Resolution of Cytidine- and Adenosine-Terminal Transfer Ribonucleic Acids*

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ABSTRACT: Transfer ribonucleic acid (t-RNA) serine from baker's yeast, purified by repeated partition chromatography on Sephadex, was fractionated further in three different countercurrent distribution systems. In each of these, t-RNA serine was resolved into two species which were detected and distinguished in studies utilizing purified seryl-t-RNA synthetase and cytidine and adenosine monophosphate incorporating enzyme. One of the t-RNA serine species terminated at the 3′-OH end in adenosine, the other in cytidine. Analogous results were obtained for t-RNA leucine (yeast).

Conversion of the cytidine-terminal to the adenosine-terminal species of t-RNA serine was accompanied by change in the chromatographic behavior. It is concluded that differential migration of these two species is related to the nature of the 3′-OH-terminal nucleoside.

A number of observations indicate that a transfer ribonucleic acid (t-RNA) preparation, specific in its acceptor function for a single amino acid, may be resolved into two or more fractions by various chromatographic or biochemical procedures. The nature of this multiplicity of species has been explored by a number of investigators. On the basis of the important contribution of Weisblum *et al.* (1962), who found that two distinct leucyl-t-RNA's (*Escherichia coli*) would respond to different code words in a ribosomal amino acid incorporation system, it has generally been assumed that multiplicity of peaks for a given amino acid in chromatography or countercurrent distribution of t-RNA is equivalent to coding degeneracy. The extent of the difference in nucleotide composition or sequence between degenerate t-RNA species is not known; it may be recalled, however, that Berg *et al.* (1962) found that t-RNA leucine 1 and t-RNA leucine 2 (*E. coli*) differed in the sequence of nucleotides adjacent to the amino acid acceptor terminal CpCpA (cytidine–cytidine–adenosine),[1] and that Berg *et al.* (1961) and Bennett *et al.* (1963, 1965) demonstrated differential responses of multiple methionine t-RNA's, leucine t-RNA's, and serine t-RNA's (*E. coli*) to homologous and heterologous aminoacyl-t-RNA synthetases. Furthermore, Marcker (1965) has found that only one of the two degenerate methioninyl-t-RNA's (*E. coli*) was capable of being formylated. These observations indicate that differences in degenerate species of t-RNA may extend beyond the "coding site." Moreover, Zachau *et al.* (1966) have recently published the complete base sequence of t-RNA serine I and II from brewer's yeast, presumably identical at the "coding site," but differing in three nucleotides over a span of 19 nucleotides. Berquist and Robertson (1965) and Rushisky *et al.* (1965) have also reported multiple species of t-RNA serine (baker's yeast) that exhibit slightly different nucleotide compositions by partial analysis of digestion fragments.

This paper will describe a different and previously unreported type of t-RNA heterogeneity that results in the resolution of t-RNA serine and t-RNA leucine from baker's yeast into two species depending on the presence or absence of adenosine in the CpCpA-terminal nucleotide sequence. Furthermore, it will be shown that the difference in the chromatographic mobilities of the two species of t-RNA serine can be erased by addition of an adenosine nucleoside to the species terminating in CpC.

Materials and Methods

Preparation of Seryl-t-RNA Synthetase and CMP–AMP-Incorporating Enzyme. Crystalline seryl-t-RNA synthetase was prepared according to the method of Makman and Cantoni (1965). A partially purified CMP–AMP-incorporating enzyme was prepared at 2–4° as follows.

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[1] Abbreviations used are: CMP and AMP, cytidine and adenosine monophosphates; CTP and ATP, cytidine and adenosine triphosphates; CpCpA, 3′-OH-terminal nucleotide sequence common to all t-RNA's; TCA, trichloroacetic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-2-(5-phenyloxazolyl)benzene; PCA, perchloric acid.

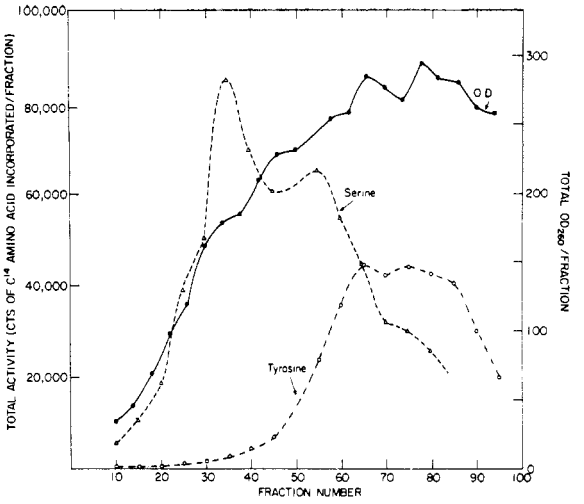

FIGURE 1: Partition chromatography of bulk yeast t-RNA on Sephadex G-25 according to the method of Tanaka *et al.* (1962). Only the early portion of the chromatogram, containing peaks of acceptor activity for serine and tyrosine is shown.

