

Different Arginine Transfer Ribonucleic Acid Species Prevalent in Shaken and Unshaken Cultures of *Neurospora*

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When the arginyl-transfer ribonucleic acid (tRNA) species isolated from unshaken and from shaken cultures of *Neurospora* were compared by co-chromatography, a marked change in the relative abundance of the two main tRNA^{arg} species was found. The two arginine tRNA species had different codon responses in ribosome binding assays. The tRNA^{arg} eluting first (prevalent in shaken cultures) bound strongly to polyadenylic-guanlyic acid [poly(A, G)] and to a lesser extent to polycytidylic-guanlyic-adenylic acid [poly(C, G, A)]. The second tRNA^{arg} species (prevalent in unshaken cultures) bound to poly(C, G, A) but not to poly(A, G). The possible significance of these observations is briefly discussed. Several modifications that improve the yield of tRNA from *Neurospora* were introduced in a standard isolation procedure.

The occurrence of multiple, chemically resolvable transfer ribonucleic acids (tRNAs) that recognize different codons for the same amino acid (isoaccepting tRNAs) has inspired several theories of cellular control at the translation level. The different models have recently been reviewed by Sueoka and Kano-Sueoka (24). In essence, the ability of a cell to translate a messenger RNA (mRNA) that utilizes a limited number of synonym codons could depend upon the availability of the isoaccepting tRNA(s) required.

There are many reports of changes in the relative or absolute amounts, or the functional competence of isoaccepting tRNAs associated with changes in growth conditions, viral infection, embryonic development, tissue differentiation, and neoplasia (24).

In the course of studies related to the possible involvement of arginyl-tRNA(s) in the regulation of the arginine biosynthetic sequence (16), a marked difference was found in the relative amounts of the two main arginyl-tRNA species present in tRNA isolated from unshaken and from shaken cultures. Since the two arginyl-tRNAs recognize different codewords, the observed changes could be involved in the control of the translation of different sets of mRNAs.

MATERIALS AND METHODS

Strains. The strains of *Neurospora crassa* used were the wild-type 74A and the *arg-10* mutant B-317. Both strains were kindly provided by the Fungal Genetics Stock Center, Humboldt College, Arcata, Calif. (FGSC 262 and 122, respectively).

Growth conditions. Medium N of Vogel (25), supplemented as indicated, was used in all experiments.

Carboy cultures were grown in 5-gal (19-liter) carboys containing 10 liters of medium. An inoculum of approximately 5×10^9 conidia was used, and the cultures were incubated at room temperature (23 ± 2 C) for 36 to 42 hr, under vigorous aeration.

Exponential cultures were grown by the procedure of Shearn and Horowitz (20) with slight modifications. The inocula were adjusted so as to give 5×10^6 to 1×10^7 conidia per ml, and the flasks were incubated for 15 hr in a gyratory shaker at approximately 200 excursions per min at 28 C.

Unshaken cultures were grown as described previously (15).

All cultures were harvested by filtration on Büchner funnels and were immediately lyophilized as previously described (15, 17). The conidial suspensions used to inoculate all cultures were 1- to 3-week-old cultures grown and harvested as described elsewhere (20).

Chemicals. ¹⁴C (U) arginine (specific activity, 306 mCi/mmole) and ¹⁴C-guanido-arginine (specific activity, 25 to 31 mCi/mmole) were purchased from

Amersham/Searle. ^3H (G) arginine (specific activity, 703 or 741 mCi/mmmole) was a product of New England Nuclear Corp., Boston, Mass., as were all other ^3H -labeled amino acids. Freon 214 was a generous gift from E. I. DuPont de Nemours and Co., Wilmington, Del. Aliquat 336 was obtained through the courtesy of the Chemical Division of General Mills, Kankakee, Ill. Macaloid was a gift from the Baroid Division, National Lead Co., Houston, Tex. Enzyme-grade ammonium sulfate and benzoylated diethylaminoethyl (DEAE)-cellulose (50 to 100 mesh) were purchased from Schwarz BioResearch, Inc., Orangeburg, N.Y.

