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Specific Degradation of a Plant Leucyl Transfer Ribonucleic Acid by a Factor in the Homologous Synthetase Preparation[1,2]

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ABSTRACT

Partially purified aminoacyl synthetase preparations from pea roots (*Pisum sativum* L. var. Alaska) contain a heat-labile factor which can degrade leucyl-tRNA$_6^{leu}$ to a new species. The singular electrophoretic and chromatographic mobilities, the isoprenoid nucleoside content, and the charging characteristics of the new species (designated leucyl-tRNA$_L^{leu}$), suggest that it is a fragment of tRNA$_6^{leu}$ containing at least that portion of the original molecule extending from the 3′ terminus to the anticodon. Conversion appears to be highly specific since neither bulk tRNA, the other leucine tRNA subspecies, nor tyrosine, phenylalanine, or tryptophan tRNAs are susceptible to degradation during incubation with the synthetase preparation.

Differentiation or dedifferentiation of organs or cell lines, nutritional stress, and exposure to pathogens are accompanied in many instances by changes in the chromatographic profiles of isoacceptor tRNA species (5, 11, 21, 26, 28). Although nontranslational functions for certain tRNA molecules are known (13, 14), those tRNAs susceptible to change have often been cast in the role of effectors of translational control. Evidence for such a role has been provided in only one case: a very early event following infection of *Escherichia coli* with T2 bacteriophage is the transient appearance of a new leucine-tRNA (tRNA$_F^{leu}$) which plays a part in the inhibition of protein synthesis of the host cell (15–17). While tRNA$_F^{leu}$ is probably produced by the action of a phage-induced nuclease on existing tRNA$_1^{leu}$ (31), the extraction or characterization of such a nuclease was not reported.

We describe below a fragmented tRNA (leucyl-tRNA$_L^{leu}$) from an entirely different source, pea roots. It has considerable similarity to tRNA$_F^{leu}$ from *E. coli* although derived apparently from a leucine-tRNA which recognizes a different code word. We also present evidence that extracts of pea roots contain a degradative enzyme responsible for the production of leucyl-tRNA$_L^{leu}$ in the manner suggested by Kano-Sueoka *et al.* (17) for tRNA$_F^{leu}$.

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MATERIALS AND METHODS

Reagents. *N*-2-Hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid was purchased from Calbiochem, Los Angeles, California; and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide from the Regis Chemical Co., Chicago, Illinois. ^{3}H-L-Leucine (1 c/mmole), ^{14}C-L-leucine (440 mc/mmole), ^{14}C-L-tyrosine (440 mc/mmole), ^{14}C-L-tryptophan (15 mc/mmole), and ^{14}C-L-phenylalanine (440 mc/mmole) were obtained from Amersham/Searle, Arlington Heights, Illinois. Peninsula Chemical Research, Inc., Gainesville, Florida, supplied 1,1,1,3-tetrachlorotetrafluoropropane and tricaprylylmethyl-ammonium chloride for reverse phase chromatography. Electrophoretically purified DNase was obtained from the Worthington Chemical Corp., Freehold, New Jersey.

Aminoacyl Synthetases. A mixed aminoacyl synthetase preparation devoid of detectable nuclease activity (as measured by the liberation of acid-soluble fragments from radioactive rRNA) was prepared from the roots of 48-hr-old pea seedlings (*Pisum sativum* L. var. Alaska) by the procedure of Scott and Morris (25), except that DEAE-cellulose chromatography was performed in the presence of 5 mM $MgCl_2$ while gel filtration on P-100 was replaced by dialysis against 10 mM potassium phosphate (pH 7.2), 5 mM $MgCl_2$, 0.1 mM dithiothreitol, and 10% (v/v) glycerol. Synthetase activities for most amino acids were stable indefinitely at −70 C although some, notably seryl synthetase, were diminished in a few weeks.

Transfer RNA. The method of preparation of tRNA was similar to that used by others for pea (8, 27) and soybean tissue (2). Roots, collected in liquid nitrogen, were powdered and then homogenized in 3 volumes of tris-Mg-DDT[4] buffer containing 0.5% (w/v) sodium dodecyl sulfate. After shaking twice with 0.5 volume of redistilled phenol saturated with tris-Mg-DDT, total RNA was precipitated from the aqueous phase by the addition of 0.1 volume of 1 M potassium acetate (pH 4.5) followed by 2 volumes of 95% ethanol cooled to −20 C. Transfer RNA was extracted from the precipitate by homogenization with 2 volumes of 1 M NaCl containing 0.1 mM dithiothreitol. A further precipitation with ethanol was followed by a brief treatment with DNase (15 min, 4 C, 50 μg/ml DNase) after which the solution was deproteinized with phenol and applied to DEAE-cellulose. Impurities were removed by washing the column with 0.2 M NaCl (in tris-Mg-DDT), and the tRNA was eluted with 1 M NaCl in this buffer. After ethanol precipitation, the tRNA was dissolved in tris-Mg-DDT to a concentration of 5 mg/ml; insoluble material was removed by

[4] Abbreviations: tris-Mg-DDT: tris Cl (10 mM, pH 7.4), $MgCl_2$ (10 mM), dithiothreitol (0.1 mM); BDC: benzoylated DEAE-cellulose.

centrifugation at 20,000*g* for 10 min, and the purified product was stored at −70 C.

