References and Notes

1. P. F. Scholander, H. T. Hammel, E. D. Bradstreet, E. A. Hemmingsen, *Science* **148**, 339 (1965); P. F. Scholander, *Proc. Nat. Acad. Sci. U.S.* **55**, 1407 (1966).
2. A. S. Crafts, H. B. Currier, C. R. Stocking, *Water in the Physiology of Plants* (Chronica Botanica, Waltham, Massachusetts, 1949).
3. J. H. Poynting, *Phil. Mag.* **12**, 32 (1881), quoted in G. N. Lewis and M. Randall, *Thermodynamics* (McGraw-Hill, New York, 1961).
4. The equation of Poynting reads: $dP_l \times \bar{V}_l = dP_g \times \bar{V}_g$, where P is pressure, l is liquid, g is gas phase, and $\bar{V}$ is molecular volume. At 24 atm, 20°C, we have: $24 \times 760 \times 18 = dP_g \times 24{,}000 \times 760/17.5$; $dP_g = 17.5/55.5 =$ 0.31 mm. At dP_l of 12 and 48 atm, dP_g would be, respectively, $17.5 \times 0.5/55.5$ and $17.5 \times 2/55.5$ mm. According to Raoult's law, dP_g is proportional to the mole fraction, and at 0.5, 1, and 2 moles would be $17.5 \times 0.5/56.0$, $17.5 \times 1/56.5$, and $17.5 \times 2/57.5$ mm.
5. G. Meschia and I. Stenikar, *J. Gen. Physiol.* **42**, 429 (1958).
6. H. B. Bull, *Physical Biochemistry* (Wiley, New York, 1951).

7. Supported by NSF grant GB-4259. I thank my colleagues on the "Billabong" expedition, which took the R.V. *Alpha Helix* to the Great Barrier Reefs in 1965, for stimulating discussions.

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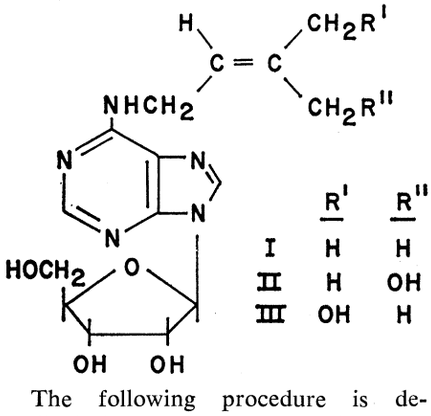
Cytokinins in the Soluble RNA of Plant Tissues

Abstract. *The cytokinin,* N^6-(Δ^2-*isopentenyl*)*adenosine occurs in the soluble RNA of yeast and mammalian tissue and has now been detected in plant soluble RNA. A hydroxylated derivative of this cytokinin 6-*(cis-*4-hydroxy-3-methylbut-2-enylamino)-9-*β*-D-*ribofuranosylpurine has also been identified as a constituent of plant soluble RNA.*

The sRNA (*1*) of yeast contains a nucleoside identified as N^6-(Δ^2-isopentenyl)adenosine (IPA) (*2–4*). This nucleoside, I, also occurs in the sRNA of several mammalian tissues (*2, 3*). It shows a selective toxicity toward various mammalian cell lines grown in culture (*5*) and possesses exceptionally high cytokinin activity (*2, 6*), that is, IPA promotes cell division, growth, and organ formation in cultured plant cells.

We have found IPA in the sRNA of spinach and garden peas. In addition, these sRNA samples and that of immature sweet corn kernels contain a hydroxylated derivative of IPA related to ribosylzeatin, III, identified as 6-(*cis*-4-hydroxy-3-methylbut-2-enylamino)-9-*β*-D-ribofuranosylpurine, II.



