Howell, D. S., Pita, J. C., Marquez, J. F., and Madruga, J. E. (1968), *J. Clin. Invest. 47*, 1121.
Jethi, R. K., Inlow, C. W., and Wadkins, C. L. (1970), *Calcif. Tissue Res. 6*, 81.
Jethi, R. K., and Wadkins, C. L. (1971), *Calcif. Tiss. Res.* (in press).
Katz, E. P. (1969), *Biochim. Biophys. Acta 194*, 121.
Posner, A. S. (1969), *Physiol. Rev. 49*, 760.
Samachson, J. (1968), *Nature (London) 218*, 1262.
Solomons, C. C., and Neuman, W. F. (1960), *J. Biol. Chem. 235*, 2502.
Thomas, W. C., Jr., and Tomita, A. (1967), *Amer. J. Pathol. 51*, 621.
Vatassery, G. T., Armstrong, W. D., and Singer, L. (1970), *Calcif. Tiss. Res. 5*, 183.
Wadkins, C. L. (1968), *Calcif. Tiss. Res. 2*, 214.

Isolation and Identification of Cytokinins Located in the Transfer Ribonucleic Acid of Tobacco Callus Grown in the Presence of 6-Benzylaminopurine*

W. J. Burrows,† F. Skoog,† and N. J. Leonard‡

ABSTRACT: From the tRNA of tobacco callus grown in the presence of the synthetic cytokinin 6-benzylaminopurine, four cytokinin-active ribonucleosides have been isolated. Of these, the two ribonucleosides present in largest amount were identified conclusively by their chromatographic properties, ultraviolet spectra, and low- and high-resolution mass spectra as the natural tRNA components 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine and 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine. A third ribonucleoside, present in smaller amount, was indicated as another natural cytokinin, 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine, on the basis of the data obtainable.

The fourth cytokinin-active ribonucleoside was identified conclusively as 6-benzylamino-9-β-D-ribofuranosylpurine, normally not a natural tRNA component. Thus, we have shown that in the presence of an unnatural cytokinin, 6-benzylaminopurine, tobacco callus tissue generates natural cytokinin-active tRNA components and in addition the related *unnatural* ribonucleoside.

Evidence for and against the incorporation of the cytokinin 6-benzylaminopurine (**1**) into tRNA has been reported (Fox, 1966; Fox and Chen, 1967; Kende and Tavares, 1968; Richmond *et al.*, 1970). It was argued that this molecule must be incorporated into tRNA if cytokinins exert their growth regulatory action as constituents of tRNA. The problem of incorporation is only part of a broader problem which had not been attacked experimentally until now, namely, the question of what cytokinin-active ribonucleosides, natural or unnatural, are actually present in the tRNA of cytokinin-dependent plant tissue grown on a defined basal medium supplemented with a synthetic cytokinin. In order to answer this question, tobacco callus dependent on exogenous cytokinin was grown on a medium with added 6-benzylaminopurine (**1**), and the tRNA was isolated, purified, and hydrolyzed to identify the constituent cytokinins.[1] We are now able to report the isolation and identification of four cytokinin-active ribonucleosides in the tRNA of cytokinin-dependent tobacco callus supplied with 6-benzylaminopurine.

Experimental Section

Extraction of tRNA. Tobacco callus which had previously been grown on basal medium (Linsmaier and Skoog, 1965) supplemented with 1 μg/l. of 6-benzylaminopurine (**1**), BAP[2] (Shell development Company SD4901) was propagated on the same medium containing 10 μg/l. or 100 μg/l. of BAP. The tissue was harvested after approximately 21-days growth and stored at −20°. The frozen tissue (47 kg) was homogenized in 0.3 volume of 0.4 M Tris-HCl buffer (pH 7.3) and 0.5 vol-

* From the Institute of Plant Development, Birge Hall, University of Wisconsin, Madison, Wisconsin 53706 (W. J. B. and F. S.), and the School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801 (N. J. L.). *Received December 18, 1970.* Supported at the University of Wisconsin by National Science Foundation Research Grants GB-6994X and GB-25812 and by the Research Committee of the Graduate School with funds from the Wisconsin Alumni Research Foundation. Supported at the University of Illinois by National Institutes of Health Research Grants GM-05829, GM-16864, and CA-11388. These results were first presented (by F. S.) at the Symposium on Control Mechanisms of Growth and Differentiation, Society of Experimental Biology, Sept 1970.

† Present address: Shell Research Limited, Woodstock Agricultural Research Centre, Sittingbourne, Kent, England.

‡ To whom to address correspondence.

