Plant Physiol. (1979) 63, 87–92
0032-0089/79/63/0087/06/$00.50/0

Purification of Leucine tRNA Isoaccepting Species from Soybean Cotyledons

II. RPC-2 PURIFICATION, RIBOSOME BINDING, AND CYTOKININ CONTENT[1]

Received for publication May 1, 1978 and in revised form August 15, 1978

BRUCE R. LESTER[2], ROY O. MORRIS[3], AND JOE H. CHERRY[4]
Department of Horticulture, Purdue University, West Lafayette, Indiana 47907

ABSTRACT

Two of the six leucine isoaccepting tRNA species from soybean (*Glycine max*) cotyledons recognize U-beginning codons, and contain cytokinin moieties in their structure. These same two isoaccepting species have been shown to undergo quantitative changes in their relative amounts upon treatment with N^6-benzyladenine *in vivo*. In addition a procedure has been developed for purification of the isoaccepting species of leucine tRNA from soybean cotyledons resulting in isoacceptors of minimum purity, calculated by amino acid acceptance capacity, of from 46 to 78% leucine tRNA.

The idea that tRNA has a regulatory function in the translation of mRNA originated with Ames and Hartman (1) and was expanded and modified by others (2, 13–15). The salient feature of this proposal was that the rate of synthesis of certain proteins was limited not by the supply of the appropriate mRNA but by the supply of specific tRNAs required to read "rate-limiting" or minor codons present in the message.

A cytokinin is present in the structure of several tRNA isoaccepting species. Because the mechanism of hormonal action of the cytokinins is not understood one can propose that the cytokinin molecule is involved in the activity of specific isoaccepting tRNA. The native cytokinin is adjacent to the anticodon of specific tRNAs from mycoplasma to man (12). In addition, it has been shown that all known cytokinin-containing tRNA species translate codons with the initial nucleotide U (8, 12). It seems clear from previous studies (5) that the presence and integrity of the N^6-Δ^2 (isopentenyl) adenosine adjacent to the anticodon is essential for proper binding to the messenger ribosome complex and thus of importance for protein synthesis.

After purification of various isoaccepting species of leucine tRNA present in soybean some fundamental questions concerning the connection between these tRNAs and cytokinin might be answered. For example, do any of the leucine tRNAs from soybean possess cytokinin activity, and if so which specific species? Does the presence of cytokinin in the particular leucine tRNA isoaccepting species correlate with the ribosome-directed binding of this tRNA to U-beginning codons? Are the cytokinin-containing tRNA isoaccepting species related to the specificities of the various tRNA synthetases? Is there a correlation between cytokinin-containing isoaccepting species and the leucine isoacceptors which undergo alteration in level upon N^6-benzyladenine treatment in soybean cotyledons? This report presents some of the results of these studies.

MATERIALS AND METHODS

***Escherichia coli* Ribosomes.** *E. coli* strain KB was grown from a lyophilized standard media containing per liter: 10 g Bactopeptone, 1 g yeast extract, 5 g NaCl, 1.36 g KH_2PO_4, 6.97 g K_2HPO_4, and 0.5% (v/v) glycerol. They were harvested in mid-log phase and washed with standard ribosome buffer prior to ribosome preparation (11). Extraction and partial purification of ribosomes were carried out at 4 C. The *E. coli* KB cells were harvested in mid-log phase by centrifugation and washed through ribosome extraction buffer (10 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 0.06 M KCl, and 6 mM 2-mercaptoethanol). The cells were disrupted by grinding with twice their wet weight of A-301 alumina at 4 C for 15 min. The resulting paste was suspended in ribosome extraction buffer (three times the cells' wet weight) and centrifuged three times at 30,000*g* for 20 and 60 min. The pellets were discarded after each centrifugation. The final supernatant (S-30 fraction) was centrifuged at 105,000*g* for 2 hr in a Beckman-Spinco model L to pellet the ribosomes. The ribosomes were washed by being suspended in ribosome extraction buffer by gentle homogenization followed by centrifugation again. The final ribosome pellet was suspended in the original volume of ribosome extraction buffer and kept at −84 C until used.

Ribosome-binding Assay. The assay procedure was essentially that of Nirenberg and Leder (11) with slight modifications. The reaction mixture (0.1 ml) consisted of 0.1 M Tris-HCl buffer (pH 7.5), 20 mM $MgCl_2$, 0.05 M KCl, 4.0 OD_{260} units of *E. coli* ribosomes, and either 40 nmol of phosphorus with respect to the polynucleotide polymer (A,U) or 0.2 OD_{260} units of the triplet U,U,G. tRNA from 600 to 18,000 cpm, depending upon the isoacceptor used, was added to each reaction mixture. The reaction mixture was kept at 4 C and the tRNA was added last to initiate the reaction. The reaction was incubated at 30 C for 15 min after which it was chilled to 4 C and 3 ml of the reaction buffer was added immediately. Cellulose nitrate membrane filters (Millipore HAWP, 0.45-μm pore size and 25-mm diameter) were washed with 5 ml of reaction buffer. The reaction mixture was added and washed with three 3-ml portions of buffer at 4 C. The filters were dried and counted in a Packard Tri-Carb liquid scintillation spectrometer.

