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Isolation and Estimation of Cytokinins and Cytokinin-Containing Transfer RNAs from *Cucumis sativus* L. var. Guntur Seedlings

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ABSTRACT

N^6-(Δ^2-Isopentenyl) adenosine antibodies were used for the isolation of free cytokinins and cytokinin-containing tRNAs from parts of *Cucumis sativus* L. var. Guntur seedlings and for the estimation of cytokinins in them. Immobilized N^6-(Δ^2-isopentenyl) adenosine antibodies retained tRNAs containing N^6-(Δ^2-isopentenyl) adenosine and N^6-(4-hydroxy-3-methylbut-2-enyl) adenosine with equal efficiencies. There were at least five cytokinins in the free form in cucumber seedlings. N^6-(4-Hydroxy-3-methylbut-2-enyl) adenosine, N^6-(Δ^2-isopentenyl) adenosine, and N^6-(Δ^2-isopentenyl) adenine were present at least to the extent of 80, 23, and 9 nanograms, respectively, in the cotyledons and 40, 6, and 3 nanograms, respectively, in the decotyledonated seedlings per gram of tissue. Only two cytokinins were found in the tRNAs of cucumber cotyledons, namely N^6-(Δ^2-isopentenyl) adenosine and N^6-(4-hydroxy-3-methylbut-2-enyl) adenosine in amounts of 12 and 318 nanograms, respectively, per gram of tissue. Immunoaffinity chromatographic analysis of radiolabeled aminoacyl tRNAs from cucumber cotyledons showed that $tRNA^{Phe}$ and $tRNA^{Tyr}$ contained cytokinins whereas $tRNA^{Ala}$ did not.

Cytokinins are of interest in view of their possible regulatory roles in several stages of plant development. Cytokinins are known to occur in free forms and as constituents of tRNAs where they appear to influence translational process (15). Free cytokinins are usually extracted from plant source with organic solvents (10). Chromatography on Sephadex LH-20 has proved to be an efficient method for the fractionation of cytokinins (2). Several reports are available about the isolation of cytokinins in tRNAs from different sources by enzymic hydrolysis of the tRNAs to nucleosides and subsequent fractionation by column chromatography on Sephadex LH-20. In these studies, the cytokinins were monitored by bioassays (15). A rapid and sensitive radioimmunoassay has been developed for N^6-(Δ^2-isopentenyl) adenosine using N^6-(Δ^2-isopentenyl) adenosine antibodies and applied for the estimation of N^6-(Δ^2-isopentenyl) adenosine in tRNA hydrolysates (13, 17, 20) and in germinating rice embryos (21). In the present work, the assay is extended to N^6-(Δ^2-isopentenyl) adenine and N^6-(4-hydroxy-3-methylbut-2-enyl) adenosine and used to determine the distribution of N^6-(4-hydroxy-3-methylbut-2-enyl) adenosine, N^6-(Δ^2-isopentenyl) adenosine, and N^6-(Δ^2-isopentenyl) adenine in the cotyledons and decotyledonated seedlings of *Cucumis sativus* L. var. Guntur. Our goal was to investigate if cotyledons, rich in reserve food materials, contain cytokinins.

From the studies on the distribution of free cytokinins in *Cucumis sativus* L. var. Guntur cotyledons, N^6-(4-hydroxy-3-methylbut-2-enyl) adenosine was found to be the major one present. So it was of interest to study the distribution of cytokinins in the tRNAs of cucumber cotyledons because free cytokinins can be formed by *de novo* synthesis or by degradation of tRNA. In the present investigations, the cucumber cotyledon tRNAs were converted into nucleosides and fractionated on a Sephadex LH-20 column, and subsequently, the cytokinins were estimated by radioimmunoassay.

N^6-(Δ^2-isopentenyl) adenosine antibodies have been used for the detection and isolation of tRNAs containing N^6-(Δ^2-isopentenyl) adenosine from yeast tRNAs (20) and for the enrichment of *Bacillus subtilis* and rabbit or rat liver tRNAs containing N^6-(Δ^2-isopentenyl) adenosine (17). It was of interest to know whether tRNAs containing N^6-(4-hydroxy-3-methylbut-2-enyl) adenosine were also retained by the anti-N^6-(Δ^2-isopentenyl) adenosine affinity column. To that end, cucumber cotyledon tRNAs were chromatographed on a column containing immobilized anti-N^6-(Δ^2-isopentenyl) adenosine. The tRNAs that bound to the column were isolated and then converted to nucleosides, which were subsequently fractionated by Sephadex LH-20 chromatography. Cytokinins were then estimated by the radioimmunoassay. The results showed that the anti-N^6-(Δ^2-isopentenyl) adenosine affinity column retained tRNAs containing N^6-(Δ^2-isopentenyl) adenosine and N^6-(4-hydroxy-3-methylbut-2-enyl) adenosine.

