Separation of Transfer Ribonucleic Acids on Polystyrene Anion Exchangers†

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ABSTRACT: The transfer RNA separation by chromatography on strong-base-polystyrene exchange materials is examined and compared with the widely used reversed-phase chromatography. Results indicate important differences in some transfer RNA (tRNA) elution patterns by the anion-exchange chromatography, as compared with the reversed-phase chromatography. Transfer RNAs containing hydrophobic groups are adsorbed more strongly. The anion exchanger has twice the number of theoretical plates. Single peaks of $tRNA_2^{Glu}$ and $tRNA_1^{Phe}$ obtained from the reversed-phase column give multiple peaks on polystyrene anion-exchange chromatography. All six leucine tRNAs (*Escherichia coli*) and differences in tRNA populations synthesized during early and late stages of the dividing lymphocytes from normal human blood can be characterized by the anion-exchange chromatography. Different separation profiles are obtained by two separation systems for tyrosine tRNAs from mouse liver and mouse-plasma-cell tumor. The results indicate that, in contrast to the reversed-phase chromatography, strong-base-polystyrene anion-exchange chromatography is capable of separating tRNAs with minor structural differences.

About 10 years ago, reversed-phase columns were introduced for the separation of tRNAs (Kelmers et al., 1965). These columns contain small beads of an inert material coated with basic quaternary aliphatic ammonium derivatives and involve both ionic and hydrophobic interactions between the solutes and the coating material. Reversed-phase chromatography has also been used to separate oligonucleotides and monomers (Egan, 1973; Singhal, 1973; Roe et al., 1973).

Altered elution profiles have been reported for several aminoacyl-tRNAs[1] from neoplastic cells (Yang and Novelli, 1968; Yang et al., 1969; Borek, 1971; Borek and Kerr, 1972; Briscoe et al., 1972, 1975a,b). Isoacceptor tRNA species typical of cancer cells have yet to be isolated and characterized for differences in their chemical structures. Today, better separation methods are required for the isolation of such isoacceptor molecules present in minute quantities. The results of a recent study indicate that the smaller nucleic acid components separate on reversed-phase columns in a manner essentially identical with that observed in the usual anion-exchange chromatography on a polystyrene matrix. The difference between reversed-phase chromatography and the conventional anion-exchange chromatography on polystyrene resins lies in the differences in the composition and physical character of the inert support holding the anion-exchange groups, which are quaternary ammonium derivatives in both cases (Singhal, 1974a). The polystyrene exchanger contains covalent functional groups of the type $[RN(CH_3)_3]^+$, where R represents cross-linked *porous* vinylbenzene lattice. But in the reversed-phase matrix, the quaternary ammonium derivative [such as methyltrialkyl (C_8–C_{10}) ammonium chloride] is immobilized on a *solid* inert support [for example, polytrifluorochloroethylene (Kel F) and polyfluoroethylene (Teflon)]. Since the aryl residues of the polystyrene are more hydrophobic (organophilic) than the simple alkyl chains of the reversed-phase column, and because of uniform, small bead size of the former material, improved resolutions were noted for oligonucleotides and monomers on the conventional anion exchanger (Singhal, 1974b).

We have now extended the use of such polystyrene anion-exchange material to the separation of tRNAs and have obtained evidence that it is superior in several aspects to the widely used reversed-phase systems.

Experimental Section

The anion-exchanger, Aminex A-28 (7–11 μm beads), was purchased from Bio-Rad Laboratories, Richmond, Calif. Reversed-phase column material (RPC-5), consisting of beads of polychlorotrifluoroethylene coated with a quaternary ammonium derivative [methyltrialkyl (C_8–C_{10}) ammonium chloride] was prepared by the standard procedure (Kelmers et al., 1973). Transfer RNA_2^{Glu} of *Escherichia coli* was purified as described elsewhere (Singhal, 1974c).

The ^{32}P-labeled tRNAs were isolated from *E. coli* $_{\chi}402$ (Dijk and Singhal, 1974). Transfer RNAs from mouse liver and mouse plasma tumor cells (MOPC-31c tumor) were prepared as described by Yang and Novelli (1968). Lymphocytes from human peripheral blood were isolated by the technique of Rabinowitz (1964). They were mixed with Eagle's minimal essential medium, supplemented with 10% fetal calf serum, 1 mM glutamine, penicillin (100 units per ml), and streptomycin (50 μg per ml), and cultured in a 37 °C, CO_2 incubator. The mitogen phytohemagglutinin (50 μg per ml) was added to this mixture. At appropriate intervals, either [5-^{3}H]uridine or [2-^{14}C]uridine was added (50 or 0.5 μCi per ml, respectively). Thus, labeled tRNAs were isolated by phenol extraction and DEAE-cellulose chromatography.

