Nature and Distribution of Cytokinins [and Discussion]

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Nature and distribution of cytokinins

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A brief historical account is given of the discovery of the cytokinins together with details of those cytokinins that have been positively identified as occurring free, or as constituents of tRNA, in plants. An account is also given of the present state of knowledge regarding cytokinin biosynthesis and metabolism in plants.

Introduction

It is not the intention in this article to provide a detailed and comprehensive account of the isolation, characterization and distribution of cytokinins in higher plants. Comprehensive reviews by Skoog & Armstrong (1970) and Hall (1973) cover the subject up until the latter date. Apart from a brief historical and general introduction the major portion of this article will be devoted to an account of some of the current problems in the field of cytokinin research.

In contrast to, for example, the gibberellins which are classified in terms of their chemical structures, the definition of a cytokinin is essentially a physiological one. A cytokinin may be defined as a compound that promotes cell division in a cultured plant callus tissue grown on a defined medium with all necessary organic and inorganic nutrients and growth factors including an exogenous supply of auxin.

The idea that specific chemical substances may control cell division in plants goes back certainly to the nineteenth century and was first given some experimental support by Harberlandt (1913) who found that phloem diffusates could cause cell division in potato parenchyma. He later (1921) coined the term 'wundhormone' to explain the cell division promoting effects of wounding since rinsing the wounded surface led to the suppression of cell division and this could be restored by the application of crushed tissue.

The modern period of cytokinin research begins with the pioneering work of Skoog in the field of plant tissue culture. During research into the growth requirements of pith tissue isolated from *Nicotiana tabacum* cv. Wisconsin No. 38, Skoog found that cell division in this tissue could only be maintained in culture if a piece of vascular tissue was in contact with the pith. As the result of an extensive search for alternative sources of cell division promoting activity, Miller *et al.* (1956) isolated and identified the first cytokinin as 6-(furfurylamino)purine (kinetin, figure 1*a*) from an autoclaved preparation of herring sperm DNA. This compound arose as an artefact of the autoclaving process due to the dehydration and migration of a deoxyribose moiety from the 9 position of adenine to the N^6 position.

Synthetic cytokinins

The identification of the first cytokinin as a 6-substituted amino purine led to the synthesis of a very large number of analogues and this work has continued up until the present time. The early work is reviewed by Strong (1958). The structure–activity relations of the synthetic

cytokinins may be summarized briefly by saying that replacement of the N^6 substituent of kinetin with a wide variety of different groups led to compounds with a range of cell division promoting activity. Most compounds tested were less active than kinetin; however, a few, the most important being 6-(3-methyl-2-butenylamino)purine (i^6Ade, figure 1 b), were more active. In general the most active compounds were found to have an N^6 side chain of four to six carbon atoms. Alterations in the purine nucleus usually led to a large reduction in activity and frequently to complete inactivation of the compounds. Recently it has been shown that modification of the purine nucleus of a cytokinin can lead to the formation of compounds which act as anticytokinins. For reviews of these compounds see Hecht *et al.* (1975) and Skoog, Schmitz, Hecht & Frye (1975).
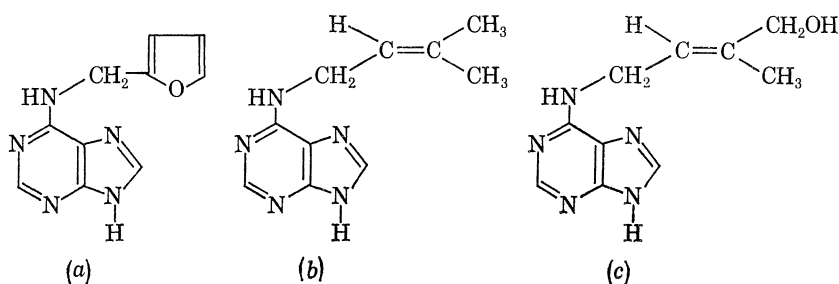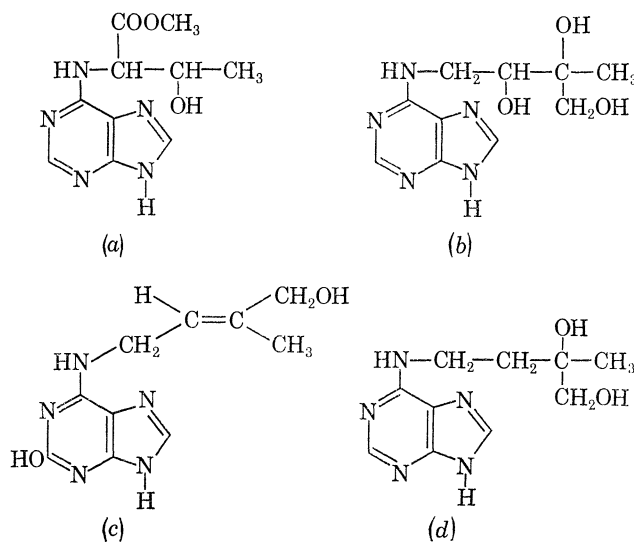