In order to detect specific tRNAs containing cytokinins, [^{14}C] aminoacyl-tRNAs were prepared and passed through a column containing immobilized N^6-(Δ^2-isopentenyl) adenosine antibodies. The tRNAs retained on the column were eluted with 10% pyridine. The results showed that $tRNA^{Phe}$ and $tRNA^{Tyr}$ from cucumber cotyledons contained cytokinins whereas $tRNA^{Ala}$ did not contain cytokinins.

MATERIALS AND METHODS

i^6A[2], i^6Ade, io^6A (predominantly *trans* isomer containing approximately 8% *cis* isomer), Sepharose 4B, and BSA were from Sigma Chemical Co. [^{3}H]i^6A trialcohol (specific activity, 6000 cpm/pmol) was prepared from i^6A by periodate oxidation and subsequent reduction with NaB^3H_4 (18). Nitrocellulose filters (0.45 μ; MD1 filters) were from Microdevices, Ambala, India. Bacterial alkaline phosphatase and snake venom phosphodiesterase were from Worthington Biochemical Corp. Sephadex LH-20 was from Pharmacia Fine Chemicals, Sweden. L-[^{14}C]Alanine (60 mCi/mmol), L-[^{14}C]phenylalanine (180 mCi/mmol), and L-[^{14}C]tyrosine (270 mCi/mmol) were from Bhaba Atomic Research Centre, Bombay, India. The seeds of *Cucumis sativus* L. var. Guntur were

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[2] Abbreviations: i^6A, N^6-(Δ^2-isopentenyl) adenosine; i^6Ade, N^6-(Δ^2-isopentenyl) adenine; io^6A, N^6-(4-hydroxy-3-methylbut-2-enyl) adenosine or zeatin riboside; bzl^6A, N^6-benzyladenosine; kinetin riboside, N^6-furfuryladenosine; TBS, Tris-buffered saline (0.01 M Tris-HCl, pH 7.5, containing 0.14 M NaCl and 0.02% NaN_3).

provided by Karnataka Seed Corp., Bangalore, India. All other chemicals used were of analytical grade available commercially.

Preparation and Purification of i^6A Antibodies. i^6A antibodies were raised in rabbits by injecting BSA-i^6A conjugate (11, 17, 19). The antibodies were purified by affinity chromatography on an AH-Sepharose-5′-phosphoryl-N^6-(Δ^2-isopentenyl) adenosine 3′,2′-cyclic phosphate column using i^6A for elution (19). They did not bind to any of the normal nucleosides or modified nucleosides like N^6-[9-β-D-(ribofuranosyl)purin-6-yl carbamoyl]-L-threonine, but bound to the cytokinins i^6A, i^6Ade, kinetin riboside, bzl^6A, and io^6A.

Sepharose-Anti-i^6A. The purified i^6A antibodies were coupled to cyanogen bromide-activated Sepharose 4B according to the procedure of March *et al.* (16). About 3.5 mg antibodies were found coupled per ml, wet weight, Sepharose. The specificity of immobilized antibodies was tested by packing it in a 4-ml column and chromatographing [^{3}H]i^6A trialcohol (30 pmol), io^6A (28 nmol), and [^{3}H]Ado trialcohol (38 pmol) separately. [^{3}H]Ado trialcohol did not bind to the column, whereas 74% of io^6A and 72% of [^{3}H]i^6A trialcohol bound to the column; both were eluted with TBS containing 10% pyridine.

Conditions of Germination and Growth of Cucumber Seedlings. The washed cucumber seeds were allowed to germinate on moist filter paper in Petri plates at room temperature for 4 d in dark. The seedlings were collected and the cotyledons and the decotyledonated seedlings were excised, washed with distilled H_2O blotted, and processed separately.

