for my parents

IAN and ESTHER

ROSA SINE SPINA
STUDIES OF CYTOKININ METABOLISM
IN RELATION TO SEED GERMINATION
AND SEEDLING DEVELOPMENT

A thesis submitted for the degree of
Doctor of Philosophy in the
Australian National University.

by

CHARLES HAMILTON HOCART

December, 1985
Statement

This thesis presents an account of research carried out by myself in the Department of Developmental Biology, Research School of Biological Sciences, The Australian National University, Canberra. Assistance and collaboration are recorded under acknowledgements and in the text.

Charles Hocart
Acknowledgements

I should like to record my gratitude to the many people who made a contribution to the production of this thesis. Firstly, I thank Dr. D.S. Letham for the privilege of working under his supervision, for his interest, guidance and patience throughout the course of my research and writings. I am also grateful for permission to quote his hitherto unpublished work undertaken in collaboration with Mr Tao Guo-qing, Mr. Zhang Ren and Mr Santokh Singh. Professor B.E.S. Gunning is also thanked for the use of facilities in the Department of Developmental Biology and for his interest and support. Dr. L.M.S. Palni's initial interest in the work described in Chapter 4 is also acknowledged.

Dr D.S. Letham and Mr. C.W. Parker helped with the chromatography in Sections 4.3 and 4.5 and both Dr D.S. Letham and Mr. Tao Guo-qing collaborated in the work described in Chapter 5. All RIA were performed by Dr J. Badenoch-Jones and Mr C.W. Parker, and mass spectrometry by Dr R.E. Summons and Dr O.C. Wong.

A number of people made their expertise freely available to me in the course of my studies and I thank them also, in particular, Dr J. Badenoch-Jones, Dr R.J. Capon, Mr C.W. Parker and Dr. R.E. Summons. Their comments were invariably helpful and much appreciated, as have been Ms B. Piper's efforts in the typing of the final product.

Financial support for this work was provided by a Commonwealth Post-Graduate Award from the Commonwealth Department of Education, the Department of Social Security and travel grants by the Australian National University.

Finally, a thank you to my family and friends for their support, but especially to my Mother and Father, and also to my Auntie Hilda and Reg.
Chapter 2 - Several new chromatographic methods were developed, including reverse phase TLC and a normal phase cellulose TLC system. The latter was incorporated into a scheme for purifying and isolating cytokinin prior to mass spectral analysis or RIA. Cytokinins could be readily detected in HPLC eluates by the qualitative use of RIA. This was particularly useful where the elution of other UV-absorbing compounds obscured the UV absorption of the cytokinins or where radio-labelled cytokinins were not available. New stable derivatives of the cytokinin bases (tBuDMS) and ribosides (tBuDMS-isopropylidene) were made for use in mass spectral quantitation. Their mass spectral fragmentation was rationalised and their chromatographic properties recorded.

Chapter 3 - A series of 3-substituted xanthines, a 2-substituted 9-methyl-BAP and a derivative of oxazolopyrimidine were synthesised as potential inhibitors of cytokinin N-glucosylation. The alkylation conditions used in the preparation of the 3-substituted xanthines gave significantly improved yields over the method previously employed. The 2-substituted 9-methyl-BAP was prepared from 2,6-dichloropurine by a synthetic route that optimised the yield of the 9-methyl derivative over that of the 7-methyl derivative. 7-Benzylaminooxazolo[5,4-d]pyrimidine (24) was prepared from 7-aminooxazolo[5,4-d]pyrimidine, in poor yield, by direct alkylation in hexamethylphosphoramide with benzyl bromide. A number of other possible synthetic routes were investigated, but proved to be completely unsatisfactory.

Chapter 4 - The cytokinins in dry Zea mays seeds were identified and quantified by stable isotope dilution and RIA using methods and
derivatives described in Chapter 2. The principle cytokinins in embryos were zeatin riboside (ZR), isopentenyladenosine (iPA) and zeatin-9-glucoside (Z9G). $[3\text{H}]$-ZR of high specific activity was synthesised and used to study the metabolism and translocation of ZR in germinating Zea mays seed. The major metabolites identified were adenosine (Ado), adenine (Ade) and adenine nucleotide (Ado-5'-P). No reduction of the sidechain double band was observed and only traces of zeatin (Z) were detected. When $[3\text{H}]$-ZR was fed to the radicle tip a significant proportion of the extracted radioactivity was identified as Z9G. However, a significant amount of ZR was translocated, especially to the shoot and embryo-remnant. During the initial stage of germination, movement of ZR from the embryo to the endosperm was pronounced but little movement occurred in the reverse direction.

When $[14\text{C}]$-Ade was supplied to the embryos of germinated seed, a low degree of incorporation into zeatin nucleotide was observed indicating that the embryo is capable of cytokinin biosynthesis.

The metabolism of $[3\text{H}]$-ZR in Zea mays seedling leaves was found to be quite complex with only some 58% of the extracted radioactivity being accounted for in terms of identified metabolites, mostly Ade, Ado and Ado-5'-P. In contrast, the metabolism of $[3\text{H}]$-BAP in the same tissue proved to be relatively simple, the principle metabolite being 6-benzylaminopurine-9-glucoside (BAP-9-G). Consequently, the compounds synthesised previously (Chapter 3) were tested for their ability to inhibit the N-glucosylation of BAP, rather than ZR, in maize leaf segments. The BAP analogue, 24, was found to be the most effective inhibitor tested, inhibiting the formation of BAP-9-G and raising the level of free BAP, even at 0.2mM.
Chapter 5 - Zeatin-7-glucoside (Z7G) and ZR were unequivocally identified and quantified in radish cotyledons. Increasing amounts of Z7G were accumulated over time. 2-(2-Hydroxyethylamino)-9-methyl-6-benzylaminopurine (27), has previously been found to inhibit the N-glucosylation of BAP in radish cotyledons. However, its effectiveness was probably limited by conjugation in vivo. This metabolite has now been identified by mass spectral studies, enzymic hydrolysis and chromatographic data as the O-α-D-glucoside, 30. The N-glucosylation of [3H]-BAP in radish leaves was suppressed most effectively by 1,7-dimethyl-3-(3-methylbutyl)xanthine (7), 1,7-dimethyl-3-(5-hexenyl)xanthine (10) and 1,7-dimethyl-3-(3-methyl-2-butenyl)xanthine (13). 7 and 10 were also effective inhibitors in radish cotyledons and elevated the level of both free BAP and BAP nucleotide.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
</tr>
<tr>
<td>Ade</td>
<td>adenine</td>
</tr>
<tr>
<td>Ado</td>
<td>adenosine</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine-5'-monophosphate</td>
</tr>
<tr>
<td>amu</td>
<td>atomic mass units</td>
</tr>
<tr>
<td>BAP</td>
<td>6-(benzylamino)purine</td>
</tr>
<tr>
<td>BAP-3-G</td>
<td>BAP-3-β-D-glucopyranoside</td>
</tr>
<tr>
<td>BAP-7-G</td>
<td>BAP-7-β-D-glucopyranoside</td>
</tr>
<tr>
<td>BAP-9-G</td>
<td>BAP-9-β-D-glucopyranoside</td>
</tr>
<tr>
<td>br</td>
<td>broad</td>
</tr>
<tr>
<td>BAP-R</td>
<td>BAP-9-β-D-riboside</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BSTFA</td>
<td>N,0-bis-(trimethylsilyl)trifluoroacetamide</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine-3'-5'-monophosphate</td>
</tr>
<tr>
<td>CDCl₃</td>
<td>deuterо-chlorоform</td>
</tr>
<tr>
<td>cf.</td>
<td>compare</td>
</tr>
<tr>
<td>CI</td>
<td>chemical ionisation</td>
</tr>
<tr>
<td>ci</td>
<td>curie,</td>
</tr>
<tr>
<td>mCi</td>
<td>millicurie</td>
</tr>
<tr>
<td>μCi</td>
<td>microcurie</td>
</tr>
<tr>
<td>cis-Z</td>
<td>cis-zeatin</td>
</tr>
<tr>
<td>cis-ZR</td>
<td>cis-zeatin riboside</td>
</tr>
<tr>
<td>CK</td>
<td>cytokinin</td>
</tr>
<tr>
<td>conc.</td>
<td>concentrated</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>cv</td>
<td>cultivar</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>d</td>
<td>doublet</td>
</tr>
<tr>
<td>D₆DMSO</td>
<td>hexadeutero-dimethylsulphoxide</td>
</tr>
<tr>
<td>diam.</td>
<td>diameter</td>
</tr>
<tr>
<td>DI-OH-Z</td>
<td>6-(2,3,4-trihydroxy-3-methylbutylamino)purine</td>
</tr>
<tr>
<td>DLA</td>
<td>dihydrolupinic acid</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-dimethylaminopyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DMPS-5X</td>
<td>dimethylpolysiloxane</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>D₂O</td>
<td>deuterium oxide</td>
</tr>
<tr>
<td>dpm</td>
<td>disintegrations per minute</td>
</tr>
<tr>
<td>DTBS</td>
<td>di-tert-butylsilene</td>
</tr>
<tr>
<td>DZ</td>
<td>dihydrozeatin</td>
</tr>
<tr>
<td>DZR</td>
<td>dihydrozeatin riboside</td>
</tr>
<tr>
<td>DZR-5'-P</td>
<td>dihydrozeatinriboside-5'-phosphate</td>
</tr>
<tr>
<td>EI</td>
<td>electron impact</td>
</tr>
<tr>
<td>EtOAc</td>
<td>ethyl acetate</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>pg</td>
<td>picogram</td>
</tr>
<tr>
<td>GA</td>
<td>gibberellin</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>GC-CIMS</td>
<td>combined gas chromatography-chemical ionisation mass spectrometry</td>
</tr>
<tr>
<td>GC-EIMS</td>
<td>combined gas chromatography-electron impact ionisation mass spectrometry</td>
</tr>
<tr>
<td>GC-MS</td>
<td>combined gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HOAc</td>
<td>acetic acid</td>
</tr>
<tr>
<td>HO-BAP</td>
<td>6-(2-hydroxybenzylamino)purine</td>
</tr>
<tr>
<td>HMPA</td>
<td>hexamethylphosphoramide</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HPTLC</td>
<td>high performance thin layer chromatography</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>HRMS</td>
<td>high resolution mass spectrometry</td>
</tr>
<tr>
<td>IAA</td>
<td>indole-3-acetic acid</td>
</tr>
<tr>
<td>iP</td>
<td>isopentenyladenine</td>
</tr>
<tr>
<td>iPA</td>
<td>isopentenyladenosine</td>
</tr>
<tr>
<td>iPA-5'-P</td>
<td>isopentenyladenosine-5'-phosphate</td>
</tr>
<tr>
<td>IPP</td>
<td>isopent-2-enylpyrophosphate</td>
</tr>
<tr>
<td>i-ProH</td>
<td>isopropanol</td>
</tr>
<tr>
<td>l</td>
<td>litre</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>µl</td>
<td>microlitre</td>
</tr>
<tr>
<td>LA</td>
<td>lupinic acid</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>µM</td>
<td>micromolar</td>
</tr>
<tr>
<td>nM</td>
<td>nanomolar</td>
</tr>
<tr>
<td>M⁺⁺</td>
<td>molecular ion</td>
</tr>
<tr>
<td>m</td>
<td>metre</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
</tr>
<tr>
<td>mm</td>
<td>millimetre</td>
</tr>
<tr>
<td>µm</td>
<td>micrometre</td>
</tr>
<tr>
<td>m</td>
<td>multiplet</td>
</tr>
<tr>
<td>MBTFA</td>
<td>N-methyl-bis(trifluoroacetamide)</td>
</tr>
<tr>
<td>MCFW</td>
<td>methanol-chloroform-formic acid-water</td>
</tr>
<tr>
<td>Me</td>
<td>methyl</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>MFW</td>
<td>methanol-formic acid-water</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MID</td>
<td>multiple ion detection</td>
</tr>
<tr>
<td>min.</td>
<td>minute</td>
</tr>
<tr>
<td>m.p.</td>
<td>melting point</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MTBSTFA</td>
<td>N-methyl-N-t-butyldimethylsilyltrifluoroacetamide</td>
</tr>
<tr>
<td>n-BuOH</td>
<td>n-butanol</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NPG</td>
<td>N-(purin-6-yl)glycine</td>
</tr>
<tr>
<td>OGDZ</td>
<td>0-β-D-glucopyranosyldihydrozeatin</td>
</tr>
<tr>
<td>OGDZZR</td>
<td>0-β-D-glucopyranosylzeatin riboside</td>
</tr>
<tr>
<td>OGZ</td>
<td>0-β-D-glucopyranosylzeatin</td>
</tr>
<tr>
<td>OGZR</td>
<td>0-β-D-glucopyranosylzeatin riboside</td>
</tr>
<tr>
<td>pH</td>
<td>$\log_{10}[H^+]$</td>
</tr>
<tr>
<td>PPO</td>
<td>2,5-diphenyloxazole</td>
</tr>
<tr>
<td>RCM(_{100})</td>
<td>radial compression module (Waters)</td>
</tr>
<tr>
<td>R(_f)</td>
<td>ratio of travel of compound to travel of solvent front</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td></td>
<td>mRNA messenger RNA</td>
</tr>
<tr>
<td></td>
<td>tRNA transfer RNA</td>
</tr>
<tr>
<td></td>
<td>sRNA soluble RNA</td>
</tr>
<tr>
<td>tBuDMS</td>
<td>tert-butyldimethylsilyl</td>
</tr>
<tr>
<td>s</td>
<td>singlet</td>
</tr>
<tr>
<td>THP</td>
<td>tetrahydropyranyl</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TMCS</td>
<td>trimethylchlorosilane</td>
</tr>
<tr>
<td>TMS</td>
<td>trimethylsilyl</td>
</tr>
<tr>
<td>UDPG</td>
<td>uridine diphosphate glucose</td>
</tr>
<tr>
<td>UV</td>
<td>ultra-violet</td>
</tr>
</tbody>
</table>
Z  
zeatin

Z7G  
zeatin-7-β-D-glucopyranoside

Z9G  
zeatin-9-β-D-glucopyranoside

ZR  
zeatin riboside

ZR-5'-P  
zeatin nucleotide
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dedication</td>
<td>i</td>
</tr>
<tr>
<td>Title</td>
<td>ii</td>
</tr>
<tr>
<td>Statement</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iv</td>
</tr>
<tr>
<td>Summary</td>
<td>v</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>viii</td>
</tr>
</tbody>
</table>

## CHAPTER ONE: CYTOKININ BIOCHEMISTRY AND METABOLISM IN RELATION TO PLANT DEVELOPMENT - AN INTRODUCTORY CHAPTER

1.1 Chemical identity of the cytokinins                                  7
1.2 Metabolism of the free cytokinins                                     10
1.3 Structural requirements for cytokinin activity                       15
1.4 Cytokinin biosynthesis                                               18
   1.4.1 Biosynthesis of tRNA cytokinins                                  18
   1.4.2 Biosynthesis of free cytokinins                                  19
   1.4.3 Sites of biosynthesis of the free cytokinins                    21
1.5 The significance of cytokinin metabolism in relation to plant development  25
1.6 The regulatory role of cytokinins in the developed plant             33
   1.6.1 Cell division and organogenesis                                 34
   1.6.2 Root-shoot interactions                                         35
1.7 Role of cytokinins in seed germination and early seedling development 36
   1.7.1 Phytohormone involvement in dormancy and germination            37
1.7.2 Phytohormone directed reserve mobilisation
1.7.3 Other evidence for cytokinin involvement in seed germination
1.7.4 Cytokinins and early seedling development.
1.8 Future studies of cytokinin metabolism - the present investigations

CHAPTER TWO: QUANTIFICATION OF CYTOKININS

2.1 Introduction
2.1.1 Bioassay
2.1.2 Radioimmunoassay
2.1.3 Gas Chromatography - Mass Spectrometry
2.2 Results and discussion
2.2.1 Extraction
2.2.2 Chromatography
2.2.3 Derivatisation and GC-MS of the cytokinin bases
2.2.4 Derivatisation and GC-MS of the cytokinin ribosides
2.2.5 Mode of ionisation
2.3 Experimental
2.3.1 Extraction
2.3.2 Chromatography
2.3.3 Cytokinin hydrolysis
2.3.4 Derivatisation procedures
2.3.5 Scintillation counting
2.3.6 Mass spectrometry
2.3.7 UV spectrometry
2.3.8 RIA
2.3.9 Synthetic cytokinins

CHAPTER THREE: THE SYNTHESIS OF INHIBITORS OF CYTOKININ N-GLUCOSYLATION 121
3.1 Introduction 121
3.2 3-Substituted xanthines 122
3.2.1 Introduction 122
3.2.2 Results and discussion 126
3.3 Oxazolopyrimidines 134
3.3.1 Introduction 134
3.3.2 Results and discussion 137
3.4 Benzylaminopurine derivatives 138
3.4.1 Introduction 138
3.4.2 Results and discussion 142
3.5 Experimental 142
3.5.1 General 142
3.5.2 Alkyl derivatives of 1,7-dimethylxanthine and 1-methylxanthine 143
3.5.3 Synthesis of 7-benzylaminoazolo[5,4-d]-pyrimidine 149
3.5.4 Derivatives of benzylaminopurine 154
CHAPTER FOUR: THE IDENTIFICATION, QUANTIFICATION, BIOSYNTHESIS AND METABOLISM OF CYTOKININS IN GERMINATING ZEA MAYS CARYOPSES AND METABOLISM OF CYTOKININS IN SEEDLING LEAVES

4.1 Introduction

4.2 Cytokinin identification and quantification
   4.2.1 Stable isotope dilution assay
   4.2.2 RIA
   4.2.3 Results and discussion

4.3 Biosynthesis of cytokinins

4.4 Metabolism and translocation of [3H]-ZR in germinating seeds
   4.4.1 Synthesis of [3H]-ZR
   4.4.2 Metabolism of [3H]-ZR
   4.4.3 Translocation of [3H]-ZR
   4.4.4 Results and discussion

4.5 Metabolism of [3H]-ZR and [3H]-BAP in Zea mays seedling leaves.
   4.5.1 Metabolism of [3H]-ZR
   4.5.2 Metabolism of [3H]-BAP
   4.5.3 Metabolism of [3H]-BAP in the presence of potential inhibitors of N-glucosylation
   4.5.4 Results and discussion
CHAPTER FIVE: THE CYTOKININS IN RAPHANUS SATIVUS COTYLEDONS:
IDENTIFICATION, QUANTIFICATION AND METABOLISM

5.1 Introduction

5.2 Identification of the endogenous cytokinins in radish cotyledons

5.3 The identification of a metabolite of 2-(2-hydroxyethylamino)-9-methyl-6-benzylaminopurine (27)

5.4 Metabolism of [3H]-BAP in the presence of potential inhibitors of N-glucosylation

5.4.1 Radish leaves

5.4.2 Radish cotyledons

5.5 Results and discussion

CHAPTER SIX: CONCLUSIONS AND FINAL DISCUSSION

6.1 Purification and quantification of cytokinin

6.2 Cytokinin metabolism in germinating maize seed and seedling leaves

6.3 Inhibition of N-glucosylation in maize and radish tissue

REFERENCES
1.1 The chemical identity of the cytokinins.