	R'	R''
I	H	H
II	H	OH
III	OH	H

The following procedure is described for the extraction of sRNA from corn. Frozen fresh sweet corn kernels (46 kg) were immersed in liquid nitrogen and, when thoroughly frozen at this temperature, were ground in a meat grinder. A constant stream of liquid nitrogen was poured over the corn during the grinding process. The frozen flour was warmed to 0°C and suspended in 100 liters of distilled water at 25°C, and the mixture was stirred with a high-speed stirrer until all the lumps of frozen material had disappeared (20 minutes). During this process, the solution was heated with a 1650-watt immersion heater to prevent the temperature from falling below 12°C. The suspension was allowed to stand 30 minutes at room temperature, and the supernatant was then siphoned off, filtered through muslin, and diluted with two volumes of 95 percent ethanol. The mixture was kept at 4°C for 24 hours and centrifuged at 10,000*g* for 10 minutes. The precipitate was dissolved immediately in 10 liters of 0.1*M* tris-HCl (*p*H 7.5), and the solution was centrifuged at 20,000*g* for 20 minutes. The following procedures were carried out at 4°C. The milky solution was run onto a DEAE-cellulose [Cl$^-$] column (10 by 30 cm). The column was washed with 0.1*M* tris-HCl (*p*H 7.5) until the effluent was clear and the OD at 260 mμ was less than 0.25. The material on the column was eluted with 1.0*M* sodium chloride in 0.1*M* tris-HCl (*p*H 7.5) as described by Holley (*7*). The fraction containing the RNA was diluted with three volumes of ethanol, and the mixture was kept for 24 hours at 4°C. The sRNA was recovered by centrifugation and dissolved in 500 ml of 0.1*M* tris-HCl (*p*H 7.5). The sRNA sample was chromatographed on a second column of DEAE-cellulose [Cl$^-$] (5.0 by 35 cm), with a sodium chloride gradient (0.0 → 1.0*M* NaCl) in 8 liters of 0.1*M* tris-HCl (*p*H 7.5). The fraction containing the sRNA was dialyzed for 24 hours against three changes of distilled water, and the sRNA was precipitated by addition of three volumes of cold ethanol. The recovered sRNA was washed with a mixture of ethanol and water (3:1), and then with ethanol; after being air-dried, it weighed 4.0 g. Spectrophotometric analysis (24 $OD_{260m\mu}$ units equals 1 mg) showed that the sample contained 2.0 g of RNA. This sample had a sedimentation constant $s_{20,w}$ of 2.9 (determined in the Spinco model E ultracentrifuge, schlieren optics).

The isolation of the nucleosides from sRNA was based on that devised for the isolation of IPA from yeast sRNA (*3*). In brief, the RNA was hydrolyzed by whole snake venom (*Crotalus adamanteus*) and bacterial alkaline phosphatase to its constituent nucleosides, and the nucleosides were separated by means of column partition chromatography.

The sRNA from 230 kg of corn kernels (10.05 g) was hydrolyzed, and the resultant nucleoside mixture was lyophilized and suspended in 75 ml of the lower phase of solvent E (*8*). The solution was stirred for 1 hour at room temperature, centrifuged, and mixed with 140 g of Celite 545. This mixture was packed into a glass column, 5.08 cm in diameter, containing 500 g of Celite 545 (Johns-Manville Co.) mixed with 230 ml of lower phase of solvent E (*9*). The elution pattern for this separation is shown in Fig. 1.

If any IPA were present in the sRNA hydrolyzate, it would be eluted in fraction 1 (*3*). Fraction 1 was concentrated in a vacuum to 0.5 ml, and the concentrate was chromatographed on Whatman No. 1 paper in solvent D. A sample of authentic IPA (*2*) was chromatographed on the same sheet. No IPA was detected even though as little as 10 to 15 μg was detectable. The eluate corresponding to fraction 2 was evaporated in a vacuum, and the residue was chromatographed on Whatman No. 3 MM paper in solvent D. Elution of the ultraviolet-absorbing band (R_F 0.73) with water yielded 305 $OD_{270m\mu}$ units. This material was chromatographed in solvent B, and two ultraviolet-absorbing bands were obtained, R_F 0.72 and 0.79. The slower-moving compound (182 $OD_{270m\mu}$ units) was identified as N^6,N^6-dimethyladenosine. The faster-moving compound

(109 $OD_{270m\mu}$ units) was chromatographically homogeneous and was identified subsequently as compound II. The R_F values of this and related compounds are presented in Table 1. The ultraviolet-absorption maxima (λ_{max}) for compound II are at *p*H 1.0, 265 mμ; at *p*H 7.0, 268 mμ; at *p*H 12.0, 268 mμ. These spectra are identical with those of IPA and the values for molar extinction are assumed to be the same (*3*) (Table 2).

A crystalline sample of this nucleoside was obtained as follows. A portion of the above sample (50 $OD_{270m\mu}$ units) was chromatographed on washed Whatman No. 1 paper in solvent E. The ultraviolet-absorbing band was eluted with water, and the eluate was treated with neutral charcoal, filtered, and evaporated in a stream of nitrogen to 0.20 ml. This solution was kept at 4°C for several days; during this time crystals appeared. The crystals were collected by centrifugation, washed with water, and dried at 65°C at reduced pressure. Crystals showed the following characteristics: m.p. 206°C; $[\alpha]_{546m\mu}^{27°} = -98°$ (*c* was 0.023, H_2O). Shaw *et al.* (*10*) report that 6-(*trans*-4-hydroxy-3-methylbut-2-enylamino)-9-β-D-ribofuranosylpurine (ribosylzeatin), III, melts at 180° to 182°C. A partial NMR spectrum (100 Mc) obtained on compound II in D_2O (*11*) showed an unsplit peak at $\delta = 2.36$ which corresponds to a methyl group adjacent to a double bond.