Other Materials and Methods. Plant material, tRNA extraction,

[1] Journal Paper No. 6633 of the Purdue Agricultural Experiment Station.

[2] Present address: Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030.

[3] Present address: Department of Agricultural Chemistry, Oregon State University, Corvallis, Oregon.

[4] To whom correspondence should be addressed.

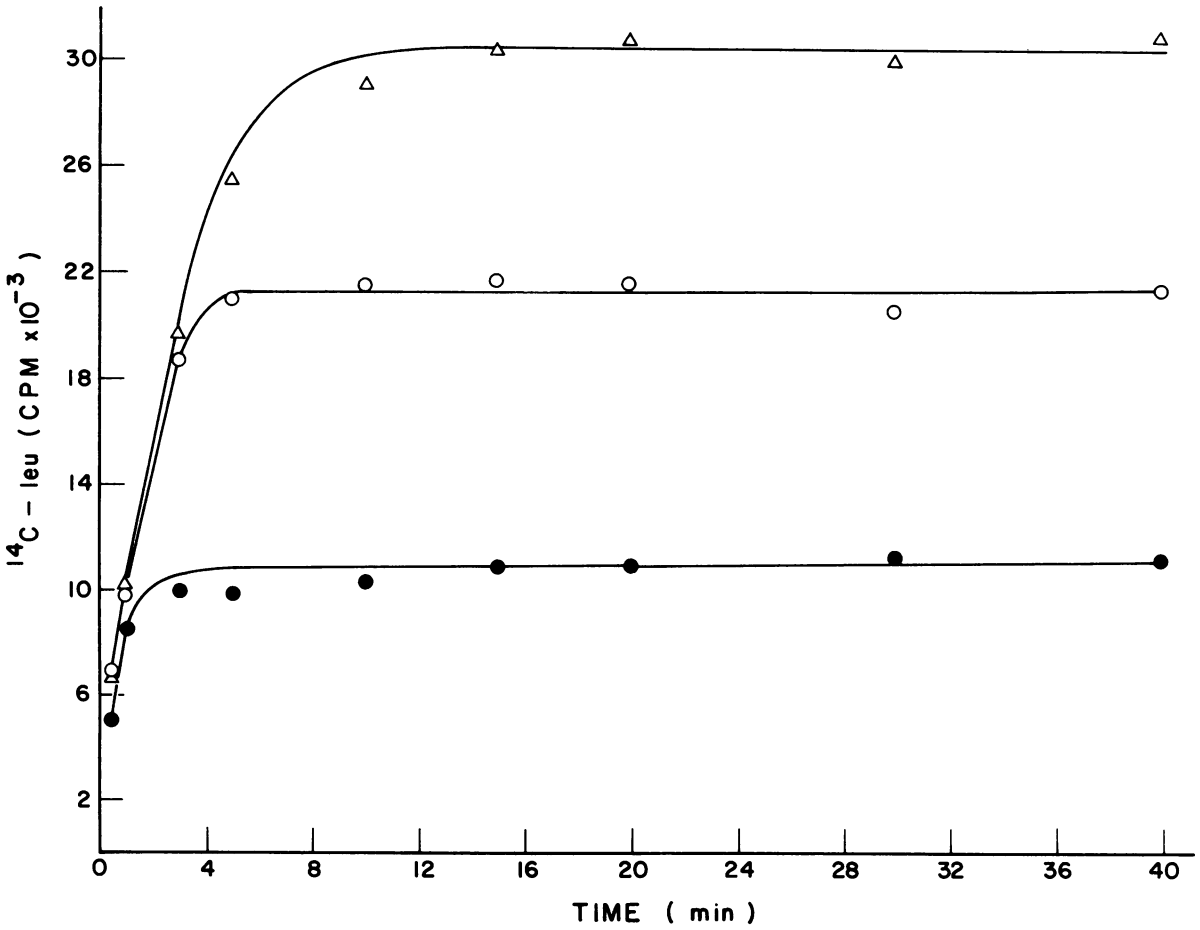

FIG. 1. Stoichiometric acylations of different concentrations of tRNA at optimum enzyme concentrations. Various tRNA concentrations were acylated as outlined in reference 10. The 0.2-ml reaction mixture contained 0.04 ml leucine tRNA synthetase (4.14 mg protein/ml) and 4.0 OD_{260} units (●), 8.0 OD_{260} units (○), and 12.0 OD_{260} units (△) of tRNA per reaction mixture.

leucine tRNA synthetase purification, tRNA aminoacylation assay column chromatography, and phenoxyacetal modification are as described in reference 10. The nucleotide triplet U,U,G and the polynucleotide co-polymer, poly(U,A), were gifts from P. T. Gilham, Purdue University.

