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Cytokinin Metabolism and the Control of Cytokinin Activity

B. A. McGaw and R. Horgan

Department of Botany and Microbiology, University College of Wales, Aberystwyth, Dyfed SY23 3DA, Wales, U.K.

Abstract. The roles of the different cytokinin structures are discussed in relation to our knowledge of their biological activities, endogenous occurrence and metabolism.

Zeatin (Z) was first isolated 20 years ago (Letham 1963) and over 30 naturally occurring cytokinins have now been unambiguously identified from a variety of plant sources (Letham and Palni 1983). The recent advances in HPLC and GC/MS instrumentation and the development of internal standard/selected ion monitoring techniques (Summons *et al.* 1977, 1978, Scott and Horgan 1980) have made it possible to identify and accurately quantify cytokinins at the ng level. Despite this there are only a handful of tissues for which the endogenous cytokinins and their levels are known with any certainty (McGaw *et al.* 1984c). The cytokinins of *Nicotiana tabacum* crown gall tissue (Scott and Horgan 1984) and *Lupinus luteus* seed (Summons *et al.* 1981) where, respectively, 11 and 10 internal standards were employed, are the most completely understood.

In a recent review (Letham and Palni 1983) it was postulated that the various cytokinin structures may be: active forms, translocation forms, storage forms, detoxification products, deactivation products (formed to reduce cytokinin activity levels) or inactivation products (formed as a result of their utilisation).

The accurate identification and quantitation of the cytokinins is an important step forward to our understanding of the significance of these compounds. If these data are examined in the light of our knowledge of the metabolism and the biological activities of the various cytokinins it is possible to tentatively assign functions within the above framework.

Biological Activity of the Cytokinins

There are many bioassay systems that have been employed in the detection and quantitation of the cytokinins. The diversity of these systems (*i.e.*: *Amaranthus* β-anthocyanin production, oat leaf senescence, radish cotyledon expansion *etc.*) reflect the many aspects of plant growth and development in which the endogenous cytokinins may be important. Perhaps significantly,

there is a general agreement between the various bioassays as to which cytokinins are active (or inactive) and at what levels they exert their effects (LETHAM *et al.* 1983). Thus it has been shown that the cytokinin bases (*i.e.*: Z, dihydrozeatin (DHZ) and N^6-(Δ^2-isopentenyl) adenine (2iP)), the O-glucosides (*i.e.*: ZOG and DHZOG) and their corresponding 9-β-D-ribosides (*i.e.*: ZR *etc.*) are extremely active at physiological levels; whilst other N-substituted conjugates, namely 7- and 9-β-D-glucosides (*i.e.*: Z7G and Z9G) and the 9-alanyl derivatives (*i.e.*: 9A1Z), are generally inactive.

TABLE 1

The distribution of metabolites following feeds with Z or 2iP to various tissues (+++ = major, + = minor metabolite)

Plant	Organ	Feed	Metabolites					Reference
			O-glucosides	N-glucosides	N-alanyl compounds	ribosides/ ribotides	oxid. products	
Phaseolus vulgaris	1° leaves	Z	+	–	–	–	+++	PALMER *et al.* 1981b
Zea mays	Kernels	Z	++	–	–	+	+++	SUMMONS *et al.* 1980
Vinca rosea	Crown gall	Z	++	–	–	++	+++	PALNI 1980
Nicotiana tabacum	Cell susp.	2iP	–	+	–	+	+++	LALOUE *et al.* 1977
Lupinus angustifolius	Shoots	Z	+++	+	+++	+++	+++	PARKER *et al.* 1978
Raphanus sativus	Seedlings	Z	+	+++	–	–	–	MCGAW *et al.* 1984b

It is possible that 'biological activity' observed for an exogenously supplied cytokinin may be the result of metabolism (*i.e.*: hydrolysis of ZR to Z) that may not, for a variety of reasons (*i.e.*: compartmentation) occur in nature. Accordingly, the biological activity of the nucleotides has not been tested, but their rapid 'turnover' would probably render any results meaningless.

Cytokinin Metabolism

In this paper only metabolic studies on tissues where the endogenous cytokinins have been accurately quantified are considered. These data are given in Tables 1 and 2.

