Plant Physiol. (1981) 67, 1185–1189
0032-0889/81/67/1185/05/$00.50/0

Cytokinin-Active Ribonucleosides in *Phaseolus* RNA.

II. DISTRIBUTION IN tRNA SPECIES FROM ETIOLATED *P. VULGARIS* L. SEEDLINGS[1]

Received for publication July 28, 1980 and in revised form November 24, 1980

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ABSTRACT

The distribution of cytokinin-active ribonucleosides in tRNA species from etiolated *Phaseolus vulgaris* L. seedlings has been examined. *Phaseolus* tRNA was fractionated by benzoylated diethylaminoethyl-cellulose and RPC-5 chromatography, and the distribution of cytokinin activity was compared with the distribution of tRNA species expected to correspond to codons beginning with U. *Phaseolus* $tRNA^{Cys}$, $tRNA^{Trp}$, $tRNA^{Tyr}$, a major peak of $tRNA^{Phe}$, and a large fraction of $tRNA^{Leu}$ were devoid of cytokinin activity in the tobacco bioassay. Cytokinin activity was associated with all fractions containing $tRNA^{Ser}$ species and with minor $tRNA^{Leu}$ species. In addition, several anomalous peaks of cytokinin activity that could not be directly attributed to U group tRNA species were detected.

The cytokinin-active ribonucleosides that occur as constituents of tRNA molecules appear to be restricted in distribution to those tRNA species that respond to codons beginning with U, in which they occur once per molecule at the position adjacent ot the 3'-end of the anticodon (10, 15, 18). The most detailed investigations of the distribution of cytokinin moieties with respect to individual tRNA species have been conducted with tRNA from microbial sources, but all available evidence concerning cytokinin distribution in tRNA from higher plant and animal tissues is consistent with this concept (2, 6, 12, 19–21). Not all tRNA species within the U group necessarily contain a cytokinin moiety. For this reason, the exact distribution of cytokinin moieties with respect to particular tRNA species may vary in different organisms.

The cytokinin constituents of a number of plant tRNA preparations have been isolated and identified (10, 15, 18), but information concerning the distribution of cytokinins in particular plant tRNA species is much more limited. The distribution of cytokinins in wheat germ tRNA species has recently been examined in our laboratory (20). Cytokinin activity was associated with wheat germ $tRNA^{Ser}$ species and a minor $tRNA^{Leu}$ species, but all other wheat germ tRNA species expected to respond to codons beginning with U ($tRNA^{Cys}$, $tRNA^{Phe}$, $tRNA^{Trp}$, and $tRNA^{Tyr}$) were devoid of cytokinin activity in the tobacco callus bioassay. Cytokinin moieties have been reported to be present in minor $tRNA^{Leu}$ species from peas (2, 6) and soybeans (12), but the other U-group tRNA species have not been examined in these plant materials. In *Euglena* (4, 11) and *Phaseolus vulgaris* (8, 9), the cytokinin 2-methylthio-N^6-(Δ^2-isopentenyl)adenosine has been identified as a constituent of the corresponding chloroplast $tRNA^{Phe}$ species.

We report here the results of an examination of the distribution of cytokinins in tRNA species from etiolated *Phaseolus vulgaris* seedlings. Etiolated material was selected for this study to minimize the contribution of plastid tRNA species to the distribution of cytokinin activity and to provide a more direct comparison with the results obtained earlier for wheat germ tRNA. As in the case of wheat germ tRNA (20), the distribution of cytokinin moieties in tRNA species from etiolated bean seedlings appears to be more restricted than in microbial systems.

MATERIALS AND METHODS

Materials. Seeds of *Phaseolus vulgaris* cv. Bush Blue Lake 274 were kindly supplied by Asgrow Seed Company. ^{14}C-Amino acids were purchased from New England Nuclear. BD-cellulose[4] was prepared as described by Gillam *et al.* (7). The RPC-5 column packing material was purchased from Astro Enterprises, Inc., Powell, TN.

Growth of Etiolated Bean Seedlings. *P. vulgaris* seeds were planted in vermiculite and grown for 7 days in the dark at 25 C as previously described (5).

