The Isolation and Identification of Two Cytokinins from *Escherichia coli* Transfer Ribonucleic Acids*

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ABSTRACT: Two nucleosides responsible for cytokinin activity of *Escherichia coli* transfer ribonucleic acids have been isolated and identified as 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine and 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine. The structures of these compounds were assigned on the basis of their ultraviolet and mass spectra, which were identical with those of the corresponding synthetic compounds. The ribosides were about equally active in the tobacco bioassay. They were detected at *ca.* 5×10^{-3} μM and gave maximum responses at *ca.* 7×10^{-1} μM. The free base 6-(3-methyl-2-butenylamino)-2-methylthiopurine was detected at *ca.* 2×10^{-3} μM as compared with *ca.* 1×10^{-4} μM as an average value for 6-(3-methyl-2-butenylamino)purine.

The presence of cytokinin-active ribosides in both unfractionated sRNA and in specific tRNA species is well documented (for references, see Skoog and Leonard, 1968; Armstrong *et al.*, 1969a,b).

In an earlier publication Skoog *et al.* (1966) were able to detect cytokinin activity in the acid hydrolysates of sRNA from *Escherichia coli*. Following the report that 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine [N^6-(Δ^2-isopentenyl)adenosine] was absent in enzymic hydrolysates of *E. coli* sRNA (Fittler *et al.*, 1968a) it was of special interest to identify the compound(s) responsible for this activity. Following the preliminary report on the isolation and identification of the 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine [N^6-(Δ^2-isopentenyl)-2-methylthioadenosine] as one of the active cytokinins (Burrows *et al.*, 1968), we now report fully on the isolation, identification, and biological activity of two active cytokinins from *E. coli* sRNA.

Experimental Section

Hydrolysis of sRNA. Partially degraded *E. coli* B sRNA (30 g) (Schwarz BioResearch Inc.) containing 9.7 OD_{260} units/mg was dialyzed for 24 hr at 4° prior to enzymic hydrolysis with lyophilized snake venom (*Crotalus adamanteus*, Sigma) and alkaline phosphatase (type II, calf intestinal mucosa, Sigma) (Hall, 1964). The incubation mixture contained 6 mg/ml of sRNA, 0.001 ml/ml of 1 M $MgSO_4$, 1 mg/ml of snake venom, and 0.025 mg/ml of alkaline phosphatase. The pH was adjusted to 8.6 with NaOH, and the incubation mixture was saturated with toluene. The mixture was stirred gently at 37° and the pH was adjusted to 8.6 at 30-min intervals during the first 8 hr. At this point more enzyme (0.3 mg/ml of snake venom and 0.0125 mg/ml of alkaline phosphatase) and 0.0005 ml/ml of M $MgSO_4$ were added. Incubation was continued for another 16 hr, after which time there was no further uptake of NaOH and the hydrolysis was judged complete. The mixture was then heated to 60° for 30 min, adjusted to pH 6.0, and stored overnight at 4°.

Separation of the Ribosides. Following centrifugation at 10,000*g* for 60 min the clear supernatant was lyophilized and the riboside mixture was fractionated by partition chromatography on Celite-545 columns (Hall, 1962, 1965); the upper phase of a mixture of ethyl acetate–methoxyethanol–water (4:1:2, v/v) was used for elution. Three replicate columns were run, each loaded with the equivalent of 10.0 g of sRNA hydrolysate. The eluates from this and all subsequent columns were monitored at 265 nm. The apparent high background represents a base-line adjustment; the actual absorption was negligible. Further fractionation was achieved on acid-washed Whatman No. I paper. The composition of the 95% ethanol eluates from the chromatograms was monitored by thin-layer chromatography on cellulose $MN300F_{254}$ plates and by bioassays of the chromatograms. The cellulose was scraped into 5 ml of distilled water, acid hydrolyzed by a standard procedure (Armstrong *et al.*, 1969a), and incorporated into the bioassay medium (Skoog *et al.*, 1966). For determination of cytokinin activity in fractions from a column, samples of eluate were reduced to dryness, dissolved in 5 ml of distilled water, and treated as above.