Extraction of Free Cytokinins from Cucumber Cotyledons and Decotyledonated Cucumber Seedlings and Purification by Affinity Chromatography. The material (35 g fresh weight) was homogenized in a Waring blender at 4°C with 95% ethanol (150 ml) (6), stored at −20°C for 12 h, and then centrifuged at 30,000*g* for 30 min at 4°C. The ethanol-soluble fraction was evaporated under vacuum. Five ml of water was added and then extracted with water-saturated ethyl acetate (25 ml × 3) (2). The pooled ethyl acetate fractions were evaporated under vacuum and then dissolved in 5 ml TBS.

Sepharose-anti-i^6A was packed in a column (4 ml) and washed with TBS. The cytokinin extract in TBS (5 ml) was loaded onto the column, washed with the same buffer (100 ml), and then eluted with 10% pyridine in TBS. (50 ml). Radioimmunoassay showed absence of cytokinins in the washings. Pyridine was removed from the eluate by repeatedly evaporating it to dryness under vacuum with intermittent addition of water. It was redissolved in 2 ml of water and re-extracted with ethyl acetate (3 × 10 ml). The pooled ethyl acetate extracts were evaporated to dryness and dissolved in 1 ml 35% ethanol and used for chromatography on Sephadex LH-20.

Isolation of tRNAs from Cucumber Cotyledons. Total tRNA was extracted from cucumber cotyledons by a modification of the procedure described for *Spinacia oleracea* L. leaves by Vreman *et al.* (23). Forty g (fresh weight) of cucumber cotyledons were homogenized in 50 ml 0.1 M Tris-HCl (pH 7.4) containing 0.5% SDS in a Waring blender at 4°C and stirred with an equal volume of phenol saturated with 0.1 M Tris-HCl for 30 min at 4°C. The resulting emulsion was then centrifuged at 2000 rpm for 30 min. The phenol treatment (using 15 ml phenol) and centrifugation were repeated twice. The nucleic acids from the clear aqueous phase were precipitated by adding 0.1 volume of 20% (w/v) potassium acetate and 2 volumes of ethanol precooled to −20°C. After 12 h at −20°C, the precipitate was collected by centrifugation at 10,000 rpm for 20 min. The tRNAs were extracted five times from the precipitate with 20 ml each of 0.4 M sodium acetate using centrifugation. 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. The crude tRNA (2312 A_{260} units) was dissolved in 2 ml 0.1 M Tris-HCl, pH 7.5, and loaded on a DEAE-cellulose column (1.5 × 10 cm). After washing the column with the above buffer, the tRNA was eluted with 1 M NaCl in 0.1 M Tris-HCl, pH 7.5. The resulting salt eluates were combined and 2 volumes of cold ethanol were added. The tRNA was precipitated overnight and then collected by centrifugation. The resulting preparation, contained 792 A_{260} units of crude tRNA. The contaminating high mol wt components were removed by gel filtration on Sephadex G-200 (2 × 40 cm) (14, 24). The whole preparation yielded 672 A_{260} units.

Isolation of Cytokinin-Containing tRNAs. The cucumber cotyledon tRNA fraction (180 A_{260} units in 0.5 ml) was applied to a Sepharose-anti-i^6A column (4 ml) at the rate of 2 ml/h and washed with the same buffer (50 ml). The unbound tRNA did not show the presence of cytokinins when tested by radioimmunoassay. The bound tRNAs were eluted with 10% pyridine in TBS and 1-ml fractions were collected. To each fraction, twice the volume of cold ethanol was added and stored overnight at −20°C; the fractions were then centrifuged at 30,000*g* for 20 min. The alcohol was discarded and the contents in the tubes were washed twice with cold ethanol. TBS (1 ml) was added to each tube and the $A_{260\ nm}$ was determined. Fractions having $A > 0.05$ (fractions 5–7) were pooled and passed through a small DEAE-cellulose column (0.2 ml) pre-equilibrated with 0.1 M Tris-HCl, pH 7.5, containing 0.1 M NaCl. Elution was carried out with the same buffer (0.5 ml containing 1 M NaCl). To the eluate, 1 ml ethanol was added to precipitate the tRNA. It was stored at −20°C overnight and centrifuged at 30,000*g* for 20 min. The precipitated tRNA was stored at −20°C as such or after dissolving in TBS (2.25 A_{260} units).

