

**EFFECT OF AUXIN ON 6-(BENZYLAMINO)PURINE  
METABOLISM IN SUSPENSION CULTURES**

By

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FRONTISPIECE

*Dianthus zeyheri* subsp. *natalensis* in flower, Pietermaritzburg, Natal.  
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"And life alone has organisation that acts in harmony. It is the hormones that execute that harmonious action. Everywhere directing development elsewhere, they bring the organism along its way with unfaltering timing. Many-voiced, intermingling, accurate on each beat, the parts of the plant command one another's progress, leaf inducing root to form, root controlling the growth of leaves, one organ exciting another yet at the same time inhibiting a third. So from the splitting acorn rises the oak's majestic complexity. Hormones played out the symphony of its growth. But it is life that holds the baton.....

Out of admiration for all this springs the impulse to supplant an older awe with the assertion that the fiat of life is simply chemical. One touch of the right hormones to a plant, and it gushes roots; it lifts the startling finger of the shoot. But paint a rock all over with these lifeless chemicals, and it is barren still."

Donald C. Peattie (1939)  
in 'Flowering Earth'

## PREFACE

I hereby declare that this thesis, submitted for the degree of Doctor of Philosophy in the Faculty of Science, University of Natal, Pietermaritzburg, except where the work of others is acknowledged is the result of my own investigation.

A handwritten signature in blue ink that reads "Neil Crouch". The signature is written in a cursive style with a large initial 'N'.

Neil Robert Crouch

December, 1993

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## ABSTRACT

A review of the literature indicated that the purine cytokinin 6-(benzylamino)purine (BA) may be converted to a wide range of metabolites. Although the functional significance of these metabolites remains obscure, cytokinin physiologists have essentially classed them as either active or inactive. Inactivation of cytokinins is considered to proceed via catabolic oxidation (side-chain cleavage), or N-conjugation with glucose or alanine moieties.

The literature survey was hampered by the confusing array of synonyms which have been coined for cytokinin metabolites. Accordingly, a working system of (semi-systematic) abbreviations was devised which accommodated all groups and classes of purine cytokinins.

Prior to commencing metabolic interactive studies, it was necessary to resolve the contentious issue associated with the successful extraction of cytokinin nucleotides. Five-week-old soybean callus was fed [8-<sup>14</sup>C]BA and subsequently extracted using four widely used cytokinin extraction techniques. Techniques compared were a modified Bielecki method, 80% ethanol with tissue homogenisation, 80% ethanol without homogenisation, and boiling ethanol. All four procedures produced similar results, showing that all metabolites of BA, including the nucleotide, were adequately extracted. It was concluded that the extraction of nucleotides with Bielecki solvents did not warrant the inconvenience.

Auxins have been shown to interact with cytokinins in the regulation of many physiological processes, although little is known of the mechanisms of interaction which proceed at the metabolic level. Previous investigators have shown that auxin promoted cytokinin degradation through catabolic oxidation. Shoot-apex and seed-derived cell suspensions of *Dianthus zeyheri* subsp. *natalensis* were incubated with [8-<sup>14</sup>C]BA for between 30 minutes and 48 hours in the presence of both low (2 mg l<sup>-1</sup>) and high (4 mg l<sup>-1</sup>) levels of exogenously supplied 2,4-dichlorophenoxyacetic acid (2,4-D). In both systems, the auxin 2,4-D was shown to promote BA

inactivation through 7-glucosylation (N-conjugation). This observation represents the first report of auxin-promoted cytokinin N-conjugate formation. The auxin effect on metabolism was transient in the case of shoot-apex, but not in seed-derived systems over a 48 hour period. Formation of the 7-glucoside of BA was dose-dependent in apex-derived cultures. Further studies were undertaken with indole-3-acetic acid (IAA) and  $\alpha$ -naphthaleneacetic acid (NAA). It was found that auxin-promoted 7-glucosylation of BA was only minimally effected by these two auxins.

In comparable studies with soybean suspension cultures (*Glycine max* cv. Acme), 2,4-D-promoted catabolic oxidation was observed between 18 and 48 hours, following application of phytohormones. The main catabolite was tentatively identified as adenosine-5<sup>1</sup>-monophosphate (AMP), based on chromatographic characteristics.

Carrot (*Daucus carota*) cell suspensions similarly supplied with 2,4-D and BA maintained a large active cytokinin pool. Neither substantial oxidative nor N-conjugative processes were observed. Instead, there was a transient effect by 2,4-D on the relative formation of the riboside and the 7- and 9-glucosides of BA.

The effect of auxin on the metabolism of BA thus varied with the species and system investigated. Generally, auxin promoted (rather than inhibited), the formation of inactivated metabolites and catabolites of BA, possibly by the induction of relevant enzyme systems.

Transient auxin effects on the metabolism of BA are discussed in relation to the role of the auxin/cytokinin balance in the induction of developmental processes.

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## GENERAL INTRODUCTION AND OBJECTIVES

The extensive use of plant growth regulators in agriculture, especially auxins in the capacity of rooting promoters or as herbicides, is a well-established practise. Such regulation of growth differentiation and overall plant development is known to involve complex interactions between two or more types of growth substances (WAREING, 1978). In particular, the correlative influence of auxins and cytokinins in the control of apical dominance (MILLER, 1961; WOOLLEY & WAREING, 1972), leaf senescence, transport of metabolites (WICKSON & THIMANN, 1960; OSBORNE & BLACK, 1964; FOX & WEIS, 1965; SETH, DAVIES & WAREING, 1966), cell division (GAZIT & BLUMENFELD, 1970) and morphogenesis in cultured cells and explants (SKOOG & TSUI, 1948; SMIGOCKI & OWENS, 1989) is well documented. Yet much of our knowledge on plant hormone interaction is descriptive rather than causal. In particular, the biochemical and molecular basis of this interaction is poorly known. Only in the last decade has any significant progress been made in this field.

Both normal and disrupted plant development involves interaction of auxins and cytokinins. The use of the hormonal herbicide 2,4-D (2,4-dichlorophenoxyacetic acid) illustrates how compounds are commercially exploited without a full understanding of actual principles and mechanisms of their action. A better understanding of basic physiological processes will likely improve on their safety in use. Greater knowledge of such interactive metabolism will contribute to our ability to modify plant development in both a desirable and controlled manner for the benefit of several disciplines. The field of plant cell culture offers a good example. When a novel system is being studied, the media components required for successful suspensions are currently found only by trial-and-error (DODDS & ROBERTS, 1985) or alternatively, in 'a systematic study' (BLIGNY & LEGUAY, 1987). Amongst ring-substituted aminopurines, BA is the most active cytokinin (MATSUBARA, 1990). Consequently, and in line with its cost-effectiveness, BA is currently the most frequently used compound in micropropagation (THOMAS & BLAKESLEY, 1987).

Knowledge of the metabolic fate of cytokinins, particularly BA with its widespread application, should help to rationalise the use of cytokinins in tissue culture and micropropagation systems. Such knowledge could permit researchers to potentiate the action of known plant hormones and their analogues (PARKER, ENTSCH & LETHAM, 1986), given that plant growth regulators may act as competitive inhibitors blocking hormone inactivation (ZHANG, LETHAM, WONG, NOODÉN & PARKER, 1987). In addition, manipulation of horticultural crops using plant growth regulators would likely be more profitable if hormonal interaction at the metabolic level was better understood.

Hormonal interaction at the physiological level has been classified as synergistic, antagonistic, or qualitative (WAREING, 1978). At the level of metabolism, both synergism and antagonism have been demonstrated, but apparently not qualitative interaction.

As a cytokinin base, BA has recently been found to naturally occur (NANDI, LETHAM, PALNI, WONG AND SUMMONS, 1989), in limited abundance and distribution. This compound is one of more than twenty free cytokinins, which to date have been detected in higher plants. These include those which contain a ring substituent in the  $N^6$ -sidechain (e.g. BA), and cytokinins with an isopentenyl-type sidechain. From a number of studies, it is clear that following exogenous application of BA, only a proportion of the range of BA metabolites are produced by any single system (LETHAM, 1978). Some metabolites may even be restricted taxonomically. It is increasingly clear that different species (BLAKESLEY & CONSTANTINE, 1992), lines, and even organs (BAYLEY, VAN STADEN, MALLETT & DREWES, 1989) metabolise cytokinins differently. Studies at the metabolic level are complicated by a number of factors. Environmental parameters and endogenous phytohormone levels are known to influence the metabolism of exogenously applied cytokinins. Further, the extent to which the metabolism of externally applied cytokinins mimics the metabolic fate of the endogenous compounds is an important problem which has not yet been resolved. Clearly then, studies in the field of hormonal interaction are complex, multivariate investigations. Accordingly, the effect of auxin on BA metabolism in one system would be expected to differ to interactive effects observed in another.

An understanding of the mode of action of phytohormones still seems very distant, despite the intervention of molecular techniques. Current signal transduction hypotheses remain highly speculative. As a prerequisite to understanding the events leading to morphogenesis or any other physiological response, one needs a better knowledge of the uptake, metabolism and subsequent distribution of applied substances (VOGELMANN, BORNMAN & NISSEN, 1984). Without elucidating metabolic conversions in a system and interpreting the significance of the individual metabolites produced, observed interactive effects at the level of metabolism remain meaningless.

Plant cell suspension cultures are useful experimental tools, which have been selected for current investigations. Such cultures are useful model systems for studying secondary metabolism, enzyme induction, gene expression, degradation of xenobiotics (DIXON, 1985), and the metabolism of plant hormones. In the current study, a range of cell suspensions were used to probe the effect of 2,4-D on the metabolism of BA.

On the basis of the above, this work has sought to demonstrate at the metabolic level some reflection of the observed correlative influences of auxins and cytokinins recorded at the physiological level. To achieve this end, the effect exerted by the synthetic auxin 2,4-D on the metabolism of the cytokinin 6-(benzylamino)purine (BA) in four different cell suspensions has been considered.

The main areas of research included:

1. The initiation of suspension cultures of a wild plant species for metabolic comparisons with cultures from domesticated plants;
2. A study to determine the effect of 2,4-D on BA metabolism in soybean cell suspensions, of a line known to exhibit catabolic oxidation of cytokinins;
3. Identical treatment of cell cultures of another domesticated species, carrot, in which no previous hormonal interactive studies have been performed;

4. Comparison of 2,4-D-affected BA metabolism in soybean and carrot (domesticated) systems with effects observed in two cell lines of a wild plant species, *Dianthus zeyheri* Sond. subsp. *natalensis* Hooper;
5. Manipulation of auxin effects in a system where BA is clearly affected by 2,4-D, using inhibitors, other auxin types, and altered auxin/cytokinin ratios.

Chapter 1 reviews the occurrence, distributions, inter-conversions, activities, and proposed roles of the range of metabolites of BA. The effect of auxin on cytokinin metabolism is reviewed separately in the subsequent chapters.

## CHAPTER 1

### LITERATURE REVIEW

#### 1.1 Introduction

This review primarily considers the purine cytokinin 6-(benzylamino)purine (BA) which is still generally considered a synthetic compound, despite its recent identification as a naturally-occurring plant product (NANDI, PALNI, LETHAM & WONG, 1989). To place in context the activity, significance, and metabolism of BA, analogies have been drawn from studies which have involved both naturally-occurring (e.g. 6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)purine (zeatin)) and synthetic (e.g. 6-(furanosylamino)purine (kinetin)) purine cytokinins.

Although the biochemical and physiological effects of cytokinins are well documented (LETHAM & PALNI, 1983) and structure-activity patterns have emerged (VENIS, 1985; MATSUBARA, 1990), their precise action remains unknown. One prerequisite for progress in the understanding of the molecular basis of cytokinin action would seem to be a detailed knowledge of cytokinin uptake and metabolism in plant cells (DOREE & GUERN, 1973). 'Multilevels of experimental approach' have been advocated (CHEN, 1981) for the elucidation of the mechanism of cytokinin action. The determination of the active form(s) of cytokinin is probably the most significant unsolved problem in cytokinin metabolism. It is not even known if cytokinin activity *in vivo* occurs specifically at the level of the base, riboside, or ribotide(s) (TERRINE & LALOUE, 1980). Cytokinins may not be active as such, but only after metabolic transformation into other substances (FOX, DYSON, SOOD & McCHESNEY, 1972). Such substances may not necessarily be cytokinins of another form either, in view of the limited success (BRINEGAR, COOPER, STEVENS, HAUER, SHABANOWITZ, HUNT & FOX, 1988) of hormone receptor studies to date.

Although application of a cytokinin metabolite to a bioassay system may promote an

active response, each of these 'active' compounds are themselves rapidly metabolised to an extensive range of products, many of which are deemed active in the same bioassays (VAN STADEN & DREWES, 1991). MOK, MARTIN, MOK and SHAW (1992) observed that the activity of a particular cytokinin may depend on the bioassay system used. Activity differences between metabolites were attributed by these authors to reflect uptake, compartmentation, sensitivities to enzymes, or binding site specificities; in different bioassays these components may change, so altering the effectiveness of particular metabolites in inducing a response. In an alternative experimental approach, studies of endogenous cytokinin levels during different phases of plant growth (PETERS & BECK, 1992) have provided insight into *in vivo* biological activity. The further manipulation of endogenous cytokinin levels (in transformed plants) has also been considered (AINLEY, McNEIL, HILL, LINGLE, SIMPSON, BRENNER, NAGAO & KEY, 1993) to be more revealing of natural processes than exogenous applications to isolated organs or calli.

Plant tissues convert exogenous BA into a great diversity of metabolites which include products of ring substitution (ribosides, nucleotides, N-glucosides), and products of sidechain cleavage (e.g. adenine (Ade), adenosine (Ado), and adenosine-5<sup>1</sup>-monophosphate (AMP)) (LETHAM & PALNI, 1983).

The functional significance of these metabolites remains obscure (WAGNER & BECK, 1993), but it has been suggested (LETHAM & PALNI, 1983) that these compounds could be:

1. Active forms of cytokinin, i.e. the molecular species which bind to a receptor to evoke a growth or physiological response;
2. Translocation forms;
3. Storage forms which would release free cytokinin when required;
4. Detoxification products formed following exogenous cytokinin application at

toxic levels;

5. Deactivation products formed to lower endogenous (active) cytokinin levels; and
6. Inactivation products, formation of which is coupled with cytokinin action (formed as a result of cytokinin utilisation).

McGAW and HORGAN (1985) observed that an understanding of compartmentation with respect to the mechanisms and sites of cytokinin action needs to occur before the exact roles of various cytokinins may be assigned. Until this knowledge is obtained and activities can be measured directly at the site of action (MOK, MARTIN, MOK & SHAW, 1992), prescribed roles will remain mainly speculative.

CHEN (1981) considered fundamental control mechanisms to be those operating at the level of enzymic regulation of metabolism (biosynthesis, interconversion, and degradation). Several major enzymic pathways compete for cytokinins, by which they are inter-converted and degraded (CHEN, 1981). BURCH AND STUCHBURY (1987) noted that enzymes metabolising Ade derivatives (CHEN & KRISTOPEIT, 1981; 1981a; CHEN, MELITZ & CLOUGH, 1982) exhibit a low degree of specificity for the exact structure of the purine ring and hence the same enzymes will actively metabolise  $N^6$ -substituted cytokinins. The fate of a cytokinin may be attributed to the relative activities of cytokinin metabolic enzymes, which in turn are affected by the relative concentrations and distribution of the hormone and its precursors in the plant cell (CHEN, 1981). Given the lack of specificity of some cytokinin-metabolising enzymes, metabolism of cytokinins may be limited by competition for the enzymes. Hence BURCH and STUCHBURY (1987) were led to state that 'interpretation of many aspects of cytokinin biochemistry is dependent on a much better understanding of the relationship of their metabolism to that of other purines'.

Much is known of cytokinin metabolism, but no common metabolic pattern has emerged. Several factors may have contributed to this complexity. The stage of plant development (DAVEY & VAN STADEN, 1976; LEE, MOK, MOK, GRIFFIN & SHAW, 1985;

BURCH & STUCHBURY, 1987), physiological condition (PALMER, HORGAN & WAREING, 1981; FUBEDER, ZIEGLER, PETERS & BECK, 1989), organ type (BURCH & STUCHBURY, 1987; BAYLEY, VAN STADEN, MALLETT & DREWES, 1989), plant species used (BLAKESLEY & CONSTANTINE, 1992), concentration of supplied compounds (VAN STADEN, 1983), and method of application (VAN STADEN & MALLETT, 1988) have all been shown to affect the metabolism of exogenous and endogenous cytokinins.

In the following survey, the occurrence, distribution, activity, enzymatic formation, metabolic inter-conversions, and roles of the metabolites of BA are reviewed:

## 1.2 Inter-conversions within the active cytokinin pool

The free base, nucleoside and nucleotide forms of cytokinins all appear to be readily inter-convertible in plant tissues (LETHAM & PALNI, 1983; VAN STADEN & BAYLEY, 1991). These cytokinin species are considered the functional forms (LETHAM, TAO & PARKER, 1982). The early formation of the 9-riboside ([9R]BA) and 9-ribotide of BA ([9R-MP]BA) by many systems as the principal metabolites of BA (FOX, DYSON, SOOD & MCCHESENEY, 1972; LETHAM, TAO & PARKER, 1982; VAN STADEN & MALLETT, 1988; AUER, LALOUE, COHEN & COOKE, 1992a; NIEDERWIESER, VAN STADEN, UPFOLD & DREWES, 1992; VAN STADEN & DREWES, 1992) could be a mechanism for maintaining a high active cytokinin pool. This would ensure a continued supply of precursors for subsequent conversion to the base (or active form). Di- and tri-nucleotides of cytokinins do not appear to contribute significantly to this active pool (LALOUE, TERRINE & GAWER, 1974).

Should the biosynthetic pathway for BA, like that for 6-(3-methylbut-2-enylamino)purine (iP), proceed at the nucleotide level (TAYA, TANAKA & NISHIMURA, 1978), then subsequent conversion to base and riboside would be expected. Cytokinin bases can be continuously catabolised by various enzymes to form Ade and other degradation compounds (MCCALLA, MORRÉ & OSBORNE, 1962; FORSYTH & VAN STADEN, 1986), resulting in a loss of cytokinin base. Such a deficiency may necessarily be replenished in order to maintain the levels of available cytokinin (the so called 'active form') (CHEN & KRISTOPEIT, 1981). De-glucosylation (ESTRUCH,

CHRIQUI, GROSSMANN, SCHELL & SPENA, 1991), de-alanylation (ZHANG & LETHAM, 1989), deribosylation (CHEN & KRISTOPEIT, 1981a), and de-phosphoribosylation (VAN STADEN & DREWES, 1992a) would provide the needed cytokinin base.

The enzymes catalysing inter-conversions within the active pool are likely not cytokinin-specific, but rather those which catalyse analogous reactions for Ade, Ado, and AMP (LETHAM & PALNI, 1983; MOFFATT, PETHE & LALOUÉ, 1991). Other workers (VAN STADEN & DREWES, 1992a) have viewed such enzymes as cytokinin-specific.

BURCH and STUCHBURY (1987) listed a series of reactions and the enzymes responsible for their inter-conversions (Table 1.1). The analogous conversions of the cytokinin 6-(benzylamino)purine are also shown.

Two mechanisms for the incorporation of purines into nucleotides have been proposed:

1. A two-step process involving first nucleoside phosphorylase to yield a cytokinin riboside and then adenosine kinase to catalyse nucleotide formation.
2. A one-step process involving a direct transfer of a ribose-5<sup>1</sup>-monophosphate group from  $\alpha$ -5-phosphoribosyl-1-pyrophosphate (PRPP) to the base, catalysed by an adenine phosphoribosyltransferase.

A one-step phosphoribosylation is not universally accepted. Evidence exists to support both the one-step (DOREE & GUERN, 1973; LALOUÉ & PETHE, 1982) and two-step (VAN STADEN, BAYLEY, UPFOLD & DREWES, 1990) pathways. The two-step route may be limited by the restricted occurrence of nucleoside phosphorylase, rather than by adenosine kinase activity, which appears ubiquitous in plants (DOREE & TERRINE, 1973).

Table 1.1 Inter-conversions within the active cytokinin pool, catalysed by non-specific enzymes of adenine metabolism

Enzyme	Class	Reaction catalysed for Ade	Analogous BA conversion
5 <sup>1</sup> -nucleotidase	EC 3.1.3.5	$\text{AMP} + \text{H}_2\text{O} \rightarrow \text{Ado} + \text{Pi}$	$[\text{9R-MP}]\text{BA} + \text{H}_2\text{O} \rightarrow [\text{9R}]\text{BA} + \text{Pi}$
Adenosine nucleosidase	EC 3.2.2.7	$\text{Ado} + \text{H}_2\text{O} \rightarrow \text{Ade} + \text{ribose}$	$[\text{9R}]\text{BA} + \text{H}_2\text{O} \rightarrow \text{BA} + \text{ribose}$
Adenine phosphoribosyl transferase	EC 2.4.2.7	$\text{Ade} + \text{PRPP} \rightarrow \text{AMP} + \text{PPi}$	$\text{BA} + \text{PRPP} \rightarrow [\text{9R-MP}]\text{BA} + \text{PPi}$
Adenosine phosphorylase	EC 2.4.2.1	$\text{Ade} + \text{R-1-P} \rightarrow \text{Ado} + \text{Pi}$	$\text{BA} + \text{R-1-P} \rightarrow [\text{9R}]\text{BA} + \text{Pi}$
Adenosine kinase	EC 2.7.1.20	$\text{Ado} + \text{ATP} \rightarrow \text{AMP} + \text{ADP}$	$[\text{9R}]\text{BA} + \text{ATP} \rightarrow [\text{9R-MP}]\text{BA} + \text{ADP}$

Pi = inorganic phosphate

PPi = inorganic diphosphate

R-1-P = ribose-1-phosphate

PRPP =  $\alpha$  5-phosphoribosyl-1-pyrophosphate

### 1.2.1 Conversion of cytokinin base to riboside (BA → [9R]BA)

Ribonucleosides have been shown to be formed in plant tissues when the corresponding base was supplied (SONDHEIMER & TZOU, 1971). The significance of adenosine phosphorylase activity in purine salvage reactions has been the subject of considerable debate. Phosphoribosylation catalysed in a single step by adenine phosphoribosyltransferase (APRT) is viewed as the main pathway (LEE & MOFFATT, 1993). However, in APRT-lacking mutants of *Arabidopsis* Heynh., limited formation of [9R-MP]BA revealed some activity of the two-step reaction involving adenosine phosphorylase (MOFFATT, PETHE & LALOUE, 1991).

Although detected in bacterial systems (HOCHSTADT-OZER & STADTMAN, 1971; SENESI, FALCONE, MURA, SGARRELLA & IPATA, 1976), the occurrence of adenosine phosphorylase in plants was initially questioned (DOREE & TERRINE, 1973), and is still viewed by some researchers (BURCH & STUCHBURY, 1987) as limited in distribution. This enzyme was purified from wheat germ cells by CHEN and PETSCHOW (1978). Conversion of base to the riboside requires the addition of ribose-1-phosphate. In the presence of inorganic phosphate, phosphorolysis of nucleosides occurs (SENESI, FALCONE, MURA, SGARRELLA & IPATA, 1976; CHEN, 1981). However, CHEN and PETSCHOW (1978) noted that the equilibrium constants for the phosphorolysis of [9R]iP and iP indicate that nucleoside formation is the favoured reaction. CHEN (1981) suggested that purine nucleosidase was the enzyme catalysing cytokinin nucleoside formation, as distinct from purine nucleoside phosphorylase which is generally considered to be inactive towards Ade, Ado, and cytokinin nucleosides (ZIMMERMAN, GERSTEN, ROSS & MIECH, 1971). SENESI, FALCONE, MURA, SGARRELLA and IPATA (1976) were the first to clearly distinguish adenosine phosphorylase from purine nucleoside phosphorylase. Adenosine was not a substrate for purine nucleoside phosphorylase, unlike the nucleosides of hypoxanthine and guanine.

### 1.2.2 Conversion of riboside to base ([9R]BA → BA)

Conversion of the ribotide to the base may involve a dephosphorylation to the free

riboside (VAN STADEN, 1973; LALOUE, PETHE-TERRINE & GUERN, 1981) or may be a direct (reversible) dephosphoribosylation (MIERNYK & BLAYDES, 1977). Cytokinin base has been reported as a metabolite formed from the nucleoside (PAČES, 1976; VAN STADEN & BAYLEY, 1991), and may represent an activation step (ROLLE & CHISM, 1989).

The product of the hydrolytic nucleosidase from *Lactobacillus pentosus* was shown by WANG (1955) to be the purine, and the free ribose. This author did not expect this hydrolytic enzyme to exist because of the wide distribution of the phosphorolytic nucleosidase in animal tissues and in micro-organisms. WHITTY and HALL (1974) termed this enzyme of WANG (1955) 'purine nucleoside hydrolase'.

Adenosine nucleosidase catalyses the irreversible deribosylation of Ado, to give Ade and ribose (BURCH & STUCHBURY, 1986). Three separate adenosine nucleosidase enzymes were partially purified from tomato roots and leaves (BURCH & STUCHBURY, 1986). These workers found the conversion of Ado to Ade to be inhibited by the presence of [9R]BA, with substantial differences in the pattern of inhibition evidenced for each of the three enzymes. Earlier, CHISM, LONG and ROLLE (1984) distinguished between cytokinin nucleosidases and adenosine nucleosidases in tomato fruits. When the  $N^6$ -amino group of Ado was replaced by an isopentenyl amino sidechain in substrates of adenosine nucleosidase, the  $K_m$  value of the reaction was decreased by a factor of 1.7 (CHEN & KRISTOPEIT, 1981). The cytokinin base, BA, appeared a suitable substrate in this reaction, as the adenosine nucleosidase exhibited a specificity for Ado and  $N^6$ -derivatives of Ado. From wheat germ cells, a partially purified adenosine nucleosidase (EC 3.2.2.7) catalysed the irreversible hydrolysis of the riboside of iP ([9R]iP) to iP, and Ado to Ade (CHEN, 1981; CHEN & KRISTOPEIT, 1981). The activity of such nucleosidases appears to depend on the plant tissue investigated (PAČES, 1976; TERRINE & LALOUE, 1980). Significant differences in adenosine nucleosidase activity were detected between wild-type and domesticated plant species (LESZCZYNSKA, SCHNEIDER, TOMASZEWSKI & MACKOWIAK, 1984).

Adenosine nucleosidase activity has also been detected in soybean (MILLER & EVANS,

1955), beet (POULTON & BUTT, 1976), and barley leaves (GURANOWSKI & SCHNEIDER, 1977).

### 1.2.3 Conversion of riboside to nucleotide ([9R]BA → [9R-MP]BA)

Enzymic preparation of mono-nucleotides from  $N_6$ -substituted adenosines and ATP is catalysed by adenosine kinase (EC 2.7.1.20). Such activity has been found in both yeasts and higher plants (DOREE & TERRINE, 1973). These authors demonstrated adenosine kinase activity in buds of *Cicer arietinum* L. and in suspension-cultured cells of *Acer pseudoplatanus* Falk.

Time course studies with *Phaseolus vulgaris* L. (RAMINA, 1979) and *Dianthus caryophyllus* (VAN STADEN, BAYLEY, UPFOLD & DREWES, 1990) have indicated formation of cytokinin nucleotide from the corresponding nucleoside. More direct evidence was provided by CHEN and ECKERT (1977) who reported that cytokinin nucleoside could be converted to the nucleotide (5<sup>1</sup>-monophosphate) by adenosine kinase isolated from wheat germ cells. The phosphorylation of [9R]iP depended upon the presence of ATP and Mg<sup>2+</sup>. The enzyme activity responsible for such synthesis was considered by DOREE & TERRINE (1973) to be ubiquitous in plants.

### 1.2.4 Conversion of nucleotide to riboside ([9R-MP]BA → [9R]BA)

Following application to soybean callus, [9R-MP]Z was rapidly metabolised to both the riboside and the base (VAN STADEN & DREWES, 1992a). Ribonucleosides can be formed from the corresponding ribonucleotide or from the cytokinin base (CHEN, 1981; SKOOG & ARMSTRONG, 1970). Should the one-step pathway of nucleotide synthesis be dominant, then conversion of applied cytokinin base to the riboside may proceed through phosphoribosylation of the base to the nucleotide, followed by conversion to the riboside (CHEN & PETSCHOW, 1978).

Conversion of cytokinin ribonucleotide to its nucleoside may be catalysed by 5<sup>1</sup>-nucleotidase (CHEN & KRISTOPEIT, 1981a). Such conversions have been indicated

by time-course studies (MARTIN, 1969) and demonstrated during *in vitro* investigations (CHEN, 1981). This cytosolic enzyme consists of at least two forms, the F-1 and F-2 5<sup>1</sup>-nucleotidases which specifically hydrolyse purine ribonucleoside-5<sup>1</sup>-phosphates (CHEN, 1981). Notably, 5<sup>1</sup>-nucleotidases have been reported (CHEN & KRISTOPEIT, 1981a) to show almost equal affinity toward the mono, di- and tri-phosphates of Ado (AMP, ADP and ATP).

The extent of dephosphorylation of cytokinin ribonucleotide in plant cells by acid phosphatase and membrane-bound 5<sup>1</sup>- nucleotidase remains to be investigated (CHEN & KRISTOPEIT, 1981a).

#### 1.2.5 Conversion of base to nucleotide (BA → [9R-MP]BA)

Quick metabolism of base to nucleotide has been demonstrated for both lower (ERICHSEN, KNOOP & BOPP, 1978) and higher plants (GORDON, LETHAM & PARKER, 1974; MIERNYK & BLAYDES, 1977). A one-step purine salvage reaction catalysed by adenine phosphoribosyltransferase (APRT) is seen as the predominant pathway in plants (MIERNYK & BLAYDES, 1977; MOFFATT, PETHE & LALOUÉ, 1991), as this enzyme activity is high enough to account for the salvage of Ade into AMP. Further, some researchers (LEE & MOFFATT, 1993) maintain, despite the work of CHEN and PETSCHOW (1978), that the presence of adenosine phosphorylase in plants has not been unequivocally demonstrated. From a time-course study on *Acer pseudoplatanus* cell cultures, DOREE and GUERN (1973) provided evidence to show that the synthesis of N<sub>6</sub>-substituted nucleotides does not proceed through a two-step reaction, but rather through the direct transfer of ribose-5<sup>1</sup>-monophosphate. 6-(Benzylamino)purine was a suitable substrate. Their interpretation was supported by *in vitro* studies of enzymic systems using [7-<sup>14</sup>C]BA as substrate.

Extracts from soybean (cv. Acme) similarly yielded APRT activity, as did senescing barley (*Hordeum distichon* L. cv. Prior) leaves (NICHOLLS & MURRAY, 1968). This enzyme from soybean was inhibited by AMP (product feedback inhibition), and stimulated by ATP. The monophosphate of BA ([9R-MP]BA) was also found to inhibit AMP production, though not to the same extent (13 vs. 92%). Soybean callus

contacting kinetin in the agar medium was shown to have increased APRT activity (NICHOLLS & MURRAY, 1968).

Adenine phosphoribosyltransferase has been partially purified from tobacco pith tissue cultures (CHEN & ECKERT, 1977) and from wheat cells (CHEN, 1981). This enzyme was also extracted and partially purified from Jerusalem artichoke shoots (LE FLOC'H, LAFLEURIEL & GUILLOT, 1978; 1982). Phosphate ions and thiol-reducing substances were required to stabilise it.

However, CHEN (1981) showed that in wheat germ cells, cytokinin nucleotide is not preferentially formed by this one-step pathway. This author suggested this after finding a high  $K_m$  for iP. KRENITSKY, PAPIOANNOU and ELION (1969) had earlier reported that the enzyme binds to adenine through the 6-amino group and the 3- and 7-nitrogens. CHEN (1981) was thus not surprised to find iP (an adenine analogue with a modified 6-amino group) to show reduced ability as a substrate. This author suggested that a different form of this enzyme may exist for cytokinin bases in wheat. A later investigation revealed that APRT from the cytosol of wheat germ was capable of phosphoribosylating BA (CHEN, MELITZ & CLOUGH, 1982). However, the ratio of  $V:K_m$  indicated that Ade is approximately two-fold more efficient than BA as a substrate. LEE and MOFFATT (1993) have more recently purified and characterised an APRT from *Arabidopsis thaliana* which catalysed phosphoribosylation of BA. However, it was again not possible to fully resolve the physiological role of APRT with respect to BA.

In summary, the base, riboside and nucleotides of cytokinins appear to be readily inter-convertible within plant tissues. Enzymes responsible for catalysing these reactions are not likely to be cytokinin-specific, although in some tissues specific enzymes may be present (CHISM, LONG & ROLLE, 1984). The extent to which one-step or two-step phosphoribosylation of cytokinin bases occurs appears to be a function of the plant system investigated.

### 1.3 The metabolites of 6-(benzylamino)purine

#### 1.3.1 BA, the base

The purine cytokinin 6-(benzylamino)purine has been shown to affect growth of both animal (BECKER & ROUSSAUX, 1981) and plant (MATSUBARA, 1990) cells. The base BA is an adenine derivative with a substitution on the sixth position of the purine nucleus (SKOOG & ARMSTRONG, 1970). Recently, this cytokinin was found as a free, naturally occurring cytokinin (NANDI, LETHAM, PALNI, WONG & SUMMONS, 1989). This compound has been shown to affect plant metabolism in the capacity of a growth regulator (cytokinin), with the result that a wide variety of physiological responses have been recorded.

Besides delaying senescence (FLETCHER, 1969; GILBERT, THOMPSON & DUMBROFF, 1980), including both floral (VAN STADEN, BAYLEY, UPFOLD & DREWES, 1990) and monocarpic senescence (LINDOO & NOODÉN, 1978), BA promotes chlorophyll retention (RUSHING, 1990) and formation (DEI, 1983). This cytokinin has been shown to enhance photosynthetic activity (LEOPOLD & KAWASE, 1964; ADEDIPE, HUNT & FLETCHER, 1971; CAERS, RUDELSHEIM, VAN ONCKELEN & HOREMANS, 1985) and reduce the respiration rate (RUSHING, 1990). The application of BA has resulted in increased shoot to root ratios (KUIPER, KUIPER, LAMBERS, SCHUIT & STAAL, 1989), increased production of ethylene (RUSHING, 1990), lowered stomatal resistance (GARRISON, BRINKER & NOODÉN, 1984), promotion of leaf expansion (SCOTT & LIVERMAN, 1956), and stimulated protein synthesis (SZWEYKOWSKA, GWOZDZ & SPYCHALA, 1981). Adverse environmental conditions have been counteracted through use of BA, including heat stress (CAERS, RUDELSHEIM, VAN ONCKELEN & HOREMANS, 1985), although it was not shown whether this was due to BA itself or via an increase in the natural cytokinin levels. Similarly, a stimulative effect of BA on plant mineral nutrition (KUIPER, KUIPER, LAMBERS, SCHUIT & STAAL, 1989) was associated with an effect on the levels of endogenous cytokinins.

Applied as a cytokinin base, BA is currently the most frequently used compound in

micropropagation (THOMAS & BLAKESLEY, 1987). However, when applied in field trials, BA showed disappointing cytokinin-like activity in delaying senescence in field crops (DYBING & LAY, 1981). ZHANG, LETHAM, WONG, NOODÉN and PARKER (1987) suggested that the 'design' of cytokinins which are more field-effective than BA 'would be facilitated by a study of the metabolism of BA'.

MORRIS (1981) provided results to suggest that kinetin applied to roots may be converted to an endogenous cytokinin before export to the shoot. However, within elm shoots, [8-<sup>14</sup>C]BA was largely transported in the un-metabolised state (BIONDI, CANCIANI & BAGNI, 1984). Following metabolic conversion to [9R]BA, the riboside is generally considered the translocatory cytokinin species (HORGAN, 1984; WAGNER & BECK, 1993). Despite the report on BA transport in elms, research on the potential conversion of BA to endogenous cytokinin may prove profitable.

Much circumstantial evidence derived from bioassays exists to indicate that as a base, BA is the active cytokinin form (MILLER, 1968). MATSUBARA (1990) considered BA to be the most active cytokinin in the class of ring-substituted aminopurines. Cytokinin-binding protein studies (CHEN & KRISTOPEIT, 1981) have more directly implicated cytokinin bases as one of the active forms. Although BA is assumed to be active *per se*, there is no evidence to support this proposition (WILSON, GORDON, LETHAM & PARKER, 1974; VAN STADEN & HUTTON, 1982). To date, this issue has not been unequivocally resolved.

LALOUE and PETHE (1982) presented results on growth studies with tobacco cell cultures which indicated that conversion of cytokinin ribosides to bases is necessary for activity. Uptake of exogenously supplied BA by a variety of experimental systems was mostly linear in relation to the external BA concentration, suggesting a passive role (LAMPUGNANI, FANTELLI, LONGO, LONGO & ROSSI, 1981; MINOCHA & NISSEN, 1982; VOGELMANN, BORNMAN & NISSEN, 1984). However, uptake of cytokinin base has also been related to the rate of cytokinin metabolism inside the cell (DOREE, TERRINE & GUERN, 1972 in DEKHUIJZEN, BOSSEN & VONK, 1978).

In the soybean callus bioassay (MILLER, 1968), BA gave an optimum response when applied at a concentration of between  $10^{-6}$  and  $10^{-5}$  M (VAN STADEN, 1983). Activity in a similar range has been recorded for zeatin (VAN STADEN & PAPAPHILIPPOU, 1977). VAN STADEN (1973a) compared the activities of BA, [9R]BA and [9R-MP]BA in the same bioassay system. Of these three, BA appeared most active, with the riboside more active than the nucleotide. This author suggested that the applied cytokinins might not have been taken up by the tissue at the same rate, or that differences in the metabolism of these metabolites occurred. In this case, BA may have been taken up quicker, or metabolised more slowly if active *per se*, or quickly converted to the 'active form' of cytokinin. Problems associated with the exogenous application of cytokinins to plant systems are further discussed in Chapter 3.4.

HECHT, FRYE, WERNER, HAWRELAK, SKOOG and SCHMITZ (1975) reported that both the nucleoside and nucleotide were less active than the corresponding base. Their findings led them to suggest that exogenous bases do not require activation before the expression of cytokinin activity. Similarly, LALOUÉ, TERRINE and GUERN (1977) recorded that iP was three times as active as the corresponding nucleoside in a tobacco callus bioassay. However, MOK, MOK and TURNER (1985) reported that reversed activities of cytokinin bases and nucleosides were detected with iP and [9R]iP in some *Phaseolus* callus cultures.

PETERS and BECK (1992) reasoned that cell division-controlling substances would be expected in highest concentrations during the logarithmic phase of cell culture growth. Yet, at the start of the log phase in *Chenopodium* cell suspensions, free bases were detected in low concentrations, suggesting that bases may not be involved in the regulation of cell division activity. However, other researchers (HENDRY, VAN STADEN & ALLAN, 1982) have been of the opinion that low non-polar cytokinin levels do not necessarily reflect cytokinin inactivity in tissue, but rather implies their active metabolism, coupled to cytokinin action. Future research which considers endogenous cytokinin levels before, during, and after physiological responses, may yet result in a re-evaluation of the concept of 'cytokinin activity', and the molecular species associated with it.

### 1.3.2 BA riboside, [9R]BA

The 9-riboside of BA ([9R]BA) has been found as a naturally-occurring cytokinin in anise plant cells (ERNST, SCHÄFER & OESTERHELT, 1983). Following exogenous application of BA, [9R]BA has been identified as a prominent (DYSON, FOX & McCHESNEY, 1972) and sometimes dominant metabolite (McCALLA, MORRÉ & OSBORNE, 1962; GUERN, DOREE & SADORGE, 1968; WOOLLEY & WAREING, 1972a), extracted from a variety of species. The analogous riboside of zeatin ([9R]Z) was the main detectable metabolite when zeatin was supplied to detached leaves of *Xanthium strumarium* L. (HENSON & WAREING, 1977). In *Vinca rosea* crown gall tissue, [9R]Z and the corresponding O-glucoside ((OG)[9R]Z) were found by mass spectrometric technique (SCOTT, MARTIN, HORGAN & HEALD, 1982) to be the most abundant natural cytokinins. Other systems have been recorded as metabolising applied BA quite differently. Conversion of BA to [9R]BA was almost negligible in radish seedlings (WILSON, GORDON, LETHAM & PARKER, 1974).

The 9- $\beta$ -D-ribonucleosides of  $N^6$ -adenine derivatives have been synthesised and tested (LEONARD, HECHT, SKOOG & SCHMITZ, 1968; 1969). Their activity in the tobacco bioassay was not as high as the corresponding base. Metabolites of BA substituted at position 9 on the purine ring were less active than the base in the soybean bioassay (FOX, SOOD & McCHESNEY, 1973). These authors attributed this lower activity to the difficulty which the tissues may have in converting such compounds to the base. Riboside degradation through isoprenoid sidechain cleavage (resulting in Ado formation) has been correlated with the weak activity of [9R]iP in some tissues (*P. vulgaris* cv. Great Northern) (MOK, MOK, DIXON, ARMSTRONG & SHAW, 1982). Tissues of *P. lunatus* L. cv. Kingston converted the unsaturated riboside to the corresponding nucleotide, and activity was maintained. This investigation highlights the prominent intra-specific differences in cytokinin metabolism which occurs naturally.

The free base BA was among metabolites formed from the 9-substituted cytokinin, 6-benzylamino-9-methyl purine, suggesting that the biological activity of 9-substituted cytokinins could be accounted for by their conversion to the free base (Fox, Sood,

BUCKWALTER & McCHESNEY, 1971). Other reports confirm the relatively low activity of cytokinin ribosides, both natural and synthetic, in a variety of bioassay systems (HECHT, BOCK, SCHMITZ, SKOOG, LEONARD & OCCOLOWITZ, 1971; SCHMITZ, SKOOG, HECHT, BOCK & LEONARD, 1972; VAN STADEN, 1973a; WHITACKER & KENDE, 1974; SPIESS, 1975; LALOUE, TERRINE & GUERN, 1977; MATSUBARA, SHIOJIRI, FUJII, OGAWA, INAMURA, YAMAGISHI & KOSHIMIZU, 1977; LETHAM, 1978). Generally, a second substituent in the 9-position of  $N^6$ -substituted purines lowers, but does not eliminate cytokinin activity (SKOOG, HAMZI, SZWEYKOWSKA, LEONARD, CARRAWAY, FUJII, HELGESON & LEOPPKY, 1967; MATSUBARA, 1990).

Not all researchers consider the riboside less active than the corresponding base. BOPP and ERICHSEN (1981) viewed observed differences as more a consequence of restricted uptake than of an inefficiency of the substance. PETERS and BECK (1992) have recently considered the endogenous cytokinin patterns at all growth stages of a *Chenopodium* cell culture. These authors found that cytokinin ribosides likely control cell division, more so than free bases which have traditionally been considered the active form. It remains to be determined whether activity resides in the ribonucleosides, or is acquired only on conversion to their bases. Most evidence to date has supported the latter concept (FOX, SOOD, BUCKWALTER & McCHESNEY, 1971).

Cytokinin ribosides are generally considered the translocatory species (PALMER, HORGAN & WAREING, 1981a). Trans-membrane transport of the 9-riboside of kinetin ([9R]KIN) was determined by VAN STADEN and MOONEY (1987), using *Catharanthus roseus* (L.) G. Don crown gall callus. Earlier, LALOUE, PETHE-TERRINE and GUERN (1981) had shown ready uptake of [9R]BA by tobacco cells. Movement of cytokinins within the whole plant also occurs at the riboside level. Ribosides have been detected in the xylem sap of several species, including *Urtica* L. (WAGNER & BECK, 1993), *Phaseolus* (RAMINA, 1979), and radish (GORDON, LETHAM & PARKER, 1974). After exogenous application of BA, [9R]BA was found in senescing *Xanthium pensylvanicum* Wallr. leaves as a major product (McCALLA, MORRÉ & OSBORNE, 1962). Such evidence strengthens the view (LETHAM & PALNI, 1983; HORGAN, 1984) that the riboside is a translocatory form which is exported along with other important compounds from

leaves prior to senescence. Following supply of  $^{14}\text{C}$ -BA to *Phaseolus vulgaris* plant roots, only [9R]BA was detected in xylem sap collected from the stem (RAMINA, 1979). Similarly, the riboside was the only significant source of radioactivity in the xylem sap of radish seedlings after application of  $^{14}\text{C}$ -Z (GORDON, LETHAM & PARKER, 1974). These findings indicate that acropetal translocation of cytokinins is in the riboside form. Notably, ribosides are also major cytokinins in the phloem sap (LETHAM & PALNI, 1983), indicating that the transport of ribosides may also be basipetal in character.

A storage function for nucleosides has also been implied. A cytokinin riboside ([9R]Z) was detected in the (storage) roots of chicory by BUI-DANG-HA and NITSCH (1970). It was not determined whether this riboside was synthesised *in situ* or merely stored there.

### 1.3.3 BA nucleotide, [9R-MP]BA

Following treatment of tissues with BA, [9R-MP]BA has been identified as a metabolite of BA in *Lemna minor* L. plants (BEZEMER-SYBRANDY & VELDSTRA, 1971), and soybean callus (FOX & CHEN, 1967), and the main metabolite in *Acer pseudoplatanus* cell cultures (DOREE & GUERN, 1973). The monophosphate of BA has appeared relatively stable, as shown by its metabolic half-life in tobacco cells (8 days) (LALOUÉ, PETHE-TERRINE & GUERN, 1981). In other systems which initially produced cytokinin mono-nucleotide as the principal metabolite, levels rapidly became sub-dominant (GORDON, LETHAM & PARKER, 1974).

LALOUÉ and PETHE (1982) considered cytokinin riboside-5<sup>1</sup>-phosphates to play a central role in the regulation of the levels of the various metabolic forms of cytokinins as they are readily interconverted to the riboside, and to the base. A role of cytokinin mono-nucleotides in hormonal homeostasis is generally accepted (PALMER, LETHAM & GUNNING, 1984). The main feature of inter-conversion pathways in this active cytokinin pool is that their overall equilibrium is in favour of nucleotide formation (LALOUÉ, PETHE-TERRINE & GUERN, 1981), although such conversions may involve a

steady state maintenance of base and riboside levels (LALOUE & PETHE, 1982). When cytokinin-dependent soybean callus was fed [9R-MP]Z (VAN STADEN & DREWES, 1992a), only the corresponding base and nucleoside were produced, in support of this homeostatic notion. LALOUE, PETHE-TERRINE and GUERN (1981) claimed that nucleotide isolation and identification has been neglected. These workers proposed that more attention should be paid to ribotides as naturally occurring cytokinins with a central role. A later report by SCOTT and HORGAN (1984) which employed mass spectrometric techniques has shown that cytokinin nucleotides may be more abundant than has been previously shown. These authors demonstrated that the nucleotide is more abundant than the riboside in tissues where this was previously seen to be otherwise. SCOTT and HORGAN (1984) predicted that the application of 'new analytical techniques for cytokinin nucleotides will result in an extensive re-evaluation of the existing cytokinin literature.' Such a re-evaluation has not yet occurred.

According to ASHIHARA (1983), purine nucleotides are synthesised both from amino acids, CO<sub>2</sub>, tetrafolate derivatives and  $\alpha$ -5- phosphoribosyl-1-pyrophosphate (PRPP) (the *de novo* pathway) and from preformed purine bases and their ribonucleosides (the salvage pathway). Nothing is known of BA biosynthesis in plants (KAMINEK, 1992). Should biosynthesis of 6-(benzylamino)purine proceed at the nucleotide level, as with isopentenyl-type cytokinins (TAYA, TANAKA & NISHIMURA, 1978; LETHAM & PALNI, 1983), then [9R-MP]BA will likely play an essential role as an intermediate precursor in those tissues where naturally-occurring BA metabolites are known to occur (STRNAD, PETERS, BECK & KAMINEK, 1992; refs within).

Once in the active pool, the nucleotide may exit via the base or the riboside to N-conjugates, or oxidative catabolites. [9R-MP]BA has been implicated as the immediate precursor of [7G]BA in tobacco systems (FOX, CORNETTE, DELEUZE, DYSON, GIERSAK, NIU, ZAPATA & McCHESNEY, 1973). Conversely, [7G]BA applied to tobacco was (indirectly) converted to BA nucleotides (GAWER, LALOUE, TERRINE & GUERN, 1977). These authors suggested that such a conversion is indirect, via the transient formation of BA.

As with the other cytokinin species which contribute to the active pool, the exact role(s) of nucleotides remains to be fully elucidated.

Nucleotides have been associated with storage of cytokinins (VAN STADEN, 1973a; LETHAM & PALNI, 1983). PIETRAFACE and BLAYDES (1981) provided evidence to show that the nucleotide is a storage form in lettuce seeds before conversion to the active nucleoside.

Nucleotide formation may also be associated with cytokinin uptake (PALMER, LETHAM & GUNNING, 1984) and transport across membranes, in much the same way as phosphoribosylation plays a role in the uptake of adenine by *Escherichia coli* membranes (LETHAM & PALNI, 1983). BURCH and STUCHBURY (1987) noted that although polar cytokinins such as nucleotides are common metabolites within cells, they have rarely been identified in the culture media. This has been considered indicative of plasmalemma impermeability to [9R-MP]BA (LALOUÉ, PETHE-TERRINE & GUERN, 1981; LALOUÉ & PETHE, 1982). However, following incubation of [9R-MP]Z with soybean callus, various zeatin metabolites were extracted from cellular contents. High levels of [9R-MP]Z detected in artichoke tissues shortly after the start of culture was cited as evidence (PALMER, LETHAM & GUNNING, 1984) that the nucleotides are involved in cytokinin uptake. Yet with the aid of APRT-deficient mutants, MOFFATT, PETHE and LALOUÉ (1991) have recently shown that phosphoribosylation of BA is not a prerequisite of its uptake by *Arabidopsis* plants.

A translocatory role for this cytokinin class has been proposed. Free base applied to bean roots was recovered in the stem, partly as the nucleotide (WAREING, HORGAN, HENSON & DAVIS, 1977). Both ribosides and ribotides were identified by PALMER, HORGAN and WAREING (1981a) in stems of decapitated, disbudded bean plants. VONK and DAVELAAR (1981) suggested that the nucleotides are a cytokinin form transportable in the phloem. The highest levels of [9R-MP]BA detected in tomato plants were in the stems (BAYLEY, VAN STADEN, MALLET & DREWES, 1989), again implying a translocatory role.

SHAW, SMALLWOOD and STEWARD (1968) considered the cytokinin activity of the 3-, 7-, and 9-methyl derivatives of zeatin. The results implied that the mechanism of cytokinin activity in substituted adenines does not require prior formation of nucleotide derivatives. In contrast, LALOUE, PETHE-TERRINE and GUERN (1981) correlated division of cytokinin-requiring tobacco cells with high levels of cytokinin ribotides and low levels of cytokinin base and riboside, irrespective of whether the base or riboside was supplied. The fact that the methyl group in the 9-position does not constitute an effective block, but is easily metabolised, makes suspect the presumed stability of other 9-substituted cytokinins (FOX, SOOD, BUCKWALTER & McCHESNEY, 1971). When compared in the tobacco callus bioassay, [9R]BA and [9R-MP]BA were less active at lower concentrations ( $10^{-1}$   $\mu$ M) than BA (SCHMITZ, SKOOG, VINCZE, WALKER, KIRKEGAARD & LEONARD, 1975). More recently, similar results were obtained in the soybean callus bioassay (VAN STADEN & DREWES, 1991).

The identity of the actual 'active cytokinin' form(s) remains an unresolved issue. The nucleotide may be necessary for the expression of activity (LALOUE, PETHE-TERRINE & GUERN, 1981), but if not, is likely to contribute to the steady state maintenance of an active cytokinin pool.

#### 1.3.4 BA di- and tri-phosphates, [9R-DP]BA and [9R-TP]BA

The di- and tri-nucleotides of BA have been detected in extracts of *Petunia* leaves following incubation with BA (AUER, COHEN, LALOUE & COOKE, 1992; AUER, LALOUE, COHEN & COOKE, 1992; 1992a). These metabolites were rapidly produced by the explants and were considered by these authors to be active forms responsible for shoot induction. An earlier indication of the activity of cytokinin polynucleotides was revealed by MILLER (1967). This author extracted a cytokinin from *Zea mays* L. kernels possessing 'at least two phosphate groups', which showed some activity in a soybean callus bioassay. BEZEMER-SYBRANDY and VELDSTRA (1971) then detected mono-, di-, and tri-nucleotides of BA as metabolites in *Lemna minor* cultures. The formation of such nucleotides was considered to be a normal feature of cytokinin metabolism in plant tissues (MILLER, 1967; TZOU, GALSTON & SONDHEIMER, 1973), and

to indicate the natural occurrence of analogous endogenous nucleotides in plant tissues (LALOUE, TERRINE & GAWER, 1974).