Synthesis of 6-(3-Methyl-2-butenylamino)-2-methylthiopurine (III). A solution of 1.03 g (4.87 mmol) of 2,6-bis(methylthio)purine (II) (Montgomery *et al.*, 1959) in 4.0 g of γ,γ-di-

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[1] KE = micrograms of kinetin required to give the same response as the test sample.

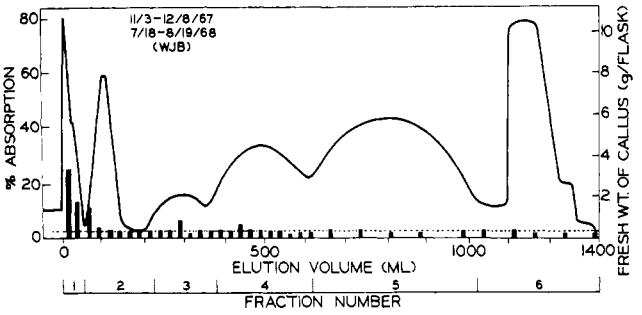
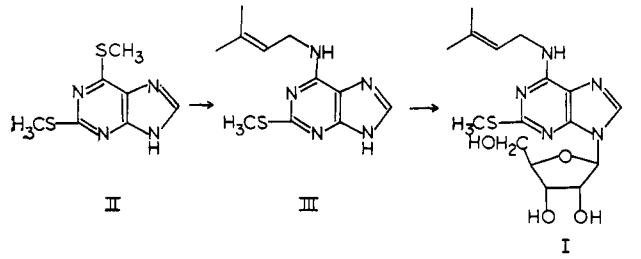


FIGURE 1: The cytokinin activity in the elution profile of an enzyme hydrolysate of 500 mg of *E. coli* sRNA. The lyophilized riboside mixture was dissolved in 8 ml of the lower phase of ethyl acetate–methoxyethanol–water (4:1:2, v/v), mixed with 16 g of Celite-545, and packed into a glass column (87 × 2.5 cm) containing 50 g of Celite-545 mixed with 25 ml of the lower phase of the same solvent. The upper phase of this solvent was used for elution. The flow rate was 150 ml/hr and 20-ml fractions were collected.

methylallylamine was heated at reflux for 30 hr. Sufficient water was added to the cooled reaction mixture to cause precipitation of the product, which was collected by filtration and air dried. Purification was effected by trituration with hot ethanol to remove traces of starting material, leaving colorless



II III I

crystals of III: yield 0.65 g (64%), mp 259–260°; λ_{max}^{EtOH} (pH 7) 242 mμ (ε 25,200) and 279 mμ (ε 15,900), λ_{min} 257 mμ (ε 9300) and 220 mμ (ε 7300); λ_{max}^{EtOH} (pH 1) 253 mμ (ε 21,700) and 292 mμ (ε 15,900), λ_{min} 277 mμ (ε 11,700) and 217 mμ (ε 7200); λ_{max}^{EtOH} (pH 10) 287 mμ (ε 14,800), λ_{min} 256 mμ (ε 4700); nuclear magnetic resonance spectra (δ) from tetramethylsilane (dimethyl sulfoxide-d_6): 1.73 (6 H, s, $(CH_3)_2C$), 2.55 (3 H, s, CH_3S), 4.2 (2 H, m, CCH_2N), 5.4 (1 H, m, C=CH), and 8.17 (1 H, s, Ad-C_8H). *Anal.* Calcd for $C_{11}H_{15}N_5S$: C, 52.98; H, 6.06; N, 28.09. Found: C, 52.58; H, 6.08; N, 28.47.

*Synthesis of 6-(3-Methyl-2-butenylamino)-2-methylthio-9-β-*D*-ribofuranosylpurine (I).* To a hot solution of 500 mg (2.0 mmol) of 6-(3-methyl-2-butenylamino)-2-methylthiopurine (III) and 80 mg (2.0 mmol) of sodium hydroxide in 20 ml of 50% aqueous ethanol was added a hot solution containing 544 mg (2.0 mmol) of mercuric chloride in 10 ml of ethanol. The combined solution was cooled, and the precipitate was filtered and dried in a vacuum oven.

To a solution of 1.20 g of 1-acetyl-2,3,5-tribenzoylribofuranose in 2 ml of dry methylene chloride was added 6 ml of 30–32% hydrogen bromide in acetic acid. The solution was allowed to stand for 1.5 hr, and acetic acid and hydrogen bromide were removed by evaporation under diminished pressure (bath temperature <35°) and by codistillation with five portions of dry toluene.

