Resolution of Aminoacyl Transfer Ribonucleic Acid by Hydroxylapatite Chromatography*

Karl H. Muench† and Paul Berg

ABSTRACT: Aminoacyl ribonucleic acid from *E. coli* has been resolved on columns of hydroxylapatite with gradient elution by potassium phosphate buffers, pH 5.8. Transfer ribonucleic acid esterified to alanine, leucine, methionine, and valine is fractionated into multiple peaks. After prior purification by partition chromatography, individual t-RNA species have been resolved to 80–90% purity by hydroxylapatite. The acyl bonds are stable enough to permit recovery of intact aminoacyl RNA from column effluents, facilitating analysis.

Column capacity is at least 24 mg of t-RNA/cm^2 of column cross-sectional area. Recoveries of t-RNA and of acceptor activities exceed 90%.

A method for fractionation of amino acid specific transfer ribonucleic acid (t-RNA) using partition chromatography on a Sephadex column was described in the previous paper (Muench and Berg, 1966). Several t-RNA species were recovered in partially purified form (25–40% purity), and additional approaches for purification were explored for further enrichment of these species. In addition to methods depending on differences in partition coefficients of t-RNA's, a variety of techniques for enrichment of specific t-RNA's have been described. These include chromatography on methylated albumin-kieselguhr (Sueoka and Yamane, 1962), diethylaminoethyl-cellulose and Sephadex (Kawade *et al.*, 1963; Cherayil and Bock, 1965), or hydroxylapatite (Hartman and Coy, 1961). There are also chemical methods converting acceptor termini of free t-RNA to dialdehydes by periodate oxidation and removing such modified chains by Schiff base formation (Zamecnik *et al.*, 1960; Zubay, 1962). Enrichment has also been achieved by coupling polypeptides to the free α-amino group of aminoacyl RNA (Mehler and Bank, 1963; Simon *et al.*, 1964).

We have found that hydroxylapatite chromatography using phosphate-gradient elution provides an encouraging approach for fractionation of specific t-RNA's. It has high resolving power and is suitable for analytic as well as for preparative purposes. Also, isotopically labeled aminoacyl RNA's can be chromatographed; therefore, the problem of determining the elution profiles for specific t-RNA's is minimized.

Experimental Section

Materials. Aminoacyl RNA synthetases, used to esterify the various amino acids to t-RNA, were prepared and assayed as described in the previous paper (Muench and Berg, 1966). Inorganic pyrophosphatase was purchased from Worthington Biochemical Corporation, Freehold, N. J., and assayed by the method of Heppel (1955). t-RNA was either the bulk, unfractionated material or fractions recovered from the partition column described in the previous paper (Muench and Berg, 1966). Radioactive and unlabeled amino acids were obtained from New England Nuclear Corporation and California Corporation for Biochemical Research, respectively; labeled amino acids were used at about 0.4–4.0 $\times$ 10^8 cpm/μmole when counted in the Nuclear Chicago or Packard scintillation spectrometers.

Methods. AMINOACYL RNA SYNTHESIS. Aminoacyl RNA synthetase activity and the amino acid acceptor activity of t-RNA were measured as already described (Muench and Berg, 1966). Labeled aminoacyl RNA's for chromatography were prepared by means of the usual assay conditions (Berg *et al.*, 1961) except that the amounts of enzyme and t-RNA were increased. Aminoacyl RNA's were recovered by adjusting the reaction mixture to 0.2 M potassium acetate, pH 5, precipitating with 2 volumes of cold ethanol, and centrifugation. Free amino acid, ATP,[1] and other reaction components were removed by repeated solution and precipitation of aminoacyl RNA in 0.2 M potassium acetate, pH 5–6, followed by ethanol precipitation as above. In some cases the ami-

* From the Department of Biochemistry, Stanford University School of Medicine, Palo Alto, California. *Received October 28, 1965.* Supported by grants from the National Institutes of Health, U. S. Public Health Service.

