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# Distribution of Cytokinin-Active Nucleosides in Isoaccepting Transfer Ribonucleic Acids from *Agrobacterium tumefaciens*†

R. O. Morris,* D. A. Regier, R. M. Olson, Jr., L. A. Struxness, and D. J. Armstrong

ABSTRACT: The cytokinin-active isoprenoid nucleosides of *Agrobacterium tumefaciens* transfer ribonucleic acid were identified by high-pressure liquid chromatography, permethylation, and mass spectroscopy. Besides the expected 6-[(3-methylbut-2-enyl)amino]-9-(β-D-ribofuranosyl)purine ($i^6A$) and its 2-methylthio derivative ($ms^2i^6A$), substantial amounts of *cis*- and *trans*-ribosylzeatin ($io^6A$) and *cis*-2-(methylthio)ribosylzeatin ($c\text{-}ms^2io^6A$) were present. These hydroxylated side chain derivatives are normally characteristic of plant tRNA. Fractionation of the total bacterial tRNA on BD-cellulose and RPC-5 allowed isolation of purified isoaccepting species whose cytokinin nucleoside contents were then determined. Distribution of the isoprenoid nucleosides among the U-group tRNA species was not uniform. *cis*-Ribosylzeatin was found almost exclusively in one $tRNA^{Ser}$ while $ms^2io^6A$ was found predominantly in $tRNA^{Phe}$, $tRNA^{Ser}$, and $tRNA^{Tyr}$. Not all cytokinin-active species were found in every member of the U-group tRNAs. The only species present in $tRNA^{Trp}$ was $i^6A$; it contained no zeatin derivatives. The hydroxylation and methylthiolation processes appear to be highly specific and dependent upon tRNA structure or sequence.

Four chemically distinct cytokinin-active nucleosides have been found to occupy the position adjacent to the 3′ side of the anticodon triplet in those transfer ribonucleic acid (tRNA)[1] species which recognize codons whose initial base is uracil. They differ in the extent to which they are chemically modified. Broadly speaking, the nature and extent of modification (Skoog & Armstrong, 1970; Leonard, 1974) depend upon whether the organism is an animal (in which case its tRNA contains $i^6A$), a bacterium ($ms^2i^6A$ and $i^6A$), or a plant ($io^6A$ and $ms^2io^6A$). An exception exists to this generalization, however, in that some plant pathogenic bacteria, notably *Agrobacterium tumefaciens* (Chapman et al., 1976; Cherayil & Lipsett, 1977) and *Corynebacterium fascians* (Einset & Skoog, 1977; Cherayil & Lipsett, 1977), have been shown to contain ribosylzeatin and $ms^2io^6A$ in their tRNA. In light of the possible involvement of cytokinins in tumorigenesis initiated by *A. tumefaciens* it was of interest to determine the relative proportions of cytokinins present in bacterial tRNA and to determine the cytokinin distribution among the U-group species. We report here the identification of $io^6A$, $ms^2io^6A$, $i^6A$, and $ms^2i^6A$ in tRNA from *A. tumefaciens* strain C58 and their distribution among the isoaccepting tRNA subspecies. The distribution of ribosylzeatin ($io^6A$) and the other species

† From the Departments of Agricultural Chemistry (R.O.M. and D.A.R.) and Botany and Plant Pathology (D.J.A., R.M.O., and L.A.S.), Oregon State University, Corvallis, Oregon 97331. *Received March 9, 1981.* This work constitutes paper no. 5762 from the Oregon State University Agricultural Experiment Station and was supported in part by National Science Foundation Grants PCM 78-22963 (to R.O.M.) and PCM 77-04266 (to D.J.A.) and by the Science and Education Administration of the U.S. Department of Agriculture under Grant No. 5901-0410-8-0190-0 from the Competitive Research Grants Office (to R.O.M.).

[1] Abbreviations used: $i^6A$, 6-[(3-methylbut-2-enyl)amino]-9-(β-D-ribofuranosyl)purine; $ms^2i^6A$, 2-(methylthio)-6-[(3-methylbut-2-enyl)-amino)]-9-(β-D-ribofuranosyl)purine; *c*-$io^6A$ and *t*-$io^6A$, *cis*- and *trans*-ribosylzeatin; $ms^2io^6A$, 2-(methylthio)ribosylzeatin; DEAE, diethylaminoethyl; BD-cellulose, benzoylated DEAE-cellulose; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; GLC–MS, gas–liquid chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; tRNA, transfer ribonucleic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; ATP, adenosine 5′-triphosphate; TLC, thin-layer chromatography.

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was found to be nonrandom within the U-group tRNAs.

## Materials and Methods

*Bacterial Strains.* Wild-type strain C58 (nopaline utilizing), its cured derivative A136, Ach5 (octopine utilizing), and transconjugant A596 = A136 $rif^R nal^R$(pTiAch5) were all gifts from Dr. E. W. Nester.