The mercury salt (908 mg, 94%) was dispersed in 20 ml of boiling xylene, and the 1-bromo-2,3,5-tribenzoylribofuranose, dissolved in 20 ml of xylene, was added. The mixture was heated at reflux for 2 hr (Davoll and Lowy, 1951). The mixture was reduced in volume and saturated with 100 ml of petroleum ether (bp 30–60°). The suspension was chilled for several hours and then filtered. The solid product was triturated with hot chloroform. The chloroform extracts were washed with 30% potassium iodide solution and water and were then dried over sodium sulfate. The extracts were concentrated to afford a reddish liquid, which was treated overnight at room temperature with 150 ml of anhydrous 15 N methanolic ammonia in a pressure bottle. The solution was concentrated and the crude product was recrystallized from ethanol–water to afford colorless crystals of I: yield 320 mg (42%), mp 194–195° $C_{16}H_{23}N_5O_4S$ (M^+ calcd: 381.1471; found: 381.144); λ_{max}^{EtOH} (pH 7) 244 mμ (ε 25,300) and 283 mμ (ε 18,100), λ_{min} 258 mμ (ε 9500); λ_{max}^{EtOH} (pH 1) 246 mμ (ε 18,600) and 286 mμ (ε 16,100), λ_{min} 265 mμ (ε 13,400); λ_{max}^{EtOH} (pH 10) 243 mμ (ε 24,900) and 283 mμ (ε 18,000), λ_{min} 259 mμ (ε 9400); mass spectrum: *m/e* 381.144 (M^+), 366.125, 249.106, 234.078, 206.048, 194.052, and 181.040; nuclear magnetic resonance (δ) (dimethyl sulfoxide-d_6–D_2O): 1.73, 1.77 (6 H, 2 s, $(CH_3)_2C$), 2.57 (3 H, s, CH_3S), 3.71 (2 H, m, C-5′ protons), 4.16 (4 H, m, CCH_2N, C-3′ and C-4′ protons), 4.71 (1 H, m, C-2′ proton), 5.38 (1 H, t, C=CH), 5.94 (1 H, d, C-1′ proton), and 8.26 (1 H, s, Ad-C_8H). *Anal.* Calcd for $C_{16}H_{23}N_5O_4S$: C, 50.38; H, 5.08. Found: C, 50.59; H, 6.32.

Results

*Isolation and Identification of 6-(3-Methyl-2-butenylamino)-2-methylthio-9-β-*D*-ribofuranosylpurine.* The elution profile of the nucleosides (Figure 1) was similar to that reported by Hall *et al.* (1965) for yeast sRNA. Cytidine and guanosine were eluted together by washing the column with distilled water. Bioassay of a 1% sample from each tube revealed high cytokinin activity associated with a peak emerging with the solvent front (fraction I, Figure 1). In the large Celite columns activity could also be detected in the methyladenosine peak and very weakly in the adenosine peak. When the fractions were tested without acid hydrolysis, activity could be detected only in fraction I. The uridine peak and water eluate were inactive, as were all fractions eluted from a control column to which a sample of enzyme solution, incubated in the absence of sRNA, had been applied.

Thin-layer chromatography of a sample from fraction I, with distilled water as the solvent, revealed four absorbing spots. Cytokinin activity was associated with two poorly resolved spots at R_F 0.06 and 0.12 (**1′** and **2′**). Two faster moving spots (**3′** and **4′**, R_F 0.64 and 0.82, respectively) were inactive as was an ultraviolet fluorescent spot at R_F 0.34. After **1′** and **2′** were resolved by thin-layer chromatography in 10% ethanol (R_F 0.10 and 0.31, respectively), only **2′** had cytokinin activity.

