka *et al.*, 1962; Muench and Berg, 1966a; Kelmers *et al.*, 1965) or chromatography on columns of hydroxylapatite (Muench and Berg, 1966b; Harding *et al.*, 1966; Pearson and Kelmers, 1966) or of MAK[1] (Sueoka and Yamane, 1962) or DEAE-cellulose (Cherayil and Bock, 1965; Baguley *et al.*, 1965).

The efficacy of these methods has been demonstrated by the separation of several tRNAs in sufficiently pure form to allow elucidation of their complete chemical structures (Holley *et al.*, 1965; Madison *et al.*, 1966; Zachau *et al.*, 1966; RajBhandary *et al.*, 1967).

This communication reports details of the preparation of a novel class of ion exchangers and their uses in the chromatographic separation of tRNAs from yeast. The exchangers are prepared from DEAE-cellulose by substitution of the hydroxyl groups of the cellulose with aromatic acids. Of the derivatives studied only those prepared by benzoylation or 1-naphthoylation have proved to be useful, and benzoylated DEAE-cellulose[2] appears to be the more convenient. Early studies, some of which will be described, utilized either materials prepared from DEAE-cellulose by incomplete benzoylation or incomplete substitution with mixed benzoyl and 1-naphthoyl groups. The more completely substituted BD-cellulose is comparable or superior to the best of these preparations and is to be preferred as it can be made with reproducible properties.

Experimental Section

Materials

DEAE-cellulose was a commercial product of capacity 0.9 mequiv/g. Pyridine was of reagent grade, dried over calcium hydride. Benzoyl chloride was of reagent grade, redistilled where this was considered necessary. 1-Naphthoyl chloride was reagent grade. sRNA from baker's yeast was prepared in the laboratory by the method of Holley (1963) or obtained commercially (Calbiochem). That from brewer's yeast was a commercial preparation (Boehringer & Soehne,

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[1] Abbreviations used: MAK, methylated albumin on kieselguhr; TCA, trichloroacetic acid; A_{260} unit, defined as that quantity of material which dissolved in 1 ml of solvent has an absorbance at 260 nm of 1; Na-ATP, sodium adenosine triphosphate; CTP, cytidine triphosphate.

[2] For convenience benzoylated DEAE-cellulose will be designated BD-cellulose and the benzoylated naphthoylated DEAE-cellulose as BND-cellulose.

Mannheim). ^{14}C-Labeled amino acids of stated specific activity were commercial products of greater than 99% purity.

Methods

BD-cellulose. DEAE-cellulose (100 g), dried overnight at 80° in a vacuum oven (90 mm), was suspended in pyridine (2.5 l.) in a 5-l. round-bottomed flask. Benzoyl chloride (190 g, 2.2 moles/mole of anhydroglucose) was added and the mixture was carefully heated to boiling under reflux with occasional swirling to prevent the viscous product from charring. After 15 min the flask was cooled slightly and more benzoyl chloride (90 g, 1.1 moles/mole of anhydroglucose) was added. The mixture was heated an additional 30 min and then cooled. The viscous red-brown mass was homogeneous but for traces of gellike fibers. Cooling was continued until the first crystals began to appear when the whole was poured slowly into water (about 35 l.) with stirring. The product precipitated as a long rope. After allowing this to set for an hour or more it was broken by further stirring and washed repeatedly with water by suspension, settling, and decantation. The material at this stage was a mass of large pieces of a porous, friable product, light tan in color. It was broken into smaller crumbs by brief treatment in a blender and washed extensively with 95% ethanol, 2 M sodium chloride in 25% ethanol, and finally 2 M sodium chloride until the absorbance of the washings at 260 nm was less than 0.04. The product was ground and sieved in the wet state through a 50 mesh (0.3-mm opening) screen and freed of very fine particles by repeated settling and decantation.

To ensure the maximal degree of benzoylation it is essential that the reagents and apparatus be dry. The use of more benzoyl chloride (a total of 4.0 moles/mole of anhydroglucose) is not harmful and allows for the presence of some water in the reaction mixture. The benzoyl chloride may be added in one batch, in which case the mixture is boiled for 45 min.

Saponification of BD-cellulose. BD-cellulose was washed as described and then with distilled water until no further chloride ion was eluted. It was dried to constant weight *in vacuo*. A sample (about 0.2 g) was dissolved in pyridine (40 ml) and standard ethanolic sodium hydroxide solution (15 ml of approximately 0.5 N) was added. The solution was allowed to stand at room temperature for 4 hr, 50% ethanol (10 ml) was added, and the mixture was allowed to stand 12 hr. Alkali remaining was titrated to the phenolphthalein end point with 0.5 N hydrochloric acid. A saponification equivalent of 159 was found. (Tri-*O*-benzoylcellulose has a calculated saponification number of 158.)

Determination of the Content of Benzoyl Groups in BD-cellulose by a Spectrophotometric Method. Dried BD-cellulose (approximately 4 mg) was dissolved in 25 ml of a solvent composed of ethanol and methylene chloride (8:92, v/v). Absorption in the ultraviolet region was recorded with particular attention to the maximum at 273 nm. From this and the molar absorption coefficients for benzoate esters (Ungnade and Lamb, 1952) values in the range 2.65–2.85 moles of benzoyl residues/mole of anhydroglucose were calculated for various samples.

Naphthoyl DEAE-cellulose. DEAE-cellulose (10 g) was allowed to react under similar conditions to those described above with 1-naphthoyl chloride (a total of 18 g, 1.5 moles/mole of anhydroglucose) in place of benzoyl chloride, then precipitated, and washed. The product was a white, flaky material.

Naphthoyl cellulose was prepared similarly from a commercial cellulose powder.

BND-cellulose. Dry DEAE-cellulose (100 g) in pyridine (2.5 l.) was heated 15 min with benzoyl chloride (172 g, 2.0 moles/mole of anhydroglucose) and 1-naphthoyl chloride (35 g, 0.3 mole/mole of anhydroglucose) as above. Further benzoyl chloride (88 g, 1.0 mole/mole of anhydroglucose) was added after cooling and the mixture was heated again and processed as for BD-cellulose.

Chromatographic Methods. Columns were loaded and eluted at room temperature (about 23°) except where specified. Columns to be developed at temperatures other than ambient were surrounded with a jacket through which water at the appropriate temperature was circulated.

BD-cellulose was packed into columns in the following manner. A slurry in 2 M sodium chloride was brought briefly to a boil at room temperature *in vacuo* to remove trapped air. It was then run into a column half filled with 2 M sodium chloride. Excess liquid was run out but the liquid level was always kept above the surface of the exchanger. Slurry was added until the desired depth of bed was obtained.

The packed column was washed with 2 M sodium chloride solution until the eluate had an acceptably low absorbance (A_{260} 0.025) and was then washed with the solution used to start the elution. The RNA, dissolved in the same solution, was applied to the column and rinsed in, and the column was washed briefly with more of the same solution. Elution with a positive salt gradient and collection of fractions were performed in the usual manner. After determining their absorbance fractions were frozen and stored at −10° until required.

Chromatography of Aminoacyl-tRNA. sRNA was aminoacylated with a chosen ^{14}C-labeled amino acid by incubation with an aminoacyl-tRNA synthetase and substrates as described below. The reaction was stopped by addition of cold ethanol, and the precipitate was recovered by centrifugation, redissolved in cold 0.05 M sodium acetate buffer (pH 5.0), and extracted with phenol, then ether, and once more precipitated with ethanol. The recovered precipitate was carefully drained and dissolved in pH 5.0 buffer for application to the column. Chromatography with eluents buffered at pH 5.0 proceeded as above. Radioactivity in eluted fractions was determined in a scintillation counter, taking 0.5 ml of eluate into a vial containing 10 ml of Bray's (1960) solution.