Isolation of *Phaseolus* tRNA—Crude *Phaseolus* tRNA was prepared according to the procedures of Murai *et al.* (14) as described previously (5).

Chromatographic Fractionation of *Phaseolus* tRNA—BD-cellulose chromatography was based on procedures described by Gillam *et al.* (7). Crude *Phaseolus* tRNA was chromatographed on an unbuffered BD-cellulose column (2.5 × 30 cm) equilibrated with 0.4 M NaCl. The tRNA sample (3,000 A_{260} units) was applied to the column in 75 ml 0.4 M NaCl and eluted with a 3000-ml linear salt gradient (0.4–1.0 M NaCl). Fractions (20 ml) were collected at a flow rate of about 1.7 ml/min. The salt gradient was discontinued at 0.86 M NaCl and the column purged with 1.0 M NaCl in 15% (v/v) ethanol.

RPC-5 chromatography was based on procedures described by Pearson *et al.* (16). Cytokinin-active tRNA fractions recovered from the BD-cellulose column eluate were chromatographed on an RPC-5 column (1.27 × 47 cm, packed at 150 p.s.i.) equilibrated with 10 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$, 1 mM β-mercaptoethanol, and 0.45 M NaCl. The tRNA samples were applied to the column in 5 ml of the equilibrating solution and eluted with a 1,680-ml linear salt gradient (0.45 or 0.50 M NaCl–0.70, 0.75, or 0.85 M NaCl) in the same buffer solution. The

[1] This research was supported by National Science Foundation Grant PCM 77-04266, by a National Science Foundation Predoctoral Fellowship to C.A.E., and by the Science and Education Administration of the United States Department of Agriculture under Grant 5901-0410-8-0028-0 from the Competitive Research Grants Office. This is Technical Paper 5579 of the Oregon Agricultural Experiment Station.

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[4] Abbreviations: BD-cellulose, benzoylated diethylaminoethyl cellulose; KE, kinetin equivalents.

column was maintained at 37 C and 120 p.s.i. during chromatography. Fractions (12 ml) were collected at a flow rate of about 2 ml/min. After most of the tRNA sample appeared to have eluted from the column, the initial gradient was discontinued and the column purged with buffer solution containing 1.2 M NaCl.

Preparation of Aminoacyl-tRNA Synthetases. All procedures were performed at 4 C. Etiolated bean seedlings (50 g) were macerated with a mortar and pestle in 50 ml 10 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$, 40 mM β-mercaptoethanol, and 20% (v/v) glycerol. The macerate was strained through cheesecloth, sonicated (Bronwill Biosonic IV, 100% at low setting for 2 min), and then centrifuged (12,000g, 15 min). The supernatant was adjusted to pH 7.5 with 0.2 M ammonium hydroxide and recentrifuged (27,000g, 1 h). The pellet was discarded and a sufficient volume of 10% (w/v) streptomycin sulfate added to the supernatant to give a final concentration of 2%. The precipitate was removed by centrifugation (27,000g, 30 min) and the supernatant made to 75% saturation with ammonium sulfate. The precipitated protein was recovered in one tube by repeated centrifugations (27,000g, 30 min) and then suspended in 3.5 ml 10 mM KH_2PO_4 buffer (pH 7.5, H_3PO_4) containing 1 mM β-mercaptoethanol and 2 mM EDTA. Any undissolved solids were removed by centrifugation (27,000g, 30 min), and the resulting supernatant was applied to a Sephadex G-25 column (1.5 × 60 cm) equilibrated with the same buffer. The column eluate was collected in 4-ml fractions, and the five fractions containing the highest concentration of protein were pooled, mixed with an equal volume of glycerol, and stored at −20 C. The activity of some of the aminoacyl-tRNA synthetases declined noticeably after a few days in storage, and amino acid acceptor assays were performed as quickly as possible after the enzyme preparation was obtained. The crude synthetase preparation was diluted 1:1 (v/v) with 8 mM DTT immediately before use in amino acid acceptor assays. In the case of serine acceptor assays, the enzyme-DTT mixture was preincubated at 30 C for 30 min immediately prior to use.

