

Incorporation of Cytokinin N⁶-Benzyladenine into Tobacco Callus Transfer Ribonucleic Acid and Ribosomal Ribonucleic Acid Preparations¹

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ABSTRACT

The incorporation of the cytokinin N⁶-benzyladenine into tobacco (*Nicotiana tabacum*) callus tRNA and rRNA preparations isolated from tissue grown on medium containing either N⁶-benzyladenine-8-¹⁴C or N⁶-benzyladenine-8-¹⁴C:benzene-³H(G) has been examined. N⁶-benzyladenine was incorporated into both the tRNA and rRNA preparations as the intact base. Over 90% of the radioactive N⁶-benzyladenosine recovered from the RNA preparations was associated with the rRNA. Purification of the crude rRNA by either MAK chromatography or Sephadex G-200 gel filtration had no effect on the N⁶-benzyladenosine content of the RNA preparation. The distribution of N⁶-benzyladenosine moieties in tobacco callus tRNA fractionated by BD-cellulose chromatography did not correspond to the distribution of ribosylzeatin activity. N⁶-benzyladenosine was released from the rRNA preparation by treatment with venom phosphodiesterase and phosphatase, ribonuclease T₂ and phosphatase, or ribonuclease T₂ and a 3'-nucleotidase. N⁶-benzyladenosine was not released from the RNA preparation by treatment with either ribonuclease T₂ or phosphatase alone or by successive treatment with ribonuclease T₂ and a 5'-nucleotidase. Brief treatment of the rRNA preparation with ribonuclease T₁ and pancreatic ribonuclease converted the N⁶-benzyladenosine moieties into an ethyl alcohol soluble form. On the basis of these and earlier results, the N⁶-benzyladenosine recovered from the tobacco callus RNA preparations appears to be present as a constituent of RNA and not as a nonpolynucleotide contaminant.

tobacco tissue cultures was reported by Fox in 1966 (7). The subsequent discovery of the natural occurrence of the highly active cytokinin N⁶-(Δ^2 -isopentenyl)adenosine (i⁶A) and related compounds as constituents of tRNA molecules focused attention on the possibility that cytokinins might function by being incorporated into RNA molecules (11, 19, 24, 25, 28). It now appears established that cytokinins do not serve as direct precursors of the structurally identical nucleosides in tRNA. The i⁶A moieties in tRNA are synthesized by the transfer of the isopentenyl group from Δ^2 -isopentenylpyrophosphate (Δ^2 -IPP) to specific adenosine residues in preformed tRNA molecules (12). The enzyme that catalyzes this reaction (Δ^2 -IPP:tRNA-isopentenyltransferase) has been partially purified from yeast (12) and *Escherichia coli* (3, 22). Although Δ^2 -IPP:tRNA-isopentenyltransferase has not been purified from a plant source, the available evidence indicates that this enzyme is present and functional in cytokinin-dependent tobacco callus tissue (5, 18).

The nature and significance of the apparent incorporation of low levels of exogenously supplied bzl⁶Ade into tobacco callus tRNA remains uncertain. The cytokinin constituents of tobacco callus tRNA prepared from tissue grown on medium containing bzl⁶Ade were isolated and identified by Burrows, Skoog, and Leonard (4). Although most of the cytokinin activity of the tobacco callus tRNA preparation could be attributed to compounds structurally related to i⁶A (*cis*-ribosylzeatin, *c*-io⁶A; 2-methylthioribosylzeatin, ms²io⁶A; and i⁶A), a small amount of N⁶-benzyladenosine (bzl⁶A) was also isolated from the tRNA preparation, and identified by mass spectroscopy. The mode of incorporation of bzl⁶Ade into tobacco callus tRNA was investigated by Walker *et al.* (27) using radioactive bzl⁶Ade labeled with ³H in the benzene ring and with ¹⁴C at the 8-position of the purine ring. It was concluded that bzl⁶Ade was incorporated into the tRNA as the intact base. The level of incorporation corresponded to about 1 bzl⁶A moiety per 10,000 tRNA molecules.

Dyson (6) observed incorporation of radioactivity from bzl⁶Ade into rRNA as well as tRNA in tobacco and soybean callus tissue but could not recover the nucleotide of bzl⁶Ade from alkali hydrolysates of the rRNA preparation.

The results of further studies on the incorporation of radioactive bzl⁶Ade into tobacco callus RNA preparations are reported here. The incorporation of bzl⁶Ade was not specific for tRNA. Ribosomal RNA prepared from tobacco callus tissue grown on medium containing radioactive bzl⁶Ade contained a greater concentration of bzl⁶A moieties than did tRNA preparations from

The apparent incorporation of the cytokinin N⁶-benzyladenine (bzl⁶Ade)³ into the soluble RNA of cytokinin-dependent

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³ Abbreviations: bzl⁶Ade: N⁶-benzyladenine or 6-benzylaminopurine; bzl⁶A: N⁶-benzyladenosine or 6-benzylamino-9- β -D-ribofuranosylpurine; ¹⁴C-bzl⁶Ade: N⁶-benzyladenine-8-¹⁴C; ³H:¹⁴C-bzl⁶Ade: N⁶-benzyladenine-8-¹⁴C:benzene-³H(G); i⁶A: N⁶-(Δ^2 -isopentenyl)adenosine or 6-(3-methyl-2-butenylamino)-9- β -D-ribofuranosylpurine; *c*-io⁶A: *cis*-ribosylzeatin or 6-(*cis*-4-hydroxy-3-methyl-2-butenylamino)-9- β -D-ribofuranosylpurine; ms²io⁶A: methylthioribosylzeatin or 6-(4-

hydroxy-3-methyl-2-butenylamino)-2-methylthio-9- β -D-ribofuranosylpurine; Δ^2 -IPP: Δ^2 -isopentenylpyrophosphate; CTAB: cetyltrimethylammonium bromide; TEAB: tetraethylammonium bromide.

the same source. The results of enzyme digestion studies support the conclusion that the bzl⁶A recovered from the RNA preparations is a constituent of the RNA molecules rather than a non-poly nucleotide contaminant.

MATERIALS AND METHODS

Materials. N⁶-benzyladenosine and polyadenylic acid were purchased from Sigma Chemical Co. N⁶-benzyladenosine-5'-monophosphate and *cis*-ribosylzeatin were synthesized as described elsewhere (20, 23).