Recovery of tRNA. Fractions containing tRNA

of interest were pooled and the RNA was concentrated by ultrafiltration (Blatt *et al.*, 1965) in the Diaflo apparatus (Amicon Corp., Cambridge, Mass.) using membrane type UM-1. RNA was precipitated from the concentrated solutions with ethanol, recovered, and dried with ether.

Preparation of Aminoacyl-tRNA synthetases. Fresh cakes of baker's yeast (Fleischmann's, Standard Brands Ltd.) were the source of extracts prepared in one of two ways.

A. LYSIS WITH TOLUENE. Cells suspended in Tris buffer (pH 7.5) were lysed with toluene at 37° as described earlier (von Tigerstrom and Tener, 1967) except that during the 1-hr period of lysis the pH of the suspension was maintained at 7.5 by addition of alkali. The mixture was then cooled to 0°. Activating enzymes were precipitated from the solution remaining after high-speed centrifugation by addition of ammonium sulfate to 80% of saturation. The precipitate was dissolved in a small volume of 0.1 M Tris buffer (pH 7.5) containing 1 mM EDTA and desalted by passage through Sephadex G-25 equilibrated with the same buffer.

B. HOMOGENIZATION WITH GLASS BEADS. Preparation of the crude activating enzymes was performed as described by Stephenson and Zamecnik (1961). The treatment with barium chloride and streptomycin was omitted. The crude preparation was centrifuged at 105,000*g* for 2 hr. The middle layer was withdrawn, brought to 80% saturation with ammonium sulfate, and processed as described above. The resultant solutions were diluted with glycerol to 40% (v/v) content and stored at −20°.

Assays for Amino Acid Acceptor Capacity. Minor modifications were made to a procedure[3] reported by Hoskinson and Khorana (1965). Eluate from the column (two volumes), a preparation of the synthetase (one volume; the solution contained glycerol), and radioactive amino acid mix (one volume) were incubated at room temperature. The composition of the mix used was Na-ATP (40 mM), magnesium chloride (40 mM), sodium cacodylate (pH 7.4, 0.2 M), and L-[^{14}C]amino acid (20 μmoles/5 ml). To terminate the reaction a sample (50 μl) was withdrawn onto a filter paper and plunged into an ice-cold solution of trichloroacetic acid (10%, w/v). Acid-soluble radioactivity was removed by repeated washes with TCA solution and the papers were dried with Hokin's solution (1.6 ml of 10 N NaOH and 125 ml of glacial acetic acid diluted to 2 l. with ethanol), then ether. Radioactivity retained in the papers was determined in a scintillation counter by immersion in a toluene-based scintillating solution. Counters were calibrated with samples of radioactive amino acids dried onto similar papers. Where salt at concentrations similar to those introduced into the incubations by the samples from the column was found to inhibit the formation of aminoacyl-tRNA, samples were diluted before assaying. Conditions of incubation (time and concentration of enzyme) were adjusted to give maximal labeling, determined beforehand with samples of crude sRNA. Usually incubation periods of from 30 to 45 min were used.

Renaturable or denaturable leucyl acceptor activities (Lindahl *et al.*, 1966) were assayed by heating (60°, 5 min) samples diluted into suitable solutions, cooling, and assaying as usual.

The mix used for the assays of acceptor activities for glutamine and asparagine contained L-[^{12}C]glutamic acid and aspartic acid, respectively, at molar concentrations ten times those of the labeled amino acids. That for assay of cysteine acceptor activities contained 2-mercaptoethanol (40 mM).

Results

BD-cellulose. Early preparations of BD-cellulose were made as described with the important exception that only 2 molar equiv of acid chloride/anhydroglucose residue was used. The product was incompletely soluble in pyridine and doubtless contained an uneven distribution of aromatic groups throughout the cellulose chains. In chromatography of a sample of sRNA from baker's yeast on a column packed with such a preparation using a sodium chloride gradient sRNA eluted over an extended range, thus indicating a basis for separation of different tRNAs. Later studies indicated that the degree of substitution was important since fully benzoylated DEAE-cellulose gave superior separations.

Other Esters of DEAE-cellulose. DEAE-cellulose reacted with other aromatic acyl chlorides (*p*-phenylazobenzoyl chloride, anisoyl chloride, and nicotinoyl chloride) and with octadecyl isocyanate and nonanoyl chloride to give materials of poor physical properties or ones which failed to produce elution patterns for sRNA comparable with those obtained with BD-cellulose.

Preparation of naphthoylated DEAE-cellulose gave a product with good physical characteristics. However, sRNA applied to a column of this material could not be eluted with salt at any concentration applied. Indeed a naphthoylated cellulose (containing no ionizable groups) was also capable of adsorbing sRNA from solution. The sRNA could be recovered from it by elution with 10% 2-methoxyethanol in water.

In a series of attempts to use the powerful attractions between naphthoyl groups and sRNA to improve the resolution obtained with columns of the early preparations of BD-cellulose, BND-cellulose was formulated. It contains an estimated 9% of naphthoyl groups on a molar basis among its content of benzoyl residues. Figure 1 shows the elution profile of a sample of sRNA from a column of BND-cellulose. Numerous peaks of optical density can be seen to be partially resolved and two peaks of histidine acceptor activity were detected.

Fractions from this experiment were pooled as indicated (Figure 1) and concentrated, and the RNA

[3] Technical Brochure 66TR1 from Schwarz BioResearch Inc., Orangeburg, N. Y.

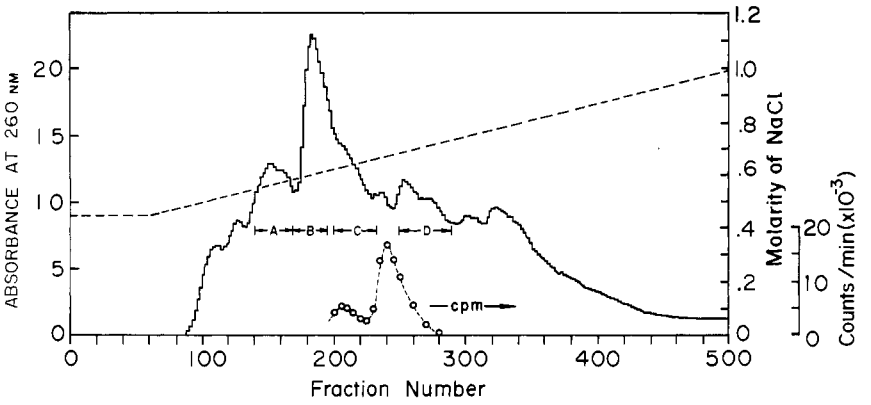


FIGURE 1: Elution profile from a column (4.3 × 110 cm) of BND-cellulose loaded with 5 g (72,000 A_{260} units) of sRNA from yeast (Calbiochem). The sample was applied in 200 ml of 0.45 M sodium chloride–0.01 M magnesium chloride, and eluted with 10 l. of sodium chloride (a gradient from 0.45 to 1.0 M)–0.01 M magnesium chloride. Fractions were 20 ml/10 min. Absorption of each fraction at 260 nm (solid line); acceptor activity for [^{14}C]-histidine (dotted line).

was recovered by precipitation with ethanol. Aliquots from each sample were redissolved and reapplied separately to a standardized column and eluted under similar conditions. The composite diagram (Figure 2) shows that each sample emerged upon rechromatography as a single peak at the concentration of salt expected to elute it. Thus the numerous peaks seen in Figure 1 represent separation of sRNA into fractions with reproducible chromatographic properties.

Chromatography of Aminoacyl-tRNA on BND-cellulose. tRNA from baker's yeast was enzymatically aminoacylated with [^{14}C]alanine and chromatographed on a column of BND-cellulose. The eluent was buffered at pH 5.0 to stabilize the aminoacyl linkage. Figure 3 shows that the pattern of elution of RNA was not substantially different from that seen when unbuffered salt solutions were used for elution (Figure 1). Figure 3 also shows that two peaks of radioactivity eluted from the column corresponding to two separable species of Ala-tRNA^{Ala}.