† Present address: Department of Medicine, University of Miami School of Medicine, Miami, Fla.

[1] Abbreviations used in this work: t-RNA$_{leu}$ or t-RNA$_{ileu}$, t-RNA which accepts leucine or isoleucine, respectively. Leu-RNA or ileu-RNA are used to designate the esterified forms of the t-RNA's. Where more than one species of t-RNA$_{leu}$ occurs, we have, in the absence of any systematic way of designating the multiple forms of RNA obtained in fractionation procedures, referred to them by the order of elution from the partition column (Muench and Berg, 1966), *e.g.*, t-RNA$_{leu,1}$ is the first peak of t-RNA$_{leu}$. Other abbreviations: PPO, 2,5-diphenyloxazole; dmPOPOP, 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene; AMP, adenosine monophosphate; ATP, adenosine triphosphate; GSH, glutathione.

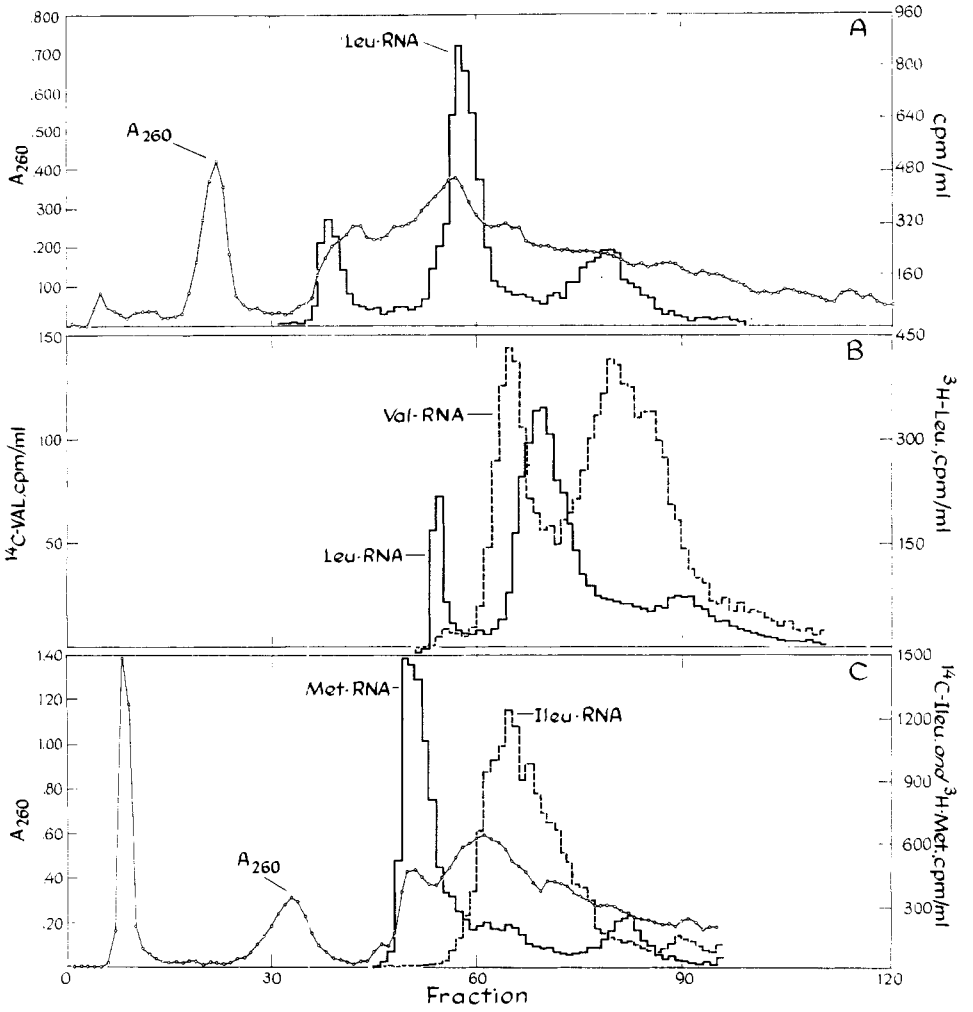

FIGURE 1: Chromatographic separation of unfractionated t-RNA. (A) Unfractionated t-RNA (14 mg), esterified with [^{14}C]leucine, was applied to a column (96 cm × 0.8 cm^2) and then eluted at 25 ml/hr with a linear gradient between 1000-ml volumes of 0.10 M and 0.25 M potassium phosphate, pH 5.8, each containing 5 ml of chloroform. Fractions were collected every 30 min. Recoveries were 99% for A_{260} and 66% for the acid-precipitable [^{14}C]. (B) Unfractionated t-RNA (19 mg), esterified with [^{3}H]leucine and [^{14}C]valine, was applied to a column (98 cm × 0.8 cm^2) and eluted first with a linear gradient between 120 ml each of 0.10 M and 0.16 M potassium phosphate, pH 5.8, at 13 ml/hr (nothing was eluted by this gradient). Then starting with fraction 35 elution was continued with a linear gradient between 750-ml volumes