Plant Physiol. (1978) 61, 296-306

Cytokinins in tRNA Obtained from *Spinacia oleracea* L. Leaves and Isolated Chloroplasts[1]

Received for publication May 24, 1977 and in revised form September 3, 1977

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ABSTRACT

Cytokinin-active ribonucleosides have been isolated from tRNA of whole spinach (*Spinacia oleracea* L.) leaves and isolated spinach chloroplasts. The tRNA from spinach leaf blades contained: 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (*cis* and *trans* isomers), 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine, and 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (*cis* and *trans* isomers). A method for isolation of large amounts of intact chloroplasts was developed and subsequently used for the isolation of chloroplast tRNA. The chloroplast tRNA contained 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine and 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (the *cis* isomer only). The structures of these compounds were assigned on the basis of their chromatographic properties and mass spectra of trimethylsilyl derivatives which were identical with those of the corresponding synthetic compounds. The results of this study indicate that ribosylzeatin was present in spinach leaf tRNA, but absent from the purified chloroplast tRNA preparation.

Cytokinin-active compounds have been demonstrated to occur in tRNA preparations from various phylogenetically divergent groups of organisms, such as bacteria, fungi, plants, and animals (25). Studies on the distribution of cytokinins in the tRNAs of these organisms show that whereas microbial tRNAs contain mainly iPA[3] and msiPA, plant tRNAs contain in addition ZR and msZR (25). However, msZR has been found in tRNA of *Pseudomonas aeruginosa* (27) and recently Chapman *et al.* (8) have reported the presence of *t*-ZR in the tRNAs of *Agrobacterium tumefaciens*. Unlike bacterial tRNA, plant tRNA contains predominantly *c*-ZR. Hall *et al.* (12) reported the occurrence of ZR and iPA in the tRNA of frozen spinach leaves. The cytokinins present in wheat germ tRNA were identified as iPA, ZR, and their methylthio derivatives (6). Vreman *et al.* (30, 31) have reported the presence of iPA, msiPA, *c*-ZR, *t*-ZR, *c*-msZR, and *t*-msZR in pea shoot tRNA. Cytokinin-dependent tobacco callus tissue was also shown to contain ZR, iPA, and msZR (7). Thus, plant tRNAs contain the greatest number of cytokinins and the presence of multiple cytokinins in the tissues of a single organism raises questions as to their origin and possible cellular and subcellular localization. Because these plant cells contain various organelles such as chloroplasts and mitochondria which have their own tRNA species (1, 19), it is conceivable that the structurally divergent cytokinins found in plant tissue tRNA might derive in part from these organellar tRNAs. Furthermore, organellar protein-synthesizing systems have been shown to resemble that of prokaryotes in drug sensitivity, chain initiation, etc. (9). If this parallelism between prokaryotes and chloroplasts were extended to the cytokinin-active bases, the normally occurring bacterial cytokinins, *i.e.* iPA and msiPA, could be expected to be present in organellar tRNA. In fact, Hecker *et al.* (13) have tentatively identified msiPA in chloroplast $tRNA^{Phe}$ of *Euglena gracilis*. The present study was undertaken to investigate whether there are cytokinins which are exclusively associated with chloroplast tRNA and whether there exists a parallelism with prokaryotic tRNA. An indirect answer to this possibility was reported by Swaminathan *et al.* (26) who have presented evidence for the exclusive localization of ZR in cytoplasmic tRNA and that of msZR in chloroplast tRNA. Furthermore, iPA might be of chloroplast origin, as relatively little of this compound has been found in nongreen plant tissues (29). There has been no report on the direct isolation and identification of cytokinins from chloroplast tRNA, mainly because of the difficulties in isolating intact

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[3] Abbreviations: ZR: ribosylzeatin: 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine. The prefix *t* or *c* is included when the isomeric configuration is known. msZR: methylthio-ribosylzeatin: 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine. The prefix *t* or *c* is included when the isomeric configuration is known. iPA: isopentenyladenosine: 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine; msiPA: methylthio-isopentenyladenosine: 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine; CTAB: cetyltrimethylammonium bromide; KE: kinetin equivalent, defined as the μg of kinetin required to give the same growth response as the test sample. Cytokinin activities are expressed as KE/22,000 A_{260} units (KE/g tRNA). Centrifugation: 580*g* for 15 min at 4 C unless noted otherwise. TMS: trimethylsilyl.

chloroplasts on a large scale. This paper details a large scale isolation method for chloroplasts from spinach leaves and reports on the identification of cytokinins occurring in spinach chloroplast tRNA, as well as in tRNA from spinach leaf blades.

MATERIALS AND METHODS

Isolation of Spinach Leaf tRNA. Leaves of commercially field-grown, mature *Spinacia oleracea* L. var. 424 were harvested, and the tRNA was extracted within 12 hr by a modification of the procedure as described (7). Each of six 1-kg batches of leaf blades was homogenized for 1 min at full speed in a 3.7-liter Waring Blendor[4] with a two-phase mixture consisting of 1 liter of cold 0.1 M tris, adjusted with HCl to pH 7.4 (tris-Cl), and 1 liter of tris-Cl-saturated phenol (pH 7.4). The combined homogenates were stirred for at least 1 hr at 4 C. All subsequent tRNA isoaltion steps were carried out at 4 C unless otherwise noted. Centrifugation of the homogenate yielded two phases, of which the phenol phase was discarded. The aqueous phase was successively stirred vigorously for 15 min with 0.3 and 0.25 volumes of tris-Cl-saturated phenol. The phases were separated by centrifugation and the interphases and phenol layers were discarded. The aqueous phase (9.1 liters) was mixed with 0.1 volume of 20% (w/v) potassium acetate, and 2 volumes of 100% ethanol. The resulting suspension was kept overnight at −20 C and the precipitate was then collected by centrifugation. The tRNA was extracted by homogenizing the precipitate for 30 sec at medium speed in a Waring Blendor (1 liter) with 3 successive volumes of 200 ml of 0.4 M sodium acetate. The remaining insoluble material was collected each time by centrifugation for 60 min and was ultimately discarded. Two volumes of 100% ethanol were added to the pooled extracts and after keeping the resulting suspension overnight at −20 C, the precipitate was collected by centrifugation for 30 min. This crude tRNA preparation, containing 40,000 A_{260} units, was further purified by the CTAB method (24). The resulting preparation, which contained 10,650 A_{260} units, was dissolved in tris-Cl and then chromatographed on a DEAE-cellulose column (120 × 50 mm) prepared from 35 g dry wt Cellex-D (Biorad Laboratories, Richmond, Calif.). The column was washed with 0.2 M NaCl in tris-Cl and the tRNA was eluted with 10 bed volumes of 1 M NaCl in tris-Cl. The eluate was mixed with 2 volumes of 100% ethanol and kept overnight at −20 C. The precipitated tRNA was collected by centrifugation and dissolved in 80 ml of distilled H_2O. This solution was dialyzed for 16 hr against two changes of 4 liters of distilled H_2O to remove salts and low mol wt material. This purified tRNA preparation (L) contained 8,500 A_{260} units and appeared to be free of DNA and high mol wt rRNA as determined by gel electrophoresis (see Fig. 2).

Isolation of Chloroplasts. Commercially field-grown mature *S. oleracea* L., var. giant Noble, depetioled leaves were harvested and the material was kept and processed at 4 C during the following 18 hr. Each of 44 1-kg quantities was homogenized for 30 sec at low speed in a 3.7-liter Waring Blendor with 2 liters of cold isolation medium containing: 0.5 M choline chloride, 0.01 M tris-Cl, and 0.005 M $MgCl_2$ (pH 7.8) (22). Each homogenate was passed through a set of the following filters: nylon screen (1 × 1 mm), nylon mesh (0.3 × 0.3 mm), and Miracloth (Van Waters and Rogers, San Francisco) to remove cell debris, nuclei etc. The filtrate was centrifuged (International refrigerated centrifuge, model 3, using 250-ml flasks), and the chloroplast pellet was suspended immediately in 100 ml of cold isolation medium, recentrifuged, and the pellet suspended in 50 ml of water and quickly added to 150 ml of 100% ethanol, containing 1% (w/v) sodium acetate (−20 C).

Isolation of Chloroplast tRNA. The precipitate from the pooled ethanolic chloroplast suspensions was collected by centrifugation and was washed consecutively, three times with 800 ml of 100% ethanol, twice with 800 ml of acetone, and once with 800 ml of diethyl ether. The precipitate was then dried under vacuum at room temperature.

It is recognized that the ethanol-acetone-diethyl ether extraction of the isolated chloroplasts prior to extraction of the nucleic acids could have affected the recovery and quality of the tRNA. However, direct extraction of tRNA from the chloroplasts with phenol-buffer was not successful as a sticky, semisolid mass was formed in an aqueous phase which contained no nucleic acids. Presumably, the choline chloride interfered with the nucleic acid extraction, because removal of choline chloride with ethanol alleviated the problem.