RESULTS

As an initial step in these studies it was necessary to isolate and purify the soybean cotyledon leucine tRNAs. BDC[5] chromatography of phenoxyacetyl-modified leucine tRNA was chosen as the initial leucine purification of the leucine species from a total tRNA extract. BDC chromatography was performed as described by Gillam *et al.* (6) with minor modifications as in reference 10.

Stoichiometric Acylation. To ensure maximal purification of leucine tRNA in the BDC step it was necessary to achieve essentially complete acylation of the leucine tRNA. This was accomplished by acylating the tRNA in a stoichiometric procedure, *i.e.* to completion and in a minimum time to limit base-catalyzed hydrolysis. Under these conditions the concentration of enzyme was greater than the concentration of substrate and the limiting component was tRNA. Completion of charging under various enzyme concentrations to determine the enzyme concentration which was optimum with respect to time and stoichiometry has been shown previously (10). Figure 1 shows that tRNA is the limiting component in this reaction which ensures stoichiometry. The plateau acylation values are linear with respect to tRNA concentration showing that stiochiometric acylation is taking place. Furthermore, the calculated values of the percentage of leucine tRNA in the total tRNA preparation are quite close to theoretical values (*i.e.* 2.4% to 4.0%).

Isolated tRNA (2,500 OD_{260} units) was acylated in two batches using the enzyme and tRNA concentrations calculated above (Fig. 1). The first reaction mixture contained the bulk of the tRNA (2,000 OD_{260} units) with unlabeled leucine. The second reaction mixture contained 500 OD_{260} units of tRNA plus labeled leucine (^{14}C). After the reaction was complete (10 min), the reaction mixtures were combined and diluted with Na-acetate buffer to yield a final concentration of 10 mM Na-acetate (pH 4.5), 10 mM $MgCl_2$, 3 mM NaN_3, and 0.3 M NaCl. This solution was added to a 13-ml DEAE column equilibrated with the above buffer and washed with buffer in 0.4 M NaCl until the OD_{260} dropped below 0.025. The bound acylated tRNA was eluted with 1.0 M NaCl in Na-acetate and precipitated in 2 volumes of cold 95% ethanol overnight. Ninety per cent of the OD_{260} units and 95% of the cpm were recovered.

Modification and Fractionation of Leucine tRNA. After the tRNA had been stoichiometrically charged, purified from the reaction components and modified as described in reference 10, it was separated into the salt and ethanol fractions on a BDC column. Ninety-five per cent of the tRNA and cpm added to the BDC column were recovered (87% of the original OD_{260} units and cpm). The fractions containing radioactivity were pooled, and

[5] Abbreviations: BDC: benzoylated diethylaminoethyl cellulose; B-DEAE: benzoylated diethylaminoethyl.

dialyzed against 4 liters of distilled H_2O and lyophilized to dryness. This fraction contained 5.3% of the starting OD_{260} units and 85.3% of the starting cpm. The BDC tRNA was characterized as to UV spectra, size, and homogeneity, acylation potential and isoacceptor integrity of leucine tRNA (10).

RPC-2 Purification of the B-DEAE tRNA. The BDC tRNA was discharged by base-catalyzed hydrolysis after precipitation by 95% ethanol. The discharged BDC tRNA, which was estimated to be at least 32 to 35% leucine tRNA, was loaded on a RPC-2 column (2.6 × 35 cm) and eluted with a 500-ml linear gradient of 0.45 M to 0.75 M NaCl in Na-acetate (Fig. 2). Two hundred 2.5-ml fractions were collected and the OD_{260} profile of the column was determined. Aliquots of the eluted fractions were utilized in postacylating the column to determine the acceptor activity profile.

Isoaccepting species I and V were separated sufficiently to be pooled, dialyzed against 4 liters of distilled H_2O, and lyophilized (Fig. 2). Isoacceptors II, III, and IV were not separated sufficiently and therefore the activities of these three peaks were pooled, concentrated, and then reapplied to the RPC-2 column and reeluted with a 500-ml linear gradient from 0.5 M NaCl to 0.65 M NaCl in Na-acetate. Figure 3 shows a significant increase in separation of these species. This column was also postacylated and the isoaccepting activities were pooled, dialyzed against 4 liters of distilled H_2O and lyophilized for storage. Isoaccepting species VI was not clearly defined on the first column (Fig. 2), therefore, fractions 140 to 180 were pooled, concentrated, and rechromatographed on RPC-2. Figure 4 shows that after postcharging, isoaccepting species VI was detected. The nature of the post-VI OD_{260} absorbing material is not known and due to its disperse character was disregarded. The activity in the sharp peak was pooled, dialyzed against 4 liters of distilled H_2O, and lyophilized for storage.