*Isolation of Bacterial tRNA.* Cells were grown in rich medium (Kado & Heskett, 1970) at 26 °C, pH 7.5, to late log phase ($A_{660} \simeq 1.0$). Cultures were chilled rapidly and harvested by centrifugation, and the cells were dispersed (Parish & Kirby, 1967) in an equal volume of buffer A [sodium 4-aminosalicylate, 6% (w/v), NaCl, 1% (w/v), sodium triisopropylnaphthalenesulfonate, 1% (w/v), phenol–cresol mixture, 3% (v/v), and β-mercaptoethanol, 5 mM]. After freezing and thawing twice, 4 volumes each of buffer A and phenol–cresol mixture [freshly distilled phenol–*m*-cresol–water = 100:14:11 (v/v) containing 8-hydroxyquinoline, 0.08% (w/v)] was added, and the whole was blended at high speed for 1 min. The phases were separated by centrifugation, the organic phase was extracted twice with fresh buffer A, and the pooled aqueous extracts were extensively deproteinized by adding solid NaCl to 2% (w/v) and shaking with phenol–cresol. Mixed nucleic acids were precipitated at −20 °C by the addition of 0.1 volume of sodium acetate (1 M, pH 4.5) and 2.5 volumes of 95% ethanol.

Purification of the tRNA was effected first by removing ribosomal RNA. The crude mixture of nucleic acids was dissolved in Tris-HCl (10 mM, pH 7.5) and $MgCl_2$ (10 mM). Solid NaCl was added to 1 M. After centrifugation, the supernatant contained tRNA contaminated with polysaccharides and fragments of DNA which were removed by Kirby's cresol procedure (Kirby, 1965) carried out at exactly 22 °C. A final purification of the tRNA over DEAE-cellulose gave ~2.5 mg of tRNA/g of fresh weight cells. Electrophoresis on 5% polyacrylamide gels indicated that it was >95% 4 S. The major UV-absorbing contaminant was 5S RNA.

*Chromatographic Fraction of RNA.* Chromatography on BD-cellulose was performed as described by Gillam et al. (1967). Approximately 3000 $A_{260}$ units of tRNA was applied in 0.35 M NaCl to an unbuffered column (2.5 × 30 cm) of BD-cellulose and eluted successively with a linear salt gradient (3 L, 0.4–1.0 M NaCl) and then with 1 M NaCl containing 15% ethanol. Fractions of 20 mL were collected.

RPC-5 chromatography (Pearson et al., 1971) was performed by applying selected fractions from the BD-cellulose column to an RPC-5 column (1.27 × 47 cm) equilibrated in Tris-HCl (10 mM, pH 7.5), $MgCl_2$ (10 mM), NaCl (0.45 M), and β-mercaptoethanol (1 mM). The column was eluted at 37 °C with a linear salt gradient (0.45–0.85 M) in the same buffer at a flow rate of 2.5 mL/min. Individual fractions (12 mL) were assayed for amino acid acceptor activity and cytokinin content as described below.

*Amino Acid Acceptor Assay.* Mixed aminoacyl synthetases were isolated from *A. tumefaciens* grown to mid log phase. The isolation protocol included cell breakage in a French press, streptomycin sulfate precipitation of nucleic acids, DEAE-cellulose chromatography, ammonium sulfate precipitation, and desalting on Sephadex G-25. The crude enzyme preparation was stable when stored at −20 °C in 50% glycerol, potassium phosphate (10 mM, pH 7.5), EDTA (1 mM), and β-mercaptoethanol (1 mM).

The composition of aminoacylation mixtures was different for each amino acid. Buffer and salt compositions for optimum acylation were determined and are as follows: Hepes (100 mM, pH 8.0) was used as buffer for Cys, Phe, Ser, and Tyr; cacodylate (100 mM, pH 7.0) for the other amino acids. Each assay mixture contained $^{14}C$-labeled amino acid (0.1 μCi/100 μL), buffer, ATP (5 mM), and $MgCl_2$ (10 mM for Leu, 15 mM for Cys, Ser, and Trp, and 20 mM for Phe and Tyr). In addition, the mixtures for Leu and Phe contained 50 mM KCl. Reaction mixtures were incubated at 30 °C for 45–60 min, and aliquots of 50 μL were applied to disks of Whatman 3MM paper or, in the case of Trp, to GF/C glass fiber disks. These were washed in 10% $Cl_3CCOOH$ and dried, and the radioactivity was estimated by liquid scintillation counting.

*Cytokinin Assay.* Suitable aliquots from total or fractionated tRNA were precipitated by the addition of 0.1 volume of $MgCl_2$ (0.6 M) and 2.5 volumes of ethanol, hydrolyzed in 0.1 M HCl (100 °C/45 min), neutralized, and included directly in the tobacco callus bioassay as previously described (Armstrong et al., 1969a,b). Cytokinin activity was expressed as kinetin equivalents, defined as the number of micrograms of kinetin required to give a response identical with that in question. The sensitivity of the method was such that it was possible to detect activity in as little as 2 $A_{260}$ units of crude *Agrobacterium* tRNA.