The aminoacylation of tRNAs was done by the method of

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[1] Abbreviations used: tRNA, transfer ribonucleic acid; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; HETP, height equivalent of a theoretical plate; BD-cellulose, benzoylated diethylaminoethylcellulose.

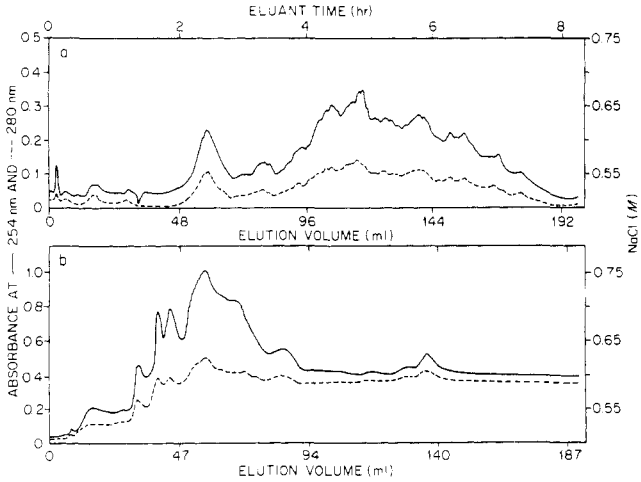

FIGURE 1: Chromatography of *E. coli* tRNAs on (a) Aminex A-28, 0.6 × 27 cm column and on (b) reversed-phase-5, 0.6 × 25 cm column. In 200 μl, 36 A_{260} units of tRNAs was applied to each column. Both were eluted with a linear gradient of 0.5 to 0.75 M NaCl, 100 ml of each solution, at 22 °C and 0.4 ml per min. The buffer used was 10 mM NaOAc, 10 mM $MgCl_2$, 1 mM EDTA, and 1 mM $Na_2S_2O_7$, pH 4.5. The resultant pressures were 245 (a) and 105 (b) psi.

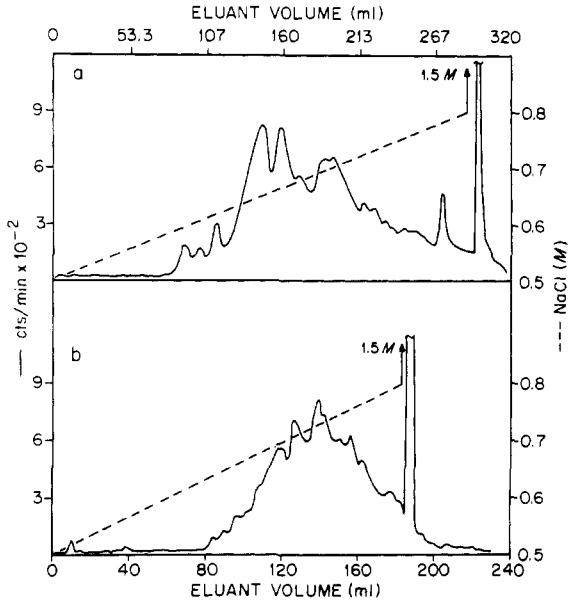

FIGURE 2: Effect of temperature on tRNA separations by anion-exchange chromatography. [^{32}P]tRNAs (*E. coli*) with carrier tRNA's were chromatographed on an Aminex A-28, 0.6 × 27 cm column at 25 (a) and 50 °C (b). The column was eluted with a linear gradient of 0.5 to 0.8 M NaCl using the same buffer as described in Figure 1, 150 ml each, pH 4.5 at 0.4 ml per min. Fractions of 2.7 ml were collected, and the acid-insoluble fraction was counted.

Rubin et al. (1967). After column chromatography, each eluent fraction was mixed with the carrier calf thymus DNA and both nucleic acids were precipitated with acid (6% HCl and 10% acetic acid). The precipitate was retained on glass-fiber filters (GF/C, 5-μm pore size, Whatman) and washed successively with 10% CCl_3COOH, ethanol, and ether. Dried filter papers were counted for radioactivity after adding a scintillation fluid.