Calculation of Minimum Purity. Aliquots of 0.02 ml from each fraction of the three RPC-2 columns (Figs. 2–4) were used in stoichiometric assays to determine the minimum purity of the leucine isoaccepting species. Reaction conditions were identical to those outlined under "Materials and Methods" with the exception that [^{3}H]leucine with a specific radioactivity of 7,000 mCi/mmol was used. This was done to increase the leucine concentration from 1.2×10^{-6} M to 8×10^{-6} M with high specific radioactivity to reduce background interference. Table I shows the calculated minimum purity of each isoaccepting species from RPC-2. Since the cpm profile is coincident with the OD_{260} profile it is not unreasonable to assume that the percentage of OD_{260} units not accounted for in these calculations is probably due to unacylatable tRNA of the same character as the isoaccepting tRNA in each peak but unable to be charged due, probably, to loss of all or part of the CCA terminus.

Codon Recognition. The isoaccepting species of leucine tRNA were tested as to their ability to recognize the two leucine U-beginning codons (U,U,A and U,U,G) in an *E. coli* ribosome-directed binding assay. An acylation reaction using 100 OD_{260} units of tRNA and [^{3}H]leucine was run according to the conditions outlined under "Materials and Methods" and the charged tRNA was loaded onto an RPC-5 column (1.0 × 15 cm). The column was eluted with a 500-ml linear gradient from 0.4 M NaCl to 0.7 NaCl in Na-acetate (pH 4.5). A typical RPC-5 profile was obtained yielding good separation of the six leucine isoaccepting tRNA species (see Fig. 2 of ref. 10). The fractions in each isoaccepting peak were pooled, concentrated on 1.0 ml DEAE-