The following enzyme preparations were purchased from Sigma Chemical Co.: lyophilized snake venom (*Crotalus adamanteus*), alkaline phosphatase (from calf intestinal mucosa, about 350 units/mg protein), ribonuclease T₁ (about 350,000 units/mg protein), ribonuclease T₂ (about 1,000 units/mg protein), pancreatic ribonuclease (about 100 Kunitz units/mg protein), 5'-nucleotidase (from *Crotalus atrox* venom, about 115 units/mg protein), and 3'-nucleotidase (from rye grass, about 44 units/mg protein).

Whatman DE-23 cellulose was used for diethylaminoethyl-cellulose (DEAE-cellulose) chromatography. Benzoylated-DEAE-cellulose (BD-cellulose) was prepared as described by Gillam *et al.* (9). Sephadex LH-20 is a product of Pharmacia.

A commercial preparation of N⁶-benzyladenine-8-¹⁴C, ¹⁴C-bzl⁶Ade, (Amersham/Searle, 24 mCi/mmol) was used in experiments employing the single labeled compound. N⁶-benzyladenine labeled with ³H in the benzene ring and ¹⁴C in the 8-position of the purine ring was kindly furnished by Professor N. J. Leonard and was prepared as described previously (27). The N⁶-benzyladenine-8-¹⁴C (17.6 mCi/mmol) and N⁶-benzyladenine-benzene-³H(G) (28.3 mCi/mmol) were synthesized independently and mixed to give a "double-labeled" preparation, ³H:¹⁴C-bzl⁶Ade, with a ³H/¹⁴C ratio of about 4.1 for the present experiments.

Growth of Tobacco Callus Tissue. Cytokinin-dependent tobacco tissue cultures (*Nicotiana tabacum* var. Wisconsin #38) were grown on RM-1965 medium (14) containing 11.4 μM indole-3-acetic acid and either 0.067 μM ¹⁴C-bzl⁶Ade or 0.067 μM ³H:¹⁴C-bzl⁶Ade. The cultures were grown in 125-ml Erlenmeyer flasks containing 50 ml of medium. Five pieces of callus tissue (about 30 mg each) were planted per flask. The cultures were harvested after 26 days growth at 28 C in dim light. At the time of harvest, the cultures were growing rapidly, and the average fresh weight per flask was 6.4 g.

Isolation of Tobacco Callus Transfer RNA. Tobacco callus tissue which had been grown on medium containing ³H:¹⁴C-bzl⁶Ade (3,175 g of tissue) or ¹⁴C-bzl⁶Ade (3,160 g of tissue) was divided into 225-g lots. Each lot was homogenized (Sorvall Omnimixer, 5 min) in 75 ml of 0.1 M tris-HCl buffer (pH 7.3) containing 2% (w/v) naphthalene-1,5-disulfonate (disodium salt) and 150 ml of phenol (buffer saturated) containing 0.1% (w/v) 8-hydroxyquinoline. The homogenate was stirred for 30 min at room temperature and then centrifuged (300g, 30 min). The aqueous phase was combined with one-third volume of a phenol/*m*-cresol mixture (10/1, v/v, buffer saturated), stirred, and centrifuged as above. RNA was precipitated from the aqueous phase of the second phenol treatment by addition of one-tenth volume of 20% (w/v) potassium acetate (pH 6, glacial acetic acid) and two volumes of cold ethyl alcohol. All subsequent steps were performed in the cold except as indicated. All centrifugations were at 7,500g for 30 min except as indicated.

The ethyl alcohol precipitate was allowed to stand overnight at -20 C, washed once by suspending it in 70% (v/v) ethyl alcohol containing 0.1 M sodium acetate (pH 6, glacial acetic acid), drained as dry as possible, and extracted twice with 2.5 M potassium acetate (pH 6, glacial acetic acid) containing 0.2%

(w/v) TEAB. Each extraction was performed by suspending the precipitate in 125 ml of the solution per kg of original tissue, homogenizing for 5 min, stirring for 25 min, and then centrifuging. The supernatants from the two extractions were combined, and RNA was precipitated from the extract by addition of two volumes of ethyl alcohol.

The RNA recovered from the potassium acetate extract was washed twice as described above, drained, and extracted twice with 0.5% (w/v) CTAB in 0.4 M NaCl containing 10% (v/v) ethyl alcohol. Each extraction was performed by stirring for 30 min in 62.5 ml of CTAB solution per kg original tissue. The supernatants from the two extractions were combined, centrifuged again (10,000g, 60 min), and the CTA-RNA complex was precipitated from the supernatant by the addition of 1 volume of 0.5% (w/v) CTAB in 10% (v/v) ethyl alcohol (final concentrations: 0.5% CTAB in 0.2 M NaCl containing 10% ethyl alcohol). The CTA-RNA precipitate was collected by centrifugation (10,000g, 30 min) and converted to the sodium form by repeated suspension in 0.4 M sodium acetate (pH 6, glacial acetic acid) followed by precipitation with ethyl alcohol. The conversion was judged to be complete when the precipitate dissolved completely in the sodium acetate solution and foam was no longer evident.

The RNA from above was dissolved in 0.1 M tris-HCl buffer (pH 7.3) containing 0.2 M NaCl and applied to a DEAE-cellulose column (about 5 ml bed volume per kg original tissue) equilibrated with the same buffer. The column was then washed with at least 20 bed volumes of 0.1 M tris-HCl (pH 7.3) containing 0.2 M NaCl, and the tRNA was eluted with the buffer containing 1 M NaCl (about 10 bed volumes). The tRNA was recovered from the column eluates by ethyl alcohol precipitation. The final yields of ¹⁴C-labeled tRNA and ³H:¹⁴C-labeled tRNA were about 1,200 A₂₆₀ units each.

Isolation of Tobacco Callus Ribosomal RNA. The residue from the 2.5 M potassium acetate extraction step used in tRNA purification was washed twice in cold 70% (v/v) ethyl alcohol containing 0.1 M NaCl and 0.2% (w/v) TEAB. The washed pellet was suspended in cold 0.025 M tris-HCl buffer (pH 7.3) containing 0.1 M NaCl and 0.2% TEAB (250 ml of solution per kg of original tissue). The suspension was stirred for 30 min and then centrifuged (10,000g, 30 min). The supernatant was brought to room temperature and made 0.6 M with respect to NaCl. CTAB was added to a final concentration of 0.5% (w/v), and any precipitate that formed was removed by centrifugation at 10,000g for 30 min. Ribosomal RNA was precipitated from the supernatant (at room temperature) by the addition of one-half volume of 0.5% CTAB containing 0.2% TEAB (final concentrations: 0.5% CTAB in 0.4 M NaCl containing 0.2% TEAB). The CTA-RNA precipitate was recovered by centrifugation and converted to the sodium form as described for tRNA. The final yields of rRNA were 5,600 A₂₆₀ units from tobacco callus tissue grown on medium containing ³H:¹⁴C-bzl⁶Ade and 6,100 A₂₆₀ units from tissue grown on medium containing ¹⁴C-bzl⁶Ade.