FIGURE 1

FIGURE 2

FREE CYTOKININS IN PLANTS

Although cytokinin-like activity had been demonstrated in extracts from a wide variety of plant sources, a number of years elapsed between the discovery of kinetin and the isolation and identification of a cytokinin from a higher plant source. This was mainly due to the extreme technical difficulties of the purification of minute quantities of biologically active compounds from large quantities of plant material and the determination of structure with physical methods of limited sensitivity. However, Miller (1962) partially characterized a substance with very high cytokinin activity from immature corn kernels and Letham (1963) further purified the

substance from the same source and determined its structure as 6-(4-hydroxy-3-methylbutanyl-amino) purine (zeatin, figure 1c). Letham (1966) also identified the nucleoside and nucleotide of zeatin from the same source. More recently Letham (1973), in what can only be described as a 'phytochemical *tour de force*', identified several more cytokinins from corn kernels. These compounds which are less active than zeatin are shown in figure 2. The compound shown in figure 2a was also isolated as the 9-riboside of the free acid.
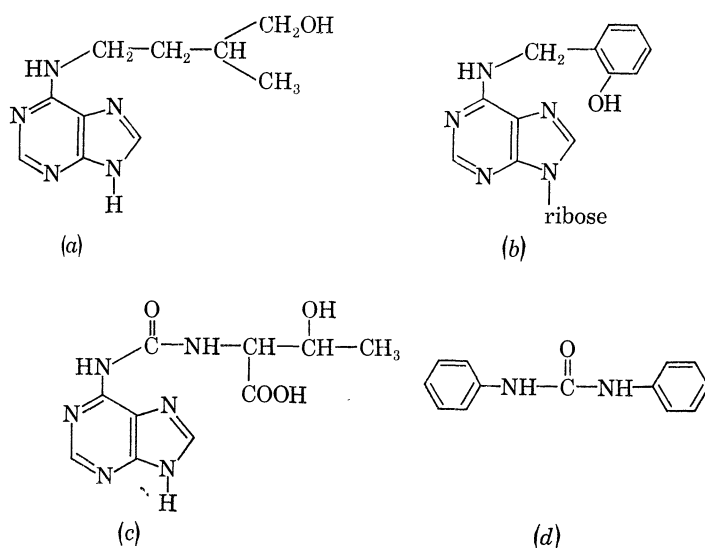

FIGURE 3

Although there is an extremely voluminous literature describing the existence of biological activity in extracts from higher plants with chromatographic properties similar to known cytokinins and many tentative identifications based upon this type of evidence, the number of unambiguous identifications of cytokinins from plant sources is relatively small. As has previously been mentioned this is mainly due to the technical difficulties of purification and structure determination. Even with sophisticated, modern, analytical instrumentation, the isolation and purification of compounds that may be present at levels as low as 1 µg/kg of plant material presents a formidable problem. In contrast the ease with which 'cytokinin-like activity' may be detected in plant extracts by bioassay methods has led to the almost exclusive use of this technique to detect and measure levels of cytokinins in plants. The dangers inherent in quantitative use of bioassays are too well known to warrant further discussion here; however, in the case of cytokinins the complex nature of the bioassay response tends to exacerbate the problem.