Amino Acid Acceptor Assays. Aminoacylation reactions catalyzed by the *Phaseolus* synthetase preparation were inhibited by NaCl concentrations equivalent to those present in chromatographic fractions from the BD-cellulose and RPC-5 columns. Therefore, tRNA was precipitated from an aliquot part (0.5 ml) of each column fraction by the addition of 0.1 volume 0.6 M $MgCl_2$ and 2.5 volumes of cold ethanol (17). The precipitated tRNA was allowed to stand at −20 C for 24 h and then recovered by centrifugation (27,000g, 30 min). Each pellet was redissolved in 0.5 ml distilled H_2O. The tRNA samples obtained in this manner were used to assay for amino acid acceptor activities.

Aminoacylation reactions were carried out in 0.1 ml reaction volumes containing the components listed in Table I. Each reaction volume was prepared from 50 μl of a concentrated assay mixture (containing the components listed in Table I at twice the concentrations specified there), 25 μl of the appropriate tRNA sample, and 25 μl of the aminoacyl-tRNA synthetase preparation diluted with DTT as described above. ATP was neutralized (pH 7.5) before incorporation into the assay mixtures. The reaction volumes were incubated at 30 C for 60 min except in the case of serine acceptor assays, in which they were incubated for 120 min. At the end of the incubation period, 50-μl aliquots were removed and applied to 2.3-cm-diameter Whatman 3MM filter paper discs (serine, leucine, and phenylalanine acceptor assays) or to 2.4-cm-diameter Whatman GF/C glass fiber discs (cysteine, tryptophan, and tyrosine acceptor assays). The discs were immediately placed in cold 10% (w/v) trichloroacetic acid (10 ml/disc) and kept in this solution until 10 min after the last disc in an assay series had been collected. The discs were then washed with a fresh volume of 10% trichloroacetic acid (10 ml/disc, 10 min) followed by three washes (5 min each) with cold 5% trichloroacetic acid (5 ml/disc) and two washes (10 ml/disc, 10 min and 5 ml/disc, 5 min) with cold Hokin's Reagent (125 ml of glacial acetic acid and 1.6 ml of 10 N NaOH diluted to 2 liters with ethanol). The discs were dried, placed in scintillation vials with 5 ml of a toluene-based scintillation fluid (Omnifluor, New England Nuclear), and counted in a Packard model 2405 scintillation counter. Counting efficiencies were determined by applying known amounts of ^{14}C-amino acids to filter discs.

Table I. *Concentration of Acceptor Assay Components in Final Reaction Volumes*

[^{14}C]Amino Acid (0.1 μCi/0.1 ml)	Hepes (pH 8)	$MgCl_2$	ATP	KCl	DTT[a]
			mM		
Cys	50	25	5	0	3
Leu	100	10	10	0	1
Phe	100	10	5	50	1
Ser	50	10	5	0	1
Try	100	10	5	0	1
Tyr	50	10	5	0	1

[a] These values include the DTT contributed to the reaction volumes by the synthetase preparation after dilution with DTT solution.

Determination of Cytokinin Activity. The cytokinin activities of tRNA fractions were determined in the tobacco callus bioassay (13). The tRNA samples were recovered from the column eluates by precipitation with $MgCl_2$ and ethanol as described above for the acceptor assay samples. The sample size used for bioassay varied from 20% of the tRNA recovered from appropriately pooled column fractions (BD-cellulose fractionation) to 50% of the RNA recovered (RPC-5 fractionations). All bioassay samples were acid hydrolyzed in 5 ml 0.1 N HCl (100 C, 45 min) prior to bioassay. The neutralized hydrolysates were incorporated into 100 ml RM-1965 medium containing 2 mg/l indole-3-acetic acid and tested in 5-fold serial dilutions in the tobacco bioassay as described by Armstrong *et al.* (1). Cytokinin activities are expressed as Microgram Kinetin Equivalents defined as the μg of kinetin (6-furfurylaminopurine) required to give the same growth response as the test samples under the specified bioassay conditions.