Table 1. Paper chromatography (*8*). Whatman No. 1 paper was used.

Nucleoside	Solvent system				
	A	B	C	D	E
N^6-(Δ^2-isopentenyl)adenosine	0.78	0.86	0.75	0.83	0.83
Compound II	.69	.79	.63	.72	.57
Ribosylzeatin, III	.67	.76	.60	.73	.53
N^6,N^6-Dimethyladenosine	.68	.72	.57	.71	.62

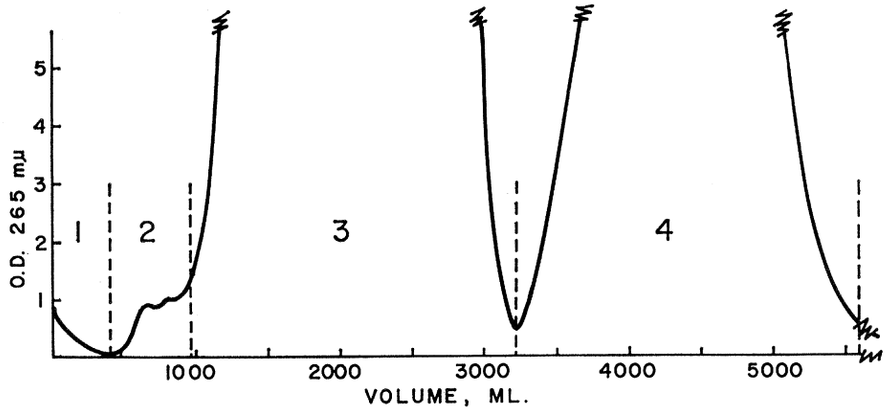


Fig. 1. Partition chromatography of an enzymic digest of 10.05 g of corn sRNA on Celite 545 (first part of elution only), solvent E, flow rate 600 ml/hr.

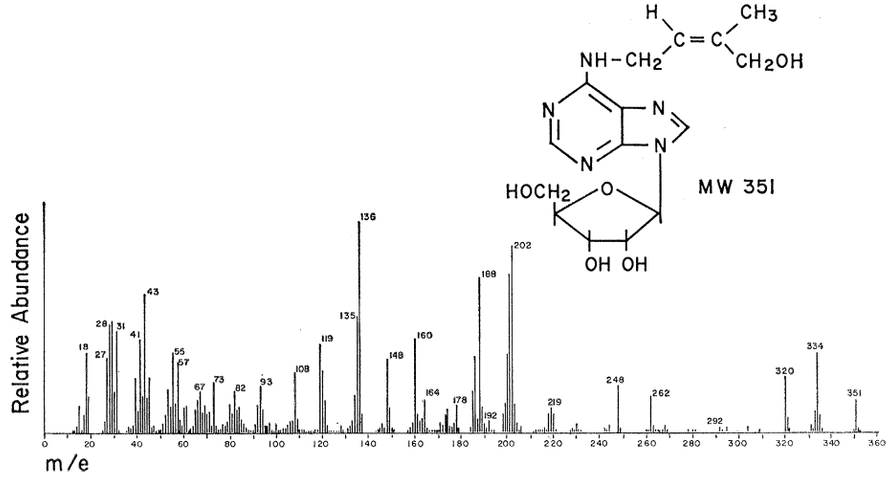
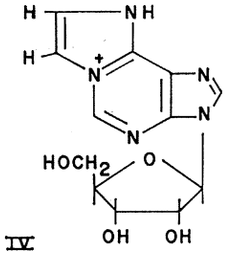


Fig. 2. Mass spectrum of the crystalline sample of nucleoside isolated from corn sRNA. Spectrum obtained on a Hitachi Perkin-Elmer RMU-6D spectrometer, direct inlet, 150°C, 70 ev.