*Cytokinin Identification.* Pooled fractions from RPC-5 columns were made 0.3 M in KOH and incubated under nitrogen at 37 °C for 24 h. The pH was adjusted to 9.2 with Tris-HCl, and the nucleotides were dephosphorylated with calf intestinal phosphatase (2 units/mL) in the presence of 10 mM $MgCl_2$ at 37 °C for 24 h. Cytokinin-active nucleosides were extracted into the organic phase of a mixture of ethyl acetate–*t*-butyl alcohol–0.1 M Tris-HCl, pH 8.8 (4:2:1 v/v), which was then dried with $Na_2SO_4$, evaporated, and fractionated by HPLC.

For HPLC, samples were applied to the column ($C_{18}$ μ Bondapak, Water Associates) in ammonium acetate (0.02 M, pH 3.5) containing 10% ethanol. Elution was with a linearly increasing gradient of ethanol (10–60% over 20 min) at 2 mL/min provided by a Varian Instruments 5010. Absorbance of the eluate was monitored at 254 nm (Waters Associates 440 detector), and appropriate fractions were collected for permethylation and GLC–MS.

Permethylation was by the dimethyl sulfoxide anion–methyl iodide technique as described previously (Morris, 1977). Reaction was essentially complete within 30 s at room temperature. After the addition of water, permethylated derivatives were extracted into chloroform, washed, dried in vacuo, and subjected to GLC on a short (4-ft) packed column of Dexsil 300 (3%) on Chromosorb W which was programmed from 180 to 310 °C at 6 or 10 °C/min with He as the carrier gas.

The GC outlet was split between a flame ionization detector and a glass jet separator (Finnegan Instruments Corp.) connected to a Varian MAT7 magnetic sector spectrometer. Electron-impact spectra were acquired continuously at 70 eV under computer control (System Industries) and stored on disc for processing. Because of the affinity of cytokinins for hot metal surfaces, it was necessary to construct all chromatographic columns and transfer lines from glass or glass-lined stainless steel tubing and to inactivate all surfaces by coating with Dexsil 300 followed by gas phase silanization.

## Results

*Cytokinin Nucleosides in Unfractionated tRNA.* We have shown previously (Chapman et al., 1976) that tRNA from *A. tumefaciens* contains *trans*-ribosylzeatin (*t*-io$^6$A) and a methylthiolated ribosylzeatin derivative. In order to confirm this and to quantitate the amounts of each cytokinin present, we isolated total tRNA (3000 $A_{260}$ units) from late log phase *A.*

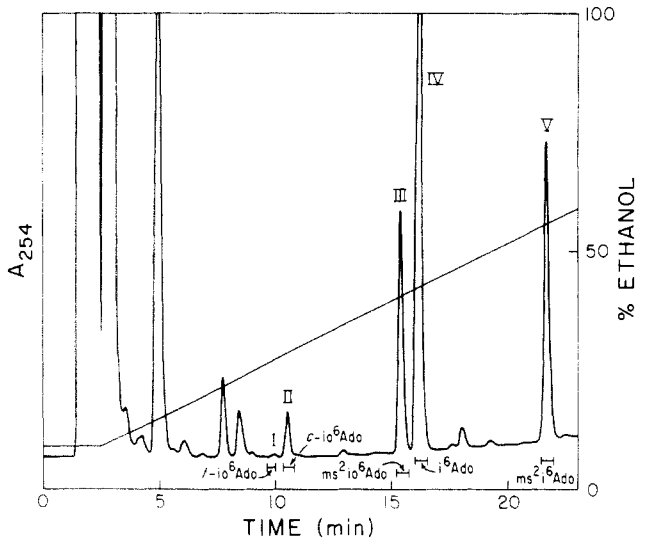


FIGURE 1: High-performance liquid chromatography of total tRNA hydrolysate. Cytokinin-active nucleosides from ~3000 $A_{260}$ units of strain C58 tRNA were fractionated on $C_{18}$-silica. The buffer was 0.02 M $NH_4OAc$, pH 3.5, and the flow rate 2 mL/min, with a gradient of 10–60% ethanol over 20 min. Peak I, *t*-io$^6$A; peak II, *c*-io$^6$A; peak III, *c*-ms$^2$io$^6$A; peak IV, i$^6$A; peak V, ms$^2$i$^6$A. Retention times of authentic standards are indicated by horizontal bars.

*tumefaciens*, strain C58. After hydrolysis and dephosphorylation the cytokinin-active nucleosides were extracted and fractionated by HPLC on $C_{18}$-silica by using an increasing gradient of ethanol for elution. Figure 1 illustrates a typical chromatogram obtained. In the range of ethanol concentrations where most cytokinins elute, there were four major peaks of absorbance at 254 nm (II–V), each of which corresponded in mobility to a known cytokinin. These were as follows: II, c-io$^6$A; III, ms$^2$io$^6$A; IV, i$^6$A, and V, ms$^2$i$^6$A.