[1] We were encouraged in undertaking the cultivation of tobacco callus and preparation of tRNA on the large scale required for the identification of cytokinins by the results of preliminary experiments by P. K. Evans, N. Murai, and J. J. McDonald in these laboratories relating to the possible incorporation of 6-(3-methyl-2-butenylamino)purine, double labeled with ^{3}H and ^{14}C, and of 6-benzylaminopurine into tRNA. These experiments indicated the presence of cytokinin activity in tRNA ribonucleosides of tobacco callus cultured on media with a single exogenous cytokinin, and, although they failed to give conclusive evidence for the incorporation of the exogenous cytokinin into tRNA, this possibility was not excluded.

[2] Abbreviations used are: BAP, 6-benzylaminopurine (**1**): BAPA, 6-benzylamino-9-β-D-ribofuranosylpurine (**5**).

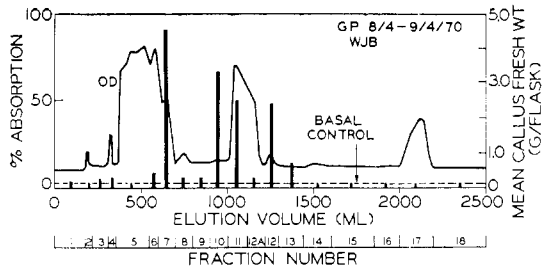


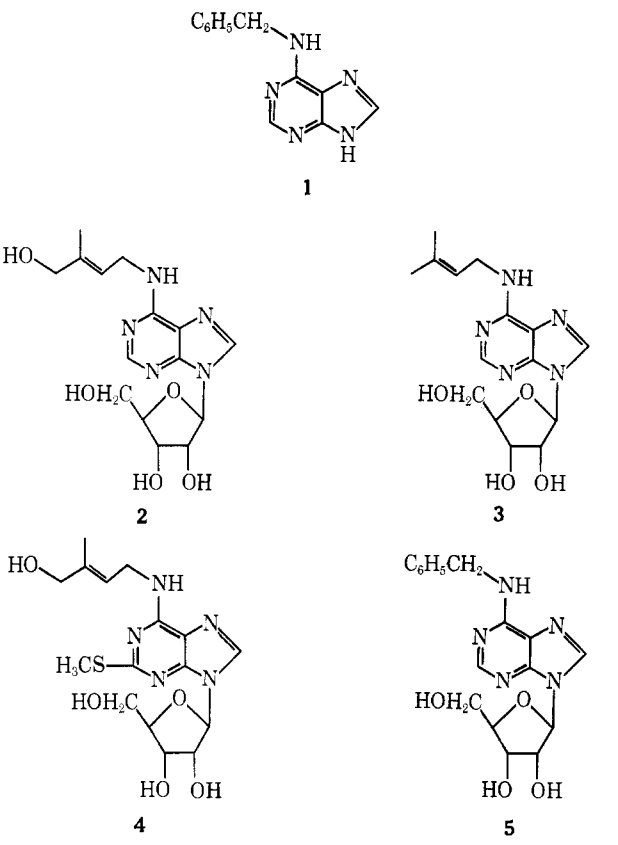
FIGURE 1: Cytokinin activity in the elution profile of the ethyl acetate soluble ribonucleosides from tobacco callus tRNA hydrolysate. The ribonucleoside mixture from 54,340 A_{260} units was dissolved in 5.5 ml of 35% aqueous ethanol and applied to a Sephadex LH-20 column (150 g, 52 × 3.65 cm) equilibrated with the same solvent. The column was eluted with 35% aqueous ethanol. The flow rate was 70 ml/hr, and fractions of 10 ml were collected. Cytokinin activity (mean fresh weight of tobacco callus) is represented by vertical bars.

ume of buffer-saturated phenol. The aqueous phase was reextracted with buffer-saturated phenol (0.5 volume/volume of supernatant), adjusted to 0.1 M NaCl, and the nucleic acids were precipitated with 2.5 volumes of cold 95% ethanol. The precipitate was then extracted four times with cold 3 M aqueous sodium chloride solution (Kirby, 1965) (100 ml of 3 M NaCl/4 kg of callus). The combined supernatant was diluted twice with distilled water, and the nucleic acids were precipitated with 2.5 volumes of cold 95% ethanol. The precipitate was washed twice with cold 95% ethanol, dissolved in 0.1 M Tris-HCl buffer (pH 7.3), and applied in the cold to a DEAE-cellulose column which had been equilibrated with the same buffer (1-ml column volume/2 kg of callus). The column was washed with 30 volumes of 0.1 M Tris-HCl buffer (pH 7.3) containing 0.2 M NaCl, and the tRNA was eluted with the same buffer containing 1.0 M NaCl (Brunngraber, 1962). The tRNA was precipitated from the eluate with 2.5 volumes of cold 95% ethanol and washed twice with cold 90% ethanol. Polyacrylamide gel electrophoresis indicated that the preparation referred to as tRNA corresponds mainly to 4S RNA. No high molecular weight RNA or DNA was detected.