RESULTS

BD-Cellulose Chromatography of *Phaseolus* tRNA. Crude *Phaseolus* tRNA was fractionated by chromatography on BD-cellulose (Fig. 1). The elution profile of cytokinin activity was compared with the distribution of tRNA species expected to correspond to codons beginning with U ($tRNA^{Cys}$, $tRNA^{Leu}$, $tRNA^{Phe}$, $tRNA^{Ser}$, $tRNA^{Trp}$, and $tRNA^{Tyr}$). The cytokinin activity eluted in the latter half of the salt gradient (pooled fractions V, VI, VII, and VIII) and in the salt-ethanol purge (pooled fraction IX). All of the cysteine and tryptophan acceptor activity eluted early in the salt gradient, in fractions that were inactive in the tobacco bioassay. A portion of the leucine and tyrosine acceptor activity also eluted in fractions devoid of cytokinin activity. Serine and leucine acceptor activities were present in all of the cytokinin-active fractions. The other U group acceptor activities associated with the cytokinin-active regions of the elution profile included the phenylalanine acceptor activity, which eluted in the salt-ethanol purge region of the profile, and a peak of tyrosine acceptor activity (pooled fraction V).

RPC-5 Chromatography of *Phaseolus* tRNA Fractions. The cytokinin-active tRNA fractions (V, VI, VII, VIII, and IX) recovered from the BD-cellulose column were rechromatographed on RPC-5 columns, and the elution profiles for cytokinin activity again compared with the distribution of the appropriate amino acid acceptor activities.

RPC-5 chromatography of BD-cellulose fraction V (containing tyrosine, leucine, and serine acceptor activities) gave the elution

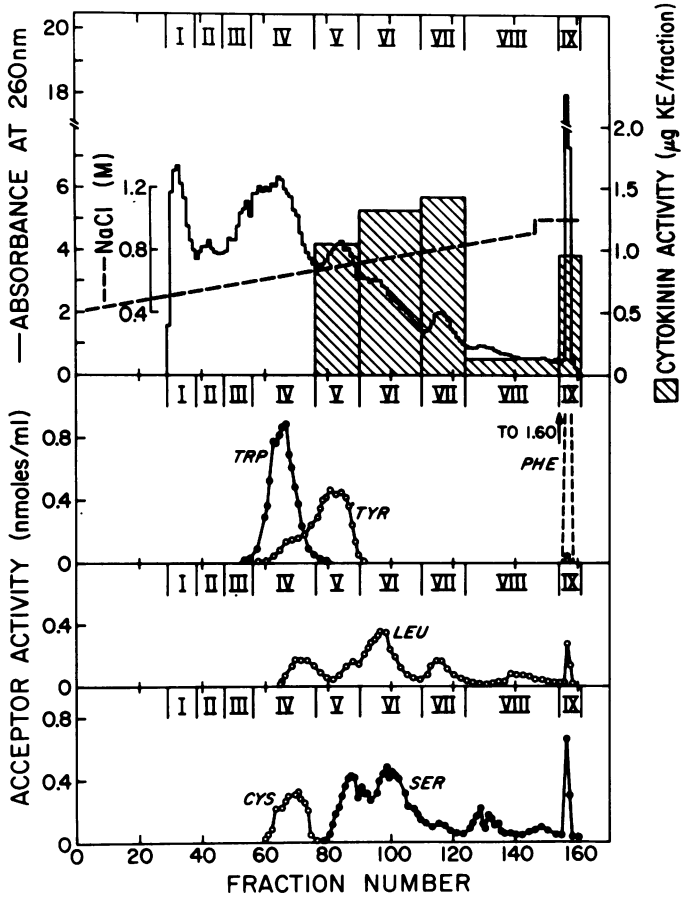

FIG. 1. BD-cellulose chromatography of crude *P. vulgaris* tRNA. Details of chromatographic procedures are described under "Materials and Methods." Fractions were pooled as indicated (I–IX) for analysis of cytokinin activity. Cytokinin activity is expressed as Microgram Kinetin Equivalents (μg KE).

