Cytokinin Activity of Ureidopurine Derivatives Related to a Modified Nucleoside Found in Transfer RNA

Abstract. *By use of a radioactive labeling technique* N-*(nebularin-6-ylcarbamoyl)threonine has been detected in plant transfer RNA. Derivatives of this nucleoside promote cell division of a cytokinin-requiring soya bean tissue.*

The modified nucleoside N^6-(Δ^2-isopentenyl)adenosine or one of its derivatives has been found only in those transfer RNA (tRNA) molecules that respond to codons starting with the letter U (uracil) (*1*, *2*). Moreover, this nucleoside occurs in a strategic position adjacent to the 3′ end of the anticodon. Data obtained by Fittler and Hall (*3*) and Gefter and Russell (*4*) demonstrate that this tRNA component is essential to the codon-anticodon interaction. Apart from its presence in the tRNA molecule, this nucleoside displays potent growth-promoting activities in plant tissue (cytokinin activity), as well as evoking certain physiological responses in experimental animal systems (*1*).

No evidence for a relation between the physiological activity of this nucleoside and its presence in tRNA has been obtained. If such a relation should exist, however, one might expect other modified nucleosides that occupy an analogous position in tRNA to be implicated in the regulation of growth. We have obtained data that bear on this proposition.

N-(Nebularin -6- ylcarbamoyl)threonine, **1** (Fig. 1), was isolated and characterized in the tRNA of yeast, *Escherichia coli*, and animal tissue (*5*, *6*). Takemura *et al.* (*7*) established its presence in the primary sequence of yeast isoleucine tRNA ($tRNA^{Ile}$) adjacent to the 3′ end of the anticodon, and Ishikura *et al.* (*8*) detected it in *E. coli* methionyl-, lysyl-, and seryl(AG_C^U)-tRNA. These findings suggest that **1** occurs only in those tRNA's responding to the codons starting with the letter A (adenine). Compound **1** is, therefore, a logical candidate for study of its growth-promoting ability.

Our initial studies were carried out in a plant system. So that we can better understand the role, if any, of this compound in the regulation of development of plant tissue, we first had to establish the existence of this compound in plant tissue tRNA. To facilitate the isolation of this relatively rare component of tRNA we used a radioactive labeling technique.

An autonomous strain of tobacco tissue (500 g) (*9*) was incubated for 10 days on medium containing 50 μc of [8-^{14}C]adenine (specific activity 50 mc/mmole). The tissue was homogenized at 0°C with 3 liters of 95 percent ethanol.

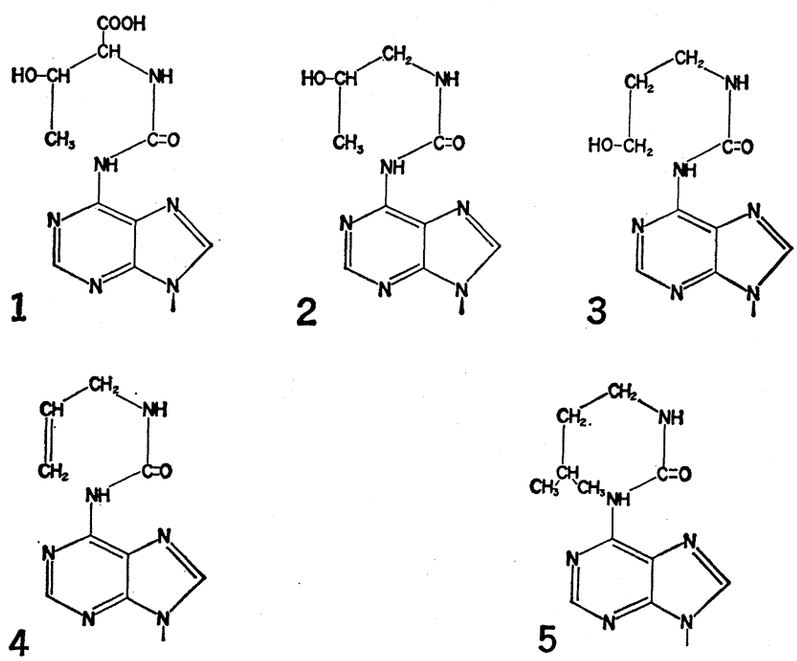

Fig. 1. Structures of *N*-(nebularin-6-ylcarbamoyl)threonine, **1**, and analogs. The 9-(β-D-ribofuranosyl) moiety has been omitted from each structure.