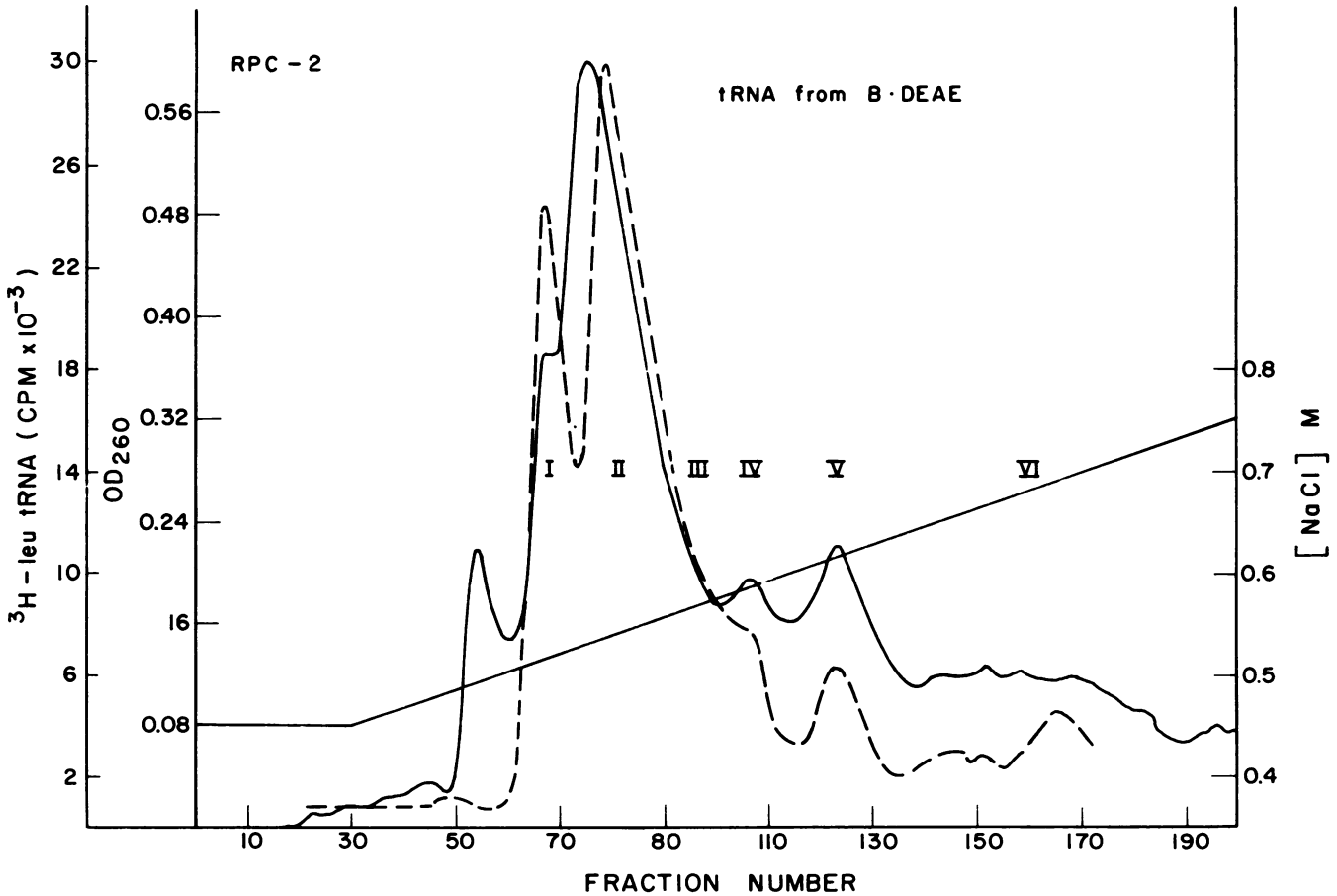

FIG. 2. RPC-2 chromatography of leucine tRNA from BDC column. Leucine tRNA purified on BDC after being discharged and concentrated was chromatographed on a RPC-2 column. OD_{260} (——) was determined and leucine tRNA acceptor activity (- - -) was calculated by postcharging as outlined in "Calculation of Minimum Purity" under "Results."

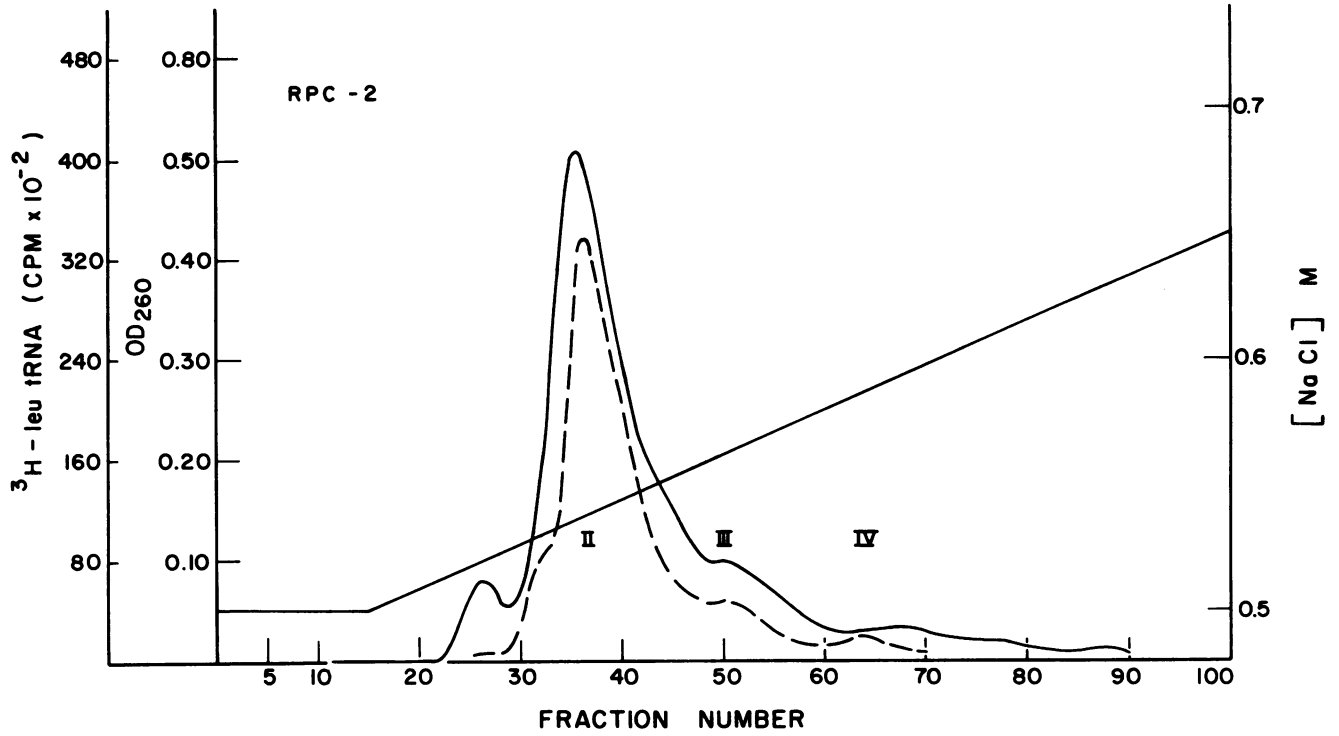

FIG. 3. RPC-2 rechromatography of leucine tRNA isoacceptors II, III, and IV from Figure 2. Conditions were the same as described in Figure 2. tRNA was pooled and diluted, followed by chromatography on new RPC-2 column. OD_{260} (——) and leucine acceptor capacity (- - -) were determined.