The tRNA (54,340 A_{260} units) was dialyzed against distilled water for 12 hr at 4° and hydrolyzed enzymatically (Burrows *et al.*, 1969). The ribonucleoside mixture was extracted and fractionated by the procedure of Armstrong *et al.* (1969a); *i.e.*, the lyophilized hydrolysate was extracted six times with 100-ml volumes of the upper phase of a mixture of ethyl acetate–water (5:1, v/v). The pooled extracts were lyophilized and the solid material was dissolved in 5.5 ml of 35% aqueous ethanol and fractionated on a Sephadex LH-20 column (150g, 52 × 2.65 cm) which had been equilibrated with the same solvent. The eluates from all columns were monitored at 265 nm. The optical density base line in Figures 1–5 has been arbitrarily raised from zero. The fractions were further purified by chromatography on acid-washed Whatman No. 1 paper. For determination of cytokinin activity in fractions from a column, or in the 95% ethanol eluates from the paper chromatograms, samples of the eluates were concentrated, dissolved in 5.0 ml of distilled water, hydrolyzed with acid by the standard procedure (Armstrong *et al.*, 1969b), and incorporated into the bioassay medium.

Identification of Cytokinins. Each of the cytokinin-active fractions was chromatographed further and finally concentrated to dryness. The ultraviolet spectrum was determined in ethanol solution. The low-resolution mass spectrum was obtained, and the composition of the molecular ion was measured by peak matching with the high-resolution mass spectrometer. Electronic absorption spectra were recorded on a Cary Model 15 spectrophotometer, and the mass spectra were determined with the Varian–MAT CH-5-3 and SM-1B mass spectrometers.

CHART I



Results

Isolation and Identification of Cytokinins. The elution profile of the ribonucleosides (Figure 1), as followed by ultraviolet absorbance, was similar to that reported for wheat germ tRNA (Burrows *et al.*, 1970). Bioassay of 4% of the eluate from each fraction revealed four peaks of cytokinin activity, fractions 7, 10, 11, and 12 corresponding to the elution volumes of 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**2**) (see Chart I), 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**3**), 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**4**), and 6-benzylamino-9-β-D-ribofuranosylpurine (**5**) under the specific column conditions.

FROM FRACTION 7. The combined solid material from fraction 7 was dissolved in 2.0 ml of distilled water and fractionated on a Sephadex G-10 column (150g, 65.6 × 2.4 cm) which had been equilibrated with the same solvent. The cytokinin-active factor eluted from this column (Figure 2) with an elution volume corresponding to that of 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**2**). The eluates in tubes 68–75 were combined, lyophilized, and purified by ascending chromatography on paper in 20% aqueous ethanol. The ultraviolet-absorbing spot at R_F 0.60 was eluted, and the eluate was concentrated to dryness. The solid residue, when

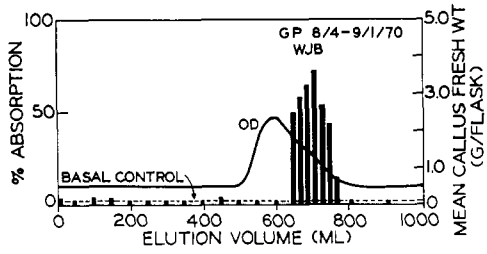


FIGURE 2: Distribution of cytokinin activity in the elution profile of the solid material in fraction 7 (Figure 1). The solid material from this fraction was dissolved in 2.0 ml of distilled water and applied to a Sephadex G-10 column (150 g, 65.5 × 2.4 cm) equilibrated with the same solvent. The column was eluted with distilled water. The flow rate was 110 ml/hr, and fractions of 10 ml were collected.

dissolved in ethanol, exhibited an ultraviolet maximum at 267 nm typical of an N^6-substituted adenosine. The low-resolution mass spectrum at 70 eV, probe temperature 180°, gave prominent peaks at *m/e* 351, 334, 320, 262, 248, 220, 219, 218, 204, 203, 202, 201, 200, 199, 198, 188, 178, 174, 160, 148, 136, and 135, *inter alia*, typical of 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**2**, side-chain stereochemistry not established) (Hall *et al.*, 1967; Hecht *et al.*, 1970). The corresponding composition of the molecular ion was confirmed as $C_{15}H_{21}N_5O_5$ (calcd 351.1543; found: 351.1542) by peak matching on the high-resolution mass spectrometer.