The solutions for column elution were prepared in 10 mM sodium acetate (pH 4.5) with 10 mM $MgCl_2$, 1 mM EDTA, and 1 mM $Na_2S_2O_7$. One A_{260} unit is the amount of material in 1 ml of solution that gives an absorbance of 1.0 when measured at 260 nm with a path length of 10 mm.

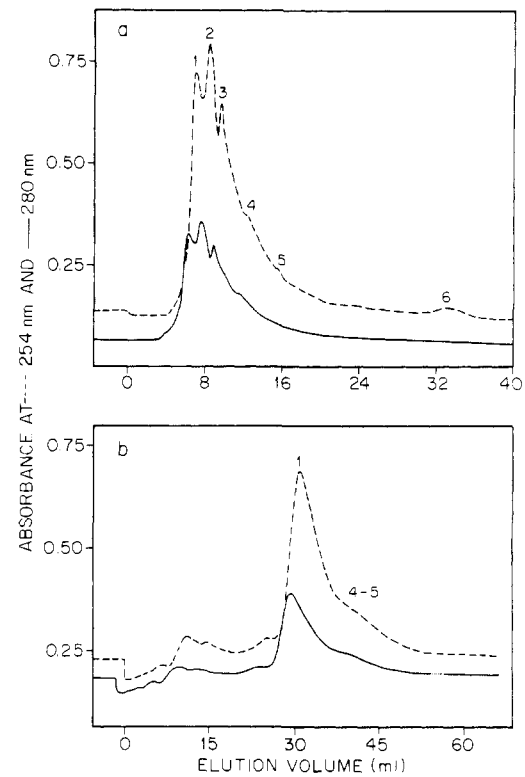

FIGURE 3: Comparison of the separation efficiency (HETP values) of (a) Aminex A-28 and (b) reversed-phase-5 columns. The Aminex column was eluted with 0.57 M NaCl, while the reversed-phase column was eluted with 0.51 M NaCl, using the same buffer as in Figure 1.

Results

The separations of mixed tRNAs (*E. coli*) by anion-exchange chromatography and reversed-phase chromatography were compared under identical conditions. They were desorbed by a linear gradient of chloride ion, from 0.5 to 0.75 molar, at 22 °C. The elution profiles, monitored at two wavelengths, indicate that, while tRNAs from the reversed-phase column elute mostly as one large ultraviolet absorbing area (mostly at the front of the gradient and some peaks in the middle of the gradient, see Figure 1b), this material on the anion-exchange column undergoes fractionation into numerous unresolved peaks throughout the salt gradient (Figure 1a). They are eluted with higher Cl^- concentration. Some tRNAs are so strongly adsorbed to the polystyrene column that their elution requires organic solvents in addition to chloride ions (such as, 10% ethanol in M NaCl). Thus, tRNAs are adsorbed more strongly by the polystyrene exchanger than by the reversed-phase matrix. The spread of tRNAs throughout the elution indicates a higher resolution power of the polystyrene anion exchanger.

The influence of temperature on the ion-exchange chromatography of tRNAs is shown in Figure 2, where separations of uniformly labeled [^{32}P]tRNAs (*E. coli*) on an Aminex (polystyrene) column are compared at 25 °C (a) with those at 50 °C (b). Though eluted under identical conditions, the resolution is enhanced at the lower temperature; that is, tRNAs are sorbed to the polystyrene more strongly at 25 than at 50 °C. (On reversed-phase columns better resolutions at 37 °C as compared with 25 °C have been reported by Kelmers et al. (1971).) Interestingly, certain tRNA species can be desorbed from the polystyrene column only by strong eluents, in this case by 1.5 M NaCl with 5% ethanol. Such tRNAs may contain