The homogenate was allowed to settle for 14 minutes and was then filtered through a Büchner funnel. The precipitate and cell debris were washed with ethanol until the filtrate was colorless. The residue was washed twice with acetone, dried in air briefly, lyophilized, and stored at −40°C.

This powder (11 g) was blended for 2 minutes with 300 ml of phenol, 300 ml of a buffer (0.1*M* tris HCl, *p*H 6.0; 0.06*M* KCl; 0.01*M* $MgCl_2$) and 45 ml of a 5.5 percent solution of sodium lauryl sulfate. The aqueous phase was separated by centrifugation (16,000*g*, 10 minutes) and extracted twice with 0.5 volume of phenol. Residual phenol was removed from the aqueous phase by extraction with ether, and the traces of ether were removed at reduced pressure.

The nucleic acid was precipitated according to the method of Bellamy and Ralph (*10*). Sodium acetate was added to the clear aqueous solution to make it 0.1*M*, then the nucleic acid was precipitated by addition of cetyltrimethylammonium bromide (1 percent solution). The precipitate was collected by centrifugation and dispersed in an excess of 70 percent ethanol and 0.1*M* sodium acetate solution in order to convert the nucleic acid to the sodium salt. The crude nucleic acid sample was washed twice with 95 percent ethanol and taken up in a minimum amount of a solution of 0.3*M* NaCl containing 0.1*M* tris HCl (*p*H 7.5).

The total nucleic acid fraction was placed on a column (20 by 2 cm) of DEAE cellulose [Cl^-] previously equilibrated with 0.3*M* NaCl solution. The column was washed with the same 0.3*M* buffer. The tRNA was eluted by a solution of 0.7*M* NaCl. The ultraviolet absorbing fraction was diluted with two volumes of ethanol. The precipitated tRNA was washed with 95 percent ethanol and then twice with acetone; it was then air-dried. This sample of tRNA was essentially free of contaminating protein as shown by the ratio of the absorbancy (*A*) at 280 to that at 260 nm, although probably it was not completely free of carbohydrate (14 A_{260} units/mg). The yield was 500 A_{260} units with a specific activity of 13,000 count/min per unit of absorbancy at 260 nm (*11*).

The tRNA sample was hydrolyzed in a solution of 1*N* HCl (3 ml) for 10 minutes at 100°C (*5*). The solution was cooled in an ice bath and a sample of unlabeled synthetic *N*-(purin-6-ylcarbamoyl)threonine (*6*) was added (0.4