Other cytokinins that have been unambiguously identified in higher plants are shown in figures 3a and b. The compound (−)-dihydrozeatin (figure 3a) was isolated from yellow lupin seeds by Koshimizu, Kusaki, Mitsui & Matsubara (1967) and its riboside has recently been found as a minor cytokinin in leaves of *Phaseolus vulgaris* (Wang & Horgan 1978). The side chain O-glucoside of dihydrozeatin has been identified as the major cytokinin in primary leaves of decapitated *Phaseolus* plants (Wang, Thompson & Horgan 1977) and the corresponding glucosides of zeatin and zeatin riboside have been isolated and identified from *Vinca rosea* crown gall tissue by Peterson & Miller (1977) and Morris (1977).

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The cytokinin *o*-hydroxybenzyladenosine (figure 3*b*) was isolated from mature leaves of *Populus robusta* (Horgan, Hewett, Purse & Wareing 1973) and its level in this tissue appears to be under phytochrome control. However, this unusual compound has not been identified from any other plant source.

Perhaps the most unusual of the naturally occurring cytokinins is diphenylurea (figure 3*d*). This compound was isolated from coconut milk (Shantz & Steward 1955), a material long known as a source of high cytokinin activity. However, Letham (1968) has shown that zeatin and zeatin riboside are responsible for most of the cytokinin activity of coconut milk.

Recently, zeatin-7-glucoside, previously only known as a metabolite of exogenously supplied zeatin, has been identified by an isotope dilution method in seeds of *Raphanus* by Summons, MacLeod, Parker & Letham (1977). The compound 6-(3-methyl-2-butenylamino)purine riboside (i⁶Ado) has been identified in an autonomous strain of tobacco callus tissue by Dyson & Hall (1972).

Although the foregoing account covers all the cytokinins that have been positively identified as occurring free in higher plants, it is not exhaustive as regards the plant materials in which these compounds have been found. However, the number of cytokinins identified and their identifications in higher plant material remains relatively small when compared with the gibberellins. Undoubtedly the number of successful isolations and identifications of cytokinins from higher plant sources will increase in step with the development of more powerful and sensitive separation and structure determination techniques.

Cytokinins in transfer RNA

The identification of i⁶Ade as a constituent of two tRNA^Ser species from brewer's yeast by Zachau, Dütting & Feldman (1966) and the subsequent demonstration that it occurred only once in the polynucleotide in a position adjacent to the 3′ end of the anticodon in both species opened an intriguing and very prolific period of research into the cytokinins. Mainly as the result of the work of Skoog and his group, the cytokinins shown in figure 4 were identified in a variety of tRNA species. The distribution of these compounds in tRNA species from different sources is of considerable interest. Only i⁶Ado has been found in tRNA from animal sources. Transfer RNAs from bacterial sources have been found to contain both i⁶Ado and 2-methyl-thio-i⁶Ado (2 ms-1⁶Ado). The hydroxylated compounds seem to be confined to plant tRNAs and with the exception of pea tRNA the side chains have the *cis* configuration. This is in contrast to the *trans* configuration exhibited by zeatin and zeatin riboside isolated as the free compounds from plants. Recently, however, it has been demonstrated that both the tRNA and culture filtrates of *Agrobacterium tumefaciens* contains *trans* zeatin riboside (Chapman, Morris & Zaerr 1976; Kaiss-Chapman & Morris 1977).