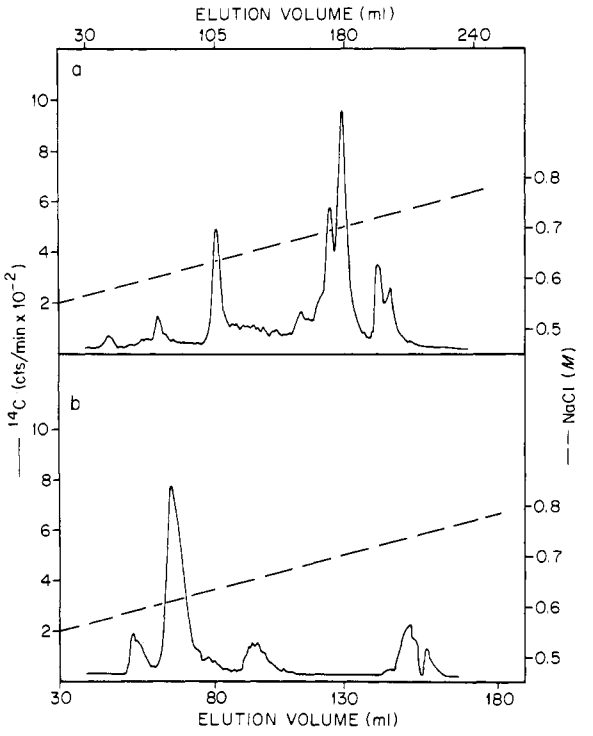

FIGURE 4: Separation of [^{14}C]leucyl-tRNAs (*E. coli*). (a) Aminex A-28, 0.6 × 32 cm column, eluted with a linear gradient of 0.55 to 0.80 M NaCl, 150 ml each at 0.5 ml per min, 200 psi, 35 °C. (b) reversed-phase, 0.6 × 30 cm column, eluted with a linear gradient of 0.5 to 0.8 M NaCl, 100 ml each at 0.5 ml per min, 100 psi, 37 °C. Two A_{260} units of [^{14}C]leucyl-tRNAs were applied to each column (for buffer composition, see legend to Figure 1).

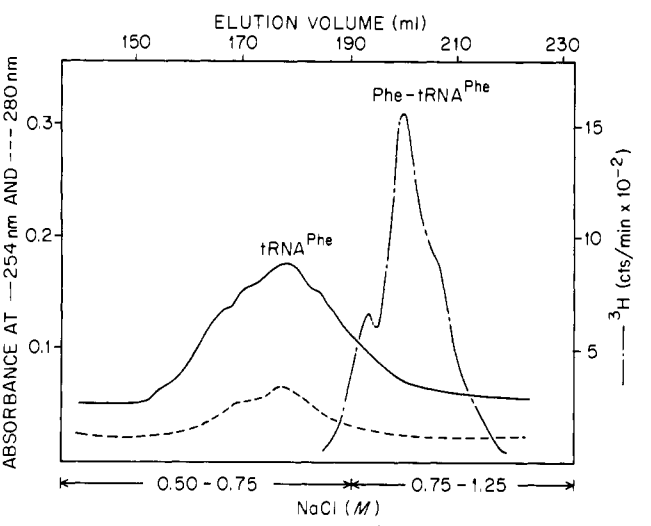

FIGURE 5: Cochromatography of tRNA$_1^{Phe}$ and Phe-tRNA$_1^{Phe}$ on an Aminex A-28, 0.6 × 27 cm column. A mixture of 3.6 A_{260} units of tRNA$_1^{Phe}$ in 80 μl and 25 000 cpm of [^{3}H]Phe-tRNA$_1^{Phe}$ (0.2 A_{260} unit) in 200 μl was applied. The column was first eluted with a linear gradient of 0.5 to 0.75 M NaCl, 100 ml each, and then with a second gradient of 0.75 to 1.25 M (plus 10% ethanol) NaCl, 50 ml each, at 0.4 ml per min, 250 psi and at 21 °C. The recovery of Phe-tRNA was 92%. (For the buffer composition, see legend to Figure 1.)

strong hydrophobic groups such as isopentenyladenine or wye (formerly "Y") base in their exposed structures.

The anion exchanger and the reversed-phase matrices were examined for separation efficiency by using a purified tRNA preparation (Figure 3). Glutamate tRNA was isolated with about 95% purity by extensive chromatography as described elsewhere (Singhal, 1974c). About 4.7 A_{260} units of this pure tRNA^{Glu} was chromatographed on above two columns of identical dimensions (0.6 × 16.5 cm) and under identical conditions (0.53 ml per min, at 30 °C). To determine the height equivalent of a theoretical plate (HETP), each column was eluted with a constant ionic strength. However, the tRNA from the two columns could not be desorbed by a common ionic strength. The separation on the reversed-phase column indicates largely one tRNA peak. But, the same material when chromatographed on the Aminex column (Figure 3a) gave at least three tRNA^{Glu} peaks.