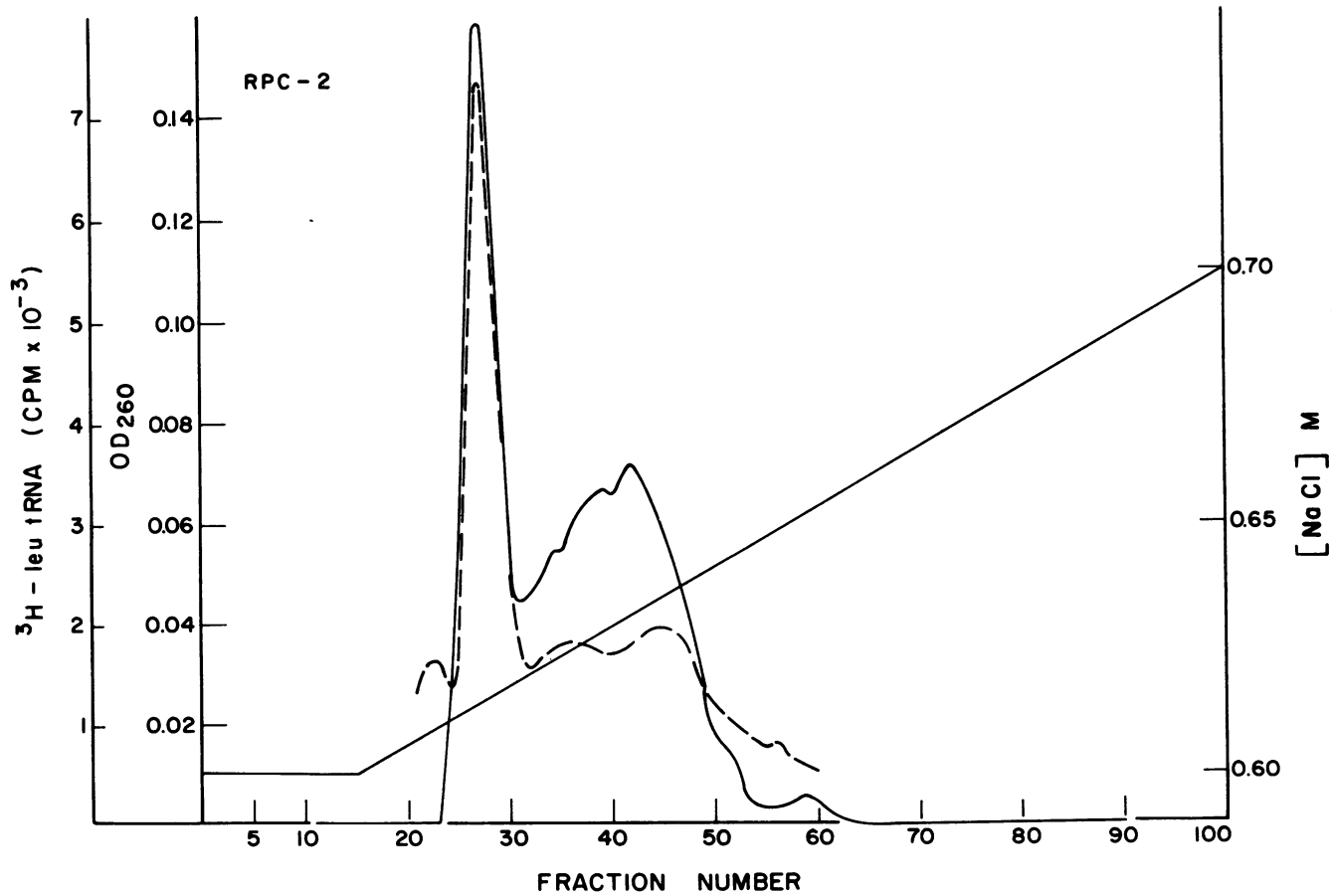

FIG. 4. RPC-2 rechromatography of leucine tRNA isoacceptor VI. Leucine tRNA isoacceptor species VI was rechromatographed on a RPC-2 column according to the conditions given for Figure 3. OD_{260} (——) and leucine acceptor capacity (- - -) were determined.