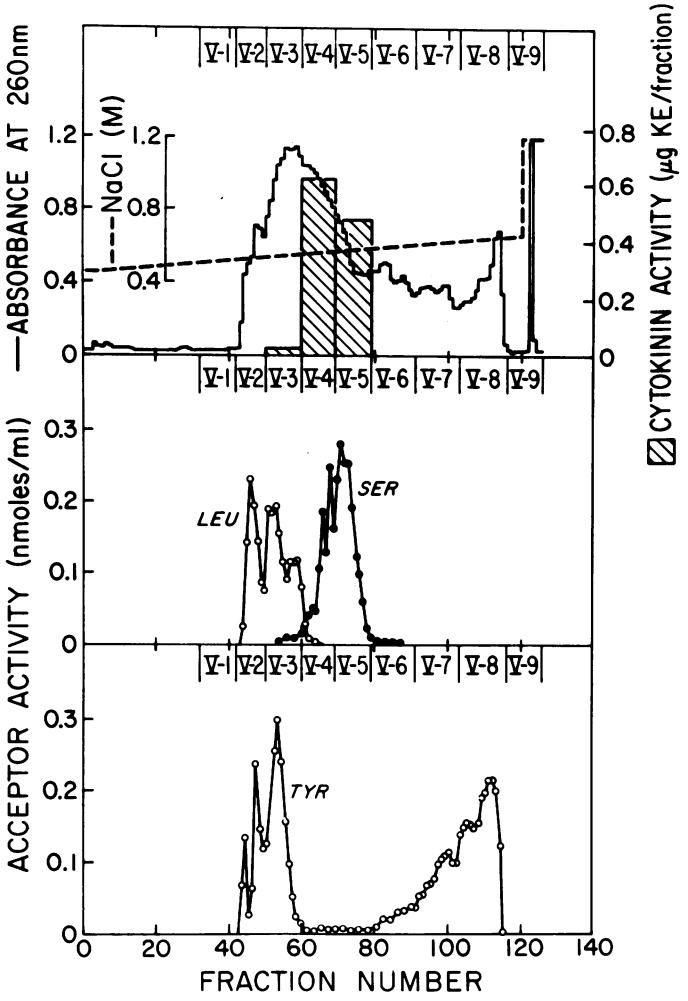

FIG. 2. RPC-5 chromatography of BD-cellulose fraction V tRNA. Fractions were pooled as indicated (V-1–V-9) for analysis of cytokinin activity.

profiles shown in Figure 2. All of the cytokinin activity was associated with a complex peak of $tRNA^{Ser}$ and was completely separated from the peaks of $tRNA^{Tyr}$ and $tRNA^{Leu}$.

The RPC-5 fractionation of BD-cellulose fraction VI (containing serine and leucine acceptor activities) is shown in Figure 3. Single peaks of serine and leucine acceptor activity eluted early in the profile and were almost completely separated from each other. The cytokinin activity was coincident with the $tRNA^{Ser}$ peak.

RPC-5 fractionation of BD-cellulose fraction VII (containing serine and leucine acceptor activities) gave a single peak of leucine acceptor activity that eluted prior to the cytokinin activity (Fig. 4). Cytokinin activity was associated with a complex $tRNA^{Ser}$ peak, but a considerable portion of the cytokinin activity eluted prior to the serine acceptor activity and was not associated with either the U group acceptor activities present in BD-cellulose fraction VII.

RPC-5 chromatography of BD-cellulose fraction VIII (containing serine and leucine acceptor activities) is shown in Figure 5. Cytokinin activity was distributed throughout the elution profile and was present in all fractions containing leucine and serine acceptor activities. In addition, a significant amount of cytokinin activity eluted late in the salt gradient and in the high salt purge region of the elution profile where U group tRNA species were not detected.