The dry chloroplast powder (195 g) was homogenized with the 1 liter of tris-Cl and 500 ml of tris-Cl-saturated phenol for 1 min at full speed in a Waring Blendor (3.7-liter). The homogenate was stirred for 1 hr. The phases were separated by centrifugation and the aqueous phase was set aside at 0 C. The phenol phase plus interphase were reextracted twice, for 60 and 30 min, respectively, with 500 ml of tris-Cl and then discarded. The three aqueous phases were combined and treated twice with 500 ml of tris-Cl-saturated phenol. The tRNA in the clear, colorloess aqueous phase was precipitated by the addition of 2 volumes of 100% ethanol which contained 1% (w/v) potassium acetate. The suspension was stored overnight at −20 C. The precipitate was collected by centrifugation for 60 min and was found to contain 52,200 A_{260} units. The RNA (42,630 A_{260} units) was extracted from the precipitate with three times 100 ml of 0.4 M sodium acetate, precipitated with 2 volumes of 100% ethanol, and further purified by the CTAB method (24). Further purification by chromatography on a DEAE-cellulose column (65g dry wt, 155 × 50 mm), and dialysis yielded 7,943 A_{260} units of chloroplast tRNA, hereafter referred to as tRNA preparation C.

Microorganism Counts. The extent of microbial contamination in the washed chloroplast preparation was estimated by plating serial dilutions on Difco plate count agar, incubating for 4 days at 28 C, and counting the colonies at 10× magnification.

Electron Microscopy. Isolated chloroplast preparations were examined by electron microscopy to determine the proportion of intact *versus* broken chloroplasts as well as to estimate the level of contamination by nonchloroplast material (mitochondria, microbodies, membrane fragments). Chloroplast suspensions in cold isolation medium, representing approximately 0.5 g of leaf tissue, were mixed in conical polyethylene microcentrifuge tubes with equal amounts of fixing medium (isolation medium containing 2% [v/v] glutaraldehyde). After 3 hr of standing at 4 C, the solid material was pelleted by centrifugation (1,200*g* for 15 min at 4 C) and the supernatant was decanted. The pellets were stored at 4 C in 4% (v/v) glutaraldehyde until processed further. They were then postfixed at 4 C for 45 min in phosphate-buffered 1% OsO_4 (21), dehydrated with 50% ethanol and 1 hr in 50% ethanol containing 1% uranyl acetate. After further dehydration at 4 C through a graded series to absolute ethanol and propylene oxide, the pellets were embedded in Epon 812 as described by Luft (18).

In the course of trimming the blocks (glass knife, Sorvall MT-2 ultramicrotome), sections 1 μm thick were cut at various orientations through the pellets and examined under 1300× oil-immersion phase contrast to assess the heterogeneity of the pellets. No gross heterogeneity was detected. Ultrathin sections, silver to silver-gold, were cut with a Du Pont diamond knife, recovered on bare, plasma-hydrophilized grids (20), and stained for 30 sec each in 2% uranyl acetate and 0.4% lead citrate

[4] Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

(28). Electron micrographs were obtained with a Hitachi HU-10.

Gel Electrophoresis. Polyacrylamide gel disc electrophoresis (16) was performed on aliquots of tRNA preparations to estimate the purity and the relative proportions of cytoplasmic to organellar tRNA. Samples containing 0.5 to 1.5 A_{260} units of nucleic acid were applied in electrophoresis buffer containing 10% (w/v) sucrose. Cytoplasmic 18S and 25S rRNA were separated from each other and from chloroplast 16S and 23S rRNA on 2.4% (w/v) gels with a constant current of 5 mamp/gel, at 45 v for 3 hr. Transfer RNA (4S) was separated from 5S rRNA on 10% (w/v) gels with a constant current of 8 mamp/gel, at 70 v for 2.75 hr. The gels were scanned immediately after electrophoresis at 265 nm in a Gilford model 240 spectrophotometer. The proportion of 4S + 5S material relative to the total RNA was estimated from the peak areas on 2.4% gel. Subsequently, the proportion of tRNA (4S) in the 4S + 5S mixture was calculated from the 10% gel by dividing the 4S peak area by the sum of the 4S and 5S peak areas. Multiplication of the percentage of 4S + 5S in the sample (from 2.4% gel) by the proportion of 4S in the 10% gel runs yielded the percentage of 4S in the tRNA preparation.

Protein Determination. The protein content of samples was determined according to the method of Lowry *et al.* (17) using BSA as a standard.

Chl Determination. The Chl content of the washed chloroplast preparation and tissue was determined by measuring the A of ethanolic extracts at 652 nm, as described by Arnon (3).

Isolation of Cytokinins. The leaf and chloroplast tRNA preparations, referred to as L and C, respectively, were hydrolyzed to ribonucleosides with *Crotalus adamanteus* venom and calf intestinal mucosa alkaline phosphatase (11). Lipophilic ribonucleosides were extracted from the lyophilized hydrolysates with water-saturated ethyl acetate (2) and fractionated on Sephadex LH-20 columns with 35% (v/v) deaerated ethanol. Progress of the ribonucleoside elution was monitored at 254 nm. Approximately 8-ml eluate portions were collected which were then combined into fractions on the basis of the peaks in the UV absorption profile and/or known elution volumes of standard cytokinins. Details concerning these fractionations are given with the results of each experiment. Aliquots (5%) of the column fractions were dried under an air stream and the solid residues were acid-hydrolyzed to obtain their respective bases which were then incorporated into tobacco callus bioassay medium for testing of cytokinin activity (15). Cytokinin activities are expressed as KE/22,000 A_{260} units. Cytokinin-active fractions were evaporated to dryness at 45 C. Fractions with elution volumes corresponding to those of ZR and iPA plus msZR were rechromatographed separately on Sephadex LH-20 columns and eluted with deaerated water. The solid material in fractions with cytokinin activity was subjected to spectral, chromatographic, and mass spectral analysis to establish their structural identities.

UV Spectroscopy. UV absorption spectra were obtained with a Cary model 15 spectrophotometer at various stages of the purification procedure to monitor the purity and quantity of the isolated cytokinins. The quantities were estimated from the absorption at the λ_{max} of the sample and the known extinction coefficient of the corresponding synthetic compound in the same solvent (14, 23, 30).

TLC. The stereoisomers of ZR and msZR were separated by applying aliquots of the isolated sample (approximately 4 μg of mixture/cm) at the origin of a 10-cm-long chromatogram (Eastman chromagram sheets, 6060 silica gel with fluorescent indicator). A mixture of the appropriate reference compounds was applied to a section of the same chromatogram, but separated from the sample by a 1-mm vertical scratch through the absorbent layer. The chromatogram was developed in a mixture of chloroform-glacial acetic acid-methanol (45:3:75:1.25) for 15 to 20 min. After drying, the chromatogram was usually developed in the same solvent for a second time to effect greater separation. The dried chromatogram was viewed under UV light (254 nm) and UV absorbing areas were outlined with a soft pencil. For bioassay, the chromatograms were cut into sections on the basis of UV absorbing areas and/or migration of the reference compounds. After acid hydrolysis, the absorbent layer of each section was incorporated into the bioassay medium.

Gas Chromatography/Mass Spectrometry of Cytokinins. The isolated cytokinin-active ribonucleosides were converted to their trimethylsilyl derivatives (TMS) prior to GC/MS. Aliquots (about 25 μg) of the isolated nucleosides were dissolved in 25 μl of pyridine and mixed with 25 μl of N,0-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA). Silylation was carried out at room temperature for 30 min in a desiccator.

Low resolution mass spectra of the TMS derivatives were obtained with a Du Pont 21-491B mass spectrometer interfaced with a Varian 2700 gas chromatograph. The GC was fitted with a coiled glass column (180 × 0.2 cm) packed with 3% OV-1 on 100–200 mesh Aeropak. Helium was used as a carrier gas with a flow rate of 30 ml/min. The column temperature was kept at 275 C.