In order to establish their chemical identities unequivocally, we converted each of the four fractions into its volatile permethylated derivative which was then subjected to GLC–MS. Two representative mass spectra are shown in Figure 2. HPLC peak II gave a mass spectrum (Figure 2A) having fragment ions at $m/e$ = 421 ($M^{+\cdot}$), 390, 348, 216, and 174, identical with those of an authentic sample of permethylated ribosylzeatin. Both the retention time of the material on HPLC and of its permethylated derivative on GLC indicated that it was the cis isomer. Similarly, peak III gave a mass spectrum (Figure 2B) identical with that of authentic ms$^2$io$^6$A [$m/e$ = 467 ($M^{+\cdot}$), 452, 436, 278, 262, 248, and 220].

The particular column and gradient illustrated in Figure 1 are capable of resolving the geometric isomers of ribosylzeatin (retention times for the trans and cis isomers were 9.8 and 10.6 min, respectively), but not the geometric isomers of ms$^2$io$^6$A. However, fractionation of ms$^2$io$^6$A by TLC on silica in chloroform–methanol–triethylamine acetate (0.1 M, 60:40:10) indicated that it was predominantly the cis isomer. $R_f$'s were as follows: peak III, 0.58; *c*-ms$^2$io$^6$A, 0.59; *t*-ms$^2$io$^6$A, 0.54.

Peaks IV and V gave mass mass spectra (not shown here) identical with those of authentic i$^6$A [ions at $m/e$ = 391 ($M^{+\cdot}$), 348, 217, 216, 202, and 174] and authentic ms$^2$i$^6$A [ions at $m/e$ = 437 ($M^{+\cdot}$), 394, 262, 248, and 220].

In addition to peaks II–V, the hydrolysate also contained a very small peak (I) at 9.8 min having a retention time identical with that of authentic *trans*-ribosylzeatin. The amount present was too low to provide a full mass spectrum but it did exhibit characteristic fragment ions ($m/e$ = 390 and 216) on permethylation and GLC–MS at the retention time expected for *t*-io$^6$A. Confirmation of its presence was sought by examination of a closely related strain A596. Here, the amount of peak I was greater, and a full scan mass spectrum was readily obtained. It was identical with that shown in Figure 2A.

The ratio of *t*- to *c*-io$^6$A was small and variable. In some strains it was as low as 0.054 and in others as high as 0.175. In addition, the ratio and the total amount of io$^6$A were dependent upon the time at which cultures were harvested. Early in the growth curve, the relative amounts of *t*-io$^6$A/*c*-io$^6$A/ms$^2$io$^6$A/i$^6$A were 2.7:25.6:94:100 for strain Ach5. At late log phase, they were 5.7:32.6:128:100. There was a significant increase both in hydroxylation and in methylthiolation. Irrespective of the age of the culture, however, a significant percentage of the total cytokinin-active nucleosides were hydroxylated (io$^6$A and ms$^2$io$^6$A). In this respect, *A. tumefaciens* tRNA resembles plant rather than bacterial tRNA.

*Chromatography of tRNA on BD-cellulose.* In order to determine how the cytokinins were distributed among the various isoaccepting subspecies, we fractionated total tRNA chromatographically, first on BD-cellulose and then on RPC-5, and the cytokinins were identified in individual fractions. Total mixed tRNA (3000 $A_{260}$ units) was applied to BD-cellulose and eluted first with an increasing gradient of sodium chloride and subsequently with an ethanol–sodium chloride solution. Absorbance at 260 nm, acceptance of amino acids, and cytokinin content were determined and are illustrated in Figure 3. As expected, the bulk of the tRNAs eluted at low salt concentrations and contained little or no cytokinin activity on

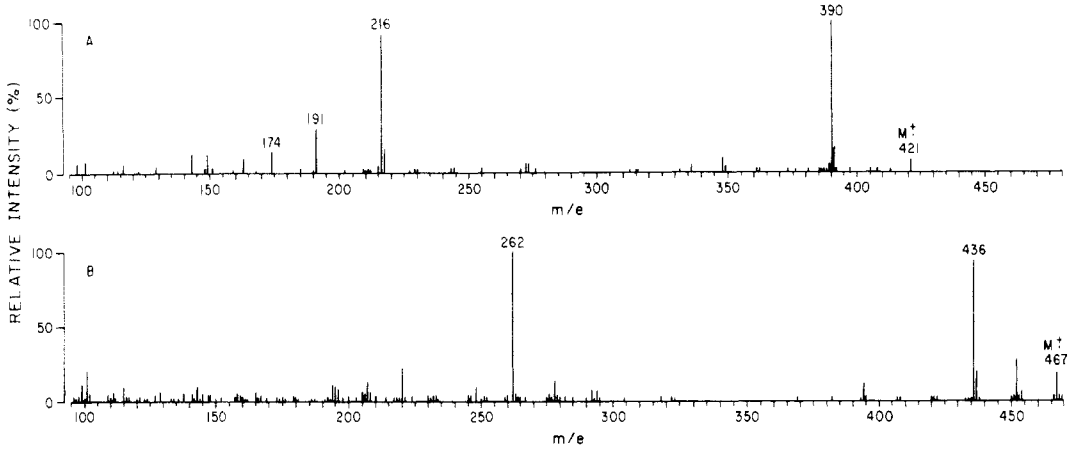


FIGURE 2: Mass spectra of selected permethylated cytokinins from tRNA hydrolysate. Spectra were recorded at 70 eV. (A) Peak II from Figure 1; spectrum identical with that of authentic io$^6$A. (B) Peak III from Figure 1; spectrum identical with that of authentic ms$^2$io$^6$A.