Digestion of tRNAs to Nucleosides. tRNAs were digested to nucleosides by the combined action of snake venom phosphodiesterase and alkaline phosphatase (13).

Preparation of Aminoacyl-tRNA Synthetases from Cucumber Cotyledons and Aminoacylation of Cucumber Cotyledon tRNAs. The procedure described for the soybean systems by Anderson and Cerry (1) was used for the preparation of aminoacyl-tRNA synthetases and for the aminoacylaton of tRNAs. The tRNAs were deacylated before aminoacylation using the method of Ehrenstein and Lipmann (8). Three separate samples of total cucumber cotyledon tRNAs were aminoacylated using [^{14}C]Ala, [^{14}C]Phe, or [^{14}C]Tyr so that each sample was labeled with one of these ^{14}C-amino acids. The aminoacyl-tRNA was isolated by phenol extraction and ethanol precipitation; the precipitate was dissolved in PBS buffer (10 mM phosphate buffer, pH 6.0; 0.14 M NaCl) and stored at −15°C until use. The specific activity ratios of the aminoacyl-tRNAs obtained (paper disc assay) per A_{260} unit of unfractionated tRNA were [^{14}C]Ala-$tRNA^{Ala}$, 634 cpm; [^{14}C]Phe-$tRNA^{Phe}$, 600 cpm; and [^{14}C]Tyr-$tRNA^{Tyr}$, 1125 cpm.

RESULTS

Radioimmunoassay for Cytokinins. The inhibition of the binding of [^{3}H]i^6A trialcohol to i^6A antibodies in the presence of varying amounts of io^6A, i^6Ade, or i^6A was studied using the nitrocellulose filter assay (11). By plotting percentage of inhibition against the amount of the inhibitor in the assay mixture, standard curves (Fig. 1, a, b, and c) were obtained for the assay of these cytokinins. For the detection of the cytokinins, all the regions of the curves have been used. The minimum amounts of cytokinins detectable under the conditions of the experiments are 1 ng in the case of i^6A or i^6Ade and 250 ng for io^6A. For the quantitation of the cytokinins inhibition regions of the curves used were 40 to 60% for io^6A, 40 to 70% for i^6Ade, and 50 to 74% for i^6A. To obtain the required concentrations, the samples were either pooled and concentrated or diluted after preliminary screening.

Free Cytokinins. The cytokinins from 35 g cucumber cotyledons and 35 g decotyledonated cucumber seedlings extracted by alcohol

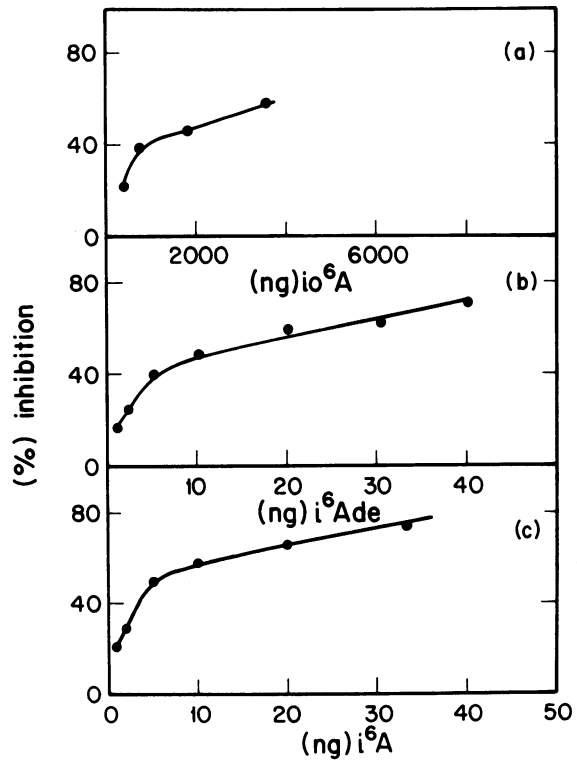

FIG. 1. Standard curves for the estimation of cytokinins io^{6}A (a), i^{6}Ade (b), and i^{6}A (c). The reaction mixture (0.4 ml) contained 1:500 anti-i^{6}A serum in TBS (0.1 ml), 3 pmol [^{3}H]i^{6}A trialcohol in TBS (0.1 ml), and various quantities of nonradioactive io^{6}A, i^{6}Ade, or i^{6}A in TBS (0.2 ml). Incubations were done at 37°C for 10 min. The mixtures were filtered on prewetted nitrocellulose membrane filter and washed with 5 ml buffer at 4°C. The wet filters were then transferred into glass scintillation vials and dried at 80°C, cooled to room temperature, and counted for radioactivity in Beckman LS100 liquid scintillation counter using 0.5% 2,5-diphenyloxazole in toluene.