The mid-log *E. coli* W cells used for the isolation of ribosomes were obtained from General Biochemicals Corp., Chagrin Falls, Ohio. Polyadenylic-guanilyc acid [poly(A, G), 1.0:1.0] was from General Biochemicals Corp. Polycytidylic-guanilyc-adenylic acid [poly(C, G, A), 1.0:1.2:1.17] was purchased from Miles Laboratories, Elkhart, Ind.

Neurospora tRNA. Unless stated otherwise, all steps in the isolation procedure were carried out at 0 to 4 C, and centrifugations were for 20 min at $8,000 \times g$. A 50-g amount of lyophilized powdered mycelium was suspended in 1,200 ml of 0.03 M tris(hydroxymethyl)aminomethane (Tris)-acetate buffer (pH 8.0) containing 10 mM 2-mercaptoethanol, 0.1 mM Na_2 ethylenediaminetetraacetic acid (EDTA), and 0.15% washed Macaloid (22). After 5 min of vigorous stirring, 950 ml of water-saturated phenol (containing 0.1 mM Na_2 EDTA) was added, and stirring was continued for 60 to 90 min. After centrifugation at $10,000 \times g$ for 30 min, the aqueous layer was aspirated, by use of a 50-ml syringe, down to approximately 8 mm from the interphase. The nucleic acids were precipitated from the aqueous phase by the addition of 0.1 volume of 2 M potassium acetate (pH 5.0) followed by 2.5 volumes of 95% ethanol. After 15 hr at -20 C, most of the clear supernatant fluid was aspirated off and discarded; the remaining suspension was centrifuged and the pellets were washed with 70% ethanol. The pellets were dissolved in 0.2 M glycine-NaOH (pH 9.1) to a total volume of 125 ml. This solution was incubated at 37 C for 60 min, and the nucleic acids were precipitated with 0.2 volume of 2 M potassium acetate (pH 5.0) and 2.5 volumes of 95% ethanol. After centrifugation and washing, the pellets were suspended in 100 ml of 1.1 M NaCl, stirred for 1 hr, and centrifuged. The extraction was repeated with 50 ml of 1.1 M NaCl. The combined supernatant solutions were mixed with ethanol, centrifuged, and washed as above; the pellets were dissolved with 0.3 M sodium acetate (pH 7.0) to a total volume of 150 ml. Isopropanol fractionation was performed at 20 C as described by Zubay (30) except that the amount of isopropanol added was reduced to 0.41 volume (62 ml). The pellets were dissolved in 0.3 M sodium acetate (pH 7.0) to a total volume of 80 ml, and the isopropanol precipitation (0.41 volume, 32 ml) was repeated under the same conditions. From the combined supernatant fluids, kept in an ice-water bath, the precipitation of tRNA was completed by the addition of 0.50 volume of isopropanol. The precipitate was collected

by centrifugation, dissolved in a small volume of water, frozen, and lyophilized. The powder was dissolved with water to a concentration of 50 mg per ml, and the solution was stored at -20 C. The yield was 7 to 10 mg of powder per g of mycelium. The isolation scheme described above was derived from the procedure used by Zubay (30) for the isolation of tRNA from *Escherichia coli*. Two modifications should be pointed out. (i) The NaCl extraction was performed after the incubation at pH 9.1. Inversion of the order of these two steps consistently improved the yield of tRNA two- to threefold. (ii) A lower concentration of isopropanol was used to precipitate deoxyribonucleic acid and other impurities. This modification resulted in a threefold increase in yield, yet the final product was diphenylamine-negative (*see below*).

Different tRNA preparations had A_{260}/A_{280} ratios of 1.96 to 2.05. Assuming an $E_{1\text{cm}}^{1\%}$ value of 200 (in 0.01 N KOH at 260 nm), the nucleic acid content of different preparations ranged from 50 to 91%. Protein content, measured by the method of Lowry et al. (13), was less than 1%. DNA, determined by the diphenylamine reaction of Burton (3), was less than 1%. The major contaminant was an anthrone-positive polysaccharide (5) that was eliminated by chromatography on benzoylated DEAE-cellulose or Freon columns.