FROM FRACTION 10. The combined eluate was lyophilized, and the solid product was dissolved in 2.0 ml of distilled water and purified by chromatography on a Sephadex LH-20 column (40g, 31.5 × 2.4 cm), previously equilibrated with the same solvent. The solid material in fraction 3′ (Figure 3) eluting with a volume identical with that of 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (N^6-(Δ^2-isopentenyl)adenosine) (**3**) was chromatographed ascending on paper in 20% aqueous ethanol, the ultraviolet-absorbing band at R_F 0.63 was eluted, and the eluate was evaporated to dryness. The ultraviolet spectrum of this fraction, λ_{max}^{EtOH} 267 nm, indicated that the N^6-substituted adenosine present was in smaller amount than that in fraction 7. The low-resolution mass spectrum at 70 eV, probe temperature 185°, included a series of prominent peaks at *m/e* 335, 320, 305, 292, 246, 232, 205, 204, 203, 188, 160, 136, and 135, *inter alia*, typical of 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**3**) (Zachau *et al.*, 1966; Biemann *et al.*, 1966; Robins *et al.*, 1967; Madison and Kung, 1967; Madison *et al.*, 1967; Staehelin *et al.*, 1968; Hecht *et al.*, 1969b, 1970; Burrows *et al.*, 1969). The composition of the molecular ion was confirmed as $C_{15}H_{21}N_5O_4$ (calcd: 335.1594; found: 335.1592) by peak matching at high resolution.

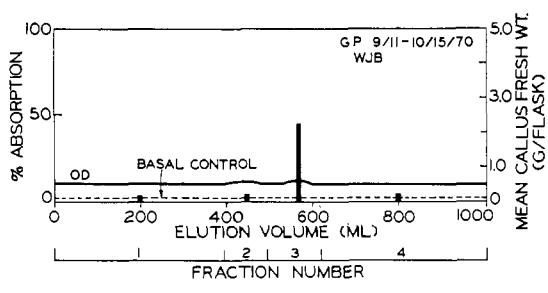


FIGURE 3: Cytokinin distribution in the elution profile of the solid material in fraction 10 (Figure 1). The solid was dissolved in 2.0 ml of distilled water and chromatographed on a Sephadex LH-20 column (40 g, 31.5 × 2.4 cm) equilibrated with the same solvent. The column was eluted with distilled water. The flow rate was 30 ml/hr, and fractions of 10 ml were collected.

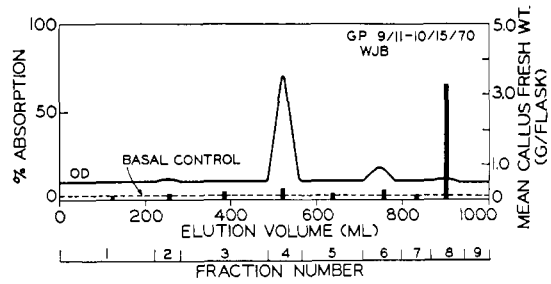


FIGURE 4: Cytokinin distribution in the elution profile of the solid material in fraction 11 (Figure 1). Conditions as in Figure 3.

FROM FRACTION 11. The combined solid in this fraction was dissolved in 2.0 ml of distilled water and fractionated on a Sephadex LH-20 column (40g, 31.5 × 2.4 cm) previously equilibrated with the same solvent. The cytokinin in fraction 8 (Figure 4) eluted with a volume identical with that of 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**4**). The solid in this fraction was further purified by ascending paper chromatography in 20% aqueous ethanol, the ultraviolet-absorbing spot at R_F 0.70 was eluted, and the eluate was evaporated to dryness. The ultraviolet spectrum, which showed λ_{min}^{EtOH} at 258 nm and a composite peak at longer wavelength, with apparent maximum at 278 nm, was suggestive of an N^6-substituted-2-methylthioadenosine (Burrows *et al.*, 1968–1970). The low-resolution mass spectrum at 70 eV, probe temperature 220°, included a series of prominent peaks at *m/e* 397, 382, 341, 339, 313, 295, 267, 265, 264, 250, 248, 247, 246, 239, (?), 234, 232, 206, 200, 194, 182, 181, 165, *inter alia*, as for 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**4**, side-chain stereochemistry not established) (Hecht *et al.*, 1969a; Burrows *et al.*, 1970). The structure was not fully established in the present case by high-resolution mass spectrometry because the mass of the molecular ion (calcd for $C_{16}H_{23}N_5O_5S$: 397.1420) was not found.

FROM FRACTION 12. The combined eluate was lyophilized, and the solid was dissolved in 2.0 ml of distilled water and chromatographed on Sephadex LH-20 equilibrated with the same solvent. The cytokinin activity, fraction 4′ (Figure 5), eluted with an elution volume similar to that of 6-benzylamino-9-β-D-ribofuranosylpurine. The solid was further purified by ascending paper chromatography in 20% aqueous ethanol, the ultraviolet-absorbing spot at R_F 0.74 was eluted,

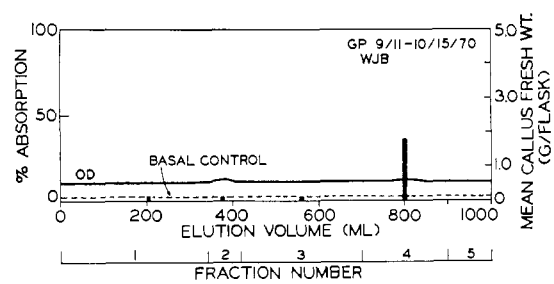


FIGURE 5: Cytokinin distribution in the elution profile of the solid in fraction 12 (Figure 1). Conditions as in Figure 3.