cellulose columns, dialyzed, and used for ribosome-directed triplet-binding assays as outlined in "Ribosome-binding Assay" under "Materials and Methods." Table II gives the results of these binding assays. It appears that only isoaccepting species V and VI bind to U-beginning codons. Isoaccepting species V appears to bind primarily to the codons (U,U,G); however, there is some (U,U,A) binding also (12%). Each isoaccepting species probably does not represent a single codon even though there are six leucine codons and six leucine isoaccepting tRNA species from RPC. Therefore, it would not be unusual to find that each isoaccepting tRNA species of leucine isolated by RPC-5 recognizes more than one codon. Isoaccepting species VI seems to recognize both codons equally well; however, the wobble hypothesis prediction of ambiguous recognition in the third position of some codons must be taken into account (4). For example, A in the third position of a codon recognizes only U in the first position of the anticodon but G in the third position of a codon recognizes either C or U; therefore, the triplet codon U,U,A will only recognize the anticodon U,A,A but the triplet codon U,U,G will recognize both its natural anticodon being detected in species VI were U,U,A. Alternatively, it might be, as mentioned above, that there are equal amounts of the anticodons U,U,A and C,A,A represented in isoaccepting species VI. The important datum is that only isoaccepting species V and VI appear to possess the ability to bind to U-beginning codons, which probably all contain a natural cytokinin in the position 3′ to the anticodon (8). Charging specificities of unacylated tRNA from the salt and ethanol fraction of the BDC column tend to substantiate these results and vice versa (10). Isoaccepting species V and VI of leucine tRNA seem to contain intrinsic hydrophobic character, which might represent the presence of cytokinin in the structure. Additionally, these species are able to recognize U-beginning codon triplets in a ribosome-directed binding assay, again an indication of cytokinin presence in the structure.

Cytokinin Content. The purified leucine tRNA isoaccepting species were assayed as to their cytokinin content by gas chromatographic methods previously described (3). It was found that leucine isoaccepting species I, V, and VI showed hydrolysis products which migrated in the positions of standard cytokinin-like compounds. Peak I contained a hydrolysis product similar to zeatin riboside; comparison of the amount of this hydrolysis product with the purity of the sample showed that it was present in amounts too high by severalfold. We concluded that peak I tRNA probably does not contain cytokinin compounds. Peaks V and VI contained hydrolysis products analogous to hypermodified bases and in amounts commensurate with their purities. Peak V hydrolysis products migrated in positions on GLC coincident to zeatin riboside or 2-methyl,thiol-N^6)Δ^2-isopentenyl) adenosine. Peak VI appeared to have hydrolysis products which migrated in GLC coincident to both N^6, (Δ^2-isopentenyl) adenosine and zeatin riboside.

Table I. Minimum purity calculations from post-charged tRNA in Figures 2, 3 and 4.

Acylation Capacity	Leucine Isoaccepting Species					
	I	II	III	IV	V	VI
p moles leu/OD of tRNA	1,265	1,330	93	1,090	716	920
% minimum purity*	76	79	56	66	43	55

*1667 p moles leucine/OD tRNA = 100%

DISCUSSION

One of the aims of this research was to ascertain if there was a possibility of coincidence between hormone treatments, increases, and decreases in the abundance of minor tRNA isoacceptor species, and effects on plant differentiation and/or aging. Previous work from this laboratory has shown that soybeans, if treated with a synthetic cytokinin, N^6-benzyladenine, showed an increase in the abundance of leucine tRNA isoaccepting species V and VI and a concomitant decrease in isoaccepting species I in both cotyledons and hypocotyls; a deferral of the onset of senescence in cotyledons, and no effect on the leucine tRNA synthetases (2). In addition it was found that soybean hypocotyl tissue contained very little leucine tRNA isoaccepting species V and VI and that it contained almost no leucine tRNA synthetase specific for acylating these isoaccepting species (2, 9). These results demonstrated for the first time in plants that application of a hormone influenced the abundancy of a specific tRNA isoaccepting species. It was an interesting possibility that these changes in isoaccepting tRNA species were a direct result of cytokinin treatment. Since the codons for leucine include two U-beginning triplets it was not unreasonable to assume that one or more of the leucine tRNA isoacceptors from RPC-2 contain cytokinin in its structure, probably 3′ to the anticodon. All tRNAs which recognize U-beginning codons were found to contain cytokinin or a close derivative in that position (7, 12). The problems were to ensure that this was the case in plants and to ascertain which isoaccepting leucine tRNAs from RPC-2 were the cytokinin-containing species. The ribosome-binding studies show that leucine tRNA isoaccepting species V and VI recognize U-beginning triplet codons in the *E. coli* ribosome-directed binding assays. These results do not demonstrate that leucine tRNA isoaccepting tRNA Species V and VI contain only U-beginning codon recognition capacity but they do show that no other isoaccepting species is capable of recognizing U-beginning codons. In addition, it has been shown that the purified isoaccepting species of leucine tRNA contain hypermodified bases as an integral constituent of their structure. Specifically, isoaccepting species V contains zeatin riboside and possibly 2-methylthioisopentenyl adenosine, while isoaccepting species VI was shown to contain zeatin riboside and isopentenyl adenosine. From our data, no other isoaccepting species of leucine tRNA contained any hypermodified bases except isoaccepting