RESULTS

Preliminary Studies on Chloroplast Isolation. Preliminary experiments were conducted to establish the conditions for a practical, large scale chloroplast isolation procedure to be used with the available equipment. Of primary concern were the integrity, purity, and yield of the isolated chloroplast preparation. The isolation medium described by Mukohata and coworkers (22) was used as a starting point on the basis of their findings that the medium's main ingredient, choline chloride, maintained membrane integrity and thus protected the chloroplasts from deterioration after isolation. In pilot experiments, 1-kg spinach leaf blades were homogenized at 4 C for 30, 60, and 90 sec at low speeds in a Waring Blendor (3.7-liter) with 2 liters of isolation medium. Homogenization longer than 30 sec (*i.e.* 60 and 90 sec) increased the chloroplast dry wt yield (by 9 and 18%, respectively) but decreased the percentage of highly refractile (intact) chloroplasts as observed under the phase contrast microscope. Filtering the homogenate through nylon screen and nylon mesh removed tissue and cell debris and prevented the Miracloth filter from plugging up. Miracloth retains nuclei, but passes chloroplasts and smaller particles. The filtrate was then subjected to differential centrifugation by varying centrifugation speed and time. The major portion of intact chloroplasts appeared to sediment well at values of 580g and lower. Centrifugation for 5, 10, 15, and 30 min resulted in a linear increase in dry wt yields (15, 30, and 75%, respectively, increase over the 5-min run). Observations of these preparations under the phase contrast microscope indicated that centrifugation at 580g for 15 min yielded an acceptable preparation, *i.e.* approximately 60% chloroplasts intact and little contamination with nonchloroplast material. Furthermore, wet volume, dry wt, Chl content, nucleic acid content (A_{260} units), and gel electrophoresis of isolated nucleic acids indicated that centrifugation at 580g for 15 min gave the best preparation in terms of integrity, purity, and yield. Electrophoresis of nucleic acids, extracted from the crude chloroplast preparation, indicated the presence of a considerable amount of cytoplasmic rRNA. Therefore, the chloroplasts were rinsed once with isolation medium.

Quality of Chloroplast Preparation. Forty-four kg of spinach leaf blades yielded 1,600 g wet wt chloroplasts and 195 g dry weight after ethanol-acetone-diethyl ether extraction. The chloroplasts contained 10.9 g of Chl and 16.4 g of protein. On the basis of Chl measurements it is estimated that 34% of the

chloroplasts in the leaf tissue were isolated. Samples withdrawn at the beginning and end of the isolation showed that a washed chloroplast preparation, isolated from 1 kg of fresh leaves, contained 2×10^8 microorganisms and 247 mg of Chl or 1.2×10^{11} chloroplasts (based on the value of 2×10^{-12}g Chl/plastid [5, 10]). Thus, the preparation contained approximately one microorganism/600 chloroplasts.

Observation of electron micrographs of washed chloroplasts (*e.g.* Fig. 1B) indicated that about 50% of the chloroplasts were class II, lacking outer membrane and stroma, but the other 50% were apparently intact (class I). The washed preparations were largely free of the finely fragmented cytoplasmic material and small thylakoid pieces which were abundant in the filtered, whole homogenate (Fig. 1A). In addition to occasional starch grains and lipid bodies, the washed chloroplast preparation also contained a few small objects tentatively identified as mitochondria or microbodies. The incidence of these organelles relative to chloroplasts was considered negligible.

Characterization of tRNA Preparations. A gel electrophoretogram of an aliquot of the 40,000 A_{260} units of sodium acetate-soluble nucleic acids, isolated from spinach leaves, is presented in Figure 2, L-1. The proportions of 23S/25S and 16S/18S, obtained from this gel scan, were 0.18 and 0.70, respectively. The final, purified, leaf tRNA preparation (8,500 A_{260} units) contained approximately 67% 4S + 5S RNA (Fig. 2, L-2), and 63% or 5,355 A_{260} units of 4S tRNA (Fig. 2, L-3).

The first ethanol precipitate of the nucleic acids extracted from the isolated chloroplasts was also fractionated by gel electrophoresis and the profile is presented in Figure 2, C-1. The calculated proportions of 23S/25S and 16S/18S were 2.6 and 3.6, respectively. When these values are compared with those obtained from the leaf nucleic acid preparation, it appears that a 14-fold enrichment of 23S over 25S was achieved and a 5-fold enrichment of 16S over 18S by separating the chloroplasts from the cytoplasm. Although the chloroplast tRNA relative to cytoplasmic tRNA need not necessarily be enriched in a similar fashion, the above calculations may yield an indirect approximation of the magnitude of enrichment of chloroplast tRNA species over those occurring in the cytoplasm. The final purified chloroplast tRNA preparation (7,943 A_{260} units) contained essentially 4S plus 5S RNA (Fig. 2, C-2) of which 75% or 5,957 A_{260} units are 4S tRNA (Fig. 2, C-3). The remainder is 5S RNA and a RNA slightly heavier than the 4S tRNA. This component is also observed when *Escherichia coli* tRNA is fractionated under the same conditions.

Cytokinins from Leaf tRNA. The ethyl acetate-soluble ribo-

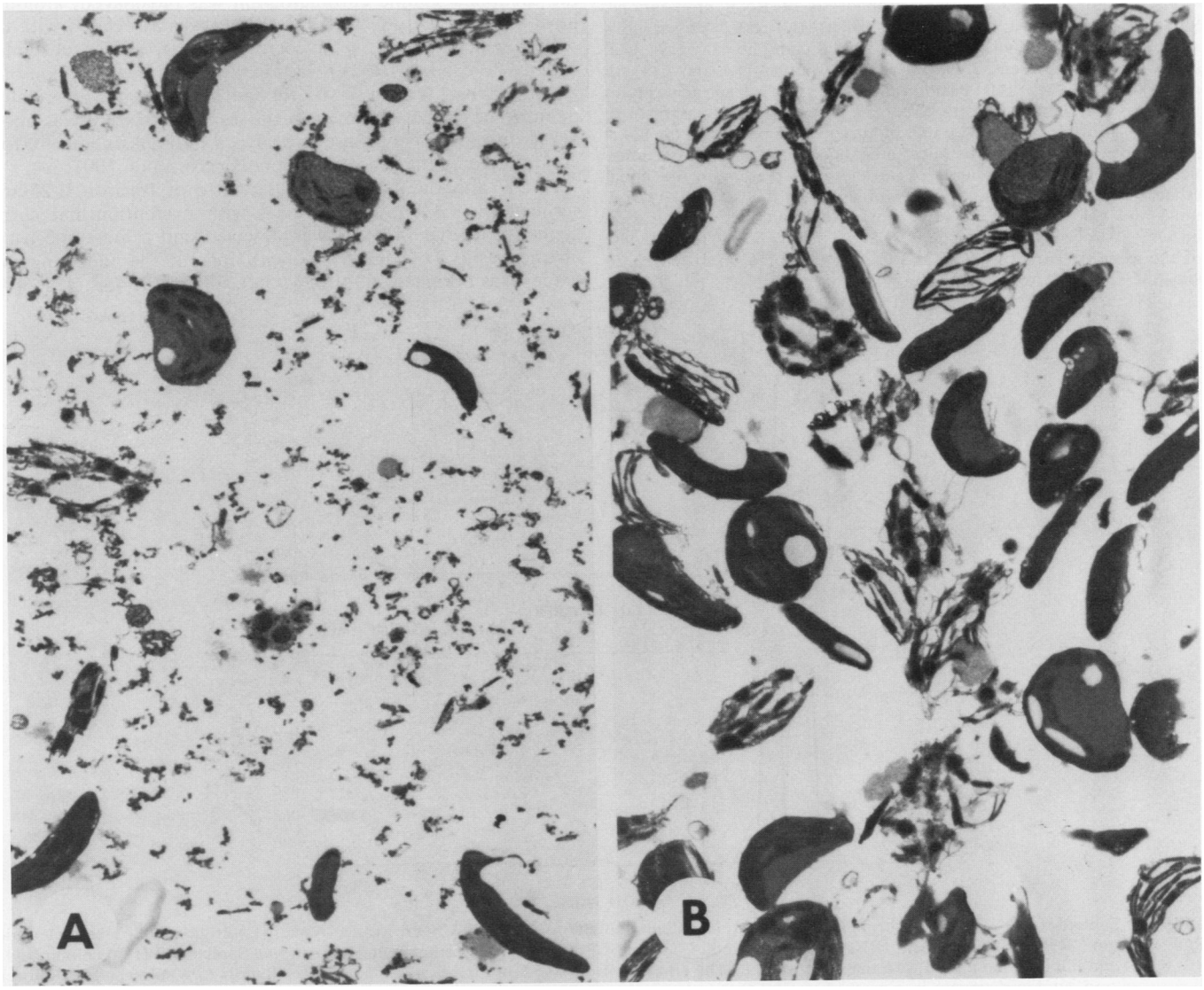

FIG. 1. Thin section electron micrographs of spinach leaf homogenate fractions (×5,400). A: Leaf homogenate after filtration; B: washed chloroplast fraction.