TABLE I: Major Fragment Ions in Mass Spectra.[a]

Fraction 12[b]		BAPA (5)[c]		BAP (1)[d]		Fragment Ions	
m/e	% Total	*m/e*	% Total	*m/e*	% Total	Composition	Type
		358	0.67			$C_{17}H_{20}N_5O_4$	$(M + 1)^+$
357	0.50	375	1.60			$C_{17}H_{19}N_5O_4$	M^+ (BAPA)$^+$
		269	0.68			$C_{14}H_{15}N_5O_4$	$(M + 1 - C_3H_5O_3)^+$[h]
268	0.50	268	0.95			$C_{14}H_{14}N_5O$	$(M - C_3H_5O_3)^+$
		267	0.46			$C_{14}H_{13}N_5O$	$(M - 1 - C_3H_5O_3)^+$
		255	0.72			$C_{13}H_{13}N_5O$	$(M + 1 - C_4H_7O_3)^+$[h]
254	0.66	254	1.95			$C_{13}H_{12}N_5O$	$(M - C_4H_7O_3)^+$
266	0.91	226	2.95	226	1.96	$C_{12}H_{12}N_5$	$(BAP + 1)^+$
225	4.16	225	9.19[e]	225	10.85	$C_{12}H_{11}N_5$	$(BAP)^+$ $(B + 1)^+$[h]
224	1.95	224	3.34	224	4.91	$C_{12}H_{10}N_5$	$(BAP - 1)^+$
		223	0.65	223	0.75	$C_{12}H_9N_5$	$(BAP - H_2)^+$
209	0.39	209	0.76	209	0.54	$C_{12}H_9N_4$	$(BAP - NH_2)^+$
197	0.49	197	0.70	197	0.61	$C_{11}H_9N_4$	$(BAP - 1 - HCN)^+$
				170	0.64	$C_{10}H_8N_3$	$(C_{11}H_9N_4 - HCN)^+$
169	0.44			169	0.73	$C_{10}H_7N_3$	$(C_{12}H_9N_5 - 2HCN)^+$
148	0.44	148	0.64	148	1.68	$C_6H_6N_5$	$(C_5H_4N_5CH_2)^+$
142	0.42			142	0.73	$C_9H_6N_2$	$(C_{12}H_9N_5 - 3HCN)^+$
135	0.49	135	0.63			$C_5H_5N_5$	$(Ade)^+$
121	0.58	121	1.10			$C_7N_5N_4$	$(Ade + 1 - NH)^+$
120	1.16	120	2.00	120	2.25	$C_5H_4N_4$	$(Ade + 1 - NH_2)^+$
119	0.54	119	0.78	119	1.79	$C_5H_3N_4$	$(Ade - NH_2)^+$
106	5.92	106	7.16[f]	106	10.95	C_7H_8N	$(C_7H_6NH_2)^+$
91	3.80	91	7.20[g]	91	7.52	C_7H_7	$(C_7H_7)^+$

[a] Determined at 70 eV on the Varian–MAT CH-5-3 mass spectrometer. Compositions of the ions were verified by peak matching on the SM-1B mass spectrometer. [b] Probe temperature 170°. Peaks recorded for $\geq 0.33\%$ total abundance above *m/e* 160 and selected peaks below 160. [c] Probe temperature 160°. Peaks recorded for $\geq 0.33\%$ total abundance above *m/e* 119 and $>1\%$ below 119. [d] Probe temperature 70°. Peaks recorded for $\geq 54\%$ total abundance above *m/e* 110 and $>1\%$ below 110. [e] Base peak. [f] 78% of base peak. [g] 78% of base peak. [h] Designation of fragment ions by Biemann and McCloskey (1962).

and the eluate was evaporated to dryness. The ultraviolet spectrum showed λ_{max}^{EtOH} 268 nm, and the low-resolution mass spectrum at 70 eV, probe temperature 170°, included a set of prominent peaks at *m/e* 357, 268, 255, 254, 226, 225 (70% of base peak), 224, 209, 198, 197, 148, 142, 135, 121, 120, 119, 116, 106 (base peak), and 91 (64% of base peak) and at *m/2e* 112.5 and 111.5, parallel of those of authentic 6-benzylamino-9-β-D-ribofuranosylpurine (**5**) (Table I). The composition of the molecular ion was confirmed as $C_{17}H_{19}N_5O_4$ (calcd: 357.1440; found: 357.1443) by peak matching on the high resolution mass spectrometer. The structure can be assigned unequivocally as **5**. The low-resolution mass spectrum of recrystallized, authentic 6-benzylamino-9-β-D-ribofuranosylpurine (Calbiochem 65061) served as a standard. Where there was some doubt as to the elemental composition assignable to the fragment ions, these were checked by peak matching on the high resolution mass spectrometer (Table I). 6-Benzylaminopurine (**1**) used in the growth experiments was checked for purity and for the fragment ions due to the base moiety of **5**. The low-resolution *m/e* figures have also been related to the formulas in Table I by peak matching on the high-resolution mass spectrometer. These results have extended our knowledge of the composition and type of ion resulting from electron bombardment of the synthetic cytokinin **1** (Budzikiewicz *et al.*, 1967).