The mass spectrum of compound II, obtained on the crystalline sample, is shown in Fig. 2. The salient features are as follows. (i) With respect to the side chain, there are peaks at *m/e* of 336 (−15), loss of CH_3; 334 (−17), loss of OH; 333 (−18), loss of H_2O; 292 (−59), loss of $C(CH_3)CH_2OH$ and H; 219 (−132), free base of structure II. The peak at 292 deserves comment since this peak, characteristic of the mass spectrum of N^6-(Δ^2-isopentenyl)adenosine, I (*3*), is probably due to the ion, structure IV.



IV

Letham *et al.* (*12*) suggested a similar structure for a prominent peak which appears in the spectrum of zeatin at *m/e* 160. The characteristic fragmentation of the side chain is repeated, starting from the free base (*m/e* 219). (ii) The parent nucleoside, adenosine, can be deduced from the overall fragmentation pattern. The peak at *m/e* 135 is due to adenine. Fragmentation of the ribose portion of the nucleoside gives rise to two peaks at *m/e* 262 and 248 which correspond to the *M*-89 and *M*-103 fragments reported by Biemann and McCloskey (*13*) to be characteristic of ribonucleosides. These characteristic *M*-89 and *M*-103 peaks also occur in the mass spectrum of IPA (*3*). The prominent peaks at *m/e* 178 (*M*-89) and 164 (*M*-103) in the spectrum of adenosine (*13*) also appear in the spectrum of II.

We obtained the mass spectrum of ribosylzeatin (*10*), and it is identical with that of the isolated compound II, except for the following points. A peak in the spectrum of ribosylzeatin at *m/e* of 192 is barely perceptible while peaks at *m/e* 228 and 331 are five times more intense

Table 2. Amount of compound II and IPA isolated from sRNA.

Source of sRNA	sRNA taken (g)	Yields	
		Compound II (mg)	IPA (mg)
Sweet corn kernels	10.05	1.9	0
Garden peas	5.24	0.27	0.13
Spinach	2.42	.25	.34

than those in the spectrum of compound II.

The sRNA was extracted from 45 kg of frozen spinach and 45 kg of frozen garden peas by a procedure identical with that used for the corn (Table 2). These samples were hydrolyzed and fractionated on partition columns, and elution patterns similar to the one shown in Fig. 1 were obtained.

Fraction 1 of the pea and of spinach sRNA eluates was chromatographed in solvent E, and pure IPA was obtained in each case. The isolated nucleoside was identified by its ultraviolet spectrum and cochromatography with a synthetic sample (*2*) of IPA. Compound II was isolated from the fraction 2 of each sample of sRNA and identified by ultraviolet spectroscopy and by chromatographic comparison with the nuceloside obtained from corn sRNA. The yields are recorded in Table 2.

On the basis of the procedure used and the sedimentation coefficients obtained, the RNA samples appear to consist, principally, of sRNA. These samples, however, could contain some degraded ribosomal RNA, and if one makes the assumption that compound II occurs only in the sRNA then the values recorded in Table 1 may be lower than actual amounts of this nucleoside in the sRNA. In mammalian tissue, IPA occurs only in the sRNA fraction (*3*).

The evidence for assignment of structure II to the nucleoside isolated from plant sRNA can be summarized as follows. (i) The ultraviolet absorption spectra are those of an N^6-(alkyl substituted)adenosine. (ii) The mass spectrum conforms to structure II. (iii) The NMR spectrum shows that compound II contains a methyl group attached to a vinyl carbon which does not have an attached proton. (iv) The specific optical rotation of II is similar to that of IPA (*3*) and is identical with that of a synthetic sample of compound III measured in the same instrument under the identical conditions. Therefore II is the β anomer. (v) In a comparison with ribosylzeatin, III, the melting point of II is 206°C compared to 181°C for III (*10*). On paper chromatography, compound II migrates slightly faster than III in several systems (Table 1).

On the basis of these data we conclude that this nucleoside isolated from plant sRNA consists of a 4-hydroxy-3-methylbut-2-enly side chain attached to the N^6 position of adenosine. Since the isolated nucleoside is not identical with the synthetic compound III we have assigned the *cis* configuration, structure II, to this component of sRNA.

Letham *et al.* (*12*) isolated from an extract of immature corn kernels a purine base identified as 6-(*trans*-4-hydroxy-3-methylbut-2-enylamino)purine, zeatin (*10*) (the base of structure III). This compound also has been obtained as its nucleoside and nucleotide (*14*), and the evidence suggests that these compounds exist in the corn kernels in an unbound form.

Compound II represents the second isoprenoid adenosine to be identified in sRNA. A third such derivative has been detected in the sRNA of yeast and mammalin tissue (*15*). These findings indicate that a series of isoprenoid nucleosides occurs in the sRNA although all members of this series do not necessarily occur in the sRNA of a given species.

