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On the Significance of Cytokinin Incorporation into RNA

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Abstract. **The clarification of the following 2 questions was attempted: (a) are cytokinins precursors in the formation of sRNA, (b) is the observed incorporation of cytokinins into sRNA related to the action of the hormone? Although *Escherichia coli* contains cytokinins in its sRNA, no cytokinin auxotroph mutants of *E. coli* could be found and the statistical probability for the existence of such mutants is extremely low. This suggests that cytokinins are not precursors in the synthesis of sRNA. A radioactive cytokinin, 6-benzylamino-9-methylpurine was synthesized and it was tested whether or not it is incorporated into sRNA of soybean callus tissue. Masking the 9-position of the purine inhibited the incorporation of this cytokinin into RNA while not affecting its biological activity. This is taken as an indication that the observed incorporation of cytokinins such as benzyladenine into sRNA is not related to the action of this hormone.**

Substances with cytokinin activity have been isolated from the hydrolysates of the soluble RNA (sRNA) of microorganisms, animals and plants (1, 3, 12, 13, 20, 24, 26). The minor base 6-(γ,γ-dimethylallylamino)purine (= *N*-6-isopentenyladenine or IPA) was found in seryl transfer RNA (tRNA) of yeast at the position following the presumed anticodon (3, 31). This compound has high cytokinin activity (14) and is, chemically, closely related to the naturally occurring cytokinin zeatin, 6-(4-hydroxy-3-methylbut-*trans*-2-enyl)aminopurine (21). IPA was also isolated from sRNA of spinach and peas and the *cis*-isomer of zeatin from the sRNA of the same 2 plants and of corn (12). The presence of cytokinins seems to be restricted to sRNA since no such compounds were found in hydrolysates of ribosomal RNA (26).

Recently, evidence was presented that soybean and tobacco callus cultures, which require exogenous cytokinins for growth, incorporate one such growth regulator, 6-benzylaminopurine (benzyladenine), into sRNA (7). Chromatography of the labeled sRNA on *O*-benzoyldiethylaminoethyl cellulose showed that only 1 of the 6 fractions obtained was radioactive, and the bulk of this radioactivity appeared to be associated with the nucleotide of benzyladenine (8).

The incorporation of a cytokinin into sRNA and the natural occurrence of cytokinins in sRNA led to the question whether this hormone is exerting its biological effect through its presence in certain RNA species. As one possibility, it was suggested that the incorporation of benzyladenine into sRNA may confer amino acid transfer activity to certain tRNAs (7). Regulation at the level of amino acid transfer is well known in microorganisms (27) and similar control mechanisms can be visualized in plants.

There are, however, serious arguments against the hypothesis that cytokinins act by being incorporated into RNA, either at very specific positions or, more generally, in place of methylated bases as suggested by Fox (7). From microbial systems it is known that tRNA is first synthesized by polymerization of purine and pyrimidine bases and that methylation occurs on the finished RNA chain (4). There is also evidence that pseudouridine is formed by intramolecular rearrangement within the polymerized sRNA (30). If similar biosynthetic mechanisms are functional in plants, cytokinins are not precursors in the formation of tRNA. In this respect, it is interesting to note that ethanolic extracts of corn kernels contain the *trans*-isomer of zeatin (21) while the sRNA hydrolysates from the same source yield the *cis*-isomer (12), suggesting that zeatin is not a precursor in the synthesis of sRNA. There is, however, one possibility which could reconcile the contradictions discussed above: cytokinins may not be incorporated as a whole into RNA but the side-chain may be transferred to an adenine in the RNA polymer (7).

The following 2 questions need to be answered at this point: A) are cytokinins precursors in the formation of RNA, and B) is the incorporation of benzyladenine into sRNA related to the action of the hormone? An attempt was made to answer the first question by screening for cytokinin-requiring mutants of *Escherichia coli* which contains cytokinins in its sRNA (26). Such a mutant would be a useful tool for studying the formation of cytokinin-containing sRNA species. In order to answer the second question, experiments were designed by which one might separate the hormonal action of cytokinins from their incorporation into RNA. A radioactive analogue of benzyladenine, 6-benzyl-

amino-9-methylpurine, was synthesized and its biological activity was established. It was hoped that, by masking the 9-position of the purine ring with a methyl group, formation of a nucleotide and hence incorporation into RNA could be prevented.