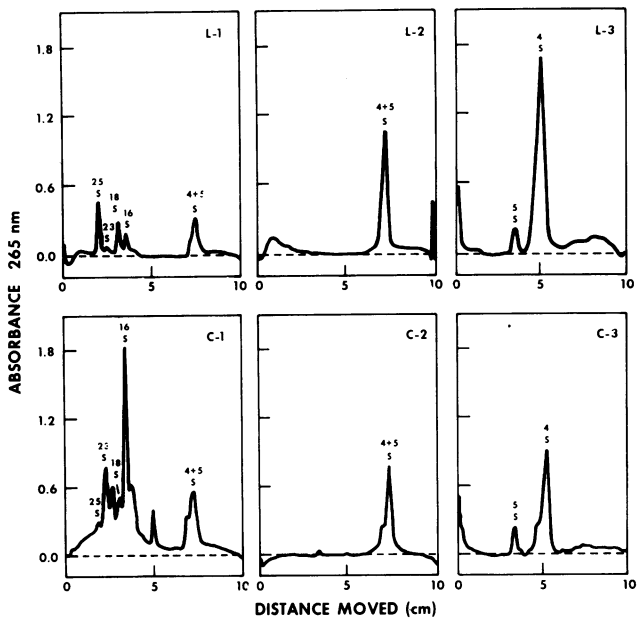

FIG. 2. Polyacrylamide gel electrophoresis of RNA preparations. Spinach left (L) and spinach leaf chloroplast (C) tRNA preparations were extracted, purified, and subjected to electrophoresis. L-1, L-2, and C-1, C-2 represent 2.4% (w/v) gels whereas L-3 and C-3 are profiles of 10% (w/v) gels. Positions of the relevant nucleic acid species in the gel are identified by the Svedberg values commonly ascribed to them. L-1: Profile of 0.45 A_{260} unit of nucleic acids present in the 0.4 M sodium acetate extract of the initial ethanol precipitate; C-1: fractionation profile of 1.52 A_{260} units of nucleic acids present in the initial ethanol precipitate; L-2 and C-2: profiles of purified leaf (0.49 A_{260} unit) and chloroplast (0.46 A_{260} unit) tRNA preparations prior to enzyme hydrolysis; L-3 and C-3: 0.92 and 0.95 A_{260} units, respectively, of the same preparations (L-2 and C-2) fractionated on 10% gels to separate 4S tRNA from 5S tRNA.

nucleosides obtained from the hydrolysate of 240 mg of leaf tRNA were fractionated on a Sephadex LH-20 column eluted with 35% (v/v) ethanol. The compounds separated as shown in Figure 3A. The eluates were pooled into fractions as indicated in the figure and 5% aliquots of the fractions were bioassayed. Cytokinin activity was detected in fractions L-5, 6, 7 (21, 75, 4 KE/g tRNA, respectively) and in fractions L-9, 10, 11 (50, 4, 42 KE/g tRNA, respectively) corresponding to the elution volumes of ZR and iPA plus msZR. Fraction L-7 was discarded because it contained relatively little activity and much contaminating, UV absorbing material. Fractions L-5 and L-6 were combined, lyophilized, and rechromatographed on a Sephadex LH-20 column eluted with water (Fig. 4A). Only fraction L-25, corresponding to the elution volume of ZR, contained growth-stimulating material (44 KE/g tRNA) when 5% aliquots were bioassayed. The material in this fraction yielded a UV spectrum characteristic of ZR. On the basis of UV absorption at λ_{max}, the quantity of ZR is approximately 100 μg. A 7.5% aliquot of fraction L-25 along with *t*-ZR and *c*-ZR standards was chromatographed twice on TLC (Fig. 5). Three UV absorbing spots were observed, one of which (IV) had a R_F roughly corresponding to *c*-ZR but no UV absorbing spot was detected in the region with an R_F corresponding to *t*-ZR (II). However, when the sectioned sample chromatogram was bioassayed, although most of the activity (121 KE/g tRNA) was associated with the section which had a R_F value corresponding to *c*-ZR, substantial activity (105 KE/g tRNA) was also detected in the region of *t*-ZR. Although section II (t-ZR) had high activity, the relative quantity of the *trans* isomer in the *cis/trans* mixture was about 2% (w/w). This is because hydrolyzed *t*-ZR has about 40-fold greater activity than the corresponding *cis* isomer (30).

A 2% aliquot of the TMS derivative of fraction L-25 was subjected to GC/MS. Only a peak with a retention time corresponding to that of *c*-ZR was detected and a mass spectrum, characteristic for $ZR(TMS)_4$, with ions at the following m/e values was obtained: 639, 624, 550, 536, 508, 483, 421, 406,

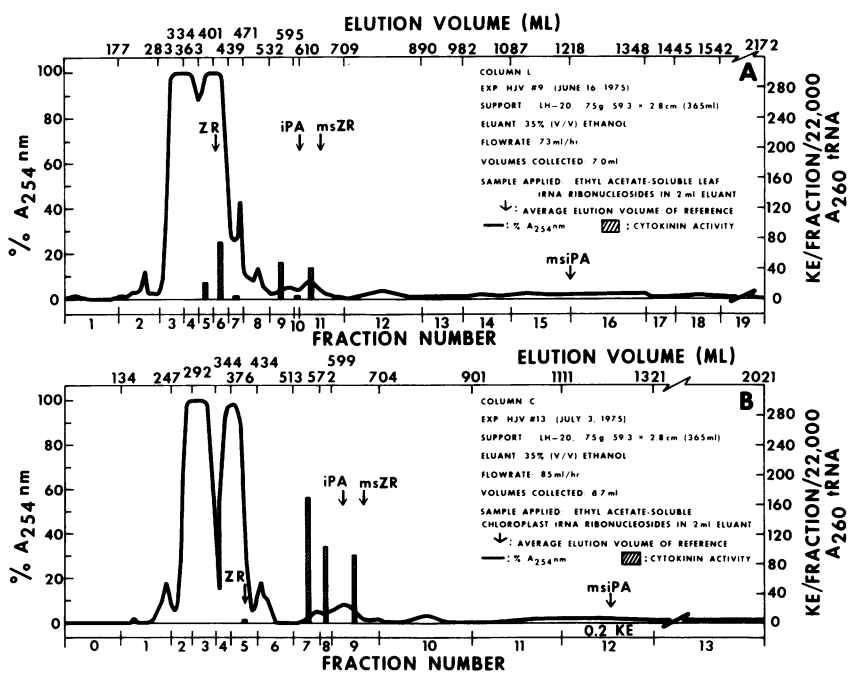

FIG. 3. Sephadex LH-20 column chromatography of spinach leaf and chloroplast tRNA hydrolysates. A: Ethyl acetate-soluble ribonucleosides obtained from 5,355 A_{260} units of leaf tRNA were dissolved in 2 ml of 35% (v/v) aqueous ethanol and applied to a Sephadex LH-20 column (59.3 × 2.8 cm) equilibrated and eluted with the same solvent. The eluates were pooled into fractions as indicated. Aliquots (5%) of each fraction were bioassayed and cytokinin activity is expressed as KE/22,000 A_{260} units of tRNA and is represented by the shaded vertical bars. Twenty-two A_{260} units of tRNA are assumed to represent 1 mg of tRNA. B: Ribonucleoside mixture obtained from 4,765 A_{260} units of chloroplast tRNA was chromatographed and processed as under A.