The existence *in vivo* of cytokinin nucleoside-5<sup>1</sup>-triphosphate is of theoretical importance as such compounds could be incorporated into tRNA molecules (FOX, 1966; LALOUE, TERRINE & GAWER, 1974) to provide a basis for cytokinin action. Such incorporation of BA into RNA was demonstrated by Fox (1964). ARMSTRONG, MURAI, TALLER and SKOOG (1976) similarly viewed [9R-TP]BA as an important intermediate in a pathway for the incorporation of BA into RNA species. The incorporation of cytokinin bases into polynucleotides (CHEN & ECKERT, 1977) indicates that the 5<sup>1</sup>-monophosphate is an intermediate metabolite in the reaction. Preferential incorporation of label into the guanine fraction of soluble RNA hydrolysates from soybean and tobacco callus cultured on media containing <sup>14</sup>C-BA was recorded by FOX (1966). In soybean callus, [9R-MP]BA appeared as the major metabolite (FOX & CHEN, 1967). Cytokinin-dependent tobacco callus supplied with BA incorporated this compound in low levels in both tRNA and rRNA though mainly in the rRNA (ARMSTRONG, MURAI, TALLER & SKOOG, 1976). JOUANNEAU and TEYSSENDIER DE LA SERVE (1981) considered this to occur through a direct insertion process.

Despite the large number of BA metabolic studies in plant tissues, detection of di- and tri-nucleotides has rarely been reported. When identified, these compounds are normally minor metabolites (LALOUE, TERRINE & GAWER, 1974). *Acer* cultures supplied with N<sup>6</sup>-substituted nucleosides did not phosphorylate these compounds beyond the monophosphate level (DOREE & GUERN, 1973; DOREE & TERRINE, 1973). In contrast, 3 hours after application of <sup>14</sup>C-BA to tobacco cell cultures, 6% of the radioactivity was associated with [9R-DP]BA and [9R-TP]BA (LALOUE, TERRINE & GAWER, 1974). The monophosphate ([9R-MP]BA) represented 28%, the base 30%, and [9R]BA was unrepresented. To explain differences in the metabolism of BA observed between *Acer* and tobacco, LALOUE, TERRINE and GAWER (1974) suggested that the cytokinin inactivation through sidechain cleavage noted for *Acer* (DOREE & GUERN, 1973) would restrict formation of the di- and tri-nucleotides.

The hydrolytic action of 5<sup>1</sup> nucleotidases which show equal affinity for mono-, di- and

tri-phosphates of Ado (CHEN & KRISTOPEIT, 1981a), are likely responsible for release of [9R]BA from [9R-DP]BA and [9R-TP]BA. Such a conversion could produce a more active cytokinin species, either in the form of [9R]BA, or after de-ribosylation of [9R]BA to the base.

### 1.3.5 N-conjugation of BA

Collectively, the N-alanyl conjugates and N-glucosides of cytokinins are referred to as the N-conjugates.

N-conjugates are stable both when applied externally and when found as metabolites (PARKER & LETHAM, 1973; PARKER, LETHAM, GOLLNOW, SUMMONS, DUKE & MACLEOD, 1978) and are generally incapable of further metabolism back to the base (McGAW, HEALD & HORGAN, 1984; McGAW & HORGAN, 1985). For this reason N-conjugates are regarded as detoxification or inactivation products (GAWER, LALOUE, TERRINE & GUERN, 1977; LETHAM & PALNI, 1983) rather than storage forms; a role proposed for the O-glucosides of zeatin (FUBEDER, ZIEGLER, PETERS & BECK, 1989). Hence N-conjugation may result in the irreversible loss of cytokinin activity.

In reducing levels of cytokinin activity, plants may oxidise the 'active compound' (SUMMONS, ENTSCH, LETHAM, GOLLNOW & MACLEOD, 1980), or alternatively glucosylate/alanylate. McGAW and HORGAN (1985) distinguished an 'oxidative-type' metabolism from a 'glucosidase-type' metabolism to describe either oxidative cleavage of the  $N^6$ -sidechain, or conjugation of exogenously supplied cytokinin. In many tissues BA is resistant to attack by cytokinin oxidase (refer to Chapter 1.5.1). Consequently, N-conjugation may provide the only mechanism by which the biological activity of this cytokinin might be controlled. Oxidation of N-conjugates (PALNI, PALMER & LETHAM, 1984) may be a means of reducing still further the activity of these detoxification products.

### 1.3.5.1 *Glucosides of BA*

#### 1.3.5.1.1 Introduction

The metabolites of BA include a group of N-glucosides in which the sugar moiety is linked to a purine ring nitrogen atom. These are [3G]BA, [7G]BA, and [9G]BA, of which the 3- and 7-glucosides are particularly unusual (LETHAM & PALNI, 1983). Transglucosylation reactions in which glucose is transferred enzymically from one glucoside to BA to yield a different glucoside do not appear to occur (LETHAM & GOLLNOW, 1985).

Cytokinin O-glucosides, where glucose is substituted in the  $N^6$ -sidechain of a molecule such as (2OH)BA, have not been observed for BA, as they have for zeatin (HORGAN, 1975; VAN STADEN & DAVEY, 1977). The O-glucosidic linkage in such compounds as (OG)Z and (OG)DHZ confers a greater lability to acid and  $\beta$ -glucosidase hydrolysis (VAN STADEN & PAPAPHILIPPOU, 1977; VAN STADEN, 1979; PALMER, SCOTT & HORGAN, 1981; VAN STADEN & DREWES, 1991) than has been observed with any N-glucosidic bonds. As a result, O-glucosides probably serve as translocatory (VAN STADEN, 1976; VAN STADEN & DAVEY, 1981) and storage forms (VAN STADEN, 1976a; FUBEDER, ZIEGLER, PETERS & BECK, 1989), unlike the less labile N-glucosides. As the presumed roles of these two glucoside types fundamentally differ, analogies which may be made are limited. Accordingly, cytokinin-O-glucosides are not currently reviewed. However, it is noteworthy that not only glucose, but xylose has also been identified (from *Phaseolus vulgaris* embryos) as a cytokinin conjugate ((OX)Z) (MOK & MOK, 1987; MOK, MOK, MARSDEN & SHAW, 1987). TURNER, MOK, MOK and SHAW (1987) isolated and partially characterised the enzyme (UDP xylose: zeatin-xylosyl transferase) catalysing such conjugation, and showed its specific requirement for UDP-xylose. To date, cytokinin-O-xylosides have only been detected in members of the family Leguminosae. Should the natural occurrence of an O-glucoside of (2OH)BA or (2OH)[9R]BA be indicated, then the existence of an analogous O-xyloside of BA in selected (papilionaceous) species is conceivable. In this regard, the finding of (2OH)[9R]BA in *Populus x robusta* Schneid (HORGAN, HEWETT, PURSE & WAREING,

1973) and the recent identification of (2OH)BA as a natural cytokinin (STRNAD, PETERS, BECK & KAMINEK, 1992) makes the hypothetical existence of (OG)BA and (OX)BA more feasible.

Both [7G]BA and [9G]BA have been confirmed by synthesis (COWLEY, DUKE, LIEPA, MACLEOD & LETHAM, 1978) to be  $\beta$ -D-glucopyranosides. PARKER, WILSON, LETHAM, COWLEY and MACLEOD (1973) considered the identification of the glucosides of zeatin and BA to be the first unequivocal evidence for the occurrence of purine glucosides in living tissues. LETHAM, SUMMONS, ENTSCH, GOLLNOW, PARKER and MACLEOD (1978) later demonstrated that glucosylation of such purines is not restricted to only  $N^6$ -substituted adenines with strong cytokinin activity.

Cytokinin N-glucosides have not been detected in xylem sap, and hence are not supplied to the leaf from the root (DUKE, LETHAM, PARKER, MACLEOD & SUMMONS, 1979). These N-glucosides are apparently much less active in bioassays than the O-glucoside or the parent molecule (HENSON & WHEELER, 1977; LETHAM, 1978). They have alternatively been described as having 'enhanced metabolic stability' (PARKER & LETHAM, 1973; LETHAM, TAO & PARKER, 1982; HUTTON & VAN STADEN, 1984). If these metabolites are the functional form of BA, then they would probably exhibit high cytokinin activity. The stability of N-glucoside metabolites is possibly due to their resistance to degradative enzymes (LETHAM, WILSON, PARKER, JENKINS, MACLEOD & SUMMONS, 1975) or to their compartmentation (LETHAM, TAO & PARKER, 1982).

LETHAM, TAO and PARKER (1982) found that formation of the 3-, 7-, and 9-glucosides of BA was not dependent on BA concentration, in which case formation of the metabolites may not simply be a mechanism for inactivating physiological excesses of BA. Similarly, the rate of BA glycosylation in radish cotyledons (LETHAM, TAO & PARKER, 1982) and tobacco cells (GAWER, LALOUE, TERRINE & GUERN, 1977) was found to be relatively insensitive to large differences in the concentration of supplied BA (PALMER & PALNI, 1986).

ENTSCH and LETHAM (1979) claimed that the physiological significance of the 7- and

9-glucosides of cytokinins is uncertain, although it has been suggested (PARKER & LETHAM, 1973) that they are storage forms of the hormone rather than the product of a detoxification pathway (GAWER, LALOUE, TERRINE & GUERN, 1977). ENTSCH, PARKER, LETHAM and SUMMONS (1979) proposed that cytokinin glucosides may simply be waste-products formed by glucose transferases, which catalyse the formation of glucoside metabolites characteristic of a particular species.

In summary, the significance of cytokinin metabolite formation, in particular glucosylation, has been variously associated with a detoxification mechanism, a method of storage, and a mechanism for lowering endogenous cytokinin levels (SKOOG & SCHMITZ, 1979).

#### 1.3.5.1.2 The 3-glucoside of BA, [3G]BA

When supplied to de-rooted radish seedlings, BA was principally converted to 7-, and 9-glucosides. A third minor metabolite exhibited cytokinin-like activity markedly greater than that of these glucosides (WILSON, GORDON, LETHAM & PARKER, 1974). It was identified (LETHAM, WILSON, PARKER, JENKINS, MACLEOD & SUMMONS, 1975) as the first compound with a glycosidic linkage at position 3 of a purine ring to be isolated from a plant tissue. This compound was 6-benzylamino-3- $\beta$ -D-glucopyranosylpurine ([3G]BA).

The 3-glucosides have not been isolated as endogenous cytokinins from any source, although [3G]DHZ appeared as a minor metabolite when DHZ was exogenously applied to de-rooted radish seedlings (McGAW, HEALD & HORGAN, 1984). The 3-glucoside of zeatin may have been found in *Populus x robusta* by HEWETT and WAREING (1973), although this was more likely the O-glucoside.

LETHAM, PALNI, TAO, GOLLNOW and BATES (1983) considered a number of cytokinin bioassays and compared the activities of 3-, 7-, and 9-glucosides of BA. Cytokinin activity was markedly reduced by 7- and 9-glucosylation in nearly all bioassays, but 3-glucosylation of BA had little effect on activity. The 3-glucoside of BA, produced as a minor metabolite of BA in *D. caryophyllus* flowers, showed higher

senescence-delaying activity than either of the 7- or 9- glucosides (VAN STADEN, BAYLEY, UPFOLD & DREWES, 1990). Since 3-alkyl derivatives of BA are essentially inactive (SKOOG, HAMZI, SZWEYKOWSKA, LEONARD, CARRAWAY, FUJII, HELGESON & LEOPPKY, 1967), the high activity of [3G]BA in diverse bioassays (LETHAM, WILSON, PARKER, JENKINS, MACLEOD & SUMMONS, 1975; LETHAM, PALNI, TAO, GOLLNOW & BATES, 1983; VAN STADEN & DREWES, 1992) is probably due to cleavage of the 3-glucoside moiety to release free BA. Such cleavage has been demonstrated in radish cotyledons (LETHAM, TAO & PARKER, 1982; LETHAM & GOLLNOW, 1985) and soybean callus (VAN STADEN & DREWES, 1992). The 3-glucoside of BA supplied to cytokinin-dependent soybean callus was rapidly metabolised to mainly BA, and another unidentified bioactive compound (VAN STADEN & DREWES, 1992). Release of appreciable amounts of BA from [3G]BA was considered by LETHAM and GOLLNOW (1985) to account for the high activity of this glucoside in cytokinin bioassays. The 3-glucoside has been shown susceptible to hydrolysis by almond  $\beta$ -glucosidase (LETHAM, WILSON, PARKER, JENKINS, MACLEOD & SUMMONS, 1975; PARKER, LETHAM, WILSON, JENKINS, MACLEOD & SUMMONS, 1975; VAN STADEN & DREWES, 1992). The 3-glucoside was hydrolysed slowly by this enzyme whereas the 7- and 9-glycosyl metabolites of BA were not hydrolysed at a detectable rate by either  $\alpha$ - or  $\beta$ -glucosidase (LETHAM, WILSON, PARKER, JENKINS, MACLEOD & SUMMONS, 1975). Of the three N-glucosides of BA, [3G]BA was most readily hydrolysed by acid (VAN STADEN & DREWES, 1992). These authors proposed that if steric factors cannot adequately explain the greater lability of the N-C glucosidic bond of [3G]BA in the presence of  $\beta$ -glucosidase, then compartmentation of the various glucosides may differ.

The enzyme(s) responsible for [3G]BA formation have not yet been characterised.

Although more active than [7G]BA and [9G]BA as a cytokinin, [3G]BA is still only weakly active relative to the corresponding base when applied at physiological levels in bioassays (VAN STADEN & DREWES, 1992). Accordingly, [3G]BA has been considered an inactive N-conjugate in the current study.

### 1.3.5.1.3 The 7-glucoside of BA, [7G]BA

Raphanatin ([7G]Z) was the first purine glucoside to be correctly identified from nature (PARKER & LETHAM, 1973). It was earlier isolated from radish cotyledons by PARKER, LETHAM, COWLEY and MACLEOD (1972) who reported on its activity in the radish cotyledon bioassay. Prior to the report by PARKER and LETHAM (1973), DELEUZE, McCHESNEY and FOX (1972) had isolated the 7-glucoside of BA from sliced potato tubers and proposed a glucofuranosyl structure, based on spectral evidence. The glucose ring size and stereochemistry of the sugar linkage of [7G]BA was later investigated (DUKE, LIEPA, MACLEOD, LETHAM & PARKER, 1975) and found to be a 7- $\beta$ -D-glucopyranoside. Similarly, although FOX, CORNETTE, DELEUZE, DYSON, GIERSAK, NIU, ZAPATA and McCHESNEY (1973) reported on the existence of the 7-glucofuranoside of BA in potato tuber tissue, this was later shown (LETHAM, GOLLNOW & PARKER, 1979) to be the pyranoside. The 7-glucoside of BA ([7G]BA) has been shown to be the major metabolite in tobacco, another solanaceous species (GAWER, LALOUE, TERRINE & GUERN, 1977; VAN DER KRIEKEN, CROES, BARENDSE & WULLEMS, 1988).

The base appears to be the precursor for 7-glucosylation, as expected from the consideration of the 7- and 9-tautomeric positions of the purine ring, although kinetic studies (FOX, CORNETTE, DELEUZE, DYSON, GIERSAK, NIU, ZAPATA & McCHESNEY, 1973) indicated that the ribonucleotide is the immediate precursor.

The roles of cytokinin-7-glucosides in controlling hormone activity remains unclear. The 7-glucosides of cytokinins are metabolically stable (PARKER & LETHAM, 1973; GAWER, LALOUE, TERRINE & GUERN, 1977), and weakly active relative to the unsubstituted cytokinin (LALOUE, 1977; WILSON, GORDON, LETHAM & PARKER, 1974). McGAW and HORGAN (1985) considered [7G]BA as a deactivation or detoxified cytokinin form, which was biologically inactive. LALOUE, PETHE-TERRINE and GUERN (1981) considered 7-glucosylation of BA as a (terminal) inactivation step as its formation was 'practically irreversible' and the rate of reutilization extremely slow (GAWER, LALOUE, TERRINE & GUERN, 1977). When the amount of [7G]BA present in the

tobacco cells did not increase proportionately following further addition of BA, GAWER, LALOUE, TERRINE and GUERN (1977) reasoned that 7-glucosylation may not be a detoxification pathway.

In contrast to the proposed terminal inactivity of [7G]BA, several researchers have viewed the 7-glucosides as storage forms of cytokinins (PARKER & LETHAM, 1973; LALOUE, 1977; LALOUE & PETHE, 1982; LETHAM & GOLLNOW, 1985). LALOUE (1977) suggested that they are storage forms as they are stable with respect to degradation that occurs upon  $N^6$ -sidechain removal (LALOUE, TERRINE & GUERN, 1977) and because they can be converted to cytokinin nucleotides (GAWER, LALOUE, TERRINE & GUERN, 1977). These authors suggested that this conversion is indirect, via the transient formation of BA. As  $\beta$ -glucosidases do not substantially hydrolyse [7G]BA, then the existence of an enzyme which removes the glucosyl moiety at position 7 of the purine ring and simultaneously attaches a phosphoribosyl group should be considered.

LETHAM and GOLLNOW (1985) suggested that the cytokinin-7-glucoside of zeatin may be a translocation and a storage form, given its resistance to degradation, its production at sub-optimal levels (hence not a detoxification form), and movement in radish seedlings (LETHAM, TAO & PARKER, 1982).

LALOUE (1977) considered the effect of [7G]BA on cell division in suspension cultures of *Nicotiana tabacum* L. This author found that cytokinin-7-glucosylation was not involved in the expression of the biological activity of cytokinins. This report conflicted with the view of FOX, CORNETTE, DELEUZE, DYSON, GIERSAK, NIU, ZAPATA and McCHESNEY (1973) who considered cytokinin-7-glucosides to be the 'active forms', as this metabolite was the only cytokinin species containing the intact cytokinin moiety that remained in actively growing cytokinin-requiring tobacco tissue in the long term. These authors had reasoned that as [7G]BA was not degraded through sidechain cleavage, the 7-glucoside may thus be the active form of the cytokinin. Additionally, substantial growth was detected in the soybean callus bioassay.

Soybean callus degraded [7G]Z to [7G]Ade (PALNI, PALMER & LETHAM, 1984), showing

that oxidation of [7G]BA does in fact occur. McGAW and HORGAN (1985) found that [7G]Z was metabolised to Ade, Ado and [7G]Ade within two days. By inference, one would expect [7G]BA to be susceptible to a  $\beta$ -glucosidase, although this has not yet been demonstrated. VAN STADEN and DREWES (1992) later showed partial degradation by this enzyme, although this was much less than the more labile [3G]BA.

The lack of agreement on the biological significance of cytokinin-7-glucosides highlights the confusion surrounding most cytokinin metabolite roles. A lack of uniformity with respect to systems investigated and particularly bioassays employed, has probably been a major cause for such apparent contradictions. However, when one considers the wide array of bio-responses elicited by cytokinins, the varied experimental approaches are placed in context.

In summary, although early evidence permitted the interpretation of [7G]BA as an active or storage form of cytokinin, this compound is currently widely viewed as an inactive product of deactivation or detoxification mechanisms. This glucoside may be degraded further to oxidation products.

#### 1.3.5.1.4 The 9-glucoside of BA, [9G]BA

LETHAM, GOLLNOW and PARKER (1979) demonstrated the production of [9G]BA by potato tuber tissue. A prominent amount of this compound was found by ZHANG, LETHAM, WONG, NOODÉN and PARKER (1987) to be produced in soybean leaves. The 9-glucoside of BA is a stable metabolite in radish cotyledons (WILSON, GORDON, LETHAM & PARKER, 1974). This glucoside was detected by VAN STADEN, BAYLEY, UPFOLD and DREWES (1990) as a major metabolite in cut carnation flowers. Cytokinin base (BA) supplied to the stems was later recovered partly as [9G]BA in the stem, petals and receptacle.

The partially characterised cytokinin extracted from rice roots and presumed to be a 9-glucoside (YOSHIDA & ORITANI, 1972) is more likely O-glucosylzeatin (HORGAN, 1975; WANG, THOMPSON & HORGAN, 1977; SUMMONS, ENTSCH, LETHAM, GOLLNOW & MACLEOD,

1980). Following this report, LETHAM (1973) identified a 9-glycoside of zeatin from corn kernels, although the identity of the sugar moiety was not established. Subsequently, the 9-glucoside of zeatin was isolated as a major metabolite from roots of *Zea mays* by PARKER and LETHAM (1974).

Oil palm (*Elaeis guineensis* Jacq.) callus supplied with kinetin produced [9G]KIN as the major metabolite (JONES & HANKE, 1992). As oil palm cultures do not require added cytokinins, these authors suggested inactivation of an excessive cytokinin load had occurred. JONES and HANKE (1992) noted that in cytokinin-autonomous *Elaeis* cultures, added cytokinins have not been shown to improve callus growth. Cytokinin-9-glucosides may then be formed in an inactivation or detoxification pathway. Earlier reports (PARKER & LETHAM, 1973) have considered [9G]BA to be a storage form.

The stability of [9G]BA has been demonstrated: [9G]BA was resistant to enzymic degradation by *Escherichia coli* nucleoside phosphorylase and a nucleoside hydrolase from *Cicer arietinum*, unlike [9R]BA which was susceptible (GUERN, DOREE & SADORGE, 1968). VAN STADEN and DREWES (1992) similarly found [9G]BA to be a stable, inactive metabolite in soybean callus. In 1968, when GUERN and co-workers were experimenting with [9G]BA, no purine nucleotides were known to occur naturally. Recently, [9G]BA has been identified as a naturally occurring cytokinin (NANDI, LETHAM, PALNI, WONG & SUMMONS, 1989).

Following injection of [9G]BA into *Cicer arietinum* seedlings, this metabolite was apparently readily translocated without appreciable enzymic modification (GUERN, DOREE & SADORGE, 1968). However, cytokinin-9-glucosides have not been detected as endogenous translocatory forms.

As [9G]BA is weakly active relative to BA (WILSON, GORDON, LETHAM & PARKER, 1974; LETHAM, PALNI, TAO, GOLLNOW & BATES, 1983; VAN STADEN & DREWES, 1991; 1992), specific inhibitors of the glucosylating enzymes may constitute a mechanism for elevating endogenous cytokinin levels. Although the N-glucosides (7- and 9-particularly) are generally inactive forms of cytokinins (LETHAM, TAO & PARKER, 1982),

[9G]BA has been found similarly active to free BA in retarding the senescence of radish leaf discs (LETHAM, PALNI, TAO, GOLLNOW & BATES, 1983).

In summary, the generally low activity of [9G]BA in a variety of cytokinin bioassays indicates that this compound is probably an inactivation or detoxification form.

#### 1.3.5.1.5 Glucose-ribose conjugate, [9R-G]BA

A new hexose (probably glucose) conjugate of [9R]Z, susceptible to  $\beta$ -glucosidase cleavage, was detected in Douglas-fir (*Pseudotsuga menziesii* (Mirbel) Franco, Pinaceae) by MORRIS, DOUMAS, MORRIS and ZAERR (1990). Immuno-affinity and mass spectral techniques indicated that this compound was not (OG)[9R]Z. These authors suggested that the hexose moiety was attached to the purine ring or to the ribose group, but favoured the later position owing to the ease of hydrolysis by  $\beta$ -glucosidase. However, N-conjugation does not preclude hydrolysis by  $\beta$ -glucosidase (VAN STADEN & DREWES, 1992a). Earlier, TAYLOR, KOSHIOKA, PHARIS and SWEET (1984) had detected a novel ribosyl zeatin glycoside which could be the same compound further characterised by MORRIS, DOUMAS, MORRIS and ZAERR (1990), but in a second coniferous species (*Pinus radiata* D. Don, Pinaceae). These workers suggested that the hexose moiety was glucose. VAN STADEN and MALLETT (1988) and later VAN STADEN and BAYLEY (1991) detected a glucosylated form of [9R]BA following BA application to tomato shoots. Further structural characterisation of this unknown metabolite was not attempted.

A disaccharide of BA, 6-(benzylamino)-9-(glucosylribosyl)purine was identified by BLAKESLEY, LENTON and HORGAN (1990) as the major metabolite in *Gerbera jamesonii* Bolus callus. The exact position of the ribose-glucose linkage was not determined. Unaware of the report on *Gerbera*, AUER and COHEN (1993) reported on [9R-G]BA formation in *Petunia* leaves, and proposed a linkage of the ribose at the 3- position to glucose at the terminal (1) position. MORRIS, DOUMAS, MORRIS and ZAERR (1990) suggested a possible storage role for this metabolite. In citing the observed activity of [9R-G]Z in the soybean hypocotyl bioassay (TAYLOR, KOSHIOKA, PHARIS & SWEET,

cytokinin O-xylosyltransferase (TURNER, MOK, MOK & SHAW, 1987).

The metabolism of exogenously applied zeatin and other cytokinins, including BA (BLAKESLEY, LENTON & HORGAN, 1990; VAN STADEN & DREWES, 1992) indicates the presence of other, as yet uncharacterised enzyme systems.

#### 1.4.1 Cytokinin-7-glucosyl transferase

Radish cotyledon extracts yielded a single enzyme system (collectively known as cytokinin-7-glucosyl transferase) comprised of two enzymes/isozymes. These converted BA into 7- and 9-glucosides when uridine diphosphate glucose (UDPG) was supplied as a glucose donor (ENTSCH, PARKER & LETHAM, 1978; ENTSCH & LETHAM, 1979). Cytokinin-7-glucosyl transferase produced the two glucosides in different proportions; the major isozyme favoured production of the 7-glucoside, and the minor glucosyl transferase formed the 7- and 9-cytokinin glucosides in similar proportions (ENTSCH, PARKER, LETHAM & SUMMONS, 1979). ENTSCH and LETHAM (1979) expressed surprise to find that the 7- and 9-glucosides were not formed by separate enzymes, especially considering the small size of the enzyme (46 000 daltons).

However, in view of the many systems in which cytokinin-9-glucosides are produced as the major metabolite (BLAKESLEY, 1991; MOFFATT, PETHE & LALOUE, 1991; JONES & HANKE, 1992), it is possible that a separate cytokinin-9-glucosyl transferase exists, or a 'cytokinin-7-glucosyl transferase' which forms both the 7- and 9-glucosides, yet favours production of the latter. As with cytokinin oxidase-type systems (CHATFIELD & ARMSTRONG, 1988; KAMINEK & ARMSTRONG, 1990), different enzymes or isozymes of cytokinin-7-glucosyl transferase with a similar function are likely to occur in a range of plant tissues.

Although a trace enzyme, the glucosyl transferase studied by ENTSCH, PARKER, LETHAM and SUMMONS (1979) could exert a regulatory role in metabolism since cytokinins occur in trace amounts, evoking key responses at the sub-nanomolar level.

ENTSCH, PARKER, LETHAM and SUMMONS (1979) considered that inhibitors of cytokinin-7-glucosyl transferase merit study, as 'a stable, effective, and specific inhibitor *in vitro* could be a valuable physiological tool and a means of elevating endogenous free cytokinin levels by suppressing formation of the very weakly active 7-glucosides'. Several studies have been undertaken in this regard (ELLIOTT & MURRAY, 1975; PARKER, ENTSCH & LETHAM, 1986; HOCART, LETHAM & PARKER, 1991; TAO, LETHAM, HOCART & SUMMONS, 1991).

#### 1.4.2 $\beta$ -Glucosidase

Hydrolysis of O-glucosides of isopentenyl-type cytokinins may function in controlling cytokinin activity. In this regard  $\beta$ -glucosidases would play an important role (VAN STADEN, 1979). Up until recently, non-specific  $\beta$ -glucosidases were considered to be involved in cytokinin metabolism. However, the existence of a specific 'cytokinin- $\beta$ -glucosidase' has recently been reported. ESTRUCH, CHRIQUI, GROSSMANN, SCHELL and SPENA (1991) transformed tobacco tissues with a *rolC* oncogene from the T-DNA of *Agrobacterium rhizogenes*. This gene coded for a 'cytokinin- $\beta$ -glucosidase' which was capable of hydrolysing [9G]BA to its free base. Bioassays confirmed the release of active cytokinins from an inactive form. Despite this report, KAMINEK (1992) considered such hydrolases to be either absent or inactive in normal plant cells.

Almond  $\beta$ -glucosidase did not hydrolyse cytokinin 7- or 9-glucosides *in vitro* (DUKE, LETHAM, PARKER, MACLEOD & SUMMONS, 1979), although limited cleavage of [7G]Z has been reported in *Raphanus* L. tissues (LETHAM, TAO & PARKER, 1982). The resistance to hydrolysis was presumed due to their C-N glycosidic linkages (HORGAN, 1975), although enzymic degradation of just such a bond in [3G]BA (VAN STADEN & DREWES, 1992) remains unaccountable.

$\beta$ -glucosidases have functions unrelated to growth (HUGHES, 1968), so their activity in regard to cytokinin metabolism is not surprising. HUGHES (1968) found two distinct  $\beta$ -glucosidases produced by clover callus. This author showed that  $\beta$ -glucosidase activity and concentration varied both between plants and between organs of the

same plant. Genetic variation and the environment were cited as causal factors of this. McCREIGHT, PHARR, LOWER and SOX (1976) later found different forms of  $\beta$ -glucosidase in different plant organs of the same species.

There are several reports of  $\beta$ -glucosidase occurrence in test systems where cytokinin-glucoside hydrolysis would be expected, or has been demonstrated. High  $\beta$ -glucosidase activity was found in the stems of *Yucca* Dill. ex Linn. plants (VONK & DAVELAAR, 1981), maize kernel embryos (SMITH & VAN STADEN, 1978), and the meristematic region of plants (DATKO & MACLACHLAN, 1970). Further circumstantial evidence has linked  $\beta$ -glucosidase activity to cytokinin metabolism. Soybean callus supplied with the O-glucoside of zeatin accumulated zeatin (VAN STADEN & PAPAPHILIPPOU, 1977); the presence of this enzyme has also been inferred from studies with potatoes (VAN STADEN & DIMALLA, 1978), and soybean (VAN STADEN & DREWES, 1992).

$\beta$ -glucosidase purified from germinating *Phaseolus vulgaris* seeds was highly specific for the anomeric configuration of the glycosidic linkage (i.e.  $\beta$ -, not  $\alpha$ -) (AGRAWAL & BAHL, 1968).

Histochemical methods capable of determining the inter- and intra-cellular distribution of  $\beta$ -glucosidase are available (ASHFORD, 1970; ASHFORD & McCULLY, 1970); it remains now to determine the sub-cellular compartmentation of [3G]BA, and to relate this to its relatively high lability.

#### 1.4.3 $\beta$ -(9-Cytokinin)-alanine synthase

The enzyme which converts cytokinin bases to considerably less active (ZHANG, LETHAM, WONG, NOODÉN & PARKER, 1987) alanine conjugates is known as lupinic acid synthase or  $\beta$ -(9-cytokinin)-alanine synthase. This enzyme is classed as a C-N-ligase (EC 6.3.2). O-acetyl-L-serine was apparently the source of the alanine moiety in lupinic acid ([9Ala]Z) (MURAKOSHI, IKEGAMI, OOKAMA, HAGINIWA & LETHAM, 1977; ENTSCH, LETHAM, PARKER, SUMMONS & GOLLNOW, 1979). Enzyme-catalysed formation

of lupinic acid was determined by  $^{14}\text{C}$  incorporation from *o*-acetyl-L-serine-3- $^{14}\text{C}$  as a substrate into lupinic acid. MURAKOSHI, Ikegami, OOKAMA, HAGIWA and LETHAM (1977) demonstrated that enzymes from different plant species which catalysed the synthesis of  $\beta$ -substituted alanines from *O*-acetyl-L-serine had different specificities; not all enzymes recognised zeatin as a substrate.

$\beta$ -(9-cytokinin)-alanine synthase was isolated from developing lupin seeds by ENTSCH, PARKER and LETHAM (1983). In this report, a number of adenine derivatives were shown to serve as substrates, although preference was shown for compounds with high cytokinin activity, including BA and kinetin. In the reverse direction, a small amount of base was formed (PALNI, PALMER & LETHAM, 1984). Although indole auxins have a similar ring structure to purines, IAA was not a substrate for lupinic acid synthase (ENTSCH, PARKER & LETHAM, 1983).

## 1.5 Oxidative catabolism of BA

### 1.5.1 Cytokinin sidechain cleavage

Cytokinin activity is conferred on (intact) purine molecules through the possession of a suitably-structured  $N^6$ -sidechain (VENIS, 1985; MATSUBARA, 1990). When this sidechain is (oxidatively) removed, relatively inactive degradation products are formed. Further loss of activity occurs on disruption of the adenine moiety (BLACK & OSBORNE, 1965; SKOOG & ARMSTRONG, 1970). The cytokinin-specific enzyme responsible for such sidechain cleavage is cytokinin oxidase, an enzyme common to both lower (ARMSTRONG & FIRTEL, 1989) and higher (WHITTY & HALL, 1974) plant orders.

The activity associated with oxidative catabolism was detected in many early cytokinin metabolic studies, involving the degradation of cytokinin bases and ribosides of both naturally-occurring (CHEN, LOGAN, McLENNAN & HALL, 1968; PAČES, WERSTIUK & HALL, 1971; MIURA & HALL, 1973) and synthetic cytokinins (e.g. kinetin and BA) (McCALLA, MORRÉ & OSBORNE, 1962; FOX, 1964; 1966; EL-SAIDI, 1971; FOX, DYSON, SOOD & McCHESNEY, 1972). FOX, DYSON, SOOD and McCHESNEY (1972) even found

revealed that the metabolism of Ade varies according to the species. The presence of enzymes responsible for such catabolism have been demonstrated in plants (TRACEY, 1955; LE FLOC'H, LAFLEURIEL & GUILLOT, 1982). The purine degradation pathway leads eventually to the production of urea from Ade. Urea formed is eventually hydrolysed rapidly to CO<sub>2</sub> and ammonia by urease, a widely distributed higher plant enzyme (ROSENTHAL, 1982). Following cytokinin application to a plant system, BIONDI, CANCIANI and BAGNI (1984) accounted for the loss in detectable radioactivity by suggesting that <sup>14</sup>C was lost as <sup>14</sup>CO<sub>2</sub>. The apparent loss of radioactivity from plant material/medium in a closed system (NORDSTROM & ELIASSON, 1986) was also thought to be due to release of <sup>14</sup>C as <sup>14</sup>CO<sub>2</sub>, or some other volatile compound. However, no detectable amounts of <sup>14</sup>CO<sub>2</sub> were found trapped in carbosorb.

## 1.6 Concluding remarks

Cytokinin metabolites possess functional, though somewhat obscure roles in plants (LETHAM & PALNI 1983; WAGNER & BECK, 1993), contributing to either an active or inactive pool. Inactivation of cytokinin occurs through sidechain cleavage or alternatively N-conjugation, which proceeds through 9-alanylation or 7- and/or 9-glucosylation (McGAW & HORGAN, 1985; KAMINEK, 1992). The 3-glucoside is more biologically active than other cytokinin N-glucosides (LETHAM, WILSON, PARKER, JENKINS, MACLEOD & SUMMONS, 1975; LETHAM, PALNI, TAO, GOLLNOW & BATES, 1983), and appears reversibly sequestratable (LETHAM & GOLLNOW, 1985; VAN STADEN & DREWES, 1992), suggesting a storage role. Internal levels of free, non-metabolised bases appear important in the initiation of physiological responses (VAN DER KRIEKEN, CROES, BARENDSE & WULLEMS, 1988). Nucleosides and nucleotides are also considered as active forms (LETHAM & PALNI, 1983; LETHAM & GOLLNOW, 1985), given their ready conversion to cytokinin bases (VAN STADEN & BAYLEY, 1991; VAN STADEN & DREWES, 1992a).

From a physiological viewpoint, cytokinin metabolism may be classified (HORGAN, 1987) under three headings:

1. Irreversible loss of biological activity through oxidative degradation of the  $N^6$  sidechain (products here referred to as 'oxidation products').
2. Irreversible conjugation with alanine or glucose with loss of, or reduction in activity (products here referred to as 'N-conjugates').
3. Reversible conjugation to (inter-convertible) compounds which are themselves active, or serve as storage forms which may be converted to active cytokinins (here referred to as the 'active pool').

Subsequent investigations which are detailed in the following chapters have often considered pools of metabolites associated with these three physiological routes of metabolism. Particular attention has been given to the effect of auxin on cytokinin activation /inactivation.

The above survey was hampered by the confusing array of synonyms which have been generated for BA metabolites. Accordingly, a working system of (semi-systematic) abbreviations was devised, based on a proposal by LETHAM (1978). This scheme accommodates all groups and classes of purine cytokinins.

## CHAPTER 2

### PURINE CYTOKININ NOMENCLATURE: A WORKING SYSTEM OF ABBREVIATIONS

#### 2.1 Introduction

Synonymy allows for variety in the expression of a language by providing interchangeable words. Naturally, this latitude has also extended to scientific writing. However, given the international character of science, it has been necessary to define systems of nomenclature which should be universally applied. These principles are embodied in a published system of nomenclature, proposed in 1970 by the IUPAC-IUB Commission on Biochemical Nomenclature (ANON, 1970). These recommendations delimited symbols and abbreviations for nucleic acid derivatives, polynucleotides and their constituents, which include cytokinins. This publication amended a set of earlier tentative rules (ANON, 1967). These frames of reference were designed to facilitate communication, and to stem the tide of abbreviations indiscriminately coined by biochemists. However, some twenty years later, the literature still teems with synonymy which often confuses, and at the very least hinders, new researchers to the field. The variety of terms generated can limit the effectiveness of abstracting services, thereby limiting communication between workers. Synonyms have been generated for systematic and semisystematic, as well as trivial names. Table 2.1 is illustrative of this excess, evidenced when considering only a single cytokinin base. 6-(Benzylamino)purine, labelled with  $^{14}\text{C}$  at position 8 in the purine ring, has also been considered to emphasise the point (Table 2.2). The attention of the reader is drawn to the indiscriminate use of hyphens, spaces, and parentheses for the compounds listed. Further lists of synonyms of BA metabolites have been tabulated by CROUCH, SMITH and VAN STADEN (1993). These lists of examples are by no means exhaustive, and some compounds listed may or may not occur naturally.

#### 2.2 Proposal and discussion

Systematic cytokinin names are normally communicated as a  $N^6$ -substituted adenine

Table 2.1 Synonyms of 6-(benzylamino)purine (BA), including systematic, semisystematic and trivial names found in the literature.

TERM USED	REFERENCE
3A	CHISM, LONG & ROLLE (1984)
3AP	MONETTE (1987); MALIK & SAXENA (1991)
benzyl adenine	MCGAW & HORGAN (1985)
benzyladenine	VAN STADEN & DAVEY (1979)
bzAde	JOUANNEAU & TEYSSENDIER DE LA SERVE (1981)
benzylaminopurine	SCOTT & LIVERMAN (1956)
bz <sup>6</sup> adenine	LALOUE (1977)
bz <sup>6</sup> Ade	MORRIS (1981)
bz <sup>6</sup> Ade	LALOUE & PETHE (1982)
b <sup>6</sup> Ade	CHEN (1987)
b <sup>6</sup> Ade	CHATFIELD & ARMSTRONG (1987)
N <sup>6</sup> -benzyladenine	HALL (1973)
N <sup>6</sup> -benzyl-adenine	PAIS & CHAVES NEVES (1982/83)
N <sup>6</sup> -benzylaminopurine	HALL (1973)
N <sup>6</sup> -benzylamino purine	PALNI, HORGAN, DARRAL, STUCHBURY & WAREING (1983)
N-(phenylmethyl)-1 <i>H</i> -purin-6-amine	CLARK, KELLY & PEMBERTON (1991)
N <sup>6</sup> (benzyl) adenine	HORGAN (1984)
N <sup>6</sup> -benzyladenine	CHATFIELD & ARMSTRONG (1986)
N <sup>6</sup> ,6-benzyladenine	FOX (1966)
6-BAP	NARAIN & LALORAYA (1974)
6-benzyladenine	METIVIER & PAULILO (1980)
6-benzylamino purine	DYSON, FOX & MCCHESENEY (1973)
6-benzylamino-purine	MATSUBARA (1990)
6-benzyl-aminopurine	FOX, SOOD & MCCHESENEY (1973)
6-benzylaminopurine	YOSHIDA & ORITANI (1972)
6-(benzylamino)purine	DYBING & LAY (1981)
6-N-benzyladenine	SONDHEIMER & TZOU (1971)
C-BA	COWLEY, DUKE, LIEPA, MACLEOD & LETHAM (1978)

Table 2.2 Synonyms of 6-(benzylamino)purine labelled with  $^{14}\text{C}$ . Terms include systematic, semisystematic and trivial names found in the literature.

TERM USED	REFERENCE
BAP-8- $^{14}\text{C}$	MOZES & ALTMAN (1977)
BA-8- $^{14}\text{C}$	FORSYTH & VAN STADEN (1987)
BA-8-C $^{14}$	FOX (1966)
benzyladenine-8- $^{14}\text{C}$	KOEVENIG (1973)
benzyladenine- $^{14}\text{C}$	VEEN & JACOBS (1969)
benzyl-8- $^{14}\text{C}$ adenine	MINOCHA & NISSEN (1982)
C $^{14}$ -BAP	WDOLLEY & WAREING (1972)
C $^{14}$ labeled benzyladenine	FOX (1966)
N $_6$ -benzyladenine- $^{14}\text{C}$	KOEVENIG (1973)
N $^6$ -benzyladenine-[8- $^{14}\text{C}$ ]	LALOUÉ, TERRINE & GAWER (1974)
N $^6$ -benzyladenine-8- $^{14}\text{C}$	ARMSTRONG, MURAI, TALLER & SKOOG (1976)
N $^6$ -benzyl[8- $^{14}\text{C}$ ]adenine	BORNMAN & VOGELMANN (1984)
N,6-benzyladenine-8- $^{14}\text{C}$	FOX (1966)
N,6-benzyladenine-8-C $^{14}$	FOX (1966)
N $^6$ -benzyladenine-[8- $^{14}\text{C}$ ]	LALOUÉ, TERRINE & GAWER (1974)
6-benzylaminopurine-8- $^{14}\text{C}$	BEZEMER-SYBRANDY & VELDSTRA (1971)
6-benzylaminopurine $^{14}\text{C}$	PILET, GUERN & HURON (1967)
6-benzylamino-[8- $^{14}\text{C}$ ]purine	DOREE & GUERN (1973)
[8- $^{14}\text{C}$ ]BA	RAMINA (1979)
[8- $^{14}\text{C}$ ]-BA	HOFMAN, FORSYTH & VAN STADEN (1986)
8- $^{14}\text{C}$ -BAP	BEZEMER-SYBRANDY & VELDSTRA (1971)
[8- $^{14}\text{C}$ ] benzyladenine	VAN STADEN & BAYLEY (1991)
[8- $^{14}\text{C}$ ]benzyladenine	VAN STADEN & BAYLEY (1991)
[8- $^{14}\text{C}$ ]benzyladenine	MCCALLA, MORRE & OSBORNE (1962)
8- $^{14}\text{C}$ -benzyladenine	LALOUÉ & PETHE (1982)
[8- $^{14}\text{C}$ ]benzylaminopurine	RAMINA, PIMPINI, BONIOLLO & BERGAMASCO (1979)
[8- $^{14}\text{C}$ ]benzylaminopurine	RAMINA (1979)
[8- $^{14}\text{C}$ ]-benzylaminopurine	NORDSTROM & ELIASSON (1986)
[8- $^{14}\text{C}$ ]bzl $^6$ Ade	MORRIS (1981)
$^{14}\text{C}$ -BA	BORNMAN & VOGELMANN (1984)
[ $^{14}\text{C}$ BA]	RAMINA (1979)
[ $^{14}\text{C}$ ]-BA	KOBAYASHI, ZBELL & REINERT (1981)
[ $^{14}\text{C}$ ]BA	MINOCHA & NISSEN (1982)
[ $^{14}\text{C}$ ]-BAP	NORDSTROM & ELIASSON (1986)
$^{14}\text{C}$ -BAP	WOOLLEY & WAREING (1972)
[ $^{14}\text{C}$ ]-benzyladenine	HENSON & WAREING (1977)
[ $^{14}\text{C}$ ]-benzylaminopurine	PHILLIPS (1975)
$^{14}\text{C}$ -benzylaminopurine	YOSHIDA & OHJAN (1972)
$^{14}\text{C}$ -bzl $^6$ Ade	ARMSTRONG, MURAI, TALLER & SKOOG (1976)
$^{14}\text{C}$ -6-benzylaminopurine	BEZEMER-SYBRANDY & VELDSTRA (1971)
[ $^{14}\text{C}$ ]-labelled benzylaminopurine	NORDSTROM & ELIASSON (1986)
$^{14}\text{C}$ -labelled 6-benzylaminopurine	VAN STADEN (1973)

or a substituted 6-aminopurine (HORGAN, 1984), as most members of this class are substituted purines. Further, the position of substitution in the purine ring is numbered according to the standard numbering of the purine nucleus (Figure 2.1). Even so, the format for naming cytokinins, even at a systematic level, has not been standardised. The tabulated synonyms demonstrate that workers in the field of hormone physiology need be concerned as much with the unabridged as with the abbreviated terms.

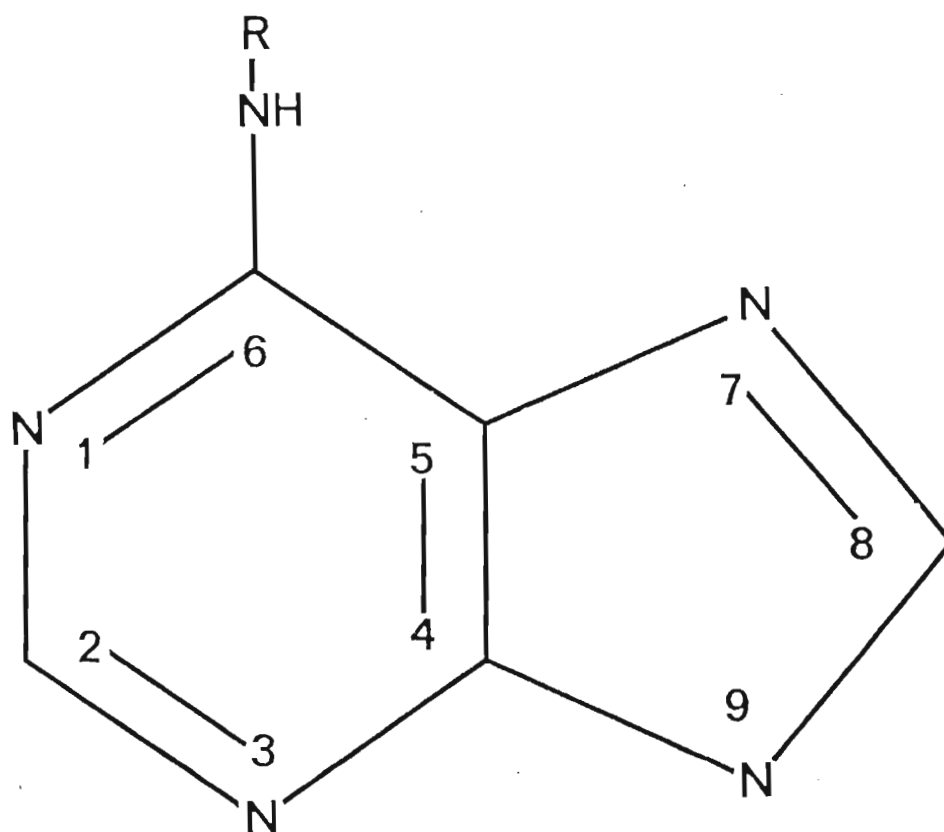


Figure 2.1 Structure and numbering scheme of the purine ring.

It has been clearly demonstrated (Tables 2.1 and 2.2; CROUCH, SMITH & VAN STADEN, 1993, tables within) that cytokinin physiologists have not adopted the IUPAC-IUB system of abbreviated structural formulae. This is likely because although brief and unambiguous, such symbols for cytokinins are not 'user-friendly'. The meanings of symbols are not immediately clear to those encountering them, and are an ordeal for the typist and typesetter. In preference physiologists have used, and in future will continue to use, semi-systematic or trivial names. In the process, more non-

systematic abbreviations could be coined and further confusion generated. Although not in line with IUPAC-IUB recommendations, the scheme of LETHAM (1978) is both systematic and clear and should be promoted for these reasons.

In this system the abbreviation assigned to the base stems from a trivial name e.g. KIN for kinetin and Z for zeatin. Substitutions in the purine ring are indicated using square brackets and modifications/substitutions in the sidechain with round parentheses. In this way the systematic name 6-(4-hydroxy-3-methylbut-*cis*-2-enylamino)-9- $\beta$ -D-ribofuranosylpurine becomes abbreviated to (*cis*)[9R]Z. The base 6-(benzylamino)purine is assigned the letters BA; hence the riboside is [9R]BA, the ribotide [9R-MP]BA, and the 3-glucoside [3G]BA. Where the benzyl sidechain has been modified as in the ortho-hydroxylated sidechain derivative of BA, the abbreviated form should be (2OH)BA. Accordingly, the riboside of this compound would be (2OH)[9R]BA. An explanation of terms used in this modified system is given in Table 2.3.

The cytokinin 6-(4-hydroxy-3-methylbutylamino)purine is represented by DHZ and not by (diH)Z (LETHAM, 1978), despite modification (hydrogenation) of the sidechain. This allows for greater representational clarity when further substitutions in the molecule are considered; e.g. (OG)[9R]DHZ compared with (diHOG)[9R]Z. Hyphens are proposed to indicate substitutions in substituent groups e.g. [9R-MP]Z for a phosphate moiety substituted in the ribose, and [9R-G]BA for a glucosyl group attached to the ribose substituted at position 9. Hyphens have also been used for the radiolabel abbreviation for the sake of clarity, when indicating the position of the  $^{14}\text{C}$  substitution. This proposed system is semi-systematic, and consequently some, but not all, structural information is conveyed. For this reason it is imperative that authors must give the full systematic name when the abbreviation is first defined in an article. In this manner accurate structural details are unambiguously presented.

Table 2.3 Abbreviations assigned for cytokinin nomenclature.

After LETHAM (1978).

SYMBOL	UNABBRIDGED FORM
G	glucosyl
MP	monophosphate
DP	diphosphate
TP	triphosphate
R	riboside (furanosyl)
X	xylosyl
OH	hydroxyl
6OG	O-glucosyl
OX	O-xylosyl
MeS	methylthio
Ala	alanyl
[ ]	substitution in the adenine nucleus
( )	substitution in the $N^6$ -sidechain
<i>cis</i>	<i>cis</i> isomeric form
<i>trans</i>	<i>trans</i> isomeric form (normally inferred by default)
-	indicates substitution within a substituent
[8- $^{14}\text{C}$ ]	$^{14}\text{C}$ Carbon substituent at position 8 in purine ring
Ade	adenine
Ado	adenosine
BA	6-(benzylamino)purine (benzyladenine)
KIN	6-(furfurylamino)purine (kinetin)
iP	6-(3-methylbut-2-enylamino)purine (isopentenyl adenine)
DHZ	6-(4-hydroxy-3-methylbutylamino)purine (dihydrozeatin)
Z	6-(4-hydroxy-3-methylbut- <i>trans</i> -2-enylamino)purine (zeatin)

For each of the principally-encountered purine cytokinins, abbreviations according to this modified system are given (Tables 2.4 to 2.8). These abbreviations are given alongside the less self-explanatory IUPAC-IUB symbols. Such IUPAC-IUB names have previously been suggested for cytokinins (HORGAN, 1984), but have not been widely followed. In that proposal, the symbol selected to represent the isopentenyl sidechain of zeatin is 'io'. However, this symbol has been assigned to designate 'iodo' in the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (section N-4.1.; (ANON, 1970)). However, [for comparative purposes and] in order to present IUPAC-IUB symbols recognisable to physiologists, the symbol 'io' has been maintained (Tables 2.4 to 2.8). The symbol 'G' for glucose has been used as it is unlikely to be confused with guanosine in this context (section 9.; (ANON, 1967)).