Discussion

The present mass spectrometric results show conclusively that the ribonucleoside of 6-benzylaminopurine is present in tRNA when cytokinin-dependent tobacco callus is cultured on a medium supplied with this base as the only exogenous source of cytokinin. (Prior to this work, chromatographic evidence had been obtained for the presence of N^6-benzyladenosine in RNA of tobacco callus grown under these conditions (Fox, 1966; Fox and Chen, 1968).) Of the total amount of the four cytokinins isolated, BAPA was the second least abundant. On the basis of available evidence of methylation of tRNAs (see Borek and Srinivasan, 1960) and incorporation of mevalonate to form isopentenyladenosine in *Lactobacillus* tRNA (Peterkofsky, 1968; Fittler *et al.*, 1968) and in tobacco callus tRNA (Chen and Hall, 1969), the biosynthesis of the benzylaminopurine ribonucleoside (**5**) may involve removal of the benzyl group from the BAP and benzylation of the appropriate adenosine in preformed tRNA rather than incorporation of **5** intact in a nonrandom manner or by a repair mechanism. Repeated attempts to answer the question of incorporation *in toto* of the endogenous cytokinin 6-(3-methyl-2-butenylamino)purine, doubly labeled with ^3H and ^{14}C in different proportions, have been unsuccessful to date (footnote, p 5). Whether or not the benzyladenosine is adjacent to

the anticodon in specific tRNA species, as would be expected from analogy with the known position of isopentenyladenosine in certain tRNA species in orher organisms, or occurs at random and is without physiological significance, remains to be determined.

Enzymes which catalyze the *in vitro* transfer of the Δ^2-isopentenyl group of Δ^2-isopentenyl pyrophosphate to adenosine of "suitably treated" tRNA or of "modification-deficient" tRNA (*e.g.*, from *Mycoplasma* sp. (Kid)) have been reported (Fittler *et al.*, 1968; Kline *et al.*, 1969; Bartz *et al.*, 1970). Such an enzyme may transfer, less specifically, the benzyl moiety through a suitable intermediate. Experiments of this type are projected.

The ^{14}C label of mevalonic acid, long recognized as the precursor of biological isoprene units through isopentenyl pyrophosphate, appears in 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**3**) isolated from tobacco callus tRNA (Chen and Hall, 1969). Mevalonic acid has been found not to substitute, however, for auxin or cytokinin requirement in KX tobacco tissue, while it does markedly increase the rate of growth when incorporated into the normal media (McChesney, 1970).

Since no one had previously approached the question of what "natural" cytokinin-active ribonucleosides are actually present in the tRNA of dependent tobacco tissue grown on a medium supplemented with a *synthetic* cytokinin, the isolation and identification of the other active tRNA components produced in the present experiment was regarded as of special interest. We were able to characterize the "natural" cytokinin-active ribonucleosides isolated from the tRNA of tobacco callus grown on medium supplemented with 6-benzylaminopurine as 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**2**), 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**3**) and 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**4**). The last of this group (**4**) was present in the smallest amount. The first of the group, or ribosylzeatin (**2**) was present in greatest amount.

The origin of the three "natural" cytokinin-active ribonucleosides (**2**, **3**, **4**) in the cytokinin-dependent callus cultures supplied only with BAP (**1**) indicates the stimulation of processes for their synthesis, since the benzyl group cannot of course provide directly for the isopentenyl chains in **1**, **2**, and **3**. It is known that when tobacco pith or callus tissue is cultured *in vitro*, occasional pieces acquire the capacity to synthesize cytokinin. Such habituated tissue can be subcultured indefinitely on a medium without added cytokinin and may also release cytokinins in sufficient quantities to permit growth of cytokinin-dependent tissue planted on the same medium (our unpublished results). Although occasional habituated cultures may have been included in the material that was extracted, routine tests for habituation, done by transferring samples of the callus to medium without cytokinin, were negative. It is highly improbable, therefore, that detectable amounts of cytokinin-active ribonucleosides would derive from this source.

It is more likely that the biosynthesis of the "natural" cytokinin-containing nucleosides represents a part of the increased anabolic activity brought about and maintained by the exogenous supply of cytokinin. Such increased anabolic activity, in response to cytokinin, as reflected in measurements of nucleic acids (RNA and DNA), total protein content, specific enzymes, pigments, and other cell constituents, is well known (see Skoog and Armstrong, 1970).