Analysis of RNA Preparations for bzl⁶A. Tobacco callus tRNA preparations were hydrolyzed to nucleosides with snake venom phosphodiesterase and alkaline phosphatase (10). Ribosomal RNA was hydrolyzed in the same manner except that the RNA preparations were incubated with ribonuclease T₁ (pH 7.5, 37 C, 2 hr) prior to the addition of snake venom and alkaline phosphatase. (Ribonuclease T₁ had no effect on the recovery of bzl⁶A from tRNA and was not used in the routine analysis of tRNA preparations). The hydrolysates were adjusted to pH 7. Two volumes of cold ethyl alcohol were added, and any precipitate that formed was removed by centrifugation at 15,000g for 20 min. The supernatants were evaporated to dryness under reduced pressure at 37 C.

The lyophilized hydrolysates were fractionated according to

the procedure of Armstrong *et al.* (1). The dry solids were extracted six times (15 min per extraction) with water saturated ethyl acetate. (The volume of solvent varied from 3 to 18 ml per extraction depending on the sample size.) The extracts were combined and evaporated to dryness under reduced pressure at room temperature. The ethyl acetate soluble nucleosides thus obtained were fractionated by chromatography on a Sephadex LH-20 column in 35% (v/v) ethyl alcohol. Details of chromatography are given in the legends to the figures. The elution position of bzl^6A was determined by chromatographing an authentic sample of this compound on the same Sephadex LH-20 column used for bzl^6A analysis. In a few cases, the unlabeled bzl^6A standard was added to the sample to be analyzed and cochromatographed with the sample. Column fractions were pooled, and the distribution of radioactivity determined as described below.

Analysis of tRNA Fractions for io^6A . Tobacco callus tRNA fractions were hydrolyzed to nucleosides and fractionated by ethyl acetate extraction and Sephadex LH-20 chromatography as described above for bzl^6A analysis. Sephadex LH-20 fractions corresponding to the elution position of $c\text{-io}^6\text{A}$ (Fig. 2) were pooled and tested for cytokinin activity in the tobacco bioassay (14, 21) as previously described (2). The same Sephadex LH-20 fractionations were used for both io^6A analysis and bzl^6A analysis. Yeast tRNA, added as a carrier to facilitate recovery of tobacco callus tRNA after BD-cellulose chromatography, was completely devoid of io^6A activity and did not interfere with the analysis of tobacco callus tRNA fractions for io^6A .

Radioactivity Measurements. Fractions from Sephadex LH-20 columns were pooled and evaporated to dryness in scintillation vials. The samples were counted in a Packard Tri-Carb Liquid Scintillation Spectrometer Model 3385. Aquasol (New England Nuclear) was used as the scintillation fluid (15 ml/vial). The counting efficiency for ^{14}C was about 93% in experiments using the single label. The counting efficiency for ^{14}C in double-labeling experiments was about 44% in the ^{14}C -channel and about 36% in the ^3H -channel.

RESULTS

Transfer RNA and ribosomal RNA were isolated from cytokinin-dependent tobacco callus tissue grown on medium containing $^3\text{H}:^{14}\text{C}\text{-bzl}^6\text{Ade}$ or $^{14}\text{C}\text{-bzl}^6\text{Ade}$ as described above. Gel electrophoresis was used to monitor the composition of the RNA preparations. Typical gel patterns are shown in Figure 1. The ribosomal RNA preparations contained primarily 18S and 25S RNA and were essentially free of tRNA. Similarly, there was no significant contamination of the tRNA preparation with rRNA. Both types of RNA preparations contained some material, presumably carbohydrate in nature, that was not degraded by phosphodiesterase treatment. In addition, the tRNA preparation contained small amounts of 5S RNA.

The results of bzl^6A analysis of the RNA preparations labeled with $^3\text{H}:^{14}\text{C}\text{-bzl}^6\text{Ade}$ are shown in Figures 2 and 3. Both the tRNA and rRNA hydrolysates contained a double-labeled compound that eluted from Sephadex LH-20 columns at the position of bzl^6A . In both cases, the $^3\text{H}/^{14}\text{C}$ ratio of the bzl^6A peak was almost identical to that of the $^3\text{H}:^{14}\text{C}\text{-bzl}^6\text{Ade}$ originally supplied to the tobacco callus tissue (Table I). Analyses of tobacco callus RNA preparations labeled with $^{14}\text{C}\text{-bzl}^6\text{Ade}$ gave results similar to those obtained with double-labeled RNA preparations. A peak of radioactivity corresponding to $^{14}\text{C}\text{-bzl}^6\text{A}$ was recovered from both the tRNA and rRNA hydrolysates.

The incorporation of radioactivity from labeled bzl^6Ade into tobacco callus tRNA and rRNA preparations is summarized in Table I. As observed previously (27), considerable randomization of the bzl^6Ade label occurred during the growth of the tissue.

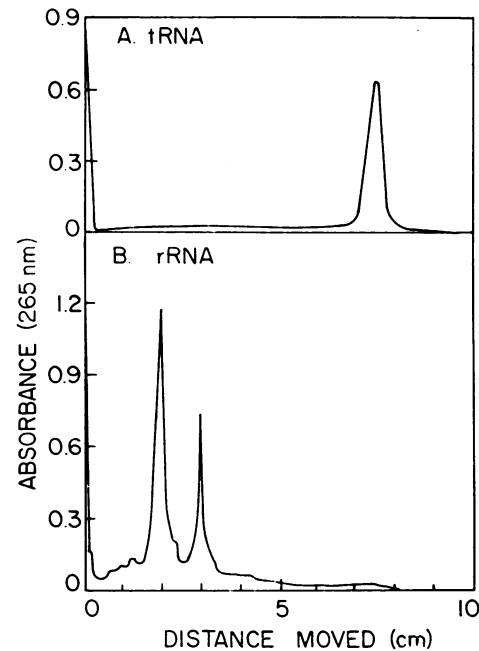


FIG. 1. UV-absorption profiles of tobacco callus RNA preparations fractionated by gel electrophoresis. The RNA samples were fractionated on 2.4% polyacrylamide gels essentially as described by Loening (15). The profiles shown here are for RNA preparations labeled with $^3\text{H}:^{14}\text{C}\text{-bzl}^6\text{Ade}$. The profiles for $^{14}\text{C}\text{-bzl}^6\text{Ade}$ labeled RNA were similar. A: tRNA preparation; B: rRNA preparation.