**Table 2.4 Suggested abbreviations for 6-(benzylamino)purine (benzyladenine) and its metabolites. These are compared with IUPAC-IUB 'shorthand' formulas.**

CYTOKININ / DERIVATIVE	ABBREVIATIONS After LETHAM (1978)	IUPAC-IUB SYMBOLS
base	BA	bzl <sup>6</sup> Ade
ribonucleoside	[9R]BA	bzl <sup>6</sup> Ado
nucleotide	[9R-MP]BA	bzl <sup>6</sup> Ado-5 <sup>1</sup> -P
dinucleotide	[9R-DP]BA	bzl <sup>6</sup> Ado-5 <sup>1</sup> -P-P
trinucleotide	[9R-TP]BA	bzl <sup>6</sup> Ado-5 <sup>1</sup> -P-P-P
3-glucoside	[3G]BA	bzl <sup>6</sup> G <sup>3</sup> Ade
7-glucoside	[7G]BA	bzl <sup>6</sup> G <sup>7</sup> Ade
9-glucoside	[9G]BA	bzl <sup>6</sup> G <sup>9</sup> Ade
9-ribosylglucoside	[9R-G]BA	bzl <sup>6</sup> 9GAde
alanine-conjugate	[9Ala]BA	Ala <sup>9</sup> bzl <sup>6</sup> Ade
ortho-OH base	(2OH)BA	2OHbzl <sup>6</sup> Ade
ortho-OH riboside	(2OH)[9R]BA	2OHbzl <sup>6</sup> Ado
meta-OH-base	(3OH)BA	3OHbzl <sup>6</sup> Ade
meta-OH-riboside	(3OH)[9R]BA	3OHbzl <sup>6</sup> Ado
ortho-OH-methylthio-9-glucoside	(2OH)[2MeS9G]BA	2OHbzl <sup>6</sup> G <sup>9</sup> ms <sup>2</sup> Ade
radiolabelled base (position	[8- <sup>14</sup> C]BA	

**Table 2.5 Suggested abbreviations for 6-(furanosylamino)purine (kinetin) and its metabolites. These are compared with IUPAC-IUB 'shorthand' formulas.**

CYTOKININ / DERIVATIVE	ABBREVIATIONS After LETHAM (1978)	IUPAC-IUB SYMBOLS
base	KIN	fzl <sup>6</sup> Ade
ribonucleoside	[9R]KIN	fzl <sup>6</sup> Ado
nucleotide	[9R-MP]KIN	fzl <sup>6</sup> Ado-5 <sup>1</sup> -P
3-glucoside	[3G]KIN	fzl <sup>6</sup> G <sup>3</sup> Ade
7-glucoside	[7G]KIN	fzl <sup>6</sup> G <sup>7</sup> Ade
9-glucoside	[9G]KIN	fzl <sup>6</sup> G <sup>9</sup> Ade
alanine-conjugate	[9Ala]KIN	Ala <sup>9</sup> fzl <sup>6</sup> Ade
Radiolabelled base (position 8 in purine ring)	[8- <sup>14</sup> C]KIN	

**Table 2.6 Suggested abbreviations for 6-(4-hydroxy-3-methylbutylamino)purine (dihydrozeatin) and its metabolites. These are compared with IUPAC-IUB 'shorthand' formulas.**

CYTOKININ / DERIVATIVE	ABBREVIATIONS After LETHAM (1978)	IUPAC-IUB SYMBOLS
base	DHZ	2,3Hio <sup>6</sup> Ade
ribonucleoside	[9R]DHZ	2,3Hio <sup>6</sup> Ado
nucleotide	[9R-MP]DHZ	2,3Hio <sup>6</sup> Ado-5 <sup>1</sup> -P
3-glucoside	[3G]DHZ	G <sup>3</sup> 2,3Hio <sup>6</sup> Ade
7-glucoside	[7G]DHZ	G <sup>7</sup> 2,3Hio <sup>6</sup> Ade
9-glucoside	[9G]DHZ	G <sup>9</sup> 2,3Hio <sup>6</sup> Ade
ala-conjugate	[9Ala]DHZ	Ala <sup>9</sup> 2,3Hio <sup>6</sup> Ade
O-glucoside of base	(OG)DHZ	OG2,3Hio <sup>6</sup> Ade
O-glucoside of riboside	(OG)[9R]DHZ	OG2,3Hio <sup>6</sup> Ado
O-xyloside of base	(OX)DHZ	OX2,3Hio <sup>6</sup> Ade
O-xyloside of riboside	(OX)[9R]DHZ	OX2,3Hio <sup>6</sup> Ado
radiolabelled base (position 8 in	[8- <sup>14</sup> C]DHZ	

Table 2.7 Suggested abbreviations for 6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)purine (zeatin) and its metabolites. These are compared with IUPAC-IUB 'shorthand' formulas.

CYTOKININ / DERIVATIVE		ABBREVIATIONS After LETHAM (1978)	IUPAC-IUB SYMBOLS
base	cis	( <i>cis</i> )Z	Z-io <sup>6</sup> Ade
	trans	Z	E-io <sup>6</sup> Ade
ribonucleoside	cis	( <i>cis</i> )[9R]Z	Z-io <sup>6</sup> Ado
	trans	[9R]Z	E-io <sup>6</sup> Ado
nucleotide	cis	( <i>cis</i> )[9R-MP]Z	Z-io <sup>6</sup> Ado-5 <sup>1</sup> -P
		[9R-MP]Z	E-io <sup>6</sup> Ado-5 <sup>1</sup> -P
3-glucoside		[3G]Z	G <sup>3</sup> io <sup>6</sup> Ade
7-glucoside		[7G]Z	G <sup>7</sup> io <sup>6</sup> Ade
9-glucoside		[9G]Z	G <sup>9</sup> io <sup>6</sup> Ade
ala-conjugate		[9Ala]Z	Ala <sup>9</sup> io <sup>6</sup> Ade
O-glucoside of base		(OG)Z	OGio <sup>6</sup> Ade
O-glucoside of riboside		(OG)[9R]Z	OGio <sup>6</sup> Ado
O-xyloside of base		(OX)Z	OXio <sup>6</sup> Ade
O-xyloside of riboside		(OX)[9R]Z	OXio <sup>6</sup> Ado
methylthio base		[2MeS]Z	io <sup>6</sup> ms <sup>2</sup> Ade
methylthio riboside	cis	( <i>cis</i> )[2MeS9R]Z	Z-io <sup>6</sup> ms <sup>2</sup> Ado
	trans	[2MeS9R]Z	E-io <sup>6</sup> ms <sup>2</sup> Ado
radiolabelled base (position 8 in purine ring)		[8- <sup>14</sup> C]Z	

**Table 2.8** Suggested abbreviations for 6-(3-methylbut-2-enylamino)purine (isopentenyl adenine) and its metabolites. These are compared with IUPAC-IUB 'shorthand' formulas.

CYTOKININ / DERIVATIVE	ABBREVIATIONS After LETHAM (1978)	IUPAC-IUB SYMBOLS
base	iP	i <sup>6</sup> Ade
ribonucleoside	[9R]iP	i <sup>6</sup> Ado
nucleotide	[9R-MP]iP	i <sup>6</sup> Ado-5 <sup>1</sup> -P
3-glucoside	[3G]iP	G <sup>3</sup> i <sup>6</sup> Ade
7-glucoside	[7G]iP	G <sup>7</sup> i <sup>6</sup> Ade
9-glucoside	[9G]iP	G <sup>9</sup> i <sup>6</sup> Ade
ala-conjugate	[9Ala]iP	Ala <sup>9</sup> i <sup>6</sup> Ade
methylthio riboside	[2MeS9R]iP	i <sup>6</sup> ms <sup>2</sup> Ado
radiolabelled base (position 8 in purine ring)	[8- <sup>14</sup> C]iP	

In the decade since the call for 'a generally accepted system of abbreviations' (LETHAM, 1978), it is obvious that little heed has been taken. However, it is hoped that this proposal will provide a standard for future abbreviations of cytokinins and their metabolites. The proposed system, which limits the generation of confusing synonyms, can only be an improvement.

## CHAPTER 3

### GENERAL MATERIALS AND METHODS

#### 3.1 Experimental approach

The experimental work in this study involved the exogenous treatment of suspension cultures with plant growth regulators. Following incubation for set periods, cytokinin metabolites were extracted and subsequently identified. General materials and methods used are described in this chapter. The initiation of four cell suspension culture lines from three different species is detailed. General analytical techniques used in the application, extraction, identification and quantification of the radio-labelled metabolites of 6-(benzylamino)purine are also described.

#### 3.2 Initiation of suspension cultures used as experimental systems

A suspension culture consists of a relatively homogeneous population of cells and aggregates of cells dispersed and growing in moving liquid medium (STREET, 1973). These cells are readily accessible to exogenously applied chemicals (DIXON 1985) and grow under defined, aseptic conditions. Unlike cell cultures, whole plants are not normally axenic, so allowing for micro-organisms to contribute to the metabolism of exogenously applied chemicals.

HORGAN (1987) noted that the metabolism of cytokinin applied to soybean systems differed depending on whether solid-grown (callus) cultures or cell suspensions were used. Metabolic differences were attributed to differences in the rates of supply of cytokinin to the growing cells. Agar has been shown to influence the availability of BA to the plant material (DEBERGH, 1983; BORNMAN & VOGELMANN, 1984). Horgan (1987) recorded that in callus tissue, cells in intimate contact with the solid medium received a high initial dose, which was attenuated across the tissue. Such a substrate gradient over time would likely be reflected in a gradient of metabolic products over that period, especially in short-term studies. Thick plant tissues would be expected to confuse rapid kinetic studies, as it becomes difficult to elucidate which pathways

are affected before which. In the soybean investigation of HORGAN (1987), suspension cultured cells received a uniform dose of cytokinin and rapidly took up the hormone, so circumventing the complications noted for solid-grown tissues. PALNI, PALMER and LETHAM (1984) suspended small callus pieces in an incubation solution for uniform uptake of cytokinins, and in order to minimise metabolic gradients within the tissue.

In summary, suspended single cells or small aggregates are more likely ensured equal exposure to the substrate, so simplifying metabolic studies.

In the current studies the physiological status of cell cultures has been defined more by their phase of growth than by their age and morphology. During the course of a batch-propagated cell suspension, both morphological and cytological changes in the cells have been reported to occur. This is reflected in Bergmann plating efficiencies which vary between phases of culture growth (THOMAS & DAVEY, 1975). Enzymic activities similarly vary through the course of the accompanying cell cycles (MITCHISON, 1981). ZILKAH and GRESSEL (1978) found the stage of development of cell cultures affected their reactivity to applied growth regulators. Uptake of BA by tobacco cell cultures was found less intense during the stationary phase than during the exponential phase (LALOUE, PETHE-TERRINE & GUERN, 1981). Similarly, FUBEDER, ZIEGLER, PETERS and BECK (1989) showed that the metabolism of both a cytokinin base and riboside by *Chenopodium* L. suspension cultures was more pronounced during the active growth phase than the stationary phase. Hence in order to make valid comparisons between different cultures, BA was, as far as practical, applied to cultures at a similar phase of growth.

Single cells or small aggregates of cells growing in batch cultures may present a useful experimental system. However, the question arises: is the cytokinin metabolism expressed in cell cultures representative of metabolism in intact plants?

All plant species share one common feature: they have or have at some time in their evolutionary history been represented by a single cell. This single (totipotent) cell must therefore contain all the genetic information necessary for the organism to grow

and reproduce in its environment. The metabolic potential of a whole plant or an organ should also be present in each single cell, though not necessarily expressed. An example of this potential would be the spontaneous appearance of cytokinin-autonomous cells in cultures which previously depended on the exogenous supply of these hormones. Such habituation indicates that all cells have the potential to synthesise cytokinins, yet only in variant cells is the capacity for synthesis 'turned on' (MIURA & MILLER, 1969). KAMINEK (1992) regarded habituation as playing a natural role in the initiation of new sites of cytokinin biosynthesis in both time and space.

Differentiation of cells *in vivo* does not result in the permanent inactivation of genetic material. Some obvious exceptions exist, namely with non-living mature cells such as velamen and xylem vessels. The production of embryos from somatic cells in suspension culture (KARTHA, 1982) demonstrates that the potential totipotency of differentiated cells has, at least in some species, not been irreversibly altered in the process of differentiation.

Responses at the cellular level may be translated into similar responses in the intact plant. On this basis, screening programs using cell cultures have been run to select for desirable traits (DIX, 1990). The metabolism of herbicides in such cultures has, in some species, shown a close parallel to that noted for whole plants (GRESSEL, 1980). This author recommended that metabolic pathways be elucidated in cell cultures first, before rechecking the pathways and products with intact plants. After considering the affect of herbicides on different systems, ZILKAH, BOCION and GRESSEL (1977) concluded that cell cultures reflect the whole plant, albeit in a complicated way. In terms of their physiology, GRESSEL (1980) considered cell cultures to have 'multihomogeneous' states; at different phases of culture growth, metabolic responses were analogous to those of a range of differentiated plant organs. In considering differential expression of genes which regulate cytokinin metabolism over time (essentially during plant development), LEE, MOK, MOK, GRIFFIN and SHAW (1985) suggested that analyses be made with both organised (intact) and unorganised (callus or cells) tissues of the same genotype to address the problem.

Although whole plant varietal differences in herbicide detoxification mechanisms has been shown to carry through to soybean suspension cultures (ZILKAH, BOCION & GRESSEL, 1977), it has also been demonstrated that soybean leaves (ZHANG, LETHAM, WONG, NOODÉN & PARKER, 1987) and tissue cultures (FORSYTH & VAN STADEN, 1986) metabolise applied BA in a manner which qualitatively differs. It should perhaps be noted that these two studies made use of different soybean cultivars.

In conclusion, it is likely that in a cell culture the genes expressed are not all the same as those operating in an intact plant. Studies involving cell cultures may illustrate the potential of intact plants of a particular species to metabolise cytokinins, at least in some plant organs during the course of their development.

Three different species were selected for use in the current investigations. A soybean (*Glycine max* (L.) Merrill cv. Acme) system was chosen as production of the unusual alanine conjugate of BA is supposedly restricted to the leguminosae (LETHAM, SUMMONS, PARKER & MACLEOD, 1979). Additionally, this particular cultivar has been shown to oxidatively catabolise applied BA (FORSYTH & VAN STADEN, 1986), in a manner which conflicts with current perceptions of cytokinin oxidase substrate-specificity.

Carrot (*Daucus carota* L.) cell cultures were employed as little is known of BA metabolism in this (apiaceous) species, despite use of carrot callus in cytokinin bioassays (MATSUBARA, 1990).

ENGVILD (1972) encouraged the use of carnation suspension cultures in biochemical studies after finding them easy to establish and predictable in their pattern of growth. Accordingly, cell suspensions of a native South African species, *Dianthus zeyheri* (Caryophyllaceae), were established. This species had not previously been domesticated, so allowing for a comparison of BA metabolism between a wild species and its domesticated relative, *D. caryophyllus* L.

*Glycine*, *Daucus*, and *Dianthus* represent not only three genera but also three plant

families which are evolutionary diverse. Thus hormonal studies made using these systems were likely to reveal either wide ranging, or alternatively, conservative interactive effects between auxin and BA.

### 3.2.1 *In vitro* culture of *Dianthus zeyheri* subsp. *natalensis*, a South African carnation

#### **3.2.1.1 Introduction**

The modern day carnation (*Dianthus caryophyllus* L.) is a 'man-made plant' (HOLLEY & BAKER, 1963) whose floral integrity has diminished at the expense of horticulturally desirable traits. Differences in floral integrity (NICHOLS, 1977) and rooting responses (STONE, 1963), which are evident between cultivars, suggest that physiological aspects of the plant may also have changed during cultivation.

*D. caryophyllus* is widely used in physiological studies, especially with respect to flower senescence (SACALIS, 1986; COOK & VAN STADEN, 1988). Despite this frequent use, cell suspension cultures of this north temperate species have seldom been employed as tools in such fundamental investigations. This is surprising, given the ease of establishment of such systems (ENGVILD, 1972). Unlike cell suspensions of *D. caryophyllus*, those of *D. zeyheri* could be considered 'natural', for the species has never been domesticated.

Of the approximately three hundred *Dianthus* species, only a few have been introduced to tissue culture (GEORGE, PUTTOCK & GEORGE, 1987), with apparently none from the southern hemisphere. *Dianthus zeyheri* Sond. subsp. *natalensis* Hooper is one of fifteen species indigenous to central and southern Africa. HOOPER (1959) described robust specimens with up to twenty pink flowers comprising a subcorymbose terminal inflorescence. This summer-flowering perennial exhibits a geophytic habit, retiring to a thickened and fusiform taproot during periods of drought. Although attractive, the horticultural potential of *D. zeyheri* is unknown. Its potential may lie as a genetic source of such desirable traits as resistance to disease and water stress. Interspecific somatic hybridisation by protoplast fusion using techniques specifically developed for *Dianthus* (NAKANO & MII, 1993; 1993a) would offer an

alternative hybridisation method to conventional sexual techniques.

The current investigation aimed to establish cell suspensions from a wild *Dianthus* species for experimental studies, and to establish the extent to which the 'behaviour' *in vitro* of *D. zeyheri* resembled that of its domesticated relative.

### 3.2.1.2 *Materials and methods*

#### 3.2.1.2.1 Axenic seed germination

Seed from dehiscent capsules was collected from a native population at Pietermaritzburg in Natal and stored in paper packets at 25°C. Preliminary investigations revealed that no dormancy mechanisms were in operation.

Half strength MURASHIGE and SKOOG (1962) media supplemented with sucrose (10 g  $\ell^{-1}$ ) was prepared and adjusted to pH 5.7. No growth regulators were included in the medium. Aliquots of 10 ml were dispensed to 25 x 150 mm rimless tubes and solidified with Unilab agar (9 g  $\ell^{-1}$ ). Seeds were soaked in 3.5% NaOCl solution (with 0.02% Tween 20) for 15 min during which period the testae bleached considerably. Alternatively, seeds were surface sterilised with 0.2% HgCl<sub>2</sub> for 10 min. After three successive 5 min rinses in sterile distilled water, each seed was transferred to a culture vessel. These were capped with Cap-O-Test aluminium caps, sealed with 'Parafilm' (Gallenkamp Ltd), and placed under cool white fluorescent light at a level of 46.3  $\mu\text{mol m}^{-2} \text{s}^{-1}$ .

#### 3.2.1.2.2 Meristem culture

Pot-grown seedlings were cultured in the greenhouse for 8 weeks, whereafter shoot tips were harvested 2 cm from the apex. All leaves on shoots were removed by hand, excepting those less than 5 mm in length. The remaining sections were sterilised with 96% ethanol for 30 sec, and rinsed in sterile distilled water for 30 min. Explants were prepared by teasing away leaves and leaf primordia surrounding the meristem, and excising the stem  $\pm 0.6$  mm below the apical dome. Shoot apices from seedlings

grown in axenic culture were also used as explants. These explants were transferred apex-up to one of two media; a callus-inducing medium containing 2,4-D ( $2 \text{ mg } \ell^{-1}$ ) and a multiple shoot-forming medium containing  $\alpha$ -naphthaleneacetic acid (NAA) ( $1 \text{ mg } \ell^{-1}$ ) and kinetin (KIN) ( $0.5 \text{ mg } \ell^{-1}$ ). Both media used are modifications of those used by EARLE and LANGHANS (1975) for *Dianthus caryophyllus*. The basal medium of MURASHIGE and SKOOG (1962) was supplemented with Unilab agar and  $30 \text{ g } \ell^{-1}$  sucrose as a source of organic carbon (Table 3.1). For the multiple shooting medium agar was supplied at  $12 \text{ g } \ell^{-1}$  and for callus induction at  $8 \text{ g } \ell^{-1}$ . Before autoclaving the pH was adjusted to 5.7. Tubes were sealed with 'Parafilm'. Shoot-induction was stimulated in the light and callus initiation promoted in the dark, at  $25 \pm 1^\circ\text{C}$ .

#### 3.2.1.2.3 Suspension cultures

The nutrient medium employed was identical to that of the solid medium with the omission of agar. The auxin 2,4-D ( $2 \text{ mg } \ell^{-1}$ ) was present in the medium. Diced callus (20 g in 3 mm cubes) was added to 100 ml of liquid medium in a 500 ml Erlenmeyer side-arm flask. Flasks were sealed with cotton wool bungs and the necks covered with tin foil. The flasks were agitated at 120 rpm on an orbital shaker (lateral displacement 3.8 cm). Batch cultures were incubated under cool white fluorescent lights ( $1 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ) for 24 hours at  $25 \pm 1^\circ\text{C}$ . Suspensions were routinely subcultured every 7 days, in the late linear or early stationary phase of growth. In subculturing, 30 ml cells and stale medium were transferred to 70 ml fresh medium. The settled cell volume (SCV) method was used for the measurement of growth of cultured plant cells. In this case the SCV represents the proportion of the culture occupied by cell aggregates settled out after 10 minutes. Each day, culture growth was measured as the average SCV of 5 separate cultures. The viability of cultures was monitored using a fluorescein diacetate (FDA) stain (WIDHOLM, 1972); fluorescence was observed under ultraviolet light (Zeiss IM35 inverted microscope).

Table 3.1 Relative levels of minerals, salts, vitamins, phytohormones and carbohydrate sources included as components of media employed in current studies. Original formulations are provided for comparison.

Media components*	System Investigated								
	GAMBORG, MILLER & OJIMA (1968) B5	Carrot callus and suspensions	Soybean suspension	MURASHIGE & SKOOG (1962)	<i>D. zeyheri</i> callus and suspensions	<i>D. zeyheri</i> meristems	Carrot callus initiation	MILLER (1967)	Soybean callus
$\text{NH}_4\text{NO}_3$	-	-	-	1650	1650	1650	1650	1000	1000
$\text{KNO}_3$	2500	3000	2500	1900	1900	1900	1900	1000	1000
$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	-	-	-	-	-	-	-	500	500
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	150	150	150	440	450	450	450	-	-
$\text{CaCl}_2$	-	-	-	-	-	-	-	65	65
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	250	500	250	370	370	370	370	71.5	71.5
$\text{H}_2\text{PO}_4$	-	-	-	170	170	170	170	300	300
$(\text{NH}_4)_2\text{SO}_4$	134	134	134	-	-	-	-	-	-
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	150	150	150	-	-	-	-	-	-
$\text{H}_3\text{BO}_3$	3	0.3	3	6.2	6.2	6.2	6.2	1.6	1.6
$\text{InSO}_4 \cdot \text{H}_2\text{O}$	10	1	-	-	-	-	-	-	-
$\text{ZnSO}_4 \cdot 4\text{H}_2\text{O}$	-	-	13.2	22.3	22.3	22.3	22.3	14	14
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	2	0.2	2	8.6	8.6	8.6	8.6	3.8	3.8
$\text{CuSO}_4$	0.75	0.075	0.75	0.83	0.84	0.84	0.84	0.8	0.8
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25	0.025	0.25	0.25	0.26	0.26	-	-	-
$(\text{NH}_4)_2\text{MoO}_7 \cdot 4\text{H}_2\text{O}$	-	-	-	-	-	-	-	0.1	0.1
$\text{NiSO}_4$	0.025	-	-	-	-	-	-	-	-
$\text{NiSO}_4 \cdot 5\text{H}_2\text{O}$	-	0.0025	0.025	0.025	0.026	0.026	0.026	-	-

Media components <sup>a</sup>	System Investigated								
	GAMBORG, MILLER & OJIMA (1968) B5	Carrot callus and suspensions	Soybean suspension	MURASHIGE & SKOOG (1962)	<i>D. zeyheri</i> callus and suspensions	<i>D. zeyheri</i> meristems	Carrot callus initiation	MILLER (1967)	Soybean callus
Cu(NO <sub>3</sub> ) <sub>2</sub> ·3H <sub>2</sub> O	-	-	-	27.85	-	-	-	0.35	0.35
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.025	0.0025	0.025	0.025	0.026	0.026	0.026	-	-
FeSO <sub>4</sub> ·7H <sub>2</sub> O	-	-	-	27.85	-	-	-	-	-
Na <sub>2</sub> EDTA(2H <sub>2</sub> O)	-	-	-	37.25	-	-	-	-	-
NaFe EDTA <sup>b</sup>	28	28	28	-	40	40	40	13.2	13.2
Inositol	100	100	100	100	100	100	100	100	100
Nicotinic acid	1	1	0.5	0.5	0.5	0.5	0.5	0.5	2
Thiamine.HCl	10	10	0.1	0.1	0.1	0.1	0.1	0.1	0.8
Pyridoxine.HCl	1	1	0.5	0.5	0.5	0.5	0.5	0.1	0.8
Glycine	-	-	-	2	-	-	-	-	-
Sucrose	20000	20000	20000	30000	30000	30000	30000	30000	30000
2,4-D	2	2	2	-	2	-	-	-	-
NAA	-	-	-	-	-	1	-	2	2
IAA	-	-	-	1-30	-	-	10	-	-
KIN	-	-	-	0.04-10	-	0.5	0.1	variable	0.5
pH	5.5	5.7	5.7	5.75	5.65	5.65	5.7	5.8	5.8

<sup>a</sup>All values in mg l<sup>-1</sup>

<sup>b</sup>NaFe ethylenediaminetetraacetate

### 3.2.1.3 Results

#### 3.2.1.3.1 Seed culture

Within 5 days, 80% of seeds treated with NaOCl had germinated to produce pathogen-free seedlings. Seeds surface-sterilised with 0.2% HgCl<sub>2</sub> for 10 min showed no germination after one month.

After 30 days a vitrified seedling was observed in culture. Vitrification in *Dianthus caryophyllus* has previously been documented (ZIV, MEIR & HALEVY, 1983; LESHEM, 1986; MESSEGUER, ARCONADA & MELE, 1993). Some two to three per cent of seeds bore achlorophyllous mutants, all of which lost viability in culture despite the provision of sucrose as a carbohydrate source.

After 60 days in culture the seedling crowns callused and several shoots developed from the base, in a 'multiple shooting' type response. The establishment of multiple shoots from seedlings *in vitro* provides several axenic shoot tips for further shoot multiplication, an important consideration when greenhouse material is heavily contaminated. Callus was excised from seedling crowns (interface of plants and agar between roots and shoots), and transferred to a basal medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) (2 mg l<sup>-1</sup>). This quickly proliferated a mass of off-white friable callus.

With the germination of one seed, the testa detached from the juvenile plant and fell onto a cotyledon which lay appressed to the agar. This seed coat never directly contacted the semi-solid medium. Callus was observed emerging from the inside of this testa, possibly from residual endosperm or perisperm (BUELL, 1952). Callus production from seed of *Dianthus* has previously been recorded on a medium supplemented with 2,4-D, *p*-chlorophenoxyacetic acid (*p*CPA) and KIN (SCHENK & HILDEBRANDT, 1972), when callus tissue was derived from the seed mesocotyl. In this investigation the slow-growing callus was transferred to the 2,4-D supplemented medium, on which it grew rapidly in the light. When exposed to light for periods exceeding 6 weeks, the normally light coloured callus became green and more

compact. Subculturing at four weekly intervals yielded a friable callus suitable for the establishment of cell suspension cultures. PALET, RIBAS-CARBO, ARGILES and AZCON-BIETO (1991) established heterotrophic callus from cut carnation petals. These authors induced cell greening by supplying a quantum flux density of  $90 \mu\text{mol m}^{-2} \text{s}^{-1}$ . These authors simultaneously altered the phytohormone addenda to initiate this photomixotrophic line. In the current investigation the gelling agent was shown to affect the greening process. Gelrite ( $2 \text{ g l}^{-1}$ ) promoted greening whilst Unilab agar ( $8 \text{ g l}^{-1}$ ) did not.

#### 3.2.1.3.2 Meristem culture

On the KIN and NAA supplemented medium multiple shoots, but no roots formed from the top of the explants. However, most of these shoots appeared vitrified after 4 weeks. A small amount of compact callus was produced from the base of the explants. Rooting of shoots was not attempted, although carnation shoots produced *in vitro* have previously been rooted on media free of plant growth regulators (JELASKA & SUTINA, 1977; LESHEM, 1986) or with phytohormones included (PETRU & LANDA, 1974). Explants transferred to a basal medium containing 2,4-D ( $2 \text{ mg l}^{-1}$ ) proliferated a creamy callus of a fairly friable nature. It was necessary to select a friable line of this apex-derived callus to initiate a cell suspension.

#### 3.2.1.3.3 Suspension cultures

Diced callus readily dispersed to form small aggregates in suspension culture. However, with repeated subcultures the aggregates increased in size, especially in the case of the seed-derived cell culture. Growth curves for these cultures (Figures 3.1 and 3.2) indicate a typical S-curve pattern for both seed and crown-derived cultures. In the case of suspensions initiated from apical meristem callus, the lag phase of growth was barely discernible. Cell cultures of *D. caryophyllus* had a doubling time of 2 days, considering dry weight as a growth parameter (ENGVILD, 1972). From SCV determinations, those of *D. zeyheri* cultures were 4.1 days, 2.6 days, and 3.4 days for seed, shoot apex and crown-derived cultures respectively.

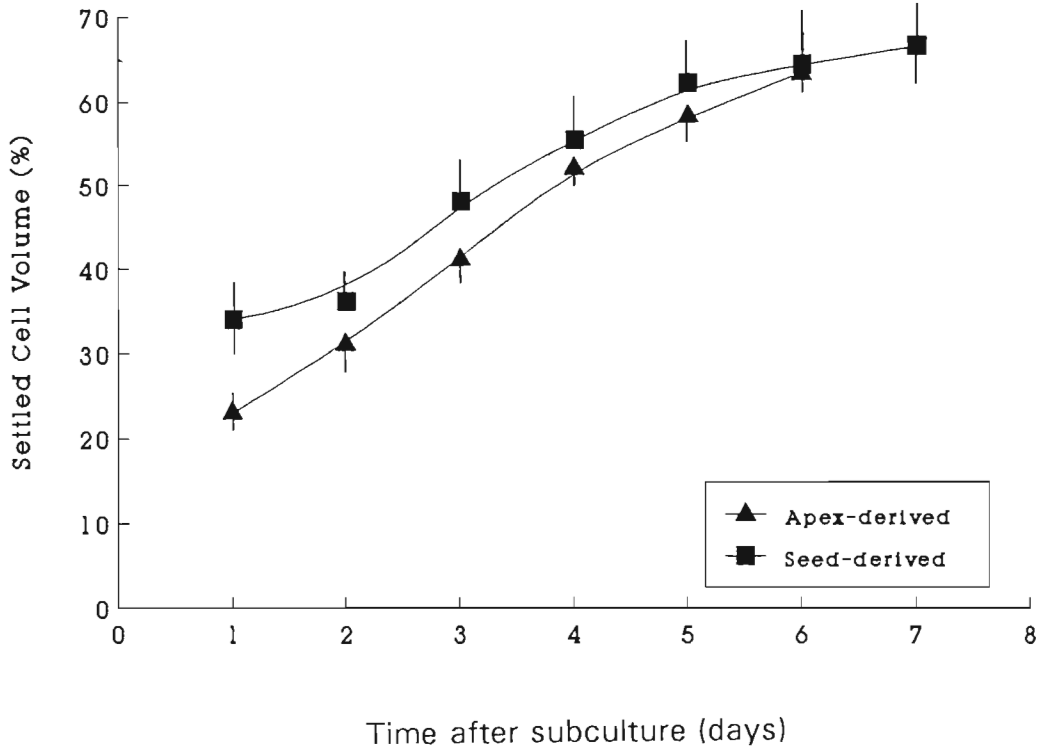


Figure 3.1 Growth curve of suspension cultures derived from seed and apex callus cultures of *D. zeyheri*, in their fourth passage. Growth is plotted on an arithmetic scale. Bars denote standard errors.

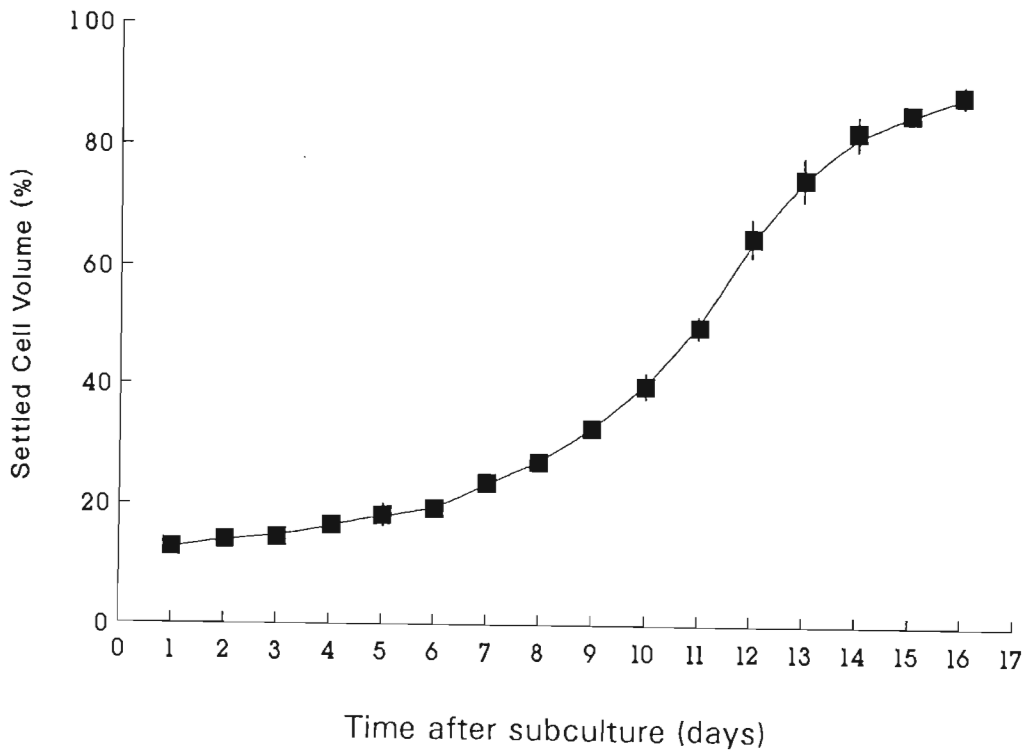


Figure 3.2 Growth curve of suspension cultures derived from crown callus of *D. zeyheri*, in their second passage. Growth is plotted on an arithmetic scale. Bars denote standard errors.

Successful subculture of apex-derived suspensions was achieved at initial SCV densities as low as  $1.89 \pm 0.46\%$ .

When assessing viability, esterases present in the incubation medium led to spurious fluorescence in reaction with the fluorescein diacetate, making it necessary to transfer cells to a fresh solution before staining. For all three types of suspension culture, friable callus lawns were established from filtered suspensions plated on the callus-inducing medium.

#### 3.2.1.4 Discussion

The cut carnation *Dianthus caryophyllus* has been cultured *in vitro* in several different systems (GEORGE, PUTTOCK & GEORGE, 1987). A suspension culture of this species (cv. 'White Sim') was established by ENGVILD (1972) in a 2,4-D medium from stem pith-derived callus. In agreement with ENGVILD (1972), a lag phase was absent in the growth curve of apex-derived cultures (Figure 3.1), regardless of whether subculturing occurred in the late lag phase or in the early stationary phase.

Only a few members of the genus have been cultured *in vitro*. SPINSKI, BECK and McCOWN (1974) cultured both *D. plumarius* L. and *D. caryophyllus* callus on the same basal medium. In several ways the response of *D. zeyheri* subsp. *natalensis* may be likened to that of the cut carnation; viz. multiple shoot and callus formation on the media of EARLE and LANGHANS (1975), vitrification (ZIV, MEIR & HALEVY, 1983), calli greening (PALET, RIBAS-CARBO, ARGILES & AZCON-BIETO, 1991) and suspension culture growth in a 2,4-D medium without a significant lag phase (ENGVILD, 1972). The almost parallel response of *D. zeyheri* to procedures developed for *D. caryophyllus* suggests that other *Dianthus* species could be similarly cultured.

ENGVILD (1972) found carnation suspension cultures to behave so predictably that this author encouraged their use in biochemical work. The results of this present study lend support to this suggestion.

### 3.2.2 *In vitro* culture of carrot (*Daucus carota* L. cv. Keystone Danvers 126)

#### **3.2.2.1 *Callus initiation***

Whole tap roots of carrot (*Daucus carota* cv. Keystone Danvers 126) supplied by 'Dawnfresh Carrots' were peeled and cut into 10 mm thick cylinders. Segments were soaked for 10 minutes in a mild sterilant (0.35% NaOCl solution with 0.02% Tween 20) followed by three successive 5 minute rinses in sterile distilled water. Cylinders 7 mm in diameter were punched from the cambial zone (using a cork borer), and transferred to sterile distilled water. The damaged ends were excised and 3 mm explant discs prepared. Four explants were placed in each jar containing 50 ml sterile culture media; the MURASHIGE and SKOOG (1962) basal medium containing 30 g  $\ell^{-1}$  sucrose and Unilab agar (8 g  $\ell^{-1}$ ) was further supplemented with 10 mg  $\ell^{-1}$  IAA and 0.1 mg  $\ell^{-1}$  KIN. In the dark, a fairly compact callus was derived from explants within 21 days. After transfer of the callus to a modified B5 medium (Table 3.1) (GAMBORG, MILLER & OJIMA, 1968), a friable callus line was generated, selected for, and successfully maintained in the light (42  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) on a medium containing 2,4-D (0.4 mg  $\ell^{-1}$ ).

#### **3.2.2.2 *Carrot suspension cultures***

Callus readily dispersed to form small aggregates in suspension culture. A modified GAMBORG, MILLER and OJIMA (1968) B5 medium was again used for suspension culture establishment; 2,4-D at 2 mg  $\ell^{-1}$  was included (Table 3.1). Inocula of 15 g friable callus were transferred to 100 ml of media in 500 ml Erlenmeyer flasks. Flask necks were stoppered with cotton wool bungs and covered in foil, and cultures agitated at 120 rpm on an orbital shaker (lateral displacement 3.8 cm). Lighting was provided by cool white fluorescent lamps (1  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) over a 24 hour photoperiod, at  $25 \pm 1^\circ\text{C}$ . Within 12 hours the callus had almost fully dispersed in the moving medium. The batch cultures were routinely subcultured every 7 days, in the late linear or early stationary phases of growth; in subculturing 35 ml of cells and stale medium were transferred to 65 ml fresh medium. The settled cell volume (SCV) method of

suspension culture measurement was employed. Each day, culture growth was measured as the average SCV of 5 separate cultures. Suspensions showed a typical S-curve pattern of growth (Figure 3.3) with a doubling time of 3.88 days.

### ***3.2.2.3 Effect of the auxin 2,4-D on growth of carrot suspensions***

The effect on suspension culture growth of 2,4-D was investigated. Callus used as inocula had previously been maintained on a solidified B5 medium with 2,4-D included at  $0.4 \text{ mg l}^{-1}$ . Cultures were initiated after transfer of 15 g inocula to 100 ml media in 500 ml Erlenmeyer flasks. Auxin (2,4-D) was included at levels of 0.5, 2, and 4 mg  $\text{l}^{-1}$  before the pH was adjusted to 5.7 with NaOH. After 20 days when suspensions were clearly established, the flask contents were subcultured; 35 ml old cells and stale media were transferred to 65 ml fresh medium. As the initial SCV was relatively small ( $\leq 17\%$ ) in all cases (Figure 3.4), the lag phase was more pronounced than previously noted (Figure 3.3), when the initial SCV (%) was  $\geq 30\%$ . Cultures of relatively high initial inocula attain the stationary phase more rapidly (KAMO & HODGES, 1986). Accordingly, low inoculum cultures permitted monitoring of growth over a longer period before subculturing again became necessary. Of the levels of 2,4-D considered, it was evident that  $2 \text{ mg l}^{-1}$  was optimal for culture growth (Figure 3.4). Casual observation revealed that these cell suspensions were not composed solely of single cells, but also of cell clusters and small aggregates. Considerable variation in the morphological appearance of cells in culture was noted. This seems a common occurrence (THOMAS & DAVEY, 1975). Despite this heterogeneity, the systems were considered more physiologically homogeneous than either calli or differentiated organs (GAMBORG, 1982). Manipulation of the incubation medium, particularly with regard to phytohormone addenda (THOMAS & DAVEY, 1975) would likely have improved the dispersion of cells in culture, but in view of the standard levels of auxins required, an attempt was not made to effect this. The lack of complete dispersion probably contributed to the observed rapid growth rates, for cell division is initiated more rapidly in cell aggregates than in free cells following subculture (THOMAS & DAVEY, 1975). The characteristic growth curves obtained for these cultures reflects a typical pattern of change in incubation media of batch cultures.

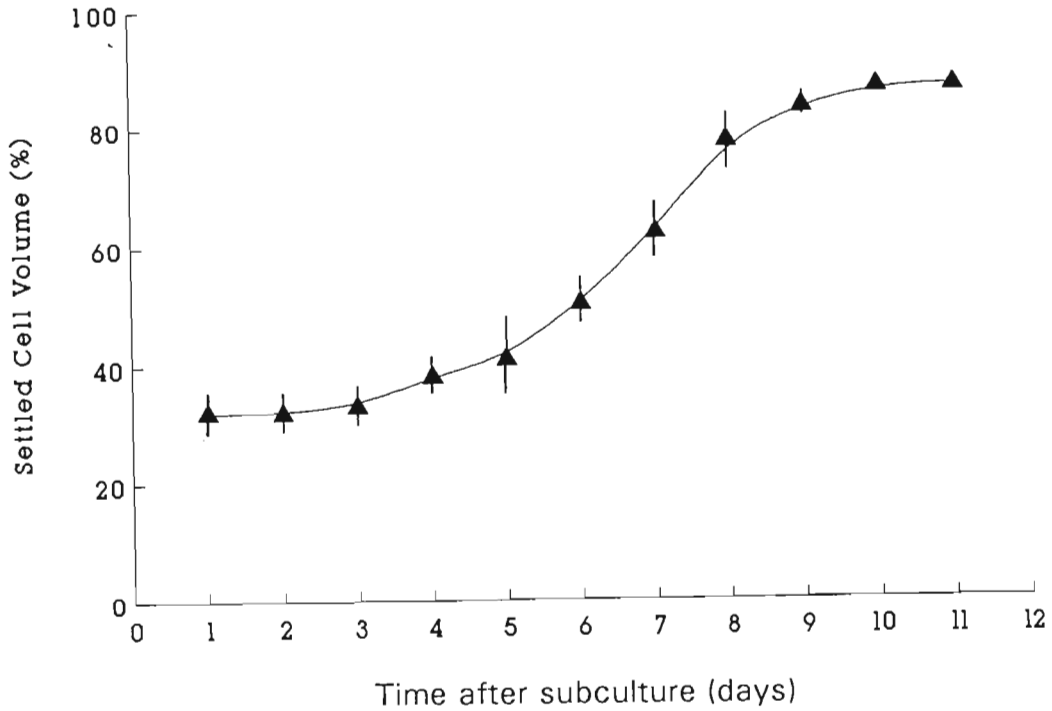


Figure 3.3 Growth curve of carrot suspension cultures in their second passage. Growth is plotted on an arithmetic scale. Bars denote standard errors.

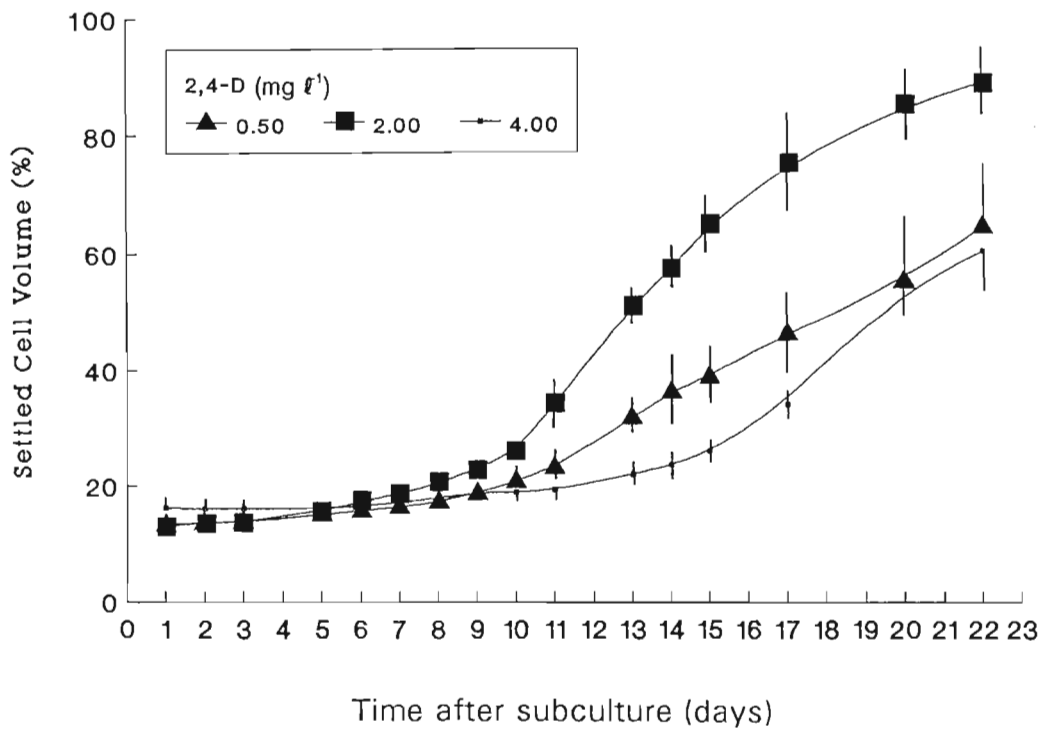


Figure 3.4 The effect of the auxin 2,4-D on growth of initiated carrot suspension cultures. Growth is plotted on an arithmetic scale. Bars denote standard errors.

### 3.2.3 *In vitro* culture of soybean (*Glycine max* (L.) Merrill cv. Acme)

#### **3.2.3.1 *Callus culture***

Soybean cotyledonary callus (*Glycine max* cv. Acme), capable of rapidly metabolising cytokinins (FORSYTH & VAN STADEN, 1986) was employed in the current studies. This cytokinin-dependent callus had been maintained on the medium of MILLER (1967), supplemented with KIN (0.5 mg  $\ell^{-1}$ ) and NAA (2 mg  $\ell^{-1}$ ) for numerous subcultures (Table 3.1). On a number of occasions in previous years, a proportion of the callus stock had inexplicably habituated, rendering it useless as a bioassay system. At such times the habituated callus was destroyed.

#### **3.2.3.2 *Soybean suspension cultures***

Cell cultures of cv. Acme have previously been established and maintained in Miller's medium containing 2 mg  $\ell^{-1}$  NAA and 1 mg  $\ell^{-1}$  BA using a 14 day subculture routine (WANG, EVERETT, GOULD & STREET, 1981). When these authors deprived their cytokinin-dependent cell lines of cytokinin, cell division was arrested. Cell viability remained high (90%) after several days, despite the lack of cell division. Growth resumed upon cytokinin reapplication. WITHAM (1968) supplied cv. Acme callus with only 2,4-D (in the absence of any cytokinin) and found that at higher concentrations of auxin (0.5  $\rightarrow$  5 mg  $\ell^{-1}$ ), substantial callus growth was detected. Prior to experimentation, stock callus cultures had been routinely subcultured on a medium identical to that used in the current studies. No habituation was detected by WITHAM (1968), to account for this growth. This author attributed callus growth to promotion of cytokinin synthesis by 2,4-D. Using cytokinin-autonomous rice callus, INOUE, MAEDA, YOSHIDA and ORITANI (1979) similarly found circumstantial evidence which indicated that auxin increases cytokinin synthesis. However, WYNDAELE, CHRISTIANSEN, HORSELE, RUDELSHEIM and VAN ONCKELEN (1988) found that cytokinin-autonomous soybean callus with low IAA content had higher cytokinin contents than lines with high IAA concentrations.

In the current studies, it was considered necessary (for comparative purposes) to grow cultures of different species on media which were uniform with respect to phytohormone addenda, viz. 2,4-D at  $2 \text{ mg } \ell^{-1}$ . The cytokinin-dependent line of soybean callus used to initiate suspension cultures could not be induced under normal culture conditions to fully habituate and grow vigorously in cytokinin-free media. As such, only sub-optimal growth of soybean suspensions was achieved (Figure 3.5). This result agrees with the report by WITHAM (1968) for soybean callus of the same cultivar. Despite poor growth, soybean suspensions remained viable and capable of metabolising supplied BA (Chapter 4) after several culture passages.

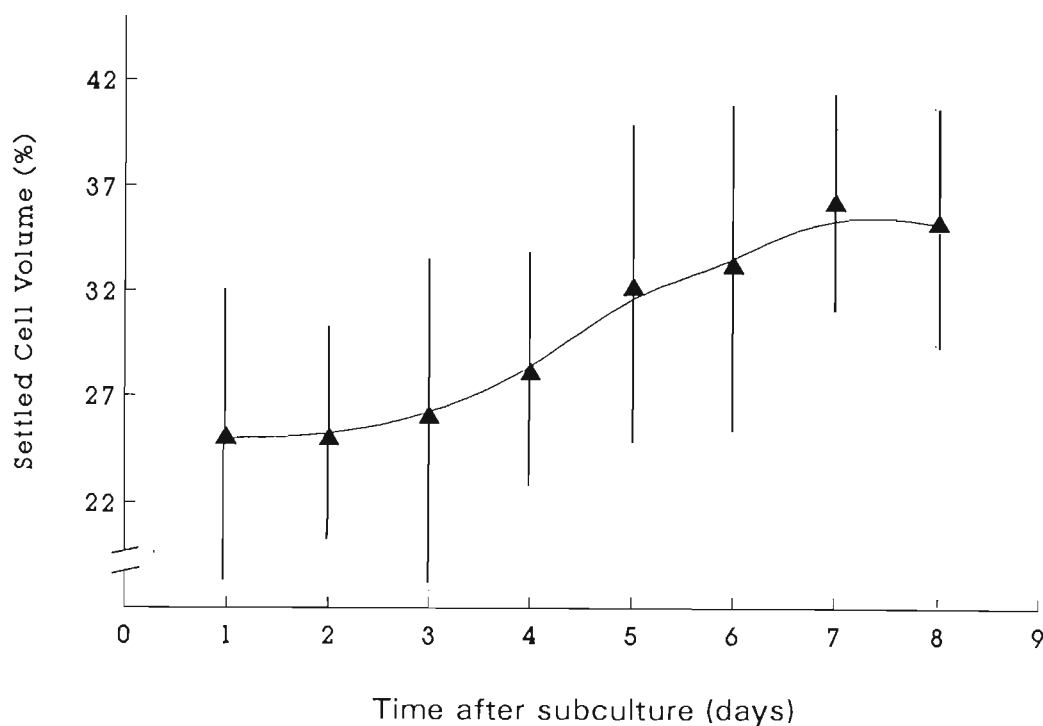


Figure 3.5 Growth curve of soybean suspension cultures in their second passage. Growth is plotted on an arithmetic scale. Bars denote standard errors.

The soybean suspensions were established in a modified B5 medium (GAMBORG, MILLER & OJIMA, 1968) which was specifically designed for growth of soybean (root) cell cultures (Table 3.1). Early attempts at establishing a viable culture using the original formulation proved unsuccessful. Only after substitution of the B5 vitamins

with those of MURASHIGE and SKOOG (1962) was browning reduced and growth recorded. Inocula of 16 g moderately friable white callus were introduced to 100 ml media in a 500 ml Erlenmeyer flask. Vessel necks were stoppered with cotton wool bungs and covered in tin foil, and cultures agitated at 120 rpm on an orbital shaker (lateral displacement 3.8 cm). Lighting was provided by cool white fluorescent lamps ( $1 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) over a 24 hour photoperiod, at  $25 \pm 1^\circ\text{C}$ . Callus dispersed over 2 days; after 7 days 40 ml fresh medium was added to each flask. Two weeks after culture initiation, suspensions were subcultured; the initial SCV (%) was reduced to approximately 25%. Each day, culture growth was measured as the average SCV of 5 separate cultures. Soybean suspension cultures were much less vigorous than other *in vitro* systems investigated, a characteristic reflected in the doubling time of 7 days (Figure 3.5).