The plate height (HETP) of the tRNA for the Aminex column was 0.46 mm, but for the reversed-phase column it was about double this value. While 98% of the applied material in this experiment was recovered from the Aminex column, only 88% could be accounted for from the reversed-phase column.

The separation of leucyl-tRNA species on the two columns was also compared. Five [^{14}C]leucyl-tRNA peaks are generally obtained by reversed-phase chromatography under the best separation conditions (Figure 4b). The anion-exchange column, not only resolves six species, but also shows heterogeneity in two of the major ones (Figure 4a). It may be noted that the elution pattern of these isoacceptors is different on the two columns.

The polystyrene anion-exchange column was examined for the separation between "free" tRNA and the aminoacyl-tRNA. A pure sample of tRNA$_1^{Phe}$ was obtained from the reversed-phase column as the first of two peaks (Weeren et al., 1972). When tRNA$_1^{Phe}$ was cochromatographed with its phenylalanyl derivative (Phe-tRNA$_1^{Phe}$), the latter could not be eluted from the ion-exchange column by the usual salt gradient (Figure 5). A second salt gradient, 0.75 to 1.25 M NaCl, was necessary for the desorption of Phe-tRNA. The presence of phenyl group at the 3′ end of the tRNA thus appears to cause a strong interaction with the ion exchanger. The tRNA$_1^{Phe}$ appears to be heterogeneous on the anion-exchange column.

Differences between tRNAs from Early and Late Stages of the Proliferating Lymphocyte. Lymphocytes from peripheral human blood were isolated (see Experimental Section). The transformation of resting lymphocytes into actively dividing lymphocytes was achieved by adding phytohemoagglutinin. Transfer RNAs were labeled with either ^{3}H- or ^{14}C-labeled uridine in vivo. Thus, lymphocytes were incubated with the mitogen and with either [^{3}H]uridine from 10 to 18 h after stimulation or with [^{14}C]uridine from 40 to 48 h after stimulation. Tritium and ^{14}C-label thus represented early and late populations of tRNAs in the cellular transformation. To determine differences, both tRNAs were cochromatographed. The reversed-phase column indicated no differences (Figure 6b), but several peaks appeared when the two tRNA populations chromatographed on the polystyrene column (Figure 6a).

Differences in Tyrosine Isoacceptor tRNAs from Normal and Tumor Cells. Transfer RNAs from mouse liver or from mouse-plasma-cell tumor (MOPC-31C) were aminoacylated with ^{14}C- or ^{3}H-labeled tyrosine, respectively. Two tRNA preparations were chromatographed (for details, see legends to Figure 7). Separations on the two columns showed very different profiles. Though a broad salt gradient (0.4 to 0.8 M NaCl) was used for the polystyrene column, peaks were clearly resolved and quantitative differences among all three peaks were clear (Figure 7a). On the other hand, the reversed-phase column, eluted with a narrow salt gradient (0.45 to 0.55 M

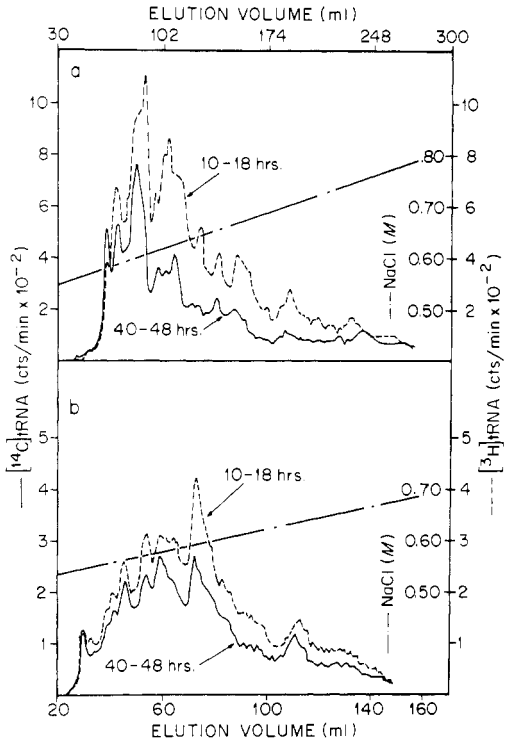

FIGURE 6: Cochromatography of tRNAs synthesized in early ^{3}H and late ^{14}C stages of the proliferating human lymphocytes. (a) Aminex A-28, 0.9 × 32 cm column, eluted with a linear gradient of 0.5 to 0.8 M NaCl, 200 ml each, at 0.3 ml per min, and at 30 °C. (b) Reversed-phase column, 0.6 × 30 cm, eluted with a gradient of 0.5 to 0.75 M NaCl, 100 ml each, at 0.4 ml per min, 150 psi, and at 37 °C. (For the buffer composition, see legend to Figure 1.)