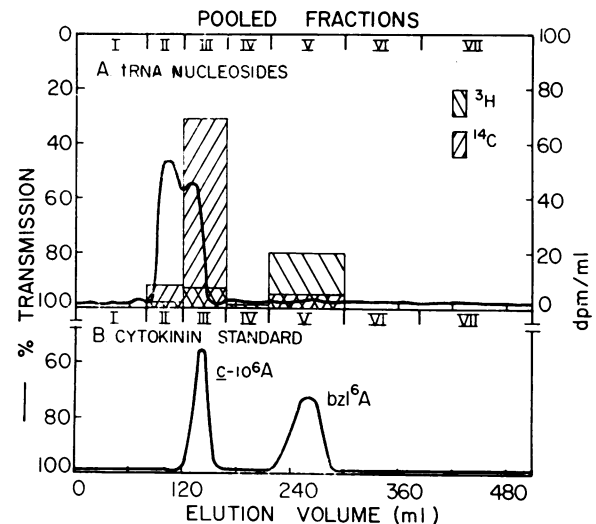


FIG. 2. Analysis of tobacco callus tRNA for bzl^6Ade incorporation. Tobacco callus tRNA (373 A_{260} units) labeled with $^3\text{H}:^{14}\text{C}\text{-bzl}^6\text{Ade}$ was hydrolyzed to nucleosides and analyzed for bzl^6A by ethyl acetate extraction and Sephadex LH-20 chromatography. The ethyl acetate soluble nucleosides extracted from the tRNA hydrolysate were dissolved in 2.5 ml of 35% (v/v) ethyl alcohol and chromatographed on a 30-g Sephadex LH-20 column ($1.9 \times$ about 42 cm) in 35% ethyl alcohol. Six-ml fractions were collected and pooled as indicated for radioactivity measurements. A: Elution profile of the ethyl acetate soluble tRNA nucleosides chromatographed on a Sephadex LH-20 column; B: elution profile of $c\text{-io}^6\text{A}$ and bzl^6A standards chromatographed on the same column.

Only a small fraction of the total ^{14}C radioactivity recovered in the RNA preparations could be attributed to actual incorporation of bzl^6Ade into bzl^6A moieties in the RNA. More than 90% of the total bzl^6A recovered from the hydrolyzed RNA preparations

was associated with the rRNA fraction. On a specific activity basis (dpm of bzI^6A per A_{260} unit RNA), the bzI^6A content of the rRNA preparation was approximately three times greater than that of the tRNA preparation.

The rRNA preparations described above were further purified by either chromatography on methylated albumin:kieselguhr columns (MAK columns) or gel filtration on Sephadex G-200. MAK chromatography of a sample of rRNA labeled with ^{14}C - bzI^6Ade gave the elution profile shown in Figure 4. Although the individual rRNA species were not resolved by this column, appropriate control experiments demonstrated that the rRNA was separated from 4S and 5S RNA as well as from DNA by this procedure. Purification by gel filtration of a sample of rRNA labeled with ^3H : ^{14}C - bzI^6Ade is shown in Figure 5. As expected, the RNA was excluded from the Sephadex G-200 gel and eluted

earlier than an *Escherichia coli* tRNA standard chromatographed separately on the same column. The RNA samples purified by the above procedures were recovered by ethyl alcohol precipitation and analyzed for bzI^6A . The results are shown in Table II. Neither MAK chromatography nor gel filtration affected the bzI^6A content of the RNA preparations.

The distribution of bzI^6A moieties in the tobacco callus tRNA preparation was investigated by BD-cellulose chromatography. A sample of ^3H : ^{14}C - bzI^6Ade labeled tRNA was fractionated on a BD-cellulose column. The tRNA fractions recovered from the column were analyzed for bzI^6A as described above. The io^6A content of each pooled tRNA fraction was determined at the same time in the tobacco bioassay (Fig. 6). The total bzI^6A con-

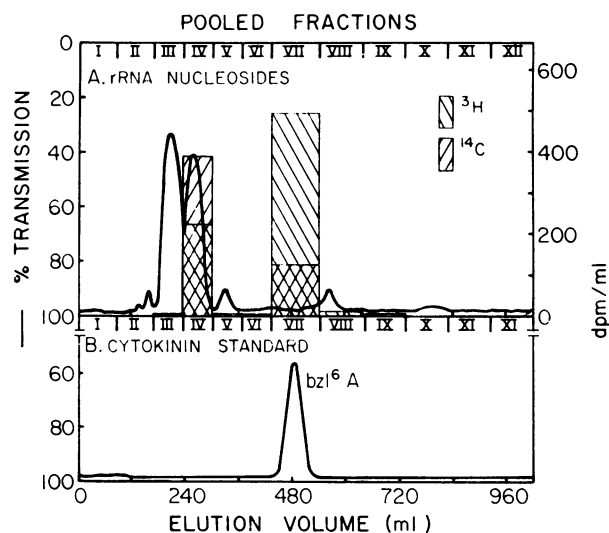


FIG. 3. Analysis of tobacco callus rRNA preparation for bzI^6Ade incorporation. Tobacco callus rRNA (3,670 A_{260} units) labeled with ^3H : ^{14}C - bzI^6Ade was hydrolyzed to nucleosides and analyzed for bzI^6A by ethyl acetate extraction and Sephadex LH-20 chromatography. The ethyl acetate soluble nucleosides extracted from the RNA hydrolysate were dissolved in 5 ml of 35% (v/v) ethyl alcohol and chromatographed on a 60-g Sephadex LH-20 column (2.6 \times about 44 cm) in 35% ethyl alcohol. Six-ml fractions were collected and pooled as indicated for radioactivity measurements. A: Elution profile of ethyl acetate soluble RNA nucleosides chromatographed on the Sephadex LH-20 column; B: elution profile of a bzI^6A standard chromatographed on the same column.