Descending paper chromatography of fraction I, with distilled water as the solvent, separated the faster moving **3′** and **4′** from the cytokinin activity, which was streaked from the origin to R_F 0.40. Compound **2′** and a large amount of fluorescent material were present in the eluate from the region of R_F 0.06–0.40. Because of the similarity in the chromato-

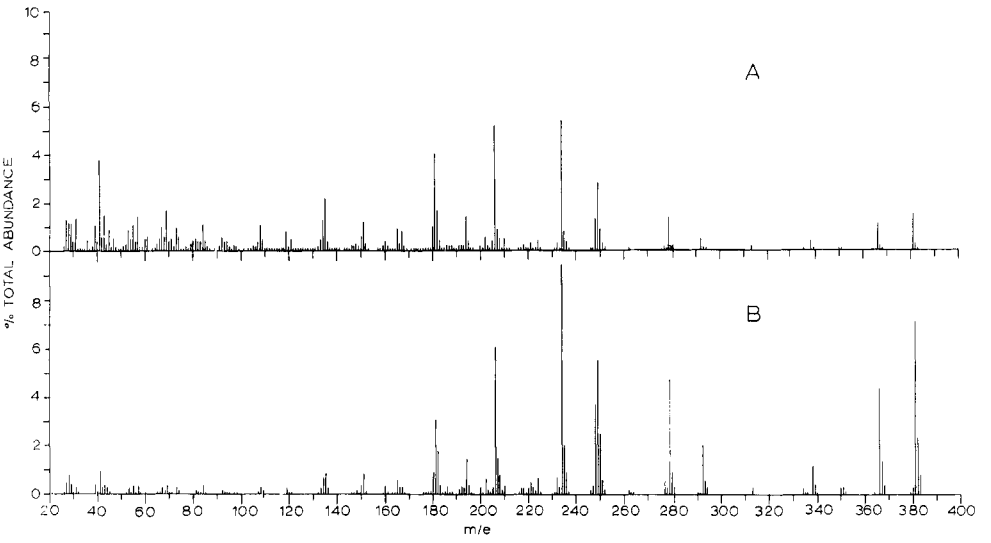


FIGURE 2: Mass spectra at 70 eV of naturally occurring (A) and synthetic (B) 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine.

graphic properties of **2′** and those of the fluorescent material on paper it was found convenient to isolate the former compound from the eluate of the region below R_F 0.06. The dried solids from this eluate were extracted twice with 1.5-ml volumes of chloroform–petroleum ether (2:1, v/v). The supernatant from this extraction contained colored material together with most of **1′** and a small amount of **2′**. The residue, consisting primarily of **2′**, was chromatographed descending in 50% ethanol, and only one ultraviolet-absorbing band (R_F 0.85) was observed. The eluate from this band was rechromatographed in 20% ethanol, and the single ultraviolet-absorbing band (R_F 0.58) was eluted and rechromatographed in the same solvent. Elution of the ultraviolet-absorbing band, R_F 0.58, yielded about 1 mg of white solids consisting of **2′** and a small amount of ultraviolet fluorescent material. This product, which contained *ca.* 300 KE of riboside, was used for the determination of the structure of **2′**.

Inspection of the low-resolution mass spectrum of **2′** revealed substantial peaks at *m/e* 381, 366, 313, 292, 278, 249, 234, 206, 194, and 181 (Burrows *et al.*, 1968). This series closely paralleled, but at 46 mass units greater, the fragmentation pattern observed for 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (Biemann *et al.*, 1966; Robins *et al.*, 1967; Hall *et al.*, 1967). In light of the considerable cytokinin activity of this material, we felt that the mass spectral data could best be accommodated by a substituted N^6-isopentenyladenosine.

The position of unsaturation in the isopentenyl side chain was deduced from the relative strengths of the peaks at *m/e* 249, 234, 206, 194, and 181. These were thought to correspond to the peaks at *m/e* 203, 188, 160, 148, and 135 in 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine and 6-(3-methyl-3-butenylamino)-9-β-D-ribofuranosylpurine and, on the basis of previous work (Leonard *et al.*, 1968), we were able to eliminate the Δ^3 location of the double bond in the isopentenyl side chain as a possibility.

The high-resolution mass spectrum showed a molecular ion peak at 381.149 (calcd 381.147), corresponding to $C_{16}H_{23}N_5O_4S$. This suggested the presence of a CH_3S substituent. The position of CH_3S substitution was deduced by comparison of the ultraviolet spectrum of the cytokinin with those of 2-methylthio- and 8-methylthio-N^6-substituted adenines (Montgomery *et al.*, 1959; Robins, 1958), which favored 2 substitution for the cytokinin. The accumulated data indicated the complete structure of the cytokinin as 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine.