ROSS H. HALL, LILLA CSONKA
HAROLD DAVID, BARRY MCLENNAN
Department of Experimental Therapeutics, Roswell Park Memorial Institute, Buffalo, New York 14203

References and Notes

1. Abreviations used are as follows: sRNA, soluble RNA; IPA, N^6-(Δ^2-isopentenyl)adenosine; DEAE, diethylaminoethyl; OD, optical density; *M*, molecular ion; *m/e*, mass-to-charge ratio; NMR, nuclear magnetic resonance spectroscopy; δ, parts per million; *c*, g/100 ml.
2. R. H. Hall, M. J. Robins, L. Stasiuk, R. Thedford, *J. Amer. Chem. Soc.* **88**, 2614 (1966).
3. M. J. Robins, R. H. Hall, R. Thedford, *Biochemistry*, in press.
4. H. G. Zachau, D. Dütting, H. Feldman, *Angew. Chem.* **78**, 392 (1966); K. Biemann, S. Tsunakawa, T. Sonnenbichler, H. Feldman, D. Dütting, H. G. Zachau, *ibid.*, p. 600.
5. J. T. Grace, Jr., M. T. Hakala, R. H. Hall, J. Blakeslee, *Proc. Amer. Ass. Cancer Res.*, in press.
6. H. Q. Hamzi and F. Skoog, *Proc. Nat. Acad. Sci. U.S.* **51**, 76 (1964).
7. R. W. Holley, *Biochem. Biophys. Res. Commun.* **10**, 186 (1963).
8. The solvent systems used were as follows. Solvent A: mixture of 1-butanol, water, and concentrated ammonium hydroxide (86:14:5); solvent B: a mixture of 1-butanol, glacial acetic acid, and water (5:2:3); solvent C: a mixture of 2-propanol, concentrated hydrochloric acid, and water (680:170:144); solvent D: a mixture of 2-propanol, water, and concentrated ammonium hydroxide (7:2:1); solvent E: a mixture of ethyl acetate, 1-propanol, and water (4:1:2).
9. For details on preparing such columns, see R. H. Hall, in *Methods Enzymol.*, in press.
10. G. Shaw, B. M. Smallwood, D. V. Wilson, *J. Chem. Soc.* **1966**(c) 921 (1966).
11. NMR spectrum by W. Jankowski of Varian Associates.
12. D. S. Letham, J. S. Shannon, I. R. McDonald, *Proc. Chem. Soc.* **1964**, 230 (1964).
13. K. Biemann and J. A. McCloskey, *J. Amer. Chem. Soc.* **84**, 2005 (1962).
14. D. S. Letham, *Life Sci.* **5**, 551, 1999 (1966); C. O. Miller, *Proc. Nat. Acad. Sci. U.S.* **54**, 1052 (1965).
15. R. H. Hall, B. Hacker, L. Kline, *Fed. Proc.*, in press.
16. Supported by grants from NIH (CA-04640) and the American Cancer Society. We thank Sister M. E. Caley, OSF, for assistance and J. Mozeiko, T. Mulcahy, and L. Stasiuk for technical help. We thank Dr. Shaw for a sample of synthetic ribosylzeatin.

6 February 1967

Apple Fruit-Set: Evidence for a Specific Role of Seeds

Abstract. *An extract of immature Wealthy apple seeds, containing substances with gibberellin activity, was applied to unpollinated blossoms of the same variety, resulting in the production of mature seedless fruits. The implications with respect to the normal process of fruit-set are discussed.*

Since the work of Gustafson (*1*), parthenocarpy has been induced in many species with synthetic auxins. However, attempts to induce parthenocarpy in apple with auxins were relatively unsuccessful (*2*). Success awaited the discovery and use of the gibberellins, Luckwill (*3*) being the first to note the effects of gibberellic acid (GA_3) in inducing parthenocarpy in apples. Endogenous gibberellins have been found in apple seeds (*4*), and two of these gibberellins have been identified as GA_4 and GA_7 (*5*). My study was initiated to determine whether substances with gibberellin activity, extracted from immature apple seeds, were capable of inducing fruit-set in unpollinated apple flowers.

The Wealthy variety was chosen as experimental material because of its parthenocarpic tendency (*6*) and the marked response of unpollinated Wealthy flowers to gibberellins (*6*, *7*). Fruits were picked from mature trees 1–9 July 1965 (6 to 7 weeks after full bloom and shortly after cessation of the June drop). The diameter of the fruit at this time was 30 to 35 mm, the length of the seed was 8.8 mm,