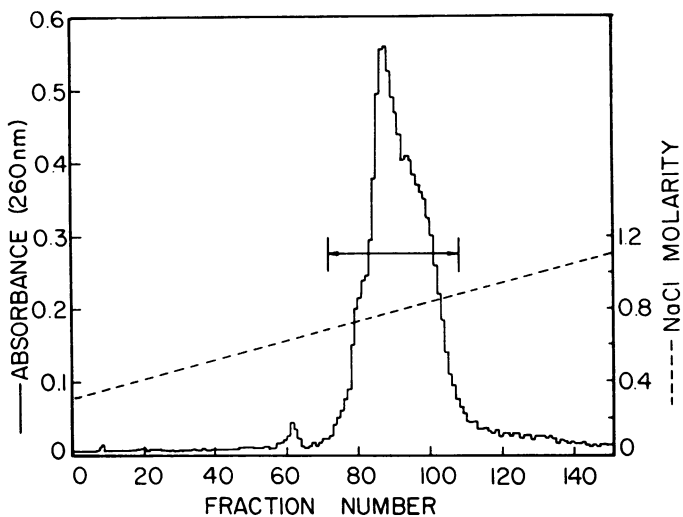


FIG. 4. Purification of tobacco callus rRNA by methylated albumin:kieselguhr (MAK) chromatography. The MAK column was prepared as described by Mandell and Hershey (16). A sample of rRNA (95 A_{260} units) labeled with ^{14}C - bzI^6Ade was dissolved in 10 ml of 0.05 M phosphate buffer (pH 6.7) containing 0.3 M NaCl and applied to a MAK column (1.6 \times 36 cm) equilibrated with the same solution. The RNA was eluted with a linear gradient of NaCl (0.3 M–1.2 M, total volume 1,200 ml) in 0.05 M phosphate buffer (pH 6.7). Fractions (7 ml) were collected at a flow rate of 7 ml per 11 min. The fractions containing the rRNA were pooled as indicated, and the RNA was recovered by ethyl alcohol precipitation after the addition of sufficient unlabeled wheat germ tRNA to bring the final RNA concentration to 2 A_{260} units/ml. The precipitate was redissolved and desalted on a Sephadex G-25 column prior to bzI^6A analysis.

Table I. Distribution of Radioactivity in Tobacco Callus tRNA and rRNA Labeled with ^3H : ^{14}C - bzI^6Ade ($^3\text{H}/^{14}\text{C} = 4.1$) or ^{14}C - bzI^6Ade

RNA Preparation (A_{260} Units/kg Tissue)	Counting Sample	DPM Eq to Sample from 1 kg Tissue		$^3\text{H}:^{14}\text{C}$ Ratio	DPM/ A_{260} Unit of RNA	
		^3H	^{14}C		^3H	^{14}C
$^3\text{H}:^{14}\text{C}$ -labeled tRNA (378 A_{260} units)	Crude RNA	2,015	17,539	0.11	5.33	46.4
	bzI^6A isolated from RNA	1,694	399	4.24	4.48	1.06
$^3\text{H}:^{14}\text{C}$ -labeled rRNA (1,764 A_{260} units)	Crude RNA	29,812	72,324	0.41	16.9	41.0
	bzI^6A isolated from RNA	25,578	6,209	4.11	14.5	3.52
^{14}C -labeled tRNA (380 A_{260} units)	Crude RNA		110,200			290.0
	bzI^6A isolated from RNA		2,064			5.43
^{14}C -labeled rRNA (1,921 A_{260} units)	Crude RNA		624,378			325.0
	bzI^6A isolated from RNA		34,328			17.9

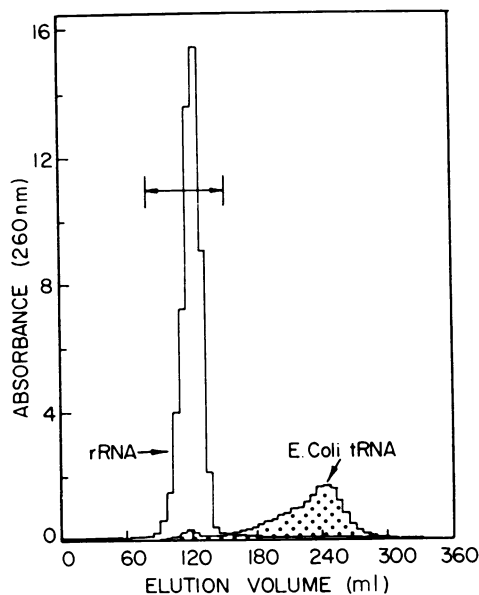


FIG. 5. Purification of tobacco callus rRNA by gel filtration on Sephadex G-200. A sample of rRNA (360 A_{260} units) labeled with $^3\text{H}:$ ^{14}C -bzl 6 Ade was dissolved in 6 ml of 0.1 M sodium acetate buffer (pH 5.5, glacial acetic acid) containing 0.1 M NaCl and applied to a Sephadex G-200 column (2.5 \times 65 cm) equilibrated with the same solvent. (A small layer of Sephadex G-25 was packed on the top and bottom of the column to protect the gel surface.) Fractions (6 ml) were collected at a flow rate of 6 ml per 11 min. Fractions containing the rRNA were pooled as indicated and the RNA recovered by ethyl alcohol precipitation. The elution profile of an *Escherichia coli* tRNA standard (125 A_{260} units applied in 6 ml of buffer) chromatographed separately on the same column is also shown.

Table II. Recovery of Radioactive bzl 6 A from Tobacco Callus rRNA Labeled with $^3\text{H}:$ ^{14}C -bzl 6 Ade ($^3\text{H}:$ ^{14}C = 4.1) or ^{14}C -bzl 6 Ade

Ribosomal RNA Sample	A_{260} Units of RNA Analyzed	Total DPM AH: 3 Recovered as Bzl 6		^{14}C Ratio	DPM of Bzl 6 A/ A_{260} Unit of RNA	
		^3H	^{14}C		^3H	^{14}C
Crude $^3\text{H}:$ ^{14}C -labeled RNA	3,675	53,258	12,944	14.5	14.5	3.52
$^3\text{H}:$ ^{14}C -labeled RNA purified by gel filtration (Fig. 5)	330	4,650	1,216	3.82	14.1	3.68
Crude ^{14}C -labeled RNA	316		5,647			17.9
^{14}C -labeled RNA purified by MAK chromatography (Fig. 4)	70		1,245			17.8

tent of the tRNA preparation was approximately equally distributed between the salt gradient and ethyl alcohol purge regions of the elution profile. No distinct peaks of bzl 6 A containing RNA were observed, although the concentration of bzl 6 A was greater in the RNA eluted by the ethyl alcohol purge than in the salt gradient. In contrast, the io 6 A activity eluted as a relatively sharp peak in the salt gradient. *c*-io 6 A is responsible for most of the cytokinin activity of tobacco callus tRNA prepared from tissue grown as described here, and the distribution of io 6 A activity was similar to the distribution of total cytokinin activity observed in previous studies of BD-cellulose fractionated tobacco callus tRNA (18).