The cytokinins shown in figure 4 seem to be confined to tRNA species that respond to codons with the initial letter U. *N*-(purin-6-yl carbamoyl)threonine (figure 3*c*), which is found in an analogous position in certain tRNA species which respond to codons beginning with A, is inactive as a cytokinin although more lipophilic ureido purines do exhibit cytokinin activity (McDonald, Leonard, Schmitz & Skoog 1971).

The significance of cytokinins in tRNA is beyond the scope of this article, although it can now be concluded that their presence is probably unconnected with their cell division inducing

properties. For a detailed account of this see Burrows (1975). However, the rôle of tRNA as a possible source of free cytokinins on higher plants raises certain important questions which will be discussed later.
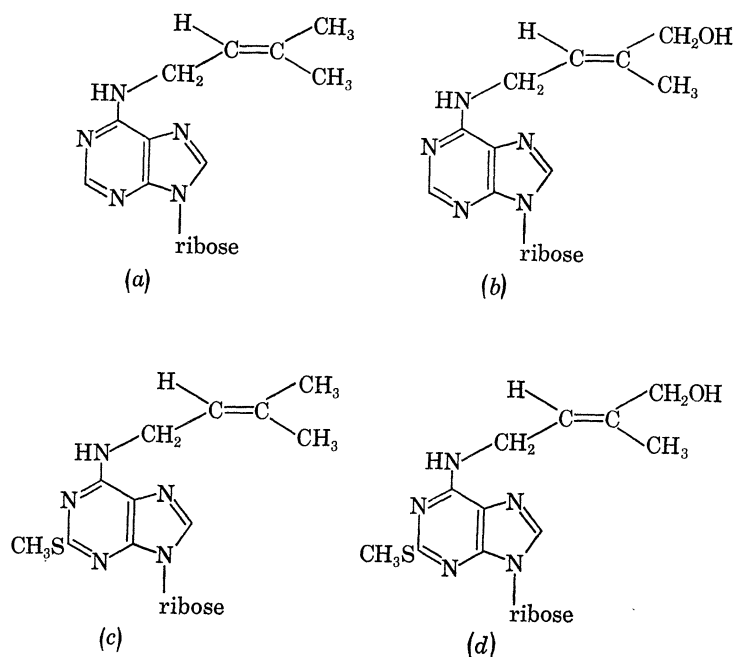

FIGURE 4

CYTOKININ METABOLISM

Although as has been stated previously our knowledge of the nature and distribution of cytokinins in higher plants is very limited, the availability of ^{14}C- and ^{3}H-labelled cytokinins has produced a considerable volume of literature regarding their metabolism. The study of cytokinin metabolism in systems which respond to cytokinins has been aimed at seeking correlations between metabolism and biological activity. From this point of view studies have involved the use of both synthetic and naturally occurring cytokinins, almost exclusively in tissue culture systems. The physiological relevance of cytokinin metabolism in these systems is obvious and does not need to be related to the endogenous cytokinin status of the tissue. On the other hand, the study of cytokinin metabolism in whole plant systems is of less physiological relevance since in most cases the endogenous cytokinins have not been identified and their physiological rôle is far from being understood. However, from the purely phytochemical point of view some interesting studies of cytokinin metabolism have been made in whole plant systems.

The observations of Fox *et al.* (1973) on the metabolism of 6-(benzylamino)purine (BAP) in plant tissue cultures of tobacco and soybean which required exogenous cytokinin for cell division led to the intriguing speculation that the metabolism of this compound to the 7-glucoside (figure 5c) was necessary for its activity as a cytokinin. Unfortunately the identification of this compound in the systems used was not exhaustive and was based on co-chromatography of metabolites with the positively identified 7-glucoside obtained by feeding BAP to potato tuber slices.

It has since been confirmed by Laloue, Gawer & Terrine (1975) that BA and i⁶Ade are metabolized to 7-glucosides in tobacco cell cultures although Laloue (1977) has also demonstrated that this metabolism is unconnected with any cytokinin activation.