of 0.16 M and 0.25 M buffer at 32 ml/hr. Fractions were collected at 30-min intervals. The recoveries were 96% for A_{260} and 76 and 68% for acid-precipitable [^{14}C]valine and [^{3}H]leucine, respectively. (C) Unfractionated t-RNA (19 mg) loaded with [^{14}C] isoleucine and [^{3}H]methionine was applied to a column (103 cm × 0.8 cm^2) which was eluted at 23 ml/hr first with approximately 180 ml of 0.10 M potassium phosphate, pH 5.8, then with a linear gradient between 1000-ml volumes of 0.10 M and 0.25 M buffer, and finally with 0.30 M buffer. Fractions were 18 ml. Recoveries were 91, 28, and 50% for A_{260} and acid-precipitable [^{3}H] and [^{14}C], respectively.

noacyl RNA was freed of the substrates and salts by passage over a Sephadex G-25 column (20 cm × 0.8 cm^2) equilibrated with 1.0 M sodium chloride–0.1 M sodium acetate, pH 5.0, followed by alcohol precipitation. The aminoacyl RNA's were dissolved in potassium phosphate buffer (0.01–0.05 M, pH 5.8) before application to the column; both [^{14}C]- or [^{3}H]-labeled aminoacyl RNA's were used in most experiments, and these were mixed after they were isolated so that two different t-RNA's could be followed on the same column.

PREPARATION AND OPERATION OF HYDROXYLAPATITE COLUMNS. Hydroxylapatite which gave reproducible resistance to flow was the material CPA prepared according to Main *et al.* (1959).[2] It was equilibrated on

[2] Commercially available hydroxylapatite, obtained from the Clarkson Chemical Company, Williamsport, Pennsylvania, or from Bio-Rad, and that made according to Tiselius *et al.* (1956) was not entirely satisfactory since columns packed with certain batches gave extreme resistance to flow.