Transfer RNA labeled with ^{32}P was prepared from peas (1 kg) which had been allowed to imbibe a small volume of a solution containing 20 mc of ^{32}P-orthophosphate at the start of germination.

Aminoacylation of tRNA. Aminoacylation was conducted with an excess of synthetase in the reaction mixture under conditions such that maximum acylation was achieved in 15 min at 30 C. For phenylalanyl-tRNA formation, optimum conditions were: 100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (pH 7.4), 12 mM $MgCl_2$, 2 mM ATP, 0.1 mM dithiothreitol, 0.06 mM phenylalanine, 0.1 mg/ml tRNA, and 0.2 mg/ml synthetase protein. For maximum attachment of leucine, conditions were identical except that the $MgCl_2$ and leucine concentrations were 1 mM and 0.02 mM respectively. In experiments where tRNA was coacylated with leucine and phenylalanine, over 90% of maximal leucine attachment was observed under conditions optimal for phenylalanine.

When the production of leucyl-$tRNA_L^{leu}$ was under study, two standard reaction mixtures were used, containing either ^{14}C-L-leucine or ^{3}H-L-leucine. As indicated later, the source of the tRNA, the synthetase level, or the time of incubation was different for each mixture. After incubation, the mixtures were adjusted to pH 4.5, pooled, and applied to a miniature DEAE-cellulose column (30). The purified aminoacyl-tRNA was eluted with 0.7 M NaCl, diluted, and examined at once by reversed phase chromatography.

Analytical Procedures. Reversed phase columns (RPC2) were prepared from tricaprylylmethylammonium chloride and 1,1,1,3-tetrachlorotetrafluoropropane by the method of Weiss and Kelmers (29). Columns (0.6 × 120 cm) were eluted with 600-ml linear gradients (0.3–0.7 M) of sodium chloride in 20 mM sodium acetate (pH 5.0), 10 mM $MgCl_2$, 1 mM EDTA, and 0.1 mM dithiothreitol at 20 ml/hr. Radioactivity was determined directly on individual fractions using a Triton X-100-toluene-based scintillation system (22).

Preparative gel electrophoresis was performed at 4 C in the Canalco prep disc apparatus (Canal Industrial Corp., Rockville, Maryland) using 3- × 5-cm 10% polyacrylamide gels polymerized in 0.02 M sodium acetate, 0.04 M tris acetate, 0.02 M EDTA (pH 4.5) and eluted with the same buffer (20). Electrophoresis required 4 to 5 hr at 200 to 250 v and 30 to 40 ma. One-milliliter fractions were collected at an eluant flow of 20 ml/hr. In other experiments, aminoacyl-tRNA was fractionated upon analytical (6- × 0.5-cm) gels. After electrophoresis, these gels were scanned at 260 nm in a Gilford linear absorbance cell (Gilford Instrument Laboratories, Oberlin, Ohio) and then sliced into 1-mm sections. Prior to counting, the slices were shaken for 1 hr at room temperature in Triton X-100-toluene scintillation mixture.

The trimethylsilyl derivatives of isoprenoid nucleosides present in leucyl-$tRNA_L^{leu}$ hydrolysates were detected by gas chromatographic examination of the appropriate partition column fractions as described previously (4). Digestion of the tRNA was carried out in 0.3 M KOH and was followed by incubation with bacterial alkaline phosphatase, removal of bulk nucleosides by partition chromatography, and trimethylsilylation with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide.

Benzoylated DEAE-Cellulose Chromatography. In many experiments, tRNA was subjected to preliminary fractionation on benzoylated DEAE-cellulose. The exchanger was prepared by the method of Gillam *et al.* (12), and up to 2700 A_{260} units of tRNA were applied to a column (0.9 × 10 cm) equilibrated against 20 mM sodium acetate (pH 5.0), 10 mM $MgCl_2$, 0.1 mM dithiothreitol, and 0.25 M sodium chloride. A linear 800-ml gradient of sodium chloride (from 0.25 M to 1.0 M) was followed by 300 ml of 1 M NaCl and finally by 100 ml of 2.5 M NaCl containing 20% ethanol.

RESULTS

Preliminary Fractionation of tRNA. When unfractionated tRNA was applied to a BDC column, 90% of the applied RNA (including all of the contaminating 5S RNA) was eluted by a linear salt gradient and an extended wash with 1 M NaCl. The remaining 10% was removed as a sharp peak by the application of ethanol in 2.5 M NaCl solution (data not presented).