The discovery of the cytokinins stems from research by Skoog and associates (Skoog and Tsui, 1948; Jablonski and Skoog, 1954) into the growth requirements of tobacco pith tissue. It was found that cell division in this cultured tissue could be induced by a piece of vascular tissue placed in contact with the callus tissue. The subsequent search for the cell division factor resulted in the isolation (Miller et al., 1955a) and identification (Miller et al., 1955b) of 6-(furfurylamino)purine (kinetin) as a potent cytokinin. When tested on the tobacco pith callus tissue in the presence of auxin, kinetin was found to induce cell division (cytokinesis) at concentrations as low as 1 µg/l (Miller et al., 1956). However, kinetin proved to be an artifact of DNA denaturation resulting from a long period of storage or autoclaving under acidic conditions (Skoog and Leonard, 1968; Scopes et al., 1976).
With the failure to isolate any naturally-occurring cytokinins, the cytokinins acquired a physiological, rather than a chemical definition. They were defined as compounds that could induce cell division in a cultured plant tissue (e.g. tobacco pith, soybean cotyledon callus or carrot secondary phloem) grown on a defined medium in the presence of an optimal amount of auxin. The bioassay thus became a key element in the detection and isolation of cytokinins from plants.

The major obstacle to the isolation and structural elucidation of the cytokinins is the very small amounts present in most plant tissues (1 µg/kg fresh weight or less). This problem is being progressively overcome with the development of new sensitive and selective chromatographic techniques for purification. These techniques include capillary gas chromatography (GC), high performance liquid chromatography (HPLC) and very recently, immunoaffinity chromatography. These developments have been matched by dramatic improvements in sensitivity of the techniques for structural determination so that meaningful mass spectra (MS) may now be obtained at the submicrogram level and Fourier transform nuclear magnetic resonance (NMR) spectra (proton) recorded from a few micrograms of compound.

The first naturally-occurring cytokinin was isolated from Zea mays caryopses by Letham (1963a) and determined to be 6-(4-hydroxy-3-methyl-trans-but-2-enylamino)purine (zeatin) (Letham et al., 1964). The nucleoside and nucleotide of zeatin were also isolated and unambiguously identified in the same tissue (Letham, 1966), along with several other weakly-active cytokinins (Letham, 1973). The structure, trivial names and abbreviations of the unambiguously-identified, common naturally-occurring cytokinins are outlined in Figure 1.1. The cytokinins have
Figure 1.1: Structures, names and abbreviations of common naturally-occurring cytokinins.

<table>
<thead>
<tr>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>Trivial Name</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>-CH₂CH=CH₂</td>
<td>-H</td>
<td>-H</td>
<td>isopentenyladenine</td>
<td>iP</td>
</tr>
<tr>
<td>-CH₂CH=CH₂</td>
<td>-H</td>
<td>-H</td>
<td>zeatin</td>
<td>Z</td>
</tr>
<tr>
<td>-CH₂CH=CH₂</td>
<td>-H</td>
<td>-H</td>
<td>dihydrozeatin</td>
<td>DZ</td>
</tr>
<tr>
<td>-CH₂CH=CH₂</td>
<td>-ribosyl</td>
<td>-H</td>
<td>isopentenyladenosine</td>
<td>iP₂</td>
</tr>
<tr>
<td>-CH₂CH=CH₂</td>
<td>-ribosyl</td>
<td>-H</td>
<td>zeatin riboside</td>
<td>ZR</td>
</tr>
<tr>
<td>-CH₂CH=CH₂</td>
<td>-ribosyl</td>
<td>-H</td>
<td>dihydrozeatin riboside</td>
<td>DZR</td>
</tr>
<tr>
<td>-CH₂CH=CH₂</td>
<td>-ribosyl-5'P</td>
<td>-H</td>
<td>isopentenyladenosine-5'-phosphate</td>
<td>iP₂-5'P</td>
</tr>
<tr>
<td>-CH₂CH=CH₂</td>
<td>-ribosyl-5'P</td>
<td>-H</td>
<td>zeatin riboside-5'-phosphate</td>
<td>ZR₂-5'P</td>
</tr>
<tr>
<td>-CH₂CH=CH₂</td>
<td>-ribosyl-5'P</td>
<td>-H</td>
<td>dihydrozeatin riboside-5'-phosphate</td>
<td>DZR₂-5'P</td>
</tr>
<tr>
<td>-CH₂CH=CH₂</td>
<td>glucosyl</td>
<td>-glucosyl</td>
<td>9-glucosylzeatin</td>
<td>Z9G</td>
</tr>
<tr>
<td>-CH₂CH=CH₂</td>
<td>glucosyl</td>
<td>-glucosyl</td>
<td>7-glucosylzeatin</td>
<td>Z7G</td>
</tr>
<tr>
<td>-CH₂CH=CH₂</td>
<td>-H</td>
<td>-H</td>
<td>0-glucosylzeatin</td>
<td>OGZ</td>
</tr>
<tr>
<td>-CH₂CH=CH₂</td>
<td>-H</td>
<td>-H</td>
<td>0-glucosylidihydrozeatin</td>
<td>ODZ</td>
</tr>
<tr>
<td>-CH₂CH=CH₂</td>
<td>ribosyl</td>
<td>-H</td>
<td>0-glucosylzeatin riboside</td>
<td>OGZR</td>
</tr>
<tr>
<td>-CH₂CH=CH₂</td>
<td>ribosyl</td>
<td>-H</td>
<td>0-glucosylidihydrozeatin riboside</td>
<td>ODZR</td>
</tr>
</tbody>
</table>
now been conclusively identified in a range of plants and from bioassay and chromatographic data, appear to be present in a great many others. Comprehensive listings of the sources of these cytokinins can be found in Letham (1978), Bearder (1980) and in Letham and Palni (1982).

As well as occurring in higher plants as free compounds, cytokinins also occur in the transfer ribonucleic acid (tRNA) of plants, animals and microorganisms, situated adjacent to the 3' end of the anticodon triplet. These component nucleosides have been identified as iPA, cis-ZR, ZR and their 2-methylthio derivatives. The major component of plant tRNA has been reported to be cis-ZR. This nucleoside, along with the 2-methylthio derivatives mentioned above, appears to be unique to tRNA and has not been shown to occur in the free form in plants (see reviews by Letham and Wettenhall, 1977; Letham and Palni, 1983).

Due to the difficulties associated with the purification and identification of the cytokinins, the scientific literature is replete with the use of bioassays. Thus, many tentative identifications and even quantifications have been made by comparing the chromatographic properties of biological activity with those of known cytokinins. The dangers inherent in the use of bioassays for these purposes are discussed in Section 2.1.1.

1.2 Metabolism of the free cytokinins.

The elucidation of the metabolic pathways of the cytokinins has been largely based on the use of exogenous radiolabelled cytokinins. Usually the exogenous compounds have been supplied in vast excess compared with the levels of the endogenous compounds. This, combined with the fact that the endogenous cytokinins may be compartmented in such a way that they do not come into contact with certain enzymes,
means that the applied compounds may not mimic the metabolic fate of the endogenous compounds. However, these studies do at least suggest the types of transformations that may be occurring with the endogenous compounds.

When applied to a plant tissue, the cytokinins are usually rapidly distributed among the respective nucleotide, nucleoside and base forms (Figure 1.2), but ultimately they are broken down by sidechain cleavage to the corresponding Ade derivatives, with irreversible loss of biological activity, or are conjugated with glucose or alanine. The formation of conjugates is a process which the cytokinins have in common with other plant hormones and xenobiotics.

Whether the enzymes that are responsible for the nucleotide-nucleoside-base interconversions are specific for the cytokinins or are perhaps the same enzymes that catalyse the analogous interconversions of AMP-Ado-Ade is not certain. A set of enzymes responsible for these types of interconversions have been isolated by Chen and co-workers. The $K_m$ of these enzymes was significantly higher for iPA-5'-P, iPA or iP, than for AMP, Ado or Ade, indicating lower affinities for the N$^6$ substituted substrates (Chen, 1981; Chen and Kristopeit, 1981a, 1981b; Chen et al., 1982) (Figure 1.2).

Biosynthetically, DZR-5'-P, DZR and DZ may be derived from the metabolic hydrogenation of the corresponding zeatin analogues. For example, Z and ZR have been converted to DZ and DZR in the embryonic axes of Phaseolus vulgaris seeds (Sondheimer and Tzou, 1971), in mature leaves of the same plant (Palmer et al., 1981a, 1981b, 1981c), and in Zea mays kernels (Summons et al., 1980). However, the ability to saturate the zeatin sidechain does not appear to be universal, as radish seedlings fail to convert Z to DZ (Parker and Letham, 1973; Gordon et
Fig. 1.2: Cytokinin biosynthesis and probable interconversion in plant tissues. The letters between compounds refer to the following enzymes: a, isopentenyltransferase; b, 5'-nucleotidase, c, adenosine nucleotidase; d, adenosine phosphorylase; e, adenosine kinase; f, adenine phosphoribosyltransferase; g, cytochrome P-450; h, unknown enzyme.
The enzymology of sidechain reduction has not been studied, but the transformation may occur at the nucleotide, nucleoside or the free base level (Figure 1.2).

In plant tissues the sidechain cleavage of Z, ZR, iP and iPA is attributed to the action of cytokinin oxidase. This enzyme was partially purified from *Zea mays* kernels, and characterised by Whitty and Hall (1974), Brownlee et al. (1975), and McGaw and Horgan (1983). The enzyme exhibited high reactivity towards iPA, iP, ZR and Z, however, DZ-type cytokinins, BAP, kinetin and the cytokinin 0-glucosides were not substrates. Cytokinin oxidase will also degrade Z7G, Z9G and lupinic acid (LA), however these compounds are very stable in vivo, indicating that they may be compartmentalised (Letham and Palni, 1983). Metabolic studies have indicated the presence of additional oxidising systems in radish and lupin seedlings and in soybean and tobacco callus where BAP is subject to sidechain cleavage, and in beans where DZ and OGDZ have also been degraded (McGaw et al., 1984a). N⁶-Dealkylation has also been detected in the microsomal fraction of cauliflowers, though the enzyme responsible is, as yet, uncharacterised (Chen and Leisner, 1984).

The conjugation of cytokinins with glucose is a commonly observed fate of both exogenous and endogenous cytokinins. All the glucosides seem to be β-D-glucopyranosides, with the sugar linked to N-3, N-7 or N-9 of the purine nucleus or to the oxygen of the zeatin sidechain (Letham, 1978). O-Glucosylation is mediated by, as yet, uncharacterised enzymes to yield OGZ, OGZR, OGDZ and OGDZR. This form of conjugation confers resistance to degradation by cytokinin oxidase (as does sidechain saturation).
The enzymology of N-glucosylation has been studied in extracts of radish cotyledons. Two enzymes were detected which converted BAP into 7- and 9-glucosides, one of which formed the 7-glucoside predominantly, while the other produced the two glucosides in similar proportions. A wide variety of naturally-occurring and synthetic cytokinins were reported as substrates, all accepting a glucose molecule from uridine diphosphate glucose (UDPG) in the presence of the major enzyme (Entsch and Letham, 1979; Entsch et al., 1979).

Conjugation of the cytokinins may also occur with alanine at the 9-position of the purine nucleus to yield lupinic acid (L-3-(zeatin-9-yl)alanine) and dihydrolupinic acid (DLA) (Parker et al., 1975; Macleod et al., 1975; Duke et al., 1978). The responsible enzyme, β-(9-cytokinin)alanine synthase, has been purified from lupin seeds, and kinetic studies have established a ping pong bi bi mechanism (Cleland nomenclature) (Entsch et al., 1983).

The ability of other hormones to influence cytokinin metabolism may be a significant feature of plant development. Thus, it is noteworthy that ethylene reduced the ability of cauliflower microsomal cytochrome P-450, to effect the trans hydroxylation of iP or iPA (Chen and Leisner, 1984); that ABA suppressed reduction of the Z sidechain (Sondheimer and Tzou, 1971); that ABA inhibited the conversion of kinetin to kinetin nucleotide in germinating lettuce seed (Miernyk, 1979); and that IAA competitively inhibited the alanine conjugation of Z by β-(9-cytokinin)alanine synthase (Parker et al., in press 1985). Thus, control of cytokinin biosynthesis, metabolism and inactivation may be exerted by a combination of one or more of the following; variations in the activities of enzymes, subcellular compartmentation, by translocation into and out of tissues, and by the mediation of other hormones.
1.3 Structural requirements for cytokinin activity.

The synthesis and testing of compounds for cytokinin activity resulted in the discovery of clear relationships between molecular structure and cytokinin activity. The most active of the naturally-occurring cytokinins in cell division bioassays has proved to be zeatin (Letham, 1967; Skoog et al., 1967).

Using the tobacco bioassay, Skoog and co-workers (1967) found that the cytokinin activity of substituted purines was limited to those compounds with a substituent at the 6-position. The 1-, 3-, 7- and 9-monosubstituted adenines proved to be inactive as cytokinins. Of the 6-substituted adenines the strongest activity was elicited by an alkyl chain of five carbon atoms attached to the purine moiety through a nitrogen atom (Skoog et al., 1967; Skoog and Armstrong, 1970). When a large alkyl group (e.g., C_{10}) is present, the N^6-substituted adenine is essentially inactive. Linkage of the sidechain through -S-CH_{2}-, -O-CH_{2}-, -CH_{2}-CH_{2}- or -NH-CO-, rather than the preferred -NH-CH_{2}-, resulted in a diminution of activity (Henderson et al., 1975). Additional substitutions around the purine moiety (e.g., alkyl substituents at positions 3 and 9) usually caused a loss of cytokinin activity except for 2-Cl and 8-methyl groups (Matsubara, 1980). Similarly, modification to the heterocyclic nucleus drastically lowered cytokinin activity or completely eliminated it (Skoog et al., 1967; Skoog and Armstrong, 1970; Matsubara, 1980).

Among the purine derivatives with an N^6-substituent of optimal size, a considerable range of activity could be obtained, depending on the functionalisation of the sidechain. A comparison of the cytokinin activities of 6-(3-methylbut-2-enylamino)purine with its saturated analogue 6-(3-methylbutylamino)purine, of zeatin with dihydrozeatin, and
of other closely-related pairs of compounds, showed that a double bond in the sidechain at the 2-position greatly increased cytokinin activity in the tobacco bioassay (Skoog et al., 1967). The formal introduction of a hydroxyl group in the sidechain of the N\textdegree_6-substituted adenines also affected the cytokinin activity. A hydroxyl group at the 4-position of either the isopentyl or 3-methylbut-2-enyl sidechain enhanced activity, but in the 2-, 3-, or 2- and 3-positions of the isopentyl sidechain, it lowered the activity ten fold (Leonard et al., 1969). The configuration of the 4-hydroxy group in the sidechain modified the cytokinin activity, the trans-forms of zeatin, isozeatin, and 2-methylthiozeatin being more active than the corresponding cis-forms (Hecht et al., 1970; Leonard et al., 1971; Schmitz et al., 1972; Vreman et al., 1974).

The R(+) and naturally-occurring S(-)dihydrozeatin were found to have similar activities in the tobacco bioassay, however, the R(+)DZ was found by Matsubara et al. (1977) to be more effective in the lettuce seed germination assay and in the radish cotyledon assays. Similarly, the synthetic R(+)DZR was found to be more active than the naturally-occurring S(-)DZR in these bioassays.

Hecht et al. (1970) considered that sidechain planarity was important to cytokinin activity. If planarity was decreased by adding substituents to the double bond of an acyclic sidechain, cytokinin activity was significantly reduced. The loss of planarity may also explain the loss of cytokinin activity observed in the saturated analogues of 6-benzylaminopurine (6-cyclohexylmethylaminopurine) (Skoog et al., 1967) and 6-furfurylaminopurine (6-tetra-hydrofuranylaminopurine) (Matsubara, 1980).

Certain synthetic O-acyl zeatins exhibit slightly greater activity than zeatin itself in some bioassays (Schmitz et al., 1971; Letham,
This was thought to be due to a steady release of zeatin by hydrolysis of the O-acyl group, maintaining relatively high levels of zeatin which may otherwise have been metabolised to an inactive form (Schmitz et al., 1971).

Thus, the expression of cytokinin activity would seem to be dependent on the dimension, distribution of polar groups and on the planarity of the N6-substituent of the purine ring.

Against this background of structure-activity relations for synthetic compounds, the activities of cytokinin metabolites may now be discussed. Among the naturally-occurring cytokinins, substitution with ribose, or ribose-5'-monophosphate at the 9-position of the purine, causes an appreciable loss of biological activity in a number of bioassays (Letham, 1978). Substitution with either glucose or alanine at this position results in an almost complete loss of cytokinin activity (Letham et al., 1983). Although the cytokinin ribosides are readily metabolised to either base or nucleotide, Z9G and the 9-alanine conjugate of zeatin (lupinic acid) are very stable metabolites in plant tissues and their lack of activity may be a reflection of the inability of most plant tissues to hydrolyse these substituents (Letham et al., 1983). Cytokinin-7-glucosides are similar to the cytokinin-9-glucosides in their lack of biological activity and their stability in plant tissue (Letham et al., 1983).