The mass spectral fragmentation pattern may thus be represented as in Scheme I. In addition to the two metastables noted in this diagram, we observed an additional metastable for the conversion 381 → 249, corresponding to a loss of the pentose moiety.

The synthesis of 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (I) was accomplished most satisfactorily by heating 2,6-bis(methylthio)purine (II) (Montgomery *et al.*, 1959) with γ,γ-dimethylallylamine. The 6-

SCHEME I: Mass Spectral Fragmentation Pattern at 70 eV of the Natural Cytokinin from *E. coli*.

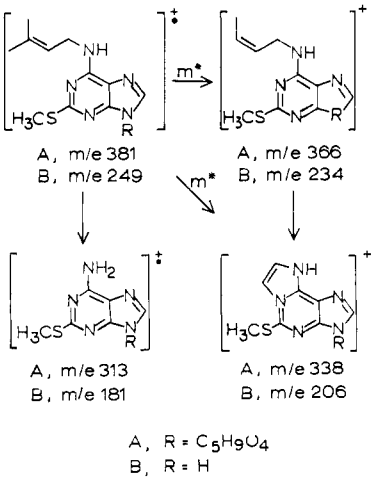


TABLE I: R_F Values of Natural and Synthetic 6-(3-Methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine and Synthetic 6-(3-Methyl-2-butenylamino)-9-β-D-ribofuranosylpurine in Different Solvent Systems on Cellulose MN300F$_{254}$ Thin-Layer Chromatography Plates.

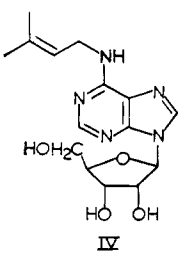
Solvent	R_F Values		
	6-(3-Methyl-2-butenylamino)-9-β-D-ribofuranosylpurine	Natural 6-(3-Methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine	Synthetic 6-(3-Methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine
Ethyl acetate–formic acid–water (60:5:35, v/v)		0.90	
Isoamyl alcohol–5% aqueous sodium citrate (1:1, v/v)		0.90	
Ethyl acetate–1-propanol–water (4:1:2, v/v)		0.97	0.97
t-Amyl alcohol–formic acid–water (4:1:2, v/v)		0.93	
Boric acid (0.03 M, pH 8.4)	0.57	0.22	0.22
Distilled water	0.55	0.16	0.15
10% ethanol	0.61	0.26	0.26
20% ethanol	0.82	0.44	0.44
50% ethanol		0.87	
95% ethanol		0.90	
1-Butanol–water (86:14, v/v)	0.91	0.91	0.91
Ethyl acetate–water, pH 7.0 (5:1, v/v)	0.97	0.97	0.97
1-Butanol–water–concentrated NH_4OH (86:14:5, v/v)	0.94	0.94	0.94
2-Propanol–1% aqueous $(NH_4)_2SO_4$ (2:1, v/v)	0.98	0.98	0.98
2-Propanol–water–concentrated NH_4OH (7:2:1, v/v)	0.98	0.98	0.98

(3-methyl-2-butenylamino)-2-methylthiopurine (III) thus obtained was converted into its chloromercuri derivative and treated with 1-bromo-2,3,5-tribenzoylribofuranose in boiling xylene (Davoll and Lowy, 1951). Treatment of the intermediate tribenzoylated nucleoside with methanolic ammonia afforded pure 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (I).

The synthetic and natural products had identical low-resolution mass spectra (Figure 2) and the empirical formula of the synthetic product was confirmed by high-resolution mass spectrometry and elemental analysis. The ultraviolet spectra of the natural and synthetic products were the same (Figure 3), as were their R_F values in thin-layer chromatography in ten solvents (Table I). The synthetic product was further characterized by its nuclear magnetic resonance spectrum.

Isolation and Identification of 6-(3-Methyl-2-butenylamino)-9-β-D-ribofuranosylpurine. The data in Figure 1 suggested that a second cytokinin was present in small quantities. This was confirmed by bioassays of eluates from a Sephadex column fractionation.