Aminoacyl acceptor activities were not distributed equally between the BDC-ethanol and BDC-salt fractions (Table I). Over 70% of the phenylalanine-accepting capacity was present in the BDC-ethanol fraction which was enriched 4-fold with respect to the phenylalanine acceptance of the original tRNA. Acceptor activities for serine and cysteine were also markedly enriched, those for leucine and tryptophan were present in significant amounts, and there was no significant retention of either tyrosine or valine acceptor capacity in the BDC-ethanol fraction.

Reversed Phase Chromatography of Aminoacylated tRNA Fractions. The BDC-ethanol fraction contains a high proportion of certain leucine isoaccepting subspecies (Fig. 1a). Aminoacylation and RPC2 chromatography of unfractionated pea root tRNA (Fig. 1b) gave rise to six well defined peaks of leucyl-tRNA (numbered consecutively as shown) which correspond closely in mobility and relative amount to those described for pea and soybean cotyledon tRNA (8). However, the small amount of leucine acceptor capacity in the BDC-ethanol fraction (see Table I) consists almost exclusively of $tRNA_{4,5,6}^{leu}$.

In addition to the six numbered peaks, a further minor peak (Fractions 20 to 30, Fig. 1a) was eluted from the RPC2 column by low concentrations of salt. This material was present in larger relative amount in the tRNA of the BDC-ethanol fraction than in unfractionated tRNA; and, when the resolution of the early region of the chromatogram was increased (by decreasing the initial salt concentration from 0.35 M to 0.30 M), it was resolved into two components (Fig. 1b). The leading component was broad (fraction 20–45), contained no acid-precipitable radioactivity (Table II), and presumably was due to traces of free leucine arising either from deacylation during chromatography or from free amino acid carried through DEAE-cellulose chromatography after charging. By contrast,

Table I. *Amino Acid Acceptance of tRNA after Benzolyated DEAE-Cellulose Chromatography*

Fractions from BDC column were pooled and acylated as described in the text. Optimal ATP concentrations (mM) and Mg^{2+}: ATP ratios for each amino acid were: Cys, 4.0, 2.0; Leu, 2.0, 0.5; Phe, 4.0, 4.0; Ser, 2.0, 5.0; Trp, 2.0, 2.5; and Tyr, 2.0, 2.5.

Amino Acid	Amino Acid Acceptance			Activity in BDC-Ethanol Fraction
	Unfractionated tRNA	BDC salt fraction	BDC ethanol fraction	
	pmoles/A_{260} unit			%
Cys/2	25	30	75	17
Leu	86	89	60	6
Phe	46	7	150	70
Ser	15	15	36	12
Trp	31	32	27	6
Tyr	12	33	10	2
Val	...	33	8	2

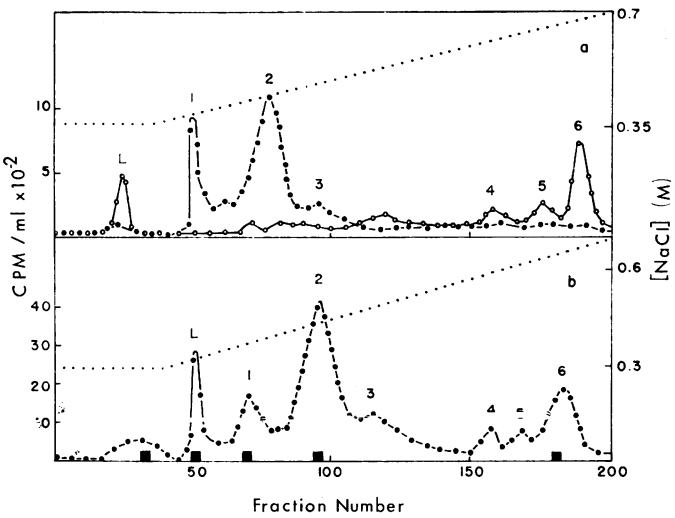

FIG. 1. Chromatography of leucine-accepting tRNA upon RPC2. a: tRNA from the BDC-salt fraction (2.5 A_{260} units) was charged with ^{14}C-leucine while tRNA from the BDC-ethanol fraction (1.5 A_{260} units) was charged with ^{3}H-leucine. After mixing and passage through DEAE-cellulose, the mixture was applied to a 0.6- × 120-cm RPC2 column and eluted with the sodium chloride gradient indicated. Radioactivity was determined directly on each fraction by dual-channel counting as described. [NaCl], ···; ^{14}C, ●——●; ^{3}H, ○——○. b: Unfractionated tRNA (20 A_{260} units) was charged with ^{14}C-leucine and applied to a RPC2 column at an initial salt concentration of 0.30 M. One-ml fractions were collected. The total acid-precipitable radioactivity in each was determined on a 0.2-ml aliquot by the addition of 1 mg of salmon sperm DNA and 0.1 ml of 50% w/v trichloroacetic acid. Those fractions indicated by the blocks on the abscissa were used for measurement of reacylation capability (Table II).

the trailing peak (fractions 50–52) was sharp, well resolved from leucyl-tRNA$_1^{leu}$ (fractions 62–72), and almost completely acid-precipitable. Because its ease of elution from RPC2 was suggestive of a fragmented molecule (L. C. Waters, personal communication), it was designated leucyl-tRNA$_L^{leu}$, and further evidence was sought as to its properties.