The nature of the apparent incorporation of bzl 6 Ade into the rRNA preparation from tobacco callus tissue was further investigated by a series of enzyme digestion experiments. In the first of these experiments, a sample of rRNA labeled with $^3\text{H}:$ ^{14}C -

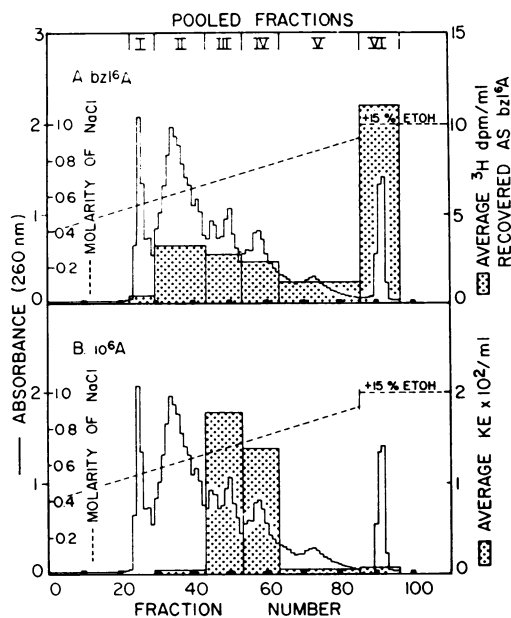


FIG. 6. Distribution of bzl 6 A and io 6 A in tobacco callus tRNA fractionated by BD-cellulose chromatography. Tobacco callus tRNA (1,000 A_{260} units) labeled with $^3\text{H}:$ ^{14}C -bzl 6 Ade was dissolved in 35 ml of 0.4 M NaCl and applied to a BD-cellulose column (1.55 \times 48 cm, about 92 ml bed volume) equilibrated with the same solution. The tRNA was eluted with a linear gradient of NaCl (0.4–1 M, total volume 1,800 ml). Fractions (18 ml) were collected at a flow rate of 18 ml per 15 min. The gradient was stopped at about 0.9 M NaCl, and the column was purged with 1 M NaCl containing 15% (v/v) ethyl alcohol. Fractions were pooled as indicated (I–VI), and sufficient yeast tRNA was added as a carrier to give a total of 400 A_{260} units in each of the six pooled fractions. The RNA was precipitated from the pooled fractions by the addition of one-tenth volume of 0.6 M MgCl_2 and two and one-half volumes of cold ethyl alcohol. The precipitates were allowed to stand overnight at -20°C and recovered by centrifugation. The RNA fractions were then hydrolyzed to nucleosides and analyzed for bzl 6 A and io 6 A. A: Bzl 6 A content of the pooled RNA fractions from the BD-cellulose column. Bzl 6 A content is expressed as DPM ^3H recovered in bzl 6 A isolated from hydrolysates of each RNA fraction. B: Io 6 A content of the pooled RNA fractions. The cytokinin activity due to io 6 A is expressed as μg kinetin equivalents (μg KE) defined as the μg of kinetin (6-furfurylamino-purine) required to give the same activity as the test sample under the specified conditions.

bzl 6 Ade was hydrolyzed to nucleosides by treatment with ribonuclease T_2 and alkaline phosphatase. A duplicate RNA sample was incubated with ribonuclease T_2 alone, and a third sample was treated with only alkaline phosphatase. All three RNA samples were then analyzed for bzl 6 A by ethyl acetate extraction and Sephadex LH-20 chromatography (Fig. 7). Treatment with ribonuclease T_2 and alkaline phosphatase resulted in the release of bzl 6 A from the RNA preparation, but no bzl 6 A was recovered after treatment with either ribonuclease T_2 or alkaline phosphatase alone.

The effect of pancreatic ribonuclease and ribonuclease T_1 on the bzl 6 A-containing component of the rRNA preparation was investigated in the following experiment. A sample of ^{14}C -bzl 6 Ade labeled RNA was incubated with the two enzymes in the presence of polyadenylic acid. At the end of a brief incubation period, the reaction was stopped by addition of cold ethyl alcohol. The poly(A) precipitate and ethyl alcohol supernatant were separated by centrifugation. Ethyl alcohol was removed by evaporation, and both fractions were digested to nucleosides with crude snake venom phosphodiesterase and alkaline phosphatase. The results of bzl 6 A analysis of the hydrolysates are shown in Figure 8. The brief treatment of the RNA preparation with the two ribonu-