Chromatography of tRNAs. The reversed phase chromatographic technique of Weiss and Kelmers (27) was used with minor modifications. Jacketed glass columns of 1.2 by 90 cm maintained at 27 C were used. The solutions employed for equilibration and elution of the column contained 10 mM sodium acetate (pH 4.6) and 10 mM MgCl_2 (buffer A) plus NaCl as indicated (*see below*). Fractions of 9 ml were collected at 12-min intervals. For chromatography of tRNA^{arg} (postchromatography charging), 40 to 70 mg of tRNA was applied to a column that had been equilibrated with buffer A containing 0.17 M NaCl. After washing with approximately 200 ml of equilibration buffer, a linear gradient (0.17 to 0.25 M NaCl, 2 liters) was started. The eluent was monitored for absorbancy changes at 260 nm, and suitable samples were assayed for amino acid acceptor capacity (*see below*).

For chromatography of arginyl-tRNAs, 0.2 to 1.0 mg of tRNA was esterified with labeled amino acid by incubation under the conditions described for determination of amino acid acceptor capacity (*see below*) except that all other components in the reaction mixture were scaled up twofold to a final incubation volume of 1.0 ml. The reaction was stopped by the addition of 0.2 ml of 1 M sodium acetate (pH 4.6) and 1 ml of water-saturated phenol (redistilled and containing 0.1 mM Na_2 EDTA) followed by 1 min of vigorous mixing; 1 mg of yeast tRNA was added as carrier, the suspension was mixed again, and the aqueous phase was separated after centrifugation. The phenol layer was re-extracted with 1 ml of buffer A, and the combined aqueous layers were mixed with 2.5 volumes of 95% ethanol. After 2 hr at -20 C, the precipitate was collected by centrifugation; it was washed twice with 4 ml of 70% ethanol

saturated with NaCl and once with 4 ml of 70% ethanol, and was then dissolved with 0.5 ml of buffer A. Not less than 50,000 counts/min of each ^{14}C - plus ^3H -labeled arginyl-tRNA were mixed and applied to a Chromosorb W-Freon column equilibrated with buffer A made 0.25 M in NaCl. The column was eluted with a 2-liter linear gradient (0.25 to 0.50 M NaCl). To each fraction, 1 mg of yeast tRNA was added; after mixing, 1 ml of 100% trichloroacetic acid made 0.1 M in lanthanum nitrate was added, and the sample was mixed again. After the mixture had stood for about 15 min in an ice-water bath, the precipitate was collected on glass-fiber filters (Whatman G F/C) and washed with 4 ml of cold 5% trichloroacetic acid. The filters were placed in scintillation vials and dried in an oven at 80 C; after addition of 10 ml of toluene-based scintillation fluid, the vials were counted in a multichannel spectrometer set so as to eliminate the ^3H pulses from the ^{14}C channel. It was found that 15% of the ^{14}C pulses were counted in the ^3H channel, and the results were corrected accordingly. The aminoacyl-tRNA radioactivity applied to the column was almost quantitatively recovered. The recovery of the eluted radioactivity on the filters was always greater than 90%. Chromatography on benzoylated DEAE-cellulose was performed according to Gillam et al. (8). A column (2 by 100 cm) equilibrated with buffer A made 0.45 M in NaCl was loaded with approximately 500 mg of tRNA and washed with one bed volume of equilibration buffer; a 6-liter linear gradient (0.45 to 0.85 M NaCl) was then applied.

Arginyl-tRNA ligase. Exponential cultures of 74A, reportedly free from nucleases (20), were used. A 20-g amount of lyophilized powdered mycelium was suspended in 400 ml of 0.03 M Tris-acetate buffer (pH 8.0) containing 10 mM 2-mercaptoethanol and 0.1 mM Na_2EDTA . The suspension was stirred for 30 min and then centrifuged for 45 min at $16,000 \times g$. To the extract, powdered ammonium sulfate (310 mg per ml) was added slowly, with constant stirring. After 1 hr at 0 C, the suspension was centrifuged for 20 min at $8,000 \times g$, and the pellets were discarded. To the supernatant fluid, ammonium sulfate (160 mg per ml of extract) was added with constant stirring. After 1 hr at 0 C, the precipitate was collected by centrifugation and dissolved with 15 ml of 0.04 M potassium phosphate (pH 7.0) containing 10 mM 2-mercaptoethanol. The preparation was dialyzed against three 1-liter portions of the same buffer over an 18-hr period. It was then applied to a column (2 by 25 cm) of DEAE-cellulose that had been pre-equilibrated and was eluted with the dialysis buffer. Fractions of 10 ml were collected every 20 min. An asymmetrical A_{280} peak, representing about one-fourth of the total protein, eluted in fractions 12 through 35; the arginyl-, aspartyl-, leucyl-, seryl-, and tryptophanyl-tRNA ligase activities were present in the descending slope of the A_{280} peak. The active fractions were pooled and dialyzed against 10 mM potassium phosphate (pH 7.0) containing 50% glycerol and 1 mM reduced glutathione. The dialyzed preparation was stored at -20 C and was stable for over 1 year.