Materials and Methods

Bacterial Mutants. According to the method of Benzer and Champe (2), rapidly growing cultures of *Escherichia coli* [C-medium (25) with 1 % glucose; approximately 5×10^8 cells per ml] were treated with 0.1 ml of diethylsulfate per 20 ml of culture. The culture was not agitated or aerated, but rather the diethylsulfate was allowed to diffuse slowly into the medium. One ml of this bacterial suspension was then transferred to 20 ml of fresh medium and allowed to reach 5×10^8 cells per ml before a second exposure to diethylsulfate was made. The mutagenic procedure was repeated for 3 consecutive treatments. Mutants were concentrated by using the penicillin method (10). The bacteria were centrifuged, resuspended in minimal medium without test substances, and were allowed to grow for 90 to 120 minutes to eliminate stored metabolites in the mutants. After this, the culture was treated with penicillin at 2000 units per ml for 90 minutes. The bacteria were then centrifuged, resuspended in 0.5 % sodium chloride solution with 5 % glycerine, and frozen. This procedure aided in breaking up the partially lysed cells. Individual mutants were selected and tested by replica plating (19). Auxotrophs were sought for the following test substances: L-isoleucine and L-arginine supplied at 0.5 mM, and adenine, kinetin (6-furfurylaminopurine) and IPA supplied at 50 μM. Cytokinins at this concentration appear to have no effect on the growth rate of the bacteria.

Synthesis of 6-Benzylamino-9-methylpurine. 6-Benzylamino-9-methylpurine-benzyl-7-^{14}C (fig 1A) was obtained by refluxing 14 μmoles of benzylamine-7-^{14}C HCl (specific activity 12 mc/mmole, International Chemical and Nuclear Corp.), 14 μmoles of 6-chloro-9-methylpurine (J. T. Baker Chemical Co.) and 0.2 ml triethylamine in 5 ml *n*-butanol for 19 hours. Addition of triethylamine facilitates the condensation reaction. The reaction mixture was taken to dryness and resuspended in water. The aqueous solution was partitioned 10 times against diethyl ether, the combined ether phases were dried with sodium sulfate and evaporated. The residue was chromatographed with water on washed Whatman No. 3 paper, and the U.V.-absorbing band was eluted with 90 % (v/v) ethanol. The purity of the product was checked by thin layer chromatography.

Growth of Callus. Callus originally derived from soybean cotyledons [*Glycine max* (L.) Merr. cv. Acme] was grown in 125 ml Erlenmeyer flasks on 20 ml of Miller's medium (23). Cytokinins were added prior to autoclaving. The tissue cultures were kept in darkness at 27° and were harvested 2 to 3 weeks after start of the experiment.

Extraction of Nucleic Acids. Nucleic acids were extracted according to Kirby (18) as modified by Johri and Varner (personal communication): 6 g of tissue was ground in a mortar in 5 ml sodium aminosalicylate (SAS), 5 ml phenol-cresol mixture and some sand. The homogenate was mixed vigorously and centrifuged at 10,000 rpm (Sorvall, head No. HB-4) for 10 minutes. The aqueous phase was removed and the extraction of the phenol phase was repeated twice using 5 ml SAS and an additional 2 ml phenol-cresol each time. The homogenates were centrifuged and the aqueous phases combined. Sodium chloride (30 mg/ml) was added to the pooled aqueous fraction which was extracted then with one-half of its volume of phenol-cresol mixture. The extract was again centrifuged and the aqueous phase was carefully separated from the phenol. The nucleic acids were precipitated with 2 volumes of ethanol-cresol mixture (−20°, 4 hr) and collected by centrifugation at 10,000 rpm for 10 minutes. The nucleic acids were resuspended and dialyzed for 20 hours against 3 changes of 1000 ml tris-HCl buffer (0.01 M, pH 7.6) containing 0.001 M $MgCl_2$ and 0.01 M KCl. Results obtained with the SAS extraction method do not differ from those obtained with the sodium laurylsulfate extraction method (16).

Chromatography of Nucleic Acids. Methylated Albumin-Kieselguhr chromatography (MAK) was performed according to Mandell and Hershey (22). The nucleic acids were eluted with a linear gradient of 0.3 to 1.6 M NaCl in phosphate buffer (200 ml each). Fractions of 3 ml were collected and the optical density at 260 nm was measured with a Beckman DB spectrophotometer.