Following the observation that zeatin was not metabolized to a 7-glucoside in cytokinin-requiring soybean tissue cultures but formed the side chain *O*-glucoside (figure 6*b*) (Horgan 1975) it was found that BA in this system was converted into the biologically inactive 9-alanyl derivative (figure 5*b*) (Horgan, unpublished results).
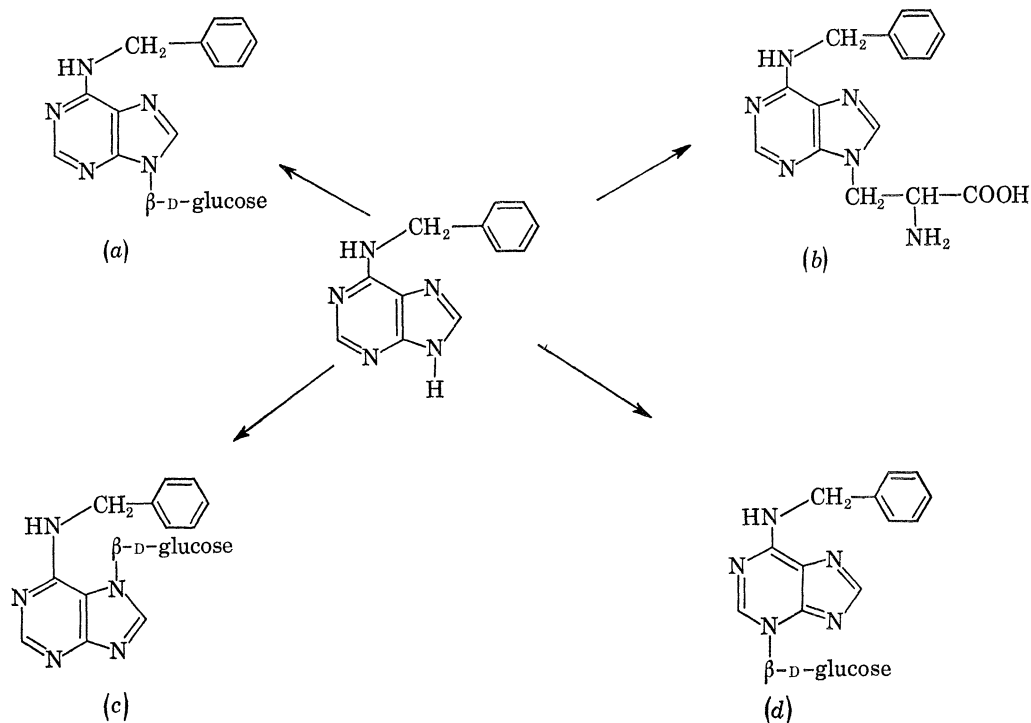

FIGURE 5.

Thus it would appear that glucosylation of cytokinins leads to inactivation rather than activation. However, the full significance of this metabolic inactivation in mediating tissue responses to applied cytokinins has to be explored further.

Letham and his group have made a detailed study of the metabolism of zeatin and BA in several plant species. In seedlings of *Raphanus sativus*, zeatin was metabolized mainly to the 7-glucoside (Parker, Letham, Cowley & MacLeod 1972). In this tissue BA was metabolized to the 9-, 7- and 3-glucosides (Parker *et al.* 1973; Parker *et al.* 1975). In plants of *Zea mays* the 9-glucoside (figure 6*c*) was the major and the 7-glucoside (figure 6*a*) the minor metabolite of zeatin. In seedlings of *Lupinus luteus* both zeatin-*O*-glucoside and 9-alanylzeatin (figure 6*d*) were identified as metabolites of zeatin (Parker *et al.* 1975). The biosynthesis of the latter metabolite in a cell free system has recently been demonstrated (Murakoshi *et al.* 1977).

In a study of zeatin metabolism in leaves of *Populus alba*, Letham *et al.* (1976) have identified the *O*-glucosides of zeatin, dihydrozeatin and dihydrozeatin riboside.