Fractions collected before emergence of these two peaks contained radioactivity above the background level due to some hydrolysis and release of [^{14}C]alanine even at pH 5.0. A slightly lower pH may offer some advantage. After elution of the Ala-tRNA^{Ala} the radioactivity found in the fractions dropped to background level, showing that no more radioactivity remained. Thus a specific aminoacylated tRNA was found in specific areas of the elution pattern.

Effect of Urea on the Chromatography of sRNA on BND-cellulose. A column packed with BND-cellulose was equilibrated, loaded with a sample of sRNA from baker's yeast, and eluted in the usual way except that all solutions used were 7 M in urea. All the sRNA eluted as a single peak, with slight tailing, around 0.4 M sodium chloride.

Effects of Other Agents on Adsorption of sRNA to BD- and BND-celluloses. The increased strength of binding of sRNA to BD- and BND-celluloses compared with that to DEAE-cellulose was also reduced by such solutes as 2-methoxyethanol or ethanol (5 or 10%, v/v). That RNA which was not eluted by solutions of salts was released when these alcohols were added to the solutions.

Effects of the Concentration of Magnesium Ion upon the Chromatography of sRNA. Crude sRNA from baker's yeast was chromatographed on columns of BND-cellulose in solutions 0.1 M in magnesium chloride or with no added magnesium ion and 0.01 M EDTA to chelate any divalent metal ions present. The results obtained in the presence of 0.1 M magnesium chloride showed the latter part of the elution diagram to correspond with that in the presence of 0.01 M magnesium chloride (Figure 3) but that the earlier peaks ran together into a single large peak. Recovery of material from this large peak and rechromatography in the system containing 0.01 M magnesium ion showed that it was compounded of the earlier peaks in this system.

Chromatography in the presence of 0.01 M EDTA gave a complex elution pattern. Slightly higher salt concentrations were needed to elute the sRNA than when magnesium was present.

Separation of tRNAs on BD-cellulose. The use of BD-cellulose for the chromatographic separation of sRNA from brewer's yeast on a preparative scale is illustrated in Figure 4. RNA was eluted from the column with increasing concentrations of salt in an unbuffered system to give the complex pattern of absorbance seen in Figure 4. As the system worked reproducibly it was preferred to omit buffers which are substrates for microbial growth (Muench and Berg, 1966b). After reaching 1 M sodium chloride the column was washed with 1 M sodium chloride in 10% 2-methoxyethanol. This eluted about 7.5% of the ultraviolet-absorbing material applied to the

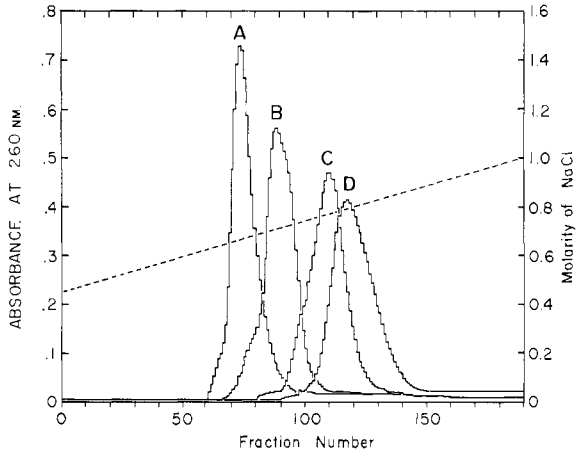


FIGURE 2: Composite elution diagram of portions of fractions A–D of Figure 1, rechromatographed under standard conditions on BND-cellulose (a column 0.7 × 100 cm). Elution was with a gradient of sodium chloride (a total of 1 l.) from 0.45 to 1.0 M, containing 0.01 M magnesium chloride. Fractions were 5 ml/9 min.

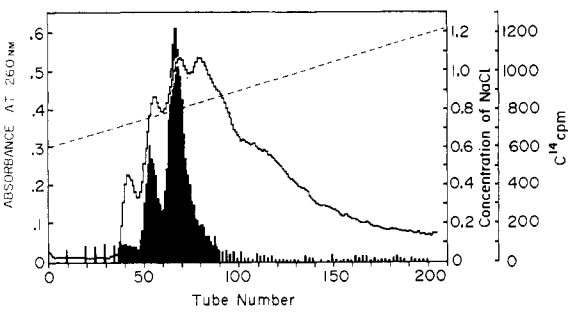


FIGURE 3: Chromatography of [^{14}C]alanyl-tRNA (19,000 cpm) prepared by the standard esterification procedure from sRNA of baker's yeast (240 A_{260} units) on a column (1 × 110 cm) of BND-cellulose. Elution was with a gradient of sodium chloride (a total of 1 l.) from 0.6 M to 1.2 M, containing 0.01 M magnesium chloride and 0.05 M sodium acetate (pH 5.0). Fractions were 5 ml/10 min. Solid line: absorbance at 260 nm; blackened area: counts per minute.

column (after tube 550). The total recovery of material applied to the column was better than 95%.

The distributions of acceptor activities for different amino acids in the fractions from this column are shown in Figure 4 and are described individually below. Toward the front of the elution diagram, where a complex pattern of sharp peaks was found, at least every fifth tube was assayed for acceptor activity. Further back in the diagram, where the peaks showed some broadening, every 10th, or toward the end, every 20th tube was assayed. Where sharply divided peaks of acceptor activity are shown close together these are the results of repeated assays of appropriately closely spaced tubes. Note that in the parts of Figure 4 the scales of acceptor activity differ from diagram to diagram. Since the various synthetases were present at different levels in the enzyme preparation and some were inhibited by salt the height of individual peaks may not necessarily indicate the true quantity of each component present.

Alanine. The enzyme system required to esterify alanine to its tRNA was often of low activity in extracts of baker's yeast prepared as described. With a sufficiently active preparation the acceptor activity of the tRNA was found to be high. There was one major peak, one lesser, and two minor ones.

Aspartic Acid. All the acceptor activity for aspartic acid eluted as a single peak. The cause of the apparent incipient splitting of this peak near its summit is not clear. Material recovered from this area (fractions 126–161), which also contains acceptor activities for leucine, isoleucine, and proline plus traces of other species, was rechromatographed under similar conditions. A single peak of absorbance, almost symmetrical in form, was observed. Acceptor activities for aspartic acid and leucine were distributed coincidentally with absorbance. When rechromatography on BD-cellulose was performed in a system buffered at pH 4.0 in the presence of 0.01 M magnesium chloride several peaks were partially separated (Figure 5). The leading and largest of these was identified with acceptor activity for aspartic acid, largely free of other detectable activities.