Assay for aminoacyl-tRNA ligases. The procedure described for the tryptophanyl-tRNA ligase (17) was used. One enzyme unit catalyzes the attachment of 1 nmole of amino acid to tRNA per hr at 35 C.

Determination of amino acid acceptor capacity. In a final volume of 0.50 ml, the reaction mixture contained: Tris-acetate buffer (pH 7.5), 60 μmoles ; MgCl_2 , 5 μmoles ; adenosine triphosphate (ATP), 2 μmoles ; ^3H - or ^{14}C -labeled amino acid, 2 to 5 nmoles; tRNA, 0.02 to 0.05 mg; and 10 to 25 units of enzyme. Blanks were reaction mixtures without tRNA or zero-time controls. Incubations were at 35 C for 15 min. The level of charging of tRNA^{arg} reached a maximum after 5 min and remained constant for at least 45 min. The reaction was stopped with 3.3 ml of 70% ethanol saturated with NaCl. The samples were mixed, and 1 mg of yeast tRNA was added as carrier. The tubes were mixed and centrifuged at -10 C. The pellets were washed, dissolved, and counted as described previously (17).

Assay of arginyl-tRNA binding to ribosomes. The procedure of Nirenberg and Leder (19) was used. The reaction mixture contained, in a final volume of 0.10 ml: 0.05 M Tris-acetate (pH 7.2), 0.05 M potassium acetate, 0.014 M magnesium acetate, 5 A_{280} units of washed ribosomes, 50 nmoles of polynucleotide (expressed as phosphate), and 5 to 10 pmoles of [^{14}C]arginyl-tRNA. The reaction mixtures were incubated at 24 C for 15 min, diluted, and washed on membrane discs (Millipore Corp.) as reported (19). Ribosomes were isolated and washed as directed by Nirenberg (18).

RESULTS

The reversed phase chromatography of tRNA^{arg} from a carboy culture is illustrated in Fig. 1; the bulk of the arginine acceptor capacity was eluted as two partially resolved components. Very similar elution patterns were obtained with tRNA isolated from exponential cultures (results not shown). Figure 2 illustrates the similarity in the elution profiles of arginyl-tRNA from an exponential and a carboy culture. When ^3H -labeled arginyl-tRNA from an unshaken culture was co-chromatographed with ^{14}C -arginyl-tRNA from a carboy culture, a marked difference in the relative proportion of the two main isoaccepting tRNAs was found (Fig. 3). The results were not changed by reversing the labels. Also, the same profiles were observed when tRNAs isolated from the *arg-10* mutant or from strain 74A grown in the presence of arginine were analyzed.

No differences were apparent in the yield of total tRNA^{arg} from the three types of cultures. In addition, the observed difference in the relative proportion of the two arginyl-tRNAs could not be related to differences in recovery; determinations of arginine acceptor capacities in all of the fractions generated in the isolation