Abbreviations used: Ade = adenine; Ado = adenosine; AMP = adenosine-5-monophosphate; 2iP = N^6-(Δ^2-isopentenyl) adenine; Z = zeatin; ZR = zeatin-9-riboside; Z7G = zeatin-7-glucoside; Z9G = zeatin-9-glucoside; ZOG = zeatin-O-glucoside; ZOG7G = zeatin-O-glucoside-7-glucoside; DHZ = dihydrozeatin; DHZOG = dihydrozeatin-O-glucoside; DHZ7G = dihydrozeatin-7-glucoside; DHZOG7G = dihydrozeatin-O-glucoside-7-glucoside; 9A1Z = 9-alanyl zeatin; BA = benzyl adenine; GAL = gluconic acid lactone.

TABLE 2

A comparison between the metabolism of 3 cytokinins in the 'oxidase-type' *Phaseolus* (from PALMER *et al.* 1981b) and the 'glucosidase-type' *Raphanus* (from MCGAW *et al.* 1984b) systems

Plant material	Feed	Unmetabolised material	O-glucosides	N-glucosides	Oxidation products
Phaseolus 1° leaves	DHZ	++	++	−	++
	ZOG	+++		−	+++
	DHZOG	+++		−	++
Raphanus seedlings	DHZ	+	−	+++	−
	ZOG	++		+++	−
	DHZOG	++		++	−

(+++ = major, + = minor metabolite)

It is clear that the biologically active Z and 2iP are rapidly metabolised by all systems. The metabolic products of these cytokinins fall into two categories: those where oxidative N^6 side chain cleavage has occurred giving adenine (Ade), adenosine (Ado) AMP and products of purine catabolism (*i.e.*: ureides) and those where conjugation has occurred. Table 1 is arranged so that in descending order N-conjugation increasingly becomes the metabolic fate of exogenously supplied cytokinin.

An enzyme capable of catalysing N^6 side chain cleavage has been partially purified from several sources (PAČES *et al.* 1971, WHITTY and HALL 1974, MCGAW and HORGAN 1983). This enzyme (cytokinin oxidase) is able to utilise a large number of different cytokinin substrates (*i.e.*: bases, N-glycosides, N-alanyl conjugates), but the absence of the Δ^2 double bond (*i.e.*: DHZ) or the presence of a side chain O-glucosyl moiety render the cytokinin resistant to oxidation. The 'protection' afforded to dihydro- and O-glucosyl- compounds is clearly seen (Table 2) in the metabolic studies of PALMER *et al.* (1981b) using detached leaves of *Phaseolus vulgaris* (an 'oxidase type system' — see Table 1). It can be seen that large amounts of ZOG, DHZ and especially DHZOG remained unmetabolised in this system.

At the opposite end of Table 1 is *Raphanus* tissue, the exogenous application of Z or 2iP to which results in almost complete conversion to the corresponding 7-glucosides. Almost no cytokinin oxidase-type metabolism can be observed in this tissue. For this reason *Raphanus* tissue has been employed as a model system for further studies on N-glucosylation *in vitro* (ENTSCH *et al.* 1979) and *in vivo* (PARKER and LETHAM 1973, LETHAM *et al.* 1978). We have recently examined the metabolism of DHZ (MCGAW *et al.* 1984a), ZOG and DHZOG (MCGAW *et al.* 1984b) in derooted *Raphanus* seedlings (Table 2). The purpose of Table 2 is to compare the metabolism of these cytokinins in the oxidase-type *Phaseolus* and the N-conjugation-type *Raphanus* tissues (these are also the only tissues for which such extensive metabolic data are available). It is clear that in both cases the O-glucosides are uniquely stable with significant quantities remaining unmetabolised in both tissues. Where

metabolism does occur it is possible to invoke the involvement of β-glucosidase enzymes in converting the O-glucosides to their corresponding bases (that this does occur in *Raphanus* is supported by the isolation of Z7G and DHZ7G and not ZOG7G or DHZOG7G).

The metabolism of DHZ in these tissues is also interesting. Since this compound is resistant to cytokinin oxidase attack(and is apparently incapable of being oxidised to Z) conjugation provides the only mechanism by which the biological activity of this compound might be controlled. Accordingly O-glucoside conjugation products are the major cytokinin metabolites of DHZ in *Phaseolus*. In *Raphanus* DHZ7G was the predominant metabolite, but the unusual 3- and 9-glucosides were also formed. These are the first examples of a naturally occurring cytokinin to be conjugated in this manner (though benzyl adenine also gives the corresponding 3-, 7- and 9-glucosides in this system (LETHAM *et al.* 1975)).

The Role of Cytokinin Metabolites

The biosynthesis of the biologically inactive N-conjugation products and N^6 side chain cleavage of exogenously supplied cytokinin can be seen as mechanisms designed to modulate the expression of biological activity.

