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trans-Ribosylzeatin

ITS BIOSYNTHESIS IN *ZEA MAYS* ENDOSPERM AND THE MYCORRHIZAL FUNGUS, *RHIZOPOGON ROSEOLUS*[1]

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ABSTRACT

When [8-^{14}C]-N^6-(Δ^2-isopentenyl)adenosine is incubated with the endosperm of corn (2 weeks after pollination), it is converted to [^{14}C]-N^6-(4-hydroxy-3-methylbut-2-*trans*-enyl)adenosine, *trans*-ribosylzeatin. This biosynthetic step, N^6-(Δ^2-isopentenyl)adenosine to ribosylzeatin, also occurs in the mycorrhizal fungus, *Rhizopogon roseolus*.

Cytokinin activity has been detected in extracts of various plants and plant organs. An active component in the extracts has been identified as *trans*-ribosylzeatin, *trans*-RZ[2] (Fig. 1), in the case of immature fruit (16, 17, 20) and chicory root (2), and as its dihydro derivative in the case of Lupin (14) (see review by Kende (12) for a more detailed account). Recently Dyson and Hall (5) reported the presence of i^{6}Ado in an extract of an autonomous strain of tobacco tissue. Of these naturally occurring cytokinins, *trans*-RZ exhibits the greatest activity in the tobacco callus bioassay system (9).

As part of a study of the metabolism of cytokinins, we have investigated the biosynthesis of *trans*-RZ, and in this paper we describe its synthesis from the precursor, i^{6}Ado. We chose systems known to contain free *trans*-RZ, a mycorrhizal fungus, *Rhizopogon roseolus* (22) and corn kernels (16, 20). Miura and Miller (24) found that *R. roseolus* converted i^{6}Ade to zeatin. We have shown that the biosynthetic step i^{6}Ado → *trans*-RZ occurs in *R. roseolus* and have obtained evidence for the same step in corn endosperm.

MATERIALS AND METHODS

Organisms. *R. roseolus* was kindly furnished by Dr. C. O. Miller, Indiana University. It was grown in the following medium in milligrams/1: NH_4NO_3, 1000; KNO_3, 1000; $Ca(NO_3)_2 \cdot 4H_2O$, 500; KH_2PO_4, 250; H_3BO_3, 5.0; KI, 0.8; $MgSO_4 \cdot 7H_2O$, 300; KCl, 50; $ZnSO_4 \cdot 7H_2O$, 7.5; $MnSO_4 \cdot H_2O$, 5.0; thiamine·HCl, 0.1; nicotinic acid, 0.5; pyridoxine·HCl, 0.5; glycine, 2.0; Na_2FeEDTA 35; *i*-inositol, 100; sucrose, 30,000. Mycelia for inoculation (1–4 mg/ml, dry weight) were prepared by blending mycelial suspensions for 5 sec in a Waring Blendor. Cultures were aerated on a gyratory shaker in the dark at 27 C.

Cultures of a cytokinin-dependent soybean strain, *Glycine max* (L.), Merril var. Acme, were used to bioassay cytokinin activity (4, 20). A strain of *Zea mays* L., NK 75, was grown in a greenhouse. A hybrid strain, W64A X W182E, was grown in a growth chamber. Table corn was also purchased from a local grocery wholesale firm.

Chemicals and Enzymes. [8-^{14}C]-i^{6}Ado (5 mc/mmole) was synthesized according to the method of Pačes *et al.* (25). Before use, the sample i^{6}Ado was rechromatographed on Sephadex LH-20 column (1). [8-^{14}C]-Adenosine (55 mc/mmole) and [8-^{14}C]-adenine (58 mc/mmole) were purchased from Schwarz BioResearch, Inc. i^{6}Ado was obtained from Starks Associates, Inc., and i^{6}Ade from Raylo Chemicals, Edmonton, Alberta, Canada. *trans*-RZ and N^6-(Δ^3-isopentenyl)-adenosine were gifts from Dr. H. Vorbrüggen, Schering A.G., West Berlin. Mixed *cis* and *trans* RZ was purchased from Calbiochem. Miracloth was obtained from Chicopee Mills, Inc., New York, DEAE-resin used was No. 70 standard (0.89 meq/g) Schleicher and Schuell, Keene, N.H. Carbowax-6000 is a product of Union Carbide Chemicals, Toronto, Canada. Adenosine aminohydrolase from chicken bone marrow was prepared by Mrs. B. Bell according to the method of Hall *et al.* (7).

Chromatography. Paper chromatography was carried out on Whatman No. 3MM paper in a descending fashion.

TLC was carried out on Eastman 6060 silica gel plates with fluorescent indicator. Solvents used were (v/v): A: 1-butanol-water-concentrated NH_4OH (86:14:5); B: *tert*-butanol-concentrated NH_4OH-water (3:1:1); C: 95% ethanol-0.1 M $(NH_4)_3BO_3$, pH 9.0 (1:9); D: 2-propanol-concentrated NH_4OH-water (7:1:2); E: ethyl acetate-1-propanol-water (4:1:2); F: $CHCl_3$-ethanol (85:15); G: 1-butanol-glacial acetic acid-H_2O (5:3:2).

Gas-Liquid Chromatography. Gas-liquid chromatography was carried out on an F&M Scientific dual-column gas chromatograph, model 402, equipped with flame-ionization detectors. A U-shaped silanized glass column, 0.3 × 120 cm, packed with DC-11 Dow-Corning silicone grease (10% w/w) on Gas-chrom Q (Kensott Ltd., Toronto, Canada) was conditioned 48 hr before use. The method for preparation of the trimethylsilyl derivatives and description of the resolving characteristics of this gas-liquid chromatography system are given by Dyson and Hall (5).

Sephadex LH-20. The method of Armstrong *et al.* (1) was

[1] This research was supported by the Medical Research Council of Canada (MT-2738).

[2] Abbreviations used: i^{6}Ado:N^6-(Δ^2-isopentenyl)adenosine; i^{6}Ade: N^6-(Δ^2-isopentenyl)adenine; RZ: ribosylzeatin, N^6-(4-hydroxy-3-methylbut-2-enyl)adenosine; the name zeatin originally designated the *trans* isomer but since both *cis* and *trans* isomers exist in nature it is helpful to specify the isomer. In this paper the prefix *trans* and *cis* are used if the sample in question has been positively identified as one or the other isomer, otherwise these prefixes are omitted.