E. coli sRNA (3 g) was enzymically hydrolyzed to ribosides and the lyophilized product was extracted six times with 25-ml volumes of the upper phase of a mixture of ethyl acetate–water (5:1, v/v). The pooled extracts were lyophilized, and the solids were dissolved in 35% aqueous ethanol and loaded on a Sephadex LH$_{20}$ column (152 g of Sephadex LH$_{20}$ packed in a glass column, 120 × 4 cm). Three columns were used, each loaded with hydrolysate equivalent to 1.0 g of sRNA. The elution profiles of the columns were identical, and the corresponding fractions from all three columns were combined. The elution profile and the distribution of cytokinin activity in 0.3% samples are shown in Figure 4. Distinct peaks of cytokinin activity were present in fractions 9 and 14 corresponding to the R_F values of 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine and 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine, respectively. The 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine peak was five times greater than the 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine peak.



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Ascending paper chromatography of the solids in fraction 9, estimated to contain 30 KE of riboside, in 10% ethanol, gave a single ultraviolet-absorbing band, located at R_F 0.55. The low-resolution mass spectrum of the solids eluted from this band compared favorably with a spectrum of synthetic 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine and showed abundant ions at *m/e* values of 335 (M^+), 246, 232, 203, 188, 160, 148, and 135, characteristic of 6-(3-methyl-2-

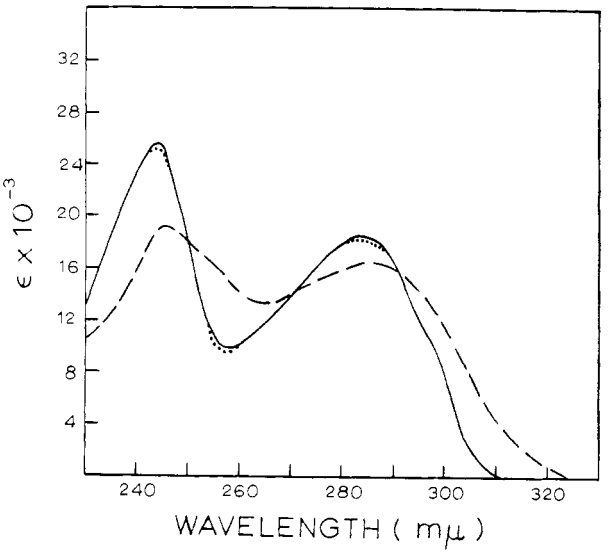

FIGURE 3: Ultraviolet absorption spectra of 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine in ethanol: - - -, pH 1; —, pH 7; and · · · ·, pH 12.

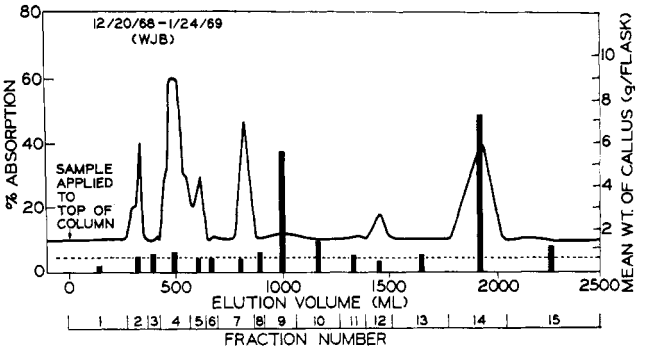

FIGURE 4: Chromatography of ethyl acetate soluble nucleosides from the *E. coli* sRNA hydrolysate. The riboside mixture from 1.0 g of sRNA was dissolved in 3.5 ml of 35% ethanol and applied to a Sephadex LH-20 column (152 g of Sephadex LH-20 equilibrated with the same solvent and packed in a glass column (120 × 4 cm)). The column was eluted with 35% ethanol; flow rate was 70 ml/hr; fractions of 10 ml were collected.