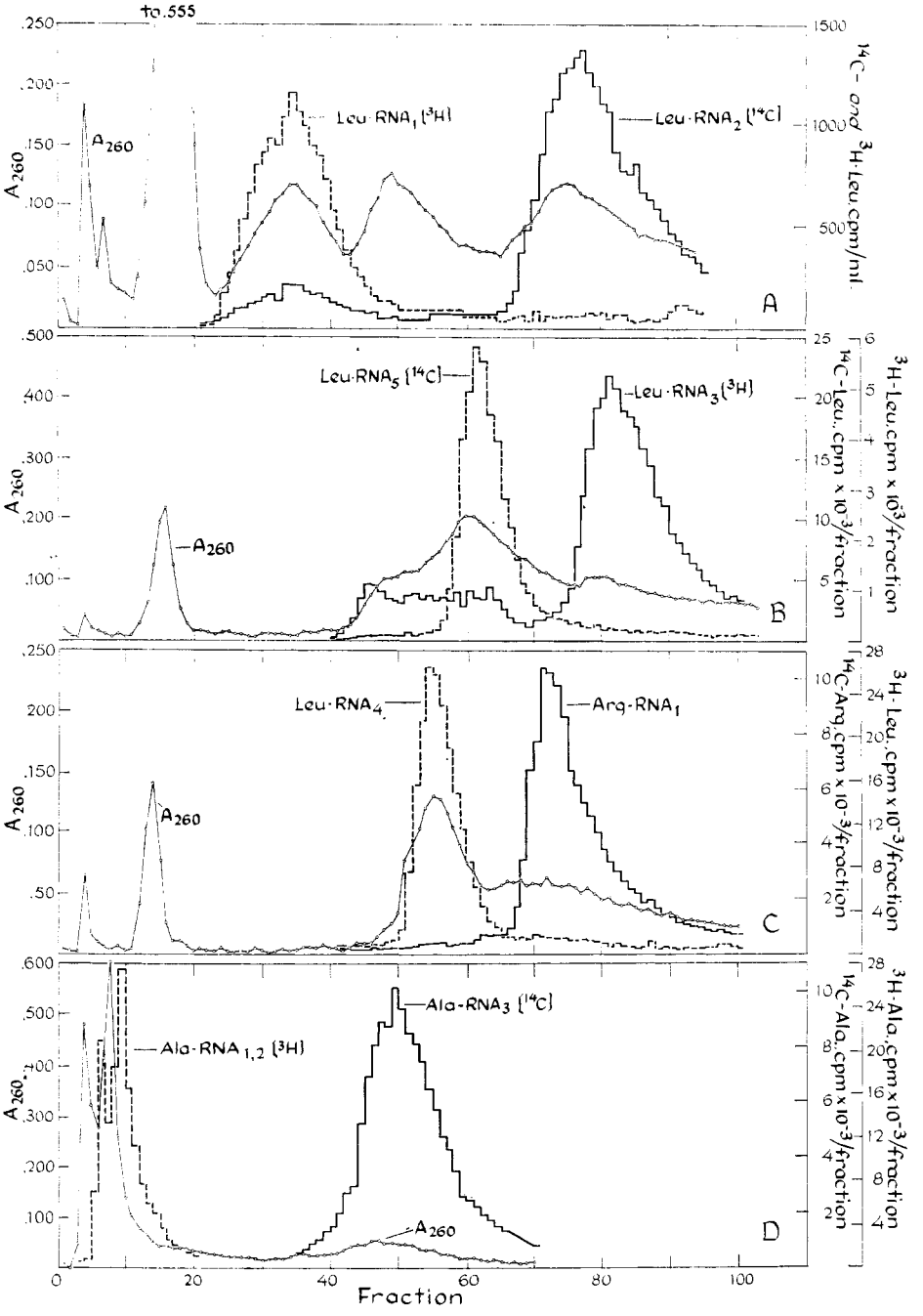

FIGURE 2: Chromatographic separation of unfractionated t-RNA. (A) t-$RNA_{leu,1}$ (3.7 mg) from fraction 42 of the partition column, esterified with [^{3}H]leucine, was mixed with 6.2 mg of t-$RNA_{leu,2}$ from fraction 52 of the partition column to which [^{14}C]leucine had been esterified. The mixture was applied to a column (100 cm × 0.8 cm^2) and eluted at 32 ml/hr first with 0.10 M potassium phosphate buffer, pH 5.8, until fraction 23, followed by a linear gradient between 1000 ml at 0.10 M and 1000 ml at 0.20 M of the same buffer until fraction 95. Continuation of the elution with a gradient between 1000-ml volumes of 0.20 and 0.40 M buffer (not shown on the figure) did not yield significant quantities of A_{260} or of [^{3}H] or [^{14}C]. Fractions were 24 ml. Recoveries were 87, 68, and 53% for the A_{260}, and acid-precipitable [^{3}H]leucine and [^{14}C]leucine, respectively. (B) t-$RNA_{leu,3}$ (2.3 mg) and t-$RNA_{leu,5}$ (6.6 mg) from partition column fractions 90 and 180, esterified with [^{3}H]leucine and [^{14}C]leucine, respectively, were applied to a column (100 cm × 0.8 cm^2) and eluted at 32 ml/hr first with 0.10 M potassium phosphate buffer, pH 5.8, until fraction 23, then with a linear gradient between 1000-ml volumes of 0.10 and 0.20 M of the same buffer until fraction 100. A continuation of the chromatography with 0.30 M buffer resulted in elution of approximately 10% of the added A_{260} and less than 5% of the added isotopes. All buffers contained chloramphenicol, 10 μg/ml. Fractions were 24 ml. Recoveries were 95, 67, and 55% for A_{260}, acid-precipitable [^{3}H]leucine, and [^{14}C]leucine, respectively. (C) t-RNA (4.5 mg) from partition column fraction 132, esterified with [^{3}H]leucine, and [^{14}C]arginine, was applied to a column (100 cm ×