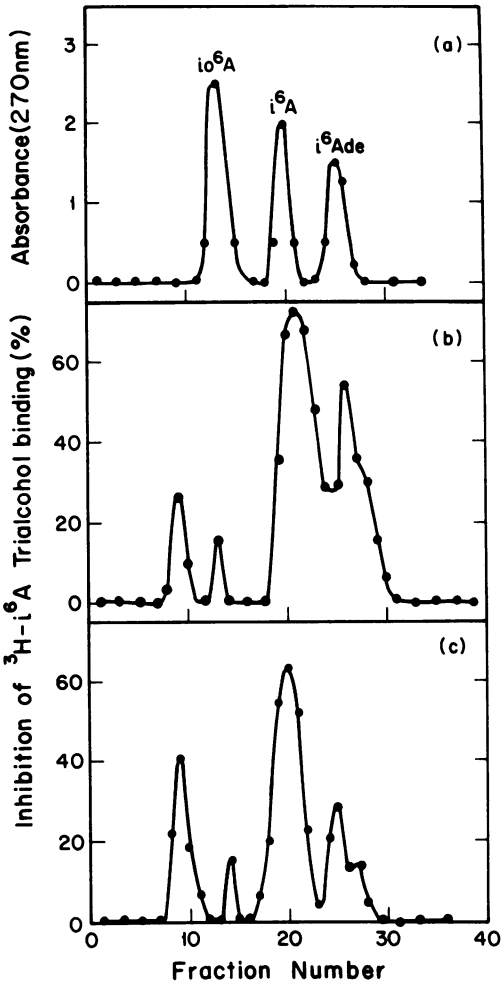

FIG. 2. Sephadex LH-20 column chromatography. a, Authentic samples of io^{6}A, i^{6}A, and i^{6}Ade; b, affinity-purified cytokinins from 35 g cucumber cotyledons; c, affinity-purified cytokinins from 35 g decotyledonated cucumber seedlings. The samples were dissolved in 1 ml 35% ethanol and chromatographed on a Sephadex LH-20 column (1 × 43 cm) using 35% ethanol for elution. Fractions (2.5-ml) were collected at a flow rate of 4 ml/h. For the detection of the cytokinins in b and c, 0.25-ml aliquots were evaporated and dissolved in 0.1 ml TBS and used for radioimmunoassay as in Figure 1.

and purified by chromatography on Sepharose-anti-i^{6}A were separately fractionated by Sephadex LH-20 chromatography (Fig. 2, b and c). The resulting fractions were tested for cytokinin activity by inhibition of the binding of [^{3}H]i^{6}A trialcohol to i^{6}A antibodies. Activity peaks 2, 3, and 4 correspond to authentic samples of io^{6}A, i^{6}A, and i^{6}Ade, respectively (Fig. 2a). Peak 1 remained unidentified. Peak 4 had a shoulder, indicating the presence of an additional cytokinin. The amounts of i^{6}A, i^{6}Ade, and io^{6}A in the pooled fractions were estimated by radioimmunoassay, using the appropriate standard curve (Fig. 1) and are given in Table I.

Identification and Estimation of Cytokinins in Cucumber Cotyledon tRNAs. About 616 A_{260} units of cucumber cotyledon tRNAs were hydrolyzed to nucleosides using snake venom phosphodiesterase and alkaline phosphatase and the nucleosides subsequently were fractionated on Sephadex LH-20 (Fig. 3). Radioimmunoassay shows only two cytokinin peaks even though there were several $A_{260\,nm}$ peaks. When io^{6}A and i^{6}A were separately chromatographed on the same column, io^{6}A eluted in the position of peak 1 and i^{6}A eluted in the position of peak 2. Fractions under io^{6}A and i^{6}A peaks were pooled and the cytokinins were estimated by radioimmunoassay using the appropriate standard curve. The amounts of io^{6}A and i^{6}A were found to be 11,550 and 420 ng, respectively.