butenylamino)-9-β-D-ribofuranosylpurine (IV) (Biemann *et al.*, 1966; Robins *et al.*, 1967; Hall *et al.*, 1967; Leonard *et al.*, 1968; Hecht *et al.*, 1969). Mass spectrometry at high resolution identified the composition of the major peaks [found (calcd)]: 335.157 (335.1594), $C_{15}H_{21}N_5O_4$ $(M)^+$; 246.138 (246.1355), $C_{12}H_{16}N_5O$ $(M - 89)^+$; 232.125 (232.1198), $C_{11}H_{14}N_5O$ $(B + 30)^+$; 240.120 (204.1249), $C_{10}H_{14}N_5$; 203.112 (203.1171), $C_{10}H_{13}N_5$ $(B + 1)^+$; 202.109 (202.1093), $C_{10}H_{12}N_5$; 188.092 (188.0936), $C_9H_{10}N_5$; 160.062 (160.0623), $C_7H_6N_5$; 148.060 (148.0623), $C_6H_6N_5$; 135.055 (135.0545), $C_5H_5N_5$; and 119.037 (119.0358), $C_5H_3N_4$. The qualitative spectra of natural and synthetic material were comparable in acid, neutral, and basic solutions and their R_F values were the same in a number of systems.

Following ascending paper chromatography of the solids in fraction 14, estimated to contain 500 KE of riboside, in 20% aqueous ethanol, the ultraviolet-absorbing band at R_F 0.51 was eluted. The ultraviolet spectrum of this cytokinin active material was the same as that of 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (Figure 3) and re-identification by mass spectrometry was considered unnecessary.

The range of activities of synthetic preparations of the ribosides and their bases tested by procedures which avoided exposure to heat (Skoog *et al.*, 1967) are shown in Figure 5. As expected, the two ribosides are about equally active with minimal and maximal responses around 5×10^{-3} and 7×10^{-1} μM, respectively.

Discussion

The results indicate that on a molar basis the sRNA contains *ca.* 17 times more 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (0.05%) than 6-(3-methyl-2-butenylamino)-9-β-D-furanosylpurine (0.003%).

By comparison with our synthetic compound Harada *et al.* (1968) have shown that the minor nucleoside adjacent to the anticodon in *E. coli* $tRNA^{Tyr}$ is in fact 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-furanosylpurine. Moreover the modified adenosine (A*) in the position adjacent to the anticodon in $tRNA^{Tyr}$ from *E. coli* amber suppressor su^+_{111} has been reported by Goodman *et al.* (1968) to be derived from adenylic acid containing both sulfur and a methyl group, and this nucleoside therefore probably is 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine. Gefter and Russell (1969) tentatively identified adenosine, 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine, and 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine in three $tRNA^{Tyr}$ from a mutant *E. coli*. In tests of ribosome binding in response to UAG the 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine-containing $tRNA^{Tyr}$ was twice as efficient as the 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine-containing $tRNA^{Tyr}$ and seven times

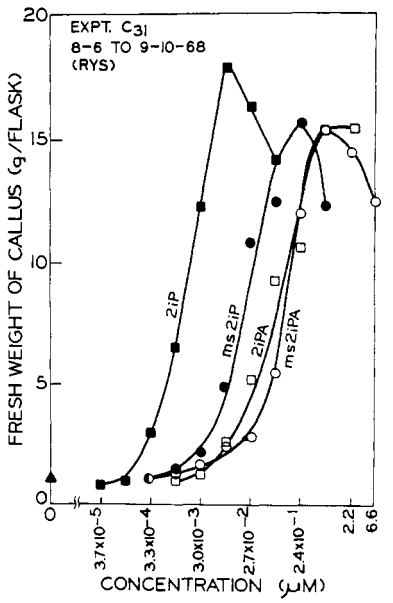

FIGURE 5: Effects of 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (ms2iPA) and 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (2iPA) and the corresponding bases (ms2iP and 2iP) on the fresh weight yields of tobacco callus.

more efficient than the $tRNA^{Tyr}$ containing the unmodified adenosine.

In the tobacco bioassay 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine and 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine have approximately equal cytokinin activity, while adenosine is completely inactive. It is interesting to note that, despite some distortion in the relative ratios, those structural features which enhance ribosomal binding efficiency also increase cytokinin activity, particularly in light of the hypothesis that the growth regulatory action depends upon the presence of a modified adenosine in tRNA.

The biosynthetic precursor of the isopentenyl chain in 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine is presumably mevalonic acid, as shown for the biosynthesis of 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine in the sRNA of *Lactobacillus acidophilus* (Peterkovsky, 1968; Fittler *et al.*, 1968a), yeast, and rat liver (Fittler *et al.*, 1968b). Harada *et al.* (1968) have suggested that the final step or steps in the biosynthesis of 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine involves methylthiation of 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine.

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