Since 3-alkyl substituted cytokinins are essentially inactive (see above), the high activity of BAP-3-G in diverse bioassays is probably due to cleavage of the 3-glucoside moiety to release the free base (Letham et al., 1983; Tao et al., 1983; Letham and Gollnow, 1985).

With the addition of an O-glucosyl moiety to the N6-sidechain of Z and ZR, one would expect to abolish cytokinin activity (see above).
However, Letham et al. (1983) have reported OGZ and OGZR to have similar activities to those recorded for Z and ZR in the radish cotyledon bioassay, tobacco callus bioassay and the Amaranthus betacyanin bioassay. In the oat leaf senescence bioassay, the activity of OGZ exceeded that of Z. As the O-glucosides are unlikely to be active per se (see above), Letham et al. (1983) suggested that the O-glucosyl moiety may have protected the sidechain from cleavage by cytokinin oxidase activity during transport to the subcellular site of action, whereupon the glucosyl residue was hydrolysed by a β-glucosidase enzyme to release the active cytokinin Z, DZ, ZR or DZR.

1.4 Cytokinin biosynthesis.

1.4.1 Biosynthesis of tRNA-cytokinins.

The formation of tRNA-cytokinins is, in contrast to the biosynthesis of free cytokinins, well established. As with other RNA base modifications, \( N^6 \)-alkylation occurs following transcription (Chen and Hall, 1969). By demonstrating the incorporation of radio-labelled precursors into tRNA in cell-free systems, it was found that the sidechain donor is isopentenyl pyrophosphate which was, in turn, derived from mevalonic acid (Murai et al., 1975; Burrows and Fuell, 1981).

Substitution of the methylthio group at the 2-position of the purine ring has been found to occur after \( N^6 \)-substitution in the tRNA, with the sulphur originating from cysteine and the methyl group being donated by methionine (Gefter, 1969; Gefter and Russell, 1969; Skoog and Armstrong, 1970). However, little seems to be known about the hydroxylation of the \( N^6 \)-sidechain, neither the stage at which it occurs, nor the stereospecificity of the enzyme or enzymes involved (Letham and Palni, 1983).
1.4.2 Biosynthesis of free cytokinins

A considerable quantity of circumstantial evidence supports the argument for the free cytokinins being derived from de novo synthesis rather than as a consequence of the degradation of the tRNAs which contain a cytokinin component. Strong evidence against tRNA being the source of the free cytokinins is the chemical identity of the particular cytokinins found both free and in tRNA. Cis-ZR occurs rarely, if at all, as a free compound, but in plants it is the predominant cytokinin of tRNA. On the other hand, ZR (trans isomer) is rarely found in plant tRNA, and all the free cytokinins of plants appear to have the trans configuration of the sidechain. Similarly, in plants, the methylthio derivatives of cis-ZR, ZR and iPA are found only in the tRNA (see review by Letham and Palni, 1983). Furthermore, the reported overall rates of tRNA turnover appear to be too low to support the known concentrations of free cytokinins (Trewavas, 1970; Hall, 1973), although it is possible that the tRNAs containing cytokinins are turned over faster than others, as has been observed in some animal tissues (Borek et al., 1977). Finally, it has been reported by Chen and Hall (1969) and by Burrows (1976) that exogenous cytokinins are required for the growth of cytokinin-dependent tobacco callus tissue, despite the fact that the tRNA of this tissue contains iPA.

The circumstantial evidence for de novo synthesis of the free cytokinins has been provided by the capacity of cell free systems, derived from higher plants (Chen and Melitz, 1979; Nishinari and Syono, 1980c) and from microorganisms (Murai, 1981; Taya et al., 1979), to synthesize cytokinins. These cell-free systems converted adenosine-5'-monophosphate (AMP) and isopent-2-enylpyrophosphate (iPP) into isopentenyladenosine-5'-monophosphate (iPA-5'-P). The enzyme
responsible, isopent-2-enylpyrophosphate:5'-AMP-isopentenyltransferase (isopentenyltransferase) (EC 2.5.1-), has been partially purified from cytokinin-autonomous tobacco callus (Chen and Melitz, 1979) and studies of substrate specificity (Chen, 1982) determined that neither adenine nor adenosine could serve as substrates for the enzyme.

*Vinca rosea* crown-gall tumour tissue has also been reported as capable of synthesising cytokinins (Stuchbury *et al*., 1979; Palni *et al*., 1983). When fed radiolabelled Ade, radioactivity was rapidly incorporated into ZR-5'-P, and to a lesser degree ZR and Z, such that a pathway via tRNA hydrolysis seemed unlikely. However, no radioactivity was found to be incorporated into iPA-5'-P, iPA or iP, the presumed intermediates in the biosynthesis of the Z-type cytokinins. It was subsequently determined that the stereospecific trans-hydroxylation proceeded very rapidly in this tissue (Palni and Horgan, 1983) such that the steady state concentrations of the iP-type cytokinins were very much lower than those of the Z-type cytokinins (Scott *et al*., 1982).

This stereospecific hydroxylation of the sidechain has also been observed in *Rhizopogon roseolus* and *Zea mays* kernels by Miura and Hall (1973). A recent report by Chen and Leisner (1984) indicated that the plant cytochrome P-450 (isolated from *Brassica oleracea*) may be the site for the hydroxylation of iP and iPA to Z and ZR respectively. The hydroxylation reaction is probably of general occurrence in plants which produce Z-type cytokinins and would represent the final step in the biosynthesis of ZR-5'-P from AMP and iPP via iPA-5'-P (see Figure 1.2).

The isolation and characterisation of isopentenyltransferase and the biosynthetic studies using *Vinca rosea* crown-gall tissue, described above, suggest that the biosynthesis of free cytokinins occurs at the nucleotide level and confirms some of the previous reports of *in vivo*
conversion of precursors to cytokinins (see review by Letham and Palni (1983). However, the induction and maintenance of crown gall disease in *Vinca rosea* is dependent upon the integration into the plant genome and expression of a DNA fragment from the Ti plasmid of *Agrobacterium tumefaciens*. This DNA fragment has been found to encode isopentenyltransferase (Morris *et al.*, 1982; Barry *et al.*, 1984). Thus, the experiments with the bacterially-transformed tumour tissue described above, may have little relationship to the cytokinin biosynthesis that occurs normally in higher plants. Similarly, the enzyme, isopentenyltransferase, was purified from a strain of tobacco tissue initially isolated by Fox (1963) who considered that the tissue had arisen following an unspecified spontaneous change to the genome.

In summary, there appear to be separate biosynthetic pathways to the tRNA cytokinins and to the free cytokinins, although the evidence is largely circumstantial.

### 1.4.3 Sites of biosynthesis of the free cytokinins

**Roots.** It is generally accepted that roots (probably the apices) are sites of cytokinin biosynthesis, although, no direct demonstration of this by labelled precursor incorporation had been reported until very recently (Chen *et al.*, 1985). Cytokinins have been extracted from the roots of many plants and their presence demonstrated in the xylem exudate of decapitated plants of diverse species (see compilation of references in review by van Staden and Davy, 1979).

Further evidence indicating that roots are a site of cytokinin biosynthesis is as follows: Firstly, the levels of cytokinins isolated in the xylem exudate of a number of plants have been shown to be dependent on the environmental conditions of the root. Thus, the stresses of salinity, heat, flooding and water deficiency all result in
the reduction of hormone production (see reviews by Skene, 1975; Torrey, 1976). Secondly, in studies of cytokinins in the leaf-cuttings of Phassolus vulgaris, Englebrecht (1972) observed marked rises in the cytokinin levels of petioles, corresponding to the appearance of roots. This was followed by increased cytokinin levels in the leaf blades and the reversal of the loss of chlorophyll and protein, associated with senescence. Thirdly, exogenous cytokinins may often completely or partially substitute for the root in producing a response in the shoot of seedlings and cuttings (Goodwin et al., 1978). For example, exogenous cytokinin was found to completely restore the coleoptile growth rate in derooted Avena sativa seedlings and to stimulate auxin production in excised coleotile tips (Jordan and Skoog, 1971). Fourthly, the level of cytokinins in sunflower xylem exudate did not decline appreciably during 4 days after detopping (Kende and Sitton, 1967). Finally, rice root tips (Yoshida and Oritani, 1974), maize and tomato roots (van Staden and Smith, 1978), and tomato root tips (Koda and Okazawa, 1978), when cultured under sterile conditions, all released cytokinin into the medium and appeared to produce free cytokinin actively. Thus, when tomato root tips were subcultured 8 times, the cytokinin level in the 8 media did not vary significantly (Koda and Okazawa, 1978).

A number of studies indicate that the cytokinin level in root apices is very much greater than that in more proximal regions of the root (see Letham, 1978). Hence, the root tip may well be the site of cytokinin biosynthesis. The studies of Feldman (1979) indicate that cytokinin biosynthesis in the maize root is the consequence of interaction between the quiescent centre and the proximal meristem. Although the root apex is a likely site of cytokinin biosynthesis, it is
not clear when the root becomes capable of such synthesis during its development.

*Other biosynthetic sites in the developed plant.* Evidence that cytokinin biosynthesis may not be limited to the roots was provided by Chen and Petschow (1978). They reported the biosynthesis of IP, iPA, Z and ZR in cultured rootless tobacco plants fed $[^{14}\text{C}]-\text{Ade}$. However, the participation of root primordia could not be definitely excluded. Peterson and Fletcher (1973) found an appreciable degree of fruit and seed development despite root removal from tobacco, tomato, pea and bean plants. This suggested that the developing fruits and seeds probably did not depend exclusively on the roots for their supply of growth regulators. However, the possibility that cytokinin may have been derived from the stems and/or the mature leaves was not excluded. For example, it is known that OGDZ is accumulated in bean leaves, and although not an active cytokinin *per se*, it might act as a storage form of cytokinin (Section 1.5).

Developing buds and the shoot apex are possible sites of cytokinin biosynthesis (Letham, 1978; Koda and Okazawa, 1980), but discussion of this is beyond the scope of this thesis.

*Developing seeds.* Developing seeds are rich sources of cytokinins and the levels of cytokinins follow the pattern of seed growth, reaching a maximum in the early stages of development and decreasing with maturity (Letham, 1963b; Burrows and Carr, 1970; Letham, 1978). The degree to which this accumulation results from biosynthesis and/or importation from neighbouring tissue is open to question. Blumenfeld and Gazit (1970b, 1971) concluded that the embryo of the avocado seed was a site of cytokinin biosynthesis, based on the high levels of cytokinin found in the embryo and the fact that cytokinins
could be extracted from cotyledonous callus grown on cytokinin-free media. In an attempt to obtain direct evidence for cytokinin biosynthesis in seeds, Hahn et al. (1974) cultured ten day-old pea pods in vitro on a cytokinin-free medium. The pea seeds developed normally to full maturity and were reported to have accumulated 3 ng of iP equivalents per seed over 18 days. However, these findings were contradicted by Krechting et al. (1978) who indicated firstly, that the cytokinin in the seed could have been transported into the seed from the pod walls and secondly, that the cytokinin content of the seeds in vitro was insignificant compared with the accumulation observed in normally-developed seeds. More substantial evidence for cytokinin biosynthesis in the developing seed has been provided by Summons et al. (1981) who reported the biosynthesis of OGDZ in lupin seeds incubated with [3H]-Ado in vitro for 75 hours. In complementary studies of translocation, it was found that radiolabelled cytokinins supplied to the stems of derooted lupin (Summons et al., 1981) and soybean (Nooden and Letham, 1984) did not move appreciably to the developing seed. In the soybean study, it was further noted that the pods do not compete with the leaves for the supply of cytokinin in the xylem and that the cytokinin transported to the leaves was not redistributed to the seeds. It has also been reported that immature soybean embryos grown in culture do not require exogenous cytokinin (Thompson et al., 1977; Egli and Wardlaw, 1980; Hsu and Obendorf, 1982). Thus, the balance of evidence available indicates that cytokinin biosynthesis is probably significant in developing seeds and that only a small proportion, if any, of the cytokinins accumulated may be root-derived.
Germinating seed. Another possible site for cytokinin biosynthesis is the germinating seed. Not only have germinating seeds been found to yield appreciable cytokinin activity (Letham, 1978), but cytokinin seems to be required in some dicotyledonous seeds for the production of a number of enzyme activities (amylolytic and proteolytic) required for reserve mobilisation. This has been suggested in a number of studies where the effect of the embryonic axis in promoting enzyme activity in the cotyledons could be largely replaced by applications of exogenous cytokinin and not by GA or IAA (Table 1.1 and references therein). The implication of these findings is that cytokinin produced in the axis diffuses out to the cotyledons to effect their promotive activity. Hence, in these plants the axis appears to be a site of cytokinin biosynthesis.

1.5 The significance of cytokinin metabolism in relation to plant development.

The significance of the metabolism of the cytokinins into the diverse array of products that are known to exist in plants (see Section 1.2) must remain speculative subject to the acquisition of much more data, particularly as regards compartmentation and turnover of the endogenous compounds. However, metabolism could be important in the control of the relative levels of active cytokinins. Cytokinin metabolism has been studied carefully in relation to growth in some systems and from this work limited conclusions can be reached regarding the role of some metabolites.
Table 1.1: The ability of exogenous cytokinins and gibberellin to replace the embryonic axis in promoting enzyme activities in cotyledons

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cotyledons</th>
<th>Degree of replacement of embryonic axis&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Effect of cytokinin on cotyledons with attached axis&lt;sup&gt;b&lt;/sup&gt;</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P (kinetin)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amylase</td>
<td>bean (Phaseolus vulgaris)</td>
<td>C (BAP)</td>
<td>N</td>
<td>Metivier and Paulilo, 1980</td>
</tr>
<tr>
<td>Proteolytic</td>
<td>bean (Phaseolus vulgaris)</td>
<td>C (kinetin or zeatin)</td>
<td>N</td>
<td>Gepstein and Ilan, 1980.</td>
</tr>
</tbody>
</table>

<sup>a</sup>C, complete replacement of axis.
<sup>b</sup>P, partial replacement of axis.
N, no appreciable effect.

<sup>b</sup>a, not recorded.
N, no appreciable effect.
R, effect much reduced relative to effect observed with excised cotyledons.
**Tobacco cells.** In studies of the dynamics of cytokinin metabolism in tobacco cells, Laloue and co-workers (Gawer et al., 1977; Laloue et al., 1977; Laloue, 1977; Laloue and Pethe, 1982) determined that exogenous cytokinin was taken up by cells in the base form, and that once in the cell, it was rapidly accumulated as the cytokinin nucleotide and cytokinin-7-glucoside. The cells were essentially impermeable to both the nucleotide and 7-glucoside. The formation of the 7-glucoside was effectively irreversible and the compound was biologically inactive. The cytokinin-7-glucoside was accumulated over a wide range of exogenous cytokinin concentrations, suggesting that it was not formed as a consequence of toxic levels of cytokinin. The cytokinin nucleotide was considered to be a sequestered form of cytokinin, incapable of diffusing out of the cell, but subject to hydrolysis to yield the riboside and base (likely active forms of the cytokinins) or subject to rapid sidechain cleavage in the case of the iP nucleotide.

**Bean plants.** In the context of the whole plant, certain functional roles seem to be indicated for some of the cytokinins. In an integrated series of studies, Waring, Horgan and co-workers (Waring et al., 1977; Wang and Horgan, 1978; Palmer et al., 1981a, 1981b, 1981c) have identified the endogenous cytokinins in the leaves and stem segments of Phaseolus vulgaris and studied their transport and metabolism. ZR, DZR and their respective nucleotides were identified in the stems of decapitated, disbudded plants as the endogenous cytokinins and also as the products of [14C]-Z fed to the roots of whole plants, suggesting that in the bean plant, cytokinins may be transported to the leaves as ribosides and/or nucleotides. In the leaves, the major endogenous cytokinin was found to be OGDZ, together with trace quantities of DZR, suggesting that the DZR could be the precursor of the
OGDZ. This was also suggested by the fate of exogenous $[^{14}\text{C}]$-Z which, when fed to the roots of the bean plant, was recovered in the leaves as OGDZ. In disbudded, decapitated bean plants, endogenous OGDZ was reported to accumulate to very high levels (500 ng/g) in the leaves. This large accumulation did not occur in detached leaves, in leaves of intact plants, or in leaves of plants that were decapitated but not disbudded. When secondary lateral buds were allowed to grow, the levels of OGDZ rapidly declined to that found in the leaves of intact plants (100 ng/g). A similar loss of OGDZ was observed in detached leaves suggesting that the reduction in OGDZ levels may be due to metabolism, rather than to export of the glucoside itself. Hence, OGDZ may represent a storage form of cytokinin in the bean plant. Despite the accumulation of OGDZ in mature bean leaves of the intact plant, this cytokinin did not prevent senescence, suggesting compartmentation in the leaf, especially since exogenous OGDZ delays senescence of leaf discs. Waring, Horgan and colleagues have also noted, that due to $\text{N}^6$-dealkylation and the conversion of Z to DZ in the stem, the plant maintained a decreasing concentration gradient of Z derivatives and an increasing gradient of DZ derivatives from the roots to the leaves. Thus, the contribution of the stem to the cytokinin economy of the bean plant may be more than a simple channel for root-produced material, however, the functional significance of these gradients is unknown.