Cytokinins in Cucumber Cotyledon tRNAs Isolated by Affinity Chromatography on Sepharose-Anti-i^{6}A Column. Even though it is known from earlier work (17, 19) that i^{6}A-containing tRNAs bind to a Sepharose-anti-i^{6}A column, such information was not available for io^{6}A-containing tRNAs. To investigate this, cucumber cotyledon tRNAs were first chromatographed on a Sepharose-anti-i^{6}A column and those tRNAs that bound to the column were eluted with pyridine as described under "Materials and Methods," then hydrolyzed to nucleosides, and finally fractionated on a Sephadex LH-20 column (Fig. 4). Radioimmunoassay shows two peaks, the first corresponding to io^{6}A and the second peak corre-

Table I. *Free Cytokinins in Cucumber Cotyledons and Decotyledonated Cucumber Seedlings*

Sample	Amount[a] of Cytokinins		
	io^{6}A (peak 2)	i^{6}A (peak 3)	i^{6}Ade (peak 4)
	ng		
Purified extract from 35 g cotyledons (Fig. 2b)	2800	805	315
Purified extract from 35 g decotyledonated seedlings (Fig. 2c)	1400	210	105

[a] The cytokinins in peak 1 and in the shoulder of peak 4 remained unidentified and were not estimated.

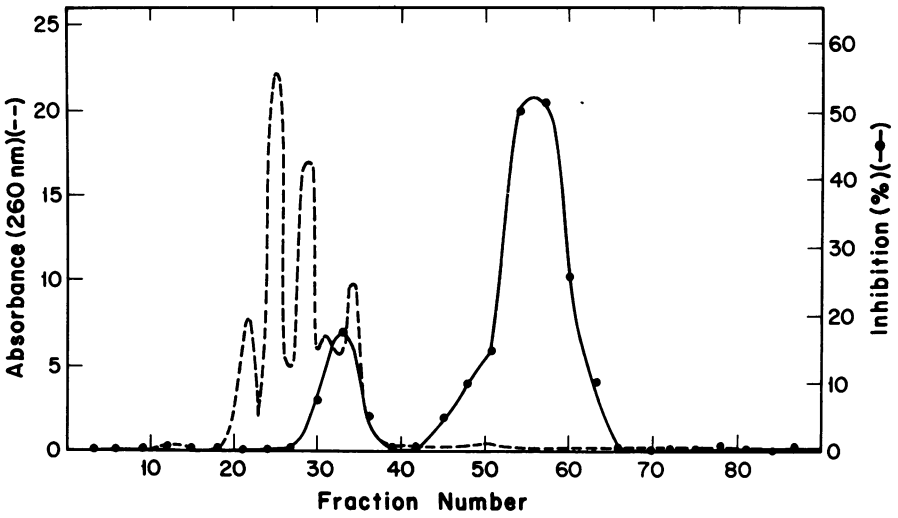

FIG. 3. Sephadex LH-20 column chromatography of nucleosides from cucumber cotyledon total tRNA. Ethyl acetate-soluble ribonucleosides obtained from 616 A_{260} units of tRNA were dissolved in 1 ml 35% (v/v) aqueous ethanol and applied to a Sephadex LH-20 column (1 × 43 cm) pre-equilibrated with 35% ethanol. It was eluted with the same solvent. Flow rate was 4 ml/h. One ml fractions were collected and were measured at $A_{260\ nm}$. Three successive fractions were then pooled, evaporated to dryness, and dissolved in 1 ml TBS and 0.05-ml aliquots were used for the detection of cytokinins by the inhibition of the binding of [^{3}H]i^{6}A trialcohol to anti-i^{6}A according to Figure 1.