RPC-5 chromatography of BD-cellulose fraction IX (containing serine, leucine, and phenylalanine acceptor activities) is shown in Figure 6. Cytokinin activity was distributed throughout the elution profile. Two major peaks of activity eluting late in the salt gradient and in the high salt purge region of the profile were not associated with acceptor activity corresponding to any of the U group tRNA species. Minor peaks of leucine acceptor activity were distributed throughout most of the elution profile. Most of the serine acceptor activity eluted near the front of the elution profile and overlapped major and minor peaks of phenylalanine acceptor activity. Rechromatography of fraction IX-2 on RPC-5 at pH 5 (not shown) separated the serine acceptor activity from the major peak of phenylalanine acceptor activity. Cytokinin activity was coincident with the $tRNA^{Ser}$ peak. The $tRNA^{Phe}$ peak was inactive in the tobacco bioassay.

DISCUSSION

The distribution of cytokinin-active ribonucleosides in tRNA species from etiolated *P. vulgaris* seedlings appears similar to that in wheat germ tRNA (20) in the sense that most of the tRNA species that correspond to codons beginning with U do not contain cytokinin moieties. Thus, as in the case of wheat germ tRNA, *Phaseolus* tRNA fractions containing $tRNA^{Cys}$, $tRNA^{Trp}$, $tRNA^{Tyr}$, a major peak of $tRNA^{Phe}$, and a large part of the $tRNA^{Leu}$ activity were devoid of cytokinin activity in the tobacco bioassay. (The $tRNA^{Leu}$ peaks that were inactive in the tobacco bioassay may represent $tRNA^{Leu}$ species that repond to leucine codons beginning with C.) In both wheat germ tRNA and the *Phaseolus* tRNA preparation examined here, cytokinin activity

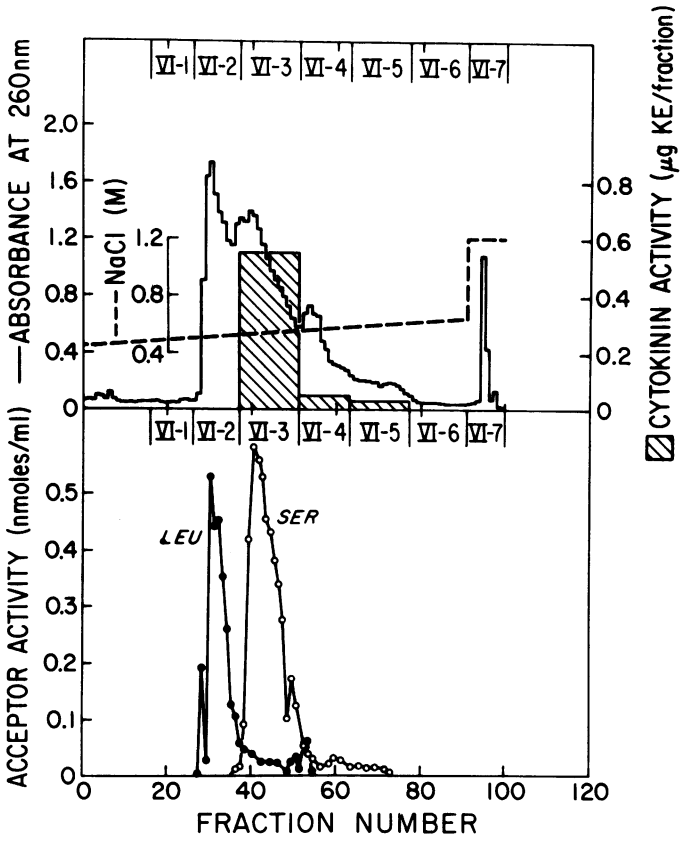

FIG. 3. RPC-5 chromatography of BD-cellulose fraction VI tRNA. Fractions were pooled as indicated (VI-1–VI-7) for analysis of cytokinin activity.