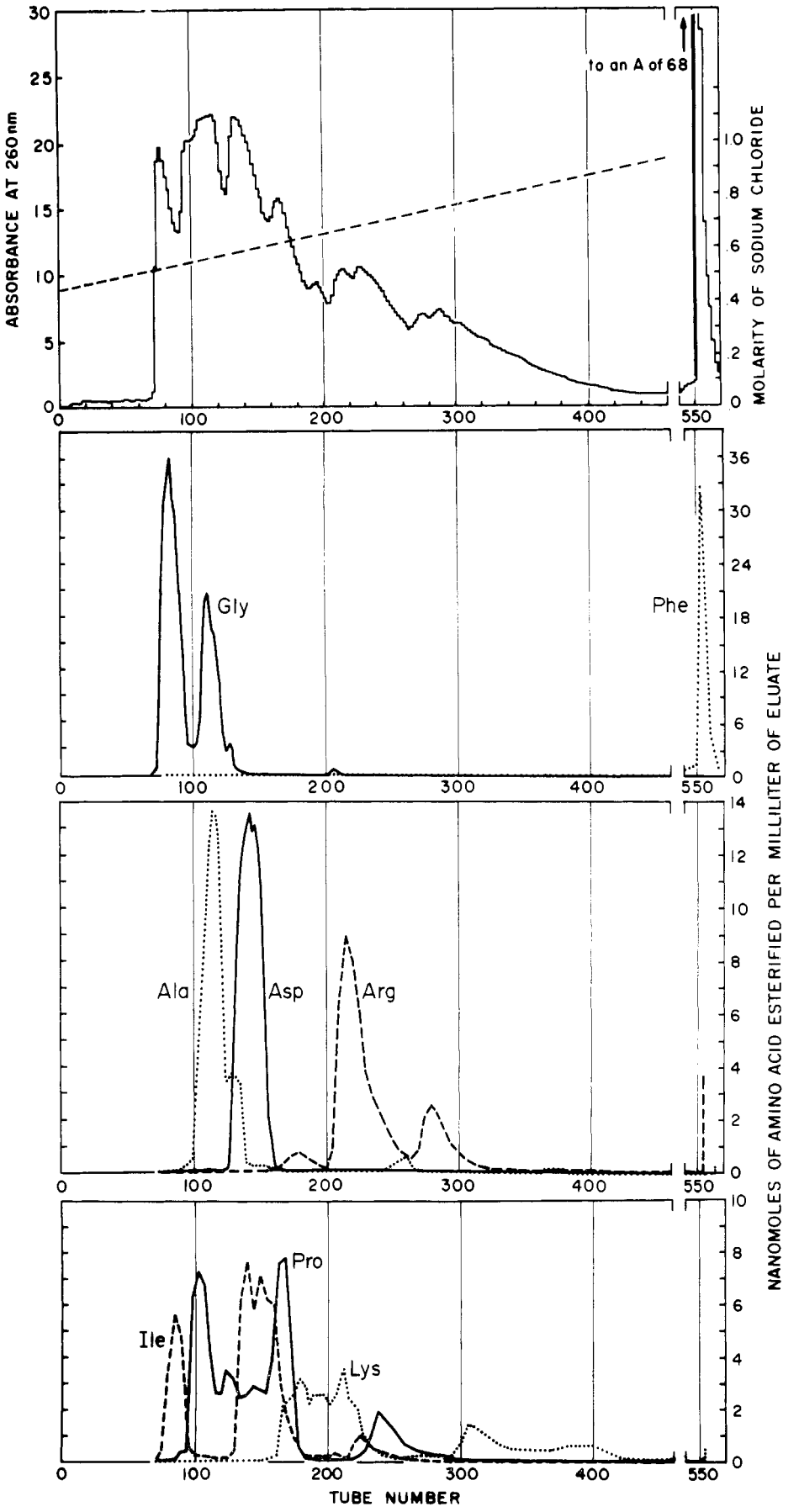
Asparagine. Three major peaks of activity for asparagine were detected, of which two were incompletely resolved. A fourth minor peak was present. No acceptor activity was found in the area where that for aspartic acid eluted.

Arginine. Three peaks of acceptor activity for arginine were resolved within the body of the elution diagram. Further activity was found in the strongly bound material eluted with the use of 2-methoxyethanol. It should be noted that the assay for arginine acceptor activity required the use of a partially purified enzyme since the crude preparation contained an enzyme which apparently destroyed arginine rapidly.

Cysteine. Mixtures for the assay of cysteine acceptor activities contained 2-mercaptoethanol but nonetheless gave higher blank values than most amino acids, possibly due to formation of disulfide bonds with proteins in the solutions. Variability of the blank values made estimation of the reality of the two small areas of acceptor activity difficult. A larger, broad peak of activity showed a clear shoulder on its leading edge. No attempt was made to determine if cystine could be activated.

Glutamic Acid. In contrast to the simple distribution of acceptor activity found for aspartic acid, that for glutamic acid was widely spread through the eluate from the column. Five larger peaks of activity were resolved, together with a possible further five minor ones, broadly spread and poorly resolved.

Glutamine. Total acceptor activities for glutamine were the lowest found for any amino acid. As for


ABSORBANCE AT 260 nm
MOLARITY OF SODIUM CHLORIDE
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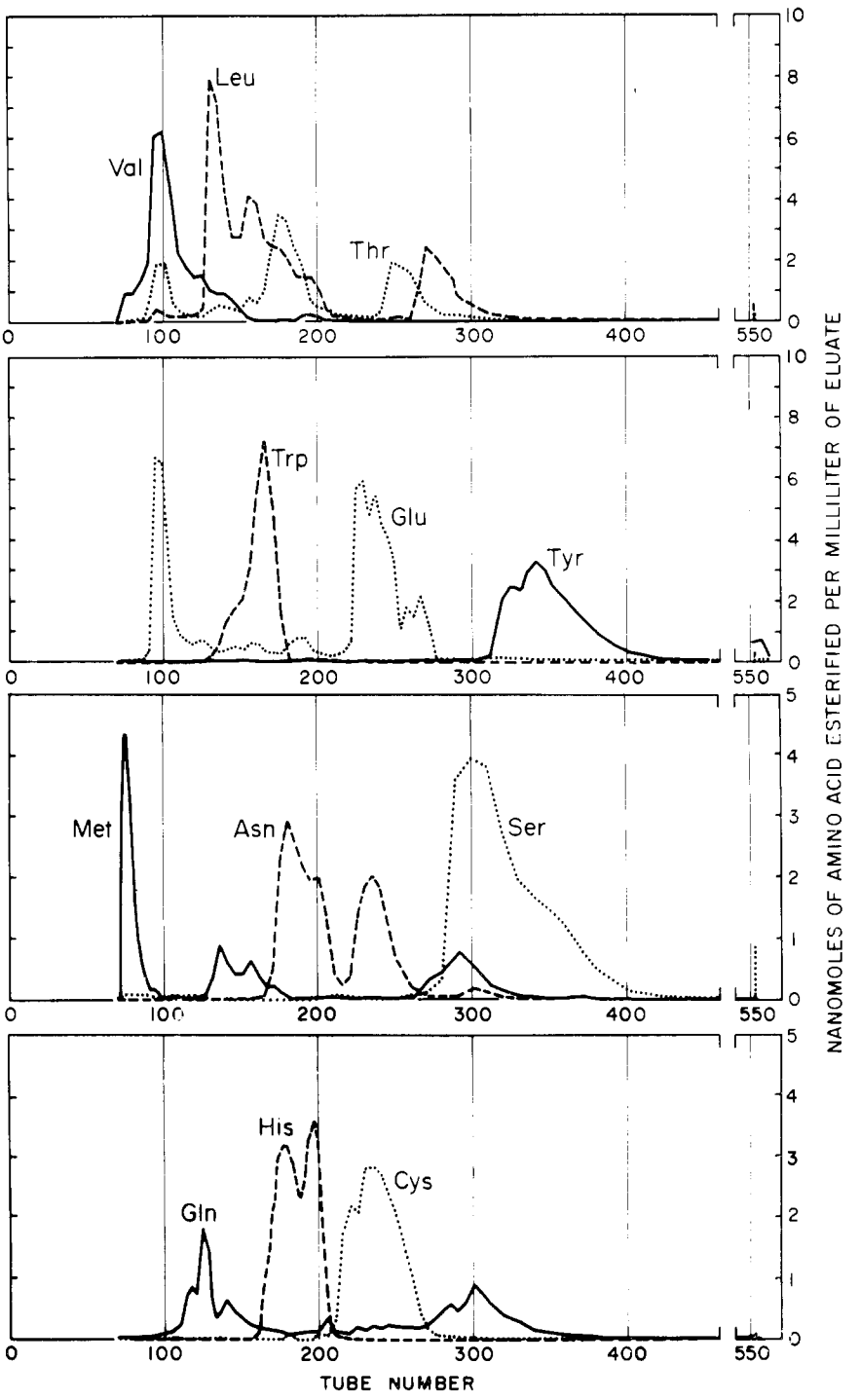

FIGURE 4: (Opposite and above) Fractionation of 5 g (70,000 A_{260} units) of sRNA from brewer's yeast (Boehringer) on a column (3.2 × 110 cm) of BD-cellulose. The sample was applied in 500 ml of 0.45 M sodium chloride and 0.01 M magnesium sulfate and eluted with the indicated (dashed line) gradient of sodium chloride solution (a total of 10 l.) containing 0.01 M magnesium sulfate. Fractions were 20 ml/15 min. At the end of the gradient (tube 497) elution was continued with 1.0 M sodium chloride–0.01 M magnesium sulfate containing 10% (v/v) 2-methoxyethanol. Total recovery, about 66,000 A_{260} units. Fractions were assayed for amino acid acceptor activity as described in the text.