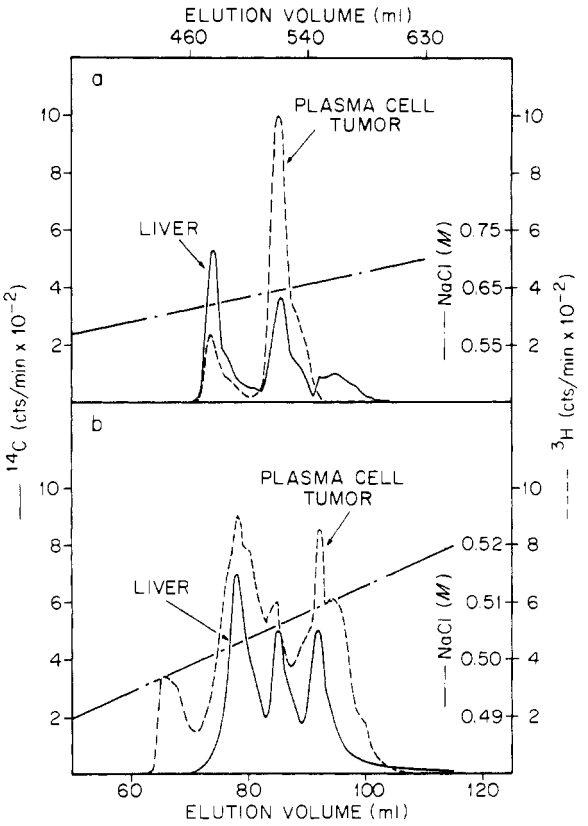

FIGURE 7: Cochromatography of mouse liver [^{14}C]- and mouse-plasma-cell tumor [^{3}H]Tyr-tRNAs. (a) Aminex A-28, 0.9 × 32 cm column eluted with a linear gradient of 0.4 to 0.8 M NaCl, 500 ml each at 35 °C. (b) Reversed-phase column, 0.6 × 30 cm, eluted with a gradient of 0.45 to 0.55 M NaCl, 100 ml each at 25 °C. (For the buffer composition, see legend to Figure 1.) (a) [^{3}H]Tyr-tRNA (4580 cpm; 1.15 pmol) and 5700 cpm (58 pmol) of [^{14}C]Tyr-tRNA were applied. And, 96% of ^{3}H and 92% of ^{14}C radioactivity were recovered from this column. (b) [^{3}H]Tyr-tRNA (27 660 cpm; 6.9 pmol) and 8100 cpm (82 pmol) of [^{14}C]Tyr-tRNA were applied. The radioactivity recoveries for ^{3}H and ^{14}C labels were 82 and 91%, respectively, for this reversed-phase column.

NaCl), showed a very different profile for the same material (Figure 7b). Thus, the last peak of the second column probably represents tyrosine tRNA species, uniquely present in normal liver and plasma-cell tumor, respectively.

Discussion

The separation of tRNAs on the anion-exchange column is superior to the reversed-phase chromatography in many ways. The chromatography of mixed tRNAs on the former indicates that different tRNAs tend to spread throughout the salt gradient. A quantitative measure of the separation efficiency (Singhal and Cohn, 1972), the actual plate heights (HETP), indicates that the Aminex anion exchanger contains twice as many theoretical plates as does a reversed-phase-5 column of the same geometry.

Some tRNA purified as single components (isoacceptors) from the reversed-phase column show heterogeneity on the anion-exchange column. Three peaks are apparent in both $tRNA_2^{Glu}$ and $tRNA_1^{Phe}$ (*E. coli*) isolated as single peaks on the reversed-phase column. Similarly, an improved resolution of six leucine tRNAs on the anion exchanger is observed. Two major leucine tRNAs show heterogeneity. The heterogeneity of such tRNA peaks perhaps does not indicate additional isoacceptors, but rather a mixture of completely and partially modified (mature and immature) tRNAs. Or, they may reflect the different stages of the growth cycle at which cells were harvested. Earlier work has shown the composition of a highly purified tRNA preparation does not agree with the primary structure (Singhal and Best, 1973); that is, a "100% pure" tRNA is not absolute with regard to its assigned structure. Another study indicates that, though the tRNA modification begins at the precursor level, it remains incomplete shortly after the maturation process (Dijk and Singhal, 1974). The heterogeneity in the tRNA peak discussed above may thus indicate incomplete modification of one or more minor constituents, and the anion-exchange chromatography is perhaps able to detect such minute differences.