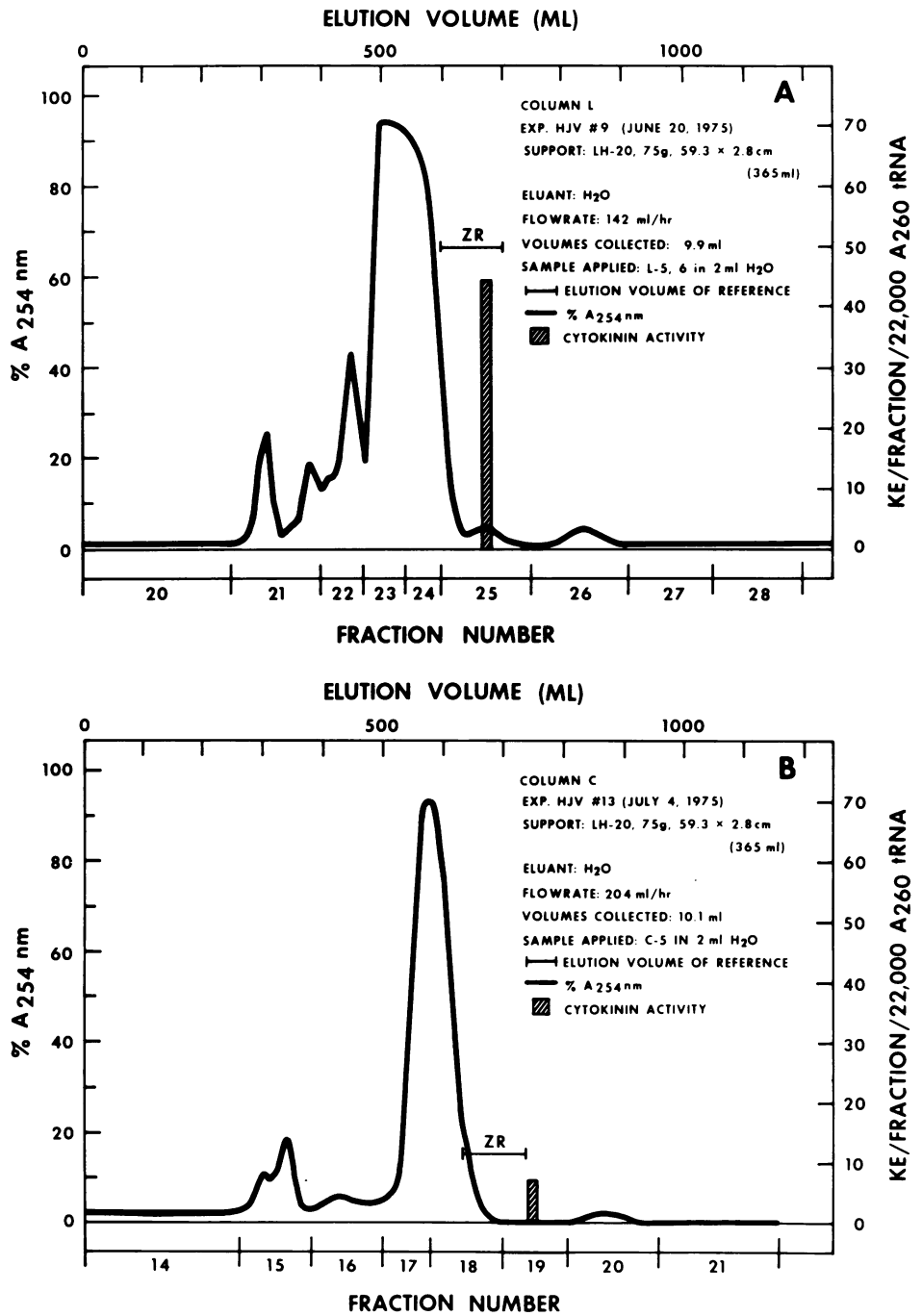

FIG. 4. Rechromatography of presumed ZR-containing fractions. Cytokinin-containing fractions L-5 and L-6 in Figure 3A were combined. The resulting fraction and fraction C-5 were evaporated to dryness. Each was dissolved in 2 ml of water and was then applied to separate Sephadex LH-20 columns (59.3 × 2.8 cm) equilibrated with water. The elution of the columns was monitored at 254 nm and resulted in separation profiles A and B, respectively. The collected volumes were pooled to fractions as indicated and 5% aliquots were removed for bioassay. Cytokinin activity, expressed as KE/22,000 A_{260} tRNA, is represented by the shaded bars.

348, 320, 290, 276, 259, 245, 230, 217, 188, and 160. The fragmentation pattern and the intensities of the fragments of the TMS derivative of ZR are presented in Figure 6.

Fractions L-9, 10, and 11 from Figure 3A were combined, evaporated to dryness, taken up in water, and rechromatographed on a Sephadex LH-20 column and eluted with water (Fig. 7A). Bioassay of 5% aliquots of each fraction indicated that fractions L-32 (168 KE/g tRNA) and L-34 (46 KE/g tRNA) contained active materials and their elution volumes corresponded to iPA and msZR, respectively. As fraction L-33 (24 KE/g tRNA) probably contained material from both fraction L-32 and L-34, it was not processed further. The UV spectrum of the material in fraction L-32 was characteristic of iPA and indicated that up to 110 μg could be present. Gas chromatography of 1% of the derivatized L-32 material showed a peak with a retention time similar to that of derivatized iPA. The mass spectrum of the TMS derivative of the material in this fraction showed ion peaks characteristic for iPA$(TMS)_3$, as follows: 551, 536, 508, 481, 348, 333, 318, 259, 245, 232, 230, 217, 203, 188, and 100. The intensities of these fragments are presented in Figure 8A.

Fraction L-34 (from Fig. 7A), when taken up in ethanol,

exhibited a UV spectrum similar to that of msZR and the estimated amount was 112 μg of msZR. Gas chromatography of the derivatized fraction L-34 yielded a peak with the retention time similar to that of derivatized synthetic msZR. The mass spectrum of the material represented by the peak showed ions at the following m/e values: 685, 670, 595, 581, 554, 529, 467, 452, 366, 349, 336, 322, 276, 247, 245, 232, 217, 207, 194, and 182; *inter alia*, typical of msZR(TMS)$_4$. The mass spectrum of the sample, showing the relative intensities of the fragments, is presented in Figure 9A.

TLC of a 7.5% aliquot of L-34 (Fig. 10A) showed two UV absorbing areas, one of which (IV) had the same R_F as *c*-msZR and the other, weaker, spot (II, III) could possibly represent the *trans* isomer. Bioassays of the marked sections showed that most active material was located in the UV absorbing areas. The activity in the section with the presumed *cis* isomer (42 KE/g tRNA) was greater than that in the section with *trans* isomer (20 KE/g tRNA). Because the relative activity of acid-hydrolized-msZR is approximately seven times that of acid-hydrolized *c*-msZR (30) it can be estimated that fraction L-34 contained approximately 6% *trans* isomer of msZR. It is unlikely that this small amount of *t*-msZR can be detected under UV light. Therefore, it is assumed that the UV absorption in areas II and III is due to the presence of a contaminant.

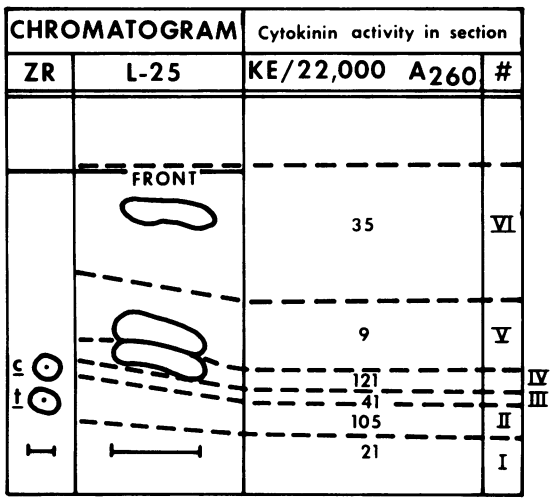

FIG. 5. TLC separation of ZR stereoisomers. Material in fraction L-25 (Fig. 4A) was chromatographed with a mixture of synthetic *c*- and *t*-ribosylzeatin and developed twice with chloroform-glacial acetic acid-methanol (45:3.75:1.25). Numbered sections of the chromatogram with the sample were bioassayed to determine the distribution of cytokinin activity. Lower UV absorbing spot in reference chromatogram represents *trans* isomer.

Cytokinins from Chloroplast tRNA. Approximately 80% of the lipophilic ribonucleosides extracted from the hydrolysate of 270 mg of chloroplast tRNA were dissolved in 35% (v/v) ethanol and chromatographed on a Sephadex LH-20 column with the same solvent (Fig. 3B). The eluates were pooled into fractions on the basis of UV absorption and the elution volumes of cytokinins known to occur in tRNA. Bioassay of 5% aliquots of these fractions indicated that presence of cytokinin-active material in fractions C-5 (15 KE/g tRNA) and C-7, 8, 9 (170, 104, 92 KE/g tRNA), respectively; which correspond to the elution volumes of ZR and iPA plus msZR. The remainder (95%) of fraction C-12, corresponding to the elution volume of msiPA, was bioassayed *in toto* but activity (0.2 KE/g tRNA) was only barely detectable. Fraction C-5 was lyophilized and the solid material, dissolved in water, was chromatographed on a Sephadex LH-20 column eluted with water (Fig. 4B). Bioassay of fractions, cut on the basis of UV absorption and the elution volume of ZR, revealed a low level of cytokinin activity in fraction C-19, roughly corresponding to the elution volume of ZR. The remaining 90% of this fraction was subsequently used for bioassay and confirmed the presence of a small amount of presumed ZR (7 KE/g tRNA). No further tests concerning unequivocal identification and stereoisomeric configuration of the active material were performed.