Counting of Radioactivity. Three consecutive chromatographic fractions were combined into one; to this, 150 μg carrier DNA and 2 ml trichloroacetic acid (50 %, w/v) were added. The nucleic acids were precipitated at 2° for 4 to 5 hours. The precipitates were collected on Millipore filters (HA 0.45 μ) and washed with an excess of ice-cold trichloroacetic acid (5 %, w/v). The filters were dried and each sample was counted twice for 20 minutes in a Packard 3375 liquid scintillation spectrometer using Bray's solution as scintillator (6). Identical results were obtained when every chromatographic fraction was dried and counted directly in Bray's solution.

Results

The Probability of Cytokinin Mutants in E. coli. The efficiency of the mutagenic treatment was tested by selecting for arginine, isoleucine, and adenine mutants, besides cytokinin auxotrophs. Table I summarizes the result of one such experiment. Assuming that the mutability of each gene is the same when treated with a "random" mutagen such as diethylsulfate, any measure of the frequency of a known

Table I. *Frequency of Arginine, Adenine, Isoleucine, Threonine, and Cytokinin Mutants in* E. coli *Treated with Diethylsulfate*

Total number of colonies tested	10,831
No. of arginine mutants	119
No. of adenine mutants	16
No. of isoleucine mutants	18
No. of isoleucine mutants that are also threonine mutants	16
No. of IPA mutants	0
No. of kinetin mutants	0

one-gene mutation will allow a statistical estimate of the probability of finding another one-gene mutation.

To estimate the probability of cytokinin mutations, a further screening for isoleucine and IPA auxotrophs was made. Any isoleucine mutant of *E. coli* which does not grow on threonine must be a single-gene mutation as threonine is the immediate precursor of isoleucine. While 240 isoleucine-requiring mutants were found which did not grow on a medium containing threonine, not 1 single IPA mutant could be isolated. Using the Poisson distribution test, the probability of finding a IPA auxotroph mutated in 1 gene would be

$$P = \frac{e^{-m}\ (m)^x}{x!}$$

where P = probability of IPA mutation, x = number of IPA mutations, and m = number of isoleucine mutants. Thus $P = e^{-m} = e^{-240}$, or $P = 1.6 \times 10^{-105}$. Therefore, the probability is very small indeed that a cytokinin auxotroph exists in *E. coli*.

The Fate of 6-Benzylamino-9-methylpurine in Soybean Calluses. 6-Benzylamino-9-methylpurine stimulated cell division in soybean tissue cultures to the same extent as or even more than benzyladenine (table II). Activation of growth of axillary buds by 6-benzylamino-9-methylpurine has been reported by Guern (11).

Table II. *Biological Activity of 6-Benzylamino-9-methylpurine*

Each figure represents the average fresh weight of 12 soybean calluses grown for 21 days.

Treatment	Fr wt per callus
	mg
Benzyladenine (1 μM)[1]	786
Benzyladenine (3 μM)	1053
Benzyladenine (10 μM)	950
6-Benzylamino-9-methylpurine (1 μM)[2]	1125
6-Benzylamino-9-methylpurine (3 μM)	1158
6-Benzylamino-9-methylpurine (10 μM)	850
Control	123

[1] Nutritional Biochemicals Corporation.

[2] Non-radioactive sample synthesized and crystallized by the authors; the purity was verified by thin-layer and gas-liquid chromatography.

In initial experiments, soybean calluses were grown on media containing benzyladenine-^{14}C or 6-benzylamino-9-methylpurine-^{14}C of the same specific activity (12 mc/mmole), and the incorporation of radioactivity into RNA was compared. Benzyladenine-benzyl-7-^{14}C was synthesized as described earlier (5) but it was further purified by chromatography on Whatman No. 3 paper using 0.01 N HCl as solvent. The total trichloroacetic acid-precipitable nucleic acids from calluses (6.4 g) grown for 2 weeks on benzyladenine-^{14}C (3 μM) showed an incorporation of 68 cpm. In the same experiment and under identical conditions, the total nucleic acid extract of calluses (6.56 g) grown for 2 weeks on 6-benzylamino-9-methylpurine-^{14}C (3 μM) contained 4.5 cpm. Similar results were obtained in other experiments.

If 6-benzylamino-9-methylpurine-^{14}C was given at the optimal concentration (1 μM), no incorporation of radioactivity into RNA was observed (fig 1B). Identical results were obtained in 5 experiments with calluses derived from different explants, with different times of incubation (14–21 days), and with different concentrations of the hormone (up to 3 μM).