Gel Electrophoresis of BDC-ethanol tRNA. After cocharging with ^{3}H-leucine and ^{14}C-phenylalanine, tRNA from the BDC-ethanol fraction was further fractionated by polyacrylamide gel electrophoresis (Fig. 2). Phenylalanyl-tRNA migrated coincidently with the major optical absorbance peak. However, leucyl-tRNA was bimodally distributed; one peak (fraction 65) led the main absorbance peak while the other (fraction 82) trailed it. The leading peak consisted principally of leucyl-tRNA$_L^{leu}$ together with small amounts of contaminating leucyl-tRNA$_{4,5,6}^{leu}$ (Fig. 3). Since more rapid migration upon polyacrylamide gels under these conditions is indicative of a lower molecular weight, the data suggest that leucyl-tRNA$_L^{leu}$ is smaller than any of the major leucine subspecies.

Attempted Recharging of Deacylated tRNA. Confirmation that leucyl-tRNA$_L^{leu}$ was a small molecule with an impaired function was sought by examining its ability to reaccept leucine after deacylation. Fractions (as indicated in the legend to Fig. 2b) were taken from the RPC2 column, deacylated by incubation at pH 7.6, dialyzed, and recharged with ^{14}C-leucine in the presence of synthetase preparation.

As shown in Table II, leucyl-tRNA$_{1\&2}^{leu}$could be fully recharged. Leucyl-tRNA$_6^{leu}$ could be recharged to the extent of 46%, in agreement with the observations of Kelmers (18), who noted that tRNAs running late on RPC2 (those which contain isoprenoid nucleosides) often show impaired amino acid acceptance following chromatography. The early running leucyl-tRNA$_L^{leu}$, however, had completely lost its capacity to accept leucine. Renaturation of the tRNA$_L^{leu}$ fraction by the procedure of Lindahl, Adams, and Fresco (19) did not restore normal function. Clearly, critical areas of the molecule are missing.

Isoprenoid Nucleoside Content of Leucyl-tRNA$_L^{leu}$. Following a three-stage sequence of purification by BDC-chromatography, polyacrylamide gel electrophoresis, and RPC2 chromatography, the specific activity of leucyl-tRNA$_L^{leu}$ was in excess of 800 pmoles of leucine/A_{260} unit. While this specific attachment of leucine suggests a purity of greater than 50% of the theoretical maximum for a tRNA molecule, this must be considered a minimal value since it reflects both the considerable

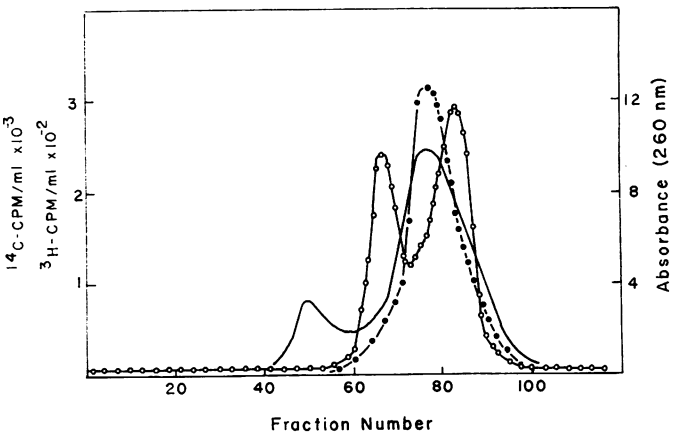

FIG. 2. Preparative gel electrophoresis of BDC-ethanol fraction tRNA charged with phenylalanine and leucine. tRNA from the BDC-ethanol fraction (150 A_{260} units), cocharged with ^{14}C-phenylalanine (150 nmoles) and ^{3}H-leucine (5.5 nmoles) was applied in a volume of 1 ml to a disc of 10% polyacrylamide gel. Electrophoresis was conducted at 250 v, 40 ma. Absorbance and radioactivity were determined directly upon fractions eluting from the base of the gel. A_{260}, ——; ^{14}C, ●——●; ^{3}H, ○——○.