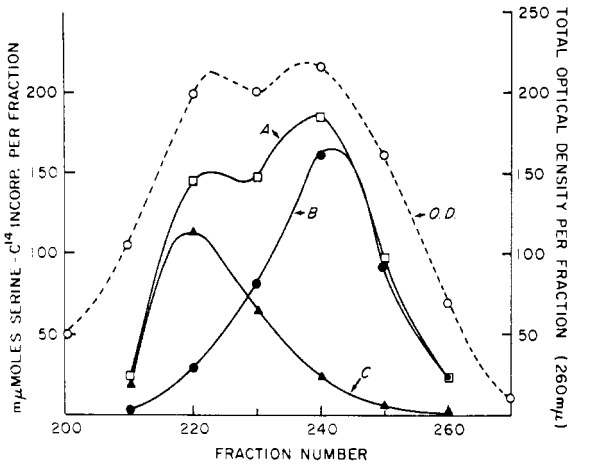

FIGURE 2: Separation of cytidine- and adenosine-terminal species of serine-specific t-RNA of yeast by countercurrent distribution in the modified Zachau solvent system; 23,000 OD_{260} units of the butylamine salt of t-RNA serine (Figure 1, fractions 20–55) was dissolved in 10 ml each of upper and lower phase, loaded into the first countercurrent tube, and transferred through 400 turns. Curve A: serine-acceptor activity with crude seryl-t-RNA synthetase. Curve B: activity with purified synthetase, indicating adenosine-terminal t-RNA. Curve C: the difference between the areas under curves A and B, indicating the amount of cytidine-terminal t-RNA present.

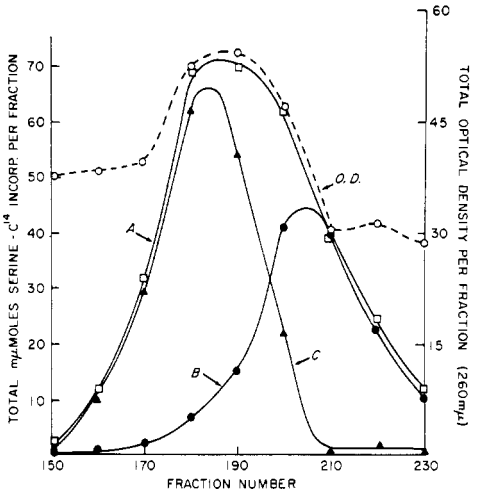

FIGURE 3: Separation of cytidine- and adenosine-terminal species of t-RNA serine (Baker's yeast) by countercurrent distribution in the system of Doctor and Connelly (1961); 1530 OD_{260} units of the potassium salt of t-RNA serine obtained by partition chromatography (Figure 1) were solubilized in 50 ml each of upper and lower phase, charged into the first five countercurrent tubes, and transferred for 300 turns. Curve A: serine-acceptor activity with pure seryl-t-RNA synthetase plus partially purified CMP–AMP-incorporating enzyme. Curve B: activity with purified synthetase alone, indicating adenosine-terminal t-RNA. Curve C: the difference between the areas under curves A and B, indicating the amount of cytidine-terminal t-RNA.

Each of four batches of 100 g of Fleishman's pressed baker's yeast was homogenized with 300 g of glass beads (Minnesota Mining and Manufacturing Co., No. 150, 75 μ, prewashed in 30% nitric acid in a steam bath until free of material absorbing at 260 mμ), 100 ml of 0.02 M PO_4 buffer (K), pH 7.6, containing 0.002 M $MgCl_2$, and 0.4 ml of *n*-octyl alcohol in a Waring Blendor for five 3-min periods with 6-min intervals on ice between successive homogenizations. Each final homogenate was mixed with 200 ml of the PO_4–$MgCl_2$ buffer and decanted after the beads had settled. The beads were washed three times with 125 ml of buffer and the washings combined with the concentrated homogenate. The pooled homogenate was then centrifuged for 1 hr at 78,000*g* in the No. 30 rotor of a Spinco Model L centrifuge.

To 1000 ml of the resultant supernatant fluid was added 20 g of crystalline streptomycin sulfate (Calbiochem) over a 30-min period with constant stirring. After 20 min of further stirring the extract was centrifuged for 30 min at 20,000*g* in the VRA head of the Lourdes LRA centrifuge.

To the supernatant fluid (980 ml), 254.8 g of solid ammonium sulfate (Merck Co.) was added over a 15-min period with constant stirring. The mixture (42.5% saturation) was allowed to stir an additional 20 min and then centrifuged in the Lourdes VRA head at 20,000*g* for 20 min. The supernatant fluid (1080 ml) was brought to 53% saturation by the slow addition of 70.2 g of ammonium sulfate. After 20 min of further stirring, the mixture was again centrifuged