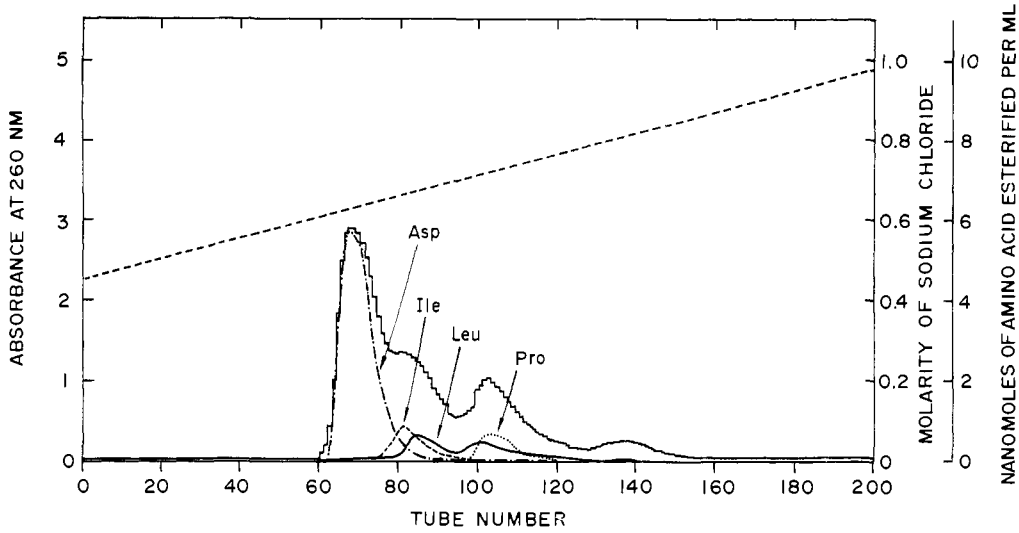

FIGURE 5: Rechromatography of a sample (25 mg) of $tRNA^{Asp}$ (recovered from fractions 126–161 of Figure 4) at pH 4.0 on a column (0.9 × 97 cm) of BD-cellulose. The sample was applied in 3 ml of 0.45 M sodium chloride, 0.01 M magnesium chloride, and 0.05 M sodium acetate buffer (pH 4.0). Elution was with the indicated (dotted line) gradient of sodium chloride containing 0.01 M magnesium chloride and 0.05 M sodium acetate buffer (pH 4.0) (total volume, 1.2 l.). Fractions were 5 ml/7 min. Assays for amino acid acceptor activity were performed as described in the text, after neutralization of the buffer.

glutamic acid, numerous peaks of activity were noted, mostly broadly spread and incompletely resolved. There was evidence of at least six such peaks, possibly more. None of the larger ones coincided with areas of major acceptor activity for glutamic acid.

Glycine. Two major and two minor peaks of acceptor activity were detected. Total acceptor activity for glycine was the highest found for any amino acid and when assaying the fractions of the major peaks it was necessary to use more glycine (of lower specific activity) than routinely. The first peak of activity was found in the first sharp absorbance peak eluted from the column. This fraction was purified further by rechromatography at pH 4 in the presence of 0.005 M EDTA (Figure 6B). Under these conditions the peak containing the glycine acceptor activity was not separated from that for isoleucine, nor was separation achieved at pH 3.5 in the presence of 0.05 M magnesium sulfate.

The second sharp peak of glycine acceptor activity (Figure 4) was well resolved but close to the first. However, on rechromatography in the presence of 0.005 M EDTA it separated into two peaks (Figure 7B).

Histidine. Two peaks of acceptor activity for histidine were incompletely resolved. The ratio of the two components differed considerably from that seen in Figure 1 where a sample of tRNA from baker's yeast was used.

Isoleucine. Acceptor activities for isoleucine appeared as a number of sharp peaks, not all of which were completely resolved. Indications of five such peaks were seen, plus one minor peak.

Leucine. Baker's yeast readily yields an enzymatic preparation active in the esterification of leucine to its tRNAs, and total acceptor activity of crude tRNA is high. In the eluate from the column several areas of leucyl acceptor activity were found. These areas are assigned letters in their order of elution for convenience in reference and to avoid confusion with the various numbered leucyl acceptor RNAs described by others. *Peak a* was a minor one centered on fraction 96. *Peak b* was the major one (around fraction 131). *Peak c* was the next largest peak (at fraction 156) and appeared to tail into *peaks d and e*, actually shoulders, around fractions 170 and 195, respectively. An area containing no acceptor activity for leucine was followed by *peak f* (about fraction 225), a region of activity slightly, but reproducibly, above the background level. *Peak g* (fraction 271) was a relatively low peak but spread rather widely. Its tail could be interpreted as containing other peaks, poorly separated. These are not assigned letters, nor is the trace of acceptor activity found in the material eluted with a solution containing 2-methoxyethanol.

Lindahl *et al.* (1966) have shown that sRNA prepared from yeast by extraction with phenol contains a renaturable leucyl acceptor RNA. Upon heating such a preparation briefly in a solution containing magnesium chloride its total acceptor activity for leucine increases considerably. By countercurrent distribution these workers were able to separate the renaturable leucyl acceptor activity ($tRNA_3^{Leu}$) from two other species (1 and 2) of leucyl acceptor RNAs. The acceptor activities of $tRNA_1^{Leu}$ and $tRNA_2^{Leu}$ are unaffected by brief periods of heating in solutions containing magnesium ion or a chelating agent.