### 3.3 Analytical techniques

#### 3.3.1 Introduction

Many analytical techniques have been employed in the quantification of cytokinins in plant tissues. These are the subject of a recent review by HEDDEN (1993).

Techniques employed in this study have focused on the analysis of alcoholic extracts containing metabolites of BA labelled with  $^{14}\text{C}$  in position 8 of the purine ring. The extraction process itself has been the subject of a protracted debate between different research groups. This is with regard to the questionable effectiveness of alcoholic solvents in inactivating non-specific plant phosphatases, such that the extract is representative of the cellular cytokinin composition at the time of harvest. It was necessary to first resolve this issue, by comparing a 'traditional' ethanolic extraction procedure, with, in particular, an extraction technique employing 'Bielecki cocktails'.

### 3.3.2 A comparison of four commonly used extraction techniques for the recovery of cytokinin nucleotides

#### **3.3.2.1 Introduction**

There is an extensive body of literature on cytokinin metabolism (LETHAM & PALNI, 1983). In such studies, extraction of metabolites from plant tissue is an obvious procedural step. However, no single extraction technique has been universally accepted. Instead one notes a wide array of extraction procedures viz.: boiling methanol (PALMER, SCOTT & HORGAN, 1981) or ethanol (MORRIS, 1981), cold perchloric acid (DOREE & GUERN, 1973), Bielecki cocktails (BIELESKI, 1964), ethanol (VAN STADEN & DIMALLA, 1978) or methanol (PALMER, HORGAN & WAREING, 1981a) at a variety of temperatures and aqueous dilutions, and these with (MIURA & MILLER, 1969) or without (PALMER, SCOTT & HORGAN, 1981) homogenisation of the material. In the absence of a standard method, disagreement occurs between the cytokinin 'schools' regarding the reliability of data generated. The reported occurrence of (*cis*)[9R]Z as a free cytokinin in plant tissues (WATANABE, YOKOTA & TAKAHASHI, 1981) is one such related issue of contention. TAY, MACLEOD and PALNI (1986) considered (*cis*)[9R]Z to be an artifact derived from enzymic breakdown of t-RNA's in tobacco shoots after extraction with ethanol. These authors could not detect this metabolite when material was extracted with Bielecki solvents. However, when rice grains were extracted with methanol, TAKAGI, YOKOTA, MUROFUSHI, SAKA and TAKAHASHI (1989) obtained good recoveries of glucoside and ribotide cytokinins, together with (*cis*)[9R]Z. These authors reasoned that if glucosidases and phosphatases had been successfully inactivated by methanol, than it would be unlikely for a nuclease (t-RNA hydrolysing enzyme) to survive the same extraction. Criticism has been levelled (DOREE & GUERN, 1973; HORGAN, 1978) particularly at those workers using traditional ethanolic extraction procedures. It was argued that workers considering the levels and ratios of ribosides and nucleotides need to concern themselves with the non-specific phosphatases present in plant tissues. The extraction method employed has to successfully inactivate such enzymes, for 'the composition of a tissue extract should reflect the state of the tissue at the moment of sampling' (BIELESKI, 1964). Since this

author advocated the use of his 'MCF cocktail', several research groups have embraced his technique. However, even after making allowance for the range of tissue types requiring extraction, it is clear (Table 3.2) that little agreement on the precise contents and ratios of cocktails has been reached. Extraction and centrifugation periods, temperatures, sample mass to solvent volume ratios and other variables are yet further considerations. On the other hand, a similarly broad range of ethanolic extraction variables is also evident in method reports, reflecting perhaps a rather indifferent approach to the whole extraction issue.

**Table 3.2 Variations on the Bielecki cocktail composition**

Solvent Cocktails		Reference
MCF(W) <sup>a</sup>	MFW <sup>b</sup>	
12:5:3 <sup>c</sup>	-	BIELESKI (1964)
12:2:1:2	6:1:4	NOODÉN & LETHAM (1984)
12:5:1:2	6:1:4	PALNI, HORGAN, DARRAL, STUCHBURY & WAREING (1983); PALNI, SUMMONS & LETHAM (1983); TAY, MACLEOD & PALNI (1986)
12:5:3	60:40:1	HORGAN (1978)
12:3:1:4	-	LALOUÉ & PETHE (1982)
-	14:1:2	NOODÉN, SINGH & LETHAM (1990)
12:5:1:2	60:1:40	PARKER, LETHAM, COWLEY & MACLEOD (1972)

<sup>a</sup>Methanol:Chloroform:Formic acid:(Water) (Parts by volume)

<sup>b</sup>Methanol:Formic acid:Water (Parts by volume)

<sup>c</sup>2 M Formic acid

This investigation considers four widely used extraction procedures and compares their effectiveness in the recovery of a cytokinin nucleotide, or viewed conversely, their effectiveness in inactivating non-specific plant phosphatases. Phosphatases

present in plant tissues convert nucleotides to ribosides. Thus high nucleoside ([9R]BA) levels relative to detectable monophosphate ([9R-MP]BA) indicates potentially high phosphorolytic activity during extraction of the tissue, particularly after homogenisation of the material. Soybean callus was used as experimental material because it has been shown that the nucleotide is formed following the application of free bases (FOX & CHEN, 1967; HORGAN, 1978).

### 3.3.2.2 *Materials and methods*

#### 3.3.2.2.1 Plant material

Soybean cotyledonary callus (*Glycine max* cv. Acme), capable of rapidly metabolising cytokinins (FORSYTH & VAN STADEN, 1986) was used in this investigation. The callus had been maintained on the medium of MILLER (1965) (Table 3.1) for numerous subcultures. Immediately prior to the start of the experiment, five-week-old callus was diced into 3 mm cubes.

#### 3.3.2.2.2 Cytokinin application

[8-<sup>14</sup>C]BA (0.167 MBq in 18.3  $\mu$ l methanol; specific activity 2.04 Gbq mmol<sup>-1</sup>) was added to 50 ml sterile nutrient solution (MILLER, 1965) in a 500 ml Erlenmeyer flask. Under aseptic conditions, the diced callus (20 g) was then added to the solution (PALNI, PALMER & LETHAM, 1984) and the whole agitated on an orbital shaker for 48 hours. (Preliminary investigations had indicated that 48 hours would be required for the production of metabolites of interest, namely [9R]BA and [9R-MP]BA). After this period the medium was filtered and the callus clumps divided into 2 g portions.

#### 3.3.2.2.3 Extraction and purification of cytokinin metabolites

Four extraction procedures were compared; two treatments were used for each method. Each treatment comprised an extraction from a 2 g callus portion. The first method was a modified Bielecki procedure as described by HORGAN (1978).

The second was a traditional 80% ethanolic extraction (SMITH & VAN STADEN, 1978) (Figure 3.6). The callus portions were immediately frozen in liquid nitrogen before being freeze dried. Dry callus was homogenised with 50 ml 80% ethanol before extraction at 4°C for 24 hours.

In a third procedure ethanol was added to make 80% with callus tissue water (HORGAN, 1978). Without homogenising the tissue, the callus was allowed to extract for 24 hours at 4°C.

The fourth method investigated the effectiveness of boiling ethanol in extracting the nucleotide intact (WOOLLEY & WAREING, 1972a; MORRIS, 1981). Callus was boiled at 85°C in 50 ml ethanol (100%) for 20 minutes. After this period the material was homogenised.

All samples were filtered through Whatman N° 1 filter paper to remove cell debris, and the filtrate taken to dryness *in vacuo* at 35°C. Residues were re-suspended in 5 ml 100% methanol and filtered successively through a 0.45 µm filter (DynaGard PP 3.9 cm<sup>2</sup> syringe filter) and a 0.2 µm nylon filter (Lida Manufacturing Corporation). The sample was reduced to dryness at room temperature in a UNIVAPO 150H vacuum centrifuge and redissolved in 300 µl HPLC grade methanol (100%) in preparation for HPLC analysis.

#### 3.3.2.2.4 HPLC analysis

Aliquots of 100 µl of each extract were injected onto a Supelcosil LC-18-DB column (C<sub>18</sub> bonded, 5 µm, 4.6 x 250 i.d., flow rate 1 ml min<sup>-1</sup>) fitted to a Varian 5000 liquid chromatograph. Separation was effected according to the procedure of LEE, MOK, MOK, GRIFFIN and SHAW (1985). Samples were eluted with a linear gradient of methanol (5-50%) over 90 minutes. Fractions (1 ml) were collected, dried, and residues redissolved in 1 ml 100% methanol. Radioactivity was quantitated in 4 ml POPOP scintillation cocktail using a Beckman LS 3801 scintillation counter.

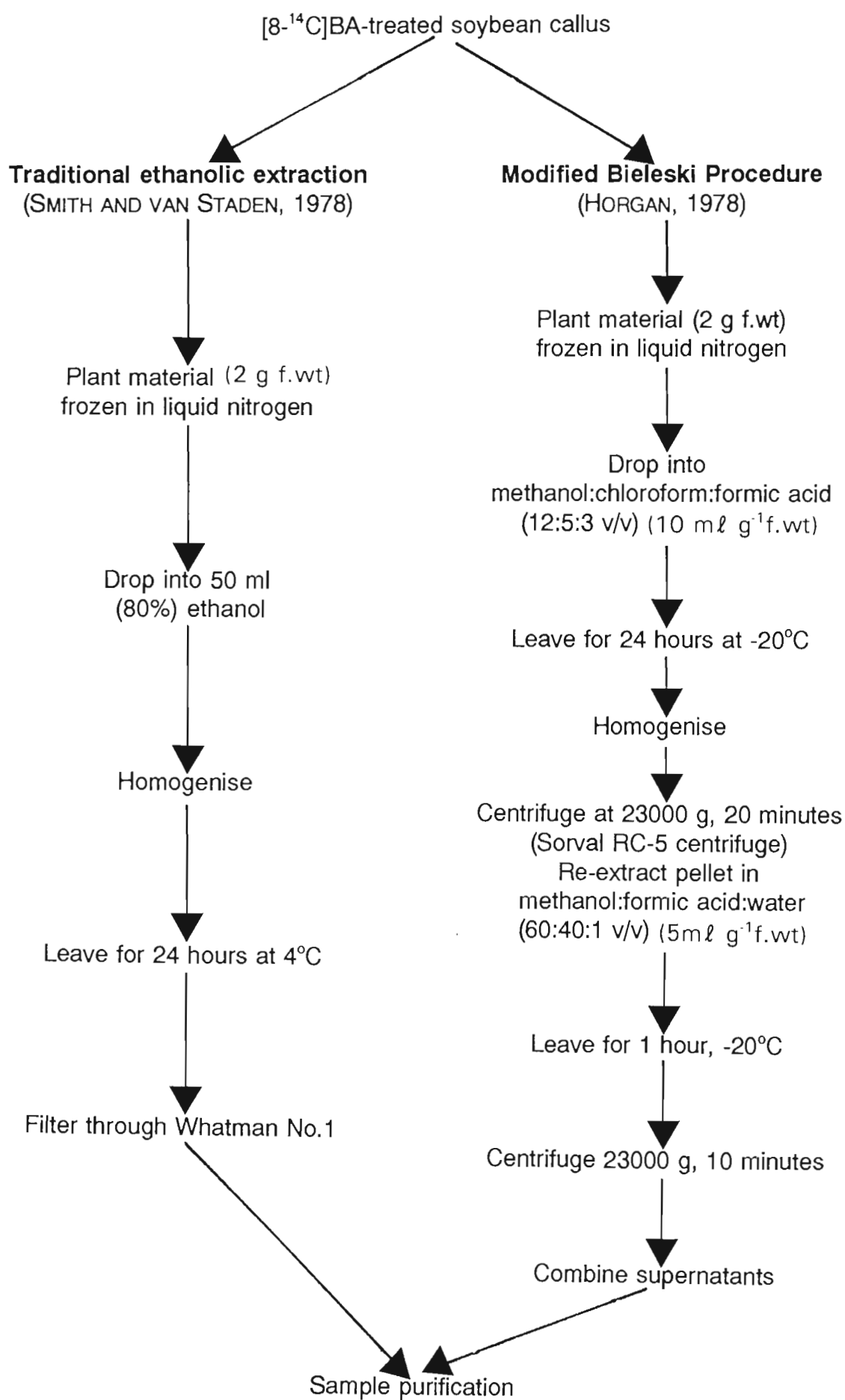


Figure 3.6 Summary of traditional ethanolic and Bielecki (1964) extraction procedures

The detected peaks of radioactivity were compared with the retention times of authentic BA derivatives separated by HPLC.

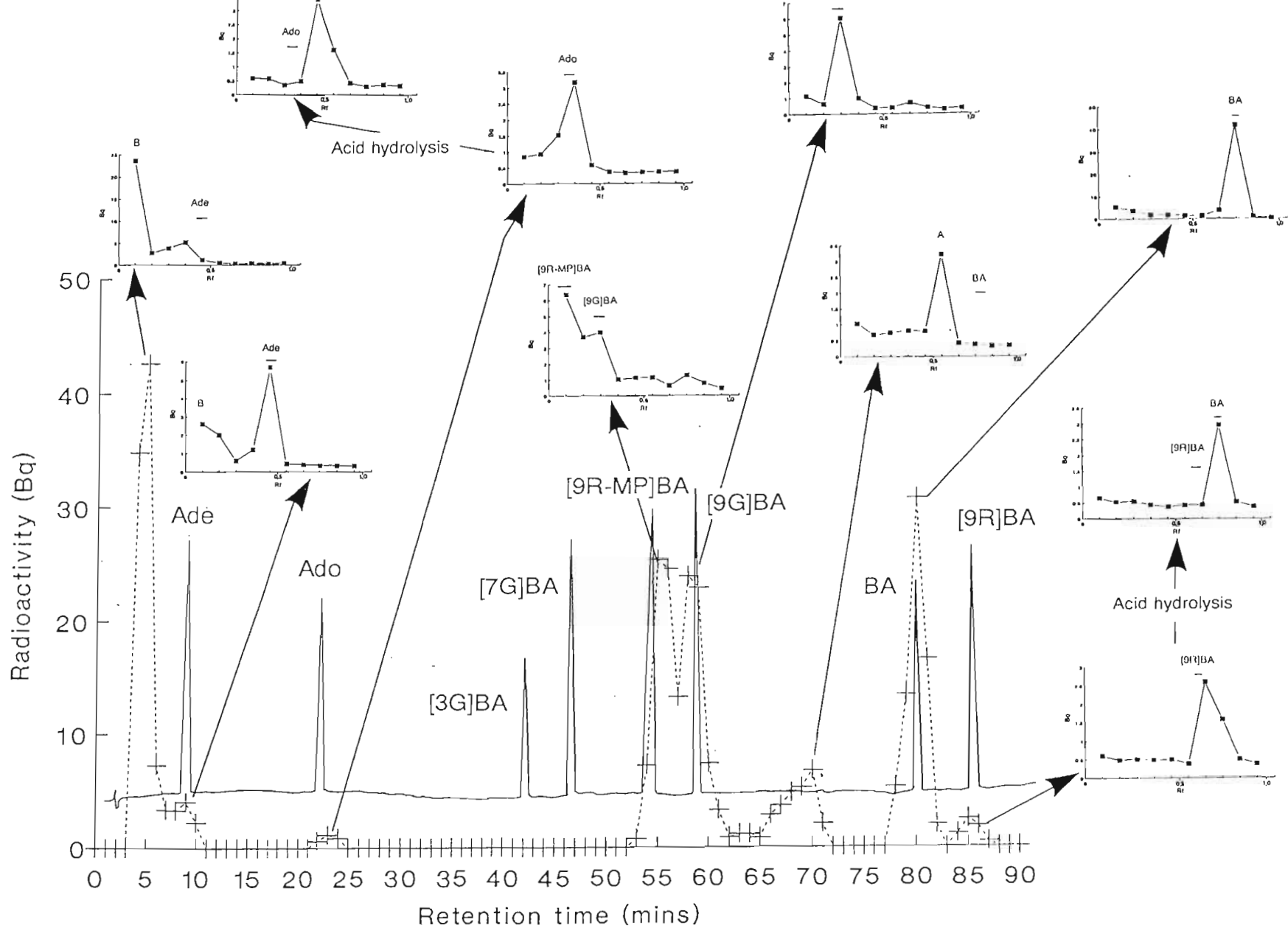
#### 3.3.2.2.5 Confirmatory techniques

Individual radioactive peaks detected were further investigated by chemical treatment with HCl (FOX, DYSON, SOOD & McCHESNEY, 1972), subjected to alkaline phosphatase treatment (MILLER, 1965), and then re-chromatographed with authentic BA derivatives using HPLC. Selected metabolites were chromatographed on Merck Silica gel 60 TLC plates ( $F_{254}$ , *n*-butanol: $\text{NH}_4\text{OH}$ :water (6:1:2) v/v, upper phase) (LETHAM, WILSON, PARKER, JENKINS, MACLEOD & SUMMONS, 1975).

#### 3.3.2.3 Results

The four extraction procedures provided similar results. Figure 3.7 shows a result typical of all four treatments. After 48 hours radioactivity was found to be associated with the retention times of authentic standards of [9R-MP]BA, [9G]BA, BA, [9R]BA, Ade and Ado, as well as with two unknown metabolites, designated compounds A and B.

The results of HPLC analyses are presented in Table 3.3. In all cases less than one third of the radioactivity was found coincident with the retention time of BA after 48 hours. [9R-MP]BA was always a major metabolite detected, as was compound B. In the TLC system described, this unidentified (polar) metabolite remained at the origin, co-eluting with authentic standards of several adenylate nucleotides (Table 3.4). Treatment of an analogous polar peak (metabolite of [8- $^{14}\text{C}$ ]BA in soybean) with alkaline phosphatase resulted in production of Ade and Ado, and treatment with acid yielded Ado and Ade (FORSYTH & VAN STADEN, 1986). Thus these authors concluded that this radioactivity consists of phosphorylated and ribosylated derivatives of Ade. This suggests that compound B is a catabolic product of, for example, [9R-MP]BA. VAN STADEN, UPFOLD, ALTMAN and NADEL (1992) have recently reported on the production of this polar BA metabolite in embryogenic cell cultures of celery.



e 3.7

Radioactivity associated with metabolites of BA (-----) following application of [8-<sup>14</sup>C]BA to soybean callus, and subsequent extraction with Bielecki solvents after 48 hours. Metabolites were separated by HPLC; the UV<sub>254</sub> trace of authentic BA derivatives is indicated (\_\_\_). Aliquots of the respective radioactive peaks detected were subsequently separated by TLC and the different R<sub>f</sub> fractions again counted for radioactivity. These are presented as inserts.

Figure 3.3 The radioactivity (expressed as a percentage of the total recovered) detected using different extraction procedures, following the application of [8-<sup>14</sup>C]BA to soybean callus.

Extraction Method	Percentage radioactivity associated with:								
	BA	[9R]BA	[9R-MP]BA	A <sup>w</sup>	[9G]BA	B <sup>w</sup>	Ade	Ado	Oxidation products <sup>x</sup>
Modified ELESKI (1964)	22.41	2.47	22.71	9.35	17.55	23.83	25.92	1.17	27.09
100% Ethanol tissue homogenised	31.92	3.63	22.95	10.86	14.99	13.39	14.02	1.64	15.6
100% Ethanol tissue not homogenised	20.15	2.78	21.22	10.00	17.91	19.31	20.53	6.59	27.12
Boiling ethanol	28.68	1.99	19.8	6.55	16.93	19.56	20.28	5.79	26.07

<sup>x</sup> unknown compound  
Ade and Ado, together with compound B (products of oxidation)

Table 3.4 Typical  $R_f$  values for authentic BA metabolites separated using Thin Layer (TLC) chromatographic techniques. High Performance Liquid Chromatography (HPLC) retention times ( $R_t$ ) of standard compounds are also shown.

BA metabolite	HPLC $R_t$	TLC $R_f$		
		Silica BAW (4:1:1) <sup>a</sup>	Cellulose BAW (4:1:1) <sup>b</sup>	Cellulose H <sub>2</sub> O (distilled)
BA	77.06	0.75	0.9	0.355
[9R]BA	80.43	0.57	0.86	0.59
[9R-MP]BA	59.07	0.05	0.39	0.905
[3G]BA	42.27	0.295	0.63	0.455
[7G]BA	49.00	0.24	0.62	0.645
[9G]BA	63.17	0.3	0.74	0.71
Ade	8.63	0.48	-	-
Ado	21.96	0.34	-	-
Xan	5.50	0.215	-	-
Hyp	5.71	0.3	-	-
c-AMP	18.3	0.17	-	-
ADP	5.51	0.01	-	-
ATP	4.66	0.05	-	-

<sup>a</sup>n-butanol:ammonium hydroxide solution:distilled water (upper phase) (Parts by volume)

<sup>b</sup>n-butanol:acetic acid:distilled water (Parts by volume)

Further indirect evidence for the oxidative origin of this compound is indicated by its poor cytokinin activity in the soybean callus bioassay ( $1 \mu\text{g l}^{-1}$  KIN equivalents) (NIEDERWIESER, VAN STADEN, UPFOLD & DREWES, 1992). VAN STADEN and DREWES (1993) had earlier found a highly polar metabolite which co-eluted with compound B in both the chromatographic systems described above. These authors isolated the compound from germinating maize embryos following application of  $[8-^{14}\text{C}]\text{Ade}$ . When treated with HCl for 30 minutes at  $100^\circ\text{C}$ , the radioactivity shifted and subsequently co-chromatographed with Ade, suggesting that the radioactivity represented adenine nucleotides. Adenylate nucleotides have earlier been identified as (oxidative) metabolites of BA (McCALLA, MORRÉ & OSBORNE, 1962; DOREE & GUERN, 1973), using a combination of chromatographic and electrophoretic techniques. Formation of such AMP may occur via the adenine salvage pathway (MOFFATT, PETHE & LALOUÉ, 1991), or through oxidation of  $[9\text{R-MP}]\text{BA}$ . However, ribonucleotides are considered resistant to attack by cytokinin oxidase (LEE, MOK, MOK, GRIFFIN & SHAW, 1985; McGAW & HORGAN, 1983). A recent report on  $[9\text{R}]\text{Z}$  and BA metabolism in radish cotyledons (TAO, LETHAM, HOCART & SUMMONS, 1991) has also reported on the existence of an 'unknown polar metabolite' which (on silica gel TLC) exhibited an  $R_f$  almost identical to that of  $[9\text{R-MP}]\text{Z}$ .

Minor metabolites were found associated with the retention times of Ado,  $[9\text{G}]\text{BA}$ ,  $[9\text{R}]\text{BA}$ , and compound A, an unknown product which eluted immediately after the 9-glucoside. In the TLC system this compound had an  $R_f$  value of 5.5 (Figure 3.7) which was not co-incident with any available standards (Table 3.4). In a similar TLC system, ELLIOTT and THOMPSON (1982) obtained an  $R_f$  value of 0.17 for authentic  $[9\text{Ala}]\text{BA}$  separated on aluminium-backed silica plates. However, it remains possible that the alanyl conjugate of BA is represented by compound A. Oxidation with L-amino acid oxidase (LETHAM, SUMMONS, PARKER & MACLEOD, 1979) would provide further information on the nature of this compound. Soybean callus has previously been shown (ELLIOTT & THOMPSON, 1982; LETHAM, PALNI, TAO, GOLLNOW & BATES, 1983) to metabolise BA to  $[9\text{Ala}]\text{BA}$ . However, the current report is at odds with several published communications from our laboratories (VAN STADEN & DREWES, 1991; 1992) which have not reported on the HPLC detection of this metabolite, from

callus of the same soybean cultivar line. However, FORSYTH and VAN STADEN (1986) did detect an unknown BA metabolite using Dowex 50 cation exchange chromatography. Further investigation of the radioactivity associated with compound A is clearly required.

Thus all four methods successfully extracted the nucleotide, [9R-MP]BA. Marginally more nucleotide was detected after extraction with cold 80% ethanol (with tissue homogenisation) than when the Bielecki procedure was employed. The latter technique also recovered less of the base and more breakdown products of oxidation (Table 3.4) than when tissues were homogenised with cold 80% ethanol (Figure 3.8).

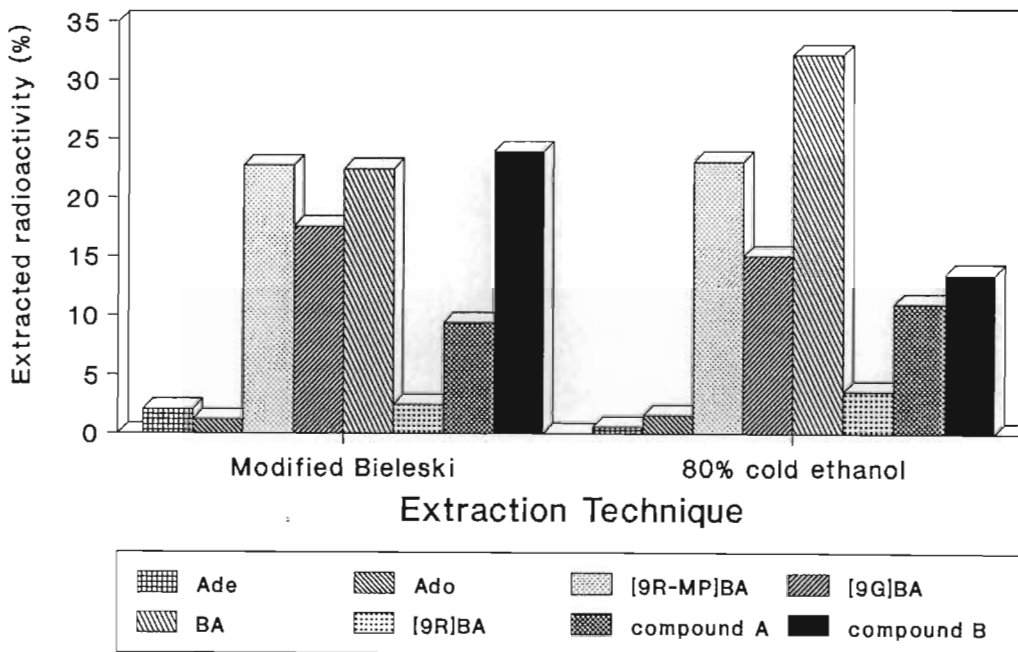


Figure 3.8 Relative levels of BA metabolites from soybean callus, detected after extraction with either Bielecki solvents or 80% cold ethanol.

### 3.3.2.4 Discussion

Metabolites of benzyladenine are adequately extracted by 80% cold ethanol. The nucleotide was not substantially degraded by non-specific phosphatases, as has been previously alleged (HORGAN, 1978; LALOUE & PETHE, 1982). There is no reason to assume that nucleotides of other cytokinins (e.g. Z, iP) will not also be properly extracted, at least from similar tissue types.

VAN STADEN and BAYLEY (1988) compared the Bielecki procedure with an 80% ethanolic extraction, also using soybean callus. Their use of RIA showed a similar recovery rate for [9R-MP]DHZ when comparing these methods. Despite this finding, some authors persist in challenging the use of traditional alcoholic extraction procedures. Recent workers (HORGAN, 1984) still advocate the use of Bielecki solvents, despite an earlier report on extracts from tobacco leaves in which MACNICOL (1972) demonstrated that such a technique may lead to 'serious underestimation' of adenine nucleotides in tissues.

The findings of this investigation are consistent with those of TURNER, MOK and MOK (1985) who, with a variety of *Phaseolus* L. tissues were similarly successful in recovering cytokinin nucleotides with an alcoholic extraction. These authors also detected a substantially higher amount of breakdown products after extraction with Bielecki solvents. Such findings suggest that Bielecki solvents, either singly or in concert directly degrade the  $N^6$ -sidechain of cytokinins. Non-metabolic degradation of cytokinins has previously been demonstrated (DEKHUIJZEN & GEVERS, 1975; SACHS, RYUGO & MESSERSCHMIDT, 1976) in which cases Ade was formed as a degradation product from kinetin. An alternative explanation is that alcoholic solvents better inactivate cytokinin oxidases so that less degradation of the base or metabolites occurs during the extraction process (especially after decompartmentation upon homogenisation).

The use of Bielecki solvents offers no advantage to cytokinin physiologists. This method appeared to extract cytokinin nucleotides only as well as cold 80% ethanol, but more breakdown products were detectable in the extract. Additionally, the

Bieleski method is more elaborate and cumbersome and lipid material may interfere with purification steps (LALOUE, TERRINE & GAWER, 1974; HORGAN, 1978). Use of Bieleski solvents does not warrant the inconvenience. As much as any other technique, cold ethanol successfully extracts cytokinin nucleotides. However, the possibility that the enzymes present in different tissue types are differentially inactivated by alcoholic and/or Bieleski extraction procedures should not be precluded.

### 3.3.3 General analytical techniques employed in current studies

#### **3.3.3.1 Chemicals**

Radioactive [8-<sup>14</sup>C]benzyladenine (specific activity 2.04 Gbq mmol<sup>-1</sup>) was supplied by Amersham International. Authentic BA standards were obtained from Apex Organics, Oxford.

#### **3.3.3.2 Sample purification**

Extracted samples were reduced to dryness under vacuum (Büchi Rotavapor RE 111) and samples re-suspended in 5 ml 80% methanol. Buchi flasks were sonicated to remove adsorbents on the vessel walls. Particulate matter was removed using a 0.2  $\mu$ m DynaGard PP syringe filter. The recovered filtrate was dried in a vacuum centrifuge. Immediately prior to HPLC analysis, samples were re-dissolved in 300  $\mu$ l HPLC grade (80%) methanol.

#### **3.3.3.3 High performance liquid chromatography (HPLC) analysis**

Aliquots of 100  $\mu$ l of each extract were injected onto a Supelcosil LC-18-DB column (C<sub>18</sub> bonded, 5  $\mu$ m, 4.6x250 i.d., flow rate 1 ml min<sup>-1</sup>) fitted to a Varian 5000 liquid chromatograph. Separation was effected using an aqueous buffer of 0.2 M acetic acid (analytical grade), adjusted to pH 3.5 with triethylamine (analytical grade) (LEE, MOK, MOK, GRIFFIN & SHAW, 1985). Samples were eluted with a linear gradient of methanol (HPLC grade) (5-50%) over 90 minutes, at an operating pressure of 180 atmospheres.

Fractions (1 ml) were collected using a time-based fraction collector (Pharmacia Frac-100). The detected peaks of radioactivity were compared with the retention times of authentic BA derivatives separated by HPLC and recorded using a Varian UV-50 variable wavelength detector ( $A_{254}$ ) linked to a Varian CDS 401 integrator. (Table 3.4; Figure 3.7). Between successive samples, the column was routinely washed with 100% methanol for 20 minutes. Occasional 10 minute washes with acetonitrile was included in this rinse stage. A set of standards were separated with each fresh buffer preparation to detect slight variations in retention times.

#### 3.3.3.4 *Confirmatory techniques*

Individual radioactive peaks so determined were further investigated by chemical treatment with HCl, subjected to alkaline phosphorolysis, or re-chromatographed with authentic BA derivatives in an HPLC or TLC system.

##### 3.3.3.4.1 Acid hydrolysis

Relevant HPLC fractions were pooled, dried under vacuum, and 1 ml 1 N HCl added to the sample in an Eppendörf tube. The vial contents were boiled at 97°C for 15 minutes and the sample reduced to dryness under vacuum (FOX, DYSON, SOOD & McCHESNEY 1972). The residue was re-constituted in 20  $\mu$ l 80% HPLC grade methanol, and applied to a TLC plate.

##### 3.3.3.4.2 Treatment with alkaline phosphatase

Suspected nucleotide fractions were collected, dried under vacuum, and hydrolysed with alkaline phosphatase (MILLER, 1965). Nucleoside products were detected using HPLC re-chromatography or TLC.

A 0.01 M MgCl 0.1 M Tris (tris(hydroxymethyl)aminomethane) solution (pH 10.5) was prepared. Calf intestinal mucosa alkaline phosphatase (BDH Biochemicals, England) (1 mg  $\text{m}^{-1}$ ) with a pH optimum of 10.5 was suspended in this buffer

solution. Dried samples were treated with 1 ml of this activated suspension, and incubated in a water bath at 37°C for 2 hours. Authentic [9R-MP]BA was similarly treated with enzyme as a control. After the hydrolytic incubation period, 3 ml 100% ethanol were added to precipitate the enzyme which was subsequently removed using a 0.2  $\mu\text{m}$  syringe filter. Samples were dried under vacuum, re-dissolved in 80  $\mu\text{l}$  80% methanol (HPLC grade) and subjected to chromatographic analysis.

#### 3.3.3.4.3 HPLC re-chromatography of HPLC fractions

Individual peaks of interest were collected, dried under vacuum (Univapo 150H vacuum centrifuge) and the residue redissolved in 80  $\mu\text{l}$  80% HPLC grade methanol. Samples were re-injected together with up to 20  $\mu\text{l}$  authentic standard, eluted according to LEE, MOK, MOK, GRIFFIN and SHAW (1985), and 1 ml fractions collected. Peaks of radioactivity were again compared with the retention time of the authentic BA derivatives.

#### 3.3.3.4.4 Thin layer chromatography (TLC) analysis

Metabolites of BA were successfully resolved (Table 3.4) when using a 'BAW' system (LETHAM, WILSON, PARKER, JENKINS, MACLEOD & SUMMONS, 1975) with Merck Silica gel 60 TLC plates ( $F_{254}$ ). Peaks of radioactivity detected after HPLC were subjected to TLC. The eluants normally employed were (proportions by volume): butan-1-ol (*n*-butanol): $\text{NH}_4\text{OH}$ :water (6:1:2) (upper phase). Analytical grade *n*-butanol was used and  $\text{NH}_4\text{OH}$  supplied as ammonia solution (25%  $\text{NH}_3$ ). Eluant (50 ml) was supplied to each two-plate glass tank. Before introduction of TLC plates, tanks were equilibrated for 16 hours. Standards of suspected compounds were applied (using a glass capillary), together with the isotopic metabolites. Enough (normally 5  $\mu\text{l}$ ) authentic derivative was applied to permit detection; ultraviolet-absorbing zones on chromatograms were located with a short-wave (254 nm) ultraviolet lamp (LETHAM, WILSON, PARKER, JENKINS, MACLEOD & SUMMONS, 1975). Each sample was divided into ten  $R_f$  bands and the fractions scraped off the glass backing. Methanol aliquots (1 ml) were added before combination with scintillation cocktails (VAN STADEN, 1981). Samples were

routinely quenched for 48 hours in the dark before quantitation of radioactivity.

### 3.3.3.5 *Scintillation counting*

HPLC fractions (1 ml) were collected and radioactivity quantitated using one of three fluors, depending on availability:

1. 3 ml Ready Value (Beckman)
2. 3 ml Ready Solv (Beckman)
3. 4 ml POPOP scintillation cocktail (10 g 2,5-diphenyloxazol; 0.5 g dimethyl-POPOP; 2.5 l toluene).

Samples were counted on one of three scintillation counters, again subject to availability:

1. Beckman LS 3800
2. Beckman LS 3801
3. Beckman LS 6000 LL

## 3.4 Critical perspective of metabolic studies

Current investigations have monitored the metabolism of exogenously applied BA as if in its interaction with auxin, it mimics the endogenous flux. Such a view is probably over-simplistic, in which case interactive (and even 'regular' metabolic) studies need to be placed in critical perspective.

The use of such compounds as 6-(benzylamino)purine is perhaps less often prompted by their real value as tools of research, than by their low cost. An analogue such as BA may cost up to six hundred times less than zeatin.

Based on available evidence, SONDHEIMER and TZOU (1971) commented that to the extent that comparisons are possible, the metabolism of synthetic and natural cytokinins are similar. An opposite view was expressed by McGAW and HORGAN (1985). These authors were of the opinion that some 'metabolic studies are essentially artificial, being the result of exogenous application of synthetic cytokinins,...observing the presence of certain enzyme systems with which the

endogenous cytokinins may never come into contact.'

DENNIS (1977) cautioned against the application of non-endogenous compounds to plants, as these can be metabolised quite differently. This author emphasised that compounds should be fed in quantities approximating endogenous levels. When zeatin was applied to soybean callus at  $10^{-3}\text{M}$ , a concentration which inhibits growth, the types of metabolites were different to the control ( $10^{-5}\text{M}$ ), demonstrating concentration-dependent metabolism (VAN STADEN, 1983). A similar inhibition of callus growth at a BA concentration of  $10^{-3}\text{M}$  was similarly noted. An exogenous hormone provided at artificially high levels interferes with the normal interaction between hormones. Such disturbance of hormonal balance is inherent in the use of 2,4-D as a weed killer (VAN OVERBEEK, 1966).

Given that BA is probably not a common naturally occurring cytokinin, the application of BA to plants, although producing various traceable and analogous metabolites, may not follow the natural metabolic route of such compounds as zeatin and iP. In this case, enzyme systems shown to operate for zeatin and iP may have little relevance for the metabolism of BA.

An assumption in metabolic studies is that the exogenously applied compound reaches the normal site of metabolism (DENNIS, 1977). This assumption is questionable, given that much contrary evidence has been presented. MILBORROW and GARMSTON (1973) for example, found that ABA synthesised *in situ* is compartmentalised, while that fed is not. BRUINSMA (1980) noted that in 'arriving' at sites other than those where the corresponding analogous hormones reside, the fate and function of exogenously applied hormones may be quite different. As evidence, this author cited differences in rates of turnover and translocation of exogenous and endogenous  $\text{GA}_{29}$  and IAA (BRUINSMA, KARSSSEN, BENSCHOP & VAN DORT, 1975). BRUINSMA (1980) envisioned the endogenous  $\text{GA}_{29}$  as localised in a compartment in which it is actively catabolised, and which is inaccessible for the exogenously applied molecules. Ideally, the compound should be applied so as not to contact membrane barriers other than those which it normally traverses in reaching a target organelle

(DENNIS, 1977), an impossible criterion to currently meet.

As a consequence of 'unnatural' supply, metabolism of externally applied natural cytokinins may not mimic the endogenous situation. High levels of zeatin and [9R]Z have been found in *Vinca rosea* L. crown gall tissue (SCOTT, MARTIN, HORGAN & HEALD, 1982) but when zeatin was applied externally, it was readily oxidised (PALNI, 1980 in McGAW & HORGAN, 1985). A similar anomaly has been recorded during work with *Ginkgo biloba* L. leaves. HUTTON and VAN STADEN (1985) demonstrated that exogenously applied metabolites were not transported according to an *in vivo* situation. This was possibly due to different compartmentation procedures, and the fact that several abnormal membrane barriers were encountered, which may have directed transport and its associated metabolism in an 'unrepresentative' manner.

Although the metabolism of zeatin has been studied in several plant species (PARKER & LETHAM, 1973; 1974), only a few attempts have been made to relate such metabolism to the cytokinins found naturally in the plant (HENSON & WHEELER, 1977; 1977a; WAREING, HORGAN, HENSON & DAVIS 1977; FUBEDER, ZIEGLER, PETERS & BECK, 1989). In these investigations, the metabolism of endogenous and exogenous cytokinins appeared to be qualitatively and quantitatively different. Interactive-metabolic phenomena between BA and auxins were not similarly observed in studies with [9R]Z and auxins (ZHANG, LETHAM, WONG, NOODÉN & PARKER, 1987), showing the somewhat limited inferences for *in vivo* situations that one may draw from metabolic studies using BA. The possibility that the metabolism of BA is unrepresentative of the endogenous situation has important considerations as there are many inferences arising from studies with BA.

However, despite these arguments, the range of endogenous cytokinin metabolites present in tobacco crown-gall tissue showed a clear correspondence to the range appearing after supplying non-tumorous, cytokinin-requiring tobacco cells with exogenous cytokinins (SCOTT & HORGAN, 1984).

Rather than seeking analogies from studies with BA for insight to endogenous cytokinin metabolism, researchers today draw analogies from studies with zeatin to explain the metabolic conversions detected with BA; to assign functions to a synthetic compound supplied in an unnatural manner to plant cells. This criticism may apply to many metabolic studies and their subsequent interpretation.

Exogenously applied hormones may also interfere with the endogenous balance (MOK, MARTIN, MOK & SHAW, 1992), to affect the synthesis, translocation, or metabolism of these substances (BRUINSMA, 1980). An interesting consideration centres itself on the interpretation of gene or metabolic responses to exogenous hormones. BANOWETZ (1992) determined that the extent of enzyme induction (of nitrate reductase) by BA was correlated with endogenous cytokinin levels at the time of application of this synthetic cytokinin. This author suggested that competition between endogenous and exogenous cytokinins for putative cytokinin receptors or binding sites influences the effectiveness of applied BA. Competition for enzymes of cytokinin metabolism has also been inferred (BURCH & STUCHBURY, 1987). Environmental factors (e.g. light) have been shown to (further) modify enzyme induction by cytokinins (LU, HE, CHEN, ZHANG & TANG, 1983, in BANOWETZ, 1992), although it is not known if this is through an effect on endogenous cytokinins (TAYLOR & WAREING, 1979).

NIEDERWIESER and VAN STADEN (1990) correlated tissue age with the interactive effects of NAA and BA in *Lachenalia* Jacq.f. ex Murrarie explants. These authors in part attributed this finding to endogenous hormone levels in the tissues, which were shown to modify the effectiveness of supplied plant growth regulators in promoting bud formation.

Physiological responses to exogenously applied cytokinins may not parallel observed metabolic conversions in plant tissues. AUER, LALOUE, COHEN and COOKE (1992) found active cytokinin forms to decrease over the period when shoots were induced in *Petunia* Juss. leaf explants. During this same period, active glucosylated conjugates accumulated. Such findings are in conflict with generally accepted metabolite roles. Studies with cytokinin-overproducing transgenic tobacco plants (AINLEY, McNEIL, HILL,

LINGLE, SIMPSON, BRENNER, NAGAO & KEY, 1993) demonstrated the consequences of controlled cytokinin perturbations in whole plants. The characterisation of previously undescribed physiological responses prompted these authors to criticise both exogenous application of cytokinins to excised organs, and comparisons made between results so obtained with the natural *in vivo* situation. Similarly, physiological effects noted after stimulation of cytokinin synthesis *in vivo* were exactly opposite to the effects brought about after exogenous application of cytokinin (CATSKY, POSPISILOVA, MACHACKOVA, SYNKOVA, WILHELMOVA & SESTAK, 1993). However, *in vivo* manipulation of the auxin/cytokinin ratio (AKIYOSHI, KLEE, AMASINO, NESTER & GORDON, 1984; SMIGOCKI & OWENS, 1988; 1989) eliminates the problems associated with uptake and transport of exogenously applied hormones.

In the current studies, direct causes for the effects noted have not been demonstrated, for mechanisms by which plant hormones interact remain obscure. All factors involved in such interaction (both biotic and environmental), have not yet been defined. Rather, in the 'black box of cellular tricks', the combined influences of endogenous and exogenous hormones, of perhaps more than two classes (GUNSÉ & ELSTNER, 1992), and environmental factors, have all played a role in producing an effect.

## CHAPTER 4

### THE EFFECT OF THE AUXIN 2,4-DICHLOROPHENOXYACETIC ACID ON THE METABOLISM OF 6-(BENZYLAMINO)PURINE IN SOYBEAN (*GLYCINE MAX* CV. ACME) SUSPENSION CULTURES

#### 4.1 Introduction

The effects of auxin on cytokinin metabolism have previously focused on promotion of oxidative catabolism. As a lowered cytokinin content in NAA-treated *Salix* L. tissues could not be attributed to an effect on cytokinin production (CHOVEAUX & VAN STADEN, 1981), these authors proposed an auxin 'effect on the activity of cytokinin oxidase-type enzymes'. Subsequently, several reports have confirmed this hypothesis, using mainly tobacco systems. PALNI, BURCH and HORGAN (1988) identified an inverse relationship between the stability of [9R]Z supplied to tobacco pith explants and the NAA concentration in the incubation medium. At higher concentrations of NAA, greater breakdown of the riboside was indicated by elevated levels of degradative metabolites (adenine, adenosine, and adenine nucleotides). Auxin-promoted degradation of cytokinin has also been recorded for artichoke tissue (PALMER, LETHAM & GUNNING, 1984). A rapid metabolism of zeatin nucleoside occurred when IAA was applied. Auxin-activated artichoke tissues treated with [9R]Z accumulated high levels of Ado and Ade nucleotides, compared with lower levels of degradative metabolites in the controls. IAA increased the BA degradation rate in carnation ovaries (FEATONBY-SMITH, VAN STADEN & COOK, 1987). The auxin effect on cytokinin metabolism appears then to be mediated, at least in part, through cytokinin oxidase. Some *in vitro* assays using this enzyme support this view. PALNI, BURCH and HORGAN (1988) found that 2,4-D at a concentration of 1 mg  $l^{-1}$  increased the activity of cytokinin oxidase by 15.6%. Weakly active  $\beta$ -NAA stimulated oxidase activity to a similar extent. Such *in vitro* stimulation of cytokinin oxidase activity by auxins paralleled *in vivo* observations. However, MOTYKA and KAMINEK (1990) could not detect increased cytokinin oxidase activity in tobacco callus after application of a variety of auxins (2,4-D was not considered). In contrast to the above reports, transient promotion of riboside

formation was observed after pulsing immobilised tobacco cells with NAA (VANKOVA, GAUDINOVA, KAMINEK & EDER, 1992). Observed discrepancies in auxin-induced metabolic effects were considered by VAN STADEN and MOONEY (1987) to arise from differences in the physiological state of the tissues investigated. These authors speculated that hormonal interaction would vary during phases of cell division, enlargement and senescence.

As cytokinin oxidase appears to utilise both BA (LALOUÉ & PETHE, 1982; FORSYTH & VAN STADEN, 1986) and isopentenyl-type cytokinins (WHITTY & HALL, 1974) as a substrate in a range of plant systems, this enzyme has been recognised as a good candidate for regulation by specific inhibitors, including auxins (PARKER, ENTSCH & LETHAM, 1986). Inhibition of oxidative catabolism could result in elevated levels of active cytokinin forms, although alternative inactivation pathways may then be promoted (McGAW, HORGAN, HEALD, WULLEMS & SCHILPEROORT, 1988). These authors determined that in transformed tobacco callus lacking functional T-DNA auxin-synthesising genes, higher levels of cytokinin were detected than in wild-type callus. Fewer cytokinin products of  $N^6$ -sidechain cleavage were detectable. However, as levels of 7-glucoside increased substantially, it is likely that 7-glucosylation was promoted as an alternative pathway for cytokinin inactivation in tobacco tissues, when functional auxin genes were not present to (indirectly) promote oxidation.

Alanine conjugation and  $N^6$ -benzyl cleavage were considered by ZHANG, LETHAM, WONG, NOODÉN and PARKER (1987) to be alternative mechanisms for BA inactivation in soybean leaves. In the shoots of another legume, *Lupinus*, both inactivation routes were evident (PARKER, LETHAM, GOLLNOW, SUMMONS, DUKE & MACLEOD, 1978). Notably, cytokinin-alanine conjugation appears restricted to the Fabaceae (LETHAM, SUMMONS, PARKER & MACLEOD, 1979), whilst broad-substrate cytokinin oxidase systems have not been demonstrated in all plant tissues.

In the current study, cytokinin-dependent soybean suspension cultures have been treated with BA in the presence of both high and low levels of the synthetic auxin 2,4-D to investigate interaction between these hormones at the metabolic level.

## 4.2 Materials and methods

### 4.2.1 Plant materials

Stock cultures of *Glycine max* cv. Acme were established and maintained on a GAMBORG, MILLER AND OJIMA (1968) B5 basal medium supplemented with 2,4-D at a concentration of 2 mg  $\ell^{-1}$  (Chapter 3.2.3.2). Stock cultures were incubated in 500 ml Erlenmeyer flasks under low intensity white fluorescent lights providing 1  $\mu\text{mol m}^{-2} \text{s}^{-1}$  with a 16 hour light regime.

### 4.2.2 Cytokinin application

Cell cultures were treated in the linear phase of growth. The [8- $^{14}\text{C}$ ]BA (0.0017 Mbq in 3  $\mu\text{l}$  methanol; specific activity 2.04 GBq  $\text{mmol}^{-1}$ ) was added to 15 ml suspension culture in a 100 ml Erlenmeyer flask. The final concentration of 2,4-D in each 15 ml replicate of cell culture was adjusted to be either 2 mg  $\ell^{-1}$  or 4 mg  $\ell^{-1}$ . In each sample, non-labelled BA (at 2 mg  $\ell^{-1}$ ) was added. Three replicates were established for each variable considered. After treating the cell suspensions, flasks were incubated under identical conditions to those of the stock cultures, except that a continual photoperiod was now maintained.

### 4.2.3 Extraction and purification of BA metabolites

After incubating for the set time period, samples were filtered through Whatman N<sup>o</sup> 1 filter paper. Cells were rinsed with 3 ml distilled water. Both cells and filter paper were immediately frozen in liquid nitrogen before freeze drying. Each sample was boiled in 20 ml 80% ethanol at 80°C for 15 minutes and left to stand for 12 hours. The extract was filtered through Whatman N<sup>o</sup> 1 to remove cell debris and the residue re-extracted in 2 ml cold 80% ethanol for ten minutes. Samples were fan dried at room temperature. Residues were re-suspended in 1.2 ml 80% methanol (HPLC grade) and filtered through a 0.20  $\mu\text{m}$  filter (DynaGard ME 5.5  $\text{cm}^2$  hollow fibre syringe filter). The sample was reduced to dryness in a UNIVAPO 150H vacuum

centrifuge and redissolved in 200  $\mu$ l HPLC grade methanol (80%) immediately prior to HPLC analysis.

#### 4.2.4 HPLC analysis

Aliquots of 100  $\mu$ l of each extract were injected onto a Supelcosil LC-18-DB column and separation effected as previously described (Chapter 3.3.3.3). Fractions (1 ml) were collected and the radioactivity quantitated in 4 ml Ready Value (Beckman) scintillation cocktail, using a Beckman LS 3801 scintillation counter. The detected peaks of radioactivity were compared with the retention times of authentic BA derivatives separated by HPLC.

#### 4.2.5 Confirmatory techniques

Individual radioactive peaks determined by HPLC were further investigated by chemical treatment with HCl (FOX, DYSON, SOOD & McCHESNEY, 1972), or re-chromatographed with authentic BA derivatives in a TLC system.

#### 4.2.6 TLC analysis

Peaks of radioactivity detected after HPLC were subjected to TLC (Merck, Silica gel 60, F<sub>254</sub>, *n*-butanol:NH<sub>4</sub>OH:water (6:1:2) v/v upper phase) (Chapter 3.3.3.4.4) and the ten R<sub>f</sub> fractions counted for radioactivity (Chapter 3.3.3.5). The results of TLC analyses and acid hydrolysis treatments of fractions were consistent with the initial identifications based on HPLC retention times.

### **4.3 Results**

#### 4.3.1 Time sequence studies

Soybean suspension cultures utilised applied BA less rapidly than both carrot (Chapter 5) and *Dianthus* (Chapter 6) cell cultures. After 30 minutes Ade was the major

product formed (Table 4.1; Figure 4.1). This was the case after all incubation periods. Other prominent metabolites were the riboside of BA and, after 18 hours incubation, BA nucleotide. Up to 18 hours most of the radioactivity remained associated with the cytokinin base, but after 48 hours the majority of [8-<sup>14</sup>C]BA had been converted to metabolites. Between 18 and 48 hours, when oxidative pathways were especially promoted, levels of Ado increased along with Ade and the unknown compound B (Figure 4.1). For a discussion on the probable identity of compound B as an adenylic nucleotide, refer to Chapter 3.3.2.3. AMP has previously been identified as a catabolite of exogenously applied cytokinins in soybean callus of the same cultivar (PALNI, PALMER & LETHAM, 1984), and in other leguminous systems (DUKE, MACLEOD, SUMMONS, LETHAM & PARKER, 1978). Further inactivation of the N-conjugates through oxidation (LETHAM, PARKER & GORDON, 1972; PALNI, PALMER & LETHAM, 1984) was not detected.

Metabolism showed, with time, a reduction in levels of active cytokinins (total radioactivity associated with base, nucleoside and nucleotide). This lowering of activity was mirrored by a concurrent increase in levels of oxidative products (Figure 4.2). Although Ado contributed to the catabolic pool (Table 4.1; Figure 4.3), Ade production accounted more completely for this lowering of activity (Figure 4.4). A slight increase (4-8%) in active cytokinin forms occurred between 6 and 18 hours (Figures 4.5 and 4.6), whether or not auxin levels were elevated. This may be accounted for by a comparable increase in base over this same period (Table 4.1). This finding suggests re-formation of the base from catabolic products.