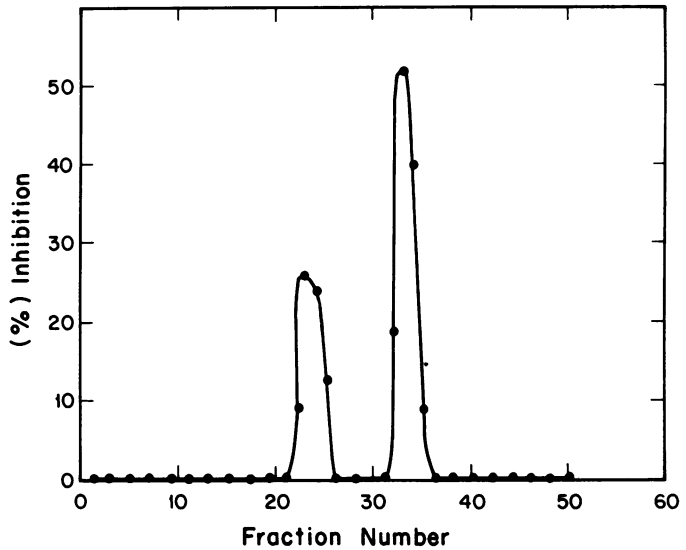

FIG. 4. Sephadex LH-20 column chromatography of nucleosides from cucumber cotyledon tRNAs that bound to the Sepharose-anti-i^{6}A column. Ethyl acetate-soluble ribonucleosides obtained from 1.25 A_{260} units of the tRNAs were dissolved in 1 ml 35% (v/v) aqueous ethanol and applied to a Sephadex LH-20 column (1.5 × 35 cm) pre-equilibrated with the same solvent and were eluted with the same solvent. Flow rate was 12 ml/h. Three-ml fractions were collected, evaporated to dryness, and dissolved in 0.3 ml TBS and 0.1-ml aliquots were used for the inhibition of the binding of [^{3}H]i^{6}A trialcohol to anti-i^{6}A as described under Figure 1.

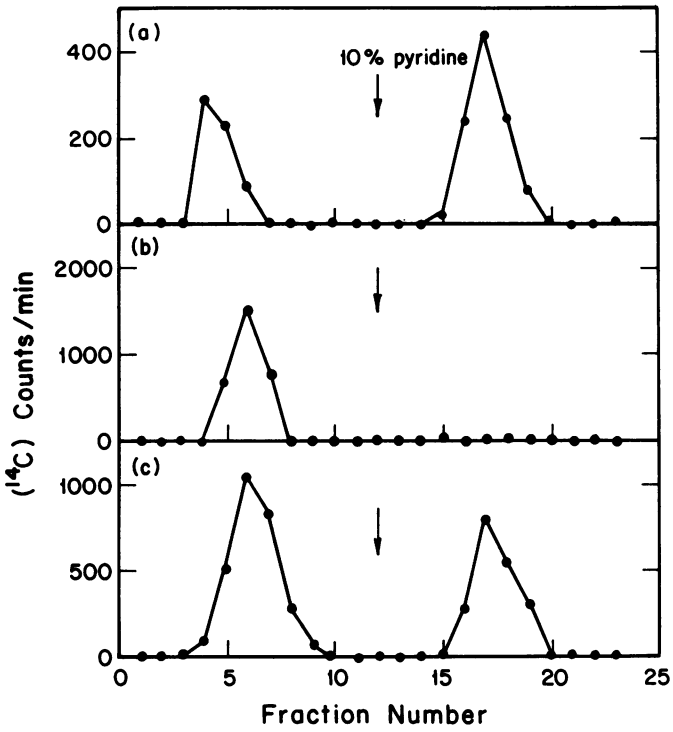

FIG. 5. Chromatography of cucumber cotyledon aminoacyl-tRNAs on the Sepharose-anti-i^{6}A column. a, Unfractionated cucumber cotyledon tRNAs (265 μg) containing [^{14}C]Phe-tRNA^{Phe} (3251 cpm). b, Unfractionated cucumber cotyledon tRNAs (405 μg) containing [^{14}C]Ala-tRNA^{Ala} (5400 cpm). c, Unfractionated cucumber cotyledon tRNAs (390 μg) containing [^{14}C]Tyr-tRNA^{Tyr} (9265 cpm). The tRNAs in 0.5 ml PBS were applied to the affinity column (4 ml) at 28°C. After 15 min, the column was eluted with 12 ml PBS, followed by 12 ml 10% pyridine in PBS. One-ml fractions were collected and applied on 2.5 × 2.5 cm Whatman no. 3 paper discs, dried, and counted for radioactivity in a Beckman LS100 liquid scintillation counter with 8 ml 0.5% 2,5-diphenyloxazole in toluene.

sponding to i^{6}A. The amounts of io^{6}A and i^{6}A in the peaks were quantitated by radioimmunoassay after pooling and were found to be 1275 and 46.5 μg, respectively.