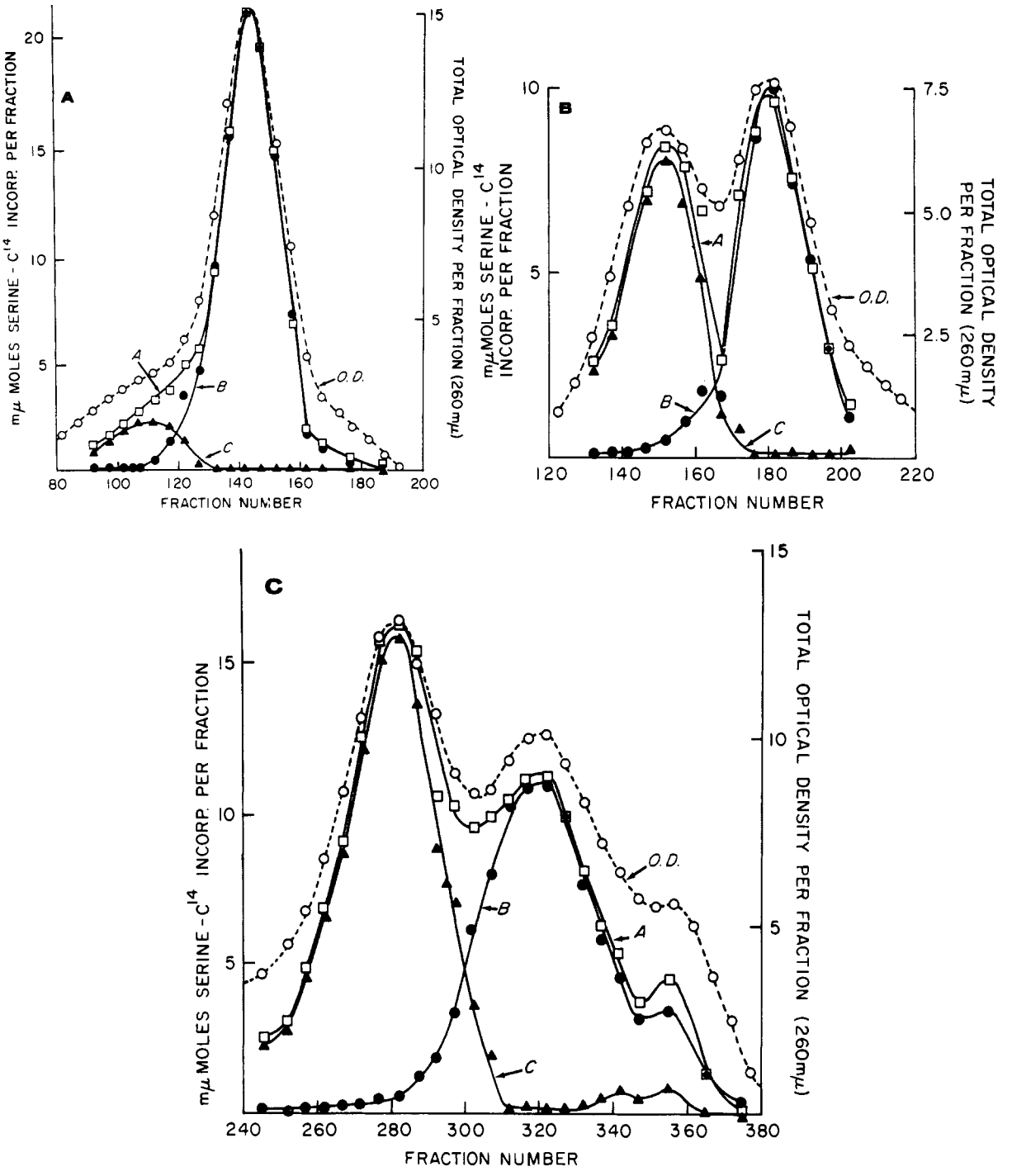

FIGURE 4: Correspondence between the proportion of cytidine- and adenosine-terminal species of t-RNA serine (yeast) in the modified countercurrent system of Zachau *et al.* (1961) (Figure 2) and that of Apgar *et al.* (1962): (A) 868 OD_{260} units of the potassium salt of t-RNA serine were pooled from tubes 240–250 of Figure 2 (95% adenosine and 5% cytidine terminal), solubilized in 30 ml each of upper and lower phase, placed in the first three countercurrent tubes, and subjected to a 200-turn distribution; (B) 840 OD_{260} units of the potassium salt of t-RNA serine from tubes 227–235 of Figure 2 (55% adenosine and 45% cytidine terminal) was similarly taken up in 60 ml of solvent system, loaded into the first three tubes, and transferred through 250 turns; (C) 1800 OD_{260} units of the potassium salt from tubes 219–227 of Figure 2 (60% cytidine and 40% adenosine-terminal t-RNA) was dissolved in 100 ml each of upper and lower phase, charged onto the first ten countercurrent tubes, and distributed through 400 turns. See legend to Figure 3 for further details. The additional peak of serine acceptor activity seen at the leading edge in 4C has been found inconstantly in distributions of primarly cytidine-terminal t-RNA, and although related to the purity of the sample, has not been characterized further.

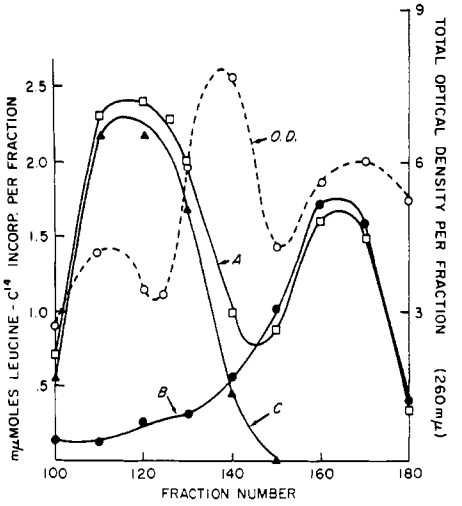

FIGURE 5: Separation of cytidine- and adenosine-terminal species of t-RNA leucine (baker's yeast) by countercurrent distribution in the system of Doctor and Connelly (1961); 810 OD_{260} units of the potassium salt of t-RNA leucine obtained from Sephadex G-25 partition chromatography were dissolved in 50 ml each of upper and lower phase, loaded into the first five tubes of the countercurrent system, and distributed through 300 turns. Curve A: assay of fractions with partially purified leucyl-t-RNA synthetase plus partially purified CMP-AMP-incorporating enzyme. Curve B: assay with partially purified synthetase alone, indicating the amount of adenosine-terminal t-RNA. Curve C: the difference between areas under curves A and B, indicating the amount of cytidine-terminal t-RNA.