Most tissues (Table 1) appear to adopt one of these approaches; the exception being *Lupinus* where both systems operate. What is the exact role of these N-conjugates? They are not biologically active and their synthesis is not a prerequisite for the expression of biological activity (LALOUE 1977). Metabolic studies with feeds of 9A1Z to *Lupinus luteus* leaves (PARKER *et al.* 1978) and Z7G to *Raphanus* cotyledons (LETHAM *et al.* 1982) (*i.e.*: to tissues where these compounds are known endogenous cytokinins) have shown that these conjugates are remarkably stable in these systems. Similarly 9A1Z (PARKER *et al.* 1978) and Z7G (PARKER and LETHAM 1973), produced as a result of Z feeds to these tissues, are also stable over a prolonged period. There is evidence that limited side chain cleavage of Z7G does occur in *Raphanus* tissues (LETHAM *et al.* 1982), but it is probable that the N-glucosides and N-alanyl conjugates are incapable of hydrolysis back to their corresponding bases *in vivo*. It is unlikely, therefore, that these N-conjugates are cytokinin storage forms. Studies with 'sub-optimal' and 'optimal' levels of benzyl adenine application to excised *Raphanus* cotyledons (LETHAM *et al.* 1982) gave similar proportions of BA3-, 7- and 9-glucosides.These data are inconsistent with N-conjugates being detoxification products. However, it must be stressed that these metabolic studies are essentially artificial, being the result of exogenous application of synthetic cytokinins and is always a possibility that we are observing the presence of certain enzyme systems with which the endogenous cytokinins may never come into contact. Z7G is an endogenous cytokinin in *Raphanus* (SUMMONS *et al.* 1977), nevertheless it is probable that the levels of N-glucosylation observed when we apply exogenous cytokinin do not mimic the controlled metabolic processes thas occur in nature. Conjugation is a common response to xenobiotics in animalt and in plants, but the occurrence of these conjugates as endogenous compounds (and only in tissues where these conjugates are formed in response to exogenous cytokinin application) is unlikely to be coincidental. It is considered, therefore, that these conjugates are probably deactivation or detoxification products.

The discrepancies between the products of externally applied cytokinins and the endogenous cytokinin picture is more obvious in oxidase-type tissues (*i.e.*: high levels of Z and ZR in *Vinca rosea* crown gall tissue (SCOTT *et al.* 1982) yet rapid metabolism of these compounds when they are applied externally (PALNI 1980) is indicative of the failure of these experiments to mimic the endogenous situation. Side chain cleavage of endogenous Z and ZR probably occurs although the rate of cytokinin biosynthesis is unlikely to be sufficient to replenish levels at the rate of oxidation observed in these metabolism experiments. Compartmentation of the cytokinins and their metabolising enzymes is obviously a factor that must be considered when comparing metabolic and quantitation data.

N-conjugation and cytokinin oxidation operate together in *Lupinus* tissues (Table 1). 9A1Z is extremely stable in *Lupinus* (both as a product of exogenously applied Z and when applied itself) yet this compound is susceptible to cytokinin oxidase attack. It is thought that rapid compartmentation of this amino-acid conjugate may be an important factor in its stability. We have fed [8-^{14}C]-Z7G to the 'oxidase-type' *V. rosea* crown gall tissue in order to ascertain whether or not this metabolite (though oxidase labile) has the stability, in these tissues, expected of a general deactivation or detoxification form. 90 % of the applied Z7G was metabolised within 2 days to compounds that co-chromatographed on HPLC with Ade, Ado, Ade7G and AMP.

The role that the O-glucosides play is not yet clear, but there is strong evidence that these compounds may be cytokinin storage forms. Storage forms must obviously fulfil two important criteria: they must be stable in certain conditions yet be readily converted to active forms as required. Evidence of their stability (both when endogenously formed from applied Z and when ZOG was applied itself) comes from feeds to *Raphanus* (MCGAW *et al.* 1981b) and *Lupinus* tissues (PARKER *et al.* 1978). O-glucosides, unlike the N-glucosyl and N-alanyl conjugates, are also metabolised to an appreciable extent in these tissues (Table 2) and others (HORGAN *et al.* 1981), presumably by the action of β-glucosidase enzymes generating the free base. Perhaps more significantly, O-glucoside levels have been observed to rise and fall at different stages of plant development (PALMER *et al.* 1981a). As would also be expected O-glucosides are produced in response to exogenous cytokinin application and are abundant endogenous cytokinins in nearly every system that has been investigated (MCGAW *et al.* 1984c).