**Radish seedlings.** In radish seedlings, cytokinins induce the expansion of excised immature cotyledons and delay the onset of senescence in mature cotyledons. Letham and colleagues have examined the cytokinin metabolism associated with these phenomena (Letham et al., 1982; Letham and Gollnow, 1985).
The metabolism of exogenous cytokinin in *Raphanus sativus* tissue has proved relatively simple, the predominant metabolites being the N-glucosides. Little oxidative sidechain cleavage occurs (see Table 1.2 and references therein). Exogenous cytokinin-3-glucosides and O-glucosides were found to be biologically active, but the activity was attributed to released free base, since BAP-3-G was found to be metabolised to BAP, BAP-7-G and BAP-9-G, while OGZ yielded Z and Z7G. BAP-7-G and BAP-9-G proved to be extremely stable metabolites, both in radish cotyledons and seedlings. With the onset of senescence, there was an associated decline in the cotyledon's ability to produce cytokinin-7-glucosides and -3-glucosides, and an increase in oxidative sidechain cleavage. The decline in production of the 7-glucosides was thought to be due to a loss of activity of the major enzyme responsible for N-glucosylation (see Section 1.2). The ability of a tissue to carry out N-3 glucosylation of the cytokinins could be associated with its capacity to respond to cytokinin by exhibiting senescence retardation. This seemed to be the case, not only for radish cotyledons, but also for oat leaf segments where BAP-3-G has been identified as a significant senescence-retarding compound (Tao *et al.*, 1983).

Letham and Gollnow (1985) found that cotyledon expansion could be related to the levels of BAP in the base, riboside and nucleotide forms, but not to the levels of the N-glucosides in the same tissue. This was determined by transferring expanding cotyledons from cytokinin-containing media to cytokinin-free media and observing the rapid drop in the levels of the nucleotide/riboside/base forms of the cytokinin in conjunction with the cessation of growth, despite the presence of the accumulated N-glucosides. Glucosylation at N-3, -7 and -9 occurred at BAP concentrations far below the optimum for growth. Hence these
### Table 1.2: Metabolism of cytokinins in radish tissue (Raphanus sativus L. cv. Long Scarlet)

<table>
<thead>
<tr>
<th>tissue</th>
<th>supplied cytokinin</th>
<th>products</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>cotyledon (mature)</td>
<td>Z7G</td>
<td>Ade-7-G</td>
<td>Letham et al., 1982.</td>
</tr>
<tr>
<td>cotyledon (mature)</td>
<td>Z (high levels)</td>
<td>Z7G (OGZ)</td>
<td>Letham et al., 1982.</td>
</tr>
<tr>
<td>cotyledon (senescent)</td>
<td>ZR</td>
<td>Z7G, Ado, OGZ</td>
<td>Letham and Gollnow, 1985.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ZR-5'-P, Ado-5'-P</td>
<td></td>
</tr>
<tr>
<td>seedling</td>
<td>BAP</td>
<td>BAP-3-G, -7-G, -9-G</td>
<td>Letham et al., 1975.</td>
</tr>
<tr>
<td>derooted seedling</td>
<td>iP</td>
<td>iP-7-G, OGZ</td>
<td>McGaw et al., 1985.</td>
</tr>
<tr>
<td>derooted seedling</td>
<td>OGDZ</td>
<td>DZ, DZ-3-G, -7-G</td>
<td>McGaw et al., 1985.</td>
</tr>
<tr>
<td>derooted seedling</td>
<td>Z (high levels)</td>
<td>Z7G (OGZ, ZR)</td>
<td>McGaw et al., 1985.</td>
</tr>
</tbody>
</table>
glucosides were not detoxification products. Furthermore, the acceptance of a 7-glucosyl residue by an adenine moiety does not seem to be related to cytokinin activity as BAP analogues, such as 2-(2-hydroxyethylamino)BAP, 2-benzylaminopurin-6-one and 3,4-dimethoxyBAP, which are weakly active or essentially inactive as cytokinins have been reported to be converted to 7-glucosides in derooted radish seedlings (Letham et al., 1978b; Letham et al., 1982). However, as neither adenine or 6-methylaminopurine were glucosylated by derooted radish seedlings, these authors concluded that 7-glucosylation was dependent on an N\(^6\)-substituent of 5-7 carbons but not on cytokinin activity per se.

**Germinating lettuce seeds.** Miernyk and Blaydes (1977) incubated lettuce seeds with \(^{14}\)C-kinetin and identified the primary metabolite as kinetin nucleotide, together with a low level of the nucleoside. A similar metabolic conversion was observed for exogenous BAP (Pietraface and Blaydes, 1981). As germination proceeded, the level of nucleotide decreased as the level of free kinetin increased. It was noted that there was a marked rise in the riboside following 8 hours of incubation. This precedes the onset of cell division which occurs from about 12 hours. It was suggested that the cytokinin nucleotide may serve as a storage form, with conversion to the nucleoside, as needed, during germination (Miernyk and Blaydes, 1977; Pietraface and Blaydes, 1981). The function of the cytokinin nucleotide during lettuce seed germination seems to be similar to the function of the cytokinin nucleotide in tobacco cell culture as postulated by Laloue and Pethe (1982) (see discussion above). It may be a sequestered cytokinin unable to move out of the cell, but subject to hydrolysis to the riboside and free base forms.
Miernyk (1979) observed that a simultaneous treatment of the lettuce seeds with ABA and kinetin resulted in an apparently specific inhibition of the conversion of the exogenous kinetin to kinetin nucleotide. The ABA did not inhibit the conversion of exogenous Ade to AMP. This inhibition may reflect an actual hormonal interaction controlling seed germination.

In summary, the study of cytokinin metabolism in tobacco cells suggested that the cytokinin base was the active form of the phytohormone. Nucleotides have been generally observed to be accumulated following the uptake of exogenous cytokinin and the uptake of cytokinin bases may be dependent on phosphoribosylation (Letham and Palni, 1983). In the tobacco cell and lettuce seed studies, it was thought that the nucleotides may also provide a short term storage form of cytokinin sequestered within the cell. The evidence for a storage role for the O-glucosides seems to be more substantial. Cytokinins often accumulate in tissues as the O-glucosides and it is known that, when supplied exogenously to plant tissue, the glucose moiety may be hydrolysed to release the cytokinin bases and ribosides (Summons et al., 1980; Palmer et al., 1981a; Letham and Palni, 1983). In addition, the levels of the O-glucosides have been seen to fall with the onset of growth, for example, during the lateral bud development in bean plants (Palmer et al., 1981a) and during the germination of maize seed (Smith and van Staden, 1978). The stability and biological inactivity of the cytokinin-7-glucosides and -9-glucosides and the 9-alanine conjugates indicates a form of inactivation that may complement the action of cytokinin oxidase-type enzymes in controlling the level of cytokinin activity. However, in tobacco cells and in radish plants, N-glucosylation is the major form of metabolism even in the presence of
suboptimal quantities of cytokinins (see above). The ribosides, on the current evidence, are a major translocational form of cytokinin in the xylem (Letham and Palni, 1983).

Before designing further studies of cytokinin metabolism, it is important to reassess the apparent role of cytokinin in plant development. Metabolism studies might then be planned to provide insight into the control of plant development.

1.6 The regulatory role of cytokinins in the developed plant.

When applied to whole plants, plant tissues and plant organs the cytokinins produce a large range of physiological effects, many of which may also be at least partially evoked by the gibberellins, auxin or ethylene. The ability of the phytohormones to produce such effects has invited speculation, among plant physiologists, that these phenomena may be controlled by the endogenous compounds. However, the extent of the regulation by phytohormones is usually obscure, as experiments with exogenous phytohormones are difficult to interpret without a comprehensive knowledge of the biochemistry of the endogenous compounds: sites of production, movement in xylem and phloem, sites of action, metabolic fate and compartmentation at the subcellular level. Additional difficulties are encountered in the interpretation of experiments on isolated organs (e.g. derooted seedlings, detached leaves and excised embryonic axes) as the observed physiological responses may be artifacts of excision, perhaps due to the loss of some controlling mechanism or reduced supply of water and nutrients. Nevertheless, from the available evidence, cytokinins appear to regulate some aspects of normal plant growth. Their roles in the developed plant which appear reasonably certain are indicated very briefly in this section, and those
in seed germination and early seedling development are discussed in
greater detail in the next section (1.7).

1.6.1 Cell division and organogenesis

In tissue culture studies, cytokinins are characteristically
associated with induction of cell division, and in conjunction with
auxins, can be used to stimulate the formation of roots (low
cytokinin/auxin ratios) or shoots (high cytokinin/auxin ratios) (Skoog
and Miller, 1957). However, there is no unequivocal evidence that these
processes are under the control of the endogenous cytokinins in normal
higher plants (crown gall tissues are probably an exception), although
it is known that cytokinins occur in high levels in meristematic
tissue. For Torrey (1976) this posed an interesting dilemma, '....cells
require cytokinin in order to divide, but actively dividing populations
of cells seem to be the source of cytokinins'. Increased levels of
endogenous cytokinins have also been associated with an increase in the
mitotic activity of synchronously-dividing tobacco cells (Nishinari and
Syono, 1980a, 1980b); however, the induction of synchrony has been
reported to generate metabolic artifacts in some instances (Mitchison,

Some observations suggest that auxin-cytokinin balance regulates
bud development, not only in tissue cultures, but also in normal
situations. Thus, the enhanced bud formation observed in the OVE
mutants of the moss Phycopertrella patens is associated with a 25 to
100-fold increase in the level of endogenous iP over that found in the
wild type (Wang et al., 1981). From a series of studies with Begonia
plants and cuttings (Heide, 1972 and numerous references therein;
Zieslin et al., 1984) it was found that a reduction of temperature or
day length elevated the endogenous cytokinin levels and lowered auxin
levels (both determined by bioassay) and also induced bud formation. Under non-inductive conditions (long days or higher temperatures), exogenous cytokinin was also found to induce bud formation. A low exogenous auxin/cytokinin ratio mimiced low temperature or short days and a high ratio had the same effect as high temperature or long days. Studies of the effects of exogenous auxin and cytokinin on epiphyllous bud formation in *Bryophyllum* under inductive and non-inductive photoperiods also support the concept of control of bud formation by auxin-cytokinin interaction (Skoog and Schmitz, 1979).

### 1.6.2 Root-shoot interactions

Several aspects of shoot development, both in developing seedlings and mature plants, are affected markedly by the roots and there is experimental evidence that these phenomena are mediated by cytokinins which are known to be biosynthesised in the roots and which are present in significant quantities in the xylem sap (see Section 1.4.3). Cytokinins often completely substitute for the effect of root on shoot development and physiology, and sometimes gibberellins act in this way. Reduction in the level of root-produced cytokinins in the xylem sap appears to be a key factor which leads to leaf senescence (Goodwin *et al.*, 1978; Stoddart and Thomas, 1982; Sexton and Woolhouse, 1984; Nooden, 1984). This process may be reversed by topical applications of cytokinin (Goodwin *et al.*, 1978; Nooden *et al.*, 1979), or in the case of excised leaves of *Phaseolus vulgaris*, by the development of roots along the petiole (Englebrecht, 1972). This root development is associated with an accumulation of cytokinin in the leaves.

While there is considerable evidence that root-derived cytokinin plays an important role in control of leaf senescence, more equivocal evidence suggests that root-produced cytokinin may also regulate other
aspects of shoot development, for example, lateral bud development, branching of the inflorescence, DNA synthesis in the shoot apex and development of the activities of photosynthetic enzymes (Goodwin et al., 1978).

1.7 Role of cytokinins in seed germination and early seedling development

Physiologically, a seed which germinates immediately when provided with adequate water and oxygen at optimal temperatures is termed quiescent or non-dormant, and seeds which require some additional factor before germinating are defined as dormant. Dormancy has evolved as a mechanism of delaying germination until conditions are favourable for the establishment of the seedling and is a common phenomena in plants which grow in regions with pronounced seasons.

The mechanisms by which dormancy is mediated are poorly understood. In the case of seed coat imposed dormancy, the dormancy may be overcome by mechanical disruption of the seed coat, while other seeds may be released from their dormant state by such environmental triggers as light and temperature (stratification) or by storage for a period in the dry state (after-ripening). The sensors by which the seed recognises and interprets its environment are virtually unknown and although phytochrome seems to be implicated by its reaction to light of certain frequencies, the way this initiates germination is unclear.

The germination process is generally accepted as commencing with the inhibition of water, culminating in the emergence of the radicle through the outer seed covering. The pattern of water uptake is generally triphasic. There is an initial rapid uptake of water followed by a lag period. Dead and dormant seeds also experience this pattern of
water uptake but unlike germinating seeds do not enter the third phase which is associated with growth of the axis. Protein and RNA synthesis begins shortly after the commencement of inhibition. Cell division and DNA synthesis follow at about the time of germination. The subsequent growth and development of the seedling is then dependent, firstly, on the hydrolysis and mobilisation of the seed reserves and, later, on the development of adequate roots and photosynthetic apparatus (Bewley and Black, 1978, 1982; Mayer, 1980/81; Berrie, 1984).

1.7.1 Phytohormonal involvement in dormancy and germination.

It has been postulated that the phytohormones may have a role in the imposition of dormancy in seeds, the release of seeds from dormancy, in the mobilisation of the seed reserves and in the subsequent development of the seedlings. These suggestions have been based on the responses of seeds to the applications of both naturally-occurring and synthetic phytohormones. However, it has not always been possible to correlate the physiological status of the seed with its endogenous hormone content. Furthermore, the interpretation of results is complicated by the fact that very high non-physiological doses of phytohormones may have been applied. Despite these qualifications, cytokinins are now generally accepted as important regulators of dormancy, germination and seedling development, along with gibberellins and a variety of inhibitors such as abscisic acid. Ethylene, with some similar properties to the cytokinins, has also been implicated (Khan and Tao, 1978; Khan, 1980/81; van Staden, 1982; Waring, 1982; Khan and Samimy, 1982).

The concept that germination is controlled by interaction between the phytohormones is based on the responses of seeds to exogenous phytohormones applied singly, in combination, or in sequence. A general
scheme for these interactions has been put forward by Khan (1971, 1975). It was proposed that GA was essential for germination and that the effects of inhibitors, if present, were countered by cytokinin. Thus, dormancy in seeds could be imposed, both by the presence of inhibitors and by the absence of promotors, such as gibberellins or cytokinins. Similarly, germination could occur, not only in the presence of gibberellins, but also in the presence of inhibitors if cytokinin were present to counter the action of inhibitors (Table 1.3).

Table 1.3: The dependence of seed status on endogenous phytohormones.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Seed</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA</td>
<td>CK</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The role of cytokinin in the Khan model is based on the ability of cytokinin, but not GA, to alleviate inhibitions of germination, in
diverse species, caused by exogenous ABA. This inhibitor–cytokinin interaction is also evident at the biochemical level, as exogenous ABA can inhibit the development of specific enzyme activities, as well as ion movements in germinating seeds. However, although ABA is known to occur in seeds, there is no conclusive evidence that the endogenous ABA is involved in these processes (Walton, 1980/81).

1.7.2 Phytohormone-directed reserve mobilisation.

The aleurone layer system. It was demonstrated by Paleg (1960) and Yomo (1960) that an increase in amylolytic enzyme activity could be induced in barley seeds following treatment with gibberellic acid (GA$_3$). Subsequently it was shown that the effect of the embryo on the release of hydrolytic activity in the endosperm could be replaced by GA$_3$. It was concluded by Varner and Chandra (1964) that GA was synthesised in the embryo and that the aleurone layer was the target tissue for the GA$_3$. The aleurone layer of rice (Ogawa, 1966), wheat and oats (Paleg et al., 1962) were found to behave in a similar fashion to that of barley. The GA$_3$-treatment induced the de novo synthesis (Varner and Chandra, 1964; Varner, 1964; Filner and Varner, 1967; Chrispeels and Varner, 1967a) of four $\alpha$-amylases (Jacobsen et al., 1970), with a lag period of 6 to 8 hours during which the mRNA required for the $\alpha$-amylase synthesis was produced (Chrispeels and Varner, 1967a; Filner and Varner, 1967; Higgins et al., 1976). Cytokinin was shown by Eastwood et al. (1969) to have some promotive effects on the appearance of $\alpha$-amylase activity in wheat. It was found that the aleurone tissue isolated free of starchy endosperm from quiescent wheat grains was dependent on a pre-treatment with endosperm extract or kinetin before the applied GA could effectively stimulate the secretion of $\alpha$-amylase.

The application of ABA inhibited the formation of $\alpha$-amylase in
barley aleurone with little effect on the overall rate of total protein biosynthesis but slightly depressing RNA synthesis (Chrispeels and Varner, 1967b, 1967c). This inhibition could be only partially relieved by the application of additional GA₃ or by ethylene, but applied together, the ABA inhibition could be almost completely eliminated (Jacobsen, 1973). The ABA inhibition of α-amylase formation in intact barley grains could also be abolished by treatment with either kinetin or BAP (Khan and Downing, 1968).

In the aleurone system, the application of GA₃ enhances the activity of many other enzymes apart from α-amylase. These include protease, ribonuclease, phosphatase, glucanase, xylanase, enzymes of fatty acid β-oxidation and enzymes of the glyoxylate pathway (for a comprehensive listing see reviews by Zeroni and Hall, 1980; and by Laidman, 1982). Other processes in the aleurone layer are unaffected by GA₃ and may be under the control of other hormones. Thus, exogenous cytokinin can initiate the mobilisation of part of the triglyceride reserve and promote the retention of mineral ions in the aleurone layer (Tavener and Laidman, 1972a). Also a combination of IAA and glutamine can induce neutral lipase activity and the mobilisation of another part of the triglyceride reserve (Tavener and Laidman, 1972b). All known groups of plant growth regulators appear to be implicated in the aleurone layer system, but the nature of the primary action of these phytohormones is unknown.

Dicotyledonous seeds. In many dicotyledonous seeds food reserves are stored in the cotyledons. The control of reserve mobilisation has been studied in only a few species, however, it seems the activity of some of the hydrolytic enzymes is promoted by cytokinin released from the growing embryo. The evidence for this is based on the ability of
the cytokinins to replace the axis in its action on reserve mobilisation, an ability not usually shown by the other phyothormones (see discussion in Section 1.4.3, Table 1.1 and references therein, and review by Ilan and Gepstein, 1980/81). This argument is supported by work on germinating bean seeds (Hutton et al., 1982; Hutton and van Staden, 1982a, 1982b) and pumpkin seeds (Englebrecht et al., 1976; Rybicka et al., 1977) which indicated that the endogenous cytokinins are produced in the embryonic axis from where they are exported to the cotyledons.