columns at 4° or 23° with 0.1 M potassium phosphate buffer, pH 5.8 (10 parts KH_2PO_4 and 1 part K_2HPO_4), until the pH of the effluent remained constant at pH 5.8. Each batch was tested on a 1 cm × 0.8 cm^2 column to ascertain initial and final phosphate concentration to be used in elution, since there was some variation in adsorption properties. The hydroxylapatite, suspended in dilute phosphate buffer (0.01–0.05 M), was poured into the appropriate size column containing a sintered-glass base plate covered by 1 cm of glass beads (200 μ diameter). Columns (usually 100 cm × 0.8 cm^2) were packed by gravity flow and then equilibrated with 0.01 M potassium phosphate buffer pumped at rates of 15 to 32 ml/hr with a Beckman Accu-Flo pump. Columns were usually jacketed and maintained at −2° to 2° with a Beckman refrigerated fraction collector (Model 133); the buffer reservoirs and pump were at room temperature.[3] Prior to use each column was tested for uniformity of flow by passage of a methyl orange marker (Levin, 1962). After each run the column was washed with a concentration of potassium phosphate buffer, pH 5.8, sufficient to elute all t-RNA and then reequilibrated with 0.01 M phosphate, pH 5.8, for further use. Columns were used one to three times before repacking.

The appropriate labeled aminoacyl RNA's in 1–2 ml were applied to the column, and the column was washed with approximately 500 ml of 0.10 M potassium phosphate, pH 5.8, at 32 ml/hr. At that time one of several linear gradients of phosphate buffer concentrations, specified in the legend to each figure, was started. Attempts at stepwise elution resulted in asymmetric peaks and spurious multiplicity of peaks, whereas gradients steeper than those depicted led to poor separations. No difference in resolution of t-RNA's was noted with flow rates between 15 and 32 ml/hr.

[3] This proved to be undesirable because of difficulty in preventing bacterial growth in the buffers, in the lines to and from the column and in the pump. The growth could be partially curtailed by adding chloroform (5 ml/l.), caprylic acid (1 ml/l.), chloramphenicol (10 μg/ml), or penicillin G (1 unit/ml) to sterile buffers. Even under these conditions there was some inactivation of t-RNA during chromatography. The problem was circumvented later when the entire system was kept in a cold room (4°), and for this the LKB-Miniflow Micropump Model 4501A-P was used to maintain constant flow rates.