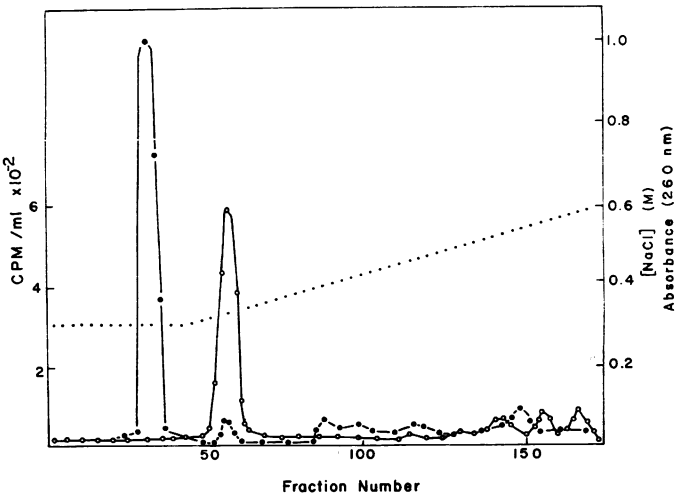

FIG. 3. RPC2 chromatography of leucyl-tRNA$_L^{leu}$. Fractions 60 to 70 (Fig. 3) containing high mobility ^{3}H-leucyl-tRNA from preparative gel electrophoresis were pooled and analyzed directly by RPC2 chromatography. A total of 15.5 A_{260} units was applied, and elution was begun with 0.3 M sodium chloride. Besides leucyl-tRNA$_L^{leu}$ (fractions 50–62) traces of leucyl-tRNAs$_{4,5,6}$ were noted (fractions 140–175). A_{260}, ●——●; ^{3}H, ○——○; [NaCl], ····.

Table II. *Discharge and Reacylation of RPC2 Fractions*

Specific 1-ml fractions were collected from the RPC2 column illustrated in Figure 2b. The total acid-precipitable radioactivity was determined on a 0.2-ml aliquot after the addition of 1 mg of salmon sperm DNA and 0.1 ml 50% (w/v) trichloroacetic acid. To another 0.3-ml aliquot, 0.15 ml of 1 M tris Cl (pH 7.6) was added, and the mixture was incubated for 30 min at 37 C. A portion (0.2 ml) was precipitated while duplicate aliquots were removed and reacylated in the standard 0.2-ml reaction mixture without added buffer or $MgCl_2$. After 15 min at 30 C these were also precipitated. The precipitates were collected on membrane filters, washed with 10% trichloroacetic acid, and dried; and the radioactivity was determined by liquid scintillation counting.

Fraction No.	Trichloroacetic Acid-precipitable Radioactivity			Reacylation
	Before discharge	After discharge	On reacylation	
	cpm			%
32	22	7	1	...
51	492	14	5	<1
68	344	18	263	77
94	561	11	537	96
180	243	7	112	46

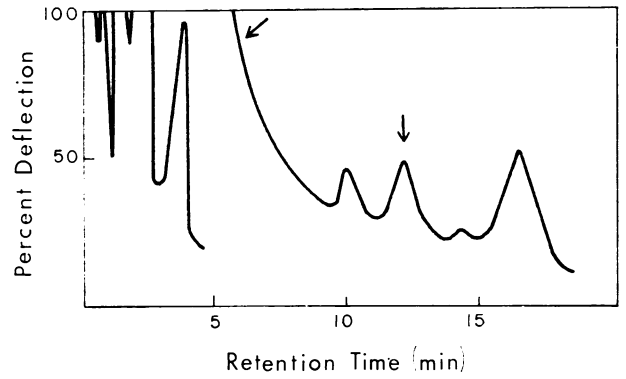

FIG. 4. Gas chromatographic analysis of $tRNA_L^{leu}$ hydrolysate. $tRNA_L^{leu}$ (0.62 A_{260} units) was hydrolyzed, dephosphorylated, and the lyophilized mixture of nucleosides was applied to a 0.3- × 60-cm partition column. The first five fractions from the column (which contained all the isoprenoid nucleosides of the hydrolysate) were lyophilized and treated with 30 μl of a mixture of equal volumes of N,O-bis(trimethylsilyl)trifluoracetamide and pyridine. Aliquots of 3 μl were examined at a column temperature of 255 C. Prior to a retention time of 5 min, the attenuation factor was × 64; subsequently it was × 4. The two arrows at retention times of 6 and 12.5 min indicate the points at which the trimethylsilyl derivatives of N^6-(3-methylbut-2-enyl) adenosine and N^6(*cis*-4-hydroxy-3-methylbut-2-enyl) adenosine would be expected to appear.

deacylation that occurs during purification and any loss of acceptor activity that may occur prior to aminoacylation.

After lyophilization and dialysis, a sample of this purified leucyl-$tRNA_L^{leu}$ was digested, and the isoprenoid nucleosides in the hydrolysate were determined by gas chromatographic analysis of the trimethylsilyl derivatives. The results (Fig. 4) indicated that the hydrolysate contained a substance (retention time 12.5 min) which cochromatographed with the trimethylsilyl derivative of authentic N^6-(*cis*-4-hydroxy-3-methylbut-2-enyl) adenosine (*cis*-ribosylzeatin). The combination of partition and gas-liquid chromatography allows a rather positive identification to be made, particularly since we have unequivocally shown (4) that this substance is the major isoprenoid nucleoside of unfractionated pea root tRNA. The other unidentified peaks in Figure 5 were due to solvent contamination or unknown minor components of the hydrolysate. Based on the assumption of homogeneity of the purified leucyl-$tRNA_L^{leu}$ sample, the content of *cis*-ribosylzeatin (1.7 $\mu g/A_{260}$ unit) supports the conclusion that leucyl-$tRNA_L^{leu}$ is about half the size of an intact tRNA.