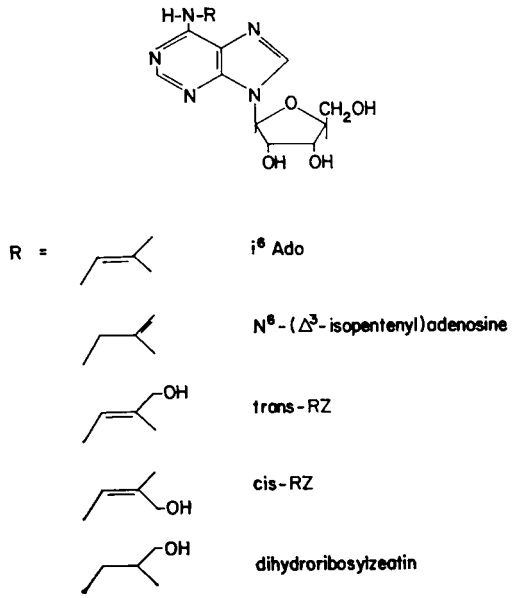

FIG. 1. Structures of cytokinins mentioned in text.

followed. The column (2.5 × 40 cm) contained 50 g of Sephadex LH-20 resin (Pharmacia) swollen in 35% aqueous ethanol. The column was eluted with this solvent; 11-ml fractions were collected.

Partition Chromatography. A standard column, 1.9 cm (diameter), containing 40 g of Celite-545 (Johns-Manville Co.) (5, 6) was used for the first stage isolation of i⁶Ado and RZ. It was eluted with solvent system E. The first 15 ml of eluate (fraction 1) contain i⁶Ado and i⁶Ade, and the next 100 ml (fraction 2) contain *trans*- or *cis*-RZ and zeatin.

Radioactivity. This was determined on a Nuclear-Chicago Mark I liquid scintillation system. For paper chromatograms, sections (1-cm) were placed in vials containing scintillation fluid made up of 5 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in 1 liter of toluene. For solutions a 1-ml aliquot was added to 15 ml of a solution of 5 g of 2,5-diphenyloxazole, 0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, and 100 g of naphthalene in 1 liter of 1,4-dioxane.

RESULTS

Conversion of i⁶Ado to *trans*-RZ in *R. roseolus*. Miura and Miller (24) showed that when this organism is incubated in the presence of radioactive i⁶Ade, the precursor is converted to zeatin. We have now shown that *R. roseolus* can convert i⁶Ado to *trans*-RZ.

A 2-day-old culture of *R. roseolus* (5 ml) was incubated for 8 days with [8-^{14}C]-i⁶Ado (3.0×10^5 cpm). The mycelia were filtered off and the filtrate was evaporated to dryness. The residue was dissolved in 2 ml of lower phase of solvent E and mixed with 4 g of Celite-545. This mixture was packed on top of a partition column of Celite-545 and the column was eluted with the upper phase of solvent E. Fraction 2 which contained RZ was rechromatographed on a Sephadex LH-20 column. A peak of radioactivity containing 1×10^5 cpm was detected between the 198- and 220-ml mark of the eluate. This fraction was evaporated to dryness *in vacuo* and the material is referred to as product 1. The identity of product 1 as *trans*-RZ is based on the following evidence.

1. Paper chromatography of product 1 in solvent D showed a discrete radioactive spot migrating with authentic RZ. On chromatography on TLC in solvent F the radioactive spot migrated coincidently with *trans*-RZ (Fig. 2). This latter system resolves completely the *cis* and *trans* isomers (26).

2. Authentic *trans*-RZ (0.3 mg) was added to product 1 (1000 cpm) in 0.3 ml of water and the solution was treated with 0.8 ml of 0.01% $KMnO_4$ for 15 min at 25 C (18, 23). Excess $KMnO_4$ was destroyed by adding ethanol and allowing the mixture to stand 15 min. The reaction mixture was chromatographed on paper in solvent B. Three ultraviolet-absorbing products were obtained, N-(9-ribosylpurin-6-yl)glycine, adenosine, and an unidentified compound (18, 23) (see Fig. 7 for profile of a similar experiment). The three spots were eluted with water and the optical density and radioactivity measured. The amount of the three degradation products normalized to 100% was N-(9-ribosylpurin-6-yl)glycine, 21.4, adenosine, 72.3, and the unknown product, 6.2%. The values for the radioactivity of the corresponding spots, normalized to 100%, were 20.0, 75.2, and 4.8%. The almost identical ratios for the two sets of degradation products support the identity of the

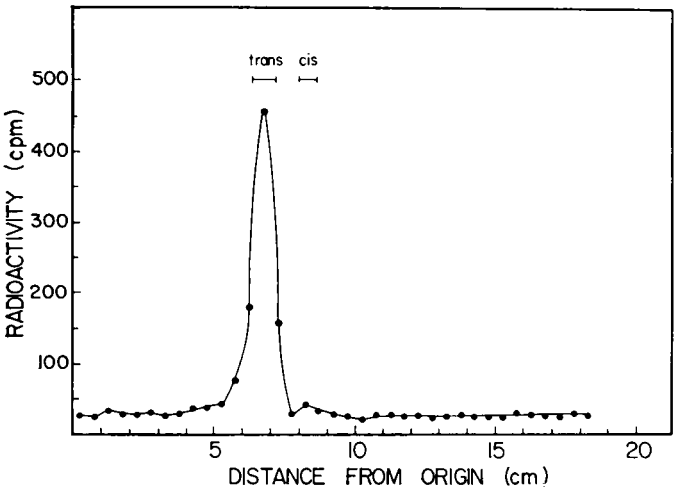

FIG. 2. Chromatography of *trans*-RZ (radioactive) isolated from *R. roseolus* after incubation with [8-^{14}C]-i⁶Ado ("Conversion of i⁶Ado to *trans*-RZ in *R. roseolus*"). The radioactive sample was chromatographed together with authentic *cis*- and *trans*-RZ on TLC, solvent F.