ASSAY OF FRACTIONS FROM COLUMN. Fractions recovered from the column were assayed for total RNA (A_{260}) and for the particular aminoacyl RNA as follows: After A_{260} was recorded, an aliquot of each fraction was mixed with about 1 mg of carrier yeast RNA and 0.2 volume of cold 6 N HCl, and the mixture was filtered through a Whatman glass fiber (GF/C) filter disk. The disk was dried, immersed in toluene–PPO–dmPOPOP solution, and counted in a scintillation counter with settings appropriate for single- or double-isotope counting.

If fractions were also to be analyzed for amino acid acceptor activity, the RNA was isolated by ethanol precipitation following dialysis in sterile dialysis sacs against polyethylene glycol 6000, potassium acetate, and EDTA as described earlier (Berg and Muench, 1966). Since the recovered RNA contained variable amounts of esterified amino acid, measurements of amino acid acceptor activity was made after enzymic deacylation as follows: aminoacyl RNA was incubated in a total volume of 0.5 ml with 50 μmoles of sodium cacodylate buffer, pH 7.0, 5 μmoles of $MgCl_2$, 5 μmoles of KCl, 2 μmoles of GSH, 100 μg of bovine serum albumin, 1 μmole of PP_i, 1 μmole of 5′-AMP, and 2 units of the appropriate aminoacyl RNA synthetase (1 unit forms 1 mμmole of aminoacyl RNA/10 min). After 10 min at 37°, 3 units of inorganic pyrophosphatase (1 unit liberates 1 μmole of P_i/min) was added, and an additional 10-min incubation followed to destroy the PP_i. At this point 1 μmole of ATP, 0.1 μmole of the appropriate radioactive amino acid, and 2 additional units of the aminoacyl RNA synthetase were added. Aminoacyl RNA was then measured following a 30-min incubation as already described (Berg and Muench, 1966). In each case controls were run to assure that deacylation of the t-RNA was complete. Thus, if pyrophosphatase was not added to destroy the added PP_i, or if ATP was omitted from the final incubation, no aminoacyl RNA was detectable. In appropriate reconstruction experiments there was no significant decrease (<5%) in the acceptor activity of t-RNA as a result of the two incubations

Results

Chromatography of Aminoacyl RNA's Prepared with

FIGURE 2 (caption continued)
0.8 cm^2) and eluted at 32 ml/hr first with 0.10 M potassium phosphate buffer, pH 5.8, until fraction 23, then with a linear gradient between 1000-ml volumes of 0.10 and 0.20 M of the same buffer until fraction 100. Continuation of the chromatography with 0.30 M buffer resulted in elution of approximately 7% of the added A_{260} and less than 5% of the added isotopes. All buffers contained chloramphenicol, 10 μg/ml. Fractions were 24 ml. Recoveries were 93, 74, and 56% of the A_{260}, and acid-precipitable [^{3}H]leucine and [^{14}C]arginine, respectively. (D) t-RNA_{ala} (2.7 mg) from partition column fraction 292 loaded with [^{3}H]alanine was mixed with t-RNA_{ala} from partition column fraction 342 loaded with [^{14}C]alanine and applied to a column (100 cm × 0.8 cm^2) which was eluted at 32 ml/hr first with 0.15 M potassium phosphate buffer, pH 5.8, until fraction 25, and then with a linear gradient between 1000-ml volumes of 0.15 and 0.30 M of the same buffer. Buffers contained chloramphenicol, 10 μg/ml, and penicillin G, 1 unit/ml. Fractions were 24 ml. Recoveries were 88, 48, and 56% for the A_{260}, and acid-precipitable [^{14}C]alanine and [^{3}H]alanine, respectively.