Origin of Leucyl-$tRNA_L^{leu}$. Since $tRNA_L^{leu}$ was not aminoacylated *in vitro* (Table II), it is probably not a part of the normal transfer RNA complement of the cell but may be a degradation product of one of the six major leucyl-tRNA subspecies. Because both unfractionated bulk tRNA and the tRNA from the BDC-ethanol fraction contain significant amounts of $tRNA_L^{leu}$, it is improbable that this species is an artifact of chromatography on BDC. However, since root tissues contain highly active nucleases (9), it is possible that $tRNA_L^{leu}$ is formed by nucleolytic cleavage of a precursor during preparation of tRNA from pea roots. To test this hypothesis, unfractionated tRNA was heated to 85 C for 5 min in the presence of 0.1 mM $MgCl_2$ and then cooled. These conditions would allow a "nicked" species to dissociate into its constituent nonacylatable fragments. After aminoacylation and chromatography upon RPC2, there was no significant difference between the levels of leucyl-$tRNA_L^{leu}$ present in a heated and a control preparation (results not presented). The most likely remaining possibility is that $tRNA_L^{leu}$ is formed during aminoacylation *in vitro*.

Support for this concept was obtained from experiments in which tRNA from the BDC-ethanol fraction was charged with leucine and fractionated upon RPC2. Fractions containing either leucyl-$tRNA_L^{leu}$ or leucyl-$tRNA_6^{leu}$ were then isolated and rechromatographed upon BDC. As shown in Figure 5a, leucyl-$tRNA_L^{leu}$ was eluted by the salt gradient, whereas, prior to charging, it (or its precursor) had been retained in the BDC-ethanol fraction. Since in the control experiment, leucyl-$tRNA_6^{leu}$ was retained, as expected, in the BDC-ethanol fraction (Fig. 5b), the elution of leucyl-$tRNA_L^{leu}$ by the salt gradient was not due to the attachment of leucine *per se*. It

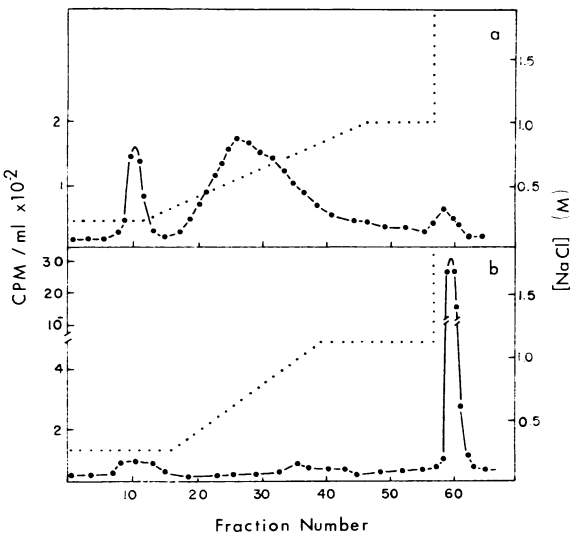

FIG. 5. Rechromatography of RPC2 fractions on BD-cellulose. tRNA from the BDC-ethanol fraction (20 A_{260} units) was chromatographed on RPC2 after charging with ^{14}C-leucine. Peaks corresponding to leucyl-$tRNA_L^{leu}$ and leucyl-$tRNA_6^{leu}$ were collected, precipitated with ethanol, and subjected to rechromatography on separate BDC columns. a: leucyl-$tRNA_L^{leu}$; b: leucyl-$tRNA_6^{leu}$. [NaCl], ···; ^{14}C, ●——●.

follows, therefore, that tRNA from the BDC-ethanol fraction does not contain significant quantities of $tRNA_L^{leu}$ prior to aminoacylation.

If a component of the synthetase preparation modifies one of the major leucine tRNA species, an increase in the ratio of synthetase to tRNA in the reaction mixture, or prolonged contact between the reactants would increase the formation of leucyl-$tRNA_L^{leu}$ and decrease the formation of that species which is its immediate precursor. Figures 6a and 6b illustrate the point. Increasing the reaction time from 15 to 45 min increased the quantity of leucyl-$tRNA_L^{leu}$ and decreased the amount of leucyl-$tRNA_6^{leu}$ formed. A similar result was obtained by increasing the ratio of synthetase to tRNA. The outcome was the same when the isotopic labels were reversed. Leucyl-$tRNA_6^{leu}$ appears, therefore, to be the precursor of leucyl-$tRNA_L^{leu}$ although final proof must await sequence studies.

All data indicate that a factor in the synthetase preparation is responsible for the conversion of the precursor tRNA into leucyl-$tRNA_L^{leu}$. Evidence that this factor is heat-labile and probably a protein was obtained by incubating ^{32}P-tRNA with normal or boiled synthetase preparations. As shown in Figure 7, leucyl-$tRNA_L^{leu}$ was produced only in the presence of an unheated synthetase preparation, thus eliminating the possibility that it was formed by a nonenzymatic process.