Glucosylation pathways were scarcely operative in soybean under the described conditions (Figure 4.7). Of the three glucosides, [9G]BA was most prominent at all times considered (Table 4.1), particularly after 48 hours.

#### 4.3.2 Effect of hormonal interaction

Oxidative catabolism pathways were promoted by the auxin 2,4-D within soybean cultures (Figure 4.2), with a concurrent reduction in cytokinin activity.

Table 4.1 Radioactivity attributable to BA and its metabolites in extracts of soybean suspension cultures supplied with [8-<sup>14</sup>C]BA in the presence of high (4 mg ℓ<sup>-1</sup>) and low (2 mg ℓ<sup>-1</sup>) levels of auxin.

Incubation hours)	2,4-D (mg ℓ <sup>-1</sup> )	Radioactivity co-eluting with standards (% of total extracted)														
		B <sup>w</sup>	Ade	Ado	[3G]BA	[7G]BA	[9G]BA	[9R- MP]BA	[9R]BA	BA	C <sup>w</sup>	D <sup>w</sup>	E <sup>w</sup>	Oxidation Products <sup>x</sup>	Active Pool <sup>y</sup>	N- conjugates <sup>z</sup>
5	2	1.56	1.92	0.09	0.64	0.08	2.34	2.08	6.86	83.50	0.03	0.60	0.29	3.57	92.44	3.06
	4	3.40	4.73	0.02	0.07	0.58	0.99	1.24	6.96	81.64	0.08	0.22	0.07	8.15	89.84	1.64
	2	6.23	7.30	1.80	0.36	0.54	1.49	3.80	10.37	66.95	0.30	0.8	0.06	15.33	81.12	2.39
	4	7.40	6.62	1.34	0.38	0.79	2.63	1.70	7.35	68.46	0.02	1.32	2.00	15.36	77.51	3.80
3	2	3.57	6.69	0.76	0.09	1.16	1.40	8.53	6.91	69.94	0.12	0.71	0.15	11.01	85.38	2.65
	4	5.21	5.92	0.68	0.00	0.14	0.36	1.54	7.97	76.59	1.29	0.32	-	11.81	86.10	0.50
3	2	7.69	13.7 2	3.53	0.05	2.08	6.51	17.2	8.34	39.88	-	0.25	0.75	24.94	65.42	8.64
	4	8.76	34.8 0	10.6 1	0.21	0.10	3.76	1.13	2.68	37.94	-	-	-	54.17	41.75	4.07

unknown compound

total radioactivity associated with B, Ade and Ado, products of oxidation

total radioactivity associated with base, nucleoside, and nucleotide

total radioactivity associated with N-glucosides

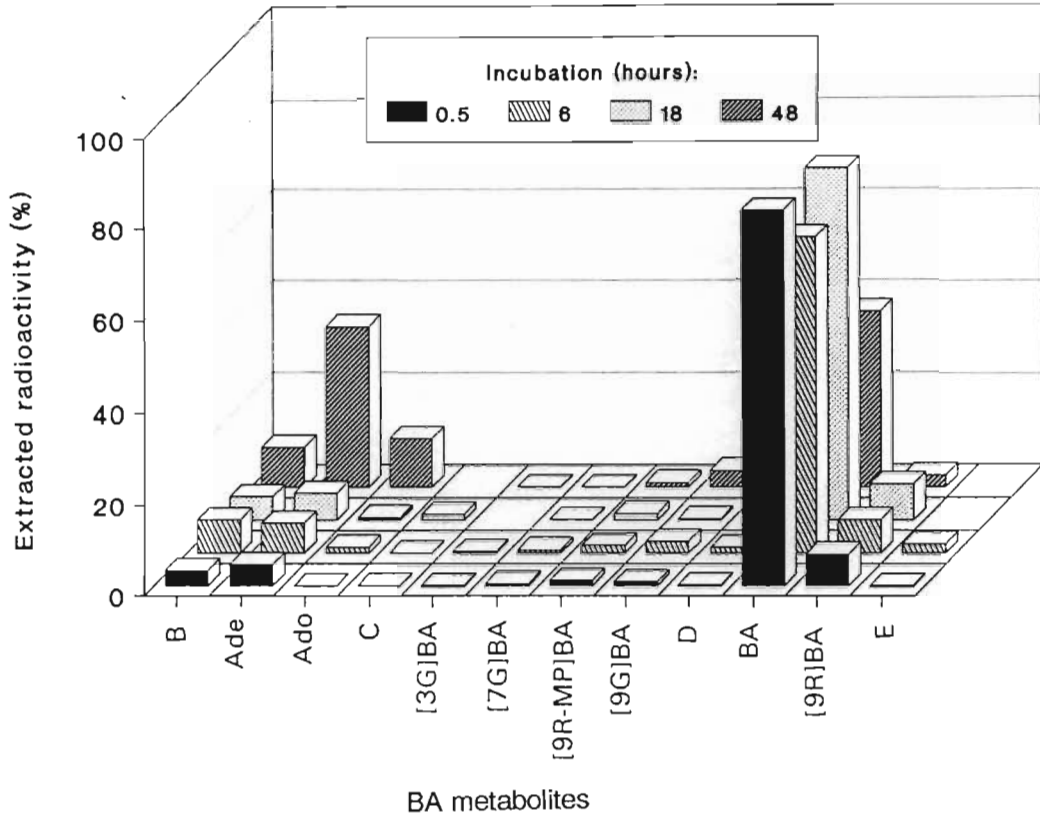


Figure 4.1 The metabolism of [8-<sup>14</sup>C]BA in soybean suspension cultures over 48 hours. 2,4-D at 4 mg l<sup>-1</sup> was included in the medium.

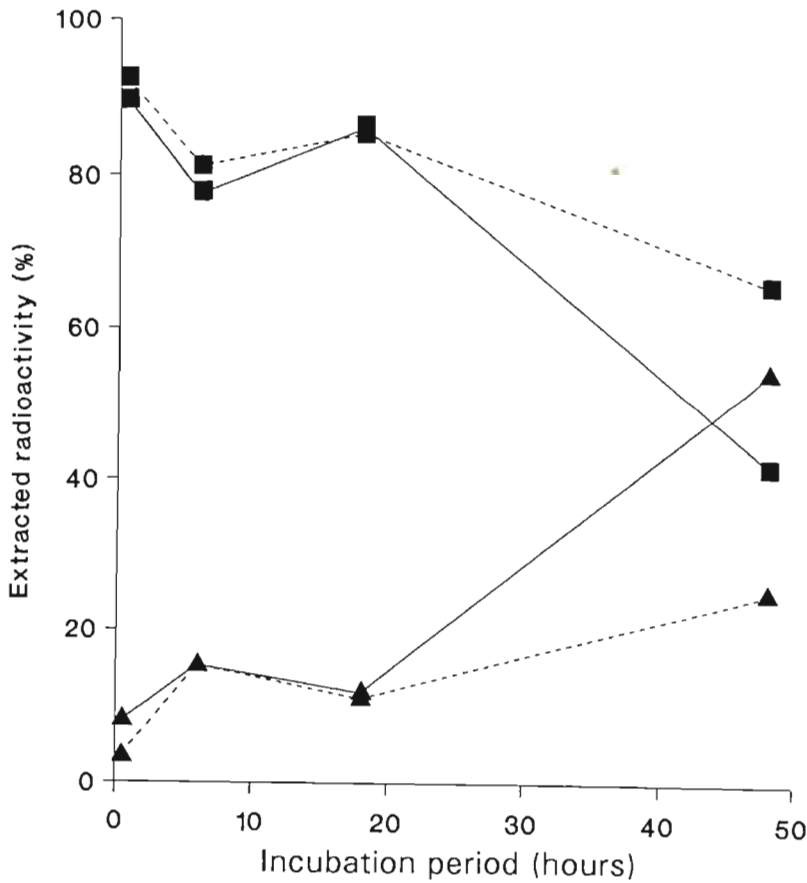


Figure 4.2 Levels of radioactivity associated with oxidation products ( ▲ ) and active cytokinins ( ■ ) in soybean suspensions of low ( ----- ) and high ( — ) auxin

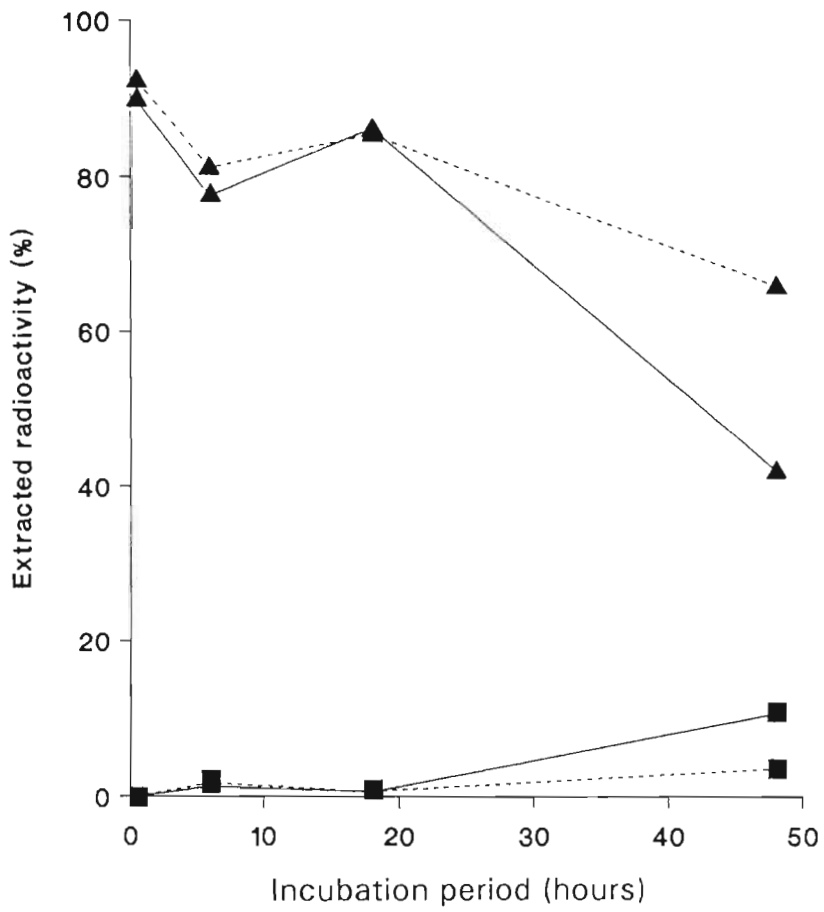


Figure 4.3 Levels of radioactivity associated with Adenosine ( ■ ) and active cytokinins ( ▲ ) in soybean suspensions of low ( - - - - ) and high ( \_\_\_ ) auxin status.

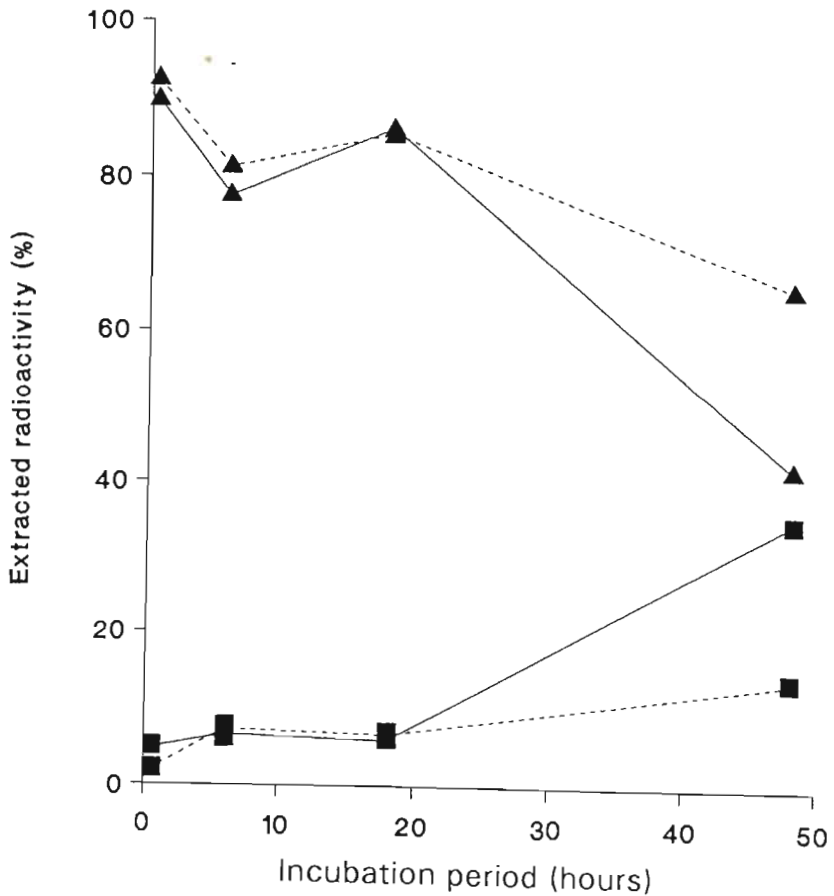


Figure 4.4 Levels of radioactivity associated with Adenine ( ■ ) and active cytokinins ( ▲ ) in soybean suspensions of low ( - - - - ) and high ( \_\_\_ ) auxin status.

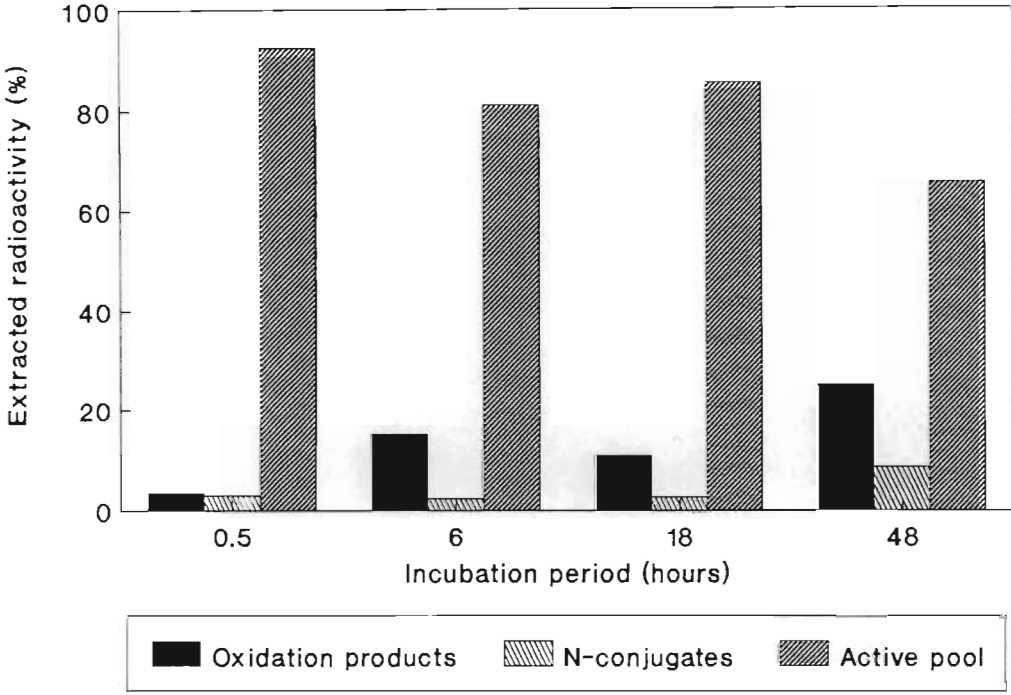


Figure 4.5 Activity fate of BA in soybean suspensions of low auxin status over 48 hours.

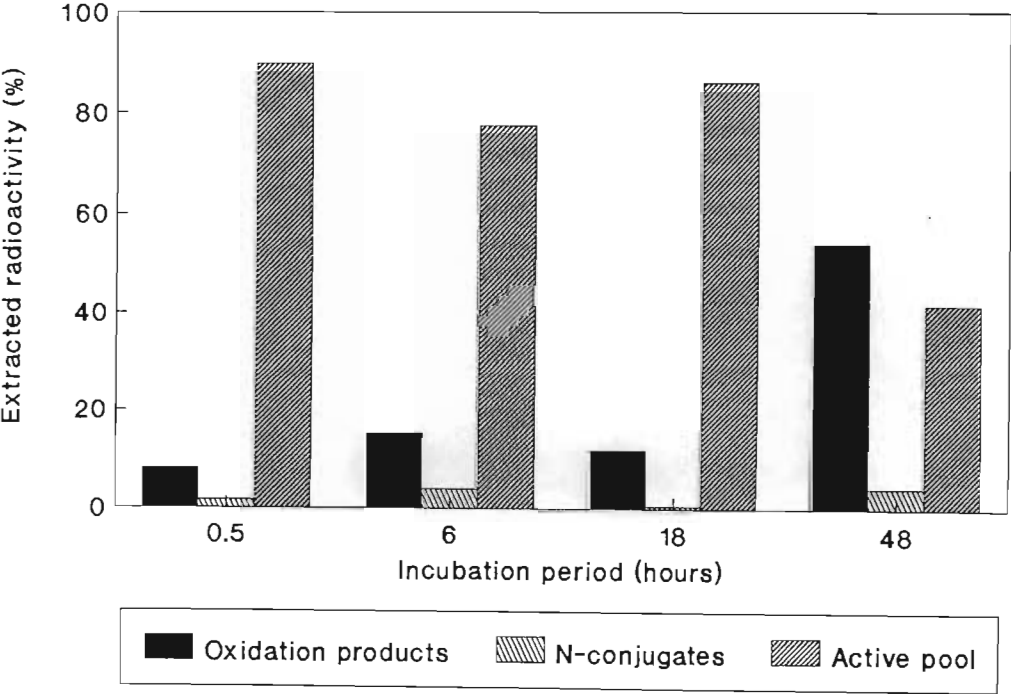


Figure 4.6 Activity fate of BA in soybean suspensions of high auxin status over 48 hours.

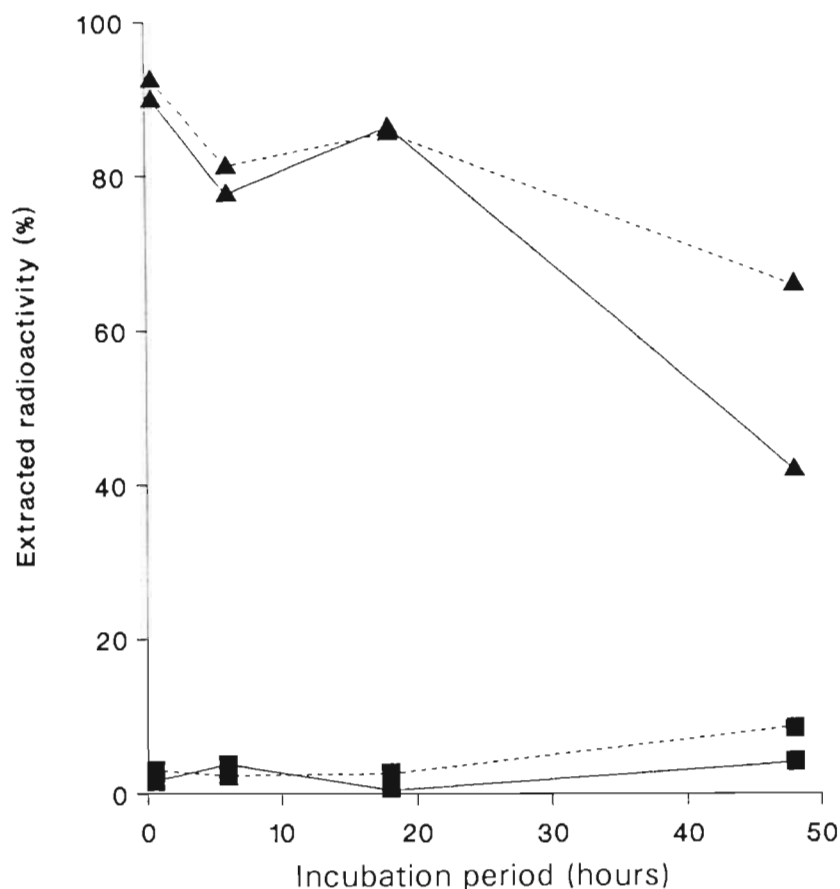


Figure 4.7 Levels of radioactivity associated with N-conjugates ( ▲ ) and active cytokinins ( ■ ) in soybean suspensions of low ( ----- ) and high ( \_\_\_ ) auxin status.

The effect of auxin became pronounced between 18 and 48 hours (Figure 4.8), when levels of both Ade (Figure 4.4) and Ado (Figure 4.3) increased markedly in cultures of high auxin status. Less glucosides were produced over this same period in cultures of high auxin status (Figure 4.7). This suggests that auxin-promoted oxidation proceeds at the expense of N-conjugation, possibly in consequence of competition for BA, a substrate which is common to both these inactivation pathways. From 18 hours to 48 hours, levels of both [9R]BA and [9R-MP]BA decreased in cultures of high auxin status (Figures 4.9 and 4.10). In systems treated with less auxin, levels of both riboside and ribotide increased over this period. In this investigation, the riboside, [9R]BA, was considered as a substrate for cytokinin oxidase (Figure 4.11).

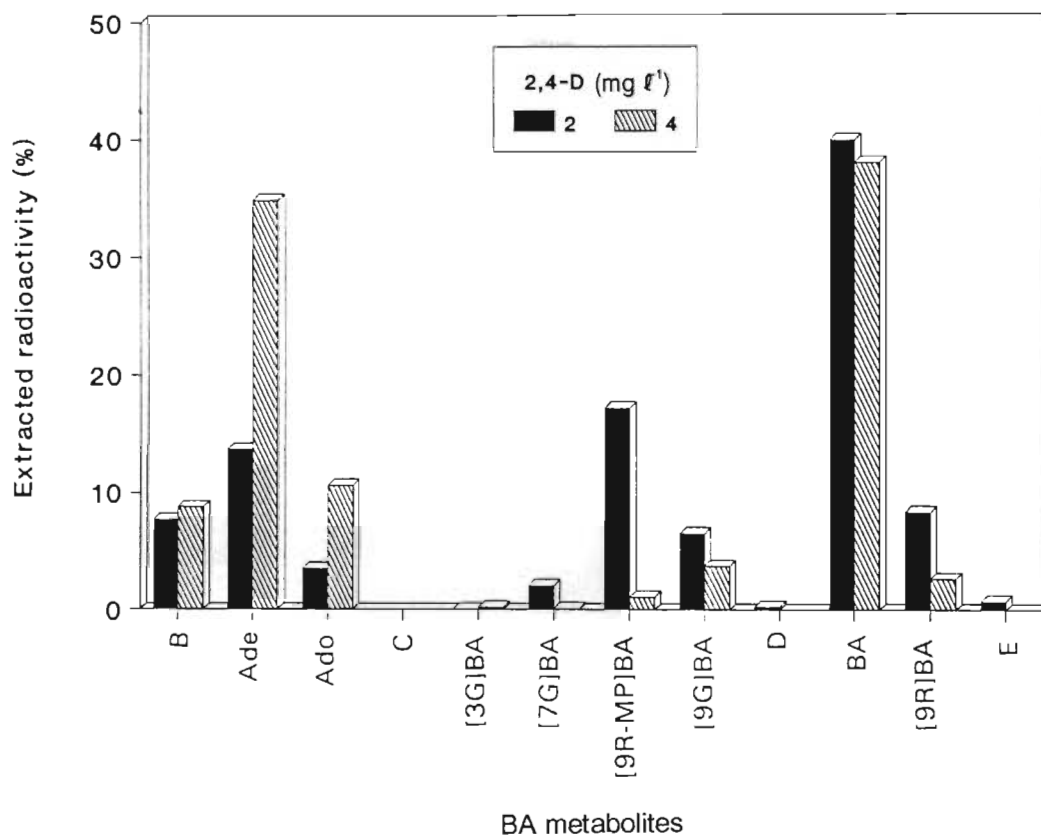


Figure 4.8 Effect of elevated auxin (2,4-D) levels on the metabolism of BA in soybean callus suspensions after 48 hours.

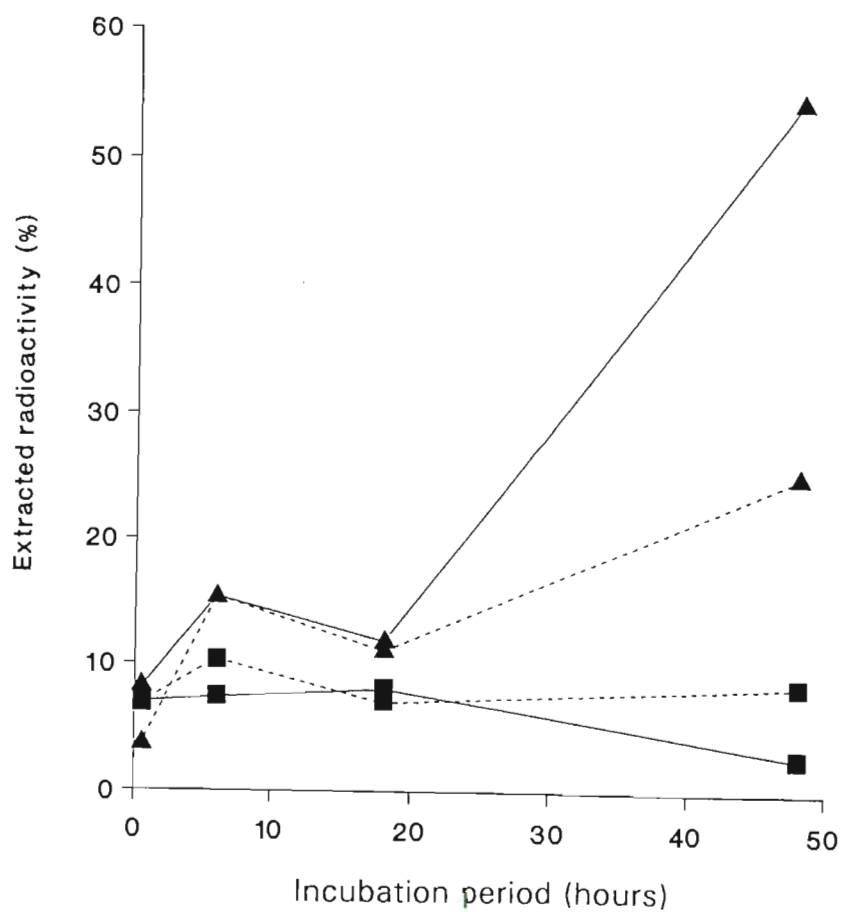


Figure 4.9 Levels of radioactivity associated with oxidation products (▲) and [9R]BA (■) in soybean callus suspensions over an incubation period of 0 to 50 hours.

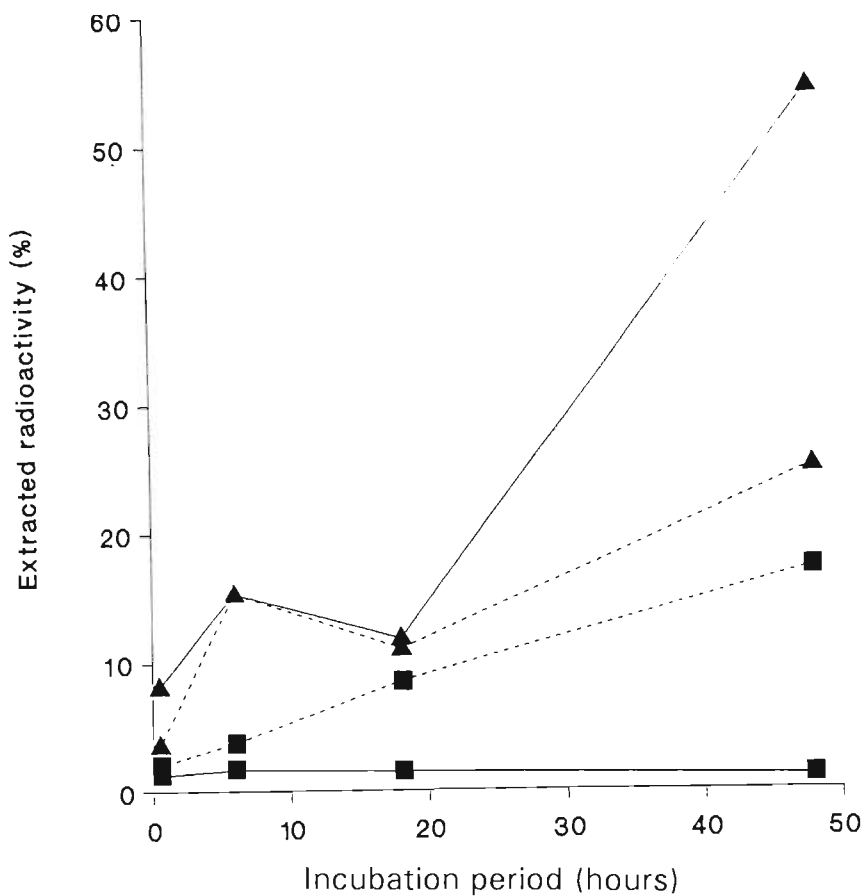


Figure 4.10 Levels of radioactivity associated with oxidation products ( ▲ ) and [9R-MP]BA ( ■ ) in soybean suspensions of low ( ---- ) and high ( \_\_\_ ) auxin status.

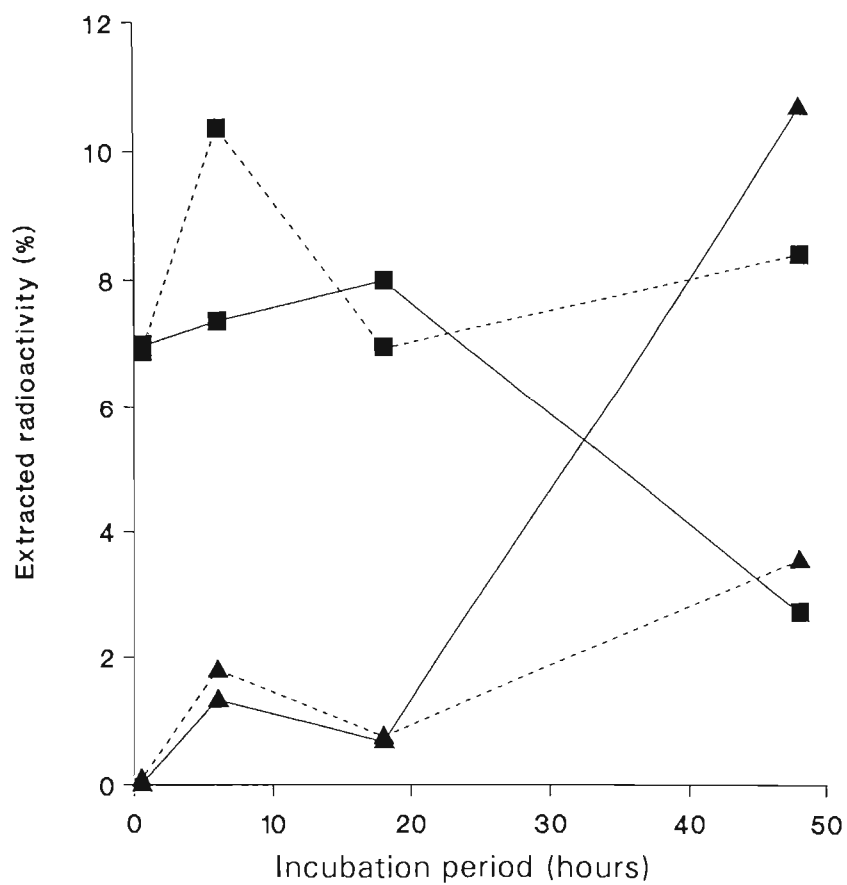


Figure 4.11 Levels of radioactivity associated with Adenosine ( ▲ ) and [9R]BA

The increase in extractable Ado between 18 and 48 hours was accompanied by a reduction in levels of [9R]BA. However, this decrease did not account for all Ado produced during this period. Oxidation at the riboside level has previously been documented (SINGH, PALNI & LETHAM, 1992). It is likely then that Ado was also derived from either the nucleotide ([9R-MP]BA) following dephosphorylation and oxidation, or through ribosylation of Ade formed from BA. Formation of unknown compound B directly from [9R-MP]BA may not occur, given that ribonucleotide formation appears to confer resistance to cytokinin oxidases (McGAW & HORGAN, 1983; LEE, MOK, MOK, GRIFFIN & SHAW, 1985).

#### 4.4 Discussion

##### 4.4.1 Auxin-affected cytokinin oxidase activity

Cleavage of the  $N^6$ -sidechain of BA applied to soybean suspensions was the prominent metabolic route of cytokinin inactivation. After 48 hours incubation, the effect of 2,4-D in promoting oxidative catabolism was evident (Figure 4.2), and was largely attributable to Ade formation (Figure 4.4). Current findings are consistent with several earlier reports (PALMER, LETHAM & GUNNING, 1984; PALNI, BURCH & HORGAN, 1988) which described auxin activation of 'cytokinin oxidase' type systems. 2,4-D at a concentration of  $1 \text{ mg } \ell^{-1}$  increased the activity of cytokinin oxidase by 15.6% *in vitro* (PALNI, BURCH & HORGAN, 1988). Current findings for the promotion of cytokinin oxidase activity by auxin contrast with recent reports with tobacco systems which described either no effect (MOTYKA & KAMINEK, 1990) or indirect inhibition (VANKOVA, GAUDINOVA, KAMINEK & EDER, 1992).

The enzyme system associated with catabolic oxidation of cytokinins is 'cytokinin oxidase'. The suitability of BA as a substrate for this enzyme has for many years been a contentious issue. All cytokinin oxidase preparations so far isolated from plant tissues do not appreciably attack BA (CHATFIELD & ARMSTRONG, 1986; KAMINEK & ARMSTRONG, 1990; refs within). Despite such reports on *in vitro* activity, many other investigators have identified catabolic products (Ade, Ado, adenine nucleotides,

xanthine, ureides etc.) after exogenous application of [8-<sup>14</sup>C]BA to a wide variety of plant tissues (McCALLA, MORRÉ & OSBORNE, 1962; DYSON, FOX & McCHESNEY, 1972; FOX, DYSON, SOOD & McCHESNEY, 1972; TERRINE & LALOUÉ, 1980; LALOUÉ & PETHE, 1982; BIONDI, CANCIANI & BAGNI, 1984; FORSYTH & VAN STADEN, 1986). 'Cytokinin oxidase', as characterised by WHITTY and HALL (1974) readily cleaves cytokinin sidechains of the (unsaturated) isopentenyl type whilst saturated aliphatic sidechains are not attacked. The presence of a double bond in the N<sup>6</sup>-sidechain renders it susceptible to oxidation. As such, BA and KIN, cytokinins with a cyclic sidechain, are supposedly not appreciably degraded by 'cytokinin oxidase' (WHITTY & HALL, 1974; HENSON, 1978; SCOTT, McGAW, HORGAN & WILLIAMS, 1982; LETHAM & PALNI, 1983). To explain observed anomalies, ZHANG and LETHAM (1989) hypothesised that N<sup>6</sup> de-benzoylation of BA 'probably involves an imino intermediate formed enzymically by elimination of a hydrogen atom from both the NH group at position 6 and the benzylic methylene'. Thus cytokinin oxidase activity may be expressed in those systems where the (hypothetical) enzyme facilitating production of the imino form is also present and active to prepare BA for attack. The same mechanism may apply to KIN degradation (EL-SAIDI, 1971; VAN STADEN & FORSYTH, 1985).

Before cytokinin oxidase was first isolated and characterised, McCALLA, MORRÉ and OSBORNE (1962) had proposed an alternative theory for benzyl group loss and [<sup>14</sup>C]Ade production from [8-<sup>14</sup>C]BA: loss of C-8 from BA to the 'one carbon' pool with subsequent re-incorporation into newly synthesised purine. However, such transcarboxylation reactions would not account for the relatively high [<sup>14</sup>C]Ade production reported for soybean (FORSYTH & VAN STADEN, 1986) and tobacco (LALOUÉ & PETHE, 1982) systems.

In their review, LETHAM and PALNI (1983) attributed benzyl cleavage reactions to an alternate enzyme system. Yet, as new experimental systems are considered, a diversity in cytokinin oxidase species is increasingly evident (KAMINEK, 1992). The variation in character and size of purified cytokinin oxidases (WHITTY & HALL, 1974; McGAW & HORGAN, 1983) indicates that distinct isozymes (CHATFIELD & ARMSTRONG, 1988; KAMINEK & ARMSTRONG, 1990) of this enzyme exist, which in some tissues appreciably catabolise BA and KIN. Given the natural occurrence of BA and

metabolites in plant tissues (STRNAD, PETERS, BECK & KAMINEK, 1992; refs within), cytokinin oxidases capable of degrading BA (as yet uncharacterised) are likely present to regulate *in vivo* metabolite levels. Assuming that the promotive influence of 2,4-D on BA degradation was at the metabolic rather than the molecular level, this auxin may have activated a BA-utilising cytokinin oxidase. Alternatively, 2,4-D may have activated the enzyme catalysing formation of the imino intermediate, so providing cytokinin oxidase with a suitably modified substrate. *In vitro* enzyme studies are required to further elucidate the level of action.

#### 4.4.2 Formation of [9Ala]BA in soybean tissues

Up to 48 hours after supplying soybean suspensions with BA, [9Ala]BA was not detected as a metabolite (Table 4.1). Although found as the major metabolite of BA in soybean leaves (ZHANG, LETHAM, WONG, NOODÉN & PARKER, 1987), [9Ala]BA has not always been unambiguously identified as a metabolite in soybean callus. ELLIOTT and THOMPSON (1982) considered this to be largely due to misidentification of [9Ala]BA as the 7-glucoside. Cell cultures of soybean (cv. Acme) supplied with [8-<sup>14</sup>C]BA rapidly converted this base to [9Ala]BA (WANG, EVERETT, GOULD & STREET, 1981), but no details were published. The auxin NAA was required for growth in this particular experimental system. In the current 2,4-D-supported system, formation of this amino-acid conjugate would not necessarily have been inhibited. However, both the findings for *Dianthus zeyheri* (Chapter 7) and of ZHANG, LETHAM, WONG, NOODÉN and PARKER (1987) for soybean leaves indicate that whilst NAA is essentially inactive in modifying BA metabolism, 2,4-D is not. Accordingly, 2,4-D present in the current experimental system may have prevented [9Ala]BA formation.

No previous reports using the described HPLC procedure have reported on an elution time for [9Ala]BA. In this study, trace metabolites appeared after approximately 31 and 72 minutes (designated Compounds C and D respectively). Compound C was apparently detected by VAN STADEN AND DREWES (1992) following application of [3G]BA to soybean callus. These authors associated this metabolite with cytokinin activity. The possibility that either of these metabolites is the alanyl conjugate should

not be excluded. Further characterisation of these metabolites was precluded by low biosynthetic rates, erratic formation (Table 4.1), and lack of available standards. During previous studies with this soybean line, unidentified cytokinin metabolites were detected (VAN STADEN & DAVEY, 1981; FORSYTH & VAN STADEN, 1986). In the latter study an unidentified BA conjugate was isolated which was not phosphorylated, glucosylated or ribosylated. Its  $R_f$  value corresponded to the value for [9Ala]BA provided by ELLIOTT and THOMPSON (1982), for a TLC system similar to that described (Chapter 3.3.3.4.4).

Strangely, although cytokinin-alanine conjugation is considered to be restricted to the legumes (LETHAM, SUMMONS, PARKER & MACLEOD, 1979), neither [9Ala]BA nor [9Ala]Z formation had been reported in metabolic studies on these systems up until 1975 (DYSON, FOX & McCHESNEY, 1972). This critical point was raised by WAREING, HORGAN, HENSON and DAVIS (1977). However, as clear differences in the metabolism of applied cytokinin in soybean callus of a single cultivar (var. Acme) have been reported (ELLIOTT & THOMPSON, 1982; FORSYTH & VAN STADEN, 1986; VAN STADEN & DREWES, 1992), it is conceivable that production of the alanyl conjugate is not a certainty in any leguminous system, whether morphologically differentiated or not. Clear differences in the array of BA metabolites between this and an earlier investigation on extraction techniques (Chapter 3.3.2) further emphasises this variation. The unidentified metabolite 'Compound A' detected earlier in tissue of the same genotype (Chapter 3.3.2.3) was conspicuously absent in this study, perhaps reflecting the inclusion of different auxins in these two experimental systems. Alternatively, the time lapse between these two studies may have allowed for epigenetic changes (MEINS, 1989) in stock callus. Compounds C and D could also correspond with a number of less common metabolites, viz. [7G]Ade, [9G]Ade or even [9Ala]Ade. The apparent high activity of a cytokinin-oxidase type system in soybean (*Glycine max* var. Acme) (FORSYTH & VAN STADEN, 1986) should allow for further degradation of N-conjugates. PALNI, PALMER and LETHAM (1984) had earlier reported on the oxidation in soybean callus of the zeatin derivatives [7G]Z, [9G]Z, and [9Ala]Z. McGAW and HORGAN (1985) similarly reported on the occurrence of [7G]Ade as a metabolite of [7G]Z.

#### 4.4.3 Biosynthesis of BA from catabolic products

Reconversion of active (benzyl) cytokinin forms from catabolic products was recorded in cultures of both low and high auxin status (Figures 4.5 and 4.6) between 6 and 18 hours. A biosynthetic route of this nature (for this class of cytokinins) is unknown. Isopentenyl-type cytokinin base formation via AMP (TAYA, TANAKA & NISHIMURA, 1978; PALNI, HORGAN, DARRALL, STUCHBURY & WAREING, 1983) or directly from Ade (HOCART & LETHAM, 1990; VAN STADEN & DREWES, 1993) has been documented. Enzymes catalysing BA synthesis have not been characterised; their existence has been inferred by the positive identification of BA and several metabolites in a number of plant species (HORGAN, HEWETT, PURSE & WAREING, 1973; DAS NEVES & PAIS, 1980; PAIS & CHAVES NEVES, 1982/83; ERNST, SCHÄFER & OESTERHELT, 1983; NANDI, LETHAM, PALNI, WONG & SUMMONS, 1989; NANDI, PALNI, LETHAM & WONG, 1989; STRNAD, PETERS, BECK & KAMINEK, 1992). Unambiguous identification of Ade or adenine nucleotides as BA metabolites will likely stimulate research into BA biosynthesis. This is especially so in view of recent findings (MULLER & HILGENBERG, 1986; VAN STADEN & DREWES, 1993) which suggest that some cytokinin biosynthesis is direct from Ade.

Whether promotive (VANKOVA, GAUDINOVA, KAMINEK & EDER, 1992) or inhibitory (HANSEN, MEINS & MILANI, 1985; HANSEN, MEINS & AEBI, 1987), auxin effects on cytokinin metabolism have appeared transient. In soybean suspension cultures no transient auxin effects were noted during the time-course considered. At longer incubation periods, this may have become apparent. Such transient effects would allow for induction of certain developmental processes (WAREING, 1978) whilst preventing long term hormonal imbalances in plant tissues (VANKOVA, GAUDINOVA, KAMINEK & EDER, 1992).

## CHAPTER 5

### THE EFFECT OF THE AUXIN 2,4-DICHLOROPHENOXYACETIC ACID ON THE METABOLISM OF 6-(BENZYLAMINO)PURINE IN CELL CULTURES OF CARROT (*DAUCUS CAROTA* CV. KEYSTONE DANVERS 126).

#### 5.1 Introduction

Members of the Solanaceae (*Nicotiana* and *Solanum* L.) are most often employed in auxin/cytokinin interactive studies (WOOLLEY & WAREING, 1972; PALNI, BURCH & HORGAN, 1988; MOTYKA & KAMINEK, 1990; VANKOVA, GAUDINOVA, KAMINEK & EDER, 1992). Modifications of alanylation by 2,4-D in soybean leaves, shown by ZHANG, LETHAM, WONG, NOODÉN and PARKER (1987), indicated the potential of soybean cell cultures as useful experimental tools. The reported production of [9Ala]BA in soybean leaves and controversy surrounding *N*<sup>6</sup>-benzyl cleavage in soybean callus further encouraged use of this leguminous system in interactive studies (Chapter 4). Results with soybean showed similarities in (oxidative catabolic) responses which were common to both solanaceous and leguminous plant groups.

To date, carrot cell cultures have apparently not been used in hormone interactive studies, despite their wide application in metabolic (ASHIHARA & NOBUSAWA, 1981) and hormonal (LETHAM, 1967; MONTAGUE, ENNS, SIEGEL & JAWORSKI, 1981a) investigations.

To determine whether similar metabolic conversions would be influenced by auxin in an unrelated (apiaceous) species, cytokinin-autonomous carrot suspensions (*Daucus carota* cv. Keystone Danvers 126) were treated with BA in the presence of both high and low levels of the synthetic auxin 2,4-D. Levels of phytohormones employed were identical to those for investigations in soybean and *Dianthus zeyheri* cultures (Chapters 4 and 6). This permitted an investigation of interaction between these hormones at the metabolic level, and provided a basis for comparison with other systems.

## 5.2 Materials and methods

### 5.2.1 Plant materials

Stock cultures of *Daucus carota* cv. Keystone Danvers 126 were established and maintained on a modified GAMBORG, MILLER & OJIMA (1968) B5 basal medium supplemented with 2,4-D at a concentration of 2 mg  $\ell^{-1}$  (Chapter 3.2.2.2). Stock cultures were incubated in 500 ml Erlenmeyer flasks under low intensity white fluorescent lights providing 1  $\mu\text{mol m}^{-2} \text{s}^{-1}$  with a 16 hour light regime.

### 5.2.2 Cytokinin application

The [8- $^{14}\text{C}$ ]BA (0.0017 Mbq in 3  $\mu\text{l}$  methanol; specific activity 2.04 GBq  $\text{mmol}^{-1}$ ) was added to 15 ml suspension culture in a 100 ml Erlenmeyer flask. The final concentration of 2,4-D in each 15 ml sample of cell culture was adjusted to be either 2 mg  $\ell^{-1}$  or 4 mg  $\ell^{-1}$ . In each sample, non-labelled BA (at 2 mg  $\ell^{-1}$ ) was added. Three replicates were established for each variable considered. After treating the cell suspensions, flasks were incubated under identical conditions to those of the stock cultures, except that a continual photoperiod was now maintained.

### 5.2.3 Extraction and purification of BA metabolites

The procedure for the extraction and purification of BA metabolites was identical to that employed in soybean studies (Chapter 4.2.3).

### 5.2.4 HPLC analysis

Extracts were subjected to HPLC analysis as for soybean studies (Chapter 4.2.4).

### 5.2.5 Confirmatory techniques

Individual radioactive peaks determined by HPLC were further investigated by

chemical treatment with HCl (FOX, DYSON, SOOD & McCHESENEY, 1972), or re-chromatographed with authentic BA derivatives in a TLC system.

#### 5.2.6 TLC analysis

Peaks of radioactivity detected after HPLC were subjected to TLC (Merck, Silica gel 60, F<sub>254</sub>, *n*-butanol:NH<sub>4</sub>OH:water (6:1:2) v/v upper phase) (Chapter 3.3.3.4.4) and the ten R<sub>f</sub> fractions counted for radioactivity (Chapter 3.3.3.5). The results of TLC analyses and acid hydrolysis treatments of fractions were consistent with the initial tentative identifications based on HPLC retention times.

### 5.3 Results

#### 5.3.1 Time sequence studies

In contrast to both the *Dianthus* (Chapter 6) and soybean (Chapter 4) systems, carrot cell cultures supplied with cytokinin base ([8-<sup>14</sup>C]BA) maintained, over 48 hours, a large active cytokinin pool (Table 5.1). High activity was maintained in systems of both low (Figure 5.1) and high (Figure 5.2) auxin status. Rapid metabolism to the riboside was evident (Figure 5.3). After 6 hours most radioactivity extracted was associated with [9R]BA. This conversion reflects the rapid utilisation of supplied BA. After 48 hours, radioactivity associated with the riboside accounted for over 70% of the BA metabolites produced. Nucleotide formation was not prominent after any incubation period, nor was N-conjugation through glucosylation (Table 5.1). Pathways of oxidative catabolism were weakly expressed in carrot cell cultures, for no more than 8% of the radioactivity was associated with oxidation products at any time (Figure 5.1).

#### 5.3.2 Effect of hormonal interaction

Auxin effects on the metabolism of 6-(benzylamino)purine were of a transient nature. After 18 hours the effect of auxin on the active cytokinin pool diminished such that by 48 hours, an effect on activity was barely perceptible (Figure 5.4).

Figure 5.1 Radioactivity attributable to BA and its metabolites in extracts of carrot suspension cultures supplied with [8-<sup>14</sup>C]BA in the presence of high (4 mg l<sup>-1</sup>) and low (2 mg l<sup>-1</sup>) levels of auxin.

Incubation Time (hrs)	2,4-D (mg l <sup>-1</sup> )	Radioactivity co-eluting with standards (% of total extracted)														
		B <sup>w</sup>	Ade	Ado	[3G]BA	[7G]BA	[9G]BA	[9R-MP]BA	[9R]BA	BA	C <sup>w</sup>	D <sup>w</sup>	E <sup>w</sup>	Oxidation Products*	Active Pool <sup>y</sup>	N-conjugates <sup>z</sup>
	2	0.56	0.48	0.10	0.05	0.33	1.12	0.01	2.92	94.28	-	0.62	-	1.14	97.21	1.15
	4	1.99	2.90	0.10	0.62	0.17	0.39	0.50	17.81	73.88	0.84	0.27	0.54	4.99	92.19	1.18
	2	3.46	0.32	1.36	0.00	0.22	2.07	1.40	56.64	34.34	0.05	0.11	0.03	5.14	92.38	2.29
	4	0.76	1.54	0.77	0.08	0.23	2.37	2.76	52.45	38.85	-	0.09	0.10	3.07	94.06	2.68
	2	2.31	0.64	0.38	0.20	1.48	2.02	5.80	71.02	15.54	0.34	0.53	0.13	3.34	92.36	3.70
	4	1.83	0.84	1.55	2.33	0.94	7.14	2.58	63.87	16.38	0.48	1.66	0.40	4.22	82.83	10.41
	2	3.77	0.52	0.94	1.52	0.75	2.04	0.83	71.59	16.65	0.05	0.85	0.49	5.23	89.07	4.31
	4	1.46	1.04	4.99	0.33	1.18	3.57	2.76	76.55	7.81	-	0.74	0.17	7.49	87.12	5.08

\* known compound

<sup>w</sup> radioactivity associated with B, Ade and Ado, products of oxidation

<sup>y</sup> radioactivity associated with base, nucleoside, and nucleotide

<sup>z</sup> radioactivity associated with N-glucosides

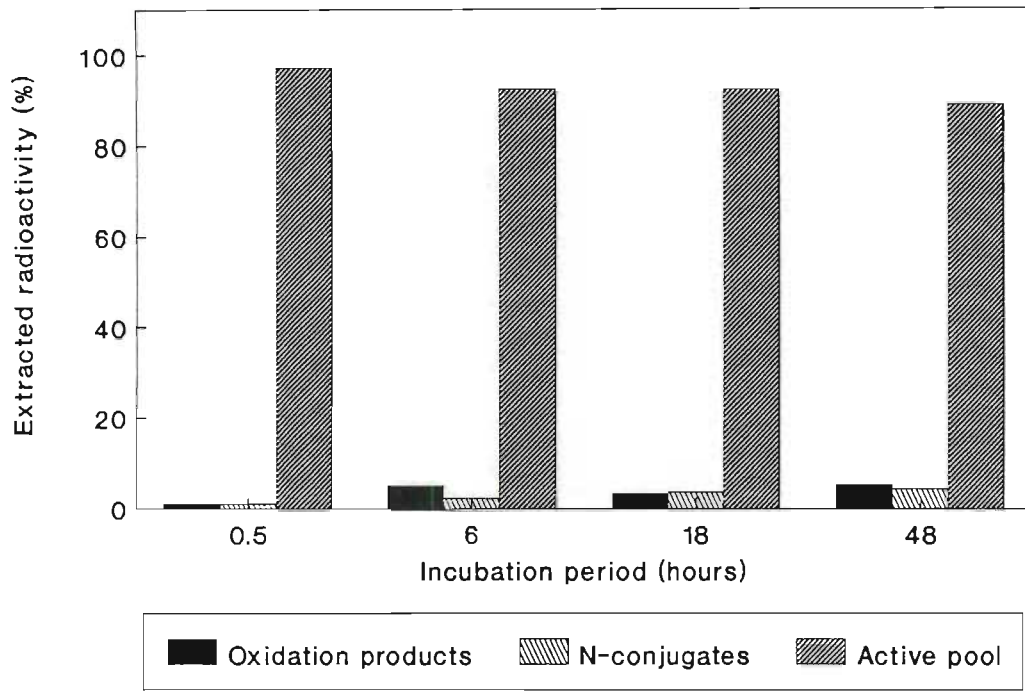


Figure 5.1 Activity fate of BA in carrot cell cultures of low auxin status over 48 hours.