Fractions C-7, 8, and 9 were combined, lyophilized, and redissolved in water. The sample was applied to a Sephadex LH-20 column and eluted with water (Fig. 7B). The elution profile contained three UV absorbing peaks, two of which had elution volumes corresponding to iPA (C-25) and msZR (C-28), respectively. The UV absorption spectrum of C-25 was characteristic of iPA and the fraction contained an estimated amount of 103 μg of presumed iPA. The fraction was lyophilized and 1.6% was derivatized and subjected to GC/MS. The sample yielded a peak with the retention time of 4.1 min

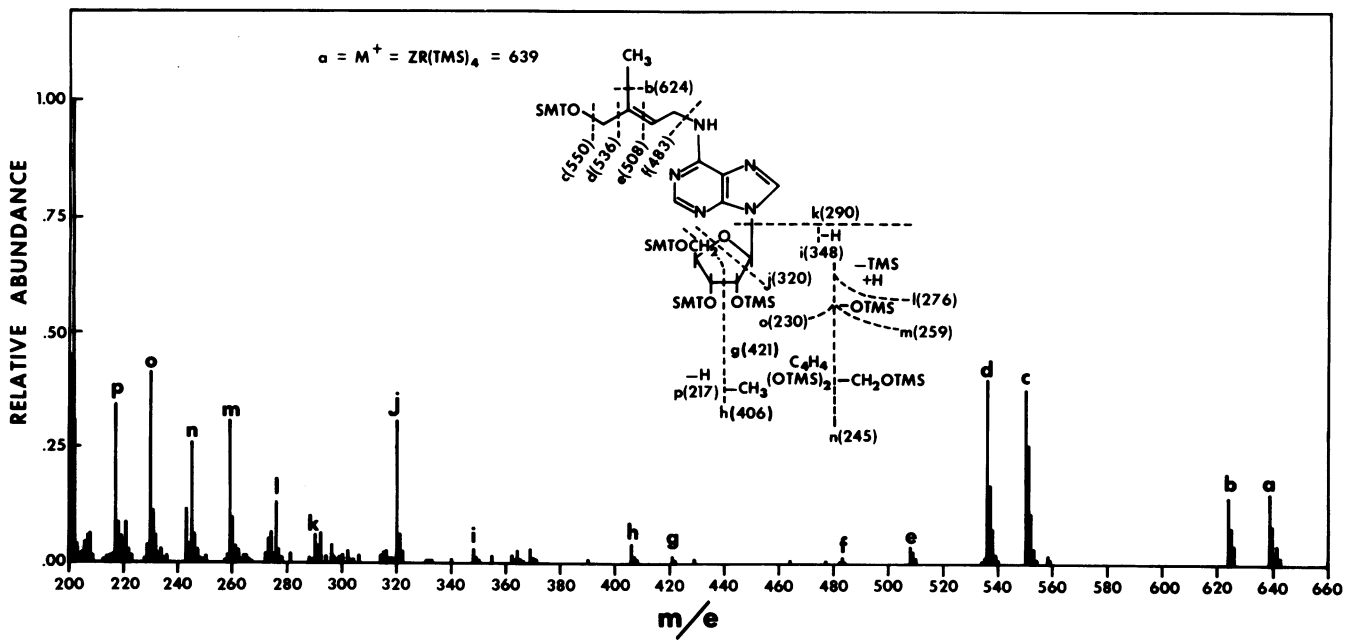

FIG. 6. Low resolution mass spectrum of TMS derivative of ZR isolated from spinach leaf tRNA.

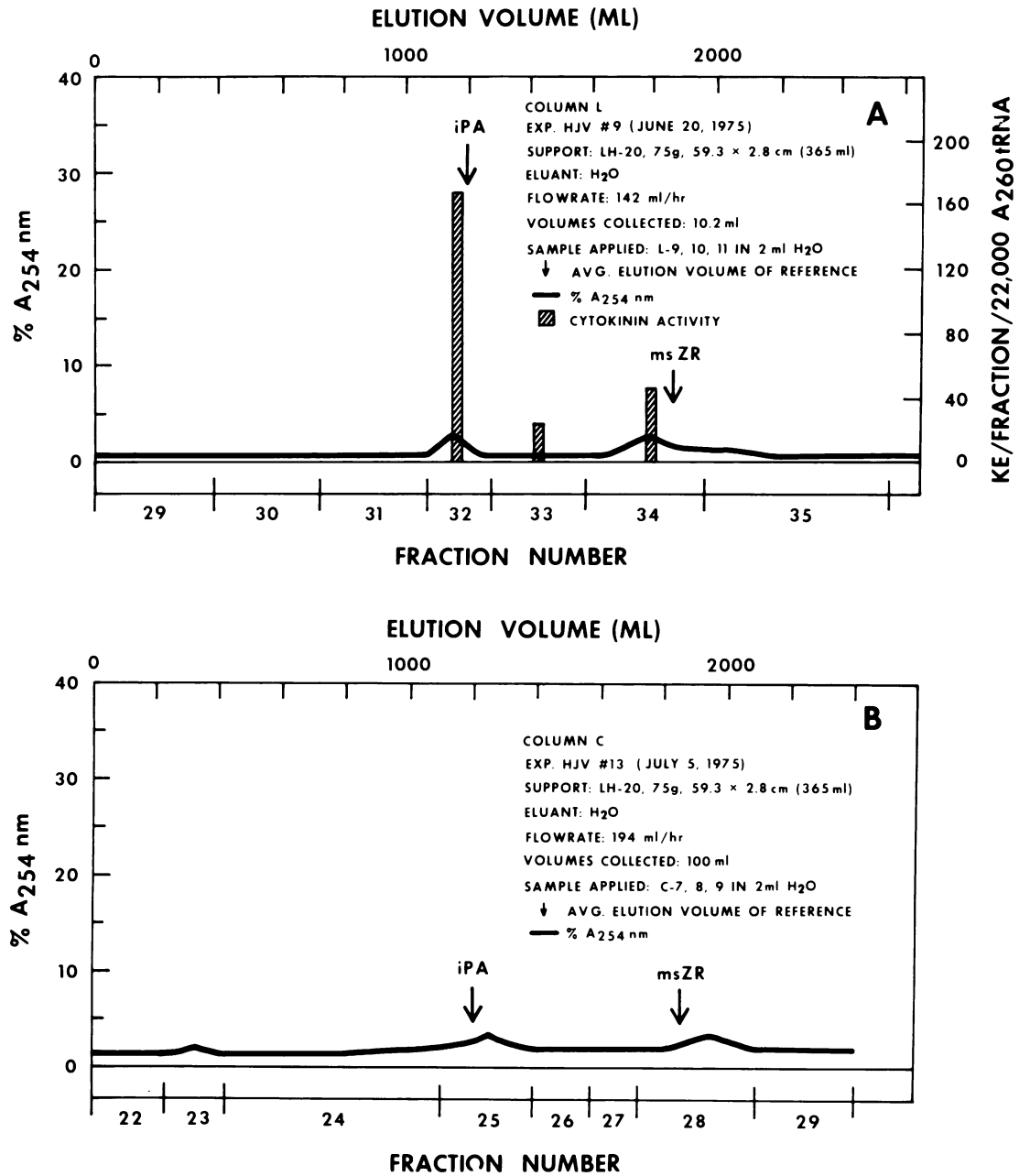

FIG. 7. Rechromatography of fractions with presumed iPA and msZR. A: Cytokinin-active fractions L-9, 10, and 11 in Figure 3A were combined and evaporated to dryness. The residue was dissolved in 2 ml of water and chromatographed on a Sephadex LH-20 column with water. Elution was monitored at 254 nm and gave the above fractionation profile. The collected eluate was pooled into fractions as shown. Five percent aliquots were removed from each fraction for bioassay to determine the cytokinin distribution. Cytokinin activity is represented by shaded vertical bars. B: Cytokinin-containing fractions C-7, 8, and 9 in Figure 3B, isolated from chloroplast tRNA, were combined and further treated as under A.

corresponding to that of iPA(TMS)$_3$. The mass spectrum, generated by this peak, presented in Figure 8B, is similar to that obtained earlier for the iPA isolated from leaf tRNA (Fig. 8A).

Fraction C-28, from Figure 7B, taken up in ethanol had a UV spectrum which resembled that of msZR and contained about 149 μg of this cytokinin. About 5% of this fraction was silylated and injected into the GC/MS. The gas chromatogram showed a peak with the retention time of 9.8 min, corresponding to msZR(TMS)$_4$. The mass spectrum of this materail is presented in Figure 9B and it contains the fragments characteristic of the TMS derivative of msZR depicted in Figure 9A. This evidence established the identity of the cytokinin in fraction C-28 as msZR.

The separation and identification of the stereoisomers in fraction C-28 (in Fig. 7B) were achieved by TLC and bioassay. Chromatography of a 7.5% aliquot of this fraction showed three UV abosrbing bands, one of which (III) was at the R_F of *c*-msZR (Fig. 10B). No UV absorption was observed in the area corresponding to the *trans* isomer. Bioassay of the marked sections of the chromatogram showed significant cytokinin activity (24 KE/g tRNA) only in the area corresponding to the R_F of *c*-msZR.