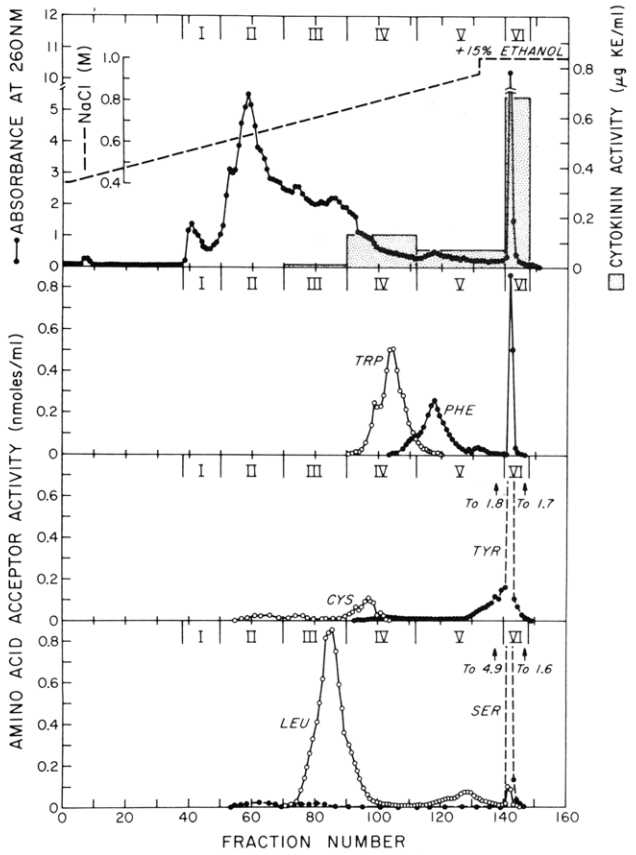


FIGURE 3: Benzoylated DEAE-cellulose chromatography of total tRNA. Fractions I–VI were collected and pooled as indicated for rechromatography on RPC-5 and measurement of total cytokinin content. Fraction size, 20 mL.

hydrolysis (fractions 40–90, 0.5–0.75 M NaCl). The cytokinin-containing species eluted toward the end of the gradient or in the ethanol–salt wash. A major peak of leucine acceptor activity eluted prior to any significant cytokinin activity, but all other species of tRNA expected to recognize U-group codons were associated with fraction having significant cytokinin content.

On the basis of amino acid acceptance, individual fractions were pooled to give three major cytokinin-containing fractions as indicated in Figure 3. Fraction IV contained all of the Cys and Trp, a portion of the Leu and Phe, and none of the Ser acceptor activity. Fraction V contained part of the Phe and Tyr activities, another Leu isoacceptor, and no Ser. Fraction VI (the ethanol–salt wash) contained only Phe, Ser, and Tyr acceptance activity. Each was examined further by RPC-5 chromatography.

*RPC-5 Chromatography.* Chromatography of BD-cellulose fraction IV on RPC-5 gave a partially resolved complex of peaks containing Leu, Cys, Tyr, Trp, and Phe tRNAs (Figure 4A). In some fractions, several amino acid acceptance activities were present. For example, fraction IV-1, which contained all of the Leu activity of IV as a double peak, also contained minor Trp and Tyr species (not shown). The lack of correspondence between the $A_{260}$ profile and the amino acid acceptance profiles suggests that the purity is low, and this was borne out by specific acceptance data, which for Leu in this fraction, were low ($\sim$0.84 nmol/$A_{260}$ unit, theoretical maximum 1.6 nmol/$A_{260}$ unit). Fraction IV-2 was homogeneous in the sense that it contained only Trp acceptor activity. However, again there was not a good correspondence with the $A_{260}$ profile. On the other hand, the Cys and Phe peaks in fractions IV-3 and IV-4 were of high purity. The apparent specific activity was reasonably constant across each fraction, although clearly, there are two $tRNA^{Cys}$ isoacceptors present in IV-3.

BD-cellulose fraction V gave similar results on rechromatography (Figure 4B). Fractions V-1 and V-2, which contained substantial cytokinin activity, were heterogenous and able to accept both leucine and tyrosine. Fraction V-4 was close to homogeneous, containing only Phe acceptance which paralleled the $A_{260}$ profile. Again, peak shapes suggest the presence of more than one Phe isoacceptor in this fraction, an interpretation which was strengthened by subsequent cytokinin analysis.