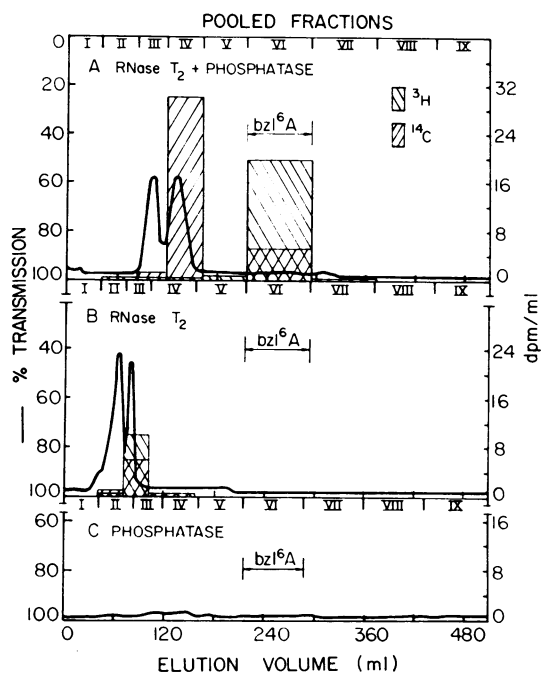


FIG. 7. Recovery of bzl^6A from tobacco callus rRNA preparation after digestion with ribonuclease T_2 and phosphatase, ribonuclease T_2 alone, or phosphatase alone. Three samples of rRNA (106 A_{260} units each) labeled with 3H : ^{14}C - bzl^6A were digested enzymically as described below. The lyophilized digests were extracted with water saturated ethyl acetate. A sample of the ethyl acetate soluble material (equivalent to 98 A_{260} units of RNA) from each digest was dissolved in 2.5 ml of 35% (v/v) ethyl alcohol and chromatographed on a Sephadex LH-20 column (30 g, $1.9 \times$ about 42.5 cm) in 35% ethyl alcohol. The elution profiles are shown above. Six-ml fractions were collected and pooled as indicated for determination of radioactivity. A: RNase T_2 and phosphatase digestion. One RNA sample was dissolved in 3.5 ml of 0.05 M potassium acetate buffer (pH 4.7, glacial acetic acid) and incubated with 59 units of RNase T_2 for 16 hr at 37 C. The pH of the hydrolysate was adjusted to 8 (dilute KOH), and 0.035 ml of 0.1 M $MgSO_4$ and 65 units of alkaline phosphatase were added. After 3 hr at 37 C, 26 additional units of alkaline phosphatase were added, and the incubation was continued for 3 hr. The digestion was terminated by adjusting the pH to 7 and adding two volumes of cold ethyl alcohol. The digest was evaporated to dryness under reduced pressure at 37 C and fractionated as described above. B: RNase T_2 digestion. The second RNA sample was hydrolyzed with RNase T_2 and treated in exactly the same manner as sample A except that alkaline phosphatase was not added during the incubation at pH 8. C: Phosphatase digestion. The third RNA sample was treated in the same manner as sample A except that RNase T_2 was not added during the incubation at pH 4.7.

cleases converted almost all of the bzl^6A moieties into an ethyl alcohol soluble form.

The products of ribonuclease T_2 digestion of RNA are 3'-nucleotides. To determine whether the bzl^6A found in the tobacco callus rRNA preparation was present as a 3'-nucleotide following ribonuclease T_2 digestion, the following experiment was performed. Duplicate RNA samples labeled with ^{14}C - bzl^6A were digested to nucleotides with ribonuclease T_2 . A quantity of adenosine-5'-monophosphate approximately equal to the total 3'-nucleotides released from the RNA was added to each hydrolysate together with a small amount of synthetic, unlabeled N^6 -benzyladenosine-5'-monophosphate. One of the hydrolysates was then treated with a purified venom 5'-nucleotidase and the other with a 3'-nucleotidase from rye grass. After an appropriate incubation period, each hydrolysate was analyzed for bzl^6A (Fig. 9). A large peak of UV-absorption corresponding to unlabeled bzl^6A released from the synthetic N^6 -benzyladenosine-

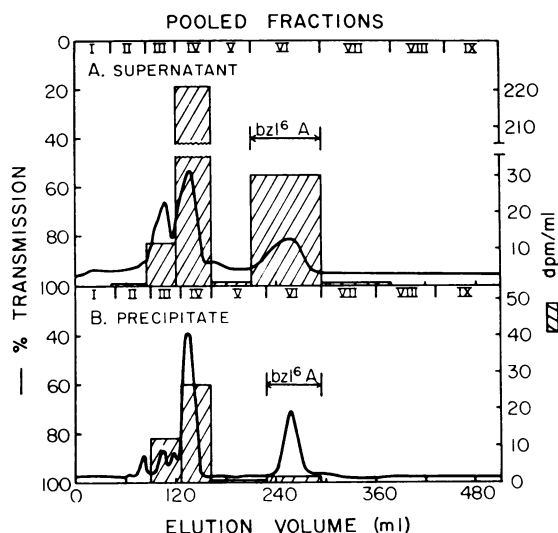


FIG. 8. Effect of ribonuclease T_1 and pancreatic ribonuclease treatment on the ethyl alcohol solubility of the bzl^6A containing component of tobacco callus rRNA preparation. Tobacco callus rRNA (106 A_{260} units) labeled with ^{14}C - bzl^6A and synthetic polyadenylic acid (53 A_{260} units) were dissolved in 3 ml of 0.01 M tris-HCl buffer (pH 7.5) containing 0.3 M KCl and was incubated with RNase T_1 (3,000 units) and pancreatic RNase (80 μg) for 30 min at 37 C. Hydrolysis was terminated by the addition of 7 ml of cold ethyl alcohol, and the RNA digest was separated with the poly(A) precipitate by centrifugation. Ethyl alcohol was removed by evaporation under reduced pressure, and both the supernatant and precipitate fractions were hydrolyzed to nucleosides with crude snake venom phosphodiesterase and alkaline phosphatase. The hydrolysates were analyzed for bzl^6A . The ethyl acetate soluble material from each hydrolysate was dissolved in 2.5 ml of 35% (v/v) ethyl alcohol containing 0.25 mg of unlabeled bzl^6A standard and fractionated on a Sephadex LH-20 column (30 g, $1.9 \times$ about 42 cm) in 35% ethyl alcohol. The elution profiles are shown above. Six-ml fractions were collected and pooled as indicated for radioactivity measurements. A: Sephadex LH-20 fractionation of the ethyl acetate soluble nucleosides from the ethyl alcohol supernatant fraction of the RNase digest. B: Sephadex LH-20 fractionation of the ethyl acetate soluble nucleosides from the ethyl alcohol precipitate fraction of the RNase digest.

5'-monophosphate was recovered from the hydrolysate treated with the 5'-nucleotidase. However, no labeled bzl^6A was associated with this peak. (The same result was obtained if adenosine-5'-monophosphate was omitted during the treatment with 5'-nucleotidase.) In marked contrast to this result, treatment of the ribonuclease T_2 hydrolysate with the 3'-nucleotidase resulted in the recovery of a large peak of radioactivity corresponding to bzl^6A , but there was only slight UV-absorption associated with this peak. From these results, it appears that the bzl^6A found in the rRNA preparation is present as a 3'-nucleotide following ribonuclease T_2 digestion of the RNA.

DISCUSSION

The incorporation of bzl^6A into tobacco callus RNA observed here was not restricted to tRNA (7, 8) and agreed with the distribution of radioactivity reported by Dyson (6). In the present study, the total bzl^6A recovered from the rRNA fraction exceeded the total recovered from the tRNA fraction by more than 15-fold. The concentration of bzl^6A in the rRNA fraction was equal to about 1 bzl^6A moiety per 3.8×10^5 nucleotide residues compared to about 1 bzl^6A per 12.8×10^5 nucleotides in the tRNA fraction. (The latter figure is equivalent to about 1 bzl^6A moiety per 16,000 tRNA molecules.)