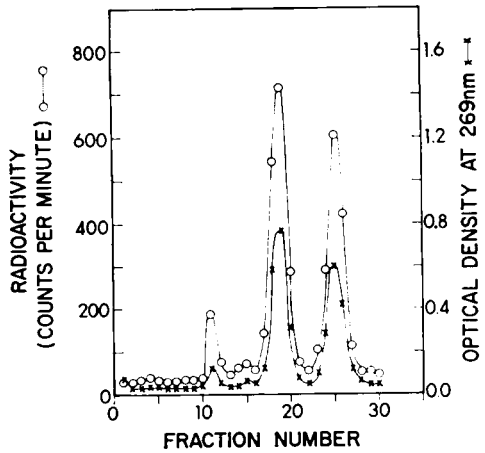

FIG. 3. Separation on a Sephadex LH-20 column of the products of acid hydrolysis of *trans*-RZ (radioactive) obtained from *R. roseolus* mixed with authentic *trans*-RZ (ultraviolet-absorbing) ("Conversion of i⁶Ado to *trans*-RZ in *R. roseolus*"). Fraction size was 11 ml. Radioactivity is for 1-ml aliquot.

radioactive product as RZ. This $KMnO_4$ oxidation test distinguishes *trans*-RZ from the closely related derivative N^6-(3-methyl-3-hydroxybutyl)adenosine and dihydro RZ since those compounds do not have double bonds.

3. When zeatin is heated in acid it degrades to two ultraviolet-absorbing products which have not been characterized (18). RZ undergoes a similar degradation. Product 1 (4×10^4 cpm) the solution was heated for 2 hr at 90 C. The HCl was removed and 1.1 mg of *trans*-RZ were dissolved in 2 ml of 1 N HCl and by evaporation *in vacuo* and the residue was fractionated on a Sephadex LH-20 column. Three ultraviolet-absorbing peaks were obtained amounting to 3.1, 52.1, and 43.5% of total ultraviolet absorption (269 nm). The radioactivity of the peaks amounted to 5.2, 49.1, and 44% of total (Fig. 3). The two sets of ratios demonstrate a similar breakdown pattern between the radioactive product from *R. roseolus* and *trans*-RZ.

Conversion of Adenosine to i^{6}Ado in *R. roseolus*. A 2-day-old culture of *R. roseolus* containing i^{6}Ado (0.17 mM) (5 ml) was incubated for 6 days with 3 μc of [8-^{14}C]-adenosine. The culture medium was fractionated in a Celite-545 column. Fraction 1 was evaporated to dryness and the residue was redissolved in 0.5 ml of 35% ethanol. This material was fractionated on an LH-20 column (Fig. 4). The fraction corresponding to peak B contained 3.5×10^4 cpm. It was evaporated to dryness. It was identified as i^{6}Ado on the basis of the following criteria.

1. It cochromatographs with authentic i^{6}Ado in solvents A, C, D, and E.

2. A sample of B (500 cpm) and 1.0 mg of authentic i^{6}Ado in 1.0 ml of water containing 0.4 μmole of $MgCl_2$ and 50 μmoles of phosphate, pH 6.4, was treated with purified chicken bone marrow N^6-(Δ^2-isopentenyl)adenosine Δ^2-isopentenylaminohydrolase according to the method of Hall *et al.* (7). The radioactive product was converted to inosine (identified by paper chromatography in solvent D).

3. On hydrolysis in 1 N HCl for 15 min at 100 C i^{6}Ado is converted to two products, N^6-(3-hydroxy-3-methylbutyl)adenine and 3H,7,7-dimethyl-7,8,9-trihydropyrimido-(2,1-*i*)-purine (27). A sample of B (2300 cpm) and 0.3 mg of i^{6}Ado was hydrolyzed in 0.25 ml of 1 N HCl. The reaction mixture was chromatographed on paper in solvents D and E. In both cases two discrete radioactive spots migrating coincidently with the two marker products were detected.

4. On treatment with $KMnO_4$, i^{6}Ado is oxidized to N^6-(2,3-dihydroxy-3-methylbutyl)adenosine, adenosine, and a third unidentified product (27). A sample of B (1500 cpm) and 0.5 mg of i^{6}Ado dissolved in 0.5 ml of water was oxidized with 0.8 ml of a 0.01% aqueous solution of $KMnO_4$ for 10 min at 25 C. The reaction mixture was diluted with excess ethanol and the mixture was left for 15 min. The reaction products were chromatographed in solvent D. Three discrete radioactive spots were detected coinciding with the three ultraviolet-absorbing products resulting from the oxidation of the marker i^{6}Ado.

5. Peak B was converted to the free base i^{6}Ade. A sample (500 cpm) was treated with 3 ml of 0.1% $NaIO_4$ solution for 1 hr at room temperature. The solution was made basic with 0.8 ml of NH_4OH and heated for 1 min at 93 C. Under these conditions the ribose is removed and the radioactive product cochromatographed with i^{6}Ade in solvent A.

Peak C was identified as i^{6}Ade by means of chromatography on paper with an authentic sample in solvents A, C, and D. Acid hydrolysis of peak C as described above yielded the two characteristic products that cochromatographed with the authentic products of acid degradation.

Peak A was not identified.

In the same experiment, fraction 2 from the Celite-545 column was chromatographed on a Sephadex LH-20 column. The RZ fraction contained 2000 cpm, compared to 28,000 cpm when i^{6}Ado is absent from the culture media.