In addition to the formation of conjugates with sugars and amino acids, cytokinins can be rapidly degraded in plant tissues. This reaction, which involves the cleavage of the isopentenyl

side chain at the double bond, results in immediate loss of cytokinin activity. Whitty & Hall (1974) have purified an enzyme from *Zea mays* endosperm, which they called 'cytokinin oxidase', that degrades cytokinins in this manner. Only cytokinins with isopentenyl type side chains are susceptible to this enzyme. Although it has yet to be demonstrated, it would seem likely that this or very similar enzymes play an important rôle in controlling cytokinin levels in plant tissues.
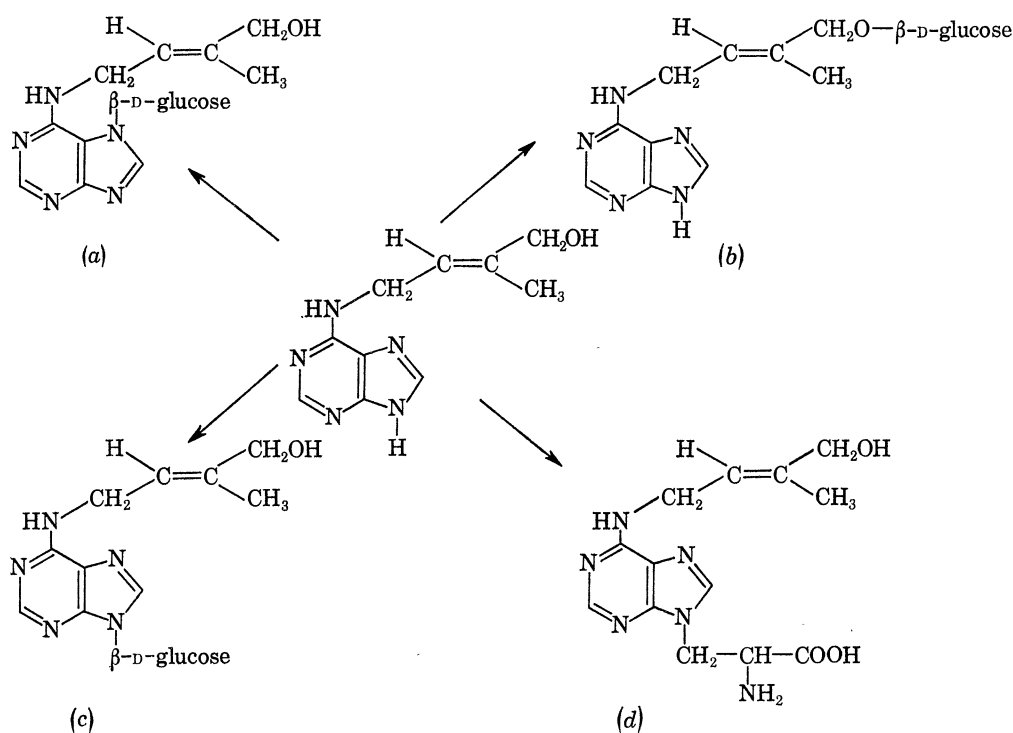

FIGURE 6

CYTOKININ BIOSYNTHESIS

There is a considerable scarcity of information on the details of cytokinin biosynthesis in higher plants. It would seem that i⁶Ade residues in tRNA are produced by the reaction between Δ^2-isopentenyl pyrophosphate and an adenine residue in the tRNA molecule. Enzymic activity of this type has been found in plant tissue (Chen & Hall 1969).