A small fraction of mixed tRNAs is strongly adsorbed to the anion exchanger requiring dilute ethanol for its elution. The fraction may represent tRNAs of high hydrophobicity. Moreover, the "charging" of $tRNA_1^{Phe}$ with phenylalanine appreciably increases its adsorption to the exchanger. The anion-exchange method, like BD-cellulose chromatography (Gillam et al., 1967), may provide a procedure for the selective separation of tRNAs containing "extra" aromatic groups from tRNAs containing little or no such groups. Reversed-phase chromatography also shows a similar, but less marked, effect of requiring a higher salt concentration to elute the aminoacyl-tRNA than that required for the "uncharged" tRNA. This is particularly true for $tRNA^{Trp}$ (Waters et al., 1975).

The results indicate that, in proliferating human lymphocytes, tRNA populations synthesized early (10–18 h) may have minor structural variations and, thus, differ chromatographically from those synthesized late (40–48 h). It would be interesting to test if the early synthesized tRNAs can be aminoacylated and to examine if new tRNAs and additional modifications may occur in the late-synthesized tRNAs.

Presently, such studies are in progress. The cochromatography of tyrosyl-tRNAs from mouse liver and mouse-plasma-cell tumor indicates differences both in the number of peaks and in the quantity of material under each peak (see legend to Figure 7). It would be interesting to examine the structure of tRNAs of tumor origin.

The anion exchanger, Aminex A-28 (7–11 μm), was used in this study. The separation on Aminex A-25 (17 μm) columns is equally satisfactory. Both exchangers are available commercially (Bio-Rad Laboratories, Richmond, Calif.). Probably any other brand of polystyrene anion exchanger of small and uniform bead size (about 10 μm) can be used for such work (see Singhal and Cohn, 1973; Singhal, 1974b). These resins can tolerate extremes of temperatures, pressure, pH, and organic solvents. But, the reversed-phase column cannot be operated at low temperatures and high pH, and with organic solvents. (Desorption of alkylamine from the inert support occurs under such adverse conditions.) The recovery of tRNAs from the Aminex column is equal or perhaps better from both ultraviolet absorption and radioactivity view points (98% vs. 88% for the reversed-phase column). Unfractionated [^{32}P]tRNAs, [^{14}C]Phe-tRNA^{Phe}, and tRNA$_2^{Glu}$ from *E. coli* were chromatographed for quantitating the tRNA recoveries from the two matrices. About 5 A_{260} units of tRNAs can be applied to each milliliter of the Aminex resin bed, whereas loads of 32 A_{260} units per ml of the reversed-phase column bed have been applied by Kelmers et al. (1971).

Since the preliminary report (Singhal et al., 1975), several workers have followed this procedure. Roe recently applied it to the purification of tRNAs from human liver and placenta (Roe, 1975; Anandaraj and Roe, 1975). It appears that two matrices (Aminex A-25, A-27, or A-28 and the reversed phase 5) can be used as complementary methods for the isolation and resolution of tRNAs.

The anion exchanger, Aminex A-28, had been unavailable from the Bio-Rad Laboratories (Richmond, Calif.) for 6–8 months. Therefore, we examined two other bead sizes of this resin, Aminex A-25 and A-27. We find any one of the two bead sizes can be replaced for the Aminex A-28 without loss of resolution. In fact, now we prefer the use of Aminex A-25 over A-28 since it causes less back pressure. The new production lot of the Aminex A-28, which is prepared by slightly different chemistry, appears to have resolution power the same as those of the earlier two lots. Several workers have experienced that the results cannot be reproduced on these resins at times. We find that the resin is extremely sensitive for alkaline pH's. A partial degradation of the resin at alkaline pH appears to be the cause of this problem. This quaternary amine divinylbenzene polystyrene resin which is about 10-μm bead size is also available from other sources (for example, DA-X8 from Durrum, Palo Alto, Calif., and HA-X8 from Hamilton Co., Reno, Nev.).

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