In germinating lettuce seeds, the initial source of carbohydrate for the growing axis is the endosperm, which is degraded prior to the mobilisation of the main food reserves in the cotyledons. The endosperm cell wall is largely composed of mannose-containing polysaccharides. These are hydrolysed by endo-β-mannanase produced in the endosperm itself and the resulting oligomannans are further hydrolysed by β-mannosidase from the cotyledons. Bewley and Halmer (1980/81) determined from a series of surgical experiments that the mannanase production was under the control of the axis, but that the control was mediated by the cotyledons. The mechanism seemed to involve gibberellins and/or cytokinins, derived from the axis, stimulating the cotyledons, which in turn, effected the lifting of inhibition (possibly imposed by ABA) on the synthesis of mannanase in the endosperm. This is in contrast to the aleurone layer system which requires a positive signal for the production of the hydrolytic enzymes, rather than a factor to counter inhibition of production.

The mechanisms responsible for the subsequent production of β-mannosidase and later for the hydrolysis of the cotyledon reserves, are unknown.
1.7.3 Other evidence for cytokinin involvement in seed germination

The culture of excised embryos in vitro provides further support for the view that cytokinin plays a role in the germination process. Radical growth in Zea mays seeds was retarded by the removal of the endosperm. Smith and van Staden (1979) reported that this radicle growth could be partially restored by a plant extract reported to contain cytokinin O-glucosides. Similarly, the elongation of the radical in isolated sycamore seeds could be induced by exogenous kinetin but not by GA (Pinfield and Stobart, 1972). It was suggested by these workers that the endogenous cytokinins may be key factors in the initiation of radicle growth.

Exogenous cytokinins have proved effective in overcoming the need for chilling to break dormancy in some seeds. Of these seeds, some experienced an increase in the levels of endogenous cytokinin, as monitored by bioassays, prior to germination (see review by van Staden et al., 1982). The endogenous cytokinins in some light-sensitive seeds have also been reported to increase following their exposure to light (van Staden et al., 1982).

1.7.4 Cytokinins and early seedling development

Exogenous cytokinin and gibberellin have been shown to substitute for the physiological influence of the roots on the growth of derooted soybean (Holm and Key, 1969) and oat seedlings (Jordan and Skoog, 1971). In the soybean, growth of the hypocotyl seemed to be regulated by a combination of cytokinin and gibberellin, while in the oat seedlings, only cytokinin was effective in restoring coleoptile growth and auxin production in the coleoptile tip. Cytokinin has also been implicated in the control of the growth of the wheat coleoptile. Wright (1961, 1968) showed that there is a sequential growth response of the
coleoptile, first to gibberellin, next to kinetin and then to IAA. He also demonstrated a sequential growth response of serial sections from the first leaf of wheat seedlings to treatment with GA, kinetin and IAA. The meristematic basal section gave a response to both GA and kinetin, the uppermost sections with the most mature tissues, showed a response to IAA only (Wright, 1968).

The most marked and best characterised response to cytokinin in immature seedlings is probably cotyledon expansion. Some cotyledons respond markedly to both GA and cytokinin (e.g. Xanthium), some markedly to cytokinin but only very weakly to GA (e.g. radish), and some only to cytokinin (e.g. cucumber in darkness and Trigonella) (see references in Goodwin et al., 1978).

Exposure of squash cotyledons to light induces a marked but transient increase in cytokinin levels (Uheda and Kuraishi, 1977). Hence the promotion of cotyledon development caused by light may be at least partially mediated by cytokinin.

1.8 Future studies of cytokinin metabolism - the present investigations.

From the preceding sections it is evident that cytokinins are implicated in the control of bud development and especially senescence in the developed plant. Furthermore, during seed germination, cytokinins appear to be involved in the mobilisation of storage reserves, in promotion of radicle growth, and in breaking dormancy normally alleviated by chilling or light. At the level of early seedling development, cytokinin may participate in the control of coleoptile and primary leaf growth in monocots, and of cotyledon development in dicots. Well designed studies of cytokinin metabolism in relation to all these aspects of development could provide new insight
into their hormonal control. Accordingly, this thesis describes studies of cytokinin metabolism in relation to certain aspects of development, namely, germination of maize seed and radish cotyledon expansion.

The identity and metabolic pathways of the naturally-occurring cytokinins have been largely established, but as yet, very few critical studies have related cytokinin biosynthesis, metabolism, and translocation to the normal development of intact plants, or attempted to examine cytokinin metabolism and location at the subcellular level.

A considerable number of studies have been conducted on the changing levels of the endogenous phytohormones during germination. However, these have usually been based on the extraction and estimation of total hormone content by bioassay, a procedure which entails clear difficulties (for example the disparity between GA estimated by GC-MS and bioassay in the same tissue, Dennis et al., 1980 and Lewak and Sinska, 1980, respectively; see also discussion in Section 2.1.1). Studies of cytokinin metabolism in relation to seed germination are confined to applications of exogenous kinetin and BAP to lettuce seeds (Miernyk and Blaydes, 1977; Miernyk, 1979, Pietraface and Blaydes, 1981; discussion in Section 1.5) and of zeatin to ash embryos (Tzou et al., 1973; zeatin could partially substitute for low temperature after-ripening but enhanced chlorophyll synthesis almost to the same extent as after-ripening), to bean axes (Sondheimer and Tzou, 1971; zeatin could partially reverse ABA-induced growth inhibition), and to maize (van Staden, 1981a, 1981b; discussion in Section 4.1). The endogenous cytokinins have not been identified unequivocally in any of the seed used in the above studies and hence the use of zeatin may not be appropriate. In some studies (e.g. maize) the metabolites formed were not identified rigorously.
The only studies of cytokinin metabolism in relation to early seedling development have been confined to intact radish seedlings, derooted radish seedling and excised cotyledons (see Section 1.5 and Table 1.2 and references therein). However, as the only endogenous cytokinin to be unequivocally identified in the radish system is Z7G in seed (Summons et al., 1977) it is difficult to relate the observed metabolism and translocation of the exogenous cytokinins to the normal development in the intact seedling with any degree of certainty. Nevertheless, the studies have yielded insight into the significance of the cytokinin metabolites including the structurally unusual 7-glucosides.

To clarify the particular role of the cytokinins in relation to seed germination and seedling development, it is necessary to:

(i) establish the identity and levels of the endogenous cytokinins in the various parts of the seed and seedlings being studied;

(ii) determine how these endogenous cytokinins are metabolised and transported in the germinating seed and seedling;

(iii) determine whether the cytokinins are synthesised de novo in germinating seed. In the present thesis these problems have been addressed.

To determine the levels of the cytokinins in plant tissue, new procedures have been developed (Chapter 2) and applied to the analysis of the endogenous cytokinins in dry Zea mays caryopses (Chapter 4). These methods should be of wide applicability. High specific activity $[^3H]ZR$ was synthesised (by a new method) and its metabolism and translocation in germinating maize seed was examined (Chapter 4). The possible biosynthesis of cytokinin in germinating maize was also studied in feeding experiments with radiolabelled precursors.
An important form of metabolism of the cytokinins in both maize seedling leaves and radish cotyledons is the formation of the cytokinin 9- or 7-glucoside, respectively, which results in inactivation of the cytokinins. The blockage of this metabolic pathway by specific inhibitors may be of considerable physiological significance since such compounds could potentiate the activity of the cytokinins. Hence potential inhibitors were synthesised (Chapter 3) and tested for their ability to inhibit N-glucosylation in maize leaves (Chapter 4) and in radish cotyledons and leaves (Chapter 5).
2. Quantification of Cytokinins

'Since we are assured that the all-wise Creator has observed the most exact proportions, of number, weight and measure, in the make of all things, the most likely way therefore, to get any insight into the nature of those parts of the creation, which come within our observation, must in all reason be to number, weigh and measure'.

Vegetable Staticks
Stephen Hales 1677-1761

2.1 Introduction

Cytokinins are implicated in the control of many aspects of plant development (see Sections 1.6 and 1.7). To define their regulatory role more precisely, it is necessary to identify and quantify the cytokinins present in plant tissues during development. The problems involved in this task are quite considerable as small quantities (1 µg/kg fresh weight or less) of cytokinins must be extensively purified from large samples of plant tissue. This requirement for large samples of plant material has placed a severe limitation on the application of physico-chemical methods to the analysis of cytokinins in physiological studies. In addition, these plant growth regulators occur in plants and microorganisms as free purine bases (Z, cis-Z, DZ and iP), purine ribosides (ZR, cis-ZR, DZR and iPA), nucleotides (of ZR, cis-ZR, DZR and iPA), glucosides (OGZ, OCGZ, OGDZ, OGDZR, Z9G and Z7G) and alanine conjugates (lupinic acid and dihydrolupinic acid). A further complication is thus created, as all or any combination of these
cytokinins may be present in any particular tissue at any one time. Ideally, any method for quantifying the cytokinins must have not only the potential for great sensitivity and accuracy, but also must be capable of analysing the full range of cytokinins.

2.1.1 Bioassay

The literature on the quantitative measurement of cytokinins is largely based on the use of bioassays, measuring a physiological response to the presence of cytokinins. These have included promotion of cell division, retardation of senescence, promotion of cell expansion and the induction of pigment synthesis. Although the bioassays are, in general, extremely sensitive, the considerable biological variability often present in plant material necessitates many replicate assays for statistically significant results to be realized. The bioassays themselves are extremely time-consuming, not only in terms of maintaining the required tissue cultures but also in the time required to obtain the physiological response, which ranges from a few days with the *Amaranthus* betacyanin assay to three weeks for the soybean callus assay.

However, the major impediment to the use of bioassays for quantification purposes is that the measured physiological response is a response to the total biological activity of the components present in the particular sample and has no chemical specificity. The biological activity may represent both growth-promoting and growth-retarding factors. For example, in the soybean callus bioassay for cytokinins it is known that high concentrations of abscisic acid inhibit the callus growth while low concentrations act synergistically with cytokinin to promote growth (Blumenfeld and Gazit, 1970a; Gazit and Blumenfeld, 1970). Similarly, the radish cotyledon expansion assay is also subject
to non-specific responses, in that growth is promoted not only by cytokinin but also by gibberellin, certain amino acids and calcium salts, and is inhibited by abscisic acid (Letham, 1971). Attempts to circumvent this problem of the specificity of response by applying further purification procedures have run into an additional difficulty of inevitable, but unknown, losses of analyte because radioactively-labelled internal standards have not been used in bioassays. These inherent problems of specificity, precision and accuracy must preclude the use of bioassays for quantifying cytokinins (Letham et al., 1978a; Brenner, 1981; Horgan, 1984).

2.1.2 Radioimmunoassay

Radioimmunoassays (RIA) of the cytokinins have been recently developed (see review by Weiler, 1984) and have been demonstrated to be a rapid and particularly sensitive assay method with the possibility of wide applicability and availability, as there is no requirement for sophisticated and expensive equipment (cf. MS methods below). The assay is based on the competition between the sample cytokinin and a fixed quantity of a radioactively-labelled cytokinin for the high affinity binding site of an antibody raised against the particular cytokinin. After equilibration, the free and bound cytokinins are separated. The amount of radioactivity in either fraction may then be used to assess the level of cytokinin present in the assayed sample, using a standard curve.

Small molecules such as the cytokinins are incapable of eliciting an immune response *per se* and must be covalently linked to some carrier molecule, usually a protein (e.g. bovine serum albumin; BSA). The BSA-cytokinin conjugate is then injected into the host animal to stimulate antibody formation. As conjugation is achieved via the ribosyl moiety
of the cytokinin ribosides (using variations of the method of Erlanger and Bieser, 1964), the prime antigenic determinant is the 6-amino substituent on the purine ring, it being most distal from the carrier molecule. Thus, anti-ZR-serum has generally been reported as being strongly cross-reactive with the 9-substituted cytokinins (Z9G, lupinic acid and Z nucleotide) and with the free base Z (for literature comparisons, see Badenoch-Jones et al., 1984), but to have little affinity for iP, DZ, cis-Z, Z7G, the O-glucosides or the respective ribosides. Similar types of cross-reactivities have been observed for the anti-iPA-, anti-DZR- and anti-cis-ZR-serums (Badenoch-Jones, personal communication).

Despite its specificity, RIA may be subject to non-specific interference from substances in extracts. These may either bind with the antibody or possibly alter its affinity constant for the cytokinins (Chapman, 1979).

All the principal naturally-occurring cytokinins may be quantified by RIA using antisera raised against ZR, cis-ZR, iPA and DZR, taking advantage of their cross-reactivities. However, an appropriate system for purifying and separating all the cytokinins must first be employed, following which the O-glucosides and Z7G must be hydrolysed prior to the RIA. Any potential problem with non-specific interferences is thus also minimised. Combinations of RIA with a variety of chromatographic techniques have been described recently; TLC/RIA (Weiler and Spanier, 1981), HPLC/RIA (MacDonald et al., 1981; and Morris et al., 1982) and ion exchange chromatography/HPLC/RIA (Badenoch-Jones et al., 1984).

Appreciable losses of cytokinins occur during the purification and separation steps and these need to be assessed. Ideally, recovery markers are required for each cytokinin one is attempting to measure,
but this would require the synthesis of high specific activity radio-labelled analogues of each cytokinin. In the study by Badenoch-Jones et al. (1984) it was assumed that the losses of Z, Z9G, DZR, lupinic acid and Z nucleotide would be similar to those of \([^{3}H]\)-ZR. This assumption proved to be reasonable when the RIA estimations were compared with those determined by mass spectral analysis using stable isotope dilution techniques.

2.1.3 Gas Chromatography - Mass Spectrometry (GC-MS)

Assays based on the use of mass spectral data must remain the method of choice for unequivocal identification and quantification of the cytokinins, combining as they do specificity, sensitivity and accuracy (Millard, 1978; Letham et al., 1978a; Horgan, 1978; Yokata et al., 1980; Brenner, 1981). Quantification is based on the addition of a standard, labelled with a stable isotope, to the extracting solvent. As the chemical and physical properties of the internal standard and the analyte will be very similar, their ratio, once established, will remain constant as no significant differential losses will occur. Overall losses may result from incomplete partitioning, entrapment in cellular debris, chemical reaction or adsorption.

The internal standard should contain three or more heavy atoms to minimise the correction required for the overlap of the mass spectra of the labelled and natural compounds. These heavy atoms should be located in non-labile positions. In general, the incorporation of deuterium labels is preferred, as deuterium is available in greater isotopic purity (> 99.9%) and is cheaper than either \([^{13}C]\) or \([^{15}N]\). However, both deuterated cytokinins (Summons et al., 1977; 1979a, 1979b; 1980; Hashizume et al., 1979) and \([^{15}N]\)-labelled cytokinins (Scott and Horgan, 1980) have been synthesised and have proved satisfactory for the
quantification of cytokinins in different tissues.

The ratio of natural compound to internal standard is obtained from the relative intensities of mass spectral peaks derived from each compound. However, it is possible that the internal standard and the analyte may not co-elute exactly during GC. These problems may be overcome by summing the series of relevant mass spectral scans, or more usually, by monitoring selected ions from the spectra of the labelled and natural compounds. Finally, knowing both the quantity of internal standard added and the ratio of internal standard to analyte, one can then determine the amount of the particular compound present in the original sample.

Effective application of mass spectral methods to the quantification of cytokinins in plant tissue has been limited by the need for large samples (> 100 g fresh weight), greatly restricting the routine use of the assay for physiological studies. With the advent of HPLC and of capillary GC interfaced with a new generation of computer-controlled fast scanning mass spectrometers, much smaller quantities of plant material may now be satisfactorily analysed. Further improvements to sensitivity should be achieved through improved methods for derivatisation of cytokinins for GC-MS, the use of chemical ionisation (CI) and the use of multiple-ion-detection (MID). These three possibilities are discussed below.

For GC-MS, a volatile derivative of the cytokinins is required. This has often been the trimethylsilyl (TMS) derivative (e.g. Summons et al., 1979b; Scott and Horgan, 1980). The advantages of using this derivative were that the procedure was simple and generally produced derivatives of good volatility in quantitative yield. However, while the TMS derivatives of the cytokinin ribosides have generally proved
satisfactory for GC-MS studies, when Z and related bases are subjected to GC as TMS derivatives, considerable losses and multiple peak formation have been observed. This is probably partly due to on-column instability; however, multiple derivative formation during trimethylsilylation is also known to occur (Young, 1977; Horgan, 1978; Kemp and Anderson, 1981; Stafford and Corse, 1982). We have observed that poor recovery of Z is particularly acute with some GC fused-silica capillary columns. Consequently, other derivatives of these compounds have been investigated in the present study in an attempt to overcome these problems and increase the sensitivity of the assay. Stable derivatives which could be subjected to HPLC and TLC would be particularly desirable.

Sensitivity may also be increased by optimising the mode of ionisation in the ion source of the mass spectrometer. Although ionisation by electron impact (EI) is very efficient, the resultant fragmentation with cytokinins and other complex molecules is extensive. As a result, the total ion current is spread over many ions, most of which are unsuitable for the purpose of analysis. CI, though less efficient than EI in an absolute sense, often offers considerable gains in sensitivity, as it produces little fragmentation of the molecule, concentrating the ion current in the pseudomolecular ion, \([\text{MH}]^+\) (Millard, 1978). In general, as reagent gases with decreasing abilities to donate protons (i.e. weaker Bronsted acids) are used, the process of protonation becomes less exothermic, so that less energy is available for the decomposition of \([\text{MH}]^+\). When the proton affinity of the analyte is less than that of the reagent gas, proton transfer will not occur and collision-stabilised adducts will form instead (Wilson and McCloskey, 1975). Thus, the intensity of \([\text{MH}]^+\) may be maximised by a
judicious choice of reagent gas. In the present study the sensitivities of El, CI (methane) and CI (ammonia) were compared for the principal cytokinins.

Finally, sensitivity may be increased substantially (10-1000 fold) by using MID, whereby only a few unique ions, characteristic of the compound being studied, are monitored (Millard, 1978; Yokata et al., 1980).