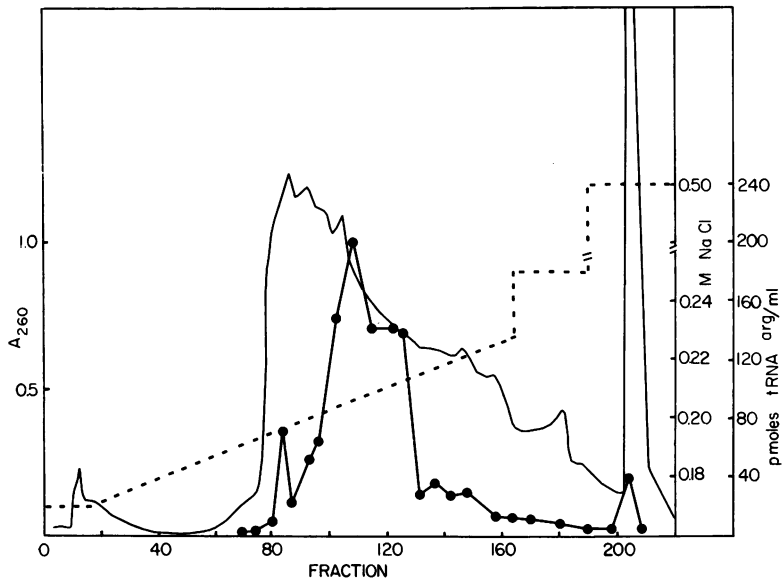


FIG. 1. Reversed phase chromatography of tRNA isolated from the wild type 74A grown on minimal medium (carboy cultures). Solid line, A_{260} ; circles, $tRNA^{arg}$; broken line, NaCl concentration in the eluent.

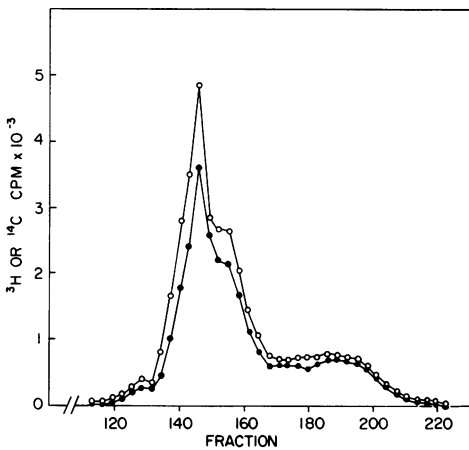


FIG. 2. Reversed phase co-chromatography of dually labeled arginyl-tRNA. The product isolated from a carboy culture of 74A was esterified with 3H -arginine (O); tRNA from an exponential culture of 74A was charged with ^{14}C -arginine (●).

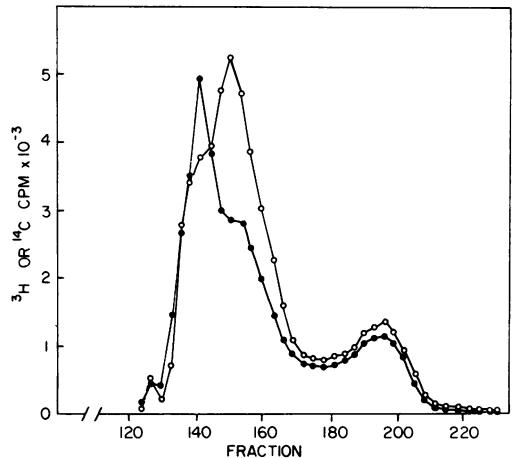


FIG. 3. Reversed phase co-chromatography of dually labeled arginyl-tRNA; tRNA isolated from a stationary culture of 74A was charged with 3H -arginine (O); tRNA from a carboy culture of 74A was charged with ^{14}C -arginine (●).

procedure (tRNA, first isopropanol precipitate, and sodium chloride-insoluble fraction) indicated that, in all cases, more than 90% of the total $tRNA^{arg}$ was present in the tRNA fraction (93, 95, and 92% for preparations from an exponential, a carboy, and an unshaken culture, respectively). Similar results were obtained for $tRNA^{trp}$.

In yeast tRNA, the acceptor capacity for several amino acids (including arginine) was

increased several-fold when the assays were conducted in the presence of tRNA nucleotidyl transferase (14). I found (*unpublished data*) that a major species of yeast $tRNA^{arg}$ lacking the 3' terminal adenosine monophosphate (AMP) is resolved by reversed phase chromatography. The arginine acceptor capacity of all *Neurospora* tRNAs used in this study increased by 7% or less when an excess of purified *Neurospora* tRNA nucleotidyl transferase

(EC 2.7.7.20; R. Hill and M. Nazario, *in preparation*) was added to the reaction mixture. The results were no different upon preincubation of the tRNA with tRNA nucleotidyl transferase and 0.1 mM cytidine triphosphate. We concluded that only a negligible proportion of the tRNA^{arg} molecules lacked the 3' terminal AMP, and therefore the differences in the elution profiles could not be due to limited degradation of the acceptor end.