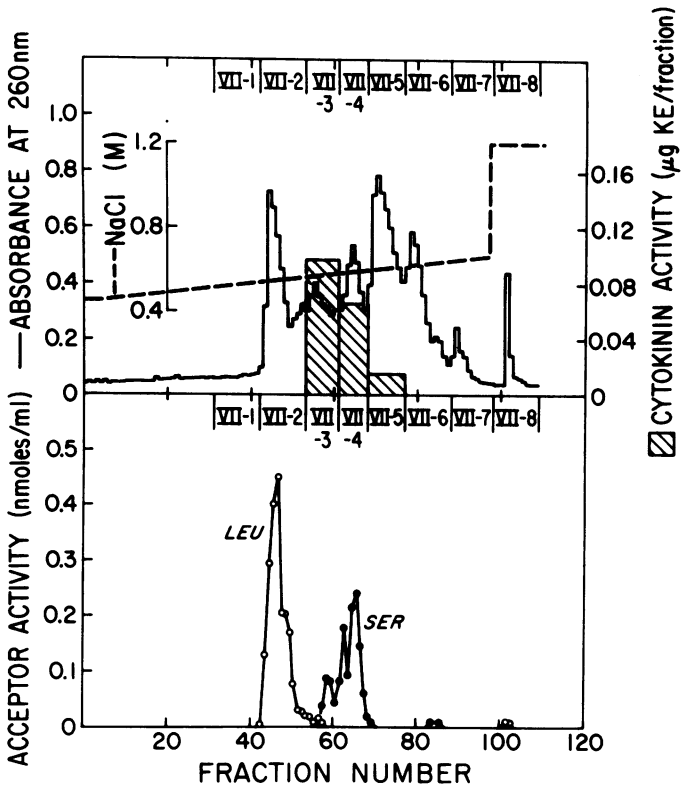

FIG. 4. RPC-5 chromatography of BD-cellulose fraction VII tRNA. Fractions were pooled as indicated (VII-1–VII-8) for analysis of cytokinin activity.

was associated with all tRNA fractions containing serine acceptor activity. Similarly, the tRNA preparations from both plant sources contained minor $tRNA^{Leu}$ species that chromatographed in cytokinin-active regions of the column elution profiles.

The distribution of cytokinin activity observed here differs from that reported for wheat germ tRNA in the sense that cytokinin activity was detected in *Phaseolus* tRNA fractions that did not contain acceptor activities corresponding to U group tRNA species. The most likely explanation for this anomalous distribution of cytokinin activity would appear to be the presence of either fragments or intact isoacceptors of U group tRNA species that were not aminoacylated in the acceptor assays. If the *Phaseolus* tRNA preparation contained tRNA species of etioplast origin, the corresponding plastid aminoacyl-tRNA synthetases may have been inactive under the assay conditions used here or not present at this particular stage of plastid development. The alternative possibility, that some of the cytokinin moieties present in plant tRNA preparations occur in tRNA species other than those that respond to codons beginning with U, cannot be completely excluded. Such a result would not be entirely without precedent. The reported occurrence of *N*-[*N*-(9-β-D-ribofuranosylpurin-6-yl)carbamoyl]threonine in $tRNA^{Tyr}$ from rat liver and silk worm has been reported (3). In prokaryotic systems, this hypermodified nucleoside is restricted to tRNA species that respond to codons beginning with A (10, 15, 18).

We have not yet examined the distribution of cytokinin-active ribonucleosides in tRNA species from green plant tissues, where chloroplast tRNA species might be expected to constitute a significant proportion of the total tRNA population. The identification of the cytokinin 2-methylthio-N^6-(Δ^2-isopentenyl)adenosine as a constituent of the chloroplast $tRNA^{Phe}$ species from *Euglena* (4, 11) and *P. vulgaris* (8, 9) suggests that the distribution of