The work described in this chapter is directed towards devising and developing new methods for purifying, derivatising and analysing the cytokinins by mass spectrometry so that smaller (< 1 g) samples may be examined in physiological studies. Particular attention is given to the development of new TLC and HPLC procedures for cytokinin purification and to the derivatisation of the cytokinin bases for GC-MS analysis. In developing extraction and chromatographic purification procedures, immature sweet corn kernels were used as a model tissue. The methods developed were tested first with standard compounds and then with the sweet corn extracts.

2.2 Results and discussion

2.2.1 Extraction

Extraction artifacts may arise from enzymatic or chemical modification of the analyte during the extraction procedure. Precautions must therefore be taken to ensure that the plant extract reflects the status of the tissue at the time of the extraction. For cytokinins, the major problem encountered is one of nucleotide hydrolysis by non-specific phosphatases. Aqueous alcohol has often been used as an extraction medium, however, phosphatase activity can persist, in either hot or cold solvent, long enough to grossly change the ratio of nucleotides to nucleosides (Letham, 1978; Horgan, 1978; Laloue and
Inactivation of these phosphatases before extraction may be achieved by the method of Bieleski (1964, 1968) whereby the experimental tissue is soaked in cold acidic organic solvents (Scheme 2.1). This procedure was used for all initial extractions in the present investigations.

The stable isotope labelled standards were added to the extracting solvent prior to homogenisation. After homogenisation, it is important that sufficient time be allowed for the internal standard and analyte to equilibrate their non-specific bindings with cell debris, otherwise the analyte and internal standard may be differentially extracted. It is also important that homogenisation be thorough, to prevent variable and incomplete extraction.

2.2.2 Chromatography

The overall strategy for the purification and isolation of the cytokinins from the crude extract is dictated by the form of the final analysis. As the GC-MS analysis was to be carried out on the t-butyldimethysilyl (tBuDMS) derivatives of the bases (see Section 2.2.3) and the trimethylsilyl (TMS) derivative of the ribosides (see Section 2.2.4), it was necessary to, firstly, fractionate the extract into cytokinin nucleotides, glucosides, bases and ribosides. The nucleotides and glucosides were then hydrolysed to their respective bases and ribosides. All this was achieved most effectively by a combination of ion exchange chromatography, TLC and HPLC prior to derivatisation for GC-MS (Scheme 2.2). This approach was also found to be suitable for RIA of extracts.

Chromatography of the crude extract on a cellulose based cation exchanger, cellulose phosphate, separated the cytokinin nucleotides from

Tissue

- Soak MCFW, -20°C, 24 hr.
- Homogenise and stir, -20°C, 24 hr.
- Centrifuge

Supernatant

- Pellet: re-suspend MFW and stir 24 hr, 4°C
- Centrifuge

Supernatant

- Pellet (discard)

Combine supernatants

Evaporate solvent

Dried extract
Scheme 2.2: General scheme for the purification and isolation of cytokinins prior to analysis by either GC-MS or RIA.

**CRUDE EXTRACT**

- Cellulose phosphate column
  - Acidic wash (nucleotides)
  - Basic eluate (bases, ribosides, glucosides)

**DEAE cellulose column**

- Water wash (discard)
- Acidic wash (discard)
- HCO₃⁻ eluate (nucleotides)

**Alkaline phosphatase hydrolysis**

- m-BuOH partition

**Cellulose phosphate column**

- Acidic wash (discard)
- Basic eluate (nucleotide-derived ribosides)

**Analysis**

- TLC
  - IPA, IP
  - Z, ZR
  - DZ, DZR

- HPLC
  - IPA
  - Analysis
  - Z, ZR
  - DZ, DZR

- 8-glucosidase hydrolysis

- Acid hydrolysis
bases and ribosides. The nucleotides were not retained by cellulose phosphate but were washed through together with the neutral and other acidic components of the extract. The nucleotides were then isolated by anion exchange chromatography with DEAE-cellulose. Enzyme hydrolysis of the nucleotides with alkaline phosphatase afforded the respective nucleosides which were again subject to cellulose phosphate chromatography. These nucleotide-derived nucleosides were then purified and quantified by the same procedures as the free cytokinins present in the initial cellulose phosphate eluate.

The use of polystyrene sulphonic acid resins is not recommended as ribosides and nucleotides bound by these resins may be hydrolysed (Zwar and Bruce, 1970; Tegley et al., 1971; Dekhuijzen and Gevers, 1975; Stafford et al., 1984) or incompletely eluted (Vreman and Corse, 1975).

High recoveries (> 90%) of cytokinins and removal of much of the unwanted solid matter was achieved with the cellulose-based ion exchangers. However, further 'bulk' purification was necessary and this was best achieved by either HPLC of the evaporated cellulose phosphate eluate on Bondapak C18/Porasil B (HPLC system L), or by reverse phase chromatography on silica gel coated with paraffin. Recoveries for both these procedures were high, > 85% and > 95% respectively.

The cytokinin fraction was then further fractionated by one-dimensional TLC. Using a silica gel TLC system (system C), together with dyes A, B, C and D, it is possible to divide the plate into four cytokinin zones; 1, iP, iPA; 2, Z, DZ, ZR, DZR; 3, Ade, Ado, Z9G; 4, Z7G, OGZ, OGDZ, OGZR, OGDZR (Fig. 2.1). The cytokinins could be eluted quantitatively from silica gel ([3H]-Z, [3H]-ZR, [3H]-OGZR; recovery > 94%), however, the co-elution of colloidal silica precluded GC-MS analysis of the eluate at this stage. This problem was also apparent
Figure 2.1: In silica gel TLC system C, the cytokinin zones are delineated by dyes A, B, C and D; zone 1, iP, iPA; zone 2, Z, DZ, ZR, DZR; zone 3, Ade, Ado, Z9G; zone 4, Z7G, OGZ, OGDZ, OGDZR.

In cellulose TLC system F, the cytokinin zones are delineated by dyes E, F, G, H; zone 1, iP, iPA; zone 2, Z, DZ, ZR, DZR; zone 3, Z9G, Z7G, OGZ, OGDZ, OGDZR.
when silica gel containing no binder (60 HF_{254}, E. Merck) was used. In addition, this stationary phase proved to be extremely friable and the dye zones were quite diffuse. It was found that colloidal silica could be removed by rechromatographing on cellulose phosphate, or on a mixed bed of QAE cellulose and boric acid gel. However, an alternative, cellulose TLC system (system F), using dyes E, F, G and H, was developed to give essentially the same fractionation of the cytokinins (Fig. 2.1). The cytokinins run on cellulose could be eluted quantitatively, as for silica gel, but without eluting any interfering contaminants from the stationary phase. By comparison with silica gel, the cellulose zones are much less compact and the sample capacity of the cellulose is much reduced so that more plates may be required for any one analysis.

The iP and iPA eluted from cellulose could be separated by any one of three HPLC systems (G, M or S, Fig. 2.2) and similarly, Z, DZ, ZR and DZR could be individually separated by HPLC system P (Fig. 2.3). Unfortunately, the excellent resolution observed when chromatographing synthetic cytokinins in these systems was not always achievable with a partially purified plant extract. With the injection of increasing fresh weight equivalent of sample, retention volumes decrease, progressively reducing resolution. This problem is particularly acute in the case of the Zorbax C-8 column and is probably due to the fact that most reverse phase columns operate by a combination of separation mechanisms depending on the number of residual adsorption sites remaining on the support material. The collection of cytokinin fractions on the basis of the retention volume of the respective standards is thus quite unreliable. Identification by UV monitoring of the HPLC effluent is also an unreliable procedure as UV absorption by other components in the sample frequently masks any cytokinin
Figure 2.2: The separation of 1, iPA and 2, iP may be achieved by using any of three HPLC systems, G, M or S.
Figure 2.3: HPLC resolution of 1, Ado; 2, Ade; 3, ZR; 4, DZR; 5, Z; 6, DZ using HPLC system P.

Figure 2.4: Separation of Z9G and Z7G using HPLC system Q.
absorption. Reliable location of the cytokinins may be achieved, however, by either co-elution with high specific activity radiolabelled standards or by the use of a qualitative RIA. Both these procedures were used in the present studies.

Treatment of the glucoside eluate from the cellulose TLC with $\beta$-D-glucosidase results in the hydrolysis of the O-glucosides. The mixture of N-glucosides and the glucoside-derived bases and ribosides (Z, DZ, ZR and DZR) may then be separated using TLC system C or F, then after elution, chromatography on HPLC systems Q (Fig. 2.4) or P, respectively. The N-glucosides may be subject to GC-MS analysis following the HPLC separation or may be acid hydrolysed prior to GC-MS analysis as a base.

2.2.3 Derivatisation and GC-MS of the Cytokinin Bases

**Derivatisation procedures.** Tetrahydropyranyl (THP), methyl, TMS and tert-butyldimethylsilyl (tBuDMS) derivatives of the cytokinin bases were examined to determine their utility for GC-MS analysis. The procedures were assessed for simplicity, yield, and the number and stability of the derivatives formed.

Although the derivatisation of iP with 2,3-dihydropyran gave a high yield and only one derivative was produced, it was not possible to reproduce this with either Z or DZ. The permethylation of Z, DZ and iP gave good yields and only single derivatives were formed, i.e. trimethyl-Z, -DZ and dimethyl-iP, respectively. These derivatives are chemically stable and may be run on TLC and HPLC but the permethylating procedure is cumbersome and time-consuming (Young, 1977; Martin et al., 1981; Horgan and Scott, 1985 in press).

The initial attempt at making a tBuDMS derivative of Z was carried out using equal volumes of the MTBSTFA reagent and acetonitrile. GC-CIMS
(methane) analysis (Fig. 2.5) of the reaction mixture demonstrated the production of two mono-derivatives and a di-tBuDMS-Z substituted at the hydroxyl group and at position N-9. The CIMS of both the mono-derivatives (see Fig. 2.5 legend) contained [MH]+ ions (m/z 334), a collision-stabilized adduct [MC₂H₅]+(m/z 362), and an ion at m/z 328 representing a loss of a methyl radical from the parent ion. However, the presence of a strong ion at m/z 316 in the MS of one of the mono-derivatives indicated the loss of a hydroxyl radical from the parent ion, and permitted the determination of the site of silylation in each of the two mono-derivatives. The use of pyridine instead of acetonitrile in the silylation reaction produced a mixture of the mono-0-silylated Z and di-tBuDMS-Z. Prolonged heating of the reaction mixture resulted in a small increase in the relative amount of the di-tBuDMS-Z.

4-Dialkylaminopyridines have been found to be very effective acylation and alkylation catalysts, forming resonance stabilized N-acylpyridinium salts. The catalytic activity of these compounds (especially dimethylaminopyridine, DMAP) has also been used to promote the silylation of alcohols to tBuDMS ethers (Scriven, 1983). When DMAP was included in the silylating solution, quantitative yields of di-tBuDMS derivatives were obtained for Z, cis-Z, and DZ, and of the N-9 tBuDMS derivatives for iP and Ade. These derivatives exhibited desirable GC characteristics (Fig. 2.6).

Mass spectra of tBuDMS derivatives. The EIMS fragmentation patterns of the tBuDMS derivatives of Z, cis-Z, iP and Ade largely parallel the patterns observed in the mass spectra of both the underivatized compounds and the corresponding TMS derivatives, taking into account the necessary mass shifts. The tBuDMS derivatives of
Figure 2.5: GC-CIMS (methane) of zeatin derivatized with MTBSTFA in acetonitrile. 1, mono-9-tBuDMS-Z: m/z (%) 374 (MC$_3$H$_5$, 3), 362 (MC$_2$H$_5$, 23), 334 (MH, base), 318 (M-15, 20), 317 (M-16, 23), 316 (M-17, 91), 315 (M-18, 5); 2, mono-0-tBuDMS-Z: m/z (%) 374 (MC$_3$H$_5$, 5), 362 (MC$_2$H$_5$, 21), 334 (MH, base), 318 (M-15, 37); 3, di-tBuDMS-Z: m/z (%) 488 (MC$_3$H$_5$, 3), 476 (MC$_2$H$_5$, 17), 488 (MH, base), 432 (M-15, 40). During GC, the temperature was increased from 250°C at 10°C/min.
Figure 2.6: GC of a mixture of di-tBuDMS derivatives of Z, cis-Z and DZ. 1, di-tBuDMS-DZ; 2, di-tBuDMS-cis-Z; 3, di-tBuDMS-Z. GC was conducted isothermally at 300°C.
hydroxyl and carboxyl groups are, in general characterized by a prominent loss of a t-butyl moiety (M-57) (Quilliam and Westmore, 1978; Quilliam et al., 1980; Wollard, 1983). However, in the case of the bases with only a 9-tBuDMS group (BAP, iP, Ade), we observed the elimination of isobutene accompanied by hydrogen transfer (M-56) followed by the loss of a methyl radical (M-56-15). Thus, the two sites of silylation in some cytokinin bases, N-9 and the isoprenoid hydroxyl, can be distinguished.

Plausible structures for the major ions in the cytokinin mass spectra (Table 2.1 and Fig. 2.7) have been assigned on the basis of studies carried out on the underivatized bases (Shannon and Letham, 1966), TMS derivatives of zeatin glucosides (MacLeod et al., 1976), the EIMS of di-TMS-[2H5]-Z, and di-tBuDMS-[2H2]-iP, and high resolution mass measurements. The [2H2]-Z and [2H2]-iP were synthesized according to Summons et al. (1979b) and the deuterium was located in the terminal methyl and methylene groups of zeatin and in the α-methylene of iP.

In accord with the literature, treatment of adenine with BSTFA-TMCS afforded the di-TMS derivative with substitution at the N-9 position and at the exocyclic nitrogen (White et al., 1972). The mass spectrum featured as principal ions, the molecular ion (M+, 279), a base peak (M-15) due to a methyl loss and an ion (m/z 192) corresponding to the loss of both a TMS with H transfer (-72), and 'CH3 (-15). However, when derivatised with MTBSTFA reagent, substitution occurred exclusively at the N-9 position. With prolonged heating, partial derivatization of the N6-amino group could be achieved (di:mono, 1:20 based on GC). The major ions observed in the spectrum of the mono derivative were M-56 and M-56-15 (Fig. 2.8a). The MS fragmentation of di-tBuDMS-Ade consisted almost exclusively of the elimination of the t-butyl radical (M-57), the M+
Table 2.1: Summary of ions observed in the mass spectra of tBuDMS and TMS derivatives of cytokinin bases.

R denotes a tBuDMS or TMS group attached to the oxygen of the isoprenoid sidechain. R' denotes these moieties when at position N-9. Ions denoted by dashed letters (e.g., hh', c') exhibit an m/z value which is 56 less than that of the ion denoted by the normal letter (hh, c). This difference is attributed to loss of isobutene from a tBuDMS group at N-9.

A. Losses of derivatizing groups by simple cleavage or elimination.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>Molecular ion (M⁺)</td>
</tr>
<tr>
<td>M-15</td>
<td>Loss of methyl radical</td>
</tr>
<tr>
<td>M-56, M-56-15</td>
<td>Losses from N-9 tBuDMS group: elimination of isobutene (-56); with loss of CH₃ (-56-15)</td>
</tr>
<tr>
<td>M-57</td>
<td>Loss of t-butyl radical from tBuDMS derivatives</td>
</tr>
<tr>
<td>a, aa</td>
<td>Loss of ROH (a) or RO (aa)</td>
</tr>
<tr>
<td>b, bb*, bb⁺, bb⁺⁺</td>
<td>Loss of ROCH₂(b) and elimination of 2H (bb); further loss of isobutene from N-9 (Fig. 2.7)</td>
</tr>
<tr>
<td>M-115</td>
<td>Loss of tBuDMS radical from M⁺</td>
</tr>
<tr>
<td>k, k⁺</td>
<td>Loss of ROH and amino radical (k), k⁺ further loss of isobutene from N-9 (Fig. 2.7)</td>
</tr>
</tbody>
</table>

B. Ions derived by side chain cleavage and fragmentation.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>c, c⁺</td>
<td>'cyclization ions' (Fig. 2.7)</td>
</tr>
<tr>
<td>d</td>
<td>Cleavage 8 to N₆</td>
</tr>
<tr>
<td>e, e⁺H, e⁺2H</td>
<td>Cleavage of N₆ substituent (e); with transfer of one H (e⁺H) (Fig. 2.7) or two H (e⁺2H) to purine moiety.</td>
</tr>
<tr>
<td>e⁺', e⁺⁺H</td>
<td>Ions formed by further elimination of isobutene</td>
</tr>
<tr>
<td>f, f⁺, ff</td>
<td>Cleavage of 6-(substituted amino) group with H transfer to purine ring (f, Fig. 2.7; f⁺) and without H transfer (ff)</td>
</tr>
<tr>
<td>g, g-57</td>
<td>Ion derived from C₅ isoprenoid substituent, formed with H transfer to amino-purine moiety (Fig. 2.7) with cleavage of t-butyl radical (g-57)</td>
</tr>
<tr>
<td>h, h⁺, hh, hh⁺⁺</td>
<td>Cleavage γ to N₆ of saturated side chain (h, h⁺); cleavage with H transfer to 8 methylene (hh, hh⁺⁺)</td>
</tr>
<tr>
<td>i⁺</td>
<td>Ion formed from tBuDMS transfer from isoprenoid oxygen to purine N-1 (Fig. 2.12) with loss of N₈ substituent</td>
</tr>
<tr>
<td>j</td>
<td>Loss of C₆H₁₃ (presumably t-butyl radical and CH₃) from N with saturated side chain</td>
</tr>
<tr>
<td>n</td>
<td>Loss of C₆H₁₃ from M with saturated side chain</td>
</tr>
</tbody>
</table>

* Formulae established by high resolution mass measurements of ions in the spectra of mono-tBuDMS-Z and mono-tBuDMS-DZ.
Figure 2.7: Probable structures for fragment ions in the cytokinin mass spectra (see Table 2.1 for letter code).
being barely detectable (Fig. 2.8b). The only other prominent peak \( m/z \) 192 corresponded to the successive losses of tBuDMS and \( t \)-butyl moieties with transfer of one H, while a weak peak, \( m/z \) 250, was indicative of successive losses of a \( t \)-butyl radical (-57) and isobutene (-56). The initial fragmentations of 9-tBuDMS-BAP were identical to those observed for mono-tBuDMS-Ade, with ions corresponding to \( M \), \( M \)-15, \( M \)-56 (base peak), \( M \)-57 and \( M \)-56-15 (Fig. 2.8c). The \( M \)-56 and \( M \)-56-15 fragmentations are also evident in the spectrum of 9-tBuDMS-iP (Fig. 2.8d), but not in those of the mono-O-tBuDMS derivatives of \( Z \), cis-\( Z \) or DZ (Fig. 2.9a, 2.9b, 2.9c), and are therefore characteristic of a 9-tBuDMS group.