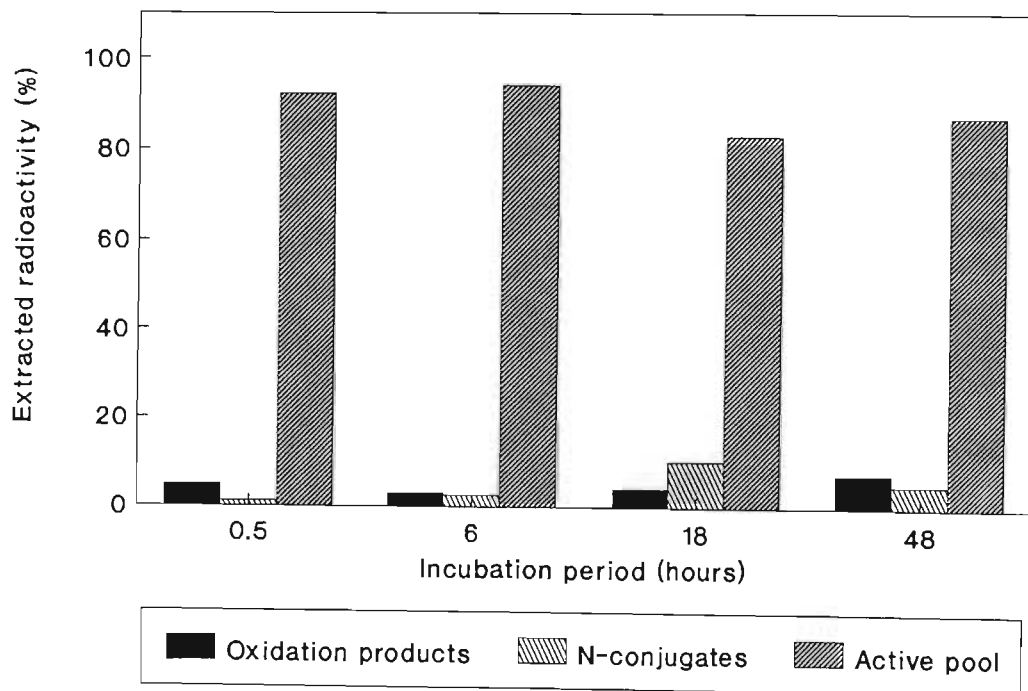


Figure 5.2 Activity fate of BA in carrot cell cultures of high auxin status over 48 hours.

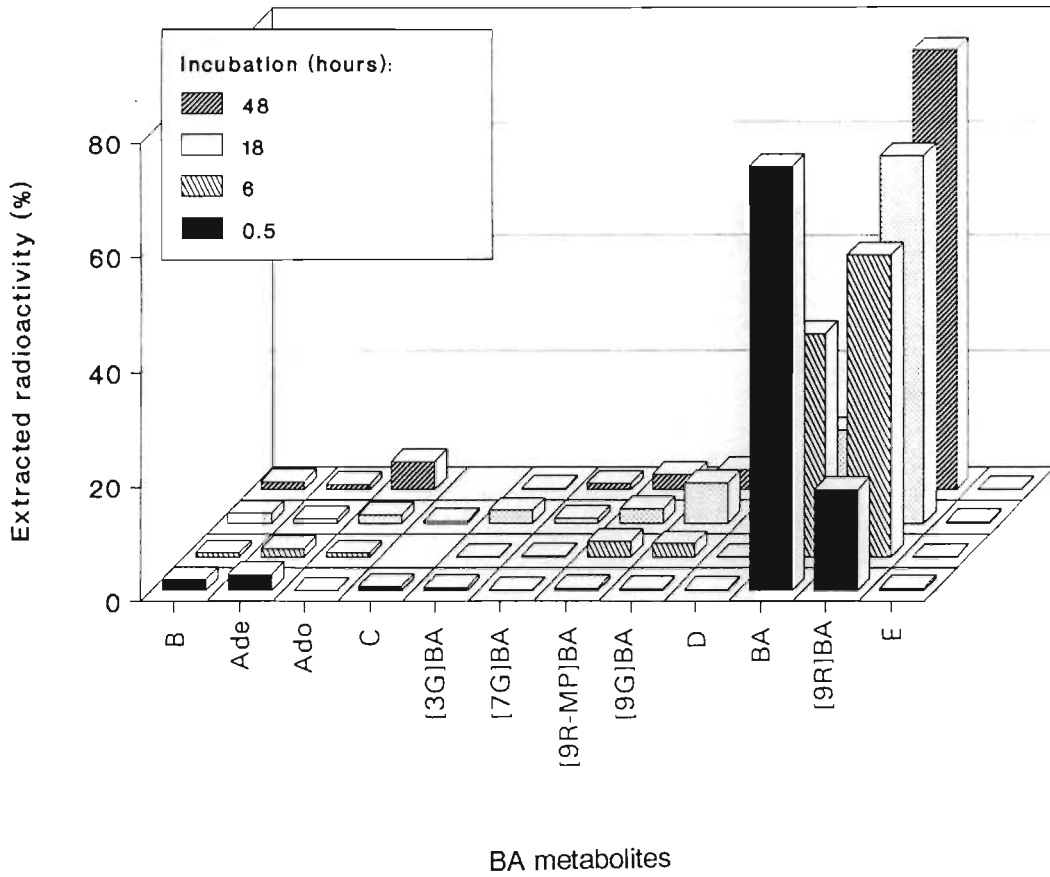


Figure 5.3 The metabolism of [8-<sup>14</sup>C]BA in carrot cell cultures over 48 hours. 2,4-D at 4 mg l<sup>-1</sup> was included in the medium.

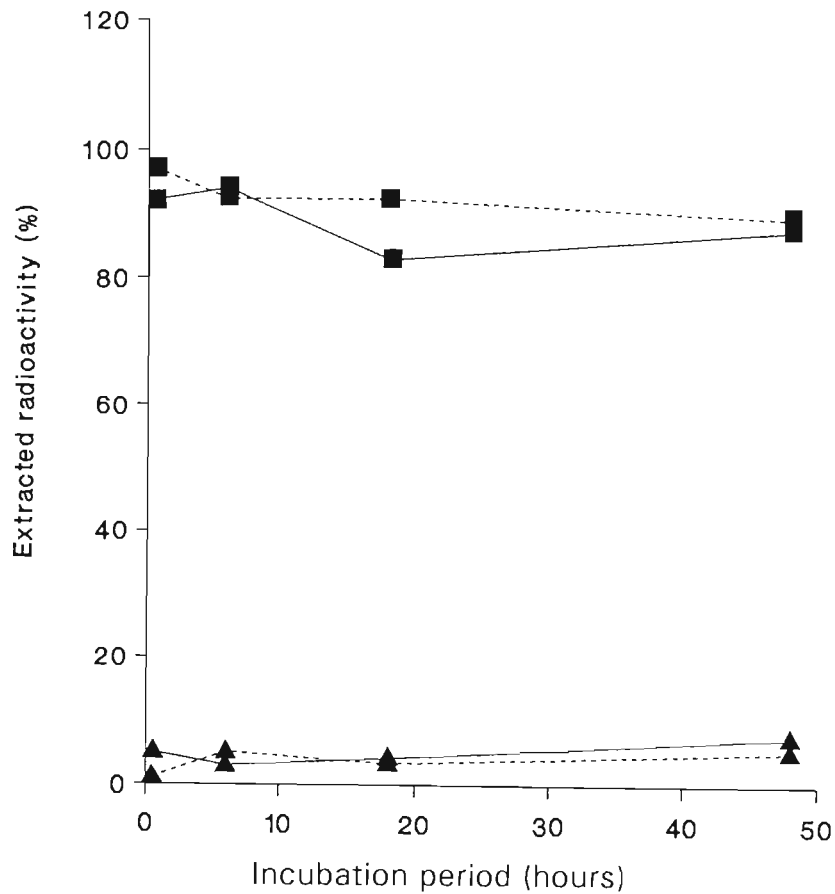


Figure 5.4 Levels of radioactivity associated with oxidation products (▲) and active cytokinins (■) in carrot cell cultures of *Daucus carota* L. (cv. 'Nantes')

However, less free base remained after 48 hours (Figure 5.5) when tissues were incubated with high auxin levels. Sidechain cleavage alone did not account for the auxin-promoted lowering of the active cytokinin pool which was most pronounced after 18 hours (Figure 5.4). Catabolic processes were only slightly promoted by 2,4-D after this period (Figure 5.4). Auxin-promoted N-conjugation (Figure 5.6), especially 9-glucosylation (Figure 5.7) after 18 hours incubation, better accounted for this lowering of active cytokinin levels. A relative increase in radioactivity associated with [9R]BA over the period 18 to 48 hours (Figure 5.8) appeared responsible for the transient effect of auxin on N-conjugation. It is probable then that 9-glucoside formed within 18 hours incubation was metabolised to [9R]BA (Figure 5.7).

A single enzyme system (two enzymes or different isozymes) was supposedly responsible for the formation of both 7- and 9-cytokinin glucosides (ENTSCH & LETHAM, 1979) by radish leaves. The effect of auxin on the relative formation of these two glucosides by cytokinin-7-glucosyl transferase has been considered (Table 5.2). At all incubation periods and at both auxin concentrations, more [9G]BA than [7G]BA was formed (Tables 5.1 and 5.2). Initially (after 30 minutes), auxin at a higher concentration in the incubation medium appeared to slightly favour promotion of [7G]BA formation over production of [9G]BA. At six hours production of neither glucoside was favoured, but after 18 hours, the ratio of 9G/7G increases nearly six-fold in systems of higher auxin status. This effect reduces such that after 48 hours 2,4-D has no effect on the relative production of these two glucosides (Table 5.2).

**Table 5.2** The effect of 2,4-D concentration on the relative formation of 9- and 7-glucosides of BA by 'cytokinin-7-glucosyl transferase' in carrot cell cultures.

Incubation (hours)	2,4-D (mg l <sup>-1</sup> )	Ratio of [9G]BA:[7G]BA	X:Z*
0.5	2	3.39	0.68
	4	2.29	
6	2	9.41	1.09
	4	10.3	
18	2	1.36	5.59
	4	7.6	
48	2	2.72	1.11
	4	3.03	

\*The ratio of [9G]BA:[7G]BA formed in systems of high auxin status (X) to [9G]BA:[7G]BA formed in systems of low auxin status (Z). The value of X:Z indicates the effect auxin has on the ratio of 9G:7G.

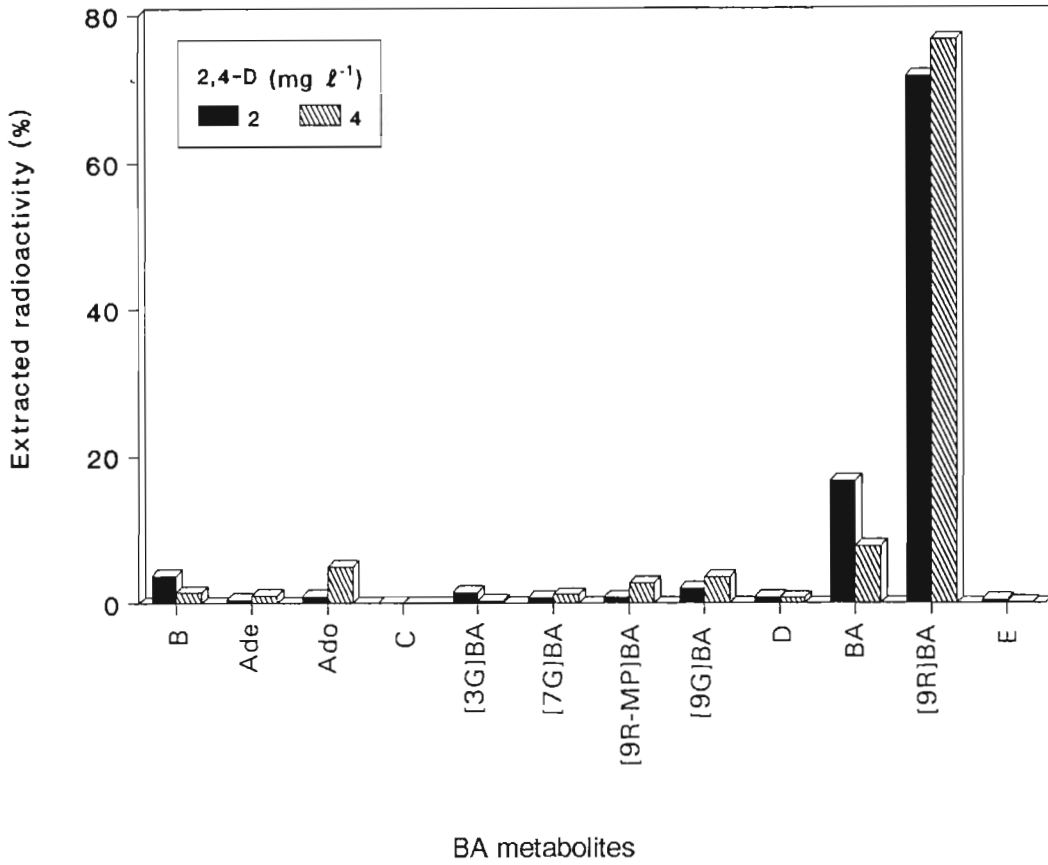


Figure 5.5 Effect of elevated auxin (2,4-D) levels on the metabolism of BA in carrot cell suspensions after 48 hours.

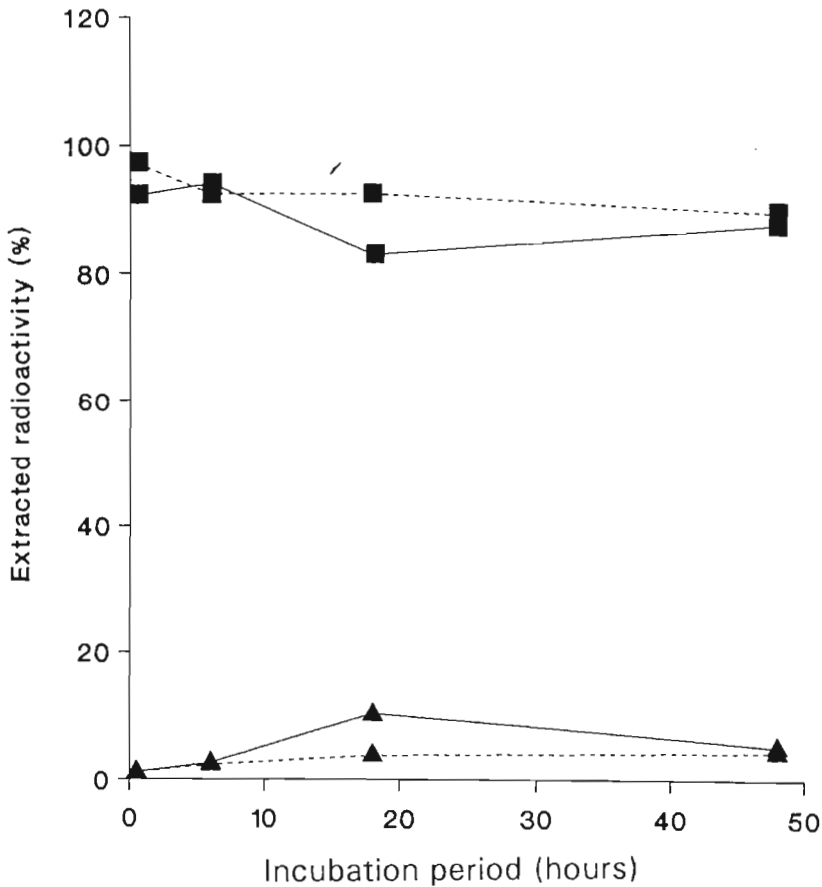


Figure 5.6 Levels of radioactivity associated with N-conjugates (▲) and active cytokinins (■) in carrot cell cultures of low (-----) and high (——) 2,4-D concentrations.

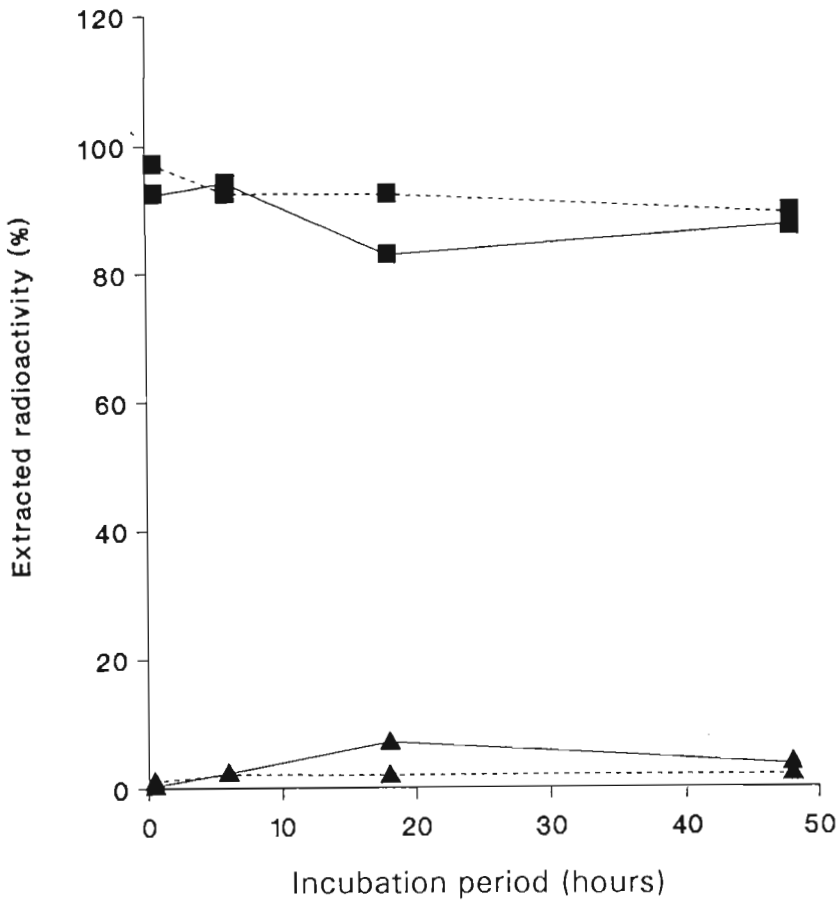


Figure 5.7 Levels of radioactivity associated with [9G]BA ( ▲ ) and active cytokinins ( ■ ) in carrot cell cultures of low ( ----- ) and high ( \_\_\_\_ ) auxin status.

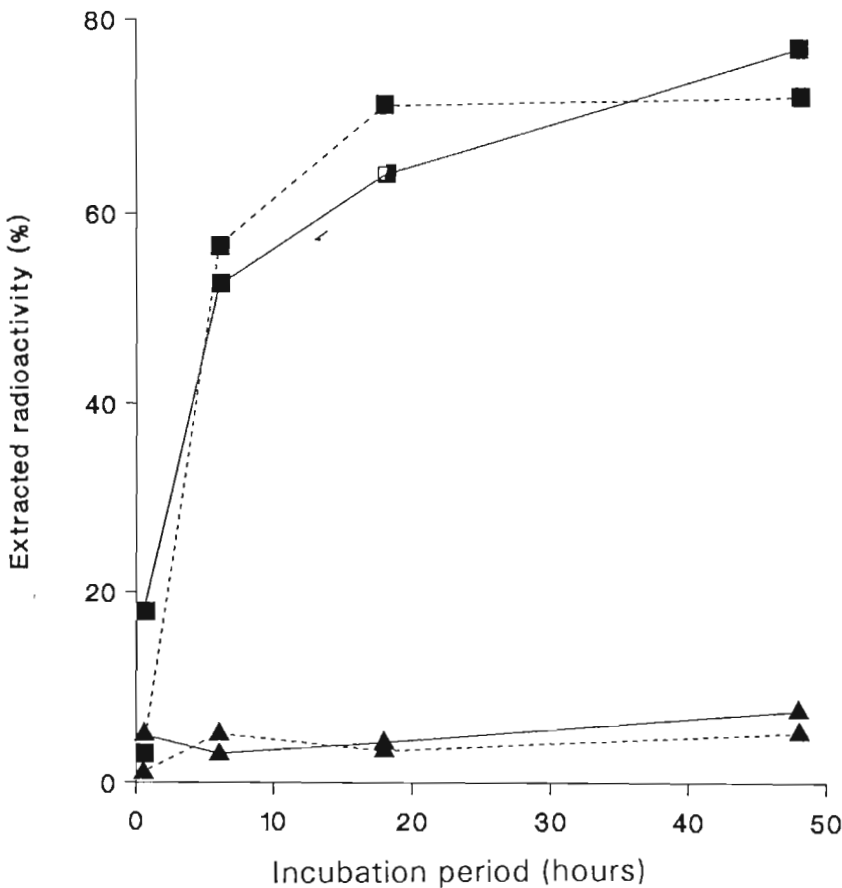


Figure 5.8 Levels of radioactivity associated with oxidation products ( ▲ ) and

## 5.4 Discussion

Carrot cell cultures supplied with BA ( $2 \text{ mg l}^{-1}$ ) maintain a large active pool, even after 48 hours incubation. Rapid riboside formation was observed; after 30 minutes, promotion by auxin of applied base to the riboside was very marked (Table 5.1; Figure 5.8). This suggests that 2,4-D directly activates the nucleoside phosphorylase responsible for ribosylation of BA. It is unlikely that auxin-induced enzyme synthesis would be expressed in so short a period. After 6 hours, little effect of auxin on [9R]BA production was evident, despite the initial promotion of ribosylation by 2,4-D. In immobilised tobacco cells, VANKOVA, GAUDINOVA, KAMINEK and EDER (1992) also observed transient auxin-promoted elevation of cytokinin riboside levels. Both these findings contrast with those of (VAN STADEN & MOONEY, 1987) who considered the effect of IAA on KIN metabolism in cytokinin-autonomous *Catharanthus roseus* callus. IAA promoted loss of the riboside in this tissue. Whether the effect was transient is unknown as a time-course evaluation was not presented. In carnation (*Dianthus caryophyllus*) ovaries, FEATONBY-SMITH, VAN STADEN and COOK (1987) similarly detected lower [9R]BA levels after 24 hours when tissues were simultaneously treated with IAA. This auxin-mediated difference in riboside levels reduced over a 28 day period, with an associated rise in levels of an unidentified oxidative catabolite (probably compound B, Table 5.1). Inactivation through N-conjugation or oxidation was not prominent at any time. Catabolic oxidation of cytokinins was poorly operative under the described conditions. A transient yet distinct promotion of N-conjugation (9-glucosylation) was evident after 18 hours (Figures 5.6 and 5.7; Table 5.2). After 48 hours this auxin effect greatly diminished. In radish cotyledons, two enzymes (or different forms of one enzyme) form both the 7- and 9-glucosides of BA. The one forms the 7-glucoside predominantly and the other the 7- and 9-glucosides in similar proportions (ENTSCH & LETHAM, 1979). Hence, in radish, the enzyme system known collectively as cytokinin-7-glucosyl transferase promoted 7- over 9-glucosylation of BA. Since the report on radish, several systems have been recorded (TAO, LETHAM, PALNI & SUMMONS, 1983; BLAKESLEY, 1991; MOFFATT, PETHE & LALOUE, 1991; JONES & HANKE, 1992) in which the cytokinin-9-glucoside is the major metabolite. To account for this, LETHAM and GOLLNOW (1985) proposed that where the ratio of 7- and

9-glucosides is in favour of the 9-, the relative activity of the former enzyme may have markedly declined. Although still only a minor metabolite in carrot cultures, more [9G]BA was produced than [7G]BA, at all variable combinations considered (Tables 5.1 and 5.2). It is possible then that a separate cytokinin-9-glucosyl transferase exists, but is limited in distribution (viz. Fabaceae-restricted cytokinin-9-alanyltransferase), and perhaps limited in time to certain developmental stages. Evidence for spacial localisation was provided by UPFOLD and VAN STADEN (1992). These workers applied BA to cut carnation stems and detected [7G]BA and [9G]BA 'specifically and separately in the various flower components'. This argues for separate enzymes for 7- and 9-glucosylation. Alternatively, there may be 'cytokinin-7-glucosyltransferase' type enzymes which catalyse both 7- and 9-glucosylation, but favour production of the 9-glucoside. Such an enzyme may be present in *Daucus* cultures. As auxin temporarily promoted 9-glucosylation of BA (Table 5.2) [an effect on [9G]BA formation effectively altered the 9G/7G ratio], it is conceivable that auxin has a role in altering the ratio of 7- and 9-glucosides produced by a single enzyme. An obvious inference of this finding is that studies with cytokinin-7-glucosyl transferase (from any source) need to critically consider pretreatment or incubation of tissues with auxin as this affects the relative formation of glucosides. Such auxin effects may be unavoidable in view of standard *in vitro* growth requirements of experimental systems. Various substituted xanthines have also been shown to affect the 7G/9G ratio (HOCART, LETHAM & PARKER, 1991; TAO, LETHAM, HOCART & SUMMONS, 1991).

The auxin-promoted 9-glucosylation (activity loss) after 18 hours seemingly contrasts with the initial auxin-promoted increase in [9R]BA (activity increase). However, auxin-promoted ribosylation did not affect the active cytokinin pool (Table 5.1). Thus the effect was not antagonistic, but rather neutral. A concurrent promotion of catabolism slightly affected this level (Table 5.1; Figure 5.4). Transient metabolic states supposedly allow for the induction of developmental processes (VANKOVA, GAUDINOVA, KAMINEK & EDER, 1992). If this is so, then both promotive and inhibitive metabolic effects may induce responses, before hormonal balance is restored to favour further development of initiated structures. The nature of these developmental processes in

cytokinin-autonomous carrot cell cultures is not known. Further, as these cultures showed no obvious requirement for supplied BA in their growth media, the maintenance of a predominantly active pool is not easily explained. Exogenously supplied cytokinin base (in excess of requirements for growth) would potentially be detoxified through inactivation pathways. This was only evident after 18 hours when 9-glucosylation was promoted (Figure 5.7). At this time, most of the cytokinins were still associated with the active pool. This tissue, which was derived from cytokinin-dependent callus, could have retained the metabolic machinery for inter-conversions within the active pool. The enzymes effecting such conversions are believed to be those inter-converting adenine bases, nucleosides and nucleotides (WAGNER & BACKER, 1992; LEE & MOFFATT, 1993). Such enzyme activity would not necessarily diminish with loss of cytokinin-dependency, as the enzymes are still required for Ade salvage.

## CHAPTER 6

### THE EFFECT OF THE AUXIN 2,4-DICHLOROPHENOXYACETIC ACID ON THE METABOLISM OF 6-(BENZYLAMINO)PURINE IN SEED AND SHOOT APEX-DERIVED CELL CULTURES OF *DIANTHUS ZEYHERI*

#### 6.1 Introduction

Where auxin has been reported to modify cytokinin metabolism, a variety of mechanisms have been observed which account for both antagonistic and synergistic physiological effects. WOOLLEY and WAREING (1972) were apparently the first to demonstrate the modification of cytokinin metabolism by exogenously applied auxin. In *Solanum andigena* Juzepczuk & Bukasov stem cuttings of low IAA status, [9R]BA was the main cytokinin formed from BA. At higher IAA levels, a 'Compound C' was the predominant metabolite. VAN STADEN and MOONEY (1987) later found IAA (2 mg  $l^{-1}$ ) to reduce the rate of ribosylation of KIN in cytokinin-autonomous *Catharanthus* crown gall callus. Similarly, HANSEN, MEINS and AEBI (1987) found accumulation of [9R]Z to be inhibited in some tobacco explants cultured on auxin-containing (NAA) medium after 14 days. However, this auxin effect diminished with time; after 28 days little effect of NAA was noticeable.

Alanine conjugation and  $N^6$ -benzyl cleavage were considered by ZHANG, LETHAM, WONG, NOODÉN and PARKER (1987) to be alternative mechanisms for BA inactivation in soybean leaves. These authors found the auxins 2,4-D and 5,7-dichloroindole acetic acid to inhibit BA metabolism to the corresponding alanine conjugate in soybean leaves. Prior to this report on soybean, PARKER, ENTSCH and LETHAM (1986) had reported on *in vitro* studies where IAA, 2,4-D, and 5,7-dichloroindole acetic acid competitively inhibited  $\beta$ -(9-cytokinin)-alanine synthase purified from lupin seed. Although formation of [9Ala]BA represents a reduction in cytokinin activity (LETHAM, PALNI, TAO, GOLLNOW & BATES 1983; ZHANG, LETHAM, WONG, NOODÉN & PARKER, 1987; ZHANG, HOCART & LETHAM, 1989), it may be a storage compound (PALNI, PALMER & LETHAM, 1984). Conversion of BA to adenine and adenosine in soybean leaf

synchronously increased when [9Ala]BA formation was inhibited by auxin; notably, levels of active forms of BA were also then slightly elevated (ZHANG, LETHAM, WONG, NOODÉN & PARKER, 1987). As a result of inhibition of 9-alanylation by aminophylline in soybean callus, levels of active BA forms were found to increase (ELLIOTT & THOMPSON, 1982). However, neither oxidative nor alternative N-conjugation processes were synchronously activated.

Promotion or inhibition by auxin of glucosylation pathways has not previously been demonstrated. On the contrary, IAA reportedly had no effect on O-glucoside formation in *D. caryophyllus* ovaries (FEATONBY-SMITH, VAN STADEN & COOK, 1987). Formation of ring-substituted cytokinin N-glucosides has appeared similarly unaffected. TAO, LETHAM, HOCART and SUMMONS (1991) could not demonstrate auxin-affected conversion of [9R]Z to [7G]Z. However, the auxins IAA and NAA were considered in this particular study, but not 2,4-D, whose affect may have been more pronounced. Detoxification of applied cytokinin through glucosylation should conceivably occur in those systems where alanylation or oxidation mechanisms are not operative.

Use has been made of a carnation species, *Dianthus zeyheri* subsp. *natalensis*, which is native to South Africa. BA metabolism has not previously been studied in this taxon, only in the north temperate species *D. caryophyllus*, and this particularly in relation to flower senescence (COOK & VAN STADEN, 1988; refs within). The use of *D. zeyheri* thus permitted a comparison of BA metabolism between a wild species and a domesticated and physiologically altered (STONE, 1963; NICHOLS, 1977) close relative. The contrasting reports on routes of BA inactivation in *D. caryophyllus* (FEATONBY-SMITH, VAN STADEN & COOK, 1987; UPFOLD & VAN STADEN, 1992) indicated that the use of *D. zeyheri* in fundamental studies was potentially rewarding. In this investigation, cytokinin-autonomous seed and apex-derived cell suspensions have been treated with BA in the presence of two levels of the synthetic auxin 2,4-D to investigate interaction between these hormones at the metabolic level.

## 6.2 Materials and methods

### 6.2.1 Plant materials

Stock cultures of both shoot apex and seed derived cell lines were established and maintained on a MURASHIGE and SKOOG (1962) basal medium supplemented with 2,4-D at a concentration of 2 mg  $\ell^{-1}$ . Stock cultures were incubated in 500 ml Erlenmeyer flasks under low intensity white fluorescent lights providing 1  $\mu\text{mol m}^{-2} \text{s}^{-1}$  with a 16 hour light regime.

### 6.2.2 Cytokinin application

Cell cultures were treated in the linear phase of growth. The [8- $^{14}\text{C}$ ]BA (0.0017 Mbq in 3  $\mu\text{l}$  methanol; specific activity 2.04 GBq  $\text{mmol}^{-1}$ ) was added to 15 ml suspension culture in a 100 ml Erlenmeyer flask. The final concentration of 2,4-D in each 15 ml replicate of cell culture was adjusted to be either 2 mg  $\ell^{-1}$  or 4 mg  $\ell^{-1}$ . In each sample, non-labelled BA (at 2 mg  $\ell^{-1}$ ) was added. Three replicates were established for each variable considered. After treating the cell suspensions, flasks were incubated under identical conditions to those of the stock cultures, except that a continual photoperiod was now maintained.

### 6.2.3 Extraction and purification of BA metabolites

The procedure for the extraction and purification of BA metabolites was identical to that employed in soybean studies (Chapter 4.2.3).

### 6.2.4 HPLC analysis

Extracts were subjected to HPLC analysis as for soybean studies (Chapter 4.2.4).

### 6.2.5 Confirmatory techniques

Individual radioactive peaks so determined were further investigated by chemical treatment with HCl (FOX, DYSON, SOOD & McCHESNEY, 1972), or re-chromatographed with authentic BA derivatives in a TLC system.

### 6.2.6 TLC analysis

Peaks of radioactivity detected after HPLC were subjected to TLC (Merck, Silica gel 60,  $F_{254}$ , *n*-butanol:NH<sub>4</sub>OH:water (6:1:2) v/v upper phase) and the ten R<sub>f</sub> fractions counted for radioactivity. [7G]BA was subjected to cellulose TLC (Merck plates) using two solvent systems. The solvents were (proportions by volume): A, *n*-butanol:acetic acid:water (4:1:1); B, distilled water. The results of TLC analyses and acid hydrolysis treatments of fractions were consistent with the initial tentative identifications based on HPLC retention times.

## **6.3 Results**

### 6.3.1 Time sequence studies

It is clear that cell culture lines originating from the same species, but distinct plant parts metabolised BA in a different manner. In both culture types, much less BA was extracted after 48 hours than after 30 minutes (Tables 6.1 and 6.2); after this longer period both systems had metabolised as much applied base to a qualitatively similar range of metabolites (Figure 6.1). A similar rate of BA utilisation was recorded (Figure 6.2). Both *Dianthus* culture types formed [7G]BA as a major metabolite. After 48 hours seed-derived cultures formed [9R]BA and [7G]BA as dominant products, in order of prominence (Figure 6.1). In contrast, apex-derived cultures formed predominantly the 7-glucoside, and to a lesser degree [9R]BA. Rapid production of [9R]BA from [8-<sup>14</sup>C]BA has also been recorded in flower stems of *Dianthus caryophyllus* (VAN STADEN, BAYLEY, UPFOLD & DREWES, 1990). However, no [7G]BA formation was evident in any floral parts, even after 12 days. This report contrasts

Table 6.1 Radioactivity attributable to BA and its metabolites in extracts of *D. zeyheri* shoot apex-derived suspension cultures supplied with  $^{14}\text{C}$ BA in the presence of high ( $4 \text{ mg } \ell^{-1}$ ) and low ( $2 \text{ mg } \ell^{-1}$ ) levels of auxin.

Incubation hours)	2,4-D ( $\text{mg } \ell^{-1}$ )	Radioactivity co-eluting with standards (% of total extracted)														
		B <sup>w</sup>	Ade	Ado	[3G]BA	[7G]BA	[9G]BA	[9R-MP]BA	[9R]BA	BA	C <sup>w</sup>	D <sup>w</sup>	E <sup>w</sup>	Oxidation Products <sup>x</sup>	Active Pool <sup>y</sup>	N- conjugates <sup>z</sup>
5	2	0.63	0.76	0.90	0.21	11.63	1.11	1.13	26.54	56.78	-	0.14	0.16	2.30	84.45	13.87
	4	0.10	0.95	1.24	0.02	13.83	4.24	0.88	24.10	54.21	-	0.42	0.02	2.28	79.19	18.95
	2	0.39	0.76	0.27	0.94	43.66	8.29	6.98	28.27	9.99	-	0.45	0.01	1.42	45.24	52.89
	4	1.16	0.07	0.55	2.23	38.91	6.01	2.19	20.77	26.78	0.99	0.21	0.17	1.78	49.74	47.15
3	2	0.45	0.17	2.00	0.74	56.71	0.28	0.33	36.09	2.86	-	0.39	-	2.62	39.28	57.73
	4	0.86	0.10	2.01	0.66	73.50	0.21	0.33	17.27	4.70	0.02	0.37	-	2.96	22.30	74.37
1	2	1.40	0.24	0.90	2.02	73.28	3.57	2.22	12.98	2.94	-	0.40	-	2.55	18.14	78.87
	4	1.95	0.43	1.40	1.48	82.77	1.30	0.58	4.94	4.73	-	0.43	-	3.78	10.25	85.55

known compound

al radioactivity associated with B, Ade and Ado, products of oxidation

al radioactivity associated with base, nucleoside, and nucleotide

al radioactivity associated with N-glucosides

Table 6.2 Radioactivity attributable to BA and its metabolites in extracts of *D. zeyheri* seed-derived suspension cultures supplied with [8-<sup>14</sup>C]BA in the presence of high (4 mg ℓ<sup>-1</sup>) and low (2 mg ℓ<sup>-1</sup>) levels of auxin.

Incubation hours)	2,4-D (mg ℓ <sup>-1</sup> )	Radioactivity co-eluting with standards (% of total extracted)														
		B <sup>w</sup>	Ade	Ado	[3G]BA	[7G]BA	[9G]BA	[9R- MP]BA	[9R]BA	BA	C <sup>w</sup>	D <sup>w</sup>	E <sup>w</sup>	Oxidation Products <sup>x</sup>	Active Pool <sup>y</sup>	N- conjugation <sup>z</sup>
5	2	1.01	0.89	0.71	0.41	3.29	0.34	8.76	23.74	58.57	-	0.94	1.36	2.61	91.07	4.04
	4	0.03	1.76	1.74	-	3.70	1.38	5.27	32.71	49.71	-	0.33	0.26	6.64	87.69	5.08
5	2	0.89	0.45	1.32	0.25	19.66	3.05	7.98	50.76	14.82	0.04	0.7	0.09	1.66	73.56	22.96
	4	0.87	0.34	0.96	0.49	20.11	2.42	8.50	48.77	17.15	-	0.39	-	2.17	74.42	23.02
5	2	0.63	0.46	2.17	1.22	22.83	0.18	1.43	69.15	1.71	-	0.19	0.04	3.25	72.29	24.23
	4	0.60	0.19	1.13	0.59	29.23	2.04	1.14	62.06	2.68	0.02	0.32	-	1.92	65.88	31.86
5	2	1.61	0.14	1.59	0.59	29.56	1.95	1.97	61.11	1.24	0.02	0.23	-	3.34	64.32	32.1
	4	1.77	0.54	3.37	1.16	40.42	1.59	1.36	48.49	1.27	-	0.03	-	5.68	51.12	43.17

known compound

al radioactivity associated with B, Ade and Ado, products of oxidation

al radioactivity associated with base, nucleoside, and nucleotide

al radioactivity associated with N-glucosides

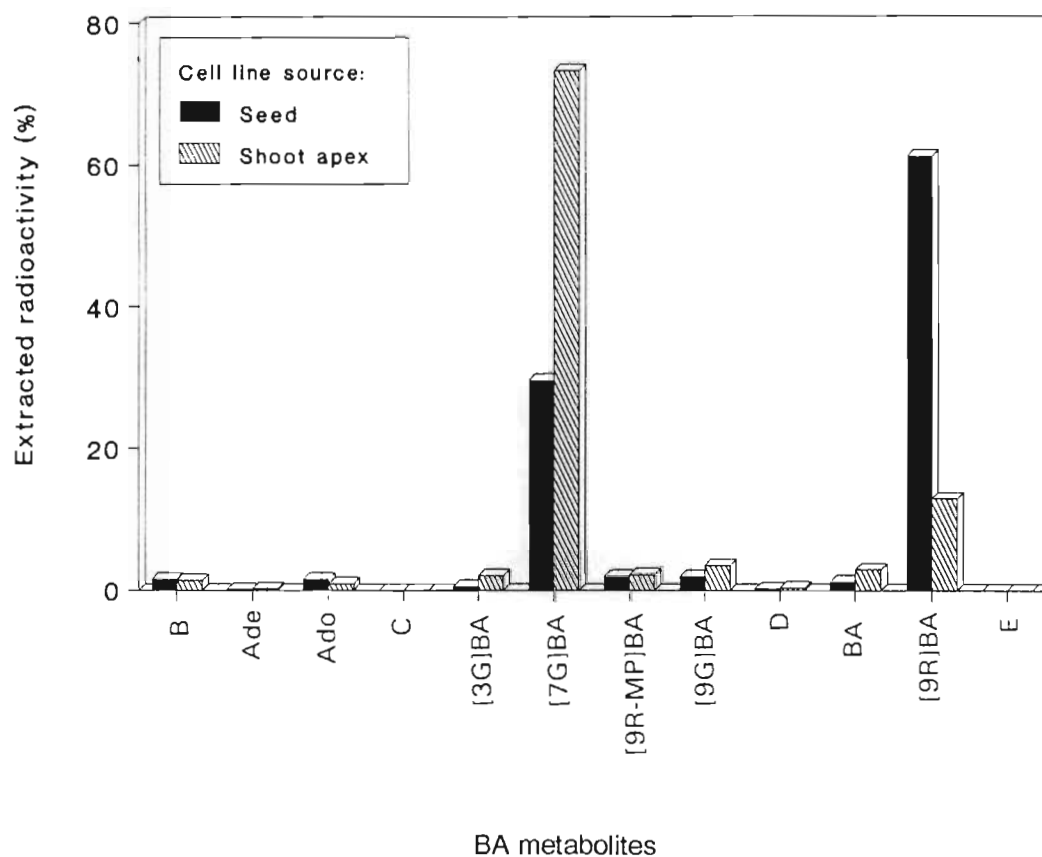


Figure 6.1 A comparison of the metabolism of  $[8-^{14}\text{C}]$ BA in two cell culture lines of *Dianthus zeyheri*. 2,4-D at  $2 \text{ mg } \ell^{-1}$  was included in the medium; incubation 48 hours.

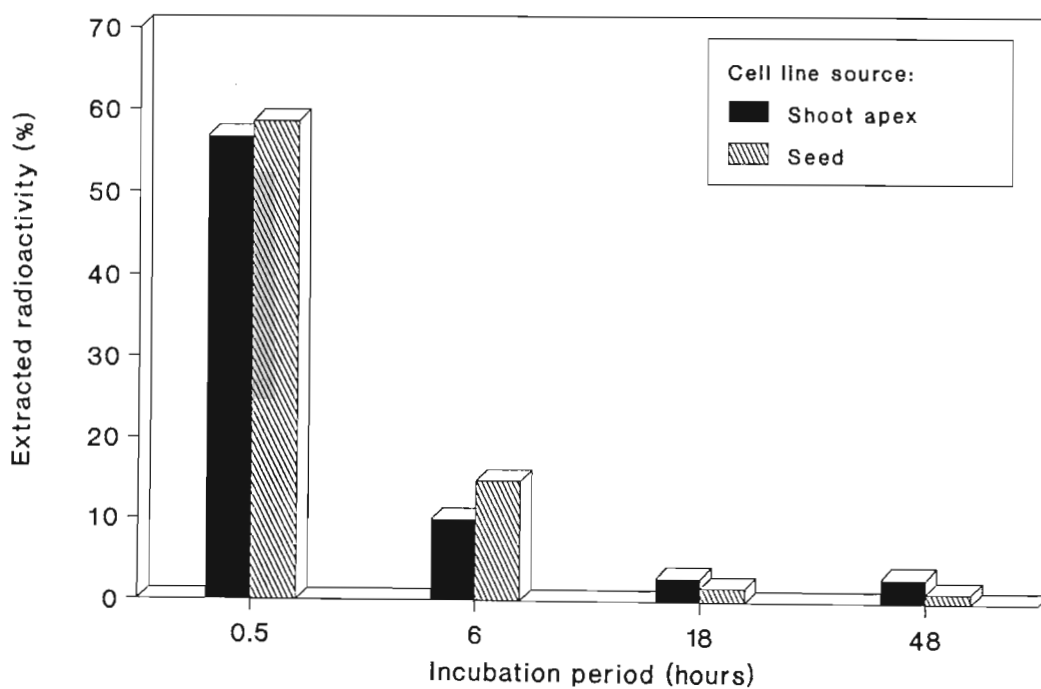


Figure 6.2 Rate of  $[8-^{14}\text{C}]$ BA utilisation by two cell culture lines of *D. zeyheri*. 2,4-D at  $2 \text{ mg } \ell^{-1}$  was included in the medium.

with a more recent investigation (UPFOLD & VAN STADEN, 1992) which identified [7G]BA as a prominent BA metabolite in the same carnation cultivar.

Metabolism in both cell lines showed, (Figures 6.3 and 6.4) with time, an overall reduction in the levels of active cytokinins (total radioactivity associated with the base, nucleoside and nucleotide). After 48 hours, most of the cytokinins in seed-derived cultures were still 'active' (Figure 6.5), whilst in the shoot-derived system, inactivated forms (mainly [7G]BA) predominated (Figure 6.6). Production of [7G]BA was mirrored by a concurrent reduction in levels of active cytokinin forms, in both cell lines, whether or not auxin levels were elevated.

Levels of detectable [9R-MP]BA were low after 48 hours. In contrast, after 6 hours, both culture lines produced substantially more of this minor metabolite (Figure 6.7). The significance of such transient occurrence is unknown. A similar pattern of 9-glucoside formation was also recorded (Figures 6.3 and 6.4), suggesting reversible sequestration of this 'stable' metabolite. Slow re-utilisation of N-glucosides has previously been recorded (GAWER, LALOUE, TERRINE & GUERN, 1977). The inherent stability of [7G]BA has been partly attributed to a resistance to degradative enzymes (LETHAM, TAO & PARKER, 1982).

### 6.3.2 Effect of hormonal interaction

The effect of auxin on BA metabolite formation in both seed and apex-derived lines was essentially similar after 48 hours incubation (Figures 6.8 and 6.9). Auxin promoted 7-glucosylation and a reduction in cytokinin activity (Figures 6.5 and 6.6). After 18 hours incubation with shoot-derived cultures (Figure 6.6), the effect of auxin was most pronounced, whilst in seed-derived cultures this effect gradually increased with time (Figure 6.5). Formation of [9R]BA and [7G]BA were at first concurrent, but after 18 hours the loss of active forms was accompanied by an increased production of [7G]BA (Figures 6.10 and 6.11).

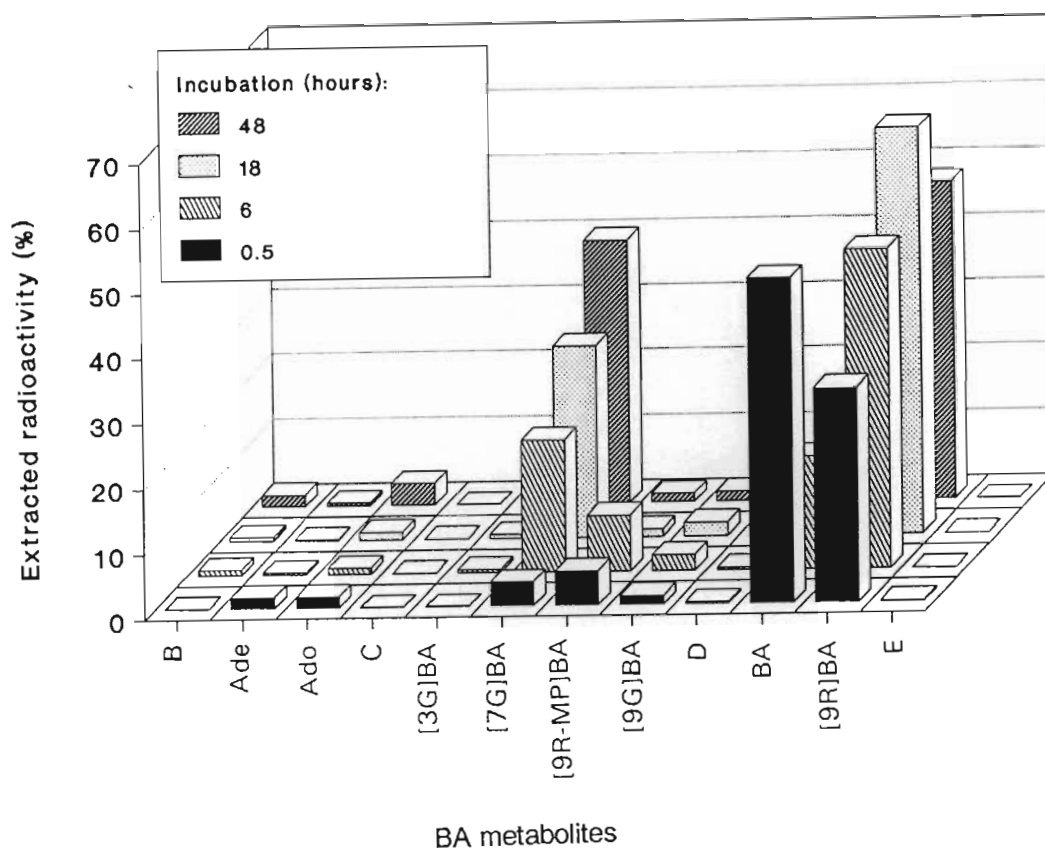


Figure 6.3 The metabolism of BA over 48 hours in seed-derived cell cultures. 2,4-D at  $4 \text{ mg l}^{-1}$  was included in the medium.

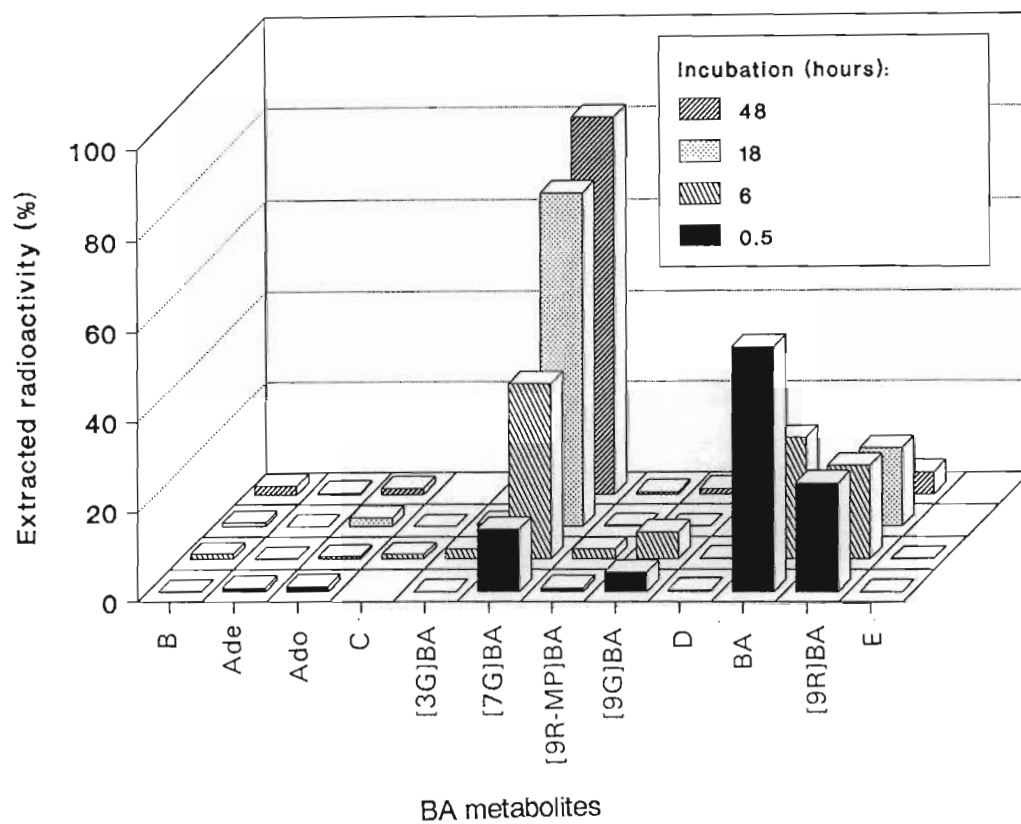


Figure 6.4 The metabolism of BA over 48 hours in apex-derived cell cultures. 2,4-D at  $4 \text{ mg l}^{-1}$  was included in the medium.