as above. The precipitate from this centrifugation was taken up in 36 ml of 0.01 M phosphate buffer, pH 7.6, with 0.002 M $MgCl_2$. This ammonium sulfate fraction was stable for at least 16 months at −80° and was used as a source of CMP–AMP-incorporating enzyme in the assay for serine acceptance described below. This protein fraction, purified over 100-fold but not homogeneous, could be used as a source of the enzyme for this assay since it contained only negligible amounts of contaminating ribonucleases and aminoacyl-t-RNA synthetases. The ribonuclease activity was somewhat variable with different enzyme preparations; however, exposure of t-RNA to the enzyme for 30 min in the serine acceptor assay usually resulted in only a 2–3% and uniformly less than 8% loss of serine-acceptor activity. Seryl-t-RNA synthetase and leucyl-t-RNA synthetase activities were not detectable at levels of enzymes 35–40 times greater than needed for maximum AMP (from ATP) incorporation. One ammonium sulfate fraction (10 ml) which exhibited no detectable ribonuclease activity (in the serine-acceptor assay during a 90-min incubation) was desalted on a Sephadex G-25 column (2.5 × 32 cm) with 0.01 M phosphate, pH 7.6, containing 0.002 M $MgCl_2$ as eluent at a flow rate of 55 ml/hr. The desalted enzyme showed no ribonuclease activity in the AMP incorporation assay at levels 20 times that necessary for maximum AMP incorporation and was used to prepare serine t-RNA-[^{14}C]AMP (see below). Further, it has been demonstrated that the enzyme preparation used does not catalyze incorporation into t-RNA of the [^{14}C]methyl group from *S*-adenosylmethionine or ^{32}P from β,γ-labeled ATP.

Serine and Leucine t-RNA Preparation. t-RNA specific for serine (baker's yeast), free of all other amino acid acceptor activities, was obtained by partition chromatography on Sephadex, as previously reported (Tanaka *et al.*, 1962; Cantoni *et al.*, 1963). t-RNA serine is eluted as an incompletely resolved double peak (Figure 1). This pattern of elution is accentuated on rechromatography of the t-RNA serine on Sephadex. A partially purified t-RNA leucine was also obtained by partition chromatography on Sephadex.

Countercurrent Distributions. t-RNA serine and t-RNA leucine were further fractionated by countercurrent distribution in three different solvent systems: (1) a modification of the system of Zachau *et al.* (1961) which utilizes *n*-butyl alcohol (Baker and Adamson, Allied Chemical Co.), glass-distilled water 0.00038 M in EDTA, redistilled tri-*n*-butylamine (Eastman Organic Chemicals), glacial acetic acid, and redistilled di-*n*-butyl ether (Eastman Organic Chemicals), 100:130:10:2.5:30; (2) that of Apgar *et al.* (1962) which consists of 1.9 M phosphate, pH 6.0, redistilled isopropyl alcohol (Fisher Scientific Co.), and redistilled formamide (Fisher Scientific Co.), 100:44:10; (3) that of Doctor and Connelly (1961) which utilizes 30% ammonium sulfate containing 0.001 M $MgCl_2$, pH 4.0, 2-ethoxyethanol (Fisher Scientific Co., purified), and formamide, 25:10:1.

The countercurrent apparatus (E. C. Apparatus Co., Philadelphia, Pa.) was filled with 10 ml of upper and lower phase per tube and the t-RNA, predissolved in the solvent system, was charged into the initial tubes of the apparatus (exact quantities of t-RNA solubilized and a number of tubes charged are given in the legends of Figures 2–6). Closed system distributions for varying numbers of transfers (see legends, Figures 2–6) at 23–24° were performed, after which the t-RNA was extracted from individual fractions by the method of Zachau *et al.* (1961) following distributions in that system, or on fractions pooled in groups of five (four for the distribution of labeled t-RNA) according to the method of Apgar *et al.* (1962) for distributions using the systems of Doctor and Connelly (1961) and Apgar *et al.* (1962). The absorbancy at 260 mμ, amino acid and AMP-acceptor activity, and [^{14}C]AMP radioactivity were determined on the extracted t-RNA.

Amino Acid Acceptor Assay. Following countercurrent distribution, t-RNA serine was localized by the serine acceptor assay used by Makman and Cantoni (1965), using either the crystalline preparation of yeast seryl-t-RNA synthetase or a cruder enzyme fraction (Sephadex-treated ammonium sulfate fraction of Makman and Cantoni, 1965). Leucine-acceptor activity was assayed similarly, using a purified leucyl-t-RNA syn-