Fragmentation of the TMS derivative of iP (Fig. 2.8e) consisted principally in cleavages of the \( N^6 \)-sidechain; partial cleavage yielded ions c (base peak) and d, while complete cleavage produced a prominent ion \( m/z \) 192, attributable to \( e+H-15 \). Loss of a \( \text{CH}_3 \) radical from the TMS group and/or the side chain of the molecular ion was also prominent. These cleavages were also intense in the spectrum of tBuDMS-iP (Fig. 2.8d), but this also exhibited additional ions due to elimination of isobutene, namely, \( M \)-56, \( M \)-56-15, c', \( e'+H \), \( e'+H-15 \) and f'. The rationalisation of the spectrum of tBuDMS-iP presented in Fig. 2.8d is in accord with the mass shifts observed in the spectrum of \([^{2}H_2] \)-iP. Di-tBuDMS-Z and di-TMS-Z, and also di-tBuDMS-DZ and di-TMS-DZ, showed significant differences in analogous ion intensity. In the spectrum of di-tBuDMS-Z (Fig. 2.10a), the unusual ion \( i \) (see below) was more intense, while ions bb and g were less intense, than in the spectrum of di-TMS-Z (Fig. 2.10b). The unusual ions j and n (see below) were present in the spectrum of di-tBuDMS-DZ (Fig. 2.10c), but were absent in that of di-TMS-DZ (Fig. 2.10d), otherwise the two spectra were basically
analogous apart from the additional fragmentation associated with the tBuDMS group.

Di-tBuDMS-Z and its cis isomer exhibit very similar spectra, although the ratio of ions a and b is appreciably different (cf. Figs. 2.10a and 2.10e). However, the direct probe spectra of the mono-O-tBuDMS derivatives of Z and cis-Z differ markedly in relative peak intensity (cf. Figs. 2.9a and 2.9b). Thus in the cis-Z spectrum, ion i (base peak) is more intense than in the spectrum of Z (trans). However, the greatest difference between the two mono-tBuDMS spectra is the intensity of the ions e+2H, b, d and ff; these ions, derived by cleavage of all or part of the 6-substituent, are intense in the spectrum of Z(97, 82, 52, 51% respectively) but only of weak to moderate intensity (22, 33, 20, 23% respectively) in the spectrum of cis-Z. The basic differences between the mass spectra of mono-O-tBuDMS-Z and its cis isomer appear to be largely a consequence of the facilitation of tBuDMS-OH elimination by the cis configuration (see Fig. 2.11). This ion reaction becomes a dominant form of sidechain fragmentation in the case of cis-Z and as a result, the stable ion a is very prominent, while those ions associated with other types of sidechain cleavage and fragmentation (e+2H, b, d, ff) are relatively much less important. It appears that the cis configuration in mono-O-tBuDMS-Z facilitates tBuDMS transfer to the purine ring giving ion i, and suppresses the hydrogen transfer from the terminal methyl or methylene positions which results in the formation of ions e+H and e+2H (Fig. 2.12).

Several ions observed in the mass spectra reported herein merit comment. The unusual ion i has been assigned a structure derived from side chain cleavage and transfer of the silyl moiety to position N-1 (see Fig. 2.12), although transfer to other positions (N6, N-7) cannot
Figure 2.8: Mass spectra of (a) mono-tBuDMS-Ade; (b) di-tBuDMS-Ade; (c) tBuDMS-BAP, (d) tBuDMS-iP, (e) TMS-iP. In Fig. 4e, the peak at $m/z$ 207 is not due to column bleed. The spectra were taken during GC and those in (a) and (e) were determined at $m/z$ values above 150. In some spectra of di-tBuDMS-Ade, M and M-15 ions were present.
Fig. 2.8b

- Si -

NH

\[ \text{M-57} \]

\[ \text{M-56} \]

129
165
192
M-57-56
250
306
350

3QS
Fig. 2.8c.
Fig. 2.8e

Figure 2.9: Mass spectra of (a) mono-0-tBuDMS-Z, (b) mono-0-tBuDMS-cis-Z, (c) mono-0-tBuDMS-DZ. The compounds were introduced into the mass spectrometer via the direct inlet probe.
Fig. 2.9b
Fig. 2.9c
Figure 2.10: Mass spectra of (a) di-tBuDMS-Z; (b) di-TMS-Z; (c) di-tBuDMS-DZ; (d) di-TMS-DZ; (e) di-tBuDMS-cis-Z. In Figs. 2.10(b) and 2.10(d), the peak at $m/z$ 207 is not due to column bleed. The spectra were taken during GC and those in (b) and (d) were determined at $m/z$ values above 150. In Fig. 2.10(a), the masses of the equivalent ions from the spectrum of di-tBuDMS-[H$_2$]Z are contained in brackets; the mass shifts of 178 to 179, 192 to 193 and 193 to 194 are about 70% complete. In the spectra shown as Figs. 2.10(a), (c) and (e), and as Fig. 2.8(d), the ion at $m/z$ 192 has been designated e+H-57. However e' may also contribute to this peak.
Fig. 2.10c
Fig. 2.10d
Fig. 2.10e
Figure 2.11: Scheme showing a six-membered transition state proposed to account for the facilitated elimination of tBuDMS-OH from cis-Z.
Figure 2.12: Proposed fragmentation and silyl transfer to form ion i.
be excluded. Ion i is prominent in spectra of both the mono- and di-tBuDMS derivatives of Z and cis-Z (especially the mono) and also in the spectrum of mono-tBuDMS-DZ. However, it is extremely weak in the spectrum of di-tBuDMS-DZ. Ions j and n occur only in the spectra of the mono- and di-tBuDMS-DZ and from high resolution mass measurements, represent the loss of C₅H₁₃ and C₈H₁₇ respectively. For ions bb and k (Table 2.1), we propose structures (Fig. 2.7) stabilized by cyclization. Corroborative evidence is provided by high resolution mass measurements and by the spectrum of di-tBuDMS-[²H₅]-Z, where both bb and k are seen to contain three ²H atoms. These ions are most prominent in the spectra of compounds with an unsaturated sidechain (Figs. 2.9a, 2.9b and 2.10a, 2.10b, 2.10e).

Stability and chromatography of tBuDMS derivatives. While the 0-tBuDMS group of Z-type compounds was stable in weakly acidic solutions (0.15M acetic acid), the 9-tBuDMS moiety was cleaved instantly even by neutral solvents containing traces of water. Thus, when the derivatising solutions of iP, Z and BAP were evaporated, and the residues dissolved in ethyl acetate, and subjected to normal phase TLC, only free iP and BAP and mono-O-tBuDMS-Z could be recovered by elution. This was evidenced by both UV and mass spectra of the eluates. The UV spectra were characteristic of N⁶-substituted adenines without a substituent at N-9 (Table 2.2).

The separation of the di-tBuDMS derivatives of Z, DZ and cis-Z by GC has been noted previously (see Fig. 2.6). HPLC and TLC methods were also devised for the separation of the stable mono-O-tBuDMS derivatives of Z, DZ, and cis-Z (Fig. 2.13 and Table 2.3). Of the 6 TLC systems compared (systems, A, K, L, Q, R and S) reverse phase chromatography on silica gel impregnated with silicone oil (system K) achieved the best
Table 2.2: UV (basic) spectra of tBuDMS derivatives (eluted from normal phase TLC) and compared with stable derivatives of the N-9 position on the purine ring

<table>
<thead>
<tr>
<th>Compound</th>
<th>λ max. (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>'tBuDMS-BAP'</td>
<td>275 with shoulder at 283</td>
</tr>
<tr>
<td>'tBuDMS-Z'</td>
<td>275 with shoulder at 283</td>
</tr>
<tr>
<td>'tBuDMS-iP'</td>
<td>275 with shoulder at 283</td>
</tr>
<tr>
<td>iP</td>
<td>275 with shoulder at 283</td>
</tr>
<tr>
<td>9-tetrahydropyran-2-yl-iP</td>
<td>269</td>
</tr>
<tr>
<td>9-cyclohexyl-iP</td>
<td>270</td>
</tr>
</tbody>
</table>

resolution. This TLC system also had the additional advantage of yielding unusually compact spots. The mono-O-tBuDMS derivative of 6-(o-hydroxybenzylamino)purine (HO-BAP) is also sufficiently stable to chromatograph using these TLC systems (see Table 2.3 for \( R_f \) values).

2.2.4 Derivatisation and GC-MS of the Cytokinin Ribosides

Derivatisation procedures. Both perTMS and permethyl derivatives have been successfully used for the GC-MS analysis of the cytokinin ribosides (see for example, methyl: Young, 1977; Martin et al., 1981; Stafford and Corse, 1982; TMS: Summons et al., 1977, 1979a, 1979b, 1980; Hashizume et al., 1979; Scott and Horgan, 1980). However, only the permethyl derivatives are sufficiently stable to be chromatographed on HPLC or TLC systems. Due to the cumbersome and time-consuming methodology of permethylation, it was decided to examine other possible derivatives of the cytokinin ribosides.

An attempt to extend the use of the tBuDMS derivative, as described in Section 2.2.3, to the ribosides was hampered by the problem of
Table 2.3: $R_f$ values for cytokinin bases and their 0-tBuDMS-derivatives in normal and reverse phase TLC systems

<table>
<thead>
<tr>
<th>Compound</th>
<th>TLC Systems</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Z</td>
<td>0.12</td>
</tr>
<tr>
<td>cis-Z</td>
<td>0.16</td>
</tr>
<tr>
<td>DZ</td>
<td>0.125</td>
</tr>
<tr>
<td>HO-BAP</td>
<td>0.31</td>
</tr>
<tr>
<td>mono-0-tBuDMS-Z</td>
<td>0.43</td>
</tr>
<tr>
<td>mono-0-tBuDMS-cis-Z</td>
<td>0.44</td>
</tr>
<tr>
<td>mono-0-tBuDMS-DZ</td>
<td>0.43</td>
</tr>
<tr>
<td>0-tBuDMS-HO-BAP</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Figure 2.13: HPLC of the mono-0-tBuDMS derivatives of Z, cis-Z, and DZ using HPLC system B. 1, mono-0-tBuDMS-Z; 2, mono-0-tBuDMS-cis-Z; 3, mono-0-tBuDMS-DZ.
completely derivatising the 2',3'-cis-diol of the ribosyl moiety. The silylation of one of the hydroxyl groups sterically hinders the approach of a second tBuDMS group even under conditions of prolonged heating in the presence of DMAP. Quilliam and Westmore (1978) solved this problem by converting their partial tBuDMS derivatives of nucleosides into mixed TMS-tBuDMS derivatives. However, for the purposes of sensitive quantification, this is an unsatisfactory solution as each cytokinin riboside is converted into a mixture of several derivatives.

The cis-1,2-diol may be specifically derivatised by reaction with acetone or a substituted boronic acid to form an isopropylidene ether or boronate ester, respectively (Dolhun and Weibers, 1970). Although, both 2',3'-O-phenylboronic esters and 2',3'-O-n-butylboronic esters of nucleosides have been reported to be hydrolysed in water or by atmospheric moisture (Yurkevich et al., 1969 and Eisenberg, 1971, respectively), we decided to examine both the n-butylboronic ester and the isopropylidene derivative of ZR.

The boronate esters were formed by heating the nucleoside and the substituted-boronic acid in pyridine. Silylation of the remaining alcohols was then readily achieved by the addition of the appropriate reagent to the reaction mixture. In this way both TMS and tBuDMS derivatives were prepared.

Many different procedures have been employed in the formation of isopropylidene derivatives and these may be classified into two groups. Historically, the first approach was a condensation of acetone catalysed by a variety of acids, notably sulphuric acid, p-toluenesulphonic acid, di-p-nitrophenyl hydrogen phosphate and hydrochloric acid, or by zinc chloride or copper sulphate (for examples, see Levene and Stiller, 1933; Levene and Tipson, 1935; Hampton, 1961;
Chladek and Smrt, 1963; McCloskey and McClelland, 1965; and Morgenlie, 1975). These procedures give variable yields and are not readily adaptable to use at the sub-microgram level as neutralisation and extraction must be carried out to remove the catalyst. More recently, isopropylidene derivatives have been made by ketal exchange using either 2,2-dimethoxypropane or its higher boiling point homologue, 2,2-diethoxypropane, in the presence of an acid catalyst (p-toluenesulphonic acid, Fieser and Fieser, 1967; HCl, Chladek and Smrt, 1963). The driving force of this transketalisation reaction is the formation of the more thermodynamically stable cyclic ketal (isopropylidene ether).

The problems entailed in the removal of the acid catalyst would be avoided if a volatile acid is employed in the procedure. Hence, trifluoroacetic acid, formic acid and concentrated hydrochloric acid were all examined, and the most effective procedure was found to be the ketal exchange with 2,2-dimethoxypropane in the presence of concentrated hydrochloric acid. This procedure was also superior to derivatisation with acetone and HCl which formed two products. The reaction mixture from the ketal exchange could be blown down under nitrogen and the isopropylidene derivative isolated by TLC (system A). Alternatively, the dried residue could be further converted to TFA and tBuDMS derivatives, following dissolution in pyridine and the addition of the appropriate reagents. The yield of the isopropylidene derivative (83-87%) was determined by recovery of radioactivity following inclusion of $[^3H]$-ZR in the reaction.

When the silylation of 2',3'-0-isopropylidene-ZR with MTBSTFA was carried out with inadequate heating and without DMAP, a mixture of a mono-tBuDMS product and the starting material was obtained and separated by TLC (system A). Evidence for this was provided by solid probe CIMS
(methane) ([MH]\(^+\), m/z 506 and [MH]\(^+\), m/z 392; respectively). From the EIMS (Fig. 2.14b) it was determined that silylation had occurred exclusively at the sidechain hydroxyl of the Z moiety as evidenced by ions derived from glucoside cleavage (B+H, m/z 333; HB-57, m/z 276), ions resulting from charge retention on the silylated isoprenoid moiety (g, m/z 198; g-57, m/z 141) and by the presence of ion i (m/z 192) formed by tBuDMS transfer from the isoprenoid oxygen to purine N-1.

The inclusion of DMAP in the silylation reaction mixture resulted in a quantitative yield of the di-tBuDMS derivatives of both the isopropylidene ethers and n-butylboronic esters of the cytokinin ribosides.

Mass spectra of cytokinin riboside derivatives. The most important fragmentation processes observed in the EIMS of the cytokinin riboside derivatives described herein are cleavage at the glycosidic linkage and in the cytokinin N\(^5\)-sidechain. The considerable charge-stabilizing ability of the purine moiety results in the dominance of ions containing the base. The relative abundance of the sugar fragment and ions derived from it are consequently low.

In addition to the series of ions derived from the mono-silylated base (B) produced by glycosidic cleavage (cf. Fig. 2.9a, 2.9b, 2.9c) are a series of ions derived by simple cleavage or elimination from the molecular ion; M-15, M-ROH, M-ROCH\(_2\) (where R = TMS or tBuDMS), M-57 (R = tBuDMS), B+30 and B+2H (see von Minden and McCloskey, 1973) (Fig. 2.14, 2.15, 2.16).

\[
\begin{align*}
\text{B}+\text{H}^+ & \xrightarrow{-\text{CO}} \text{BH}_2^+ \\
\text{B}+30 & \xrightarrow{\text{CO}} \text{B}+2\text{H}
\end{align*}
\]
By contrast, the trifluoroacetyl derivative of 2',3'-O-isopropylidene ZR (Fig. 2.17) exhibits a very simple spectrum with most of the ion current concentrated in one fragment. This ion is attributable to the loss of CF$_3$CO$_2$ (m/z 566), an allylic type cleavage characteristic of Z moieties. The ion at m/z 298 represents cleavage of the riboside moiety with H transfer and loss of CF$_3$CO$_2$. The other ions are due to simple fragmentation of the molecular ion involving either the isopropylidene moiety (m/z 664, M-CH$_3$; m/z 620, M-C$_3$H$_7$O) or the acyl group (m/z 582, M-CF$_3$CO; m/z 552, M-CF$_3$CO$_2$CH$_2$).

Stability and chromatography of the cytokinin riboside derivatives. Although numerous attempts were made to successfully chromatograph the riboside derivatives by GC, this surprisingly was not achieved. Hence, EIMS of the derivatives were obtained via the direct insertion probe. The isopropylidene and tBuDMS-isopropylidene derivatives of ZR, cis-ZR and DZR were all amenable to TLC though respective derivatives of ZR, cis-ZR and DZR (Table 2.4) were not successfully resolved. Such a separation could be achieved using reverse phase HPLC system C (Fig. 2.18).

2.2.5 Mode of ionisation

A qualitative assessment of the most sensitive mode of ionisation was made by comparing the relative intensity of ions suitable for use in a quantitative MS assay. These ions were generated by one of either EI or CI (methane) or CI (ammonia). The methyl and TMS derivatives of Z, DZ, ZR and DZR, in addition to the tBuDMS derivatives of Z and DZ, were examined and it was found that CI, using methane as the reagent gas, resulted in an intense [MH]$^+$ ion accompanied by the collision-stabilised adducts [MCH$_3$]$^+$, [MC$_2$H$_5$]$^+$ and [MC$_3$H$_5$]$^+$ and some fragmentation. Using ammonia as the reagent gas, the CIMS in the high mass region of the
Table 2.4: \( R_f \) values for the stable derivatives of the cytokinin ribosides in normal phase TLC systems.