The evidence that bzl^6A recovered from the RNA preparations

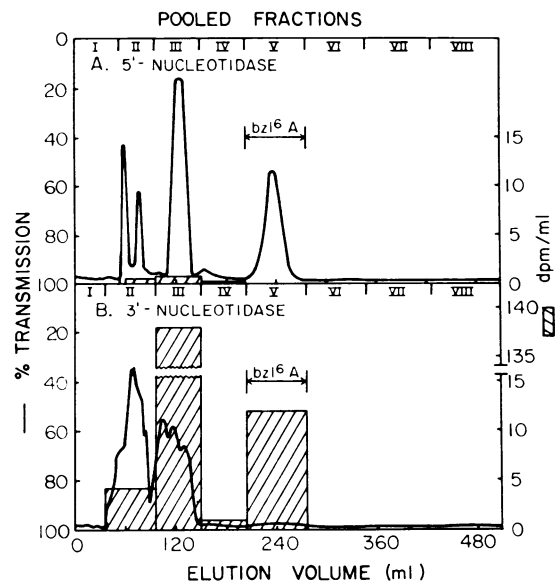


FIG. 9. Recovery of bzl^6A from ribonuclease T_2 hydrolysates of tobacco callus rRNA after treatment with 5'-nucleotidase or 3'-nucleotidase. Two replicate samples (67 A_{260} units each) of tobacco callus rRNA labeled with ^{14}C - bzl^6Ade were each dissolved in 2 ml of 0.05 M potassium acetate buffer (pH 4.7, glacial acetic acid) and incubated with RNase T_2 (52 units) at 37 C for 18 hr. Adenosine-5'-monophosphate (3 mg) and synthetic N^6 -benzyladenosine-5'-monophosphate (0.3 mg, unlabeled) were added to each hydrolysate at the end of the 18-hr incubation. The pH of one hydrolysate was adjusted to 8.5; 0.3 ml of 0.1 M MgSO_4 was added, and the hydrolysate was incubated with 4 units of 5'-nucleotidase for 30 min at 37 C. The pH of the second hydrolysate was adjusted to 7.5; 0.3 ml of distilled H_2O was added, and the hydrolysate was incubated with 4 units of 3'-nucleotidase for 30 min at 37 C. The nucleotidase digestions were terminated by adjusting both digests to pH 7 and adding 2 volumes of cold ethyl alcohol. The digests were evaporated to dryness under reduced pressure at 37 C and analyzed for bzl^6A by ethyl acetate extraction and Sephadex LH-20 chromatography. The ethyl acetate soluble material from each digest was dissolved in 2.5 ml of 35% (v/v) ethyl alcohol and fractionated on a Sephadex LH-20 column (30 g, $1.9 \times$ about 42 cm) in 35% ethyl alcohol. The elution profiles are shown above. Six-ml fractions were collected and pooled as indicated for radioactivity measurements. A: Sephadex LH-20 fractionation of the ethyl acetate soluble material from the 5'-nucleotidase digest; B: Sephadex LH-20 fractionation of the ethyl acetate soluble material from the 3'-nucleotidase digest.

is actually present as a constituent of the RNA molecules may be summarized as follows. (a) The bzl^6A moieties are released from the RNA preparations by treatment with crude snake venom phosphodiesterase and alkaline phosphatase. (b) The bzl^6A moieties remain associated with the rRNA after further purification of the crude RNA preparations by either MAK chromatography or Sephadex G-200 gel filtration. (c) Bzl^6A is released from the rRNA preparation by treatment with ribonuclease T_2 and phosphatase but not by treatment with either ribonuclease T_2 or phosphatase alone. (d) The bzl^6A moieties are converted to an ethyl alcohol soluble form by brief treatment of the rRNA preparation with ribonuclease T_2 and pancreatic ribonuclease. (e) Bzl^6A is recovered from ribonuclease T_2 digests of the rRNA preparation after treatment of the digests with a 3'-nucleotidase but not after treatment with a 5'-nucleotidase. On the basis of these results as well as earlier studies (4, 27), it appears unlikely that the bzl^6A recovered from the tobacco callus RNA preparations could be derived from a nonpolynucleotide contaminant of the preparations.

The results of double-labeling experiments indicate that bzl^6Ade is incorporated into both the tRNA and rRNA prepara-

tions as the intact base. The conversion of bzl^6Ade and the naturally occurring cytokinin i^6A to the corresponding nucleoside triphosphates by suspension cultures of tobacco and *Acer pseudoplatanus* has been reported recently (13), and may provide a pathway for the incorporation of bzl^6Ade into RNA. Evidence from earlier studies (17, 26) suggests that the formation of nucleoside triphosphate derivatives of cytokinins may be a normal feature of cytokinin metabolism in plant tissues.

The high proportion of bzl^6A recovered from the tobacco callus rRNA fraction raises the possibility that the apparent incorporation of bzl^6Ade into tobacco callus tRNA may be due to contamination of the latter preparation with rRNA fragments of high mol wt rRNA. Degradation of a label high mol wt RNA to smaller polynucleotides might provide an explanation for the apparently specific incorporation of bzl^6Ade into tobacco callus soluble RNA observed by Fox and Chen (7, 8). This interpretation is consistent with the distribution of bzl^6A in BD-cellulose fractionation of the tobacco callus tRNA preparation as well as the greater specific activity (bzl^6A residues per A_{260} unit RNA) of the rRNA compared to the tRNA. There is no compelling argument that this is actually the case. In long term labeling experiments such as those employed here, a variety of factors could be responsible for differences in the specific activity of the two RNA preparations.

Models of cytokinin action that involve the incorporation into RNA molecules are attractive, because they suggest an analogy in the function of these adenine derivatives and the corresponding nucleosides in tRNA. The physiological significance of the observed incorporation of bzl^6Ade into tobacco callus RNA is open to question. In view of the low levels of incorporation, the most plausible explanation would appear to be that bzl^6Ade is incorporated as the result of transcriptional errors. Alternatively, the low levels of incorporation could be due to the specific incorporation of bzl^6Ade into a minor polynucleotide component of the rRNA preparation or into rRNA derived from some fraction of the total ribosome population of the callus tissue. Presumably, if the incorporation is meaningful, it must occur as one of the events involved in the maturation of the RNA rather than at the level of transcription.

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