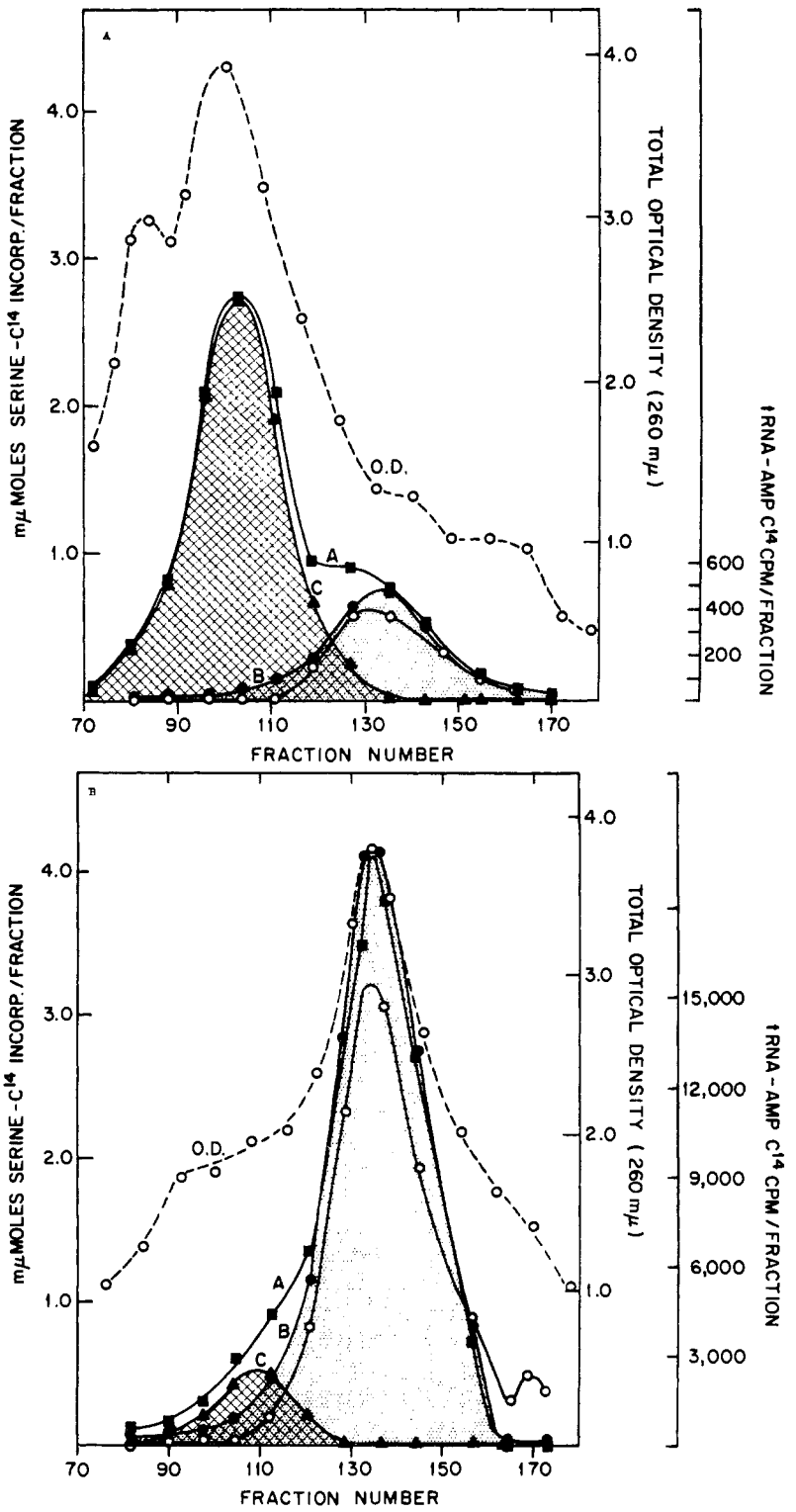

FIGURE 6: Alteration of countercurrent mobility in the system of Apgar *et al.* (1962) of cytidine-terminal t-RNA serine by conversion to adenosine-terminal t-RNA serine. Details of the distribution given in text. Curve A: serine-acceptor activity with pure seryl-t-RNA synthetase plus partially purified CMP–AMP-incorporating enzyme. Curve B (stippled): serine-acceptor activity with purified seryl-t-RNA synthetase alone, indicative of t-RNA terminating in 3′-hydroxyadenosine. Curve C (cross-hatched): the difference between the areas under curves A and B, indicating the amount of cytidine-terminal t-RNA. o———o, t-RNA [^{14}C]adenosine, identifying the precise location of the intact species of t-RNA and tracing the movement of cytidine-terminal t-RNA in tubes 90–120 in 6A to tubes 120–160 in 6B following labeling with [^{14}C]AMP by the CMP–AMP-incorporating enzyme.

thetase (Makman and Cantoni, 1965) or a crude ammonium sulfate fraction. The crystalline seryl-t-RNA synthetase or the purified leucyl-t-RNA synthetase contained on detectable CMP–AMP-incorporating activity (we could have detected incorporation of [^{14}C]AMP (from ATP) at levels of 1–2% of the serine or leucine incorporation). When t-RNA serine, labeled with [^{14}C]adenosine in the terminal nucleoside, was used as substrate for the acceptor assay, a control reaction mixture was incubated simultaneously without synthetase and the background radioactivity subtracted from the total.

[^{14}C]AMP-Acceptor Assay with CMP–AMP-Incorporating Enzyme. Assay of [8-^{14}C]AMP incorporation into t-RNA preparations consisting of a mixture of chains terminating with cytidine and adenosine was carried out in a reaction mixture of 0.225 ml containing 0.044 M Tris–HCl buffer, pH 9.0, 0.013 M $MgCl_2$, 0.0048 M [8-^{14}C]ATP (1.12 mc/mM; Schwarz Bioresearch Inc.), 1.0–5.0 OD_{260} units of t-RNA, and a suitable aliquot of the CMP–AMP-incorporating enzyme. After a 20-min incubation at 37°, 0.1 ml was pipetted onto a Whatman filter paper disk (2.3-cm diameter, No. 3) which was immediately dried in a cold air stream and placed in cold 10% TCA for 20 min. After decanting the TCA, the filter paper disks were washed once again for 20 min in 10% TCA and then rapidly in 3% perchloric acid four times, 95% ethanol four times, and ether twice. Solvent (15 ml) was used in each wash step per disk. The disks were then dried and the radioactivity was determined in the Packard Tri-Carb liquid scintillation counter using 5 ml of a scintillant of 4.0 g of PPO and 0.1 g of POPOP/l. of toluene.