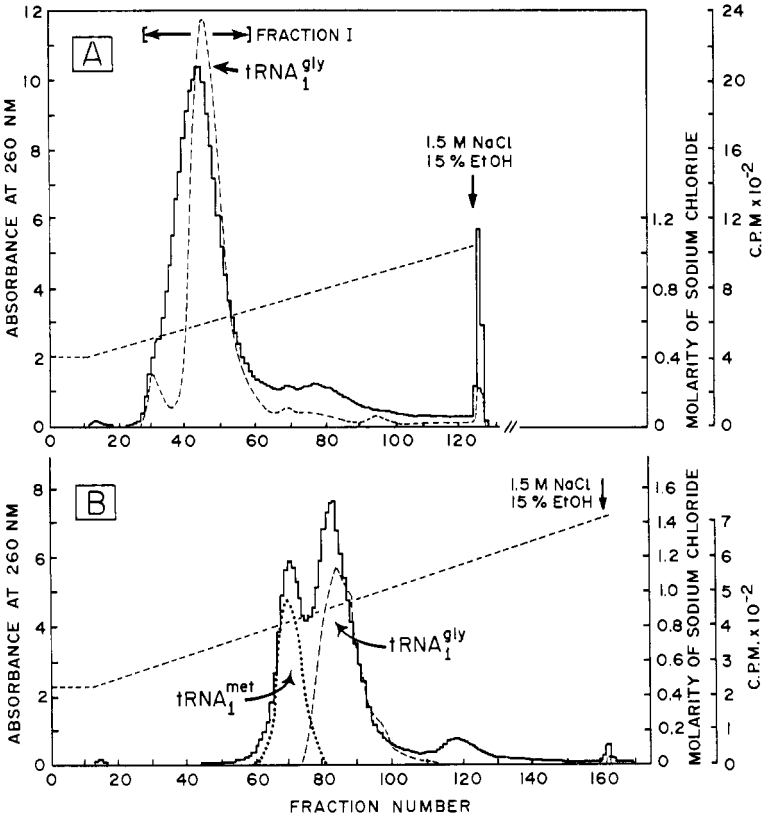

FIGURE 6: Rechromatography of $tRNA_1^{Gly}$ and fraction 1. (A) Rechromatography of $tRNA_1^{Gly}$ (fractions 73–92, Figure 4) on a column (1.2 × 119 cm) of BD-cellulose using a 2-l. gradient of sodium chloride as indicated containing 0.05 M magnesium chloride and 5 mM sodium formate buffer (pH 3.5). Fractions were 11 ml/11 min. (B) Rechromatography of fraction 1, part A, on a column of BD-cellulose (1.2 × 119 cm) with a 2-l. linear gradient of sodium chloride as shown containing 10^{-3} M EDTA and 5 mM sodium formate buffer (pH 4.0). Fractions were 11 ml/11 min. Assays were carried out using [^{14}C]glycine (1960 cpm/nmole) and [^{14}C]methionine (14,250 cpm/nmole) with a crude enzyme of low methionyl-tRNA synthetase activity.

It was of interest to determine if a peak of renaturable leucyl acceptor activity could be found in the eluate from the column. Using a sample of the crude sRNA used to load the column the effect of renaturation on acceptor activity reported by Lindahl *et al.* was readily confirmed. It was shown that the same effect occurred if the sample were heated in a solution containing 0.01 M magnesium chloride and up to 0.5 M sodium chloride. The presence of 1.0 M sodium chloride inhibited the renaturation.

Samples of fractions from the column were diluted with an equal volume of 0.01 M magnesium chloride, heated (60°, 5 min), then cooled, and assayed as usual for acceptor activity for leucine. A pattern of peaks identical with that found for unheated samples was found. Thus no renaturable $tRNA^{Leu}$ was present in fractions from the column although it was present in material applied to the column.

Samples from each of the major peaks of leucyl acceptor activity were examined for the property of being denaturable by heating in the presence of EDTA. The results, shown in Table I, indicated that the activities of peaks e and g were little affected by this treatment, while those of peaks b–d were reduced considerably. However, the activity of no peak disappeared completely upon denaturation.

The relationships of these peaks of activity as acceptors for leucine to those described by Lindahl *et al.* (1966) is not clear and has not been examined further, nor is it clear at what stage the renaturable leucyl acceptor RNA regained its activity, whether during chromatography, during storage of the fractions in the frozen state, or in the repeated cycles of freezing and thawing to which they were subjected during the long process of assaying for various acceptor activities.

Lysine. Lysyl acceptor activity was found principally in two regions (fractions 160–250 and 290–410). Within these regions activity was spread broadly though several sharp peaks emerged from the former. While it was difficult to decide how many distinct and potentially separable acceptor activities there may be for lysine it was possible to discern nine peaks and shoulders in the pattern, making the pattern for this amino acid

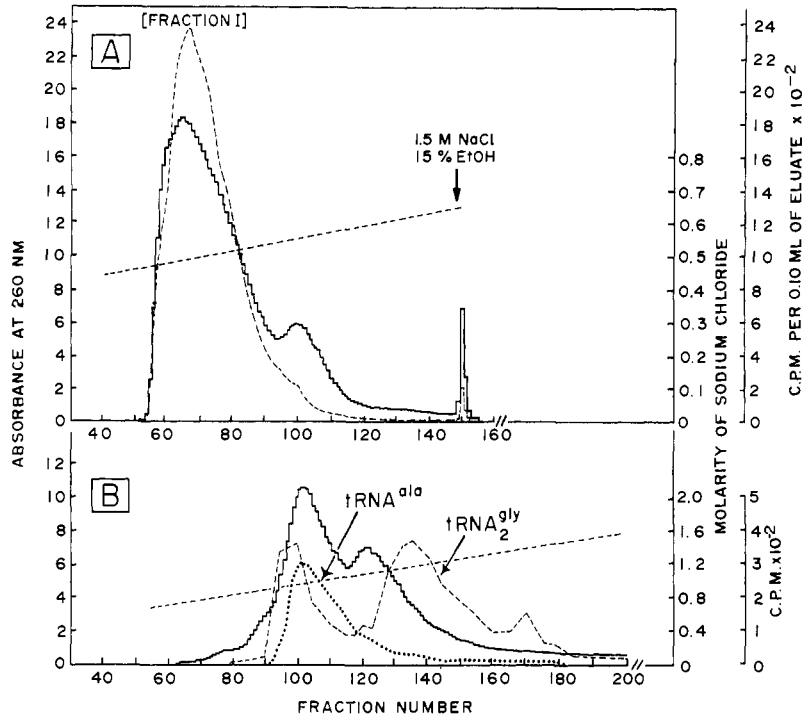

FIGURE 7: Rechromatography of $tRNA_2^{Gly}$ and fraction 1. (A) Rechromatography of $tRNA_2^{Gly}$ (fractions 103–120, Figure 4) on a column (1.2 × 119 cm) of BD-cellulose with a linear gradient of sodium chloride as shown having 0.01 M magnesium chloride and 5 mM sodium acetate buffer (pH 5.0). Fractions were 10 ml/10 min. (B) Rechromatography of fraction 1, part A, on a column (1.2 × 119 cm) of BD-cellulose with a linear gradient of sodium chloride as shown containing 10^{-3} M EDTA and 5 mM sodium formate buffer (pH 3.5). Assays were carried out using [^{14}C]glycine (1960 cpm/nmole) and [^{14}C]alanine (23,000 cpm/nmole). The values for [^{14}C]alanine were divided by four to facilitate plotting.

one of the most complex seen. Activities of individual peaks were low.

Methionine. A major peak of methionyl acceptor eluted from the column with the front of the absorption pattern and before any other acceptor activity. It was so closely followed by the much larger first peak of glycyl acceptor activity that it was not possible to obtain it substantially pure by this one step alone. This species was separated from the $tRNA^{Gly}$ by chromatography at pH 4.0 in the presence of EDTA (Figure 6B). Four other peaks and shoulders of methionine acceptor activity were noted, the last to emerge being broad.

Phenylalanine. Among the patterns of elution of acceptor activities that for phenylalanine is unique. No detectable activity was eluted by the salt gradient. A combination of 2-methoxyethanol and salt was required to release it. Fractions eluted with the aid of 2-methoxyethanol contained little other acceptor activity than that for phenylalanine and provided a 12–14-fold enriched starting material for further purification of $tRNA^{Phe}$

Proline. Two major peaks of prolyl acceptor activity were eluted relatively early from the column and four or five smaller peaks or shoulders were present. As in the case with arginine, the assay for proline acceptor activity required the use of a partially purified enzyme.

Serine. Acceptor activity for serine was eluted quite late in one broad area (a peak and a shoulder), doubtless due to incomplete separation of at least two species of $tRNA^{Ser}$. As well as the area of acceptor activity noted there were traces of activity above the background level around fractions 80, 110, and 210. The material eluted with salt and 2-methoxyethanol also contained detectable activity.

Threonine. Contrasted with that for serine, threonine acceptor activity was widely spread over the elution pattern. There were three major peaks and three minor areas of activity.

Tryptophan. Acceptor activity for tryptophan was confined to a single area. A peak with a remarkably sharp trailing edge was preceded by a lesser area of activity incompletely resolved from it. One major and at least one minor species of $tRNA^{Trp}$ were indicated.