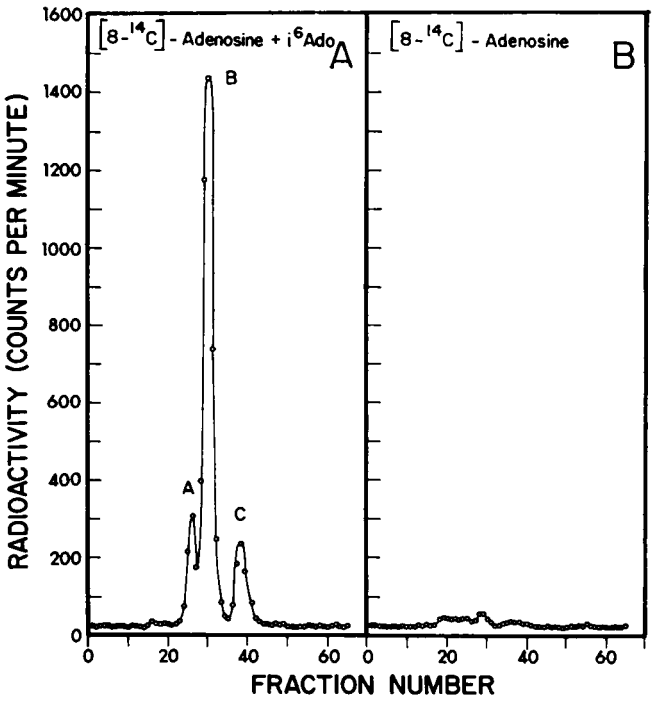

FIG. 4. A: Analysis of reaction products on Sephadex LH-20 after incubating *Rhizopogon roseolus* with 3 μc of [8-^{14}C]-adenosine in the presence of 0.17 mM i^{6}Ado ("Conversion of Adenosine to i^{6}Ado in *R. roseolus*"). Peak A: unidentified, peak B: i^{6}Ado; peak C: i^{6}Ade. Fraction size was 11 ml. Radioactivity is for 1-ml aliquot. B: Results of a similar experiment in which the i^{6}Ado was omitted.

Rapid labeling of i^{6}Ado in cultures of *R. roseolus*. The above experiment shows that in a matter of several days i^{6}Ado is converted to *trans*-RZ. On a short term basis, however, the principal product starting with radioactive labeled adenosine or adenine is i^{6}Ado. The mycelia from a 2-day-old culture (15 ml) were transferred to 5 ml of fresh medium containing 8 μc of [8-^{14}C]-adenine. The mixture was incubated for 3 hr. During this time 92% of the radioactivity disappeared from the medium. The medium with the cells was chilled to 0 C and the mixture, after being made 1 N with respect to $HClO_4$, was stirred for 5 min. The mycelia were filtered off and re-extracted with 2 ml of 1 N $HClO_4$. The combined extracts were neutralized with KOH and the $KClO_4$ was removed. The supernatant was adjusted to pH 9 with NaOH and made 10 mM with respect to $MgCl_2$. Intestinal alkaline phosphatase (1 mg/ml) was added and the mixture was incubated for 90 min at 37 C. The extract was chromatographed on Celite-545 and Sephadex LH-20 columns as described under "Conversion of Adenosine to i^{6}Ado in *R. roseolus*." Two major radioactive products were obtained, i^{6}Ado, 9800 cpm, and i^{6}Ade, 41,000 cpm. A negligible amount of radioactivity was found in the fraction corresponding to RZ.

The experiment was repeated using [8-^{14}C]-adenosine as the precursor. The results were similar except the amount of [^{14}C]-i^{6}Ade isolated was less. A negligible amount of RZ was found.

Effect of N^6-(Δ^2-Isopentenyl)adenosine on Conversion of i^{6}Ado to RZ by *R. roseolus*. The experimental conditions described under "Conversion of Adenosine to i^{6}Ado in *R. roseolus*" were repeated, except N^6-(Δ^2-isopentenyl)adenosine was added to the culture medium in place of i^{6}Ado. The culture

was analyzed as described under "Conversion of Adenosine to i^{6}Ado in *R. roseolus*." No radioactivity associated with N^6-(Δ^3-isopentenyl)adenosine was detected. The RZ fraction contained 32,000 cpm. We conclude therefore that the Δ^3 isomer of i^{6}Ado is not an intermediate in the metabolism of i^{6}Ado, nor does it have any effect on conversion of i^{6}Ado to *trans*-RZ.

Cytokinins in the tRNA of *R. roseolus*. *R. roseolus* was grown for 10 to 15 days at room temperature in 32-ounce bottles with no shaking. The mycelia were collected on cheesecloth, washed with distilled water at 0 C, and stored at −20 C. The isolation procedure was based on that of Holley (11). Frozen mycelia (250 g) were slowly added to a mixture containing 1 liter of distilled phenol and 1 liter of 0.1 M tris-HCl, pH 7.5. The mixture was stirred overnight and then centrifuged. The phenol phase was re-extracted with 300 ml of 0.1 M tris-HCl, pH 7.5. The aqueous fractions were pooled and re-extracted twice with 0.5 volume of phenol. Potassium acetate (0.1 volume of a 20% aqueous solution) and 2.5 volumes of chilled ethanol were added. The precipitate was collected by centrifugation and washed twice with ethanol and twice with acetone. The air-dried material was dissolved in 0.1 M tris-HCl, pH 7.5, and the tRNA was purified on a DEAE-cellulose column (3 × 31 cm) equilibrated with 0.1 M tris-HCl, pH 7.5. The column was washed with 500 ml of 0.3 M NaCl in 0.1 M tris-HCl, pH 7.5, and the tRNA was eluted with 1 M NaCl in 0.1 M tris-HCl, pH 7.5. It was precipitated with ethanol. The yield was 5 A_{260} units of tRNA/g mycelia (fresh weight).

tRNA (137 mg) was hydrolyzed in 5 ml of 0.3 N KOH for 24 hr at 37 C. The solution was neutralized with $HClO_4$. The $KClO_4$ was removed by centrifugation and the supernatant was adjusted to pH 9.2 with NaOH. It was made 10 mM with respect to $MgCl_2$ and intestinal alkaline phosphatase (1 mg/ml) was added. The solution was incubated for 15 hr at 37 C. The reaction mixture was chromatographed on Celite-545 and Sephadex LH-20 column as described under "Conversion of i^{6}Ado to *trans*-RZ in *R. roseolus*." Fraction 1 from the Celite-545 column after Sephadex LH-20 chromatography yielded a product that eluted in the position i^{6}Ado. This product was analyzed by gas-liquid chromatography; it yielded a single peak that corresponded to the retention time of i^{6}Ado (coinjection) (Fig. 5). Fraction 2 from the Celite-545 column on subsequent Sephadex LH-20 chromatography yielded no detectable product corresponding to *cis*- or *trans*-RZ. These results show that the tRNA of *R. roseolus* contains i^{6}Ado.