Preparation of t-RNA Labeled with [^{14}C]AMP. t-RNA serine, labeled in the 3′-hydroxyl position with [^{14}C]AMP, was prepared by incubation of 1090 OD_{260} units of the potassium salt of a t-RNA serine predominantly cytidine terminal (see below) with [8-^{14}C]ATP (1.12 mc/mM) in the presence of 29.7 mg of a partially purified CMP–AMP-incorporating enzyme in a total reaction mixture of 12.4 ml under the conditions described above for the assay of this enzyme. After a 25-min incubation at 37°, the reaction was stopped by addition of a 50-fold excess of unlabeled ATP, and the RNA was precipitated by addition of cold perchloric acid to a final concentration of 3%. After centrifugation for 10 min at 1300*g*, the t-RNA was washed three times with 5.0 ml of 0.06% PCA to remove the unbound ATP and taken up in 8.0 ml of 0.01 M Tris–HCl buffer, pH 8.0. A few drops of 0.5 N NaOH were added to effect solubilization. The t-RNA was then shaken with an equal volume of water-saturated phenol for 30 min at room temperature after which the phases were separated by brief centrifugation. The phenol phase was reextracted with 3 ml of buffer and the aqueous phases were combined. The t-RNA in 10 ml of aqueous phase was charged onto a 1.5 × 40 cm column of Sephadex G-50 and eluted with glass-distilled water at a flow rate of 36 ml/hr at 4°. The labeled t-RNA (330 cpm/OD_{260} unit) was recovered free of phenol and ATP and lyophilized to dryness prior to introduction into the countercurrent solvent system.

Results

Fractionation of t-RNA Serine. Fractionation of t-RNA serine (baker's yeast) (fractions 20–55, Figure 1) by a 400-transfer countercurrent distribution run in the modified solvent system of Zachau *et al.* (1961) is depicted in Figure 2. The OD_{260} profile now shows two incompletely resolved peaks. The profile of serine acceptor activity depends on the level of purity of the synthetase used for the assay: the profile defined by reaction with the crude synthetase (curve A) is quite broad and consists of an incompletely resolved double peak corresponding to the optical density profile; on the other hand, the profile of serine acceptor activity defined by use of the crystalline synthetase (curve B) is relatively narrow and falls under the leading half of the optical density peak. The fractions from the trailing portion of the peak (Figure 2, fractions 210–225) can accept serine when the crude enzyme is used, but are much less active when tested with the crystalline synthetase alone, whereas the fractions from the leading edge of the peak (Figure 2, fractions 245–260) show very close agreement between both assays. Curve C, obtained by subtraction of the acceptor activity defined by the crystalline enzyme from the total activity outlined by crude enzyme, corresponds to a species of t-RNA serine which can be charged with a crude synthetase fraction, but does not serve as substrate for the purified enzyme.

It is known that in yeast t-RNA the 3′-OH terminal is a mixture of adenosine and cytidine, presumably due to partial enzymatic removal of terminal nucleotide of the XpCpCpA sequence common to all t-RNA's by a yeast enzyme analogous to the CMP–AMP pyrophosphorylase discovered by Hecht *et al.* (1958), and studied in *E. coli* by Preiss *et al.* (1961), Furth *et al.* (1961), and in mammalian tissue by Daniel and Littauer (1963). Crude seryl- (or leucyl-) t-RNA synthetase preparations from yeast contain an enzyme capable of adding CMP and AMP from CTP and ATP to t-RNA (yeast) in the 3′-OH-terminal position. Since it has not been determined that this activity is in fact due to CMP–AMP pyrophosphorylase, though it appears most likely, we refer to this activity as the CMP–AMP-incorporating enzyme.

To ascertain whether the absence of the terminal adenosine from one of the two t-RNA serine peaks might be responsible for the apparent separation of t-RNA serine into a double peak, we reexamined the serine acceptor activity of the fractions from the countercurrent experiment shown in Figure 2; crystalline enzyme was used as a source of seryl-t-RNA synthetase, but now it was supplemented by the partially purified protein fraction from yeast containing CMP–AMP-incorporating activity and, as noted above, free of seryl-t-RNA synthetase activity. The profile of t-RNA serine obtained *under these conditions* matched

exactly that obtained with the cruder synthetase preparation. Addition of the CMP–AMP-incorporating fraction to crude synthetase preparations was without effect. Thus, the two t-RNA serine fractions can be designated as CMP–AMP-incorporating enzyme dependent (curve C), and CMP–AMP-incorporating enzyme independent (curve B). Provisionally, the dependent and independent species may be identified as cytidine and adenosine terminal; fuller experimental evidence for this conclusion is presented below.