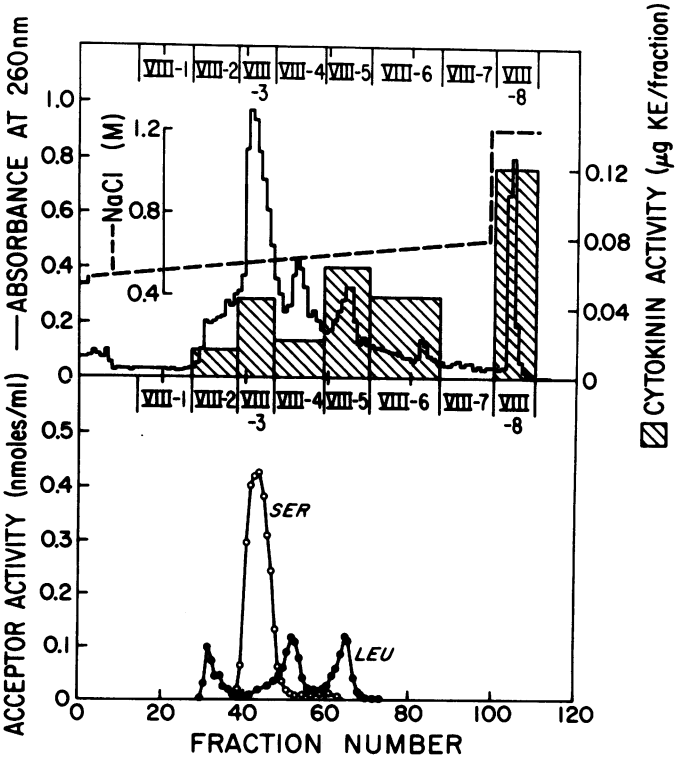

FIG. 5. RPC-5 chromatography of BD-cellulose fraction VIII tRNA. Fractions were pooled as indicated (VIII-1–VIII-8) for analysis of cytokinin activity.

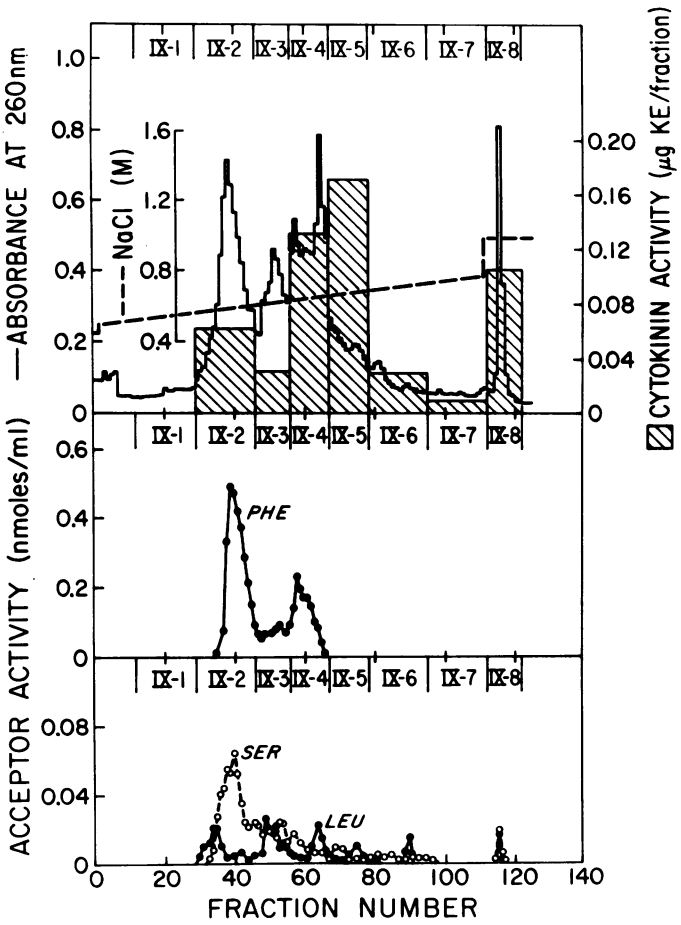

FIG. 6. RPC-5 chromatography of BD-cellulose fraction IX tRNA. Fractions were pooled as indicated (IX-1–IX-8) for analysis of cytokinin activity.

cytokinin moieties in plastid tRNA species may be similar to that in bacterial systems, where most of the tRNA species that respond to codons beginning with U contain cytokinin residues. However, on the basis of the results obtained for tRNA from etiolated *P. vulgaris* seedlings and in the case of wheat germ tRNA (20) the distribution of cytokinin moieties in cytoplasmic tRNA species from plant tissues appears to be restricted within the U group to $tRNA^{Ser}$ and minor $tRNA^{Leu}$ species.

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