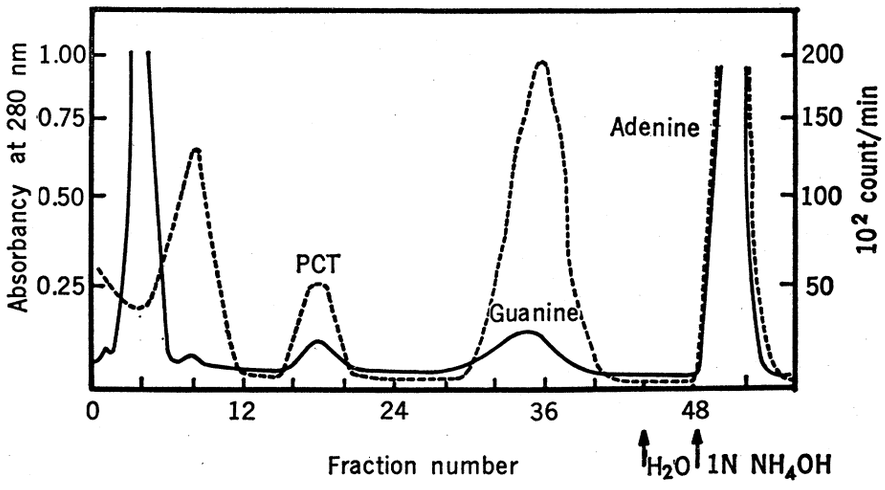

Fig. 2. Isolation of *N*-(purin-6-ylcarbamoyl)threonine (*PCT*) from an HCl digest of tobacco tissue tRNA by Dowex 50W × 8 column chromatography. A 20-μl portion of each 11-ml fraction was dried on a filter disk and counted in a liquid scintillation counter. Solid line, absorbancy; broken line, count/min.

ml of $10^{-3}M$ solution). The solution was evaporated to dryness in a rotary evaporator, and small volumes of water were added and reevaporated in order to remove traces of hydrochloric acid. The material was triturated three times with a small amount of anhydrous ether, and the residue was placed in a vacuum desiccator over KOH pellets for 4 hours.

This residue was dissolved in 3 ml of water and adjusted to *p*H 7 with 2*N* NaOH. The solution was kept at 0°C for 1 hour, and the precipitated guanine was removed by centrifugation. The clear supernatant was placed on a column (0.9 cm × 10 cm) of Dowex 50W × 8 (200 to 400 mesh) [H^+ exchange] previously equilibrated with 0.1*N* HCl. The column was washed with water (50 ml) and then developed with 500 ml of a linear gradient of HCl (0.2 → 0.8*N*). On completion of the gradient, the column was washed briefly with water, and the adenine was eluted with 1.0*N* NH_4OH. The elution profile is shown in Fig. 2.

The fraction corresponding to *N*-(purin-6-ylcarbamoyl)threonine was evaporated to dryness, and the residue was chromatographed on Whatman No. 1 filter paper in two solvent systems [1-butanol, H_2O, NH_4OH (86 : 14 : 5); 2-propanol, H_2O, NH_4OH (7 : 2 : 1)]. In both systems the radioactive spot moved coincidentally with the *N*-(purin-6-ylcarbamoyl)threonine marker.

In order to further clarify the identity of the radioactive material, the sample was converted to adenine by hydrolysis for 2 hours in a solution of 0.2*N* ammonium hydroxide at 100°C (*5*). Paper chromatography of the hydrolyzate in several solvent systems showed that the mobility of a single radioactive spot coincided with that of adenine.

A strain of soya bean tissue (*12*) that requires cytokinin and auxin was used for tests for cytokinin activity (*13*). *N*-(Nebularin-6-ylcarbamoyl)threonine (*14*) and its corresponding base (*6*) did not show any cytokinin activity in this system. Armstrong *et al.* (*15*) tested, in a tobacco tissue system, the sample of the free base that we synthesized, and they confirmed its lack of activity. It is possible, however, that cytokinin activity cannot be demonstrated in the test system, since the negative charge of the carboxyl group might prevent compound **1** from penetrating the cell. An alternative reason for the apparent lack of activity could be due to the fact that not compound **1** but rather a metabolic product exerts cytokinin activity.

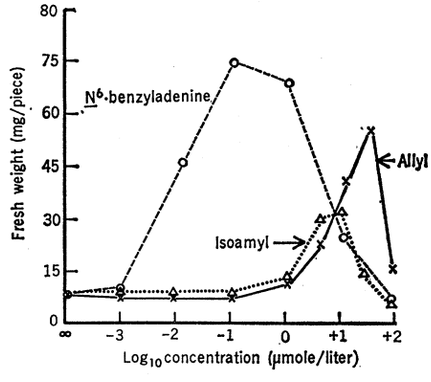

Fig. 3. Growth response of soya bean tissue (*12, 13*) to *N*-purin-6-yl-*N'*-allylurea and *N*-purin-6-yl-*N'*-isopentylurea after a 28-day incubation period, compared to the response of benzyladenine. Each point represents the average of four experiments with 12 pieces of tissue at each concentration per experiment.