However, the origin of free cytokinins in plants remains something of a mystery. There have been several accounts of the incorporation of adenine into cytokinins in plant tissues, but several of these must be questioned because of lack of purification and positive identification of the cytokinins. Miller has clearly shown that adenine is incorporated into zeatin and its riboside in *Vinca rosea* crown gall tissue. Studies in Aberystwyth (T. Stuchbury & L. M. S. Palni, personal communication) suggest that the rate of incorporation of adenine into cytokinins in this system is too fast to involve tRNA. Further evidence that free cytokinins are biosynthesized by a separate route from those in tRNA comes from the fact that zeatin in tRNA has predominantly the *cis* configuration whereas the free cytokinin, where the side chain configuration has been unambiguously determined, has the *trans* configuration. Recently, Burrows (1978) has shown that tRNA from *Populus robusta* leaves does not contain *O*-hydroxy-benzyladenosine, the major free cytokinin in the tissue, and similarly tRNA from seeds of

Lupinus does not contain dihydrozeatin. However, until details of the biosynthesis of free cytokinins have been elucidated in plants the above evidence must be considered circumstantial. The recent finding that in certain animal tumours tRNA turnover can be abnormally rapid and lead to the excretion of elevated quantities of 'odd' bases (Borek *et al.* 1977), must, however, be seriously considered with regard to the interpretation of experiments involving the use of plant tumour tissues.

CONCLUSION

The cytokinins clearly constitute an important class of plant growth regulators. The extreme potency of these compounds in causing cell division as well as a wide variety of other effects when supplied exogenously to plants makes them worthy of study, and the fact that they occur free and in tRNA in higher plants suggests an important rôle for them in controlling plant growth and development. However, until more is known about the nature of cytokinins in higher plants, their biosynthesis and metabolism, their distribution within the plant, and most importantly details of their mode of action at the molecular level, their rôle as possible endogenous plant growth regulators cannot be properly assessed.

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Discussion

D. KLÄMBT (*University of Bonn, Germany*). Regarding your statement that free cytokinins have to be synthesized via a pathway independent of tRNA degradation, I feel that the crucial experimental results are still missing. In microorganisms, for example, cytokinins are produced by degradation of tRNA.

R. HORGAN. I feel that tRNA must always be considered a potential source of cytokinins. However, experimental evidence regarding the rate of incorporation of adenine into free cytokinins obtained in our laboratory would suggest the presence of an independent biosynthetic route to free cytokinins at least in *Vinca rosea* crown gall tissue. These results must, however, be viewed in the light of the recent findings of Borek *et al.* (1977) on the rate of turnover of certain tRNA species on certain animal tumours.

In addition, the side chain stereochemistry and the presence of cytokinins in cytokinin-requiring callus cultures would tend to support an independent biosynthetic route for free cytokinins.

P. F. SAUNDERS (*Department of Botany and Microbiology, University College of Wales, Aberystwyth, Dyfed, U.K.*). I wonder if we might obtain some useful ideas by speculating a little about how a hormonal rôle for the cytokinins could have evolved. A major event in the evolution of multicellular organisms must have been the appearance of a mechanism for *preventing* cell divisions rather than for *promoting* them. If cytokinins are necessary for cell division, then it is possible that the evolution of a control mechanism would have involved the appearance of some means whereby cytokinins, released by tRNA breakdown, can be inactivated. Such an evolutionary event might well be recapitulated during apical development in higher plants, the cessation of cell division being brought about by the biochemical differentiation of cells which possess the ability to inactivate cytokinins as they are released. If division is subsequently re-initiated in such cells, the process may well be dependent on cytokinins translocated from meristematic regions. By the same token, it might be suggested that cell division can occur in certain 'autonomous' tissue cultures in the absence of exogenous cytokinins, not because the cells have acquired the capacity to produce cytokinins but because they have lost the ability to inactivate them.

R. HORGAN. Certainly the general occurrence of cytokinins on tRNA and the fact that their rôle in this context seems to have little to do with their cell division inducing properties may suggest that plants found another rôle for these compounds as controllers of cell division. Unfortunately we cannot say whether the control of cytokinin levels in plants is predominantly at the biosynthetic or catabolic level until we have more information regarding the mechanisms of these processes.