DISCUSSION

The EM photographs show that the isolated chloroplast preparation was of good quality. It contained predominantly chloroplasts, about half of which appeared to be intact. It was

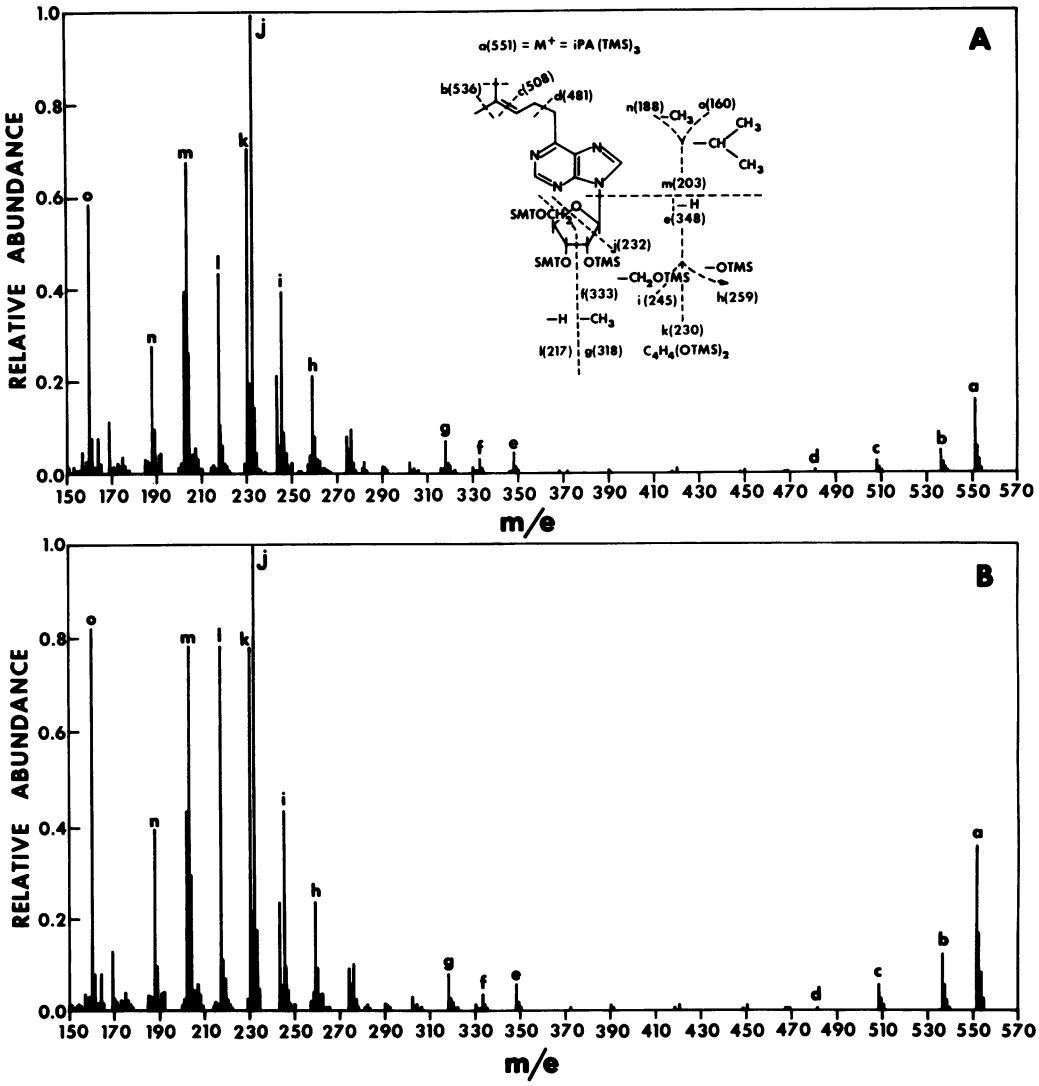

FIG. 8. Low resolution mass spectra of TMS derivative of iPA isolated from spinach. A: iPA from leaf tRNA; B: iPA from chloroplast tRNA.

largely devoid of fragmented cytoplasmic material, mitochondria, and microbodies. Microbiological assays showed that bacterial contamination was only about 0.2%. On the basis of these criteria, contamination of the chloroplast tRNA preparation by these two potential sources of extrachloroplast tRNA was judged to be negligible.

A direct biochemical assessment of contamination of the chloroplast tRNA by cytoplasmic tRNA would have required reverse phase chromatography of both the spinach leaf and chloroplast tRNA species (1, 19). We have used the presence of cytoplasmic 18S and 25S RNA as an index of cytoplasmic tRNA contamination. The results show less than a 20 to 30% contamination by these cytoplasmic rRNAs in the chloroplast tRNA preparation (Fig. 2, C-1). There was about 14-fold enrichment of 23S chloroplast rRNA over 25S cytoplasmic rRNA in the final chloroplast preparation, suggesting an enrichment of chloroplast components and possibly chloroplast tRNA as well. The contamination of chloroplast tRNA by cytoplasmic tRNA is thus probably relatively small. The low level of cytokinin activity presumably due to ZR from chloroplast tRNA (Fig. 3B) also lends support to this conclusion because this cytokinin-active compound is thought to be abundant in cytoplasmic tRNA.

Hall *et al.* (12) have analyzed tRNA from (frozen) spinach and reported the presence of both ZR and iPA with a weight ratio of 250:340, respectively. They also investigated frozen pea and corn tRNA where the ratios were found to be 270:130 and 1,900:0, respectively. Pea root tRNA also was found to contain ZR and iPA in the ratio of 750:130 (4). On the basis of these results from green and nongreen tissues, Vreman (29) suggested that iPA might be of chloroplast origin. The occurrence of iPA in nongreen tissues such as wheat germ (6) and tobacco callus (7) could be attributed to the presence of proplastids which are known to contain chloroplast-specific tRNAs. The results presented here clearly show the occurrence of iPA in chloroplast tRNA, but do not exclude its presence in cytoplasmic tRNA.

Whereas no significant amount of ZR was detected in chloroplast tRNA, a substantial amount of ZR was isolated from leaf tRNA suggesting that ZR is of cytoplasmic origin. These results correlate with the report of Swaminathan *et al.* (26) who have presented evidence for the cytoplasmic localization of ZR. The proportion of the *trans* isomer of ZR in the spinach leaf tRNA is estimated to be 2%. This level compares well with previously reported levels (2.5-5.7%) for green tissue tRNA (29, 31). Nongreen tissues have been found to contain only 0.4 to 0.8% *t*-ZR (29). The predominant occurrence of the *cis* isomer is in conformity with the isomeric distribution reported for other plant tRNA preparations. However, because of the large errors involved in the determination of relative activities of the isomers

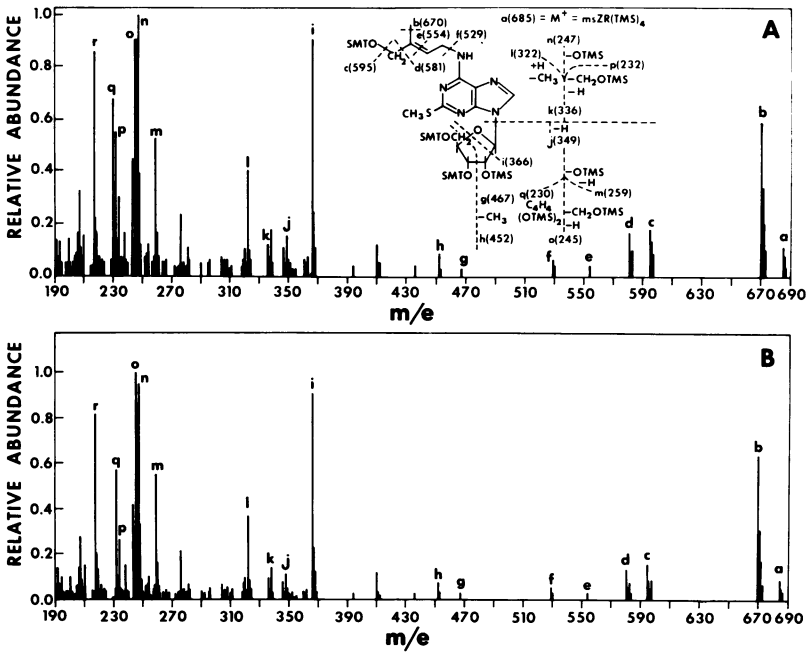

FIG. 9. Low resolution mass spectra of TMS derivative of msZR isolated from spinach. A: msZR from leaf tRNA; B: msZR from chloroplast tRNA.