It is clear that these two species of t-RNA serine can be resolved, partially at least, by use of Zachau's solvent systems, either by repeated column partition chromatography or by a combination of column chromatography and countercurrent distribution (Figures 1 and 2). A similar separation can be obtained for t-RNA serine by a combination of partition chromatography on Sephadex followed by countercurrent distribution in the system of Doctor and Connelly (1961) (Figure 3), or by partition chromatography and countercurrent distribution in the Zachau solvent system with subsequent redistribution in the countercurrent system of Apgar *et al.* (1962) (Figure 4A–C). In the latter system, it is seen that t-RNA samples pooled from successively more retarded areas of the curve of total serine acceptor activity following countercurrent distribution in the system of Zachau *et al.* (1961) (Figure 2) contain progessively less of the CMP–AMP-incorporating enzyme-independent species and more of the dependent species of t-RNA serine. Chemical analysis (see below) showed that adenosine was the terminal nucleoside in the independent species while cytidine was found predominantly as the terminal nucleoside in the dependent species.

The results of a direct assay of AMP incorporation into t-RNA serine countercurrent fractions of Figure 3 in the presence of the yeast CMP–AMP-incorporating enzyme are given in Table I. As expected, incorporation of AMP correlates with CMPA–MP-incorporating enzyme dependence in the assay for serine-acceptor activity.

That the separation of cytidine- and adenosine-terminal t-RNA's is not restricted to t-RNA serine is shown in Figure 5 which describes analogous separation of t-RNA leucine (baker's yeast) in the countercurrent system of Doctor and Connelly (1961) into two species respectively dependent on and independent of the CMP–AMP-incorporating enzyme for full activity.

TABLE I: Incorporation of [8-^{14}C]AMP into Countercurrent Fractions of t-RNA Serine (Baker's Yeast).

Countercurrent Fraction No.	mμmoles of [8-^{14}C]AMP Incorp/ OD_{260} Units of t-RNA
150	0
175	1.8
200	0.4
225	0.2

Fraction numbers correspond to those illustrated in Figure 3. Aliquots of the t-RNA fractions were assayed as described under Methods.

Effect of Terminal Nucleoside on Countercurrent Distribution. To verify more directly the role of the 3′-terminal nucleoside in the determination of the chromatographic mobility of t-RNA's, the following experiment was performed. A sample of t-RNA serine (baker's yeast) with only 21.4% of its acceptor activity independent of CMP–AMP-incorporating enzyme was divided into two aliquots: one aliquot was labeled in the 3′-terminal position with [^{14}C]AMP by incubation with the CMP–AMP-incorporating enzyme as described under Methods, while the other served as a control. The control was incubated with the enzyme in the absence of [8-^{14}C]ATP and also did not receive the addition of cold ATP; otherwise, it was in every respect treated identically with the experimental. It should be noted that acceptor activity of the control sample, assayed with crystalline synthetase supplemented with incorporating enzyme, was unchanged after incubation with the CMP–AMP-incorporating enzyme, precipitation, phenol treatment, and Sephadex gel filtration, indicating that the CMP–AMP-incorporating enzyme was completely free of ribonuclease contaminants and treatment with it did not lead to structural modifications capable of affecting acceptor activity; 270 OD_{260} units of the control RNA plus 10 OD_{260} units of labeled reconstructed RNA were loaded in tube 1 of a 400-plate instrument while 275 OD_{260} units of labeled RNA, plus 10 OD_{260} units of the control species were loaded in tube 201. Two identical, 200-transfer, closed-system countercurrent distributions in the solvent system of Apgar *et al.* (1962) were then performed simultaneously in a 400-tube apparatus. The results of this experiment are presented in Figures 6A and B, respectively. Figure 6A reveals separation of the control t-RNA into two species; the more rapidly moving species (curve B, stippled) contains about 20% of the total activity, is independent of the CMP–AMP-incorporating enzyme for acceptor activity, and contains all of the labeled tracer t-RNA; the major peak, outlined by curve C (cross-hatched), is retarded (tubes 90–120), contains about 80% of the total activity, is dependent upon the incorporating enzyme for acceptor activity, and contains no adenosine tracer. This is exactly the pattern expected since, as noted above, the starting material had only 21.4% of its total acceptor activity independent of CMP–AMP-incorporating enzyme. Figure 6B reveals how markedly the chromatographic mobility of t-RNA serine labeled with ^{14}C has been changed; peak B, the rapidly moving peak, is now a large peak corresponding to about 95% of the total acceptor activity (tubes 120–160), is totally independent of the CMP–AMP-incorporating enzyme, and terminates in labeled adenosine. Peak C corresponds to only 5% of the total activity and contains no radioactive adeno-

sine. This result establishes that the difference in migration characteristics of the two t-RNA serine peaks observed in partition chromatography in the countercurrent system of Apgar *et al.* (1962) is due to the presence or absence of the terminal adenosine.

Structural Identification of 3′-OH-Terminal Nucleosides.[2] The two t-RNA's serine (yeast), separated on the basis of their dependence or independence on the CMP–AMP-incorporating enzyme for full activity in the presence of crystalline seryl-t-RNA synthetase, have been shown conclusively to terminate in cytidine and adenosine, respectively. For the independent species, alkaline hydrolysis followed by column chromatography (Cantoni *et al.*, 1962) revealed adenosine as the main nucleoside and digestion with T_1 ribonuclease followed by column chromatography (Ishikura *et al.*, 1966) released the fragment CpCpA, but not CpC. For the dependent species, on the other hand, cytidine is the predominant nucleoside liberated on alkaline hydrolysis and CpC the 3′-terminal fragment released on T_1 digestion. In addition, t-RNA serine labeled with [^{14}C]AMP, as described in Methods, contains all of the label (96% recovery) in the [^{14}C]CpCpA fragment following T_1 digestion, demonstrating the specificity of enzymatic addition of AMP at the 3′-OH-terminal position.