The BD-cellulose ethanol–salt fraction VI was much more distinctly resolved upon RPC-5 (Figure 4C). Serine acceptance was distributed across a multiplicity of peaks between fractions VI-1 and VI-5. Of these, VI-1 was not examined

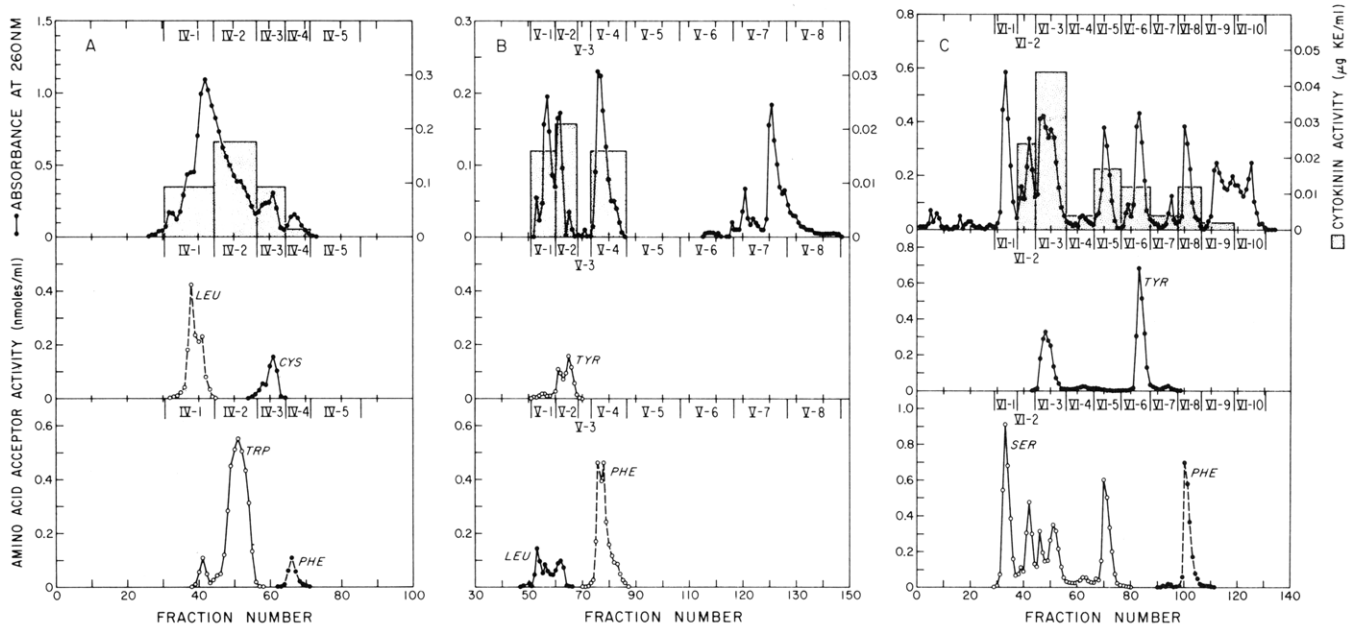


FIGURE 4: Chromatographic fractionation of tRNA on RPC-5. Individual fractions (IV–VI) from the BD-cellulose column were pooled and subjected to rechromatography on RPC-5. (A) BD-cellulose fraction IV; (B) BD-cellulose fraction V; (C) BD-cellulose fraction VI. Fraction size, 12 mL.

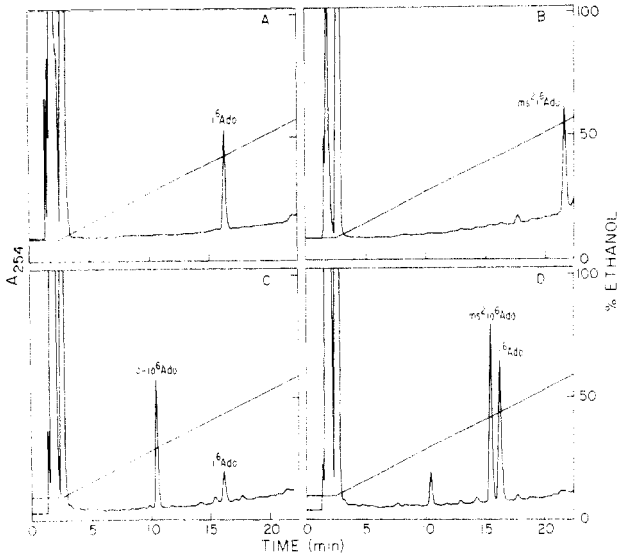


FIGURE 5: High-performance liquid chromatography of hydrolysates of individual RPC-5 fractions. (A) Fraction IV-2, $tRNA^{Trp}$; (B) fraction VI-8, $tRNA^{Phe}$; (C) fraction V-2, $tRNA^{Ser}$; (D) fraction VI-3, $tRNA^{Ser}$ + $tRNA^{Tyr}$. Elution conditions were identical with those indicated in Figure 1. Individual UV-absorbing components in the eluate were collected for permethylation and mass spectroscopy.

further because it contained no cytokinins. Serine VI-2 contained cytokinin and was close to homogeneity, but VI-3, which also contained cytokinin, accepted both Ser and Tyr in about equal amounts. The Ser (VI-5), Tyr (VI-6), and Phe (VI-8) peaks all contained cytokinin and were homogeneous.