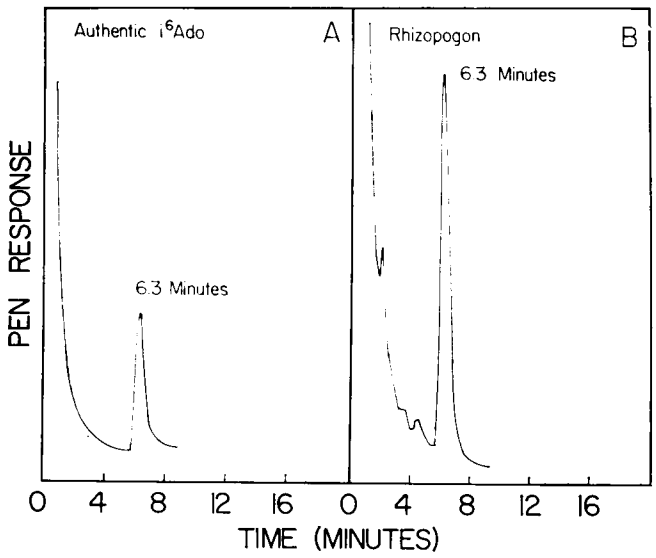

FIG. 5. Identification of component from tRNA of *R. roseolus* as i^{6}Ado by means of gas-liquid chromatography. A: Trimethylsilyl derivative of authentic i^{6}Ado (1 μg); B; trimethylsilyl derivative of the component isolated from *R. roseolus* tRNA. The analyses were run sequentially. Oven temperature was 252 C (isothermal), with a carrier gas (He) flow of 35 ml/min. Attenuation was 32.

Table I. *Incorporation of [8-^{14}C]-i^{6}Ado into trans-RZ by Excised Corn Tissues*

Amount of [^{14}C]-i^{6}Ado	Tissue Type (Variety)	Fresh Wt	Time of Incubation	[^{14}C]-*trans*-RZ
cpm		*g*	*hr*	*cpm*
8.4 × 10^5	Immature kernel slices (NK 75)	9.83	9.0	2000
6.0 × 10^5	Immature kernel slices (NK 75)	6.63	19.5	3000
1.4 × 10^6	Immature kernel slices (NK 75)	5.15	21.5	1300
6.8 × 10^5	Kernel slices (store)	6.19	12.0	4700
1.4 × 10^6	Endosperm (NK 75)	4.72	12.0	5700
5.9 × 10^5	Imbibed kernel slices (NK 75)	4.85	17.5	0
5.0 × 10^5	None (control)		23.0	0
5.0 × 10^5	None (control)		23.0	0

The sample (24 μg) recovered from the gas-liquid chromatograph stimulated the growth of soybean callus tissue 10-fold over controls.

Conversion of i^{6}Ado to *trans*-RZ by Corn Kernel Slices (Table I). Husked ears of var. NK 75, picked 2 weeks after pollination, were immersed for 5 min in a 1.2% (w/v) solution of sodium hypochlorite and washed several times with sterile water. The kernels were sliced parallel to the cob axis. The slices contained outer wall, aleurone layer, and endosperm; the scutellum was avoided. The slices were incubated (see Table I for time) in 10 ml of sterile buffer containing the [8-^{14}C]-i^{6}Ado, 500 μg of chloramphenicol, and 670 μmoles of $NaKHPO_4$ buffer, pH 7.0. The mixture was aerated on a gyratory shaker at 27 C.

The slices were filtered on miracloth, washed with 100 ml of 0.15 M NaCl, and ground in a mortar and pestle with enough 95% ethanol to give a final concentration of 70% ethanol. The extract was centrifuged at 10,000*g* and the pellets were re-extracted twice with 5 ml of 70% ethanol. A total of 200 μg of *trans*-RZ was added during the extractions to serve as a carrier. The ethanol was evaporated *in vacuo* and the residue was dissolved in 10 ml of water. The pH of the solution was adjusted to 8.5 with NaOH; $MgCl_2$ (to 10 mM) and 10 mg of intestinal alkaline phosphatase were added. The mixture was incubated for 1 hr at 37 C, after which it was evaporated *in vacuo*. The residue was chromatographed on a column of Celite-545 in solvent E. Fraction 2, containing RZ, was evaporated *in vacuo* and the residue was dissolved in 0.5 ml of 35% ethanol, and applied to an LH-20 column. The fraction containing RZ was evaporated to dryness, and the residue was chromatographed on paper in solvent D. Three peaks of radioactivity were detected (Fig. 6).

The identity of the radioactive compound, C, was confirmed by means of the following criteria. (*a*) The radioactive product was mixed with *cis*- and *trans*-RZ and chromatographed on TLC in solvent F. A pattern identical with that shown in Figure 2 was obtained. (*b*) The material was sensitive to $KMNO_4$ oxidation, yielding the characteristic products described under "Conversion of i^{6}Ado to *trans*-RZ in *R. roseolus*" (Fig. 7). (*c*) A sample of radioactive product (520 cpm) and marker *trans*-RZ (100 μg) in 0.4 ml of water was treated for 10 min with two

drops of bromine water at 25 C. The mixture was evaporated *in vacuo* and chromatographed on TLC in solvent F. A new radioactive product migrating coincidently with the brominated RZ product (Fig. 8) was observed at RF 0.34.

The identity of the radioactive peak moving coincidently with adenosine, B (Fig. 6), was confirmed as adenosine by rechromatography with a standard on TLC (solvent F) and on paper (solvents A and G). In each case the radioactive material migrated as a discrete spot together with the standard. Plant tissue contains an enzyme system that converts i^{6}Ado to adenosine (25). Unpublished results obtained in this laboratory show that corn kernels also contain this enzyme system. Thus, conversion of a portion of the i^{6}Ado to adenosine is not unexpected.

The experiment was repeated under differing conditions. In one experiment, table corn purchased from a grocery was used. Dried seeds of var. NK 75 that had been soaked in tap water for 2 days were also tried. The results are summarized in Table I.