Effects of cytokinin on the biosynthesis of thiamine and auxin, the other two growth factors ordinarily required from an exogenous source by tobacco callus, are of special interest in revealing a quantitative relationship to the cytokinin concentration. In the absence of either thiamine or auxin, the tissue will not grow on media with low or moderate cytokinin concentrations (up to 1 μM kinetin equivalents) which are adequate for optimal growth in their presence, but on media with high cytokinin concentrations (*ca.* 5 μM kinetin equivalents) the callus tissue will synthesize either thiamine (Digby and Skoog, 1966; Dravnieks *et al.*, 1969) or auxin (Jordan and Skoog, unpublished) in adequate amounts for growth. Furthermore, the biosynthesis of thiamine at rates needed for growth remained dependent on a continuous high exogenous supply of cytokinin in subcultures through 20 passages over a 2-year period (Linsmaier and Skoog, 1966).

A similar quantitative relationship may be envisaged between the exogenous cytokinin supply and the biosynthesis of the "natural" cytokinin components of the tRNA in cytokinin-dependent tissue.

On the assumption that the cytokinin components of tRNA affect its efficiency and thus modulate protein biosynthesis, the presence of several cytokinin ribonucleosides even in the tRNAs from cytokinin-dependent tissue is of interest in suggesting possible specificity in the distribution and/or action of individual species and perhaps corresponding diversity in growth regulatory functions.

The present results demonstrate clearly that cytokinins of both exogenous and endogenous origin are present as constituents of tRNA in cytokinin-dependent tobacco callus tissue cultured on media supplied with 6-benzylaminopurine.

Acknowledgment

We take pleasure in thanking Mr. J. Carter Cook and Mr. Joseph A. Wrona, University of Illinois, for their cooperation in obtaining the mass spectra and Mrs. Anna Hilden, University of Wisconsin, for technical assistance.

References

Armstrong, D. J., Burrows, W. J., Evans, P. K., and Skoog, F. (1969a), *Biochem. Biophys. Res. Commun. 37*, 451.

Armstrong, D. J., Burrows, W. J., Skoog, F., Roy, K. L., and Söll, D. (1969b), *Proc. Nat. Acad. Sci. U. S. 63*, 834.

Bartz, J. K., Kline, L. K., and Söll, D. (1970), *Biochem. Biophys. Res. Commun. 40*, 1481.

Biemann, K., and McCloskey, J. A. (1962), *J. Amer. Chem. Soc. 84*, 2005.

Biemann, K., Tsunakawa, S., Sonnenbichler, J., Feldman, H., Dütting, D., and Zachau, H. G. (1966), *Angew. Chem. 78*, 600.

Borek, E., and Srinivasan, P. R. (1966), *Annu. Rev. Biochem. 35*, 375.

Brunngraber, R. K. (1962), *Biochem. Biophys. Res. Commun. 8*, 1.

Budzikiewicz, H., Djerassi, C., and Williams, D. H. (1967), Mass Spectrometry of Organic Compounds, San Francisco, Calif., Holden-Day, Inc., p 593.

Burrows, W. J., Armstrong, D. J., Kaminek, M., Skoog, F., Bock, R. M., Hecht, S. M., Dammann, L. G., Leonard, N. J., and Occolowitz, J. (1970), *Biochemistry 9*, 1867.

Burrows, W. J., Armstrong, D. J., Skoog, F., Hecht, S. M., Boyle, J. T. A., Leonard, N. J., and Occolowitz, J. (1968), *Science 161*, 691.