The O-glucosides, however, are very active in bioassay (LETHAM *et al.* 1983) — a result not altogether consistent with the putative storage role. This acitivity may be derived from β-glucosidase attack. We have attempted to demonstrate that this is the case by the use of the β-glucosidase inhibitor gluconic acid lactone (GAL) (CONCHIE *et al.* 1967). Firstly it was demonstrated that β-glucosidase hydrolysis of applied ZOG could be inhibited by GAL in *Raphanus*. When GAL was applied at 8.4×10^{-3}M with [8-^{14}C]-ZOG ($1.2 \times \times 10^{-4}$M) the rate of hydrolysis was reduced by 50 % (derooted *Raphanus* seedlings were examined 2 days after the experiment was initiated and the metabolites were purified on HPLC and quantified by scintillation counting). Unfortunately it was not possible to exceed this level of inhibition without seriously damaging the seedlings. With this limitation in mind a *Raphanus*

cotyledon expansion bioassay was set up (LETHAM 1971) using different concentrations of GAL and ZOG. These data are presented in Fig. 1.

The results show that we were unable to reduce the biological activity of the O-glucosides with GAL. The O-glucosides may not be biologically active *per se*, but until a much greater or more specific inhibition of the β-glucosidase enzyme/s can be achieved this view will remain speculative.

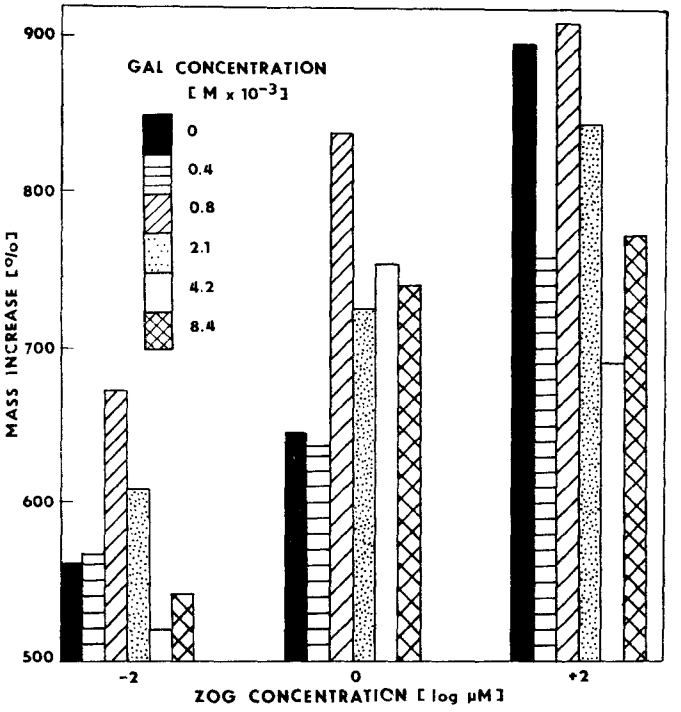

Fig. 1. Percent mass increase of *Raphanus* cotyledons at various concentrations of ZOG and with different concentrations of GAL. The bioassay was adapted from LETHAM (1971); the cotyledons being weighed before and 3 days after the experiment was initiated (10 cotyledons were used at each concentration).

The roles of the cytokinin bases and 9-ribosides are also unclear. Their biological activity and their rapid metabolism on external application suggests that one or other (or both) of these cytokinins may be the active form. The 9-ribosides are major cytokinins in the xylem and phloem sap (LETHAM and PALNI 1983) and it is thought that they may be the translocation forms. It is possible, however, that these ribosides may be artefacts of nucleotide hydrolysis during the extraction procedure. Though it is speculated that the nucleotides may be associated with uptake there is little evidence to support this view. Indeed the nucleotides (perhaps the most abundant endogenous cytokinins) have been ignored by most workers.

CONCLUSIONS

Though much progress has been made in the understanding of the roles of the various cytokinin metabolites the picture is far from clear. Modern

GC/MS instrumentation and internal standard techniques will now enable us to study, for the first time, the interconversions of the endogenous cytokinins during plant development without recourse to the 'artificial' use of exogenously applied material. Ultimately, however, the exact roles of the various cytokinins will only become clear when the compartmentation of the mechanism/s and site/s of cytokinin action are understood.

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