Conversion of i^{6}Ado to *trans*-RZ by Corn Endosperm. The tops of the kernels of a cob of var. NK 75 picked 2 weeks after

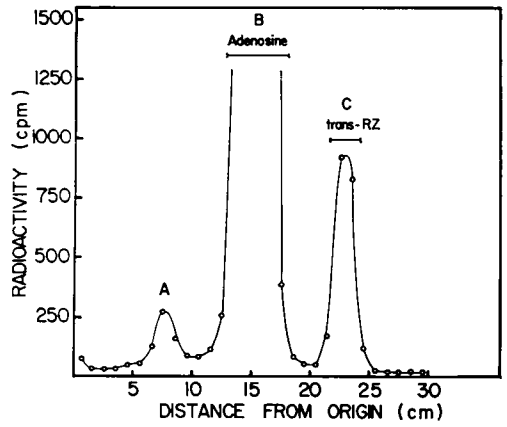

FIG. 6. Isolation of *trans*-RZ from corn kernel slices after incubation with [8-^{14}C]-i^{6}Ado ("Conversion of i^{6}Ado to *trans*-RZ by Corn Kernel Slices"). Chart shows radioactive profile of a paper chromatogram (solvent D) of fraction from Sephadex LH-20 column. Standard markers were run on same chromatogram.

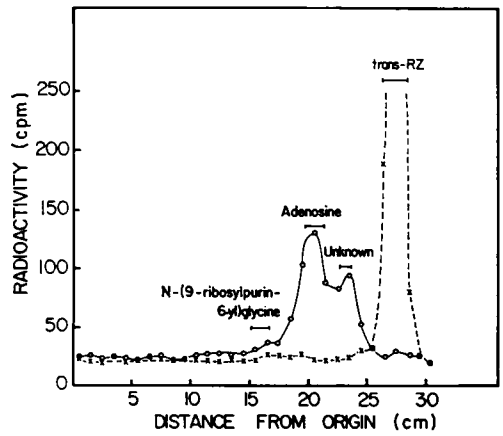

FIG. 7. Treatment of radioactive *trans*-RZ isolated from corn kernel slices after incubation with [8-^{14}C]-i^{6}Ado ("Conversion of i^{6}Ado to *trans*-RZ by Corn Kernel Slices"). The chart shows radioactive profiles of two chromatograms (solvent B) of *trans*-RZ before (×– – –×) and after (○——○) $KMnO_4$ oxidation. The radioactive materials were cochromatographed with standards.

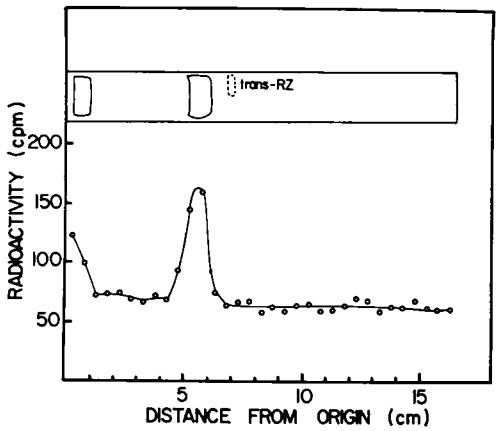

FIG. 8. Confirmation of identity of radioactive product obtained from incubation of [8-^{14}C]-i^{6}Ado with corn kernel slices as *trans*-RZ ("Conversion of i^{6}Ado to *trans*-RZ by Corn Kernel Slices"). The product was mixed with authentic *trans*-RZ and brominated. Chart shows radioactive profile of chromatograph of reaction mixture.

Table II. *Conversion of i^{6}Ado to RZ by Intact Immature Corn Kernels (NK 75)*

Experiment	Total Amount of [8-^{14}C]-i^6-Ado Applied	Fresh Wt of Kernels	Time of Incubation	Amount of RZ Isolated
	cpm	*g*	*hr*	*cpm*
Ear 1	3×10^4	9.24	15	155
Ear 2	4×10^4	10.10	2.0	145
Ear 3	1×10^5	14.00	2.0	380

pollination were sliced off and the upper portion of the endosperm was scooped out. The endosperm (4.72 g) was suspended in 10 ml of buffer (see "Conversion of i^{6}Ado to *trans*-RZ by Corn Kernel Slices.") containing [8-^{14}C]-i^{6}Ado (1.4×10^6 cpm) and the mixture was incubated for 12 hr on a gyratory shaker at 27 C. *Trans*-RZ was isolated and identified as described under "Conversion of i^{6}Ado to *trans*-RZ by Corn Kernel Slices." The results are summarized in Table I.

Incubation of [8-^{14}C]-Adenosine with Corn Kernel Slices. The experiment described under "Conversion of i^{6}Ado to *trans*-RZ by Corn Kernel Slices" was repeated in which [8-^{14}C]-adenosine (2.6×10^6 cpm) replaced [8-^{14}C]-i^{6}Ado. The incubation was carried out for 22 hr and the mixture was analyzed in an identical fashion. The final paper chromatographic profile (analogous to Fig. 8) showed a radioactive peak corresponding to adenosine but no radioactivity in the area corresponding to RZ was detected.

Conversion of i^{6}Ado to RZ in Kernels of Intact Corn Plant (Table II). Three ears of var. NK 75 about 2 weeks after pollination were used attached to the plant. The ears were husked and the endosperm of each kernel was injected with 2 μl of sterile solution containing 300 or 1100 cpm of [8-^{14}C]-i^{6}Ado. Two or fifteen hours after incubation the kernels were homogenized in a mortar and pestle with enough ethanol to make a 70% mixture. Carrier RZ (100 μg) was added during the homogenization. The extraction was repeated twice. The combined extracts were evaporated *in vacuo* and the residue was chromatographed on a Celite-545 column and then on a Sephadex LH-20 column as described under "Conversion of i^{6}Ado to *trans*-RZ by Corn Kernel Slices." The fractions which contained RZ were chromatographed on paper in solvent D. A radioactive profile similar to that shown in Figure 6 was ob-

tained. The experiment was repeated three times; the yield of radioactive products corresponding to *trans*-RZ is shown in Table II.

Attempted Conversion of [8-^{14}C]-i^{6}Ado into *trans*-RZ in Corn Leaves. *a.* Two-week-old seedlings (W64A X W182E) grown sterilely on agar medium in a growth chamber were used. A solution of [8-^{14}C]-i^{6}Ado (2×10^4 cpm) in 10 μl of 50% aqueous ethanol containing 3% (w/v) of Carbowax-6000 was applied on the upper surface of each leaf. Twenty-four plants were used.