Bulk, Unfractionated T-RNA. Chromatography of [^{14}C]leu-RNA (Figure 1A), or a mixture of [^{3}H]leu-RNA and [^{14}C]val-RNA (Figure 1B), or a mixture of [^{3}H]met-RNA and [^{14}C]ileu-RNA (Figure 1C) on hydroxylapatite columns yields a unique elution profile for each particular aminoacyl RNA. Leu-RNA appears as three well-resolved peaks (Figures 1A and 1B), and although they overlap the val-RNA profile (Figure 1B), the peaks and troughs of the two distributions are clearly distinguishable.

t-RNA$_{ileu}$ and t-RNA$_{met}$ appear in the same fractions from the partition column (Muench and Berg, 1966). However, the major components of each of these t-RNA species separate almost completely by chromatography on hydroxylapatite (Figure 1C). Moreover, the A_{260} elution pattern[4] indicates that significant purification of each of these t-RNA's might result from sequential fractionation by partition and hydroxylapatite column chromatography.

Recovery of t-RNA's from the Column. The recovery of acid-insoluble radioactivity from the column has varied from 76% for val-RNA (Figure 1B) to 28% in the case of met-RNA (Figure 1C). Losses result from hydrolysis of the ester linkage during chromatography rather than from destruction of t-RNA. This is supported first by the fact that the total t-RNA, as judged by the A_{260}, is recovered in 88–99% yield. More directly, when the [^{14}C]leu-RNA comprising the peaks in Figure 1 was precipitated, deacylated, and assayed for acceptor activity, more than 90% of the initial acceptor activity was accounted for. This implies that the chromatographic profiles of aminoacyl RNA and free t-RNA are very similar.

Resolution of Aminoacyl RNA's Prepared with Partially Purified t-RNA's Recovered from Partition Column Chromatography. It was of interest to determine the correspondence between each of the 5 t-RNA$_{leu}$ peaks found in partition chromatography and the 3 t-RNA$_{leu}$ peaks observed from the hydroxylapatite column. When [^{3}H]leu-RNA$_{leu,1}$ from the partition column was mixed with [^{14}C]leu-RNA$_{leu,2}$ and cochromatographed on hydroxylapatite (Figure 2A) the two species gave distinct elution profiles corresponding principally to the first and third peaks, respectively, seen with the unfractionated t-RNA (Figure 1A). Very little of t-RNA$_{leu,1}$ or t-RNA$_{leu,2}$ appears in the region occupied by the middle peak seen in Figure 1A. Some t-RNA$_{leu,1}$ contaminates t-RNA$_{leu,2}$ in partition fraction 52, as shown by the [^{14}C] label traveling with the [^{3}H] peak in Figure 2A.

Only approximate estimates of the purity of the 2 t-RNA$_{leu}$ chains can be made since they were not recovered and assayed for homogeneity. However, if we assume that pure t-RNA can bind about 36 mμmoles of amino acid per mg (Muench and Berg, 1966), we estimate from the A_{260} and radioactivity profiles for t-RNA$_{leu,1}$ (fractions 26–39) and t-RNA$_{leu,2}$ (fractions 72–81) that both components are at least 80% pure.[5]

When t-RNA$_{leu,3}$ and t-RNA$_{leu,5}$ are labeled with [^{3}H]- and [^{14}C]leucine, respectively, mixed, and chromatographed as already described for t-RNA$_{leu,1}$ and t-RNA$_{leu,2}$ the two species also separate; t-RNA$_{leu,3}$ appears in the position occupied by the third hydroxylapatite peak (which also contains t-RNA$_{leu,2}$) while t-RNA$_{leu,5}$ chromatographs at the position corresponding to the second hydroxylapatite peak (Figure 2B). From the A_{260} and radioactivity profiles we calculate that the procedure of mixture and chromatography of t-RNA$_{leu,3}$ and t-RNA$_{leu,5}$ leads to only slight improvement in their purities, yielding components at the peaks (fractions 58–66 and 78–86, respectively) which are each approximately 20% pure.[5]

t-RNA$_{leu,4}$ and t-RNA$_{arg}$ are eluted together on the leading shoulder of the main A_{260} peak from the partition column (Muench and Berg, 1966). Subsequent chromatography on hydroxylapatite separates them (Figure 2C). The t-RNA$_{leu,4}$ (fractions 52–58) and t-RNA$_{arg}$ (fractions 69–75) are estimated to be 54 and 39% pure, respectively.[5] t-RNA$_{leu,4}$ and t-RNA$_{leu,5}$ both chromatograph with the middle of the three t-RNA$_{leu}$ peaks eluted from hydroxylapatite.