Finally, we were not able to detect the formation of other aminoacyloligonucleotides when bulk tRNA was charged with a ^{14}C-amino acid mixture (omitting leucine) and examined by gel electrophoresis (Fig. 8a). Radioactivity migrated in a single peak coincident with the major band of absorbance, while the small amount (5% of the total) of leucyl-$tRNA_L^{leu}$ formed during aminoacylation of unfractionated tRNA with leucine was plainly visible (Fig. 8b). Neither were aminoacylated fragments seen upon RPC2 chromatography of bulk tRNA charged with tyrosine, tryptophan, or phenylalanine. A similar analysis of tRNA from the BDC-salt fraction charged with leucine also failed to reveal fragments (see Fig. 8a).

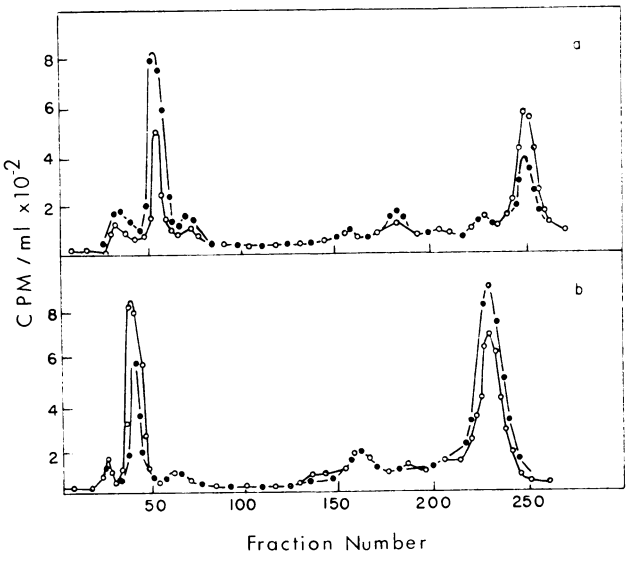

FIG. 6. Effect of time of incubation and synthetase concentration upon formation of leucyl-$tRNA_L^{leu}$. tRNA from the BDC-ethanol fraction was charged with leucine either for an extended period or with an increased synthetase level. The products were subjected to chromatography upon RPC2. a: ^{3}H-Leucyl-tRNA from a standard reaction mixture incubated for 15 min; ^{14}C-leucyl-tRNA from a standard reaction mixture incubated for 45 min. b: ^{14}C-Leucyl-tRNA prepared by incubation with one-half the normal synthetase preparation; ^{3}H-leucyl-tRNA prepared by incubation with 2.5 times the normal synthetase preparation. ^{14}C, ●——●; ^{3}H, ○——○.

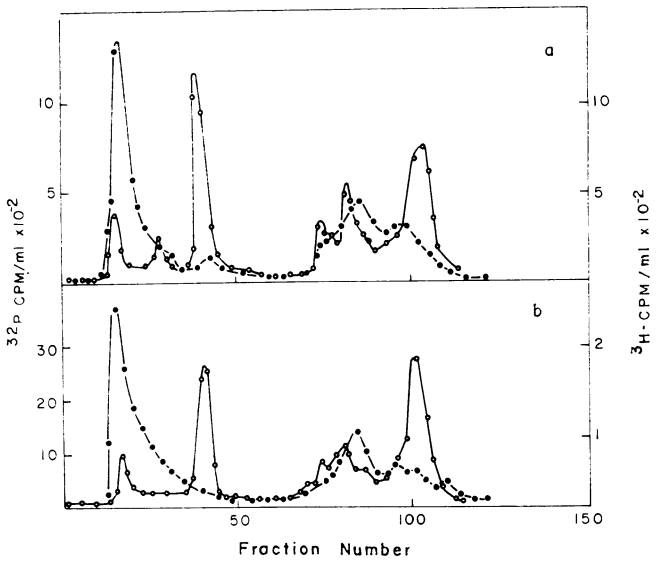

FIG. 7. Inability of heated synthetase preparation to form leucyl-$tRNA_L^{leu}$. a: RPC2 chromatogram of ^{32}P-tRNA (BDC-ethanol fraction) charged with ^{3}H-leucine in the standard reaction mixture. ^{32}P, ●——●; ^{3}H, ○——○. b: RPC2 chromatogram of ^{32}P-tRNA (BDC-ethanol fraction) incubated with heated synthetase preparation and nonradioactive leucine in the standard reaction mixture. Before chromatography an aliquot of unlabeled tRNA from the BDC-ethanol fraction was charged in the normal way with ^{3}H-leucine and added in order to be able to identify the positions of leucyl-$tRNA_L^{leu}$ and other species. The ability of the unheated synthetase preparation to produce ^{32}P-leucyl-$tRNA_L^{leu}$ (fraction 40, a) should be contrasted with the inability of the heated synthetase preparation to do so.