Twenty hours after application of the substrate, the leaves together with ground glass were ground in an ice-cold mortar with enough ethanol to make a 70% ethanol extract. The extract was centrifuged for 5 min at 10,800*g*. The pellet was re-extracted twice more with 10 ml of 70% aqueous ethanol. The combined extracts were evaporated to dryness *in vacuo* and the residue was redissolved in 2.0 ml of 10 mM $MgCl_2$ in water. The pH of the solution was adjusted to 8.0 with NaOH, and 4 mg of intestinal alkaline phosphatase were added. The solution was incubated for 1 hr at 37 C. The solution was evaporated to dryness *in vacuo*, and the residue was fractionated on columns of Celite-545, Sephadex LH-20, and paper chromatography as described under "Conversion of i^{6}Ado to *trans*-RZ by Corn Kernel Slices." No radioactivity associated with the marker *trans*-RZ was detected.

b. Ten grams of excised leaves from the week old seedlings (W64A X W182E) were cut into 1-cm sections and the sections were mixed with 100 ml of medium containing 6.7 mmoles of NaK phosphate buffer, pH 7.0, 4.7×10^5 cpm of [8-^{14}C]-i^{6}Ado, and 5 mg of chloramphenicol. The mixture was incubated for 24 hr on a rotary shaker at 27 C. The leaves were extracted and analyzed the same as above. No radioactivity associated with *trans*-RZ was detected.

DISCUSSION

The biosynthetic step, i^{6}Ado → *trans*-RZ, occurs in both *R. roseolus* and in higher plant tissue. The hydroxylation step is specific for the *trans* form. The specificity has some significance since corn kernel tRNA contains *cis*-RZ (8) and pea plant tRNA contains both the *cis* and *trans* form (31). Thus the two forms appear to play some specific role in tRNA function. In terms of cytokinin activity in cultured tobacco tissue, the *trans* form is considerably more active than the *cis* form (9). *trans*-RZ in some plants undergoes further transformations. It is converted, for example, to the dihydro form in bean axes (30); the dihydro form appears to be as biologically active as *trans*-zeatin (32).

Since i^{6}Ado is the precursor of *trans*-RZ, its origin becomes of interest. i^{6}Ado occurs in the tRNA of all species investigated (6) including corn tissue and *R. roseolus*. Certainly when tRNA breaks down it could be a source of i^{6}Ado, and there is evidence in higher organisms that an ongoing turnover of tRNA occurs (10). There is no evidence, however, that turnover of tRNA would represent a source of i^{6}Ado adequate to maintain the cytokinin needs of a particular organism. Short and Torrey (29) found in pea root tips about 27 times as much cytokinin in the free form as bound in the tRNA. Organisms probably have alternative mechanisms for the biosynthesis of i^{6}Ado that are more appropriate for its needs, or they might make use of both tRNA and an independent pathway under varying circumstances. The experiment in which [8-^{14}C]-adenosine was incubated with *R. roseolus* shows at least that adenosine is a precursor of i^{6}Ado under these conditions. It could be entering the i^{6}Ado, however, via either route.

To gain additional perspective on the i^{6}Ado-tRNA relationship we should note that the biosynthesis of i^{6}Ado in tRNA occurs at the macromolecular level; the Δ^2-isopentenyl is transferred from Δ^2-isopentenylpyrophosphate to an adenosine residue in the anticodon loop of tRNA (13). This biosynthetic process occurs in yeast, mammalian tissue, as well as in cytokinin-requiring tobacco tissue grown in culture (3, 13). The biosynthesis of practically all the modified components of tRNA has been described, and in every case it follows the general pathway in which modification occurs at the macromolecular level (6). There is a precedence, however, for the existence of biosynthetic pathways of modified components of tRNA, independent of the tRNA. 1-Methyladenine, which is a gonad-stimulating hormone in the starfish ovary, is synthesized from methionine and an adenine derivative (28). 5-Ribosyluracil can be synthesized from ribosylphosphate and uracil in a number of organisms (19).

It is important for an understanding of the significance of the biosynthesis of *trans*-RZ to comment on another metabolic reaction that i^{6}Ado undergoes. An enzyme, found in cultured tobacco cells, catalyzes the conversion of i^{6}Ado to adenosine (25). Dr. C. D. Whitty in our laboratory has extended this observation to corn kernels (unpublished results). The activity of this enzyme in the present series of experiments can be noted by the fact that a substantial proportion of the radioactive i^{6}Ado added to corn kernels is degraded to adenosine (Fig. 6). The ratios of activity of both the degradation enzyme and the hydroxylation enzyme probably vary with the maturity of the corn kernel. *trans*-RZ accumulates in larger quantities in early stages of fruiting and seems to disappear in the more mature stages (21, 23). We could not detect any of the hydroxylation activity in mature corn seeds.

The metabolism of free i^{6}Ado to either *trans*-RZ or adenosine probably accounts for the fact that i^{6}Ado has not been detected in tissues investigated for their cytokinin activity, although it may be a matter of extremely low levels. Dyson and Hall (5), however, showed that free i^{6}Ado occurs in an autonomous strain of tobacco callus tissue at a concentration equivalent to 30 nM.

With respect to the physiological sites of the biosynthesis of *trans*-RZ, it has been suggested that it is synthesized in one part of the plant and translocated to other parts; the meristem tissue of the roots has been suggested as a major site of synthesis (12). Our experiments show that corn endosperm can synthesize *trans*-RZ. Perhaps in the course of development, the site of synthesis changes at different stages. All tissue probably carries a latent capacity for cytokinin formation.

How much does the fungus *R. roseolus* tell us about cytokinin activity? The transformation, i^{6}Ado → *trans*-RZ, occurs in both the fungus and in corn tissue. The symbiotic role of the fungus suggests that under certain circumstances it serves as a source of cytokinins. The results show that the fungus secretes two cytokinins, i^{6}Ado and *trans*-RZ and, in addition, it secretes a third compound, N-[9-(β-D-ribofuranosyl-9H)purin-6-ylcarbamoyl]threonine, a potential cytokinin (see accompanying paper by Laloue and Hall (15)).

Finally, the evidence points to the conclusion that a key compound, in terms of the biosynthesis of the cytokinin *trans*-RZ, is i^{6}Ado.

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LITERATURE CITED

1. Armstrong, D. J., W. J. Burrows, P. K. Evans, and F. Skoog. 1969. Isolation of cytokinins from tRNA. Biochem. Biophys. Res. Commun. 37: 451–456.