Table II. Codon triplet recognition by the isoaccepting leucine tRNA species.*

Fraction	cpm added	cpm no polymer or triplet	Poly UA			UUG		
			cpm polymer	cpm net	% over non-specific binding	cpm triplet	cpm net	% over non-specific binding
$tRNA_1$	9,843	187±9	193±11	6	3	202±11	15	8
$tRNA_2$	18,317	3825±190	3636±171	0	0	3494±168	0	0
$tRNA_3$	3,472	427±37	439±19	15	3	397±23	0	0
$tRNA_4$	1,950	558±9	500±12	0	0	353±8	0	0
$tRNA_5$	1,176	466±7	521±13	55	1	790±9	324	70
$tRNA_6$	625	114±10	221±15	107	9	222±17	107	95

*Each value in a cpm category is the result of three experiments with four replications except for $tRNA_4$ which is the result of four experiments with four replications.

species I. The hypermodified base content was entirely too high to be accounted for by the tRNA present and could represent contamination from other aminoacyl tRNAs or breakdown products of leucine isoaccepting species V and VI (3). Isoaccepting species I probably does not contain hypermodified bases in its structure, especially when the ribosome-binding data and acylation specificity of uncharged, unmodified tRNA from BDC (10) are considered. The possibility exists that each isoaccepting species can recognize more than one codon. However, the isoaccepting species containing the specificity for U-beginning triplets and the isoaccepting species which have cytokinin present in their structure are the same in soybean leucine tRNA, namely V and VI.

LITERATURE CITED

1. AMES BN, PE HARTMAN 1963 The histidine operon. Cold Spring Harbor Symp Quant Biol 28: 349–356
2. ANDERSON MB, JH CHERRY 1969 Differences in leucyl-tRNA's and synthetase in soybean seedlings. Proc Nat Acad Sci USA 62: 202–209
3. BABCOCK DF, RO MORRIS 1973 Specific degradation of a plant leucyl-tRNA by a factor in the homologous synthetase preparation. Plant Physiol 52: 292–292
4. BRETSCHER MS, OW JONES 1967 The biochemistry of the genetic code. *In* PN Campbell, JR Sargent, eds, Techniques in Protein Biosynthesis, Vol. 1. Academic Press, London.
5. GEFTER ML, RL RUSSEL 1969 Role of modifications in tyrosine tRNA: a modified base affecting ribosome binding. J Mol Biol 39: 145–157
6. GILLAM I, D BLEW, RC WARRINGTON, M VON TIGERSTROM, GM TENER 1968 A general procedure for the isolation of specific transfer ribonucleic acids. Biochemistry 7: 3459–3468
7. HALL RH 1970 N^6-(Δ^2-Isopentenyl)adenosine: chemical reactions, biosynthesis, metabolism and significance to the structure and function of tRNA. *In* JN Davidson, EW Cohn, eds, Progress in Nucleic Acid Research and Molecular Biology, Vol. 10. Academic Press, New York, p. 57
8. HALL RH 1971 The Modified Nucleosides in Nucleic Acids. Columbia University Press, New York
9. KANABUS J, JH CHERRY 1971 Isolation of an organ-specific leucyl-tRNA synthetase from soybean seedlings. Proc Nat Acad Sci USA 68: 873–876
10. LESTER BR, JH CHERRY 1979 Purification of leucine tRNA isoaccepting species from soybean cotyledons. I. Benzoylated diethylamino cellulose fractionation. N-hydroxysuccinimide modification and characterization of product. Plant Physiol 63: 79–86
11. NIRENBERG M, P LEDER 1964 RNA codewords and protein synthesis. The effect of trinucleotides upon the binding of sRNA to ribosomes. Science 145: 1399–1407
12. SKOOG F, DJ ARMSTRONG 1970 Cytokinins. Annu Rev Plant Physiol 21: 359–384
13. STENT GS 1964 The operon: on its third anniversary. Science 144: 816–820
14. SUBAK-SHARPE H, J HAY 1965 An animal virus with DNA of high guanine + cytosine content which codes for sRNA. J Mol Biol 12: 924–928
15. SUEOKA N, T KANO-SUEOKA 1964 A specific modification of leucyl-sRNA of *E. coli* after phage T_2 infection. Proc Nat Acad Sci USA 52: 1535–1540