No changes were observed in the arginine acceptor capacities by preincubation under the "renaturation" conditions of Lindahl et al. (12) or by heating the tRNA for 2 min at 100 C in a 0.5-ml reaction mixture containing 60 μ moles of Tris-acetate (pH 7.5), 5 μ moles of MgCl₂, and 2 μ moles of ATP (see Materials and Methods). Those results indicated the absence of "denatured" or "nicked" tRNA^{arg}.

To study the codon responses of the two arginine tRNA species, it was necessary to improve on their purification and resolution. To achieve this, tRNA was first chromatographed on benzoylated DEAE-cellulose (Fig. 4). Fractions from the partially resolved second species (162 to 174) were pooled, precipitated with ethanol, and chromatographed on a Freon column (Fig. 5). The chromatographic profiles (compare Fig. 1, 2, 4, and 5) clearly indicated that the two predominant tRNA^{arg} species eluted in the same sequence from benzoylated DEAE cellulose and from reversed phase columns, and that the order of elution from the Freon column was not affected by esterifica-

tion with arginine. This was ascertained by co-chromatography of ¹⁴C-arginyl-tRNA from the first component in Fig. 5 and ³H-arginyl-tRNA from an unshaken culture: the ¹⁴C radioactivity eluted in the same position of the first main component.

As indicated in Fig. 5, the following samples were pooled: 110-122 (designated Ia), 123-140 (Ib), 186-210 (IIa), and 215-230 (IIb). After precipitation with ethanol and membrane filtration (29), suitable samples were charged with ¹⁴C-arginine as described for chromatography of arginyl-tRNA (see Materials and Methods). Each arginyl-tRNA fraction was tested in ribosome binding assays (Table 1).

Fractions Ia and Ib bound strongly to poly(A,G) and to a lesser extent to poly(C,G,A). From these data, they could be assigned the triplets AGA and AGG. Taking into account the base composition of the polynucleotides used, the frequency of AGA plus AGG triplets is 25% in poly(A,G) and 12% in poly(C,G,A); the agreement between the extent of binding and the relative frequency of AGA plus AGG reinforces the assignment of those codons to the first tRNA^{arg}.

Poly(C,G,A) stimulated the binding of fractions IIa and IIb. The slight response of fraction IIa to poly(A,G) may indicate contamination of the left part of the second component. Although no tentative codon assignments were possible in this case, it is clear that the second tRNA^{arg} component recognizes codons from a different subgroup (i.e., starting with CG) than

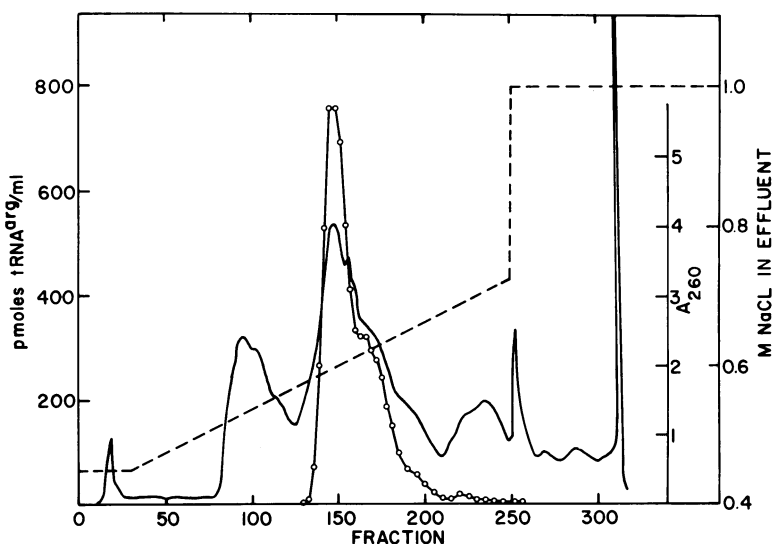


FIG. 4. Benzoylated DEAE-cellulose chromatography of tRNA isolated from carboy cultures of 74A. Solid line, A_{260} , circles, tRNA^{arg}; broken line, concentration of NaCl. For further details, see text.