Therefore, we synthesized analogs (*16*) of compound **1** that could logically be metabolic products of **1**. Each analog lacks the carboxyl group of compound **1**. The first compound in this series, **2**, represents compound **1** minus the carboxyl group. The base and nucleoside of **2** and its isomer **3** have no cytokinin activity. Abstraction of water from **2** or **3** provides the allyl derivative, **4**, which, as free base or nucleoside, has substantial cytokinin activity (Fig. 3).

This result shows that an analog of **1** has growth-promoting properties. We cannot be sure whether or not **4** exerts its activity because it represents a metabolic product of **1**. The observed structural specificity may depend more on lipid solubility as required for membrane permeability. The isoamyl derivative **5**, for example, shows activity (Fig. 2). Regardless of the exact reason for the specificity displayed by the test compounds, these data demonstrate that compounds structurally related to *N*-(nebularin-6-ylcarbamoyl)threonine have cytokinin-like growth-promoting properties.

Compounds with structures such as *N*-phenyl-*N'*-allylurea and many other phenylurea analogs induce cell division in cultured tobacco pith tissue (*17*). The phenyl group, like the purinyl group, is planar and has a resonant structure. It is possible that phenylurea derivatives have growth-promoting properties by virtue of their structural similarity to the naturally occurring purinylurea nucleoside, **1**.

In general terms, therefore, *N*-(nebularin-6-ylcarbamoyl)threonine may be implicated in a regulatory role of the type exerted by N^6-(Δ^2-isopentenyl)-adenosine. If so, this means that two modified components of tRNA, both of which occupy a similar and unique niche in tRNA structure and function, may be involved in regulatory activities.

What connection does tRNA have with such physiological activity? The locus of action of cytokinin compounds does not appear to lie in their ability to furnish a needed tRNA component. Chen and Hall (*18*), for example, showed that the tRNA of a cytokinin-requiring tobacco tissue contains N^6-(Δ^2-isopentenyl)adenosine, and further that the normal biosynthetic mechanism

for this tRNA component in this tissue remains intact and appears to function independently of exogenously added N^6-(Δ^2-isopentenyl)adenosine.

The tRNA, however, can serve as a source of these two modified nucleosides, and any degradation of tRNA would result in their release. Although alternative pathways of synthesis for these compounds may exist, Hall (*19*) has suggested that the rate of release from tRNA and the rate of metabolism of such growth regulatory compounds could constitute a means for maintenance of a physiologically active level in the tissue.

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9. This strain of tobacco tissue requires neither auxin nor cytokinin and was obtained from J. E. Fox, University of Kansas, from normal pith callus of *Nicotiana tobaccum*, variety Wisconsin 38.
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11. One A_{260} unit is defined as the quantity of material needed to give an absorbance of 1.0 at 260 nm when dissolved in 1 ml of solvent.
12. Soya bean tissue, derived from the cotyledon and requiring both an auxin and a cytokinin for growth, was used as the test organism. The tissue originated in C. O. Miller's laboratory and came to us via J. E. Fox. Pieces of tissue (2 mg), previously maintained on the basal medium plus 0.5 mg of benzyladenine per liter, were placed, three to five to a flask, on basal medium containing various concentrations of the compounds to be tested. After incubation in the dark at 25°C for 25 to 50 days the pieces of tissue were weighed, and their fresh weights were compared to those of controls grown on basal medium devoid of cytokinins.
13. For bioassay purposes, a basal medium was used, similar to that described by J. E. Fox [*Physiol. Plant.* **16**, 793 (1963)] with the addition of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ and $CuSO_4$ at 0.1 and 0.23 mg/liter, respectively.
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20. Supported by grants from the National Cancer Institute of Canada and the Medical Research of Canada (MT-2738), the National Science Foundation (6B-8690), and the U.S. Public Health Service (CA 10835). We acknowledge the assistance of Mrs. Lily Dyson and Mrs. G. R. Chen.