*Cytokinin Identification.* In order to identify the cytokinins present, we subjected selected fractions from each of the three RPC-5 columns to base hydrolysis and enzymatic dephosphorylation to release the cytokinins as free nucleosides. These were then extracted and fractionated by HPLC on microparticulate $C_{18}$-silica. Figure 5 illustrates four representative hydrolysates out of a total of eleven examined.

Of the five cytokinin-active nucleosides present in unfractionated tRNA (Figure 1), at least one was represented in each RPC-5 subfraction examined. In only two instances, however, was a given cytokinin present alone, even when the tRNA of the RPC-5 fraction was near homogeneity. These were fractions IV-2 (Trp) and VI-8 (Phe). Fraction IV-2 gave a single peak with a retention time equal to that of $i^6A$ (Figure 5A) while fraction VI-8 gave a peak identical in mobility with that of $ms^2i^6A$ (Figure 5B). Most of the other RPC-5 fractions contained one major and one minor cytokinin-active peak as typified by fraction VI-2 (Ser) (Figure 5C). It contained a major UV-absorbing component identical in mobility with that of $c\text{-}io^6A$, a smaller peak (~25%) with the retention time of $i^6A$, and a very minor peak (<2%) corresponding to $t\text{-}io^6A$. Fraction VI-2 actually exhibits a leading shoulder with some serine acceptance activity (Figure 4C), and it is possible that the $i^6A$ is contributed by this species. Other RPC-5 peaks (fraction VI-3, for example) were obviously mixtures of two tRNAs able to accept different amino acids. Fraction VI-3, which accepts serine and tyrosine with equal facility, gave on hydrolysis almost equal quantities of $ms^2io^6A$ and $i^6A$ with a minor peak of $c\text{-}io^6A$ (Figure 5D).

Because retention time on $C_{18}$-silica is a necessary but not sufficient criterion for identification, assignment of cytokinin identity to a particular HPLC peak was made in most cases by permethylation followed by GLC-MS. Table I is a compilation of the cytokinins present in each RPC-5 fraction and illustrates the relative levels present in each. The most widely distributed cytokinin was $i^6A$, which was present as the major component of six of the species examined. Next in frequency was $ms^2io^6A$, followed by $ms^2i^6A$ and $c\text{-}io^6A$.

Table I: Relative Amounts of Cytokinin-Active Nucleosides in Individual tRNAs[a]

| RPC-5 fraction | amino acid acceptance | cytokinins present (rel amount) | | | | |
|---|---|---|---|---|---|---|
| | | $t\text{-}io^6A$ | $c\text{-}io^6A$ | $ms^2io^6A$ | $i^6A$ | $ms^2i^6A$ |
| IV-1 | Leu | | 35* | | 100 | |
| IV-2 | Trp | | | | 100 | |
| IV-3 | Cys | | | 25* | 100 | |
| V-1 | Leu + Tyr | | | 21* | 100 | |
| V-2 | Tyr + Leu | | | 35* | 100* | |
| V-4 | Phe | | | 100 | 13* | |
| VI-2 | Ser | 2* | 100 | | 24 | |
| VI-3 | Ser + Tyr | | 19 | 100 | 78 | |
| VI-5 | Ser | | | 5 | 100 | |
| VI-6 | Tyr | | | 8* | 21 | 100* |
| VI-8 | Phe | | | | | 100 |

[a] All components were identified by mass spectroscopy except as indicated by an asterisk, in which case identification was made on the basis of HPLC retention time only. Relative amounts were determined by peak area measurements relative to the major cytokinin present in each fraction.

## Discussion

Unfractionated tRNA from *A. tumefaciens* contains five cytokinin-active nucleosides: $i^6A$, $ms^2i^6A$, $c\text{-}io^6A$, $t\text{-}io^6A$, and $c\text{-}ms^2io^6A$. There is a possibility that a small amount of $t\text{-}ms^2io^6A$ is also present, but current analytical methods cannot distinguish between it and the cis isomer at low levels since both isomers have identical mobilities on $C_{18}$-silica and because TLC is insufficiently sensitive to detect low amounts of the trans isomer. The presence of $i^6A$ and $ms^2i^6A$ is expected by analogy with *Escherichia coli*, *Staphylococcus epidermidis*, and *Bacillus subtilis* (Skoog & Armstrong, 1970; Vold, 1978). The presence of zeatin derivatives in *A. tumefaciens* tRNA suggests that this organism has close affinity to the higher plants. It is squarely within the set of plant-associated gall-forming prokaryotes which contain plant-like cytokinins in their tRNA. Other members of the group include *C. fascians* (Einset & Skoog, 1977), *Pseudomonas aeruginosa* (Thimmappaya & Cherayil, 1974), and *Rhizobium* spp. (Cherayil & Lipsett, 1977) but not, as Cherayil has pointed out, non-gall-forming organisms such as *Erwinia amylovora*. Whether there is a direct link between the presence of zeatin derivatives and tissue hyperplasia incited by this class of organisms remains to be demonstrated. In light of the known involvement of zeatin and ribosylzeatin in plant cell proliferation, their presence is highly suggestive.