Detection of Specific Cytokinin-Containing Cucumber Cotyledon tRNAs by Sepharose-Anti-i^{6}A Column Chromatography. The unfractionated cucumber cotyledon tRNA samples in which the tRNAs for only one amino acid were acylated with ^{14}C-amino acid in each sample was chromatographed on a Sepharose-anti-i^{6}A column. Figure 5a shows that the major part of the radioactive [^{14}C]Phe-tRNA^{Phe} was bound to the column and eluted with pyridine. On the other hand, [^{14}C]Ala-tRNA^{Ala} did not bind to the column (Fig. 5b). In the case of [^{14}C]Tyr-tRNA^{Tyr} (Fig. 5c), 40% bound to the column and was eluted with pyridine. The results show that tRNA^{Phe} and tRNA^{Tyr} contained cytokinins whereas tRNA^{Ala} did not.

DISCUSSION

Antibodies to i^6A have been used in the present studies both for purification and assay of cytokinins. Without affinity chromatography on the Sepharose-anti-i^6A column, the tissue extracts gave poor separation of the cytokinins on the Sepharose LH-20 column. Figure 2 shows that there are at least five compounds reactive to i^6A antibodies in cucumber cotyledons and decotyledonated cucumber seedlings, of which three have been identified and their amounts estimated (Table I). About 30% loss of cytokinins during work-up cannot be ruled out but preferential loss of any one is not expected. The amounts of the cytokinins are in the order $io^6A > i^6A > i^6Ade$ in both the cotyledons and decotyledonated seedlings. It may be noted that the cytokinins are more abundant in the cotyledons compared to decotyledonated seedlings to the extent of 2-, 3-, and 4-fold in the case of io^6A, i^6Ade, and i^6A, respectively. The results indicate that cotyledons may serve as storage organs for cytokinins also. It has not been established whether cotyledons are cytokinin-synthesizing centers.

There have been several studies on the distribution of io^6A and i^6A in tRNA from plant sources, and the io^6A:i^6A ratios by weight reported in some systems are as follows: spinach leaves, 250:340; pea, 270:130; corn, 1900:0; and pea root, 750:130 (5, 9). The result reported in this paper shows that the ratio is 28:1 in cucumber cotyledon tRNA. When calculated for tRNAs obtained from 1 g cotyledon tissue, the contents of io^6A and i^6A are 317.6 and 12 ng, respectively. io^6A is the major cytokinin in the free form in cucumber cotyledons and amounts to 80 ng/g tissue. The content of io^6A in tRNAs is sufficiently high to account for its formation in the free form by degradation of tRNAs but in the case of i^6A this is not so.

Earlier studies established that immobilized i^6A antibodies could be used for the isolation of i^6A-containing tRNAs (17, 20). Similarly, the isolation of tRNAs containing i^6A/2-methylthio-N^6-(Δ^2-isopentenyl) adenosine has been reported using N^6-(Δ^2-isopentenyl) adenosine 5′-phosphate antibodies (22). bzl^6A antibodies immobilized on Sepharose were shown to be useful for the isolation of cytokinins from crude extract of cabbage (7). The present studies show that io^6A-containing tRNAs are retained by this affinity column and eluted with 10% pyridine in TBS with equal efficiency as i^6A-containing tRNAs because the ratio of the amounts of io^6A and i^6A was found to be the same in both the purified and unfractionated tRNAs.

Affinity chromatography of tRNAs labeled with ^{14}C-amino acids showed that $tRNA^{Phe}$ and $tRNA^{Tyr}$ of cucumber cotyledons contained cytokinins whereas $tRNA^{Ala}$ does not. It may be noted that Phe and Tyr, unlike Ala, have codons starting with U. It has been observed by Armstrong *et al.* (3, 4) that tRNAs containing i^6A adjacent to the anticodons had codons beginning with U.

i^6A antibodies do not bind any of the normal nucleosides including adenosine but bind to the cytokinins i^6A, i^6Ade, kinetin riboside, bzl^6A, and io^6A. The N^6-substituted adenine derivative N^6-[9-β-D-(ribofuranosyl)purin-6-yl carbamoyl]-L-threonine, which does not possess cytokinin activity, does not bind to the antibodies (12, 17, 19). So it appears that the radioimmunoassay used here for the detection and estimation of cytokinin runs parallel to the generally used bioassays. The radioimmunoassay has the advantage of rapidity and sensitivity.

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