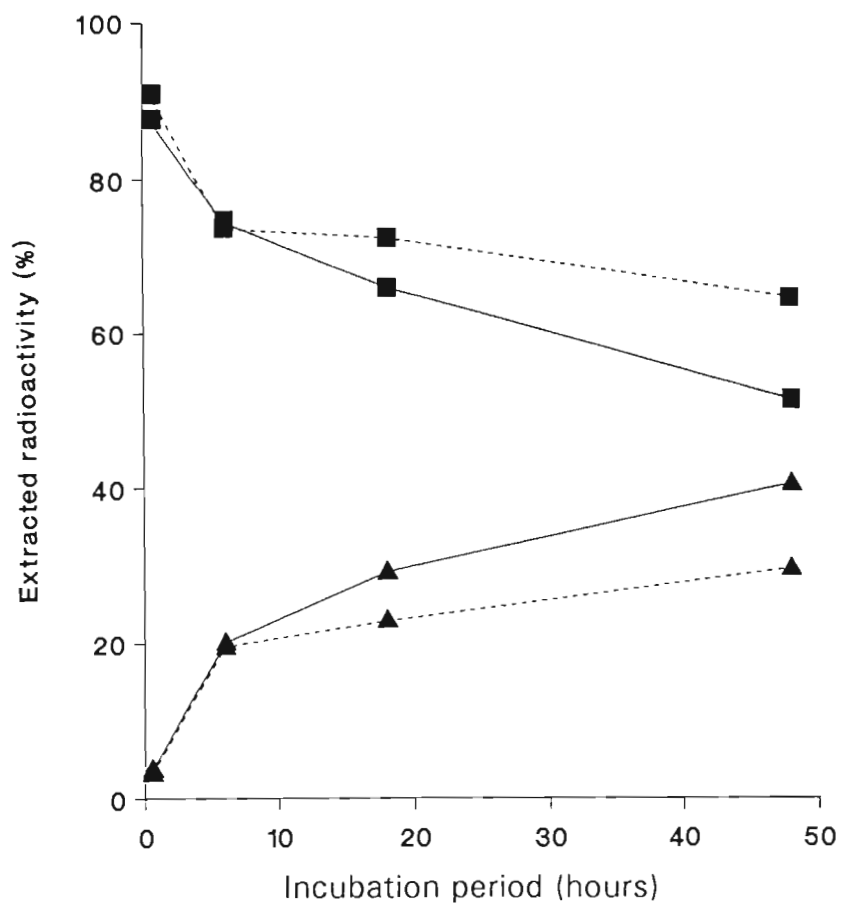


Figure 6.5 Levels of radioactivity associated with [7G]BA ( ▲ ) and active cytokinins ( ■ ) in seed-derived cultures of low ( ---- ) and high ( — ) auxin status.

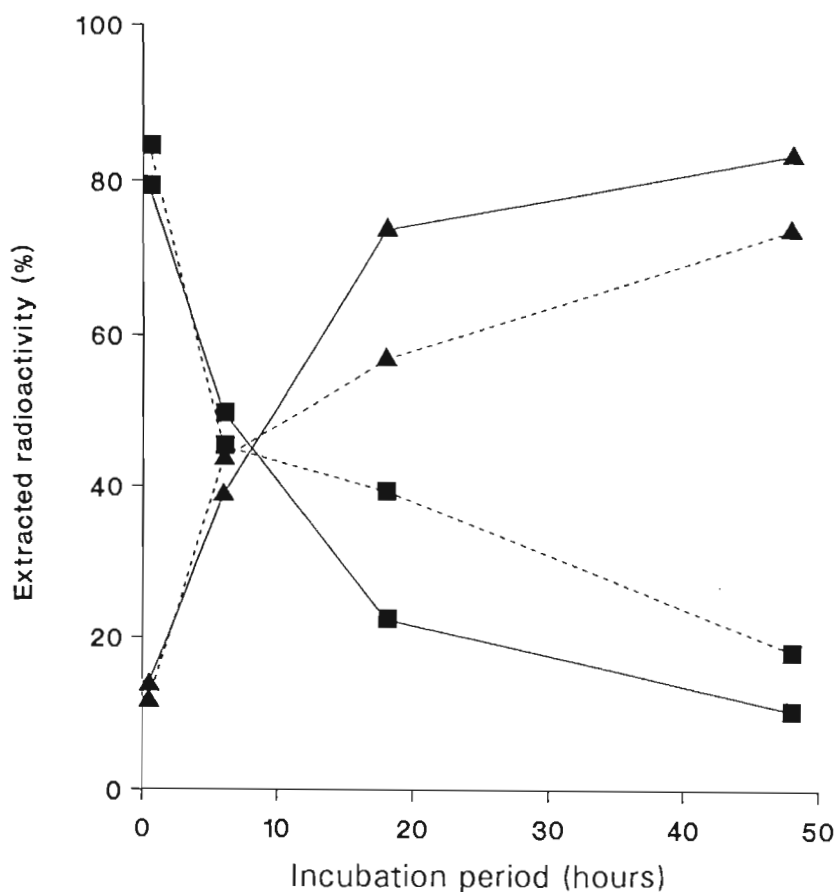


Figure 6.6 Levels of radioactivity associated with [7G]BA ( ▲ ) and active cytokinins ( ■ ) in shoot apex-derived cultures of low ( ---- ) and high ( — ) auxin status.

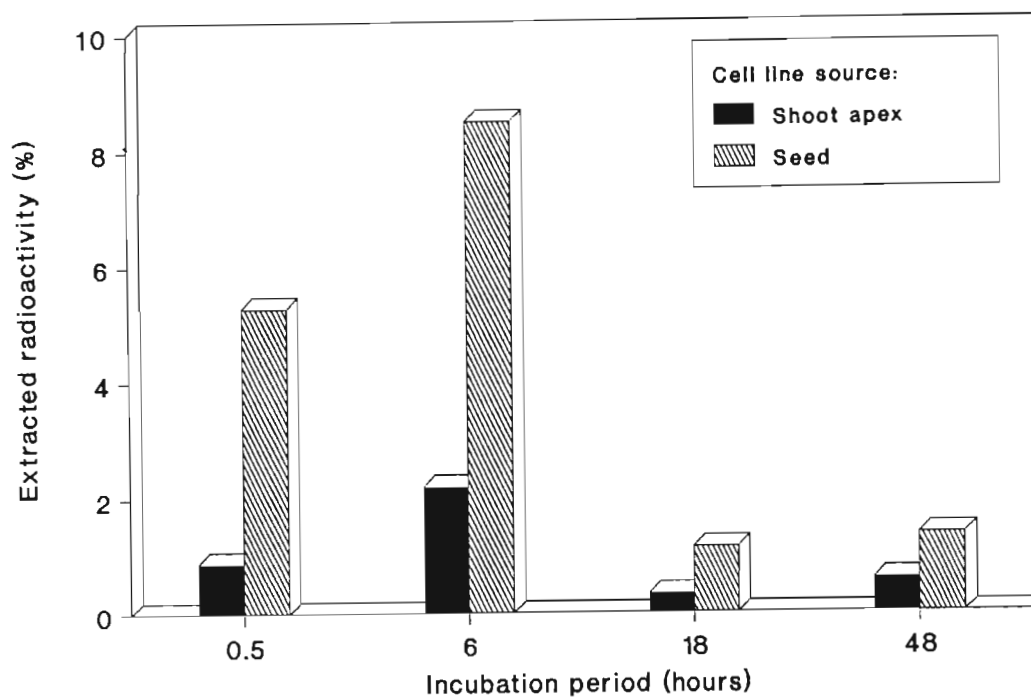


Figure 6.7 Radioactivity associated with the formation of [9R-MP]BA in seed and apex-derived cell culture lines over 48 hours. 2,4-D at  $4 \text{ mg l}^{-1}$  was included in the medium.

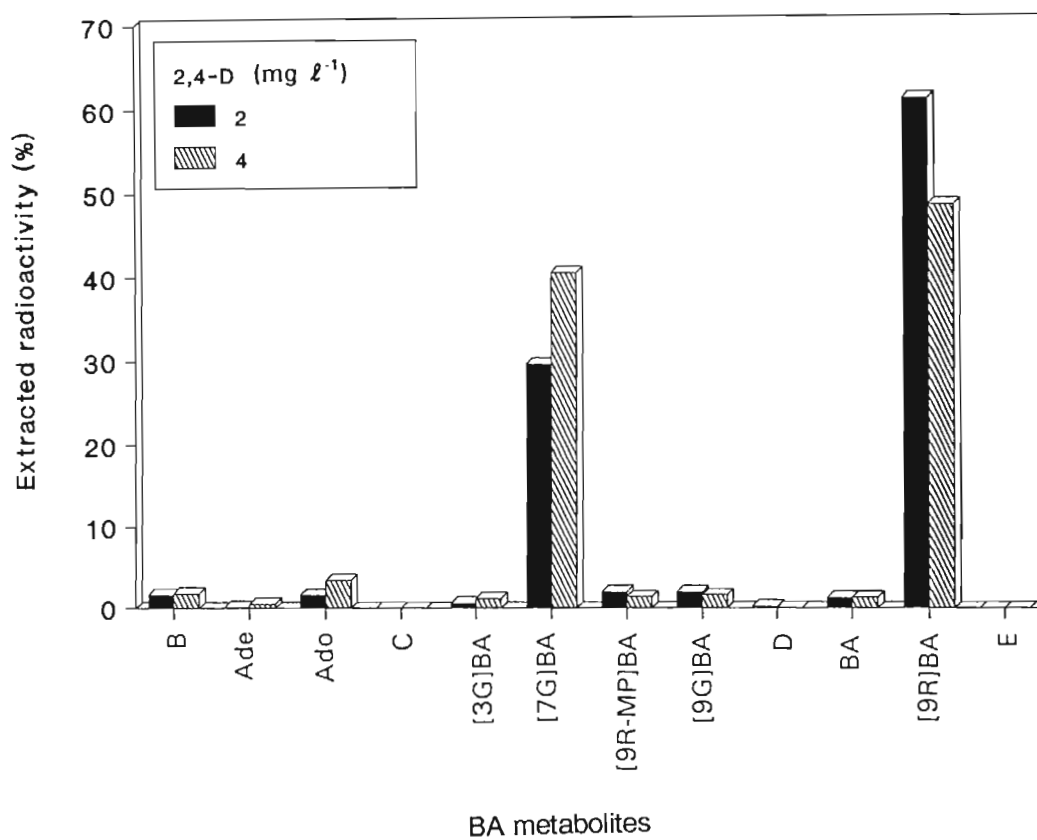


Figure 6.8 Effect of elevated auxin (2,4-D) levels on the metabolism of BA in seed-derived suspensions after 48 hours.

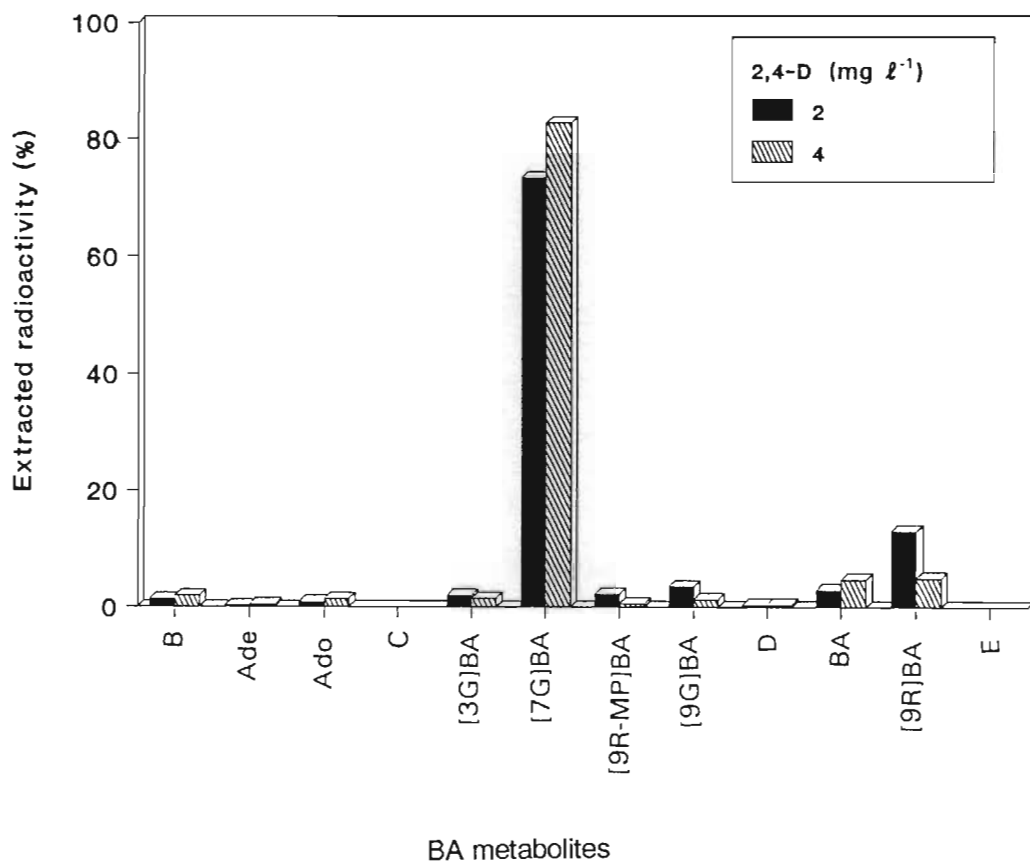


Figure 6.9 Effect of elevated auxin (2,4-D) levels on the metabolism of BA in apex-derived suspensions after 48 hours.

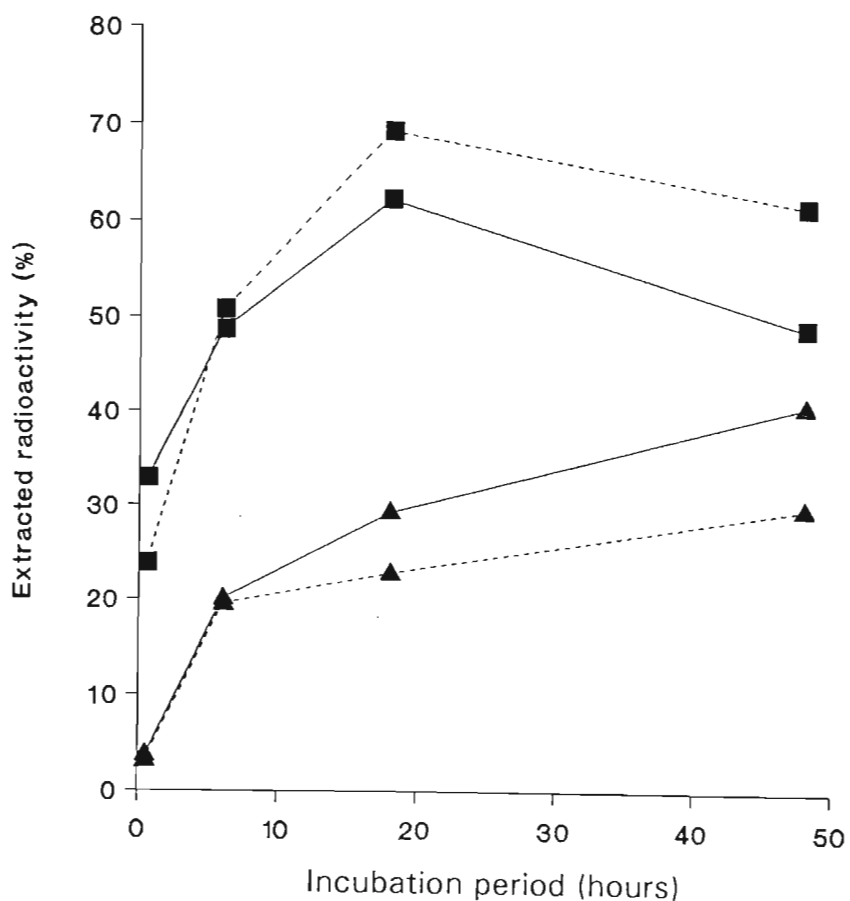


Figure 6.10 Levels of radioactivity associated with [7G]BA (▲) and [9R]BA (■) in apex-derived suspensions after 48 hours.

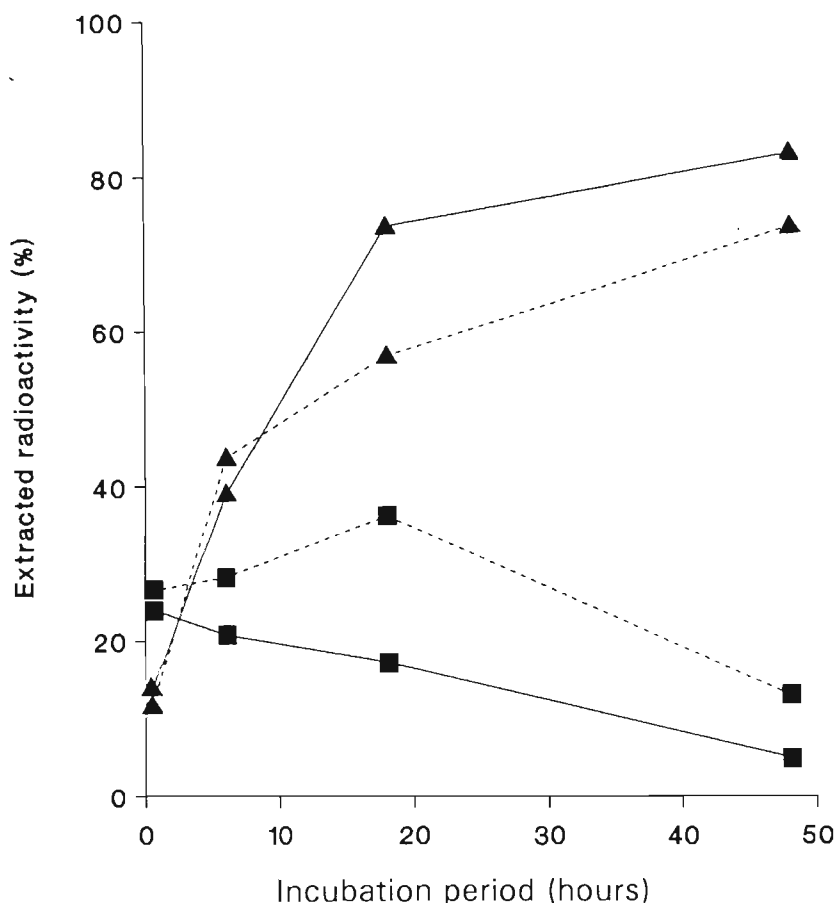


Figure 6.11 Levels of radioactivity associated with [7G]BA ( ▲ ) and [9R]BA ( ■ ) in shoot apex-derived cultures of low ( ----- ) and high ( \_\_\_ ) auxin status.

After 18 hours, levels of free BA represented less than 5% of the extractable metabolites (Figure 6.2; Tables 6.1 and 6.2), indicating that for 7-glucosylation to proceed, an alternative precursor source was required. Over this period, loss of 9-riboside appeared metabolically related to [7G]BA formation. If [9R]BA was not directly converted to [7G]BA, then conversion of BA to [7G]BA was likely a more rapid step than de-ribosylation of [9R]BA, for the levels of free base remained low (Tables 6.1 and 6.2). Prior to 18 hours, free base and mono-nucleotide were the likely substrates for [7G]BA formation.

Other than an effect on 7-glucoside formation, 2,4-D promoted [9R]BA formation in seed cultures after 30 minutes (Figure 6.10). This confirms the earlier findings of VANKOVA, GAUDINOVA, KAMINEK and EDER (1992) for transient auxin-induced promotion of cytokinin riboside in immobilised tobacco cells.

The effect of 2,4-D on catabolic processes appeared slight. Although small quantities of Ade, Ado, and unknown compound B were detected in both culture lines, only very slight auxin-promoted elevation of oxidation products was noted (Tables 6.1 and 6.2).

#### 6.4 Discussion

Inhibitors of cytokinin 7-glucosylation have previously been identified (ELLIOTT & MURRAY, 1975; HOCART, LETHAM & PARKER, 1991; TAO, LETHAM, HOCART & SUMMONS, 1991), but not promoters of this process. TAO, LETHAM, HOCART and SUMMONS (1991) found that auxins did not affect formation of [7G]Z from [9R]Z in radish cotyledons. This contrasts with the present study where auxin appeared to exert an effect on glucosylation processes in two cell culture lines of *Dianthus zeyheri*. The formation of the 7-glucoside was promoted in systems of higher auxin status, with a concurrent reduction in those metabolites associated with the active cytokinin pool (Figures 6.5 and 6.6). As [7G]BA is considered an inactivated form of BA (LALOUE, PETHE-TERRINE & GUERN, 1981; MCGAW & HORGAN, 1985), auxin is seen here to promote cytokinin inactivation. In the only previous report on auxin-affected cytokinin metabolism in *Dianthus* (FEATONBY-SMITH, VAN STADEN & COOK, 1987), ovaries inactivated applied BA through oxidative catabolism rather than N-conjugation. IAA had a transient promotive effect on oxidative processes, which was expressed in the rate of degradation.

After 18 hours, in all systems investigated, a reduction in [9R]BA levels appeared metabolically linked to biosynthesis of the 7-glucoside. Formation of [7G]BA is considered to occur via the base (LALOUE, 1977), although earlier kinetic studies by FOX, CORNETTE, DELEUZE, DYSON, GIERSAK, NIU, ZAPATA and McCHESNEY (1973) indicated that the nucleotide [9R-MP]BA was the immediate precursor. A lowering of [9R-MP]BA levels between 6 and 18 hours (Figures 6.3 and 6.4), with a concomitant increase in [7G]BA levels supports this view. Formation of 7-glucoside has been reported after exogenous application of cytokinin riboside to radish cotyledons (TAO, LETHAM, HOCART & SUMMONS, 1991). Conversion of Ade to [7G]Ade in this system indicated low substrate specificity of cytokinin-7-glucosyl transferase. However, *in*

*in vitro* specificity studies have shown that the 9-riboside is not a substrate for cytokinin-7-glucosyl transferase extracted from radish cotyledons (ENTSCH, PARKER, LETHAM & SUMMONS, 1979). Deribosylation to the base is viewed as the metabolic step necessary prior to 7-glucosylation (TAO, LETHAM, HOCART & SUMMONS, 1991), and is effected through the action of cytokinin nucleosidase (ROLLE & CHISM, 1989). The possibility of nucleotides accepting the glucose residue was not considered during *in vitro* investigations (ENTSCH, PARKER, LETHAM & SUMMONS, 1979). Assuming that in *D. zeyheri*, BA was the immediate precursor, it would appear that as nucleoside formation was quickly initiated in both systems, especially in that derived from seed (Figure 6.10), [9R]BA was later re-converted to BA before rapid 7-glucosylation.

This investigation has dealt with two cytokinin-autonomous cell culture lines which have been exogenously supplied with a synthetic cytokinin. Each line has produced a similarly wide spectrum of metabolites (Figure 6.1), each of which have been assigned roles in plants (LETHAM & PALNI 1983; refs within). It is to be expected that established 'cell machinery' should effect metabolic conversions of exogenously supplied substances. The generalised roles assigned to such metabolites may therefore be reasonably questioned, since the suspension cultures used in the present investigation showed no obvious requirement for supplied BA. The observation that a broad spectrum of metabolites were produced by these cultures was interpreted in accordance with generally accepted views on metabolite roles. It therefore seems reasonable to suggest that the biologically active nucleoside and nucleotide were produced by these cytokinin-autonomous systems to act as precursors of [7G]BA, a detoxification form.

Both synthetic and natural auxins have been shown to affect activities of enzymes of cytokinin metabolism. The extent to which this occurs appears to depend more on the type of plant system under investigation than the particular enzyme of interest. As cytokinin oxidase appears to utilise both BA (LALOUE & PETHE, 1982; FORSYTH & VAN STADEN, 1986) and natural cytokinins (WHITTY & HALL, 1974) as a substrate in a range of plant systems, this enzyme has been recognised as a good candidate for regulation by specific inhibitors, including auxins (PARKER, ENTSCH & LETHAM, 1986).

In this study, undifferentiated carnation cells produced more 7-glucoside at the higher auxin concentration tested ( $4 \text{ mg l}^{-1}$ ). The auxin effect could be mediated through the enzyme cytokinin-7-glucosyl transferase, although the level of control is unknown; auxin may stimulate enzyme induction or serve as an enzyme co-factor. Observed auxin/cytokinin interaction may be indirect. An effect of auxin on ethylene (GUNSÉ & ELSTNER 1992; LIU & REID, 1992), which may mediate cytokinin metabolic changes (BOLLMARK & ELIASSON, 1990; GROSSMANN, SIEFERT, KWIATKOWSKI, SCHRAUDNER, LANGEBARTELS & SANDERMANN, 1993) is one consideration (FEATONBY-SMITH, VAN STADEN & COOK, 1987). That such a view is perhaps over-simplistic is indicated by several reports on promotion of ethylene production by BA (BERTELL & ELIASSON, 1992; PANIZZA, MENSUALI-SODI & TOGNONI, 1993).

Alternative inactivation mechanisms appear, at least in *Dianthus zeyheri* cell cultures, to remain largely unaffected by the auxin 2,4-D. Accordingly, further inactivation of the N-conjugates through oxidation (LETHAM, TAO & PARKER, 1982; PALNI, PALMER & LETHAM, 1984) was not evident.

#### 6.4.1 Attempted identification of 'Compound C'

The current findings have been compared with the results of WOOLLEY and WAREING (1972). In *Solanum andigena*, formation of 'Compound C' was promoted in tissues with elevated levels of IAA. As this unidentified compound showed activity in the soybean callus bioassay, it is likely that 'Compound C' is not [7G]BA, which is the least active glucoside in this particular bioassay (VAN STADEN & DREWES, 1992). Authentic 7-glucoside was run on cellulose TLC plates using the two solvent systems described by WOOLLEY and WAREING (1972). With distilled water (solvent B) as eluent, authentic [7G]BA, [7G]BA from *Dianthus*, and 'Compound C' had co-incident  $R_f$  values. Using solvent A, authentic [7G]BA ( $R_f$  0.57-0.67) co-eluted with [7G]BA from *Dianthus*, but not with 'Compound C'. The 7-glucoside from *Dianthus* and 'Compound C', however, partially co-eluted in this system. In considering the non-identification of 'Compound C' by WOOLLEY and WAREING (1972), it is noteworthy that the existence of cytokinin 7-glucosides was unknown at that time. It was only later in the

same year as the *Solanum andigena* report that DELEUZE, McCHESNEY and FOX (1972) reported on its occurrence as a metabolite (albeit erroneously as the furanosyl form), notably in another *Solanum* species. The production of [7G]BA as a major metabolite in *Solanum tuberosum* Poepp. ex Walp. was confirmed by LETHAM, GOLLNOW and PARKER (1979). Although still unclear, the possibility should be raised that WOOLLEY and WAREING (1972) described auxin-promoted 7-glucoside formation in *Solanum* tissue some two decades earlier than this report.

Whether promotive (VANKOVA, GAUDINOVA, KAMINEK & EDER, 1992) or inhibitory (HANSEN, MEINS & AEBI, 1987), auxin effects on cytokinin metabolism appear transient. In *Dianthus* apex cultures, the auxin effect was greatest after 18 hours (at 48 hours less difference in 7-glucoside levels was evident). However, seed-derived cultures show an increasing effect of auxin on glucosylation up to 48 hours. Such transient effects may allow for induction of certain developmental processes whilst preventing long term hormonal imbalances in plant tissues (VANKOVA, GAUDINOVA, KAMINEK & EDER, 1992). These authors considered that the restoration of an hormonal balance may be required for further development of initiated structures. In modifying the metabolism of BA with 2,4-D, the ratio of active BA to active 2,4-D may have altered in favour of a rise in the auxin/cytokinin ratio. The extent to which auxin stimulates N-glucosylation will in itself depend on the rate of auxin metabolism within the tissue. Only after the effect of BA on 2,4-D metabolism (activation/inactivation) (MONTAGUE, ENNS, SIEGEL & JAWORSKI, 1981) is determined in the same system will the overall ratio of active cytokinin to active auxin be known. At this point the biological significance of auxin/cytokinin ratios may be clarified. Double-labelling ([8-<sup>14</sup>C]BA and [<sup>3</sup>H]IAA) experiments (e.g. PEETERS, GERARDS, BARENDSE & WULLEMS, 1991) have proven highly informative. Recently, changes in the BA/auxin ratio have been shown to affect the activity of enzyme systems (VAZQUEZ-FLOTA, QUIROZ, SCORER & LOYOLA-VARGAS, 1989). Such an influence could account for the transient nature of auxin effects, given the potential for activity feedback on the enzymes of cytokinin metabolism. In a contrasting report, PEETERS, GERARDS, BARENDSE and WULLEMS (1991) could not relate the auxin/cytokinin ratio to a physiological response. These authors found these two hormone groups to exert their effect independently; interaction

occurred beyond the level of hormone metabolism, 'in the cascade of sequential processes that they initiate'. This is in agreement with the signal transduction model proposed by GUNSE and ELSTNER (1992). VAN DER KRIEKEN, CROES, BARENDSE and WULLEMS (1988) had earlier noted the absence of interaction between BA and NAA in flower bud initiation in tobacco and concluded that these plant growth regulators affect different processes at the molecular level. Future studies in this field need to further consider the influence of non-hormonal factors (KVAALLEN & ERNSTSEN, 1993) on BA/2,4-D ratios, if suitable controls are to be applied.

## CHAPTER 7

### THE EFFECT OF A CYTOKININ 7-GLUCOSYLATION INHIBITOR, DIFFERENT AUXIN TYPES, AND AUXIN/CYTOKININ RATIOS ON THE METABOLISM OF 6-(BENZYLAMINO)PURINE IN APEX-DERIVED SUSPENSIONS OF *DIANTHUS ZEYHERI*

#### 7.1 The effect of papaverine on BA metabolism

##### 7.1.1 Introduction

Research with transgenic callus (McGAW, HORGAN, HEALD, WULLEMS & SCHILPEROORT, 1988) has had interesting implications for auxin/cytokinin interactive studies. Higher levels of cytokinin were detected in transformed tobacco tissue (lacking functional T-DNA auxin-synthesising genes) than in wild-type callus. In tissues with inactivated auxin genes, fewer cytokinin products of  $N^6$ -sidechain cleavage were detectable. As levels of 7-glucoside increased substantially, it was considered likely that 7-glucosylation was promoted as an alternative pathway for cytokinin inactivation in tobacco tissues, when functional genes were not present to promote oxidation. Promotion of oxidative catabolism would be expected when 7-glucosylation (or other N-conjugative) processes are inhibited. ZHANG, LETHAM, WONG, NOODÉN and PARKER (1987) found this to be so when alanylation inhibitors synchronously promoted the conversion of BA to Ade and Ado in soybean leaves. Inhibitors of 7-glucosylation have previously been identified (ELLIOTT & MURRAY, 1975; PARKER, ENTSCH & LETHAM, 1986), and include the substituted xanthine papaverine. Although shown to inhibit [7G]BA formation, papaverine preferentially inhibited formation of the 9-glucoside in radish cotyledons (TAO, LETHAM, HOCART & SUMMONS, 1991). Papaverine has a fused bicyclic ring system and a 'cytokinin type' sidechain (TAO, LETHAM, HOCART & SUMMONS, 1991). Total uptake of BA by *Amaranthus* seedlings was reduced by 52% when plants were synchronously provided with 5 mM aminophylline (ELLIOTT & MURRAY, 1975). Glucosyl-derivative ([7G]BA and [9G]BA) formation was inhibited by 86%. However, *in vitro* studies with cytokinin-7-glucosyl transferase (from radish

cotyledons) could not confirm this result (PARKER, ENTSCH & LETHAM, 1986). Aminophylline (5 mM) similarly reduced uptake and the extent of alanylation of BA in diced soybean callus (ELLIOTT & THOMPSON, 1982). In both systems, more radioactivity was found associated with the active forms (BA and [9R]BA), but strangely, cytokinin action (induction of betacyanin synthesis and soybean callus growth) was reduced. These results lead ELLIOTT and THOMPSON (1982) to question the relationship between cytokinin action and supposed metabolite roles.

Such inhibitors were considered by ENTSCH, PARKER, LETHAM and SUMMONS (1979) to merit study as 'a stable, effective, and specific inhibitor *in vitro* could be a valuable physiological tool and a means of elevating endogenous free cytokinin levels by suppressing formation of the very weakly active 7-glucosides.' Cytokinin-7-glucosyl transferase was most effectively inhibited by cytokinin analogues which acted competitively (PARKER, ENTSCH & LETHAM, 1986). Auxins as inhibitors of glucosylation were not considered in these early *in vitro* studies. In the current investigation, papaverine was supplied to apex-derived *Dianthus zeyheri* cells to determine the effect that inhibition of a major (N-conjugative) metabolic pathway has on the fate of exogenously supplied BA. The auxin 2,4-D, a promoter of 7-glucosylation of BA in this system (Chapter 6), was also supplied simultaneously with papaverine in an attempt to counteract expected inhibition.

### 7.1.2 Materials and methods

#### **7.1.2.1 Chemicals**

Papaverine (6,7-dimethoxy-1-veratryl-isoquinoline) hydrochloride was supplied by Sigma chemicals.

#### **7.1.2.2 Plant materials**

Stock cultures of shoot-apex derived suspensions were established and maintained on a MURASHIGE and SKOOG (1962) basal medium supplemented with 2,4-D at a

concentration of  $2 \text{ mg } \ell^{-1}$ . Stock cultures were incubated in 500 ml Erlenmeyer flasks under low intensity white fluorescent lights providing  $1 \mu\text{mol m}^{-2} \text{ s}^{-1}$  with a 16 hour light regime.

#### ***7.1.2.3 Papaverine and plant growth regulator application***

The [8- $^{14}\text{C}$ ]BA (0.0051 Mbq in  $4 \mu\text{l}$  methanol; specific activity  $2.04 \text{ MBq mmol}^{-1}$ ) was added to 15 ml suspension cultures in 100 ml Erlenmeyer flasks. The final concentration of 2,4-D in each 15 ml replicate of cell culture was adjusted to be either  $2 \text{ mg } \ell^{-1}$  or  $4 \text{ mg } \ell^{-1}$ , using  $100 \text{ mg } \ell^{-1}$  stock. In each sample, non-labelled BA (at  $2 \text{ mg } \ell^{-1}$ ) was added, again from  $100 \text{ mg } \ell^{-1}$  stock. Papaverine ( $40 \text{ g } \ell^{-1}$  aqueous stock) was supplied to culture media such that final concentrations were 0.1, 1, 5, or 10 mM. This range was selected on the basis of an earlier report (TAO, LETHAM, HOCART & SUMMONS, 1991) on inhibition in radish cotyledons. Auxin, un-labelled BA, and papaverine stock solutions were all filter-sterilised. Three replicates were established for each variable considered. After treating the cell suspensions, flasks were incubated under identical conditions to those of the stock cultures, except that a continual photoperiod was now maintained.

#### ***7.1.2.4 Extraction and purification of BA metabolites***

After incubating for 48 hours, a period suitable for substantial conversion of BA to its 7-glucoside (Chapter 6.3), samples were filtered through Whatman N<sup>o</sup> 1 filter paper. Filtrate volumes were recorded, and a 1 ml sub-sample of this incubation medium removed. Quantitation of the radioactivity associated with this fraction was achieved after adding 3 ml Ready Value. Cells were rinsed with 5 ml distilled water. Both cells and filter paper were immediately frozen in liquid nitrogen before freeze drying. Each sample was boiled in 20 ml 80% ethanol at  $80^{\circ}\text{C}$  for 15 minutes and left to stand for 24 hours. The extract was filtered through Whatman N<sup>o</sup> 1 filter paper to remove cell debris and the residue re-extracted in 3 ml cold 80% ethanol for ten minutes. Samples were fan dried at room temperature. Residues were re-suspended in 1.2 ml 80% methanol (HPLC grade) and filtered through a  $0.20 \mu\text{m}$  filter (DynaGard ME  $5.5 \text{ cm}^2$

hollow fibre syringe filter). Samples were reduced to dryness in a UNIVAPO 150H vacuum centrifuge and redissolved in 300  $\mu$ l 80% HPLC grade methanol immediately prior to HPLC analysis.

#### **7.1.2.5 HPLC analysis**

Aliquots of 100  $\mu$ l of each extract were injected onto a Supelcosil LC-18-DB column and separation effected as previously described (Chapter 3.3.3.3). Fractions (1 ml) were collected and the radioactivity quantitated in 3 ml Ready Value (Beckman) scintillation cocktail, using a Beckman LS 3801 scintillation counter. The detected peaks of radioactivity were compared with the retention times of authentic BA derivatives separated by HPLC. The total amount of radioactivity in each 100  $\mu$ l sub-sample was calculated, and from this the total radioactivity present in each cellular extract determined.

#### **7.1.2.6 Confirmatory techniques**

Individual radioactive peaks so determined were further investigated by treatment with HCl (FOX, DYSON, SOOD & McCHESNEY, 1972), alkaline phosphatase (MILLER, 1965), or re-chromatographed with authentic BA derivatives in a TLC system.

#### **7.1.2.7 TLC analysis**

Peaks of radioactivity detected after HPLC were subjected to TLC (Merck, Silica gel 60, F<sub>254</sub>, *n*-butanol:NH<sub>4</sub>OH:water (6:1:2) v/v upper phase) and the ten R<sub>f</sub> fractions counted for radioactivity. The results of TLC analyses and acid hydrolysis treatments of fractions were consistent with the initial tentative identifications based on HPLC retention times.

### **7.1.3 Results**

When supplied at concentrations of 5 mM or greater, papaverine severely inhibited

conversion of BA to all metabolites, with the exception of [9R]BA (Table 7.1; Figure 7.1). Slight promotion of Ade formation was observed (Figure 7.1). At these high levels, papaverine was clearly not a specific inhibitor of 7-glucosylation, but of both oxidative and N-conjugative pathways. At the 5 mM level, [7G]BA was only a minor metabolite (0.05%) whilst at 10 mM, production of this metabolite was inhibited altogether (Table 7.1). When papaverine was supplied at 5 mM, a doubling of the auxin concentration had little effect on metabolism of the cytokinin. Relative to the system of low auxin status, only slightly more catabolic and N-conjugative products were formed (Figures 7.2 and 7.3).

At all concentrations of papaverine less than 5 mM, the effect of the inhibitor was marginal, and in some cases even promotive (Figure 7.4). Papaverine supplied at 0.1 mM had no effect on 7-glucoside formation (Figure 7.4). When the associated auxin concentration was doubled, 7-glucoside formation increased from 15.6% to 23.3% (Figure 7.5), and levels of all oxidation products were elevated (Figures 7.3 and 7.5). Levels of both [9R]BA and [9R-MP]BA synchronously decreased. As papaverine at 0.1 mM had no effect on [7G]BA inhibition, increased auxin levels promoted 7-glucoside formation rather than counteract expected inhibition. A ten-fold increase in the papaverine level (to 1 mM) had only a slight inhibitory effect on 7-glucosylation (Table 7.1; Figure 7.4). At this higher concentration, all oxidation products increased (Figure 7.3), including unknown compound B (Figure 7.4), as did the level of radioactivity associated with [9G]BA (Figure 7.4). This suggests that oxidative and 9-glucosylation processes were alternative pathways of cytokinin inactivation to 7-glucosylation. However, these synchronous increases did not quantitatively parallel the reduction in 7-glucoside formation, but rather a decrease in the levels of active cytokinins (Table 7.1). Utilisation of the supplied base decreased as inhibitor concentrations increased (Table 7.1). Production of unknown compound B in vegetative and reproductive tissues of the cut carnation has recently been reported (UPFOLD & VAN STADEN, 1992).

Less radioactivity was recovered from viable systems than from cultures boiled immediately prior to [8-<sup>14</sup>C]BA application (Figure 7.6). At papaverine concentrations greater than 5 mM, nearly 1.5 times the amount of radioactivity was accounted for.

Figure 7.1 The effect of the xanthine derivative papaverine on BA metabolism in apex-derived cell suspensions.

Treatment			Ratio Auxin: Cytokinin	Radioactivity co-eluting with standards (% of total extracted)												
Papaverine (M)	2,4-D mg l <sup>-1</sup>	BA mg l <sup>-1</sup>		B <sup>w</sup>	Ade	Ado	[3G]BA	[7G]BA	[9G]BA	[9R- MP]BA	[9R]BA	BA	D <sup>w</sup>	Oxidation Products <sup>x</sup>	Active Pool <sup>y</sup>	N- conjugates <sup>z</sup>
	2	2	1:1	12.95	0.09	1.50	0.07	15.71	0.98	14.81	47.87	4.79	1.22	14.55	67.47	16.76
1	2	2	1:1	11.97	-	2.24	0.06	15.64	1.13	11.60	47.99	8.41	0.97	14.21	67.99	16.82
1	4	2	2:1	15.48	0.11	4.13	0.09	23.26	0.96	8.27	36.47	10.19	1.01	19.73	54.92	24.33
	2	2	1:1	16.38	0.19	4.39	0.13	13.18	5.26	2.96	43.21	12.99	1.30	20.95	59.16	18.57
	2	2	1:1	1.40	4.20	0.96	-	0.05	0.95	-	41.30	50.80	0.34	6.55	92.10	1.00
	4	2	2:1	2.50	4.45	1.47	-	0.11	1.42	-	43.52	46.22	0.30	8.42	89.74	1.53
	2	2	1:1	2.79	3.26	0.40	-	-	0.94	-	31.26	61.06	0.30	6.44	92.31	0.94

known compound

al radioactivity associated with B, Ade and Ado, products of oxidation

al radioactivity associated with base, nucleoside, and nucleotide

al radioactivity associated with N-glucosides

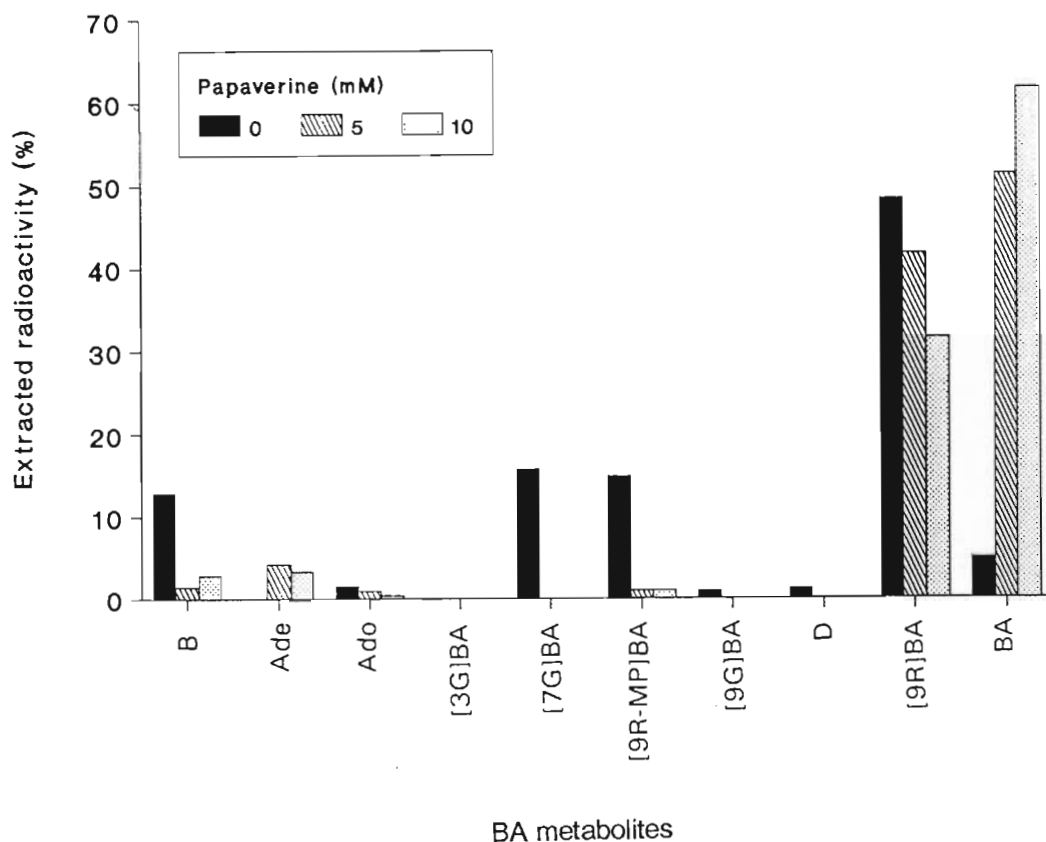


Figure 7.1 Effect of papaverine on BA metabolism in apex-derived cell suspensions. Papaverine (5 and 10 mM) exogenously supplied in the presence of 2,4-D ( $2 \text{ mg } \ell^{-1}$ ).

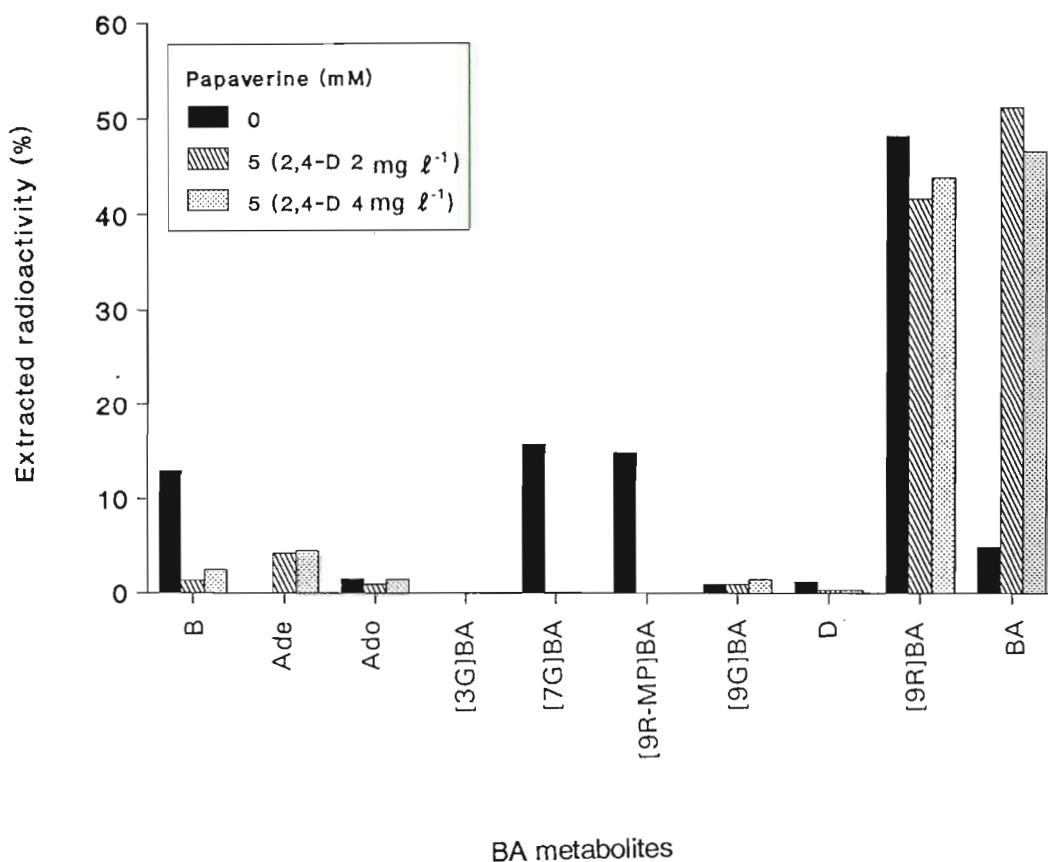


Figure 7.2 Effect of papaverine on BA metabolism in apex-derived cell suspensions. Papaverine (5 mM) exogenously supplied in the presence of both low ( $2 \text{ mg } \ell^{-1}$ ) and high ( $4 \text{ mg } \ell^{-1}$ ) concentrations of 2,4-D.

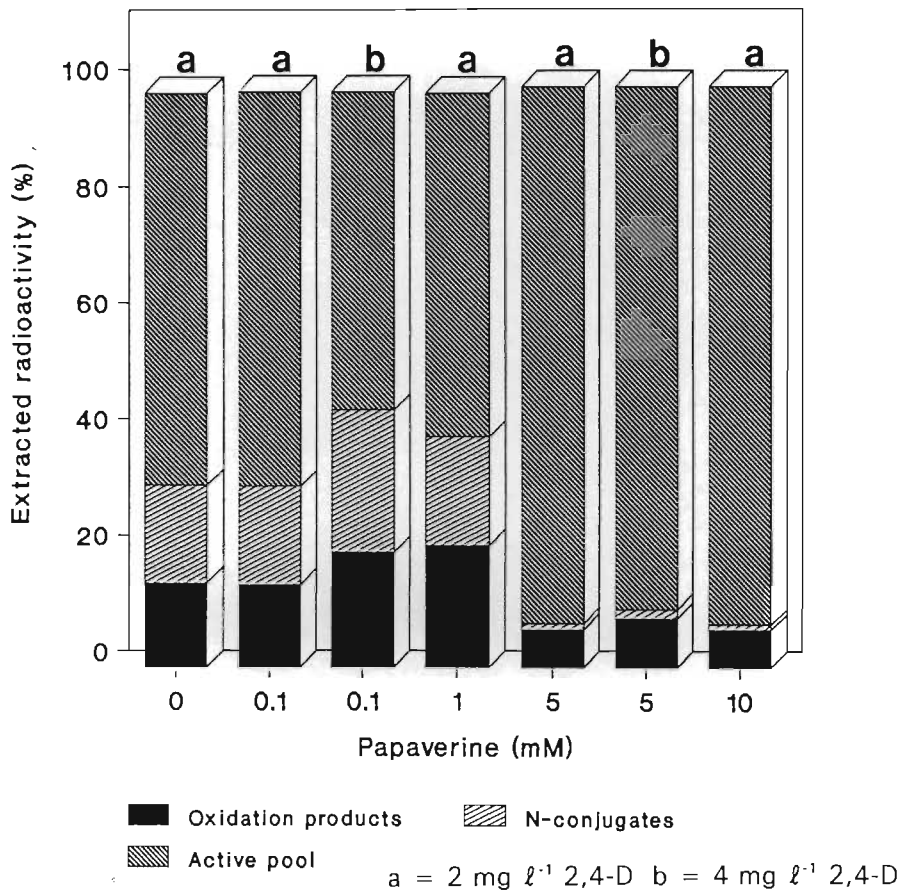


Figure 7.3 Effect of papaverine on radioactivity associated with active/inactive BA pools in apex-derived cell suspensions.

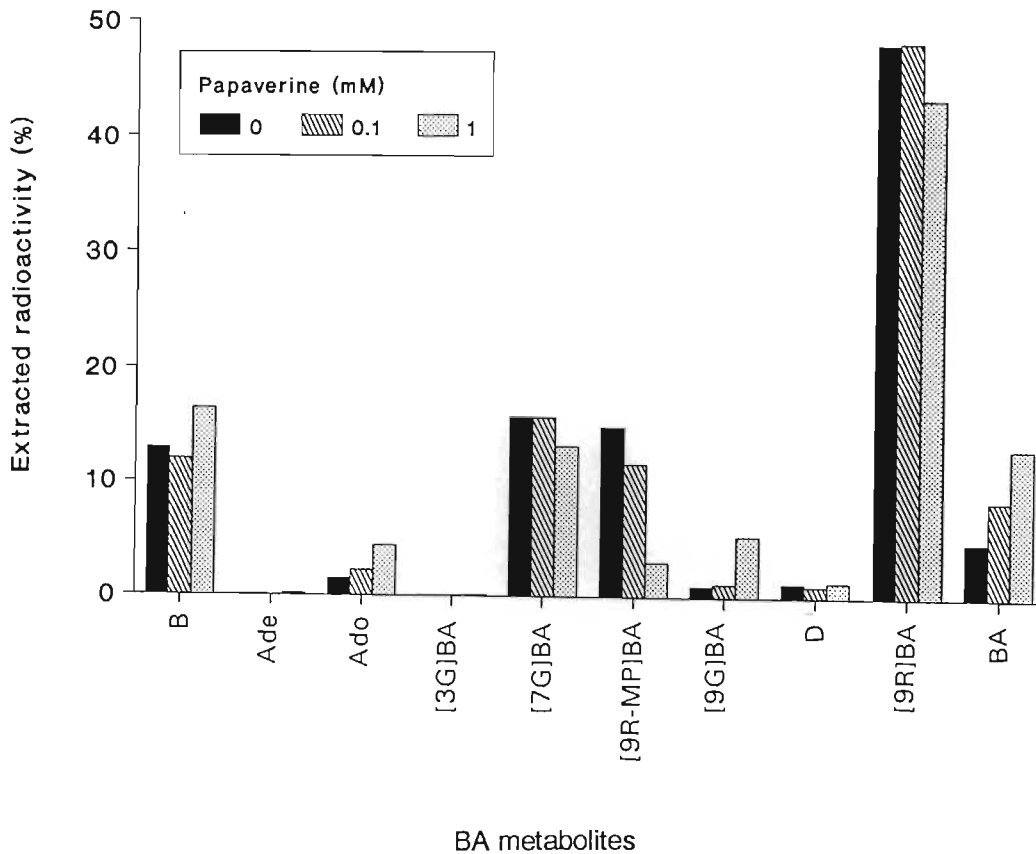


Figure 7.4 Effect of papaverine on BA metabolism in apex-derived cell suspensions. Papaverine (0.1 and 1 mM) exogenously supplied in the presence of 2,4-D ( $2 \text{ mg l}^{-1}$ ).