Tyrosine. Tyrosyl acceptor activity eluted from the column as two partially resolved peaks. The larger was the last major peak of acceptor activity to be eluted by the salt gradient though a minor part of the seryl acceptor activity was eluted in the same area.

Slight activity for acceptance of tyrosine was found in the fractions eluted with solutions containing 2-methoxyethanol. As for the seryl acceptor activity in

TABLE I: Effects of Heating with EDTA[a] on Acceptor Activities for Leucine of Samples Taken from the Peaks Shown in Figure 4.

Peak	Fraction	Acceptor Act. (% of that of the same fraction prior to treatment)
a, f		Not tested
b	131	22
	136	36
c	156	55
	161	47
d	176	39
e	196	83
g	271	100
	276	89

[a] Each sample was diluted with a solution containing 1.5 equiv of EDTA for each equivalent of magnesium ion in the sample, heated (60°, 5 min), and cooled, and a solution of magnesium chloride was added to give a final nominal concentration of magnesium (concentration of magnesium chloride minus concentration of EDTA) of 0.01 M. Acceptor activity for leucine was then assayed as usual.

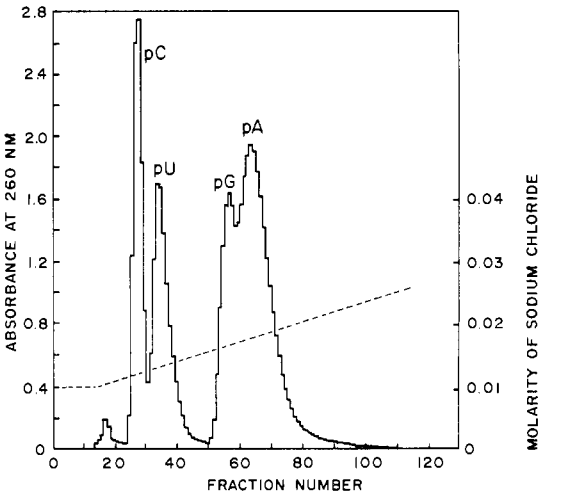

FIGURE 8: Chromatography of the four common ribonucleoside 5′-phosphates on a column of BD-cellulose (1.5 × 95 cm) with a gradient of sodium chloride as shown containing 5 mM sodium phosphate (pH 7.4). Fractions were 10.4 ml; flow rate was 1 ml/min.

the same fractions this may have represented material tailing off the main peaks that would otherwise have eluted as long but low tails to the peaks.

Valine. This acceptor was eluted early in the salt gradient. One major peak of activity was incompletely resolved from about three smaller areas which appeared as shoulders around it. A single small peak emerged later (around fraction 195).

Separation of Mononucleotides. BD-cellulose was used to separate mononucleotides (Figure 8). Adenylic acid is more strongly held than guanylic acid, a reversal of the order noted with DEAE-cellulose (Staehelin, 1961).

Discussion

The interaction of polynucleotides with DEAE-cellulose depends not only on ion exchange but also on weaker forces of attraction. The latter forces are largely a function of the base composition of the polynucleotide chains. These attractions can be reduced by inclusion of such agents as urea, formamide, or glycols (Tomlinson and Tener, 1963) in the eluting solutions. These secondary forces, which are themselves dependent upon the secondary structure of the polynucleotides, have been used to separate individual species of tRNA from crude sRNA by chromatography on DEAE-cellulose (Cherayil and Bock, 1965; Baguley *et al.*, 1965). As the various species of acceptor RNA have similar size (76–85 nucleotide residues) they should not be readily separable by the process of ion exchange alone.

The studies described here started from the idea of using increased secondary (nonionic) interactions with a modified DEAE-cellulose to separate tRNAs. The separations of tRNAs and other nucleic acids on columns of methylated albumin on kieselguhr (Sueoka and Yamane, 1962) suggested that sRNA could be fractionated by exploiting these secondary binding forces. Serum albumin is a protein with a notable capacity to bind a wide variety of lipophilic compounds, for example, steroids and dyes. Methylation of its free carboxyl groups leaves free amino and guanidino groups and after deposition on kieselguhr an insoluble anion exchanger with an affinity for lipoidal groups results. Introduction of the benzoyl groups into DEAE-cellulose produces a somewhat similar type of exchanger but of much greater capacity. From the results reported here it is clear that BD-cellulose binds polynucleotides more strongly than unsubstituted material despite the fact that substitution must decrease the capacity for ion exchange by dilution of ionic groups on the cellulose with nonionic aromatic groups. The increased secondary attractions for polynucleotides of BD-cellulose, like those of DEAE-cellulose itself, are largely eliminated in the presence of urea or alcohols, agents which disrupt both hydrogen and hydrophobic bonding.

Brief studies with a long- (nonanoyl) and a short- (acetyl) chain aliphatic ester substituent and of a long-chain aliphatic (octadecyl) urethan derivative of DEAE-cellulose showed little or no increased affinity for polynucleotides and no promise for use in separation of tRNAs. Only with aromatic esters were these in-

creased affinities found. Of those tried the simplest, BD-cellulose, gave the best results with tRNA. For applications in the separation of smaller or less strongly attracted molecules the naphthoylated DEAE-cellulose may be useful. However, it is worth noting that with BD-cellulose the increased secondary attractions are detectable at the mononucleotide level as shown by the order of elution of mononucleotides, pC, pU, pG, and pA (Figure 8), compared with the order pC, pU, pA, and pG (Staehelin, 1961) from DEAE-cellulose.

As was noted in the introduction, early studies were made with incompletely substituted DEAE-celluloses which did not become homogeneous with the pyridine during preparation. This approach led to the empirical formulation of a benzoylated naphthoylated DEAE-cellulose and its evolution into BND-cellulose, successfully used in the separation of tRNAs and other nucleic acids as noted here and by others (J. E. Fox and C. M. Chen, unpublished data; Sedat *et al.*, 1967). Though careful control of moisture allows BND-cellulose to be prepared with reasonably reproducible properties it seemed desirable to approach complete substitution of the DEAE-cellulose to give a more homogeneous and reproducible product. BD-cellulose has been prepared from batches of DEAE-cellulose from two manufacturers and has given closely similar results. It appears to be the equivalent of BND-cellulose in the number and shapes of peaks in the profiles of absorbancy eluted from columns loaded with crude sRNA. No careful comparison of the distributions of individual acceptor activities has been made but the separation on both exchangers would seem to be comparable. The reproducibility of the properties of BD-cellulose from batch to batch is likely to be limited principally by the reproducibility of the DEAE-cellulose used.

tRNAs are notable for their degree of secondary structure, which is demonstrated in the hyperchromic effects on dilution into solution of low ionic strength or upon heating. In general, complete recovery of secondary structure is effected by reversing these processes. Baguley *et al.* (1965) have shown that the secondary binding forces between DEAE-cellulose and tRNAs are increased with increasing temperature and interpreted this as being due to the stronger binding of denatured or open-stranded chains than of those with a high degree of secondary structure where individual chains are folded back upon and interact with themselves. In DEAE-cellulose where ionic groups are scattered quite sparsely over the cellulose chains a polynucleotide in the open or single-stranded form may interact with more of these sites than one of similar chain length but with a compact secondary structure.

A series of experiments with the chromatography of tRNA on partially benzoylated DEAE-cellulose and on BND-cellulose to study the effects of temperature and of the presence of various levels of magnesium ion or EDTA upon the pattern of elution gave results consistent with the hypothesis of Baguley *et al.* That is, under conditions where the secondary structure in tRNA was reduced (higher temperature and removal of magnesium ion) binding to the derivatized DEAE-cellulose was increased and higher concentrations of salt were required to elute the RNA.