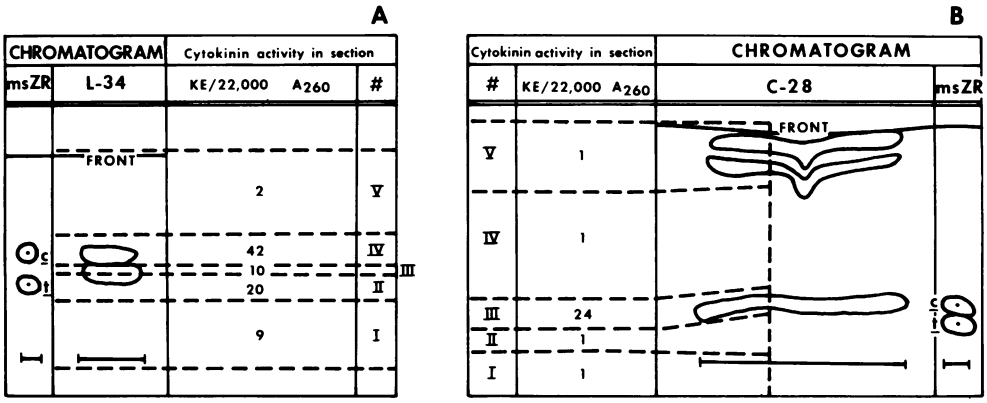

FIG. 10. TLC separation of msZR stereoisomers. A: msZR isolate from leaf tRNA, L-34, and a mixture of synthetic *c*- and *t*-msZR were chromatographed twice. Numbered sections of sample chromatogram were bioassayed. Lower UV absorbing spot in reference chromatogram represents *t*-msZR. UV absorption in sections II and III is probably due to presence of a compound other than *t*-msZR. B: msZR isolate from chloroplast tRNA was chromatographed once, as described under "Materials and Methods." Numbered sections were bioassayed and cytokinin activity is given in KE/22,000 A_{260} units.

and the additional errors involved in estimating the contents of the two isomers in a given sample, the calculated ratios should be considered to represent a relative concentration range rather than a specific value.

The results of this investigation show a parallelism between prokaryotes and chloroplasts, in that chlorplast tRNAs contain the same cytokinin-active ribonucleosides, iPA and msZR, that can be found in prokaryotes. Spinach leaf tissue tRNA contained, in addition, the *c*-ZR and *t*-ZR which are probably of cytoplasmic origin. The amounts of msZR isolated from the tRNA of whole leaves and their chloroplasts are 468 and 690 μg/g tRNA, respectively. The amount of the msZR found in leaf tRNA was much higher than would be expected if msZR were localized exclusively in the chloroplast. Considering the limitations of quantitation of this cytokinin and the extent of the observed quantitative difference, it is not possible to draw a definite conclusion as to the presence of msZR in cytoplasmic tRNA. However, Swaminathan *et al.* (26) have reported that msZR is localized in chloroplast tRNA in *Euglena gracilis*. As to the isomeric distribution, no *t*-msZR was detected in chloroplast tRNA. Generally, plant tRNA from green tissues was shown to contain considerable amounts of *trans* isomer (about 35–50% of msZR). The absence of *t*-msZR in chloroplast tRNA of spinach leaves contrasts with the distribution pattern observed earlier in green tissues of plants.

Acknowledgments—The authors express their appreciation to L. Walker for her valuable technical assistance. We wish to thank D. Michener's laboratory for performing the bacterial counts and L. Rossi for assistance in preparing the chloroplast samples for electron microscopy. We are grateful to F. Skoog for providing the Wisconsin 38 tobacco seeds and tobacco callus tissues.

LITERATURE CITED

1. ALIEV KA, II FILIPOVICH 1968 Differences between transfer ribonucleic acids and aminoacyl-tRNA synthetases from chloroplasts and from the cytoplasm of pea seedlings. Mol Biol 2: 297–304
2. ARMSTRONG DJ, WJ BURROWS, PK EVANS F SKOOG 1969 Isolation of cytokinins from

tRNA. Biochem Biophys Res Commun 37: 451-456
3. ARNON DI 1949 Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. Plant Physiol 24: 1-15
4. BABCOCK DF, RO MORRIS 1970 Quantitative measurement of isoprenoid nucleosides in transfer ribonucleic acid. Biochemistry 9: 3701-3705
5. BURKARD G, P GUILLEMAUT, JH WEIL 1970 Comparative studies of the tRNA's and the aminoacyl tRNA synthetases from the cytoplasm and the chloroplasts of *Phaseolus vulgaris*. Biochim Biophys Acta 224: 184-198
6. BURROWS WJ, DJ ARMSTRONG, M KAMINEK, F SKOOG, RM BOCK, SM HECHT, LG DAMMANN, NJ LEONARD, J OCCOLOWITZ 1970 Isolation and identification of four cytokinins from wheat germ tRNA. Biochemistry 9: 1867-1872
7. BURROWS WJ, F SKOOG, NJ LEONARD 1971 Isolation and identification of cytokinins located in tRNA of tobacco callus grown in the presence of 6-benzylaminopurine. Biochemistry 10: 2189-2194
8. CHAPMAN RW, RO MORRIS, JB ZAERR 1976 Occurrence of *trans*-ribosylzeatin in *Agrobacterium tumefaciens* tRNA. Nature 262: 153-154
9. FAIRFIELD SA, WE BARNETT 1971 On the similarity between tRNAs of organelles and prokaryotes. Proc Nat Acad Sci USA 68: 2972-2976
10. GAFFRON H 1968 Energy storage: photosynthesis. *In* FC Steward, ed. Plant Physiology, Vol 1B. Academic Press, New York p 3
11. HALL, RH 1964 Isolation of N^6-(aminoacyl)adenosine from yeast ribonucleic acid. Biochemistry 3: 769-773
12. HALL RH, L CSONKA, H DAVID, B MCLENNAN 1967 Cytokinins in the soluble RNA of plant tissues. Science 156: 69-71
13. HECKER LI, M UZIEL, WE BARNETT 1976 Comparative base compositions of chloroplast and cytoplasmic $tRNA_s^{phe}$ from *Euglena gracilis*. Nucleic Acid Res 3: 371-380
14. LEONARD NJ, S ACHMATOWICZ, RN LOEPPKY, KI CARRAWAY, WH GRIMM, A SZWEYKOWSKA, HQ HAMZI, F SKOOG 1966 Development of cytokinin activity by rearrangement of 1-substituted adenine to 6-substituted aminopurines: inactivation by N^6, 1-cyclization. Proc Nat Acad Sci USA 56: 706-716
15. LINSMAIER EM, F SKOOG 1965 Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18: 100-127
16. LOENING UE 1969 The determination of the molecular weight of ribonucleic acid by polyacrylamide-gel electrophoresis. Biochem J 113: 131-138
17. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275
18. LUFT JH 1961 Improvements in epoxy resin embedding methods. J Biophys Biochem Cytol 9: 409-414
19. MERRICK WC, LS DURE III 1972 The developmental biochemistry of cottonseed embryogenesis and germination. IV. Levels of cytoplasmic and chloroplasmic transfer ribonucleic acid species. J Biol Chem 247: 7988-7999
20. MILLARD MM, R SCHERRER, RS THOMAS 1976 Surface analysis and depth profile composition of bacterial cells by x-ray photoelectron spectroscopy and oxygen plasma etching. Biochem Biophys Res Commun 72: 1209-1217
21. MILLONIG G 1961 Advantages of a phosphate buffer for OsO_4 solutions in fixation. J Appl Physics 32: 1637
22. MUKOHATA Y, T YAGI, M HIGASHIDA, A MUTSUNO, Y SUGIYAMA 1974 Comparative studies on the properties of spinach chloroplasts prepared by media containing choline chloride or sucrose. Plant Cell Physiol 15: 619-628
23. PLAYTIS AJ, NJ LEONARD 1971 The synthesis of ribosyl-*cis* zeatin and thin layer chromatographic separation of the *cis* and *trans* isomers of riboslyzeatin. Biochem Biophys Res Commun 45: 1-5
24. RALPH RK, AR BELLAMY 1964 Isolation and purification of undegraded ribonucleic acids. Biochim Biophys Acta 87: 9-16
25. SKOOG FD, J ARMSTRONG 1970 Cytokinins. Annu Rev Plant Physiol 21: 359-384
26. SWAMINATHAN S, RM BOCK, F SKOOG 1977 Subcellular localization of cytokinins in transfer ribonucleic acid. Plant Physiol 59: 558-563
27. THIMMAPPAYA B, J CHERAYIL 1974 Unique presence of 2-methylthio-ribosylzeatin in the transfer ribonucleic acid of the bacterium *Pseudomonas aeruginosa*. Biochem Biophys Res Commun 60: 665-672
28. VENABLE JH, R COGGESHALL 1965 A simplified lead citrate stain for use in electron microscopy. J Cell Biol 25: 407-408
29. VREMAN HJ 1973 Isolation of *cis* and *trans* isomers of ribosylzeatin and 2-methylthio-ribosylzeatin from plant tRNA preparations. PhD thesis. University of Wisconsin
30. VRÊMAN HJ, RY SCHMITZ, F SKOOG, AJ PLAYTIS, CR FRIHART, NJ LEONARD 1974 Synthesis and biological activity of 2-methylthio-*cis*- and *trans* zeatin and their ribosyl derivatives. Isolation of the *cis*- and *trans* ribonucleosides from *Pisum* tRNA. Phytochemistry 13: 31-37