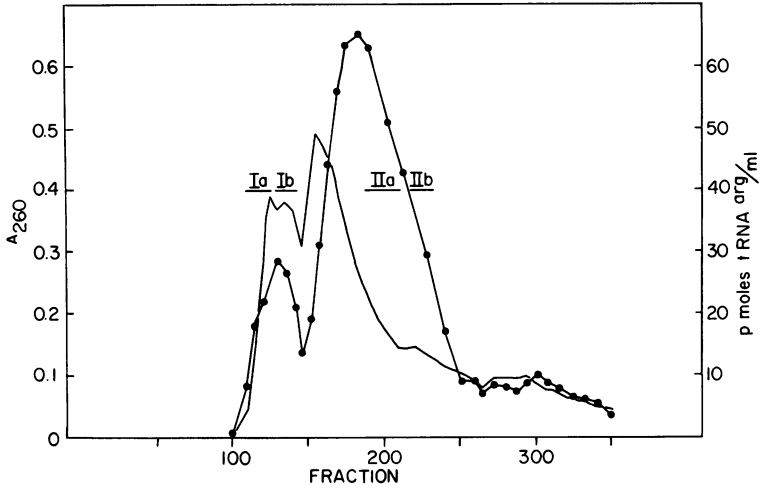


FIG. 5. Reversed phase chromatography of fractions 162-174 in Fig. 4. Solid line, A_{260} ; circles, $tRNA^{arg}$. For details, see text.

TABLE 1. Codon response of ^{14}C -arginyl-tRNA fractions

Template	Δ (p moles) ^{14}C -arginyl-tRNA bound to ribosomes ^a			
	Ia ^b	Ib ^c	IIa ^d	IIb ^e
Poly(A, G) (1.0:1.0)	3.49	3.51	0.49	0.06
Poly(C, G, A) (1.0:1.2:1.17) ..	1.56	1.53	0.78	0.65
None	1.27	1.27	1.67	1.84

^a Results are averages of duplicate bindings; Δ pmoles represents the binding of ^{14}C -arginyl-tRNA to ribosomes in presence of template minus the binding in the absence of template.

^b Samples 110-122 in Fig. 5.

^c Samples 123-140 in Fig. 5.

^d Samples 186-210 in Fig. 5.

^e Samples 215-230 in Fig. 5.

those recognized by the first $tRNA^{arg}$.

DISCUSSION

As has been shown (Fig. 3), there is a marked difference in the relative proportion of the two main arginine tRNA species between cells grown in unshaken and in shaken cultures. Some of the possible explanations for the observed differences (differential recovery, limited degradation of the 3' end, nucleases, denaturation) have been eliminated. In addition, the two arginine tRNA species differ in their codon response (Table 1).

Several reports have described changes in the chromatographic patterns of isoaccepting tRNA species associated with growth on media

of different composition (6, 11, 26, 28), anaerobiosis (10), or the stage of growth of bacterial cultures (2, 9). In some cases, however, the codeword specificities were not investigated, or the different tRNA species recognized the same codons. The chromatographic resolution of undermethylated leucine and phenylalanine tRNAs with altered codon responses was reported by Capra and Peterkofsky (4) and by Stern et al. (23), respectively. The undermethylated tRNAs were isolated from relaxed mutants starved for methionine. Gefter and Russell (7) found that a suppressor tyrosine tRNA with an unmodified adenosine adjacent to the 3' end of the anticodon was aminoacylated normally but did not bind to ribosomes. The isopentenyl adenosine derivative, however, has only been found in tRNAs that recognize codons with U in the first position (21).

It is not known whether the changes in the proportion of arginine tRNAs reported here result from lack of modification, increased rate of synthesis or degradation or a combination thereof. Whatever the mechanism, the potential regulatory significance of these observations should be pointed out. The work of Anderson (1) has shown that the rate of synthesis of proteins could be selectively regulated by the concentration of a given tRNA and the relative frequency of the corresponding codon(s) in mRNA. In many organisms, conditions such as those prevailing in our unshaken cultures (limited aeration, inefficient utilization of nutrients, etc.) have pronounced effects on cellular metabolism. A change in the relative proportion of two isoaccepting tRNAs with dif-

ferent codon specificities could, by restricting the translation of certain mRNAs (and facilitating the translation of others), play an important role in the process.

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