2. Bui-Dang-Ha, D. and J. P. Nitsch. 1970. Isolation of zeatin riboside from the chicory root. Planta 95: 119–126.
3. Chen, C.-M. and R. H. Hall. 1969. Biosynthesis of N^6-(Δ^2-isopentenyl)-adenosine in the transfer ribonucleic acid of cultured tobacco pith tissue. Phytochemistry 8: 1687–1695.
4. Dyson, W. H., C. M. Chen, S. N. Alam, R. H. Hall, C. I. Hong, and G. B. Chheda. 1970. Cytokinin activity of ureidopurine derivatives related to a modified nucleoside found in transfer RNA. Science 170: 328–330.
5. Dyson, W. H. and R. H. Hall. 1972. N^6-(Δ^2-Isopentenyl) adenosine. Its occurrence as a free nucleoside in an autonomous strain of tobacco tissue. Plant Physiol. 50: 616–621.
6. Hall, R. H. 1971. The Modified Nucleosides in Nucleic Acids. Columbia University Press, New York.
7. Hall, R. H., S. N. Alam, B. D. McLennan, C. Terrine, and J. Guern. 1971. N^6-(Δ^2-Isopentenyl) adenosine. Its conversion to inosine, catalyzed by adenosine aminohydrolases from chicken bone marrow and calf intestinal mucosa. Can. J. Biochem. 49: 623–630.
8. Hall, R. H., L. Csonka, H. David, and B. McLennan. 1967. Cytokinins in the soluble RNA of plant tissues. Science 156: 69–71.
9. Hall, R. H. and B. I. S. Srivastava. 1968. Cytokinin activity of compounds obtained from soluble RNA. Life Sci. 7: 7–13.
10. Hanoune, J. and M. K. Agarwal. 1970. Studies on the half life time of rat liver transfer RNA species. FEBS Lett. 11: 78–80.
11. Holley, R. W. 1963. Large scale preparation of yeast "soluble" ribonucleic acid. Biochem. Biophys. Res. Commun. 10: 186–188.
12. Kende, H. 1971. The cytokinins. Int. Rev. Cytol. 31: 301–338.
13. Kline, L. K., F. Fittler, and R. H. Hall. 1969. N^6-(Δ^2-Isopentenyl)-adenosine. Biosynthesis in transfer ribonucleic acid *in vitro*. Biochemistry 8: 4361–4371.
14. Koshimizu, K., S. Matsubara, T. Kusaki, and T. Mitsui. 1967. Isolation of a new cytokinin from immature yellow lupin seeds. Agr. Biol. Chem. 31: 795–801.
15. Laloue, M. and R. H. Hall. 1973. Cytokinins in *Rhizopogon roseolus*. Secretion of N-[9-(β-D-ribofuranosyl-9H) purin-6-ylcarbamoyl]threonine into the culture medium. Plant Physiol. 51: 559–562.
16. Letham, D. S. 1966. Purification and probable identity of new cytokinin in sweet corn extracts. Life Sci. 5: 551–554.
17. Letham, D. S. 1968. A new cytokinin bioassay and the naturally occurring cytokinin complex. *In:* F. Wightman and G. Setterfield, eds., Biochemistry and Physiology of Plant Growth Substances. Runge Press, Ottawa. pp. 19–31.
18. Letham, D. S., J. S. Shannon, and I. R. C. McDonald. 1967. Regulators of cell division in plant tissues. III. The identity of zeatin. Tetrahedron 23: 479–486.
19. Matsushita, T. and F. F. Davis. 1971. Studies on pseudouridylic acid synthetase from various sources. Biochim. Biophys. Acta 238: 165–173.
20. Miller, C. O. 1965. Evidence for the natural occurrence of zeatin and derivatives: compounds from maize which promote cell division. Proc. Nat. Acad. Sci. U. S. A. 54: 1052–1058.
21. Miller, C. O. 1967. Cytokinins in *Zea mays*. Ann. N.Y. Acad. Sci. 144: 251–257.
22. Miller, C. O. 1967. Zeatin and zeatin riboside from a mycorrhizal fungus. Science 157: 1055–1057.
23. Miller, C. O. and F. H. Witham. 1964. A kinetin-like factor from maize and other sources. Colloq. Int. Centre Nat. Rech. Sci. 123: I–VI.
24. Miura, G. A. and C. O. Miller. 1969. 6-(γ,γ-Dimethylallylamino) purine as a precursor of zeatin. Plant Physiol. 44: 372–376.
25. Paces, V., E. Werstiuk, and R. H. Hall. 1971. Conversion of N^6-(Δ^2-isopentenyl) adenosine to adenosine by enzyme activity in tobacco tissue. Plant Physiol. 48: 775–778.
26. Playtis, A. J. and N. J. Leonard. 1971. The synthesis of ribosyl-*cis*-zeatin and thin layer chromatographic separation of the *cis* and *trans* isomers of ribosylzeatin. Biochem. Biophys. Res. Commun. 45: 1–5.
27. Robins, M. J., R. H. Hall, and R. Thedford. 1967. N^6-(Δ^2-Isopentenyl)-adenosine. A component of the transfer ribonucleic acid of yeast and of mammalian tissue, methods of isolation, and characterization. Biochemistry 6: 1837–1848.
28. Shirai, H., H. Kanatani, and S. Taguchi. 1972. 1-Methyladenine biosynthesis in starfish ovary: action of gonad-stimulating hormone in methylation. Science 175: 1366–1368.
29. Short, K. C. and J. G. Torrey. 1972. Cytokinins in seedling roots of pea. Plant Physiol. 49: 155–160.
30. Sondheimer, E. and D.-S. Tzou. 1971. The metabolism of hormones during seed germination and dormancy. II. The metabolism of 8-^{14}C-zeatin in bean axes. Plant Physiol. 47: 516–520.
31. Vreman, H. J., F. Skoog, C. R. Frihart, and N. J. Leonard. 1972. Cytokinins in *Pisum* transfer ribonucleic acid. Plant Physiol. 49: 848–851.
32. Yamada, Y., J. Sekiya, and K. Koshimizu. 1972. Cytokinin-induced shoot formation. Phytochemistry 11: 1019–1021.