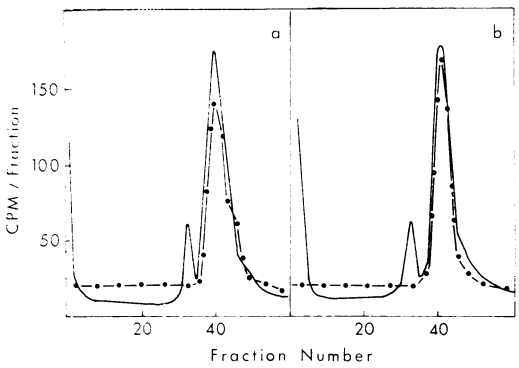

FIG. 8. Analytical gel electrophoresis of aminoacyl tRNA. a: Unfractionated tRNA (5 A_{260} units) was charged with a ^{14}C-amino acid mixture (omitting leucine) in the standard aminoacylation reaction, deproteinized, applied to DEAE-cellulose, concentrated by ethanol precipitation, and applied to an analytical scale gel. b: Unfractionated tRNA (5 A_{260} units) charged with ^{14}C-leucine and treated as above. Densitometer tracing, ——; ^{14}C cpm/slice, ●——●.

DISCUSSION

The unusual leucyl-$tRNA_L^{leu}$ species described here exhibits behavior on gel electrophoresis and RPC2 chromatography expected of a fragmented tRNA molecule, a conclusion supported by its inability to reaccept leucine after chemical deacylation. Although we have not demonstrated homogeneity

by stringent physical criteria, the isoprenoid nucleoside content of the fragment is high enough to suggest that it is homogeneous and contains 31 to 33 base residues. This is probably a low estimate since the presence of both leucine and an isoprenoid nucleoside is indicative of a fragment extending from the 3′ terminus to the anticodon. Published sequences of other tRNAs have a minimum of 40 bases between these two sites.

Our data establish that leucyl-$tRNA_L^{leu}$ is formed by the highly specific cleavage of leucyl-$tRNA_6^{leu}$ catalyzed by a factor present in the synthetase preparation. J. H. Cherry (personal communication) has obtained similar findings. A final decision as to whether this specificity is a property of the $tRNA_6^{leu}$ precursor or of a selective nuclease (1, 16, 23) must await isolation of such an enzyme. So, too, any assessment of the physiological role of $tRNA_L^{leu}$ must await a demonstration of its presence *in vivo*.

A comparison of $tRNA_L^{leu}$ with $tRNA_F^{leu}$ (16) shows that the two molecules possess considerable similarity. Both are small; both have unusual chromatographic mobility (the mobility of $tRNA_F^{leu}$ has not been reported, possibly because under the gradient conditions normally employed it is not retained by these columns); and neither can be aminoacylated once they have been discharged. However, leucyl-$tRNA_L^{leu}$ contains an isoprenoid nucleoside, and hence its precursor, leucyl-$tRNA_6^{leu}$, is probably UUPu-specific (3) (consistent with its mobility on BDC ([24] and RPC2 [18]). On the other hand, $tRNA_F^{leu}$ arises from the major $tRNA_1^{leu}$ of *E. coli* which recognizes CUG and lacks an isoprenoid nucleoside. Moreover, the site of cleavage in *E. coli* $tRNA_1^{leu}$ lies in the "extra arm" of the molecule (31), while our data indicate that pea root $tRNA_6^{leu}$ retains integrity of this region and is cleaved on the 5′ side of the isoprenoid nucleoside (probably in the anticodon region).

It is clear that differences as well as similarities exist, yet $tRNA_L^{leu}$ and a factor responsible for its production could play a part in a "codon restriction" process operating in plant development in an analogous fashion to that seen during cessation of host protein synthesis after infection of *E. coli* by T-even bacteriophage. Alternatively, leucyl-$tRNA_L^{leu}$ might be implicated in other control mechanisms unique to plant systems where free isoprenoid nucleosides possess cytokinin activity. The factor may be part of an excision-replacement-repair mechanism allowing incorporation of exogenous cytokinin into tRNA (6, 10). Or it may be that degradation of a cytokinin-containing tRNA (such as $tRNA_6^{leu}$) may control the intracellular levels of free cytokinin (7). In this regard, the observations of Cherry (2, 5, 8) concerning predictable and consistent variation in the relative levels of $tRNA_{5,6}^{leu}$ from plant sources may be significant.

If leucyl-$tRNA_L^{leu}$ is an intermediate for the production of free isoprenoid nucleosides, the process of degradation may be under feedback control by end product inhibition. Of the possible end products of such a pathway, only N^6-(3-methylbut-2-enyl) adenosine was available to test this hypothesis. It was found, however, that the formation of leucyl-$tRNA_L^{leu}$ was not affected by the addition of this cytokinin to the aminoacylation reaction mixture at a concentration of 1 mM.

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