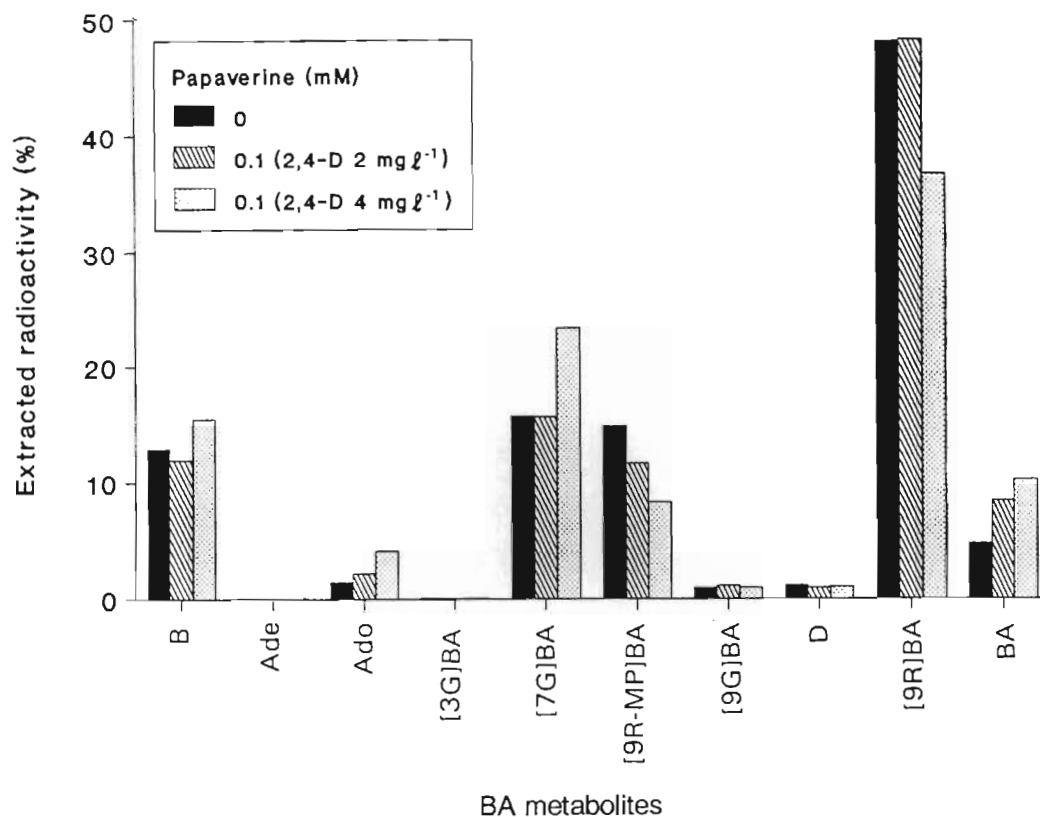


Figure 7.5 Effect of papaverine on BA metabolism in apex-derived cell suspensions. Papaverine (0.1 mM) exogenously supplied in the presence of both low (2 mg l<sup>-1</sup>) and high (4 mg l<sup>-1</sup>) levels of the auxin 2,4-D.

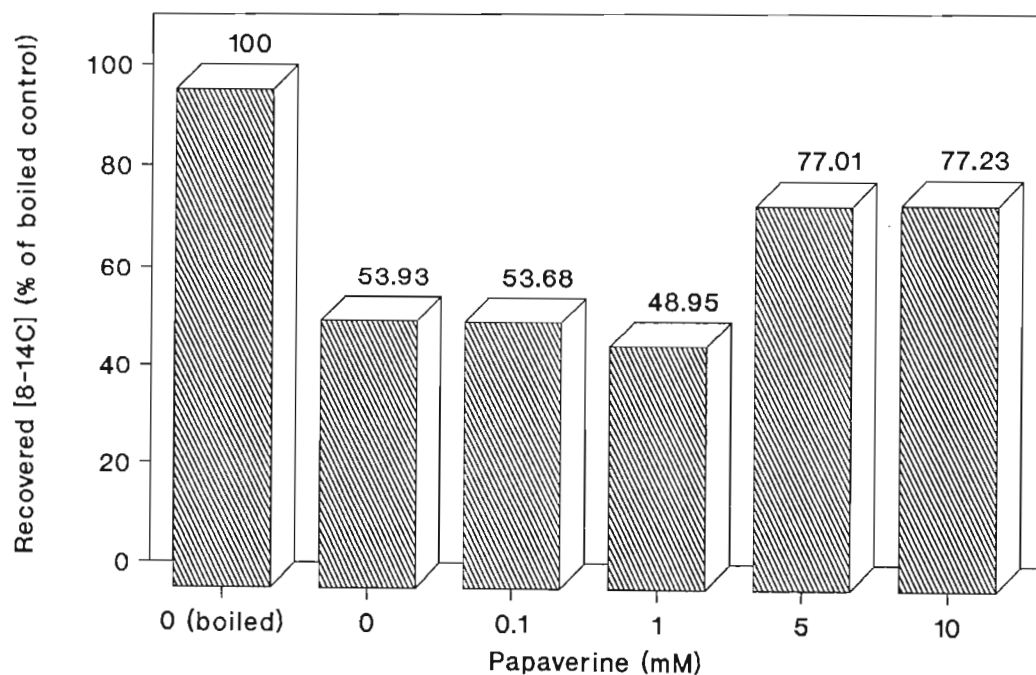


Figure 7.6 Recovery of applied radioactivity from papaverine treated apex cell cultures. Recovery expressed as a percentage of the (boiled) control.

From living cultures free of papaverine only half the amount of  $^{14}\text{C}$  was detected, relative to those which were boiled. BLAKESLEY (1991) similarly could not account for all the  $[8\text{-}^{14}\text{C}]\text{BA}$  initially applied to the experimental system. This author suggested that the observed difference was either due to incorporation of  $^{14}\text{C}$  into alcohol-insoluble compounds, or to its loss from the system as  $^{14}\text{CO}_2$ . In the current study, no attempt was made to identify the metabolites in the ethanol-insoluble fraction. Of the recovered radioactivity, living controls were found to have taken up most of the applied isotope; the majority of activity was associated with the cellular extract (Figure 7.7). In boiled systems, 74.7% of recovered radioactivity was extracted from the incubation medium after 48 hours. Similarly, the amount of radioactivity recovered from the incubation medium increased with the level of supplied papaverine (Figure 7.7). At papaverine concentrations of 5 mM or greater, more radioactivity was associated with the medium than in boiled cultures (Figure 7.7), although a higher overall recovery was obtained from the latter (Figure 7.6). TAO, LETHAM, HOCART and SUMMONS (1991) similarly found papaverine to reduce the uptake of BA by radish cotyledons (maximum reductions 45%). In considering that the observed effects on metabolism may have resulted from an effect on uptake, these authors reduced the supply of BA to mimic the inhibitor-induced uptake reduction. The distribution of radioactivity among metabolites was not appreciably altered, indicating that papaverine effects were not uptake-mediated.

#### 7.1.4 Discussion

Although a broad range of papaverine concentrations were investigated (100 fold increase), little information on inhibition of 7-glucosylation was obtained. The concentration values selected were either too low (1 mM) or too high (5 mM), resulting in either minimal inhibition, or general metabolic retardation (Figure 7.3). A papaverine concentration value of 2.5 mM in the presence of both low ( $2\text{ mg l}^{-1}$ ) and high ( $4\text{ mg l}^{-1}$ ) 2,4-D would likely have revealed more of alternative inactivation pathway switching in *Dianthus* cell cultures.

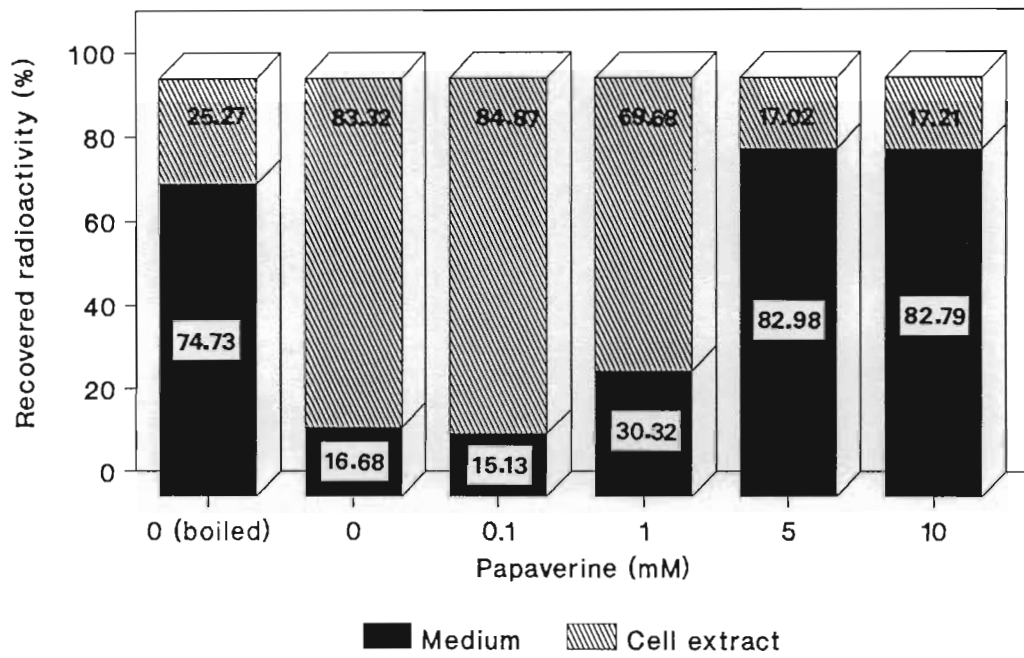


Figure 7.7 Distribution of radioactivity recovered from papaverine treated apex-derived cell cultures.

Promotion of compound B production by papaverine (1 mM) corresponds well with an earlier report (TAO, LETHAM, HOCART & SUMMONS, 1991). These authors found papaverine (5 mM) to similarly promote formation of an unidentified polar metabolite from BA, with a concomitant reduction in [7G]BA levels. Further, ABA-treated radish cotyledons inhibited conversion of [9R]Z to [7G]Z by 35%. This reduction was nearly balanced by promotion of formation of this polar metabolite, which on silica gel TLC had an  $R_f$  almost identical to [9R-MP]Z. Should this metabolite subsequently be identified as AMP, then TAO, LETHAM, HOCART and SUMMONS (1991) would have demonstrated the promotion of oxidative catabolism as an alternative inactivation mechanism to (blocked) N-glucosylation.

Values presented (Table 7.1) to describe the metabolic fate of [8- $^{14}$ C]BA are from a normalised data set based solely on the extracted activity. Consideration has not been made of  $^{14}\text{CO}_2$  produced as the final product of the adenine degradation pathway. Notably, the exact fate of Ade formed by plants appears to vary considerably (ANDERSON, 1979; BARANKIEWICZ & PASZKOWSKI, 1980), according to the species investigated. Production of  $^{14}\text{CO}_2$  from BA has previously been recorded (BIONDI, CANCIANI, & BAGNI, 1984). Despite the early work of BARNES (1959; 1961) who demonstrated the catabolism of Ade in plant leaves, only a handful of studies (McCALLA, MORRÉ & OSBORNE, 1962; BIONDI, CANCIANI, & BAGNI, 1984) have traced the fate of applied [8- $^{14}$ C]BA along such metabolic routes.

The low total recovery of applied radioactivity from cultures treated with papaverine indicates that cytokinin physiologists should be more concerned with the metabolic fate of their applied labels. Cells boiled immediately prior to cytokinin application yielded nearly twice as much radioactivity as the non-boiled control (Figure 7.6). Similarly, at papaverine levels greater than 1 mM, general inactivation of cytokinin metabolic processes (Figures 7.1) resulted in higher recovery of the applied label (Figure 7.6). These findings heighten the possibility that  $^{14}\text{CO}_2$  or other labelled volatile products are produced by *Dianthus* cell cultures in significant quantities. However, a negative effect on cell culture growth of high levels of papaverine would also explain this lowered label recovery. Given the rapid doubling time of apex-derived

cultures (2.6 days, Chapter 3.2.1.3.3), a negative effect on growth over a 2 day period would result in considerably lower cell densities at the end of the incubation period. Should radio-labelled metabolites be inextricably bound to cellular material/debris, one could expect the extent of such binding to be proportional to the number of cells, and hence recovery of radioactivity greater from slower growing cultures.

## 7.2 Effect of auxin type on BA metabolism

### 7.2.1 Experimental procedure and auxin application

To provide a valid comparison with the 2,4-D effect noted in the 2,4-D-maintained apex cultures (Chapter 6.3), it was initially intended to compare the effect of the auxin NAA on BA metabolism in cell suspensions maintained on NAA. An attempt was made to substitute the auxin source in the system (2,4-D, 2 mg  $l^{-1}$ ) with NAA (2 mg  $l^{-1}$ ). Cultures were subcultured and incubated under identical conditions to those containing 2,4-D (Chapter 3.2.1.2.3). Growth of cultures was recorded as the mean of 5 separate cultures, using the side-arm method of determination (Chapter 3.2.1.2.3). In the first subsequent culture passage, suspensions appeared to have partially adapted to the new auxin source (Figure 7.8). However, in the second passage, very poor growth was recorded (Figure 7.8), indicating culture preference for/dependence on the auxin 2,4-D. The better growth recorded during the first passage was likely due to residual 2,4-D (metabolites) present in cells and stale media transferred with the initial inoculum. Subsequent to determining the deterioration of culture vitality on an NAA-based culture medium, no attempts to grow cultures on an IAA-based medium were made. Instead, investigations into the effect of the auxins NAA and IAA on BA metabolism made use of 2,4-D-maintained apex-derived suspension cultures.

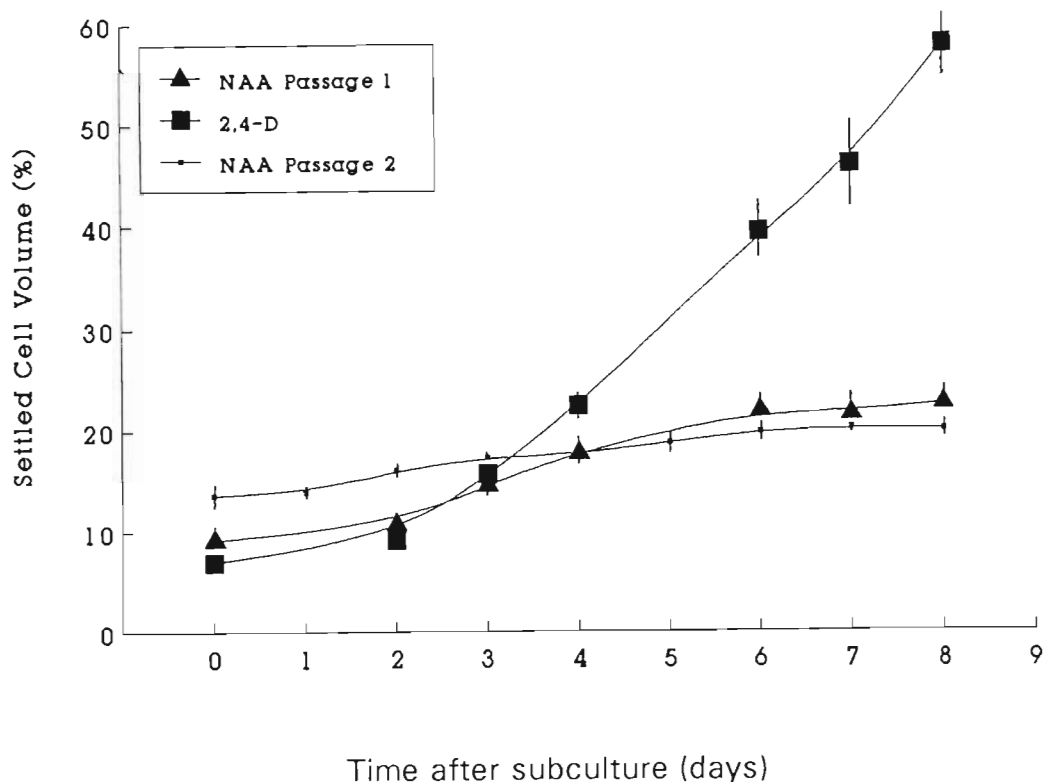


Figure 7.8 Growth of shoot-apex derived cell suspensions of *D. zeyheri* on 2,4-D and NAA based media. Bars denote standard errors.

Control cultures (15 ml in a 100 ml Erlenmeyer, 2,4-D 2 mg  $l^{-1}$ ) were supplied with [ $8-^{14}$ ]BA (0.0051 Mbq in 4  $\mu$ l methanol) and non-labelled BA (2 mg  $l^{-1}$ ). Auxin treated cultures were additionally supplied with 2,4-D, IAA, or NAA to adjust the final concentration to 4 mg  $l^{-1}$ . In a further study, the final concentration of 2,4-D was adjusted to be 8 mg  $l^{-1}$ , to consider the effect of supra-physiological levels of 2,4-D on cytokinin metabolism. All auxins and un-labelled BA stock solutions were filter-sterilised. Three replicates were established for each variable considered. After treating the cell suspensions, flasks were incubated for 48 hours under identical conditions to those of the stock cultures, except that a continual photoperiod was now maintained.

### 7.2.2 Chromatographic analyses and confirmatory techniques

Metabolite extraction, separation and identification was successfully achieved using

the same procedures as for studies on papaverine inhibition (Chapter 7.1.2.4; 7.1.2.5).

### 7.2.3 Results and discussion

Supplied BA was rapidly metabolised by the apex-derived cell suspensions. After 48 hours, less than 10% of the extracted radioactivity was found associated with the base. Elevated auxin levels slightly promoted utilization of the base (Figure 7.9). In accordance with previous findings (Chapter 6.3), doubling of the 2,4-D concentration in the medium promoted cytokinin-7-glucosylation (Table 7.2; Figure 7.9), with a concurrent reduction in active cytokinin forms (Figure 7.10). When the 2,4-D concentration was increased to  $8 \text{ mg } \ell^{-1}$ , 7-glucosylation was promoted still further. [7G]BA as the major product accounted for more than 50% of the extracted radioactivity (Figure 7.9). Control systems (2,4-D,  $2 \text{ mg } \ell^{-1}$ ) formed the riboside as the primary metabolite (34.3%) and 7-glucoside as the second most abundant product (27.7%). Neither [3G]BA nor [9G]BA were detected in significant proportions in any of the treatments (Table 7.2); [7G]BA contributed most to the N-conjugated pool.

In contrast to the (consistent) findings for 2,4-D, both NAA and IAA had little effect on BA metabolism (Figure 7.11). Only slight promotion of both oxidative and 7-glucosylation processes were recorded (Table 7.2), together with a concurrent reduction in active forms (Figure 7.12). NAA, unlike 2,4-D, has not previously been shown to affect N-conjugation of BA (ZHANG, LETHAM, WONG, NOODÉN & PARKER, 1987). The auxin NAA does, however, affect cytokinin oxidative processes (PALNI, BURCH & HORGAN, 1988). In contrast, IAA has been shown to competitively inhibit  $\beta$ -(9-cytokinin)alanine synthase purified from lupin seeds (PARKER, ENTSCH & LETHAM, 1986). WITHAM (1968) demonstrated that the specific auxin used is of importance in the growth of soybean callus. Such variety at the growth response (physiological) level resulting from auxin/cytokinin interaction follows on differences noted at the metabolic level.

Table 7.2 Effect of auxin type on BA metabolism in apex-derived cultures after 48 hours.

Treatment			Ratio	Radioactivity co-eluting with standards (% of total extracted)											
Auxin	Auxin (mg l <sup>-1</sup> )	BA (mg l <sup>-1</sup> )	Auxin: Cytokinin	B**	Ade	Ado	[3G]BA	[7G]BA	[9R-MP]BA	[9R]BA	BA	D**	Oxidation Products*	Active Pool†	N-conjugates‡
4-D	2	2	1:1	12.16	-	0.34	0.19	27.68	15.14	34.28	9.47	0.75	12.50	58.89	27.87
4-D	4	2	2:1	24.12	0.22	0.27	0.06	43.16	8.99	14.82	7.74	0.61	24.61	31.55	43.23
4-D	8	2	4:1	24.35	0.43	0.72	0.10	52.14	4.66	10.14	7.05	0.40	25.50	21.85	52.24
IA	2	2	2:1	14.04	0.13	0.67	0.07	30.54	14.23	31.88	7.97	0.47	14.84	54.08	30.60
4-D	2														
IA	2	2	2:1	13.58	0.07	0.65	0.02	30.75	11.56	34.05	8.59	0.73	14.31	54.20	30.77
4-D	2														

\* unknown compound

† total radioactivity associated with B, Ade and Ado, products of oxidation

‡ total radioactivity associated with base, nucleoside, and nucleotide

§ total radioactivity associated with N-glucosides

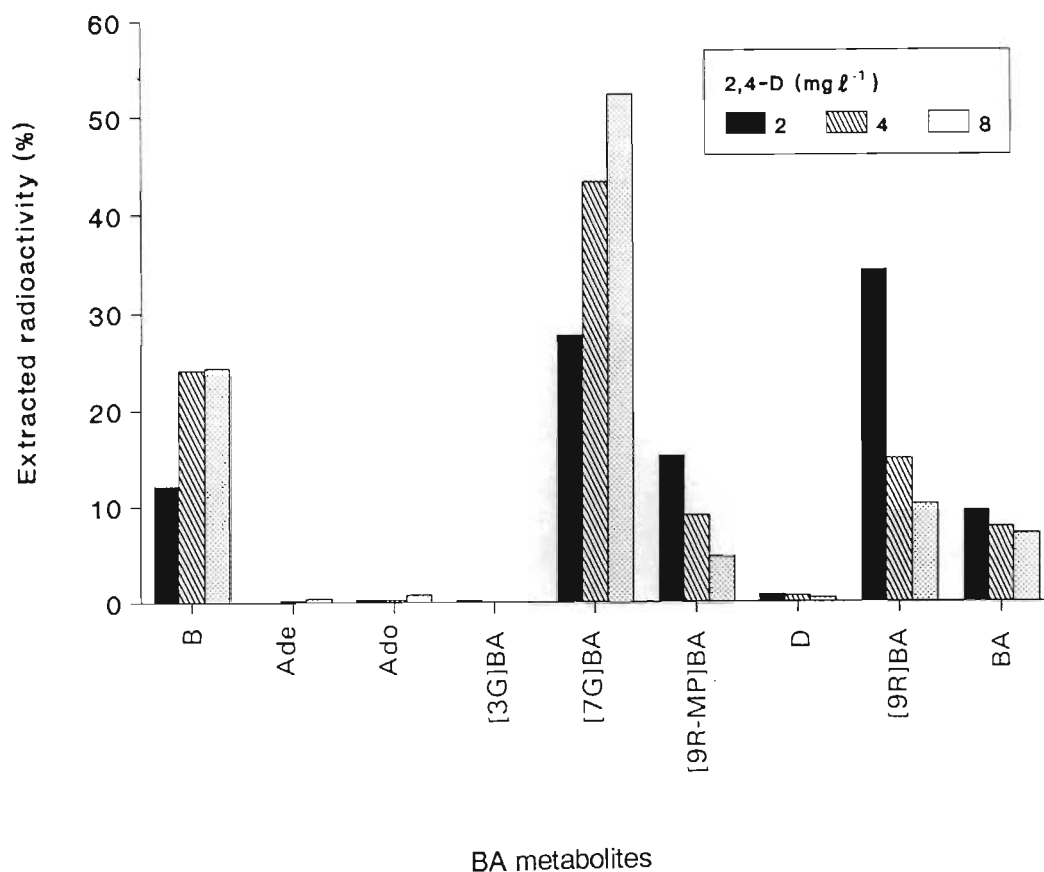


Figure 7.9 The effect of 2,4-D (2, 4, or 8 mg l<sup>-1</sup>) on BA metabolism in apex-derived cell cultures.

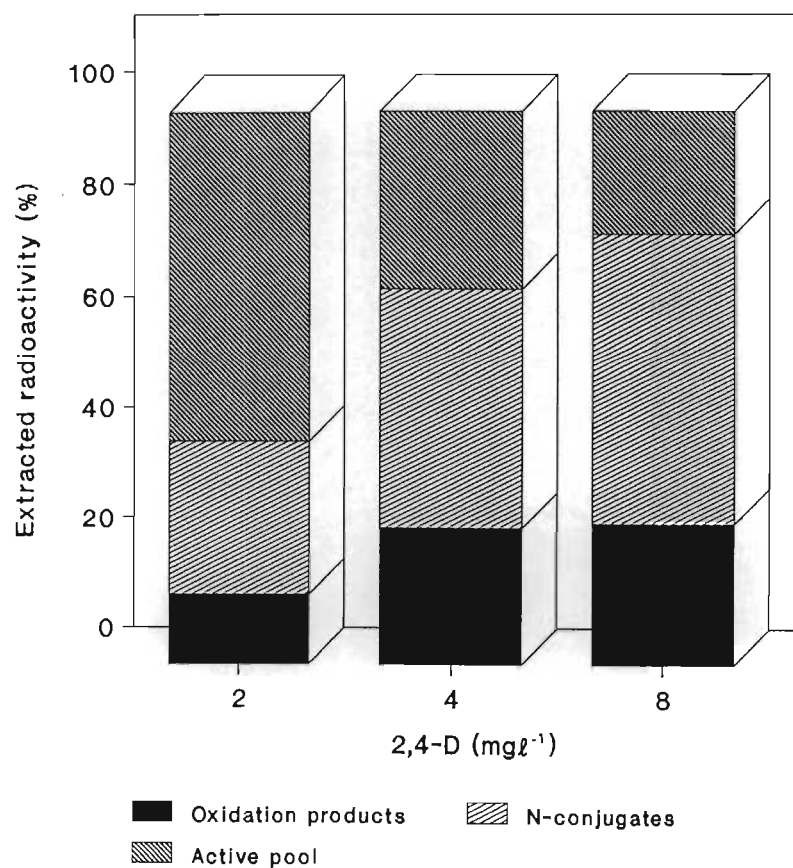


Figure 7.10 The effect of 2,4-D (2, 4, or 8 mg l<sup>-1</sup>) on radioactivity associated with

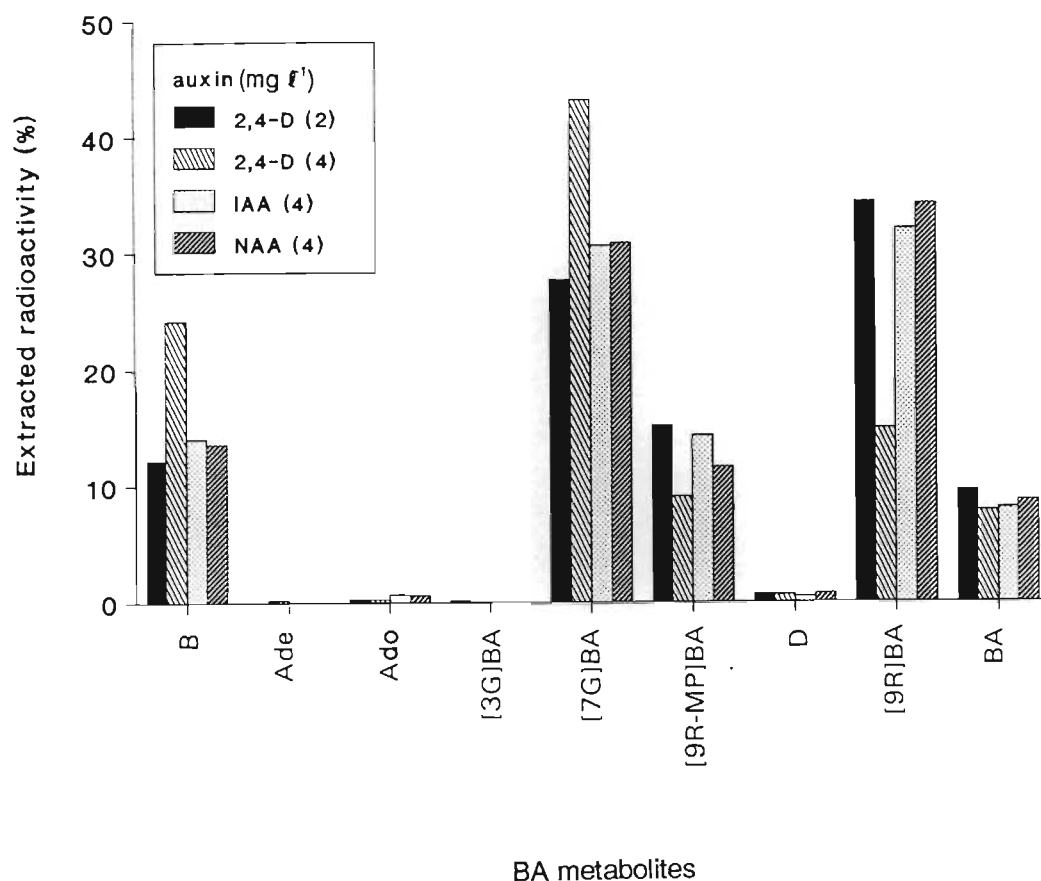


Figure 7.11 The effect of auxin type on BA metabolism in apex-derived cell suspensions. Final auxin concentrations  $4 \text{ mg } \ell^{-1}$  (including  $2 \text{ mg } \ell^{-1}$ , 2,4-D) relative to the control ( $2 \text{ mg } \ell^{-1}$ , 2,4-D).

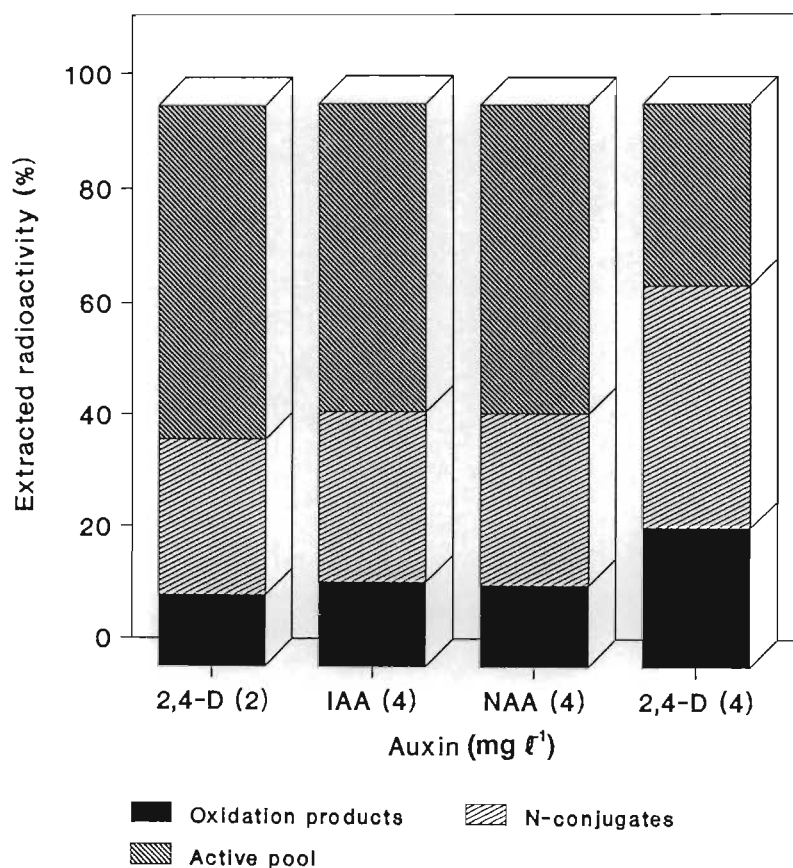


Figure 7.12 The effect of auxin type on radioactivity associated with active/inactive BA

In the current investigation, more oxidative products and less [7G]BA were formed than earlier recorded (Chapter 6.3) for similarly treated cultures under identical conditions. In particular, production of compound B (eluting on HPLC between 4 and 10 minutes) had substantially increased. For a discussion on the probable identity of compound B, refer to Chapter 3.3.2.3 Such metabolic differences may be attributed to genetic variation arising during *in vitro* culture. Habituation is one such (epigenetic) change (MEINS, 1989). BRIGHT, JARRETT, NELSON, CREISSEN, KARP, FRANKLIN, NORBURY, KUEH, ROGNES and MIFLIN (1986) suggested possible causal factors as single gene mutations, chromosome or gene rearrangements, or gene amplification or depletion. Long-term cell cultures are particularly susceptible to such changes (EVANS, SHARP & BRAVO, 1984; refs within).

### 7.3 Dose-dependent metabolism of BA

#### 7.3.1 Experimental procedure and plant growth regulator application

WAREING (1978) noted that relative concentrations of auxin and cytokinin may have a large effect on the qualitative pattern of differentiation *in vitro*. This author cited the classic study of SKOOG and MILLER (1957) as a clear example; varying the auxin/cytokinin ratio permitted control of organogenesis. MURASHIGE (1974) considered that such control of root and shoot initiation by auxin-cytokinin balances is a general phenomenon among plants, including the mosses. In the preceding chapter, an increase in the concentration of 2,4-D in the incubation medium was shown to promote 7-glucosylation of BA in apex and seed-derived *Dianthus* cell cultures. Whether this was due to a concentration effect or to relative auxin/cytokinin levels (a qualitative effect) was not determined. Varying BA/auxin ratios and a constant (100:1) BA/auxin ratio (obtained with different BA and auxin concentrations) have been shown to affect activities of enzymes of nitrogen metabolism (VAZQUEZ-FLOTA, QUIROZ, SCORER & LOYOLA-VARGAS, 1989). Conceivably then, enzymes of BA metabolism may be similarly regulated, through a feedback mechanism. Promotion of 7-glucosylation by 2,4-D would lower the BA/auxin ratio, which could differentially activate the enzymes of BA metabolism (e.g cytokinin-7-glucosyl transferase). Such

a mechanism would account for transient auxin-affected cytokinin metabolism. To investigate the importance of ratios of these two hormones, and potential dose-dependent metabolic responses, BA concentrations were increased in the presence of low ( $2 \text{ mg l}^{-1}$ ) and high ( $4 \text{ mg l}^{-1}$ ) 2,4-D levels.

Control cultures (15 ml in a 100 ml Erlenmeyer, 2,4-D  $2 \text{ mg l}^{-1}$ ) were supplied with [ $8\text{-}^{14}$ ]BA (0.0051 Mbq in  $4 \mu\text{l}$  methanol) and non-labelled BA ( $2 \text{ mg l}^{-1}$ ). Cultures with elevated auxin and/or cytokinin were additionally supplied with 2,4-D and/or BA to adjust the final concentration to  $4 \text{ mg l}^{-1}$ . Both auxin and un-labelled BA stock solutions were filter-sterilised. Three replicates were established for each variable considered. After treating the cell suspensions, flasks were incubated for 48 hours under identical conditions to those of the stock cultures (Chapter 3.2.1.2.3), except that a continual photoperiod was now maintained.

### 7.3.2 Chromatographic analyses and confirmatory techniques

Metabolite extraction, separation and identification was successfully achieved using the same procedures as for studies on papaverine inhibition (Chapter 7.1.2.4; 7.1.2.5).

### 7.3.3 Results and discussion

In doubling both the auxin and cytokinin concentrations in the experimental system, the ratio of these hormones (1:1) remained the same as the control (Table 7.3). However, a marked effect on BA metabolism was evidenced (Figure 7.13). An increase in the supply of BA (from  $2$  to  $4 \text{ mg l}^{-1}$ ) resulted in greater inactivation of supplied cytokinin (Figure 7.14). Inactivation, which proceeded primarily through 7-glucosylation rather than oxidation (Figure 7.13), was metabolically linked to a reduction in levels of riboside and [9R-MP]. When both auxin and cytokinin levels were increased to  $4 \text{ mg l}^{-1}$ , 7-glucosylation (33.5%) was even more extensive than for the control (15.7%), and oxidative processes were promoted (Table 7.3). In both systems supplied with high cytokinin ( $4 \text{ mg l}^{-1}$ ), more residual (unmetabolised) BA was detected after the 48 hour incubation period (Figure 7.13).

Figure 7.3 Dose-dependent metabolism of BA in apex-derived cultures, as affected by the auxin 2,4-D.

Treatment		Ratio	Radioactivity co-eluting with standards (% of total extracted)												
2,4-D ( $\mu\text{g l}^{-1}$ )	BA ( $\text{mg l}^{-1}$ )	Auxin: Cytokinin	B <sup>w</sup>	Ade	Ado	[3G]BA	[7G]BA	[9G]BA	[9R-MP]BA	[9R]BA	BA	D <sup>w</sup>	Oxidation Products*	Active Pool <sup>y</sup>	N- conjugates <sup>f</sup>
	2	1:1	12.95	0.09	1.50	0.07	15.71	0.98	14.81	47.87	4.79	1.22	14.55	67.47	16.76
	4	1:2	9.04	-	1.83	0.09	22.58	1.10	11.90	43.67	8.34	1.45	10.87	63.90	23.77
	4	1:1	14.98	-	0.97	0.10	33.49	0.77	10.19	29.34	8.57	1.60	15.95	48.10	34.35

\* known compound

<sup>y</sup> total radioactivity associated with B, Ade and Ado, products of oxidation

<sup>f</sup> total radioactivity associated with base, nucleoside, and nucleotide

<sup>w</sup> total radioactivity associated with N-glucosides

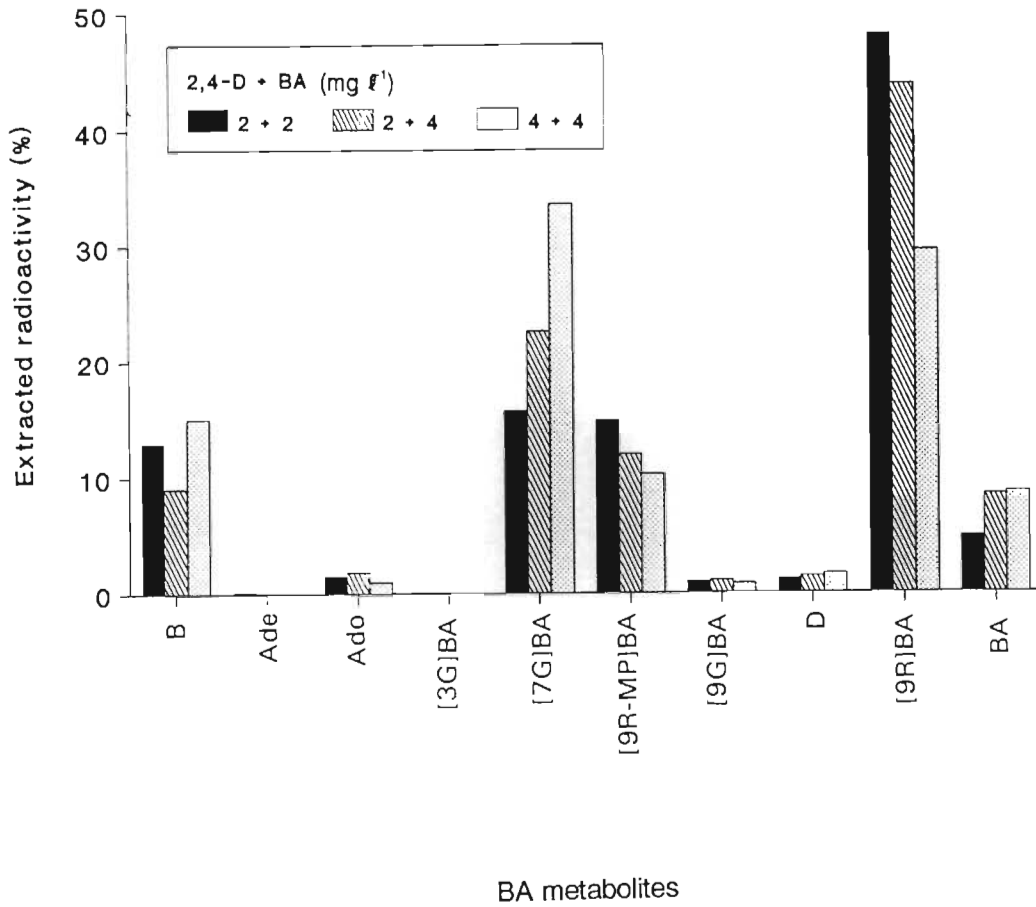


Figure 7.13 BA metabolite formation as a dose-dependent response in apex-derived suspensions, which is further modified by 2,4-D.

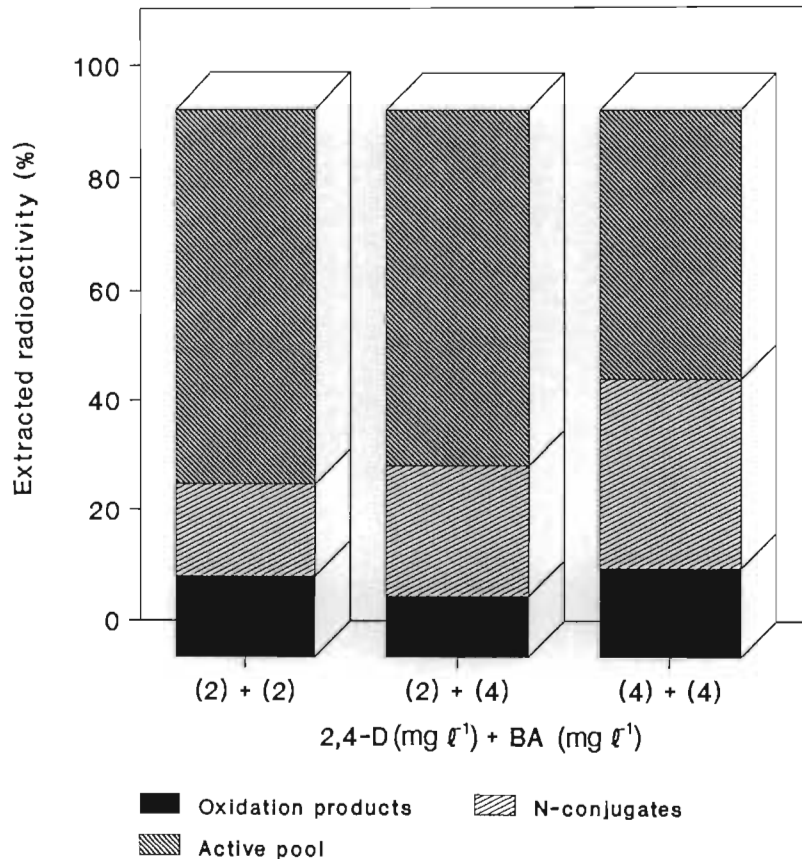


Figure 7.14 Dose-dependent effect of BA on radioactivity associated with active/inactive BA pools in apex-derived cell suspensions.

Metabolism of exogenously supplied BA by *Dianthus* cell cultures was evidently dose-dependent. More inactivation products (particularly [7G]BA) were formed at the higher BA concentrations (Figure 7.13). This occurred regardless of whether the auxin/cytokinin ratio remained constant. In control systems of low auxin status, 7-glucosylation was the preferred route for cytokinin inactivation. Oxidative catabolism was not promoted when the BA concentration was doubled, in contrast to earlier findings for tobacco (TERRINE & LALOUE, 1980). When extra auxin was synchronously provided with BA, both oxidative and N-conjugative pathways were promoted. These results support assertions that cytokinin-7-glucosides are detoxification products (McGAW & HORGAN, 1985), but are in contrast to other reports on dose responses. LETHAM, TAO & PARKER (1982) found that formation of the 3-, 7-, and 9-glucosides of BA was not dependent on BA concentration, and so suggested that formation of the metabolites was not simply a mechanism for inactivating physiological excesses of BA. Similarly, the rate of BA glucosylation in tobacco cells (GAWER, LALOUE, TERRINE & GUERN, 1977) and tobacco flower stalks (VAN DER KRIEKEN, CROES, SMULDERS & WULLEMS, 1990) was found to be relatively insensitive to large differences in the concentration of supplied BA, at least relative to active forms. *Dianthus zeyheri* suspension cultures are cytokinin-autonomous, requiring only supplied auxin (2,4-D) for cell division. Thus BA supplied in current metabolic studies may be considered superfluous, and potentially toxic to these cells. For this reason, it is likely that BA was rapidly inactivated/detoxified.

That auxin/cytokinin ratios do not wholly account for metabolic responses in these cell cultures is unlikely to have great practical relevance for tissue culturists. *In vitro* systems with a dependence on exogenously supplied auxin and cytokinin for morphogenesis (SKOOG & MILLER, 1957) are more likely to be affected by ratios of these two hormones (as well as actual amounts) than the undifferentiated cell system investigated.

## CHAPTER 8

### GENERAL CONCLUSIONS

Alcoholic extraction procedures were found to be adequate for the extraction of all metabolites of BA, including the nucleotide. Extraction with the solvents of BIELESKI (1964) was more time-consuming, elaborate, and ultimately not worth the inconvenience. In subsequent investigations, metabolites of BA were therefore extracted with ethanol.

In establishing suspension cultures of carrot, soybean, and *D. zeyheri*, an attempt was made to grow these systems on media supplemented with the same phytohormones (2,4-D; 2 mg  $\ell^{-1}$ ). Accordingly, although the basal salts and vitamins varied in each culture, the systems were essentially comparable. In addition, experimental parameters of the four cell suspension systems investigated were, as far as possible, identical. All systems were treated in the same (linear) phase of growth, for the growth phase of cell cultures has been shown to affect the uptake (LALOUE, PETHE-TERRINE & GUERN, 1981) and metabolism (FUBEDER, ZIEGLER, PETERS & BECK, 1989) of exogenously applied cytokinins. Hormonal pretreatments and subsequent treatment, extraction and analyses were identical, yet the metabolic responses varied widely. A similar result was recorded by BLAKESLEY and CONSTANTINE (1992) who had (identically) applied BA to a range of shoot cultures representing several plant families. This broad study differed fundamentally from the current investigation, for BLAKESLEY and CONSTANTINE (1992) attempted to correlate organogenic responses to BA metabolism. In the current study, long-term (embryogenic) effects in treated cell suspensions were not considered, given that the potential for organised development in 2,4-D-treated systems is low (MURASHIGE, 1974).

Following exogenous application of BA and 2,4-D, marked differences in interactive effects on BA metabolism were observed between systems.

Soybean cell cultures apparently inactivated supplied BA through sidechain cleavage. Between 18 and 48 hours this oxidative catabolism was promoted by 2,4-D. Enzyme-mediated oxidation of BA by soybean tissues has not yet been demonstrated *in vitro*. Further investigation of the sidechain cleavage system operating in soybean cell cultures will potentially lead to the characterisation of either a new form of cytokinin oxidase, or a novel enzyme which catalyses the formation of an imino-intermediate. Soybean cell suspensions may also prove profitable in studies on biosynthetic routes for 'benzylated' cytokinins, given that re-formation of BA from catabolic products was observed.

Carrot cell cultures, although cytokinin-autonomous, maintained a large active cytokinin pool, even after 48 hours incubation. An initial promotion of ribosylation by auxin was in apparent contrast to the promotion of [9G]BA formed after a longer incubation period.

The effect of 2,4-D on BA metabolism in *Dianthus zeyheri* cell cultures, whether derived from shoot-apex or seed callus, was to promote cytokinin inactivation through 7-glucosylation. This finding represents the first report of auxin-promoted cytokinin N-conjugate formation in any plant system. The effect of 2,4-D was transient in the case of apex-derived systems but not seed-derived cultures over the time period considered. In apex-derived cultures, inactivation of BA via 7-glucosylation was established to be a dose-dependent response to exogenously supplied BA. This finding contrasts with previous reports on dose-independent [7G]BA formation. Further studies with the auxins IAA and NAA revealed that auxin-promoted 7-glucosylation of BA in apex-derived cultures of *D. zeyheri* was substantially effected by only the synthetic auxin 2,4-D.

An attempt to manipulate the pathways of cytokinin inactivation with the aid of an inhibitor of 7-glucosylation, papaverine, was not successful. In apex-derived cell cultures of *D. zeyheri*, this inhibitor had little effect on [7G]BA formation when supplied at levels of 1 mM or less. At higher concentrations (5 mM or greater), papaverine almost completely inhibited general cytokinin metabolism. The critical

concentration range of 1-5 mM requires an evaluation, if inactivation pathway switching is to be demonstrated.

The time-sequence studies which were employed demonstrated that auxins affect cytokinins at the metabolic level in a transient manner. Any study in this field should similarly accord consideration to this temporal aspect of interaction. The time intervals selected were suitable for the rapid metabolism of cytokinins expected in cell suspensions. However, in order to monitor potential transient auxin-effects in soybean and seed-apex derived *Dianthus* systems, a further incubation period of 72 hours would have been appropriate.

Investigations with apex-derived cell cultures revealed that the metabolic response to supplied [8-<sup>14</sup>C]BA varied in the long term. Identical controls showed that after a period of 24 months, the cellular response towards cytokinin inactivation had changed from a predominantly N-conjugative metabolic route to one where oxidative-type catabolism was considerably more pronounced. Epigenetic changes in the parent callus tissue probably induced this shift in the relative expression of these two pathways. The classification of plant systems as characteristically expressing either 'oxidative' or 'N-conjugative' type metabolisms would therefore appear unfounded.

The contribution of transient changes in the levels of any cytokinin forms (whether active or not), to developmental processes, is unknown. It has been proposed (VANKOVA, GAUDINOVA, KAMINEK & EDER, 1992) that such transient changes allow for the induction of developmental processes, whilst preventing long-term hormonal imbalances in plant tissues.

Auxin effects on BA metabolism should not be taken out of context. In a corresponding manner, cytokinins have been shown to elevate auxin levels in tissues (JORDAN & SKOOG, 1971; NOOR SALEH, 1981; BOURQUIN & PILET, 1990), to affect the production and activity of IAA-oxidising enzymes (JAIN, KADKADE & VAN HUYSE, 1969; DARIMONT, GASPAR & HOFINGER, 1971; SCHNEIDER & WIGHTMAN, 1978), and to modify the conjugation of auxins (MONTAGUE, ENNS, SIEGEL & JAWORSKI, 1981). In the

process of mutually exerting metabolic effects, these two hormone classes effectively adjust the overall auxin/cytokinin balance in plant cells. In this regard, double-labelling experiments which permit the synchronous evaluation of mutual auxin and cytokinin-exerted effects are necessary. The exact relevance of auxin/cytokinin ratios in the control of physiological processes has not been elucidated, although several recent reports have linked auxin/cytokinin interaction at the molecular level (SHINSHI, MOHNEN & MEINS, 1987; DOMINOV, STENZLER, LEE, SCHWARZ, LEISNER & HOWELL, 1992; OUELHAZI, FILALI, DÉCENDIT, CHÉNIEUX & RIDEAU, 1993). With somewhat far-sighted reference to the 'variable gene activity' theory of development, WAREING (1978) had earlier remarked that 'qualitative interaction must in the last analysis involve hormonal regulation of selective gene expression'.

Mechanisms by which hormones interact are varied and poorly understood. In the current studies, direct causes for the effects noted have not been demonstrated. *In vitro* studies of the enzymes catalysing auxin-affected reactions are required to further elucidate the level of interaction of auxin and cytokinin. The effect of auxin may not be direct, but through an effect on enzyme co-factors or inhibitors.

Suitable controls were established in the current studies to permit the demonstration of auxin effects on BA metabolism. However, it would be expected that the combined influences of endogenous and exogenous hormones (of perhaps more than two classes), together with environmental factors, have all likely contributed to a quantifiable 'auxin effect'.

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