We previously reported (Chapman et al., 1976) the occurrence of $t\text{-}io^6A$ in tRNA from *A. tumefaciens*. Its presence is confirmed here although it is certainly not present to the extent we previously reported. In fact, the cis isomer appears to be the predominant geometric form in *A. tumefaciens* tRNA. Once again, the analogy with plant tRNA is close since $c\text{-}io^6A$ is the main isomeric form of ribosylzeatin in the tRNA of several plants (Babcock & Morris, 1970; Vreman et al., 1972). The ratio of $t\text{-}io^6A$ to $c\text{-}io^6A$ in *A. tumefaciens* depended both upon the strain examined and upon the point on the growth curve at which the cells were harvested. It was as low as 0.054 for strain C58 at late exponential phase to as high as 0.175 for strain Ach5 at the same point on the growth curve. Explanations for the variation could involve a low stereospecificity of hydroxylation, which is unlikely, or could suppose the presence of a unique tRNA, containing $t\text{-}io^6A$ and having a primary sequence different from that of the species containing $c\text{-}io^6A$. There is no evidence yet to support either

idea. The point is of some interest, in that *A. tumefaciens* is known to secrete *trans*-zeatin into the culture medium (Kaiss-Chapman & Morris, 1977) and it is possible that a bacterially coded, or Ti plasmid coded, tRNA could be the immediate precursor of the free hormone, both in culture and in the plant tumor. An excision-repair or exchange mechanism would have to be postulated, and there is evidence that such mechanisms do exist for other bases. For example, tRNA guaninetransferase which catalyzes the exchange of free guanine into a $tRNA^{Asp}$ at a point adjacent to the anticodon triplet has been described by Farkas & Singh (1973). It was originally thought to catalyze the exchange of free guanine with the highly modified base Q at this position (Okada et al., 1976), but it now appears that its primary function is the insertion of Q (or a related compound) to give Q-containing tRNA (Katze & Farkas, 1979). Irrespective of the details, it is clear that cleavage of the glycosidic linkage is possible and that exchange of a specific base without fragmentation of the polynucleotide chain can occur. Such a mechanism could act as a source of free *trans*-zeatin in *Agrobacterium*.

If an exchange mechanism of this sort exists, one would expect to find higher levels of *t*-$io^6A$ in tRNA from plasmid-containing *A. tumefaciens*. Further, if a unique tRNA is involved in the mechanism of oncogenesis, then one might expect the Ti plasmid and, specifically, that part which is transferred to the plant, the T-DNA, to hybridize with bacterial tRNA. Preliminary evidence (Morris et al., 1981) suggests that there are indeed slightly higher levels of *t*-$io^6A$ in tRNA from *Agrobacterium* strains harboring the Ti plasmid. Further, hybridization data (Morris et al., 1981) also indicate that some component of strain C58 tRNA does hybridize with the Ti plasmid. However, it has not yet been established that the hybridizable component is a tRNA, and, moreover, the hybridization is not to the T-DNA but rather to a region of the plasmid known to be necessary for oncogenicity but known not to be maintained in the plant. Studies by Cornelis et al. (1975) have shown that crown gall tRNA contains a unique $tRNA^{Phe}$. However, it is likely to have arisen by undermodification at the Y-base position. On balance, therefore, the evidence would seem to suggest that a unique tRNA containing *t*-$io^6A$ is not part of the mechanism of oncogenicity in any direct fashion, and it may be, in light of Katze and Farkas' data (Katze & Farkas, 1979), that the existence of tRNA containing *t*-$io^6A$ is a reflection of an exchange process operating in the opposite direction from preformed $io^6A$ and unmodified tRNA.

The distribution of ribosylzeatin and (methylthio)ribosylzeatin over the set of U-group tRNAs is by no means uniform. *cis*-Ribosylzeatin is found primarily in one of the $tRNA^{Ser}$ isoacceptors and $tRNA^{Leu}$. It does not occur in $tRNA^{Trp}$, which contains only $i^6A$, nor is it present in a $tRNA^{Phe}$, although $ms^2io^6A$ is present there. In this respect, the distribution resembles that seen in tRNA from wheat germ (Struxness et al., 1979), in which the zeatins were localized in $tRNA^{Ser}$ and $tRNA^{Leu}$. It may well be that the plant-associated prokaryotes have acquired genetic information from their host organisms during the course of their association.

Finally, a comparison may be made with *B. subtilis*. Vold (1978) examined tRNA from this organism and found that it contained the highest levels of $ms^2i^6A$ late in the growth cycle. The same is true for *A. tumefaciens*. Higher levels of $ms^2io^6A$ and $ms^2i^6A$ were noted in unfractionated tRNA from *A. tumefaciens* harvested toward the end of the growth curve. Vold suggested that for *B. subtilis*, the change could be correlated with the onset of sporulation. Such is not possible here, and the phenomenon may be more general, relating to the nutritional status of the cells or the extent of aeration of the cultures. In yeast (Bell et al., 1978), changes in isoacceptor profiles with, presumably, parallel changes in the modified bases have been shown to correlate with the extent of oxygenation and the stage of cell growth.

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