Discussion

This report describes the resolution of t-RNA serine and t-RNA leucine (baker's yeast) into two chromatographically distinct species. In the case of t-RNA serine, it has been shown that the two species differ chemically at their 3′-hydroxyl end terminating, respectively, in cytidine and adenosine. This difference has been confirmed enzymatically: (a) by the dependence of the cytidine species on CMP–AMP-incorporating enzyme for biological acceptor activity, and (b) by the incorporation of AMP from ATP in this species.

Enzymatic conversion of the cytidine-terminal species to the adenosine-terminal species is accompanied by changes in the chromatographic behavior. These facts lead to the conclusion that the different mobilities of the two species is determined by the presence or absence of adenosine in the terminal nucleoside. This conclusion is valid in the absence of additional information on the identity or nonidentity of nucleotide sequence in the rest of the chains.

In the case of t-RNA leucine, it has been shown that the two species show different dependence on CMP–AMP-incorporating enzyme. By analogy with the finding for t-RNA serine it is assumed (though not proved) that this chromatographic behavior also can be related to the nature of the terminal nucleoside of the two species. These findings receive support from the work of Ingram and Sjoquist (1963) who find equal amounts of cytidine- and adenosine-terminal nucleoside in unfractionated yeast t-RNA, but 90–95% terminal cytidine in the slower moving peak of the two t-RNA valine (*E. coli*) species isolated by the method of Apgar *et al.* (1962).

It should be noted that Zachau *et al.* (1961) report the isolation from brewer's yeast of two species of t-RNA serine. These two species have been shown to differ with respect to three internal nucleotides, but not with respect to the terminal nucleoside or the presumed coding site (Zachau *et al.*, 1966). Clearly, therefore, this countercurrent system is capable of separating t-RNA species on the basis of the internal sequence or of the nature of the terminal nucleoside.

Several other factors, as yet incompletely understood, are of importance in the determination of the chromatographic mobility of t-RNA. Presumably, it is the coding characteristics of the t-RNA species that determine chromatographic behavior in the experiments of Nathenson *et al.* (1965). One might suppose that the loss of one additional residue (cytidine) from the terminal CCA sequence would result in further alteration of the chromatographic mobility. Likewise, loss of the 5′-phosphate from the pGp terminal might alter the chromatographic behavior and result in a "new" t-RNA species. Caution appears to be required before separation of an amino acid specific t-RNA into multiple peaks can be safely ascribed to coding degeneracy.

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Incorporation Efficiency of Small Oligo-5′-nucleotide Initiators in the Terminal Deoxyribonucleotide Transferase Reaction*

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ABSTRACT: Radioactive oligodeoxyribo-5′-nucleotides ($d(pA)_2$, $d(pT)_2$, $d(pA)_3$, $d(pT)_3$, $d(pC)_3$, and $d(pC)_4$) have been used as initiators in the terminal deoxyribonucleotide transferase reaction under a variety of conditions with 2′-deoxyadenosine 5′-triphosphate (dATP) to give single-stranded polydeoxyribonucleotides with labeled 5′-end sequences. Per cent incorporation of initiator and 2′-deoxyadenosine 5′-monophosphate (dAMP) and also polymer length have been determined for each product. Conditions which increased initiator incorporation were low temperature, high phosphate concentration, and high enzyme specific activity. The initiators showed the preference order for incorporation: tetramer > trimer > dimer and A > T > C, and also influenced the rate of dAMP incorporation in the order A > T > C.

A reaction catalyzed by terminal deoxyribonucleotidyl transferase (hereafter referred to as addase) involves repetitive grafting of mononucleotide units from a deoxyribonucleoside 5′-triphosphate onto the 3′-terminal hydroxyl function of a growing single-stranded polymer (Bollum, 1960, 1962). The reaction requires an oligodeoxyribo-5′-nucleotide as an initiator, and the first addition takes place on its 3′-hydroxyl. The minimum size for a competent initiator is trimer (Bollum *et al.*, 1964).

We have isolated and purified addase (Yoneda and Bollum, 1965) in order to synthesize single-stranded polydeoxyribonucleotides from a variety of trimer initiators (Williams *et al.*, 1965) to serve as templates in biological information transfer studies. We have investigated the incorporation of radioactive initiators into polymers in order to understand the factors controlling the efficiency of utilization.

Materials and Methods

Oligo-5′-thymidylic-2-^{14}C acids, $d(pT\text{-}2\text{-}^{14}C)_n$,[1] and

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[1] Abbreviations used in this work: dATP, 2′-deoxyadenosine 5′-triphosphate; dAMP, 2′-deoxyadenosine 5′-monophosphate; $d(pA)_2$ and $d(pA)_3$, dimer and trimer of dAMP; $d(pT)_2$ and $d(pT)_3$, dimer and trimer of 5′-thymidylic acid; $d(pC)_3$ and $d(pC)_4$, trimer and tetramer of 2′-deoxy-5′-cytidylic acid; mono-μmole, a micromole of monomer units contained in a polymer; *E*, per cent incorporation of initiator; *Y*, per cent incorporation of dAMP; $\bar{n}$, average number of monomer units in polymer; $\bar{x}$, average number of added dpA units; PPO, 2,5-diphenyloxazole; POPOP, 2,2′-*p*-phenylenebis(5-phenyloxazole).