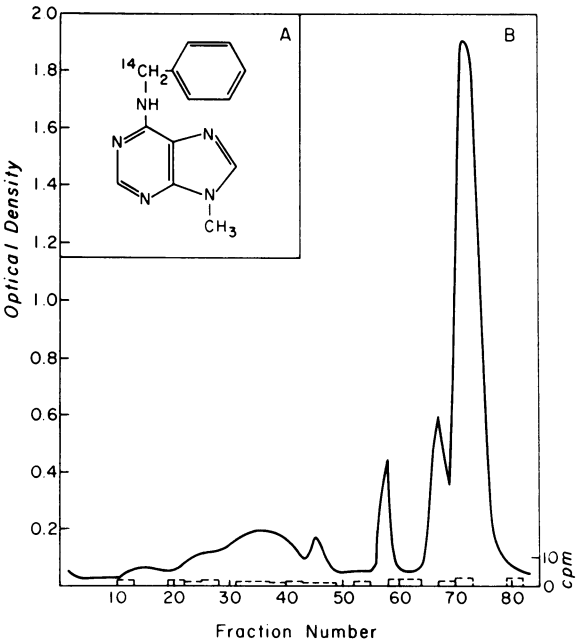

FIG. 1. A) Structure of 6-benzylamino-9-methylpurine-benzyl-7-^{14}C. B) MAK chromatography of nucleic acids extracted from 10 soybean calluses (6.336 g) grown for 17 days on a medium containing 6-benzylamino-9-methylpurine-7-^{14}C (1 μM). Weight of 10 benzyladenine-treated (1 μM) calluses grown under identical conditions 5.895 g, 10 control calluses 0.814 g. Solid line: optical density at 260 nm. Broken line: radioactivity. For the determination of the radioactivity the contents of 3 neighboring tubes were combined (fractions 11-82). All samples were counted twice and the counts were never significantly above background.

Discussion

The fact that no cytokinin auxotroph was found in *E. coli* is an indication that IPA is not a precursor in the synthesis of sRNA. This is in agreement with the hypothesis that the isopentyl group is added onto the macromolecule by substitution in a manner similar to that demonstrated for the methylation of nucleic acids (4).[1] A transfer of the side chain from IPA to an adenine in the sRNA seems also unlikely since there appears to be no need for the synthesis of IPA in *E. coli*.

The argument can be made that only kinetin and IPA were tested and that neither of these 2 compounds can substitute for the yet unidentified cytokinin in *E. coli* sRNA. However, there is evidence that IPA may be a widely occurring minor base since it has been isolated from the sRNA of mammals (13), plants (12), yeast (3, 13), and bacteria (24).

As with most negative results, the lack of a cytokinin mutant can only serve as supporting but not as conclusive evidence that cytokinins are not precursors in the synthesis of sRNA.

Methylation at the 9-position of the purine ring appears to inhibit the incorporation of this cytokinin into RNA while not affecting its biological activity. There was also no indication of the transfer of the labeled benzyl group to sRNA. The specific activity of the 6-benzylamino-9-methylpurine used was sufficient to detect the incorporation of 1 cytokinin molecule per molecule of 1 tRNA species. This was estimated from the amount of radioactivity associated with 1 tRNA species charged with a ^{14}C-amino acid, taking into account the specific activity of the amino acid and the optical density of the whole sRNA peak chromatographed on a MAK column (29).

Our experiments and the considerations discussed earlier in this paper make it seem unlikely that cytokinins exert their regulatory activities through incorporation into RNA. Results obtained in experiments on cytokinin-controlled bud formation in moss protonemata suggest that cytokinins interact with their site of action by loose, probably noncovalent bonds (5). A similar hypothesis was advanced for the binding of gibberellins (9, 17) and auxins (15) to their receptor sites. Evidence has been presented that estrogens bind to a hormone-specific protein in rat uteri most likely by non-covalent linkage (28). The finding and cellular localization of hormone-specific binding sites for any of the plant hormones would be an important step towards elucidating the mechanism of action of plant hormones.

[1] Note added in proof: Fittler, Kline, and Hall [Biochem. Biophys. Res. Commun. 31: 571–76 (1968)] reported recently that a cell-free extract from rat liver or yeast catalyzes the incorporation of mevalonic acid or isopentenyl pyrophosphate into pre-formed tRNA to form *N*-6-isopentenyladenosine.

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