Thus, considering the resolution of t-RNA$_{leu}$, the first hydroxylapatite peak contains t-RNA$_{leu,1}$, the second contains t-RNA$_{leu,4}$ and t-RNA$_{leu,5}$, whereas the third consists of t-RNA$_{leu,2}$ and t-RNA$_{leu,3}$. Studies of leucine-acceptor activity of each of the three hydroxylapatite peaks with the yeast leucyl-RNA synthetase support this conclusion; *e.g.*, the second hydroxylapatite peak has the same acceptor activity with both the yeast and *E. coli* leu-RNA synthetases; the same was true for the partition column fractions containing t-RNA$_{leu,4}$ and t-RNA$_{leu,5}$. The third hydroxylapatite peak, which contains t-RNA$_{leu,2}$ and t-RNA$_{leu,3}$, is only 60% as active as acceptor with the yeast enzyme as with the *E. coli* enzyme, since only the t-RNA$_{leu,3}$ serves as acceptor with the yeast enzyme.

When unfractionated t-RNA, esterified with labeled alanine, was chromatographed as already described, 3 peaks of acid-precipitable radioactivity were obtained. Also, when partition column fractions 292 and 342 (see Figure 5D of the previous paper (Muench and Berg, 1966)), were esterified with [^{3}H]- and [^{14}C]alanine, respectively, and cochromatographed on hydroxylapatite, 3 peaks of labeled ala-RNA were recovered (Figure 2D). The first 2 peaks are derived from partition column fraction 292 and the third peak from partition column fraction 342. We have consistently ob-

[4] The first 2 peaks of material with A_{260} in Figures 1A, 1C, 2A, 2B, and 2C are AMP and ATP which contaminate the aminoacyl RNA. They do not appear when free t-RNA or aminoacyl RNA isolated by Sephadex gel filtration is chromatographed.

[5] The purity of t-RNA's which had been fractionated sequentially on the partition and hydroxylapatite columns was estimated as follows: the specific acceptor activity, micromoles per milligrams of RNA, was determined from the specific activity of the labeled amino acid and the level of radioactivity and A_{260} in the fractions indicated. This value was corrected for the loss of esterified amino acid during the preparation and chromatography of the labeled aminoacyl RNA.

served 3 t-RNA$_{ala}$ peaks, although the resolution between the first 2 has varied in different experiments. Although the A_{260} and radioactivity profiles of the third ala-RNA peak do not coincide, the specific activity values over most of the peak agree within a factor of 2. We estimate that the t-RNA$_{ala}$ in the middle of this peak (fractions 48–57, Figure 2D) is more than 90% pure.[5]

Discussion

Hydroxylapatite chromatography appears to be a promising method for resolving different t-RNA's. It has the virtue of being applicable to aminoacyl RNA's, thereby reducing the work needed to locate particular t-RNA's, and in addition has high resolving power and yields sharp peaks. Because this resolution of t-RNA's depends upon properties other than those which determine partition coefficients, this method is useful as an adjunct to countercurrent distribution or partition column chromatography.

For example, t-RNA$_{leu,4}$ and t-RNA$_{arg}$ are eluted from the partition column together but are readily resolved from each other on hydroxylapatite. Similarly, the major t-RNA$_{ala}$ component, which is obtained in about 40% purity from the partition column (Muench and Berg, 1966), is considerably further enriched by the hydroxylapatite chromatography. Further studies are needed to delineate variables such as temperature, pH, shape of the phosphate gradients, and presence of agents such as urea (Cherayil and Bock, 1965) which may be exploited to maximize the resolution of various t-RNA's.

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