RNA prepared from yeast ribosomes was not eluted from a column of incompletely benzoylated DEAE-cellulose by solutions of sodium chloride up to 1.0 M (rRNA is not readily soluble in solutions more concentrated than this). Increasing concentrations of 2-methoxyethanol containing the same concentration of salt eluted only 30% of the RNA as a peak around 4% (v/v) 2-methoxyethanol. This material was not characterized but was perhaps the smaller (16S) species of rRNA. The strong binding of rRNA to the column can be accounted for both by its high molecular weight and its open structure.

For the resolution of the tRNAs of yeast, the combination of room temperature and 0.01 M magnesium chloride in unbuffered solutions of sodium chloride was convenient and effective. To determine patterns of elution of amino acid acceptor activities from these columns a method of assay was needed which did not require the time-consuming pooling of fractions and subsequent recovery of the RNA. In the method used the fractions were assayed directly and thus the incubation mixtures contained varying concentrations of sodium chloride. As noted by Holley *et al.* (1961) for tyrosyl-tRNA synthetase the presence of sodium chloride inhibited certain aminoacyl-tRNA synthetases, most notably those for glutamine. The addition of potassium chloride did not overcome the inhibition. Therefore before assaying for acceptor activities the kinetics of activation of the amino acid in solutions with and without sodium chloride were studied using a sample of crude sRNA of acceptor activity estimated to be equivalent to that of the most active fraction from the column. From such studies conditions of incubation (time and quantity of enzyme used) were chosen to give the maximum esterification of labeled amino acid to its acceptor regardless of possible inhibition by added salt. This approach was convenient and gave reproducible results. The values for acceptor activity for amino acids obtained are, however, minimum ones since the level of charging achieved represents a steady-state value controlled by the rate of synthesis and enzymic and chemical hydrolysis. This is particularly true for the lesser peaks of activity found for many amino acids as it is likely that some of these represent species of tRNA having different kinetics of labeling from those found for the larger peaks. Indeed it has been noted that different species of tRNA for the same amino acid require different activating enzymes (Barnett and Brown, 1967).

Lebowitz *et al.* (1966) have demonstrated that a purified seryl acceptor RNA of yeast can be further resolved by countercurrent distribution into two types of acceptor. The major component is an adenosine-terminated RNA capable of accepting serine upon incubation in a system containing crystalline seryl-tRNA synthetase, serine, and ATP. The minor component is a cytidine-terminated species which only

accepts the amino acid after reaction with ATP and tRNA–CCA pyrophosphorylase (present in crude preparations of activating enzyme) when it become inseparable from the major component. Similar effects were demonstrated with a $tRNA^{Leu}$. Since assays for acceptor activities in chromatographic fractions described here were performed with crude preparations of enzyme in the presence of ATP (but not CTP) any separation of acceptors differing only in the presence of the terminal adenosine should have resulted in the detection of two peaks. However, a high degree of resolution must be required to separate two species differing by only a single nucleotide residue and the sRNA sample used (Boehringer) contained a preponderance (90%) of adenosine as terminal nucleoside. (We wish to thank Dr. K. L. Roy for this determination.) Hence it is unlikely that the presence of adenosine- and cytidine-terminated forms of the same tRNA accounts for more than small shoulders in the distribution patterns of acceptor activities found. In all, more than 80 major and minor peaks of acceptor activities were found.

Substituted DEAE-celluloses share with the unsubstituted material and with MAK the advantage over most methods of separation based upon partition that the pH and temperature of the system may be varied at will. Variations in the nonionic interactions of tRNAs with DEAE-cellulose produced by factors which affect the secondary structures of the polynucleotides have been exploited (Cherayil and Bock, 1965; Baguley *et al.*, 1965) to separate particular acceptors by a series of chromatographic fractionations under different conditions. That this approach is applicable to fractionation on columns of modified DEAE-cellulose is indicated by the further purification of the aspartyl acceptor from the column of Figure 4 by rechromatography at pH 4.0 (Figure 5). Similarly the $tRNA_1^{Gly}$ contained in fractions 70–94 from the column of Figure 4 was further purified by rechromatography under altered conditions.

Separation was not effected at pH 3.5 in the presence of 0.05 M magnesium ion but at pH 4.0 in solutions containing 1.0 mM EDTA (Figure 6) a fraction containing $tRNA^{Met}$ was separated from a second peak containing $tRNA^{Gly}$ and $tRNA^{Ile}$.

In such purifications by rechromatography on columns of BD-cellulose the variables most likely to be useful are temperature, pH, and concentration of magnesium ion. The most effective combination must be found empirically for the particular problem under study. Alternatively fractions obtained by chromatography on BD-cellulose may be further purified by one of the existing methods of separation, preferably one which utilizes different properties of the tRNAs.

A simple analytical method for determining the number and character of acceptors for a particular amino acid in any preparation is to esterify to that acceptor the radioactively labeled amino acid, separate the mixture in a system buffered at an acid pH, and determine the distribution of radioactivity. Sueoka and Yamane (1962) chromatographed various [^{14}C]-aminoacyl-tRNAs on columns of MAK buffered at pH 6.7 to stabilize the aminoacyl esters.

An example of this procedure using BND-cellulose and buffer of pH 5.0 for the separation of tRNA esterified with [^{14}C]alanine is illustrated in Figure 3. However, acceptor RNAs for aromatic amino acids cannot be examined in this way. Stripped $tRNA^{Phe}$ requires the addition of 10% alcohol to elute it and emerges at the alcohol front. Both $tRNA^{Trp}$ and $tRNA^{Tyr}$ come off in the main area of the elution profile but when esterified with the appropriate amino acid are eluted from a column of BD-cellulose only when alcohol is incorporated into the eluting system. These observations have been used as a means of obtaining these two tRNAs in highly purified form (E. Wimmer and I. Maxwell, unpublished results). Thus the introduction of a single aromatic system causes the tRNA to be much more strongly bound to BD-cellulose. In an analogous manner *N*-phenoxyacetylalanyl-$tRNA^{Ala}$ is bound strongly to BD-cellulose and eluted only by solutions containing salt and organic solutes (Tener *et al.* (1966) and unpublished results) whereas Ala-$tRNA^{Ala}$ is eluted by salt alone (Figure 3). J. E. Fox and C. M. Chen (personal communication) have described the strong nonionic binding to BND-cellulose of a tRNA-like material which contains the synthetic cytokinin N^6-benzyladenine.

Thus it is concluded that tRNAs bind to benzoylated DEAE-cellulose by a process involving both ionic interactions and attractions between the latter's aromatic groups and certain of the bases of the tRNAs not involved in the maintenance of secondary structure, that is, in exposed or single-stranded regions of the chain. The introduction of a single extra aromatic residue at the acceptor end of the tRNA chain, presumably an exposed position, leads to a great increase in the strength of these nonionic interactions.

Of the stripped tRNAs from brewer's and baker's yeast only that for phenylalanine shows remarkably strong nonionic bonding to BD-cellulose. The structure of $tRNA^{Phe}$ of baker's yeast has been determined (RajBhandary *et al.*, 1967), but it is not immediately obvious how this tRNA differs greatly from $tRNA^{Ala}$ (Holley *et al.*, 1965), $tRNA_{1,2}^{Ser}$ (Zachau *et al.*, 1966), and $tRNA^{Tyr}$ (Madison *et al.*, 1966), all of which are of known structure. The feature which may be the cause of this unusual behavior is the unidentified nucleotide (referred to as Y) at position 37 (from the 5′-phosphate end). This is believed to be in one of the "loops" or single-stranded exposed parts of the otherwise folded structure and thus available for interaction, and to be homologous with an alkylpurine in the other tRNAs of known structure. Of particular interest is its apparent homology with the N^6-isopentenyladenine in $tRNA^{Ser}$ of brewer's yeast (Zachau *et al.*, 1966). Thus it is possible that the nucleotide Y could be a purine derivative containing a lipophilic group.

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