Burrows, W. J., Armstrong, D. J., Skoog, F., Hecht, S. M.,

Boyle, J. T. A., Leonard, N. J., and Occolowitz, J. (1969), *Biochemistry 8*, 3071.
Chen, C.-M., and Hall, R. H. (1969), *Phytochemistry 8*, 1697.
Digby, J., and Skoog, F. (1966), *Plant Physiol. 41*, 647.
Dravnieks, D. E., Burris, R. H., and Skoog, F. (1969), *Plant Physiol. 44*, 866.
Fittler, F., Kline, L. K., and Hall, R. H. (1968), *Biochemistry 7*, 940.
Fox, J. E. (1966), *Plant Physiol. 41*, 75.
Fox, J. E., and Chen, C.-M. (1967), *J Biol. Chem. 242*, 4490.
Fox, J. E., and Chen, C.-M. (1968), Biochemistry and Physiology of Plant Growth Substances, Wightman, F., and Setterfield, G., Ed., Ottawa, Runge Press, p 777.
Hall, R. H., Czonka, L., David, H., and McLennan, B. (1967), *Science 156*, 69.
Hecht, S. M., Gupta, A. S., and Leonard, N. J. (1970), *Anal. Biochem.* (in press).
Hecht, S. M., Leonard, N. J., Burrows, W. J., Armstrong, D. J., Skoog, F., and Occolowitz, J. (1969a), *Science 166*, 1272.
Hecht, S. M., Leonard, N. J., Occolowitz, J., Burrows, W. J., Armstrong, D. J., Skoog, F., Bock, R. M., Gillam, I., and Tener, G. M. (1969b), *Biochem. Biophys. Res. Commun. 35*, 205.
Kende, H., and Tavares, J. E. (1968), *Plant Physiol. 43*, 1244.
Kirby, K. S. (1965), *Biochem. J. 96*, 266.
Kline, L. K., Fittler, F., and Hall, R. H. (1969), *Biochemistry 8*, 4361.
Linsmaier, E. M., and Skoog, F. (1965), *Physiol. Plant 18*, 100.
Linsmaier, E. M., and Skoog, F. (1966), *Planta 72*, 146.
Madison, J. T., Everett, G. A., and Kung, H.-K. (1967), *J. Biol. Chem. 242*, 1318.
Madison, J. T., and Kung, H.-K. (1967), *J. Biol. Chem. 242*, 1324.
McChesney, J. D. (1970), *Can. J. Botany 48*, 2357.
Peterkofsky, A. (1968), *Biochemistry 7*, 472.
Richmond, A., Back, A., and Sachs, B. (1970), *Planta 90*, 57.
Robins, M. J., Hall, R. H., and Thedford, R. (1967), *Biochemistry 6*, 1877.
Skoog, F., and Armstrong, D. J. (1970), *Annu. Rev. Plant Physiol. 21*, 359.
Staehelin, M., Rogg, H., Baguley, B. D., Ginsburg, T., and Wehrli, W. (1968), *Nature (London) 219*, 3163.
Zachau, H. G., Dütting, D., and Feldman, H. (1966), *Angew. Chem. 78*, 392.

Photochemical Reactions of Cytosine Nucleosides in Frozen Aqueous Solution and in Deoxyribonucleic Acid*

A. J. Varghese

ABSTRACT: Irradiation of cytidine in frozen aqueous solution with 254-nm ultraviolet radiation produces two types of dimeric product, Cd_1 and Cd_2. Cd_1 has been identified as a cyclobutane-type dimer. On heating in acidic, neutral, or alkaline aqueous solutions, Cd_1 is converted mainly to cytidine, cyclobutane-type uridine dimer, and uridine, respectively. Mild acid hydrolysis of the uridine dimer obtained from Cd_1 gave cyclobutane-type uracil dimer, the infrared and ultraviolet absorption spectra of which are identical with those of the cis-syn isomer. An aqueous solution of Cd_2 showed an ultraviolet absorption maximum at 314 nm. Acid hydrolysis of Cd_2 yielded 6-(4′-pyrimidin-2′-one)uracil (PO-U) which has absorbance maxima at 305 nm (ϵ 10,800) in acidic and neutral solutions and at 325 nm (ϵ 12,000) in alkaline solutions. Irradiation of deoxycytidine in frozen aqueous solution produced two products having properties similar to those of Cd_1 and Cd_2. PO-U was also found to be present in acid hydrolysates of irradiated DNA. The proposed mechanism of formation of PO-U involves a cytosine adduct probably derived from an azetidine intermediate.

Irradiation of a mixture of thymidine and cytidine, or thymidine and deoxycytidine produced a number of products from which, after acid hydrolysis, thymine dimer, thymine–uracil dimer, uracil dimer, 6-(4′-pyrimidin-2′-one)-thymine, and 6-(4′-pyrimidin-2′-one)uracil were identified. All these products are also present in acid hydrolysates of irradiated DNA.

A major fraction of the lethal and mutagenic effects of ultraviolet light on biological systems has been attributed to photochemical transformations of pyrimidine bases in the nucleic acids (McLaren and Shugar, 1964). While considerable progress has been made in recent years in understanding the photochemical reactions of thymine and uracil derivatives, much less is known about cytosine and its derivatives (Burr, 1968; Fahr, 1968). It has been shown that irradiation of cytidine and cytidylic acid in aqueous solution with ultraviolet light (λ 254 nm) adds water across the C_5-C_6 double bond to form the 5,6-dihydro-6-hydroxy derivatives (photohydrates) (Johns *et al.*, 1965; Miller and Cerutti, 1968). However, attempts to demonstrate the formation of photohydrates in native DNA have not been successful (Setlow and Carrier, 1963). On the other hand, there is substantial evidence to indicate that cyclobutane-type dimerization of cytosine residues occurs in irradiated bacterial DNA and the number of cytosine-containing dimers is comparable to the number of thymine-containing dimers at low doses of ultraviolet

* From the Department of Biology, University of Texas at Dallas, Box 30365, Dallas, Texas 75230. *Received December 28, 1970.* Supported by U. S. Public Health Service Grants GM 13234, FRO 5646, and GM 16547.