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Amygdaloid Nucleus: New Afferent Input from the Vomeronasal Organ

Abstract. *Terminal degeneration stained by the Fink-Heimer technique was found in the medial and cortical amygdaloid nuclei in a discrete zone after lesions were inflicted in the accessory olfactory bulb but not after lesions were made in the main olfactory bulb in the rabbit. Since the accessory olfactory bulb receives the endings of the vomeronasal nerve, the mediocortical complex of the amygdala is the central projection area for the vomeronasal sensory organ. The vomeronasal organ is seen as having new potential significance in sexual and feeding behavior because the cortical amygdaloid nucleus projects to the anterior, medial hypothalamus and the ventromedial nucleus.*

Bilateral ablation of the medial preoptic-anterior hypothalamic zone abolishes copulatory behavior in the male rat (*1*). Ablation of the olfactory bulb also abolishes or impairs copulatory activity in some mammals (*2*). Among the structures likely to receive a projection from the olfactory bulb in the rat, only the posteromedial part of the cortical amygdaloid nucleus projects to the medial preoptic-anterior hypothalamic zone (*3*). However, the olfactory bulb does not actually project to the posteromedial part of the cortical amygdaloid nucleus (*4*). How, then, may ablation of the olfactory bulb impair copulation? We have evidence that the posteromedial part of the cortical amygdaloid nucleus provides a link in a chemoreceptive pathway arising from the vomeronasal organ of the snout (*5*). The peripheral part of the vomeronasal pathway is adjacent to the olfactory pathway (*6*) and is, therefore, vulnerable to injury by ablation of the olfactory bulb.

The vomeronasal organ (*5*) is a tubular sac which opens by way of a small pore either into the anterior, basal corner of the nasal chamber or into the anterior end of the oral cavity by way of the nasopalatine canal or into both oral and nasal cavities in some species. The organs, one on each side, are lined with an epithelium resembling the olfactory epithelium and, by analogy with the latter, probably have a chemoreceptive function. The openings of the vomeronasal organs are so situated that they may allow entry of moisture-borne substances picked up by the snout or taken into the mouth. The nuzzling or licking activities common to many mammals (*7*) during sexual behavior, for example, may favor stimulation of the vomeronasal organ. The vomeronasal nerve connects the vomeronasal organ to the accessory olfactory bulb (*6*), which lies in a depression on the dorsal surface of the main olfactory bulb in many mammals (Fig. 1, left). In its course to the accessory olfactory bulb, the vomeronasal nerve comes to lie closely adjacent to the main olfactory nerves and main olfactory bulb (*6*).

Although the peripheral morphology of the vomeronasal system as described above has been known for a long time, the central connections of the mammalian accessory olfactory bulb defied analysis until now (*8*). Previous investigators (*9*), who used the conventional Nauta-Gygax techniques after making lesions of the olfactory formation, were unable to detect any difference in the distribution of degenerating fibers of the lateral olfactory tract which could be attributed to damage to the accessory olfactory bulb. It now appears that in the rabbit much of the accessory olfactory bulb projection becomes thinly myelinated or unmyelinated a few millimeters before the fibers leave their main course in the lateral olfactory tract to enter their area of termination. The poorly myelinated segment of the accessory olfactory bulb efferents is readily detected, however, in sections of rabbit brain stained by the Fink-Heimer method (*10*).

We first determined the projection of the main olfactory bulb in the rabbit by charting the terminal degeneration, as revealed by the Fink-Heimer method (*10*), in seven young, female, New Zealand white rabbits which had survived surgical destruction of an olfactory bulb