<table>
<thead>
<tr>
<th>Compound</th>
<th>TLC Systems</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>isopropylidene-ZR</td>
<td>0.25</td>
</tr>
<tr>
<td>mono-tBuDMS-isopropylidene-ZR</td>
<td>0.72</td>
</tr>
<tr>
<td>di-tBuDMS-isopropylidene-ZR</td>
<td>-</td>
</tr>
<tr>
<td>di-tBuDMS-isopropylidene-cis-ZR</td>
<td>-</td>
</tr>
<tr>
<td>di-tBuDMS-isopropylidene-DZR</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 2.18: HPLC of the di-O-tBuDMS-2',3'-O-isopropylidene derivatives of 1, ZR; 2, cis-ZR; 3, DZR using HPLC system C.
spectra consisted mainly of the pseudomolecular ion \([\text{MH}]^+\), at a greater intensity compared with the methane generated \([\text{MH}]^+\). Interestingly, the \([\text{MH}]^+\) ions of per-methyl-Z7G and -Z9G produced by either CI (methane) or CI (ammonia) were of approximately equal intensity. In addition to generally yielding a more intense \([\text{MH}]^+\), the use of ammonia as a reagent gas resulted in a slower contamination of the source than when methane was employed.
Figure 2.14: EIMS of (a) TMS-borionate ester of ZR; (b) tBuDMS-borionate ester of ZR. The compounds were introduced to the mass spectrometer via the direct inlet probe.
Figure 2.15: EIMS of (a) 2',3'-O-isopropylidene ZR; (b) mono-0-tBuDMS-2'3'-O-isopropylidene ZR. The ion B in (a) results from simple cleavage of the ribosyl group with charge retention on the purine moiety. R = tBuDMS. The compounds were introduced to the mass spectrometer via the direct inlet probe.
Fig. 2.15a

![Chemical structure and mass spectrum](image)

- **e+H**: 135
- **e+2H**: 164
- **d**: 149
- **c**: 188
- **bb**: 200
- **b**: 218
- **B**: 248
- **B+30**: 302
- **M**: 391
- **M-15**: 374
- **M-CH2OH**: 360
- **M-OH**: 360
Fig. 2.15b

[Diagram with labeled peaks and molecular structures]

- M-ROCH₂
- M-ROH

Mass values:
- M-57: 57
- M-30: 30
- M-15: 15
- M: 505

Peaks:
- B+H: 333, 360, 373, 448, 475, 490, 505
Figure 2.16: EIMS of di-O-tBuDMS-2',3'-0-isopropylidene derivatives of (a) ZR, (b) cis-ZR and (c) DZR. The compounds were introduced to the mass spectrometer via the direct inlet probe.
Fig. 2.16a

![Mass Spectrum Diagram]

- M-ROH
- B-57
- M-ROCH₂
- B+2H
- M-57
- M-15
- M

Mass Values:
- 200
- 211
- 230
- 276
- 284
- 362
- 416
- 474
- 562
- 604
- 619
- 650
Figure 2.17: EIMS of trifluoroacetyl-2',3'-0-isopropylidene ZR which was introduced to the mass spectrometer via the direct inlet probe.
Fig. 2.17

M-CF₃CO₂ 566

M-CF₃CO 582

M-C₃H₇O 620

M-15 664

M 679

298

300 500 550 600 650
2.3 Experimental

2.3.1 Extraction.

**Bieleski Extraction.** The plant material was dropped into methanol-chloroform-formic acid-water (12:2:1:2, v/v) chilled at -20°C. After 24 hours at this temperature the plant material was either ground-up with a mortar and pestle or homogenised in a Waring blender and then left stirring for a further 24 hours at -20°C. The mixture was then centrifuged and the pellet re-suspended in methanol-formic acid-water (6:1:4, v/v) at 4°C. The suspension was stirred overnight, centrifuged and the supernatants combined. The solvent was removed under reduced pressure at 30°C (see Scheme 2.1).

2.3.2 Chromatography

**Cellulose Phosphate.** The extract was taken up in water (pH 3, acetic acid), clarified by centrifugation, and then passed through a column of cellulose phosphate (Whatman Pl, floc cation exchanger, in the NH₄⁺ form equilibrated to pH 3). The column was then washed with three column volumes of water (pH 3, acetic acid) and one column volume of water. The effluent containing the neutral and acidic compounds (including the cytokinin nucleotides) was collected. The basic substances (including cytokinin nucleosides, glucosides and bases) were then eluted from the column with five column volumes of 2M ammonia. The eluents were dried under reduced pressure at 30°C.

**DEAE Cellulose.** The nucleotide sample was taken up in water (pH 8, ammonia) and passed through a column of DEAE Cellulose (Whatman DE1, coarse fibrous, in the HCO₃⁻ form equilibrated to pH 8). The column was washed with five column volumes of water (pH 8, ammonia) and then eluted with five column volumes of NH₄HCO₃ solution (10%, w/v). The eluate was dried under reduced pressure and freed of bicarbonate by repeated
addition and evaporation of methanol.

**Boric-acid Gel.** The aqueous riboside solution (pH 11) was passed through a column of boric-acid gel, a copolymer of dihydroxyboryl-anilino-substituted methacrylic acid and 1,4-butanediol dimethacrylate (Sigma Chemical Co., column prepared by washing with 0.5M formic acid and then 0.2M NH₄OH). The column was then washed sequentially with 0.2M NH₄OH (3 column vols.), water (2 column vols.) and 0.5M formic acid (4 column vols.). The water and formic acid washes were evaporated separately and the residues combined to give the cytokinin riboside fraction.

**QAE Cellulose.** An aqueous solution of base cytokinins (pH 11) was passed through a column of QAE Cellulose Anion Exchanger (coarse, Sigma Chemical Co., prepared by washing with 0.5M formic acid and then 0.2M NH₃). The column was then washed sequentially with 0.2M NH₃ (3 column vols.), water (2 column vols.) and 0.5M formic acid (4 column vols.). The washes were evaporated separately and the residues combined to give the cytokinin base fraction.

**Charcoal.** The charcoal (BDH) was washed successively with water, pyridine-water (1:10, v/v), and water. Charcoal (approximately 10 mg) was then added to an aqueous solution of cytokinin standards and shaken for 5 minutes. The supernatant was discarded and the charcoal washed with water. The cytokinins were then eluted with pyridine-water (1:10, v/v).

**Paraffin/silica gel.** Silica gel (Ajax Chemical Co., 100-200 mesh) was mixed with a 6% solution of paraffin in petroleum ether, filtered and dried. The dried paraffin-impregnated silica gel was slurried with water and packed into a column (6 ml). The column was then washed with water and sample (pH 7) applied. The column was washed
with water until the eluent cleared (4 column vols.) and the cytokinin
fraction eluted with MeOH-HOAc-H₂O (10:1:10, v/v). The column could be
regenerated by washing with water.

TLC. The systems used for TLC are described in Table 2.5.
Normal phase TLC was carried out on 0.25, 0.5 or 1.0mm layers of silica
gel (Kieselgel, E. Merck, Darmstadt, FDR) or on 0.3 or 0.6 mm layers of
cellulose (Serva Feinbiochemica, Heidelberg, FDR). Woelm green
fluorescent indicator (M. Woelm, Eschwerge, FDR) was incorporated into
the cellulose (0.8%, w/w) before spreading the layer. The following
dyes were used as markers for TLC: A, meldola blue (Gurr-Searle
Diagnostic, High Wycombe, Bucks., England); B, drimarene brilliant blue
K-BL (Polysciences, Warrington, Pennsylvania, USA); C, rhodamine B; D,
toluidine blue (G.T. Gurr); E, phenol red (Elliot's and Australian Drug
Pty. Ltd. Sydney, NSW); F, lissamine flavine FFS (G. Gurr); G, malachite
green (G. Gurr); H, acridine yellow (G. Gurr); I, fast green FCF (E.
Gurr), London, England); J, bromocresol green (Aldrich); K, orange G (E.
Gurr; L, congo red (G. Gurr). In normal phase TLC, 6-(5-
hydroxypentylamino)-9-S-D-(5'-monophosphate)ribofuranosylpurine was used
as a marker for zeatin nucleotide.

Reverse phase TLC plates were made by impregnating silica gel
plates with dimethylpolysiloxane-5X (DMPS-5X, Sigma Chemical Co.) or
paraffin (Ajax Chemical Co. Sydney), by dipping the plates into a 6%
petroleum ether solution of DMPS-5X or paraffin and allowing the
petroleum ether to evaporate. The TLC plates of system N were prepared
by firstly impregnating with DMPS-5X as described above and then further
impregnating the bottom 3-4cm of the plates with paraffin by developing
with a 6% solution of paraffin in petroleum ether and allowing the
solvent to evaporate.
Table 2.5: Systems used for TLC. The silica gel layers for systems Q, R, S, I and J were prepared by the manufacturer (pre-coated). The layers for all other systems were spread and prepared in the laboratory.

<table>
<thead>
<tr>
<th>TLC System</th>
<th>Support</th>
<th>Solvent (v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) normal phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>60 PF&lt;sub&gt;254&lt;/sub&gt; silica gel</td>
<td>MeOH-CHCl&lt;sub&gt;3&lt;/sub&gt; (1:9) with a trace of NH&lt;sub&gt;3&lt;/sub&gt;</td>
</tr>
<tr>
<td>B</td>
<td>60 PF&lt;sub&gt;234&lt;/sub&gt; silica gel</td>
<td>CHCl&lt;sub&gt;3&lt;/sub&gt;</td>
</tr>
<tr>
<td>C</td>
<td>60 PF&lt;sub&gt;234&lt;/sub&gt; silica gel</td>
<td>n-BuOH-HOAc-H&lt;sub&gt;2&lt;/sub&gt;O (12:3:5)</td>
</tr>
<tr>
<td>D</td>
<td>60 PF&lt;sub&gt;234&lt;/sub&gt; silica gel</td>
<td>n-BuOH-NH&lt;sub&gt;3&lt;/sub&gt;-H&lt;sub&gt;2&lt;/sub&gt;O (6:2:1, upper phase)</td>
</tr>
<tr>
<td>E</td>
<td>60 HF&lt;sub&gt;234&lt;/sub&gt; silica gel (15µm)</td>
<td>MeOH-CHCl&lt;sub&gt;3&lt;/sub&gt; (1:9)</td>
</tr>
<tr>
<td>F</td>
<td>cellulose (Serva)</td>
<td>n-ProOH-HOAc-H&lt;sub&gt;2&lt;/sub&gt;O (5:1:1)</td>
</tr>
<tr>
<td>G</td>
<td>cellulose (Serva)</td>
<td>n-ProOH-HOAc-H&lt;sub&gt;2&lt;/sub&gt;O (7:1:1)</td>
</tr>
<tr>
<td>H</td>
<td>cellulose (Serva)</td>
<td>n-BuOH-NH&lt;sub&gt;3&lt;/sub&gt;-H&lt;sub&gt;2&lt;/sub&gt;O (6:2:1, upper phase)</td>
</tr>
<tr>
<td>2D-TLC</td>
<td>60 PF&lt;sub&gt;254&lt;/sub&gt; silica gel</td>
<td>(i) n-BuOH-HOAc-H&lt;sub&gt;2&lt;/sub&gt;O (12:3:5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) n-BuOH-NH&lt;sub&gt;3&lt;/sub&gt;-H&lt;sub&gt;2&lt;/sub&gt;O (6:2:1, upper phase)</td>
</tr>
<tr>
<td>b) HPTLC normal phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>HPTLC 60 P&lt;sub&gt;254&lt;/sub&gt; silica gel</td>
<td>MeOH-CHCl&lt;sub&gt;3&lt;/sub&gt; (1:9) with a trace of NH&lt;sub&gt;3&lt;/sub&gt;</td>
</tr>
<tr>
<td>J</td>
<td>HPTLC 60 P&lt;sub&gt;254&lt;/sub&gt; silica gel</td>
<td>CHCl&lt;sub&gt;3&lt;/sub&gt;</td>
</tr>
<tr>
<td>c) reverse phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>60 GF&lt;sub&gt;254&lt;/sub&gt; (15µm)/DMPS-5X</td>
<td>MeOH-H&lt;sub&gt;2&lt;/sub&gt;O-NH&lt;sub&gt;3&lt;/sub&gt; (22:16:1)</td>
</tr>
<tr>
<td>L</td>
<td>60 GF&lt;sub&gt;254&lt;/sub&gt; (15µm)/DMPS-5X</td>
<td>MeOH-H&lt;sub&gt;2&lt;/sub&gt;O (7:3)</td>
</tr>
<tr>
<td>M</td>
<td>60 GF&lt;sub&gt;254&lt;/sub&gt; (15µm)/DMPS-5X</td>
<td>(i) H&lt;sub&gt;2&lt;/sub&gt;O</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) MeOH-H&lt;sub&gt;2&lt;/sub&gt;O (3:7)</td>
</tr>
<tr>
<td>N</td>
<td>60 GF&lt;sub&gt;254&lt;/sub&gt; (15µm)/DMPS-5X/paraffin</td>
<td>(i) H&lt;sub&gt;2&lt;/sub&gt;O</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) MeOH-H&lt;sub&gt;2&lt;/sub&gt;O (3:7)</td>
</tr>
<tr>
<td>P</td>
<td>60 PF&lt;sub&gt;254&lt;/sub&gt;/paraffin</td>
<td>(i) H&lt;sub&gt;2&lt;/sub&gt;O</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) MeOH-H&lt;sub&gt;2&lt;/sub&gt;O (3:7)</td>
</tr>
<tr>
<td>Q</td>
<td>60 HF&lt;sub&gt;254&lt;/sub&gt; silanised</td>
<td>MeOH:H&lt;sub&gt;2&lt;/sub&gt;O (7:3)</td>
</tr>
<tr>
<td>R</td>
<td>60 F&lt;sub&gt;254&lt;/sub&gt; silanised</td>
<td>MeOH:H&lt;sub&gt;2&lt;/sub&gt;O (7:3)</td>
</tr>
<tr>
<td>S</td>
<td>60 F&lt;sub&gt;254&lt;/sub&gt; silanised/DMPS-5X</td>
<td>MeOH:H&lt;sub&gt;2&lt;/sub&gt;O (7:3)</td>
</tr>
</tbody>
</table>
UV absorbing zones on the TLC plate were detected using a short-wavelength UV-lamp. Normal phase TLC zones required for scintillation counting were scraped off the plates into scintillation vials and allowed to stand overnight in water (0.5 ml) before the scintillant (5.0 ml) was added. The reverse phase TLC zones required for scintillation counting were allowed to stand in the dark for 24 hours in a mixture of water (0.5 ml) and scintillant (5.0 ml). The cytokinins were eluted from normal phase TLC with MeOH-HOAc-H₂O (10:1:10, v/v). tBuDMS derivatives for characterisation by UV and mass spectra were eluted from normal phase TLC using EtOAc - EtOH (3:1, v/v). Dye H was best visualised after exposure to an ammonia (basic) atmosphere.

**HPLC.** HPLC was carried out with equipment supplied by Waters Associates (Milford, Mass., USA). Solvent was delivered by two constant flow-rate pumps (Model M-6000A) and controlled by a Model 680 Automated Gradient Controller. Samples were introduced through a Model U6K universal injector, and the absorbance of the column effluent was monitored at 254 and 280 nm, using a Model 440 detector with output to a dual-pen recorder. All samples were prepared in the eluting solvent and filtered prior to injection. All solvents were HPLC grade; EtOH, MeOH, CH₃CN (Waters), acetic acid (Unichrome, Ajax). HPLC systems used are as described in Table 2.6.

**2.3.3 Cytokinin Hydrolysis**

**Nucleotide hydrolysis.** The dried residue of the nucleotide sample was taken up in 0.1M 2-ethanolamine (Sigma), pH 9.5, together with 5 drops of 10mM MgCl₂ solution. Alkaline phosphatase (15 µl, EC 3.1.3.1, Type III-S from *E. coli*; Sigma) was added and the sample was incubated overnight at 37°C. The nucleosides were then extracted with water-saturated n-BuOH at pH 8-9.
<table>
<thead>
<tr>
<th>System</th>
<th>Column</th>
<th>Flow (ml/min)</th>
<th>Solvent</th>
<th>Compounds separated</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>µB C-18 semi-prep. (7.8mm x 30cm; Waters)</td>
<td>3.5</td>
<td>25% MeOH-0.01M NH₄OAc (pH7)</td>
<td>Z7G, Z9G, OGDZ, OGZ, OGZR, OGDZR.</td>
</tr>
<tr>
<td>B</td>
<td>µB C-18 analytical (3.9mm x 30cm; Waters)</td>
<td>3.0</td>
<td>45% CH₃CN-H₂O</td>
<td>tBuMS-Z, -DZ, -cis-Z.</td>
</tr>
<tr>
<td>C</td>
<td>µB C-18 analytical (3.9mm x 30cm; Waters)</td>
<td>3.0</td>
<td>75% CH₃CN-H₂O</td>
<td>tBuMS-isopropylidene, -ZR, -DZR, -cis-ZR.</td>
</tr>
<tr>
<td>D</td>
<td>µB C-18 analytical (3.9mm x 30cm; Waters)</td>
<td>3.0</td>
<td>50% MeOH-H₂O</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td>µB C-18 analytical (3.9mm x 30cm; Waters)</td>
<td>3.0</td>
<td>70% MeOH-H₂O</td>
<td>-</td>
</tr>
<tr>
<td>F</td>
<td>µB C-18 analytical (3.9mm x 30cm; Waters)</td>
<td>1.4</td>
<td>20% MeOH-0.05M NH₄OAc (pH8.2)</td>
<td>27G, 29G.</td>
</tr>
<tr>
<td>G</td>
<td>µB phenyl (3.9mm x 30cm; Waters)</td>
<td>1.5</td>
<td>45% CH₃CN-H₂O</td>
<td>1P, 1PA.</td>
</tr>
<tr>
<td>H</td>
<td>µB phenyl (3.9mm x 30cm; Waters)</td>
<td>1.5</td>
<td>15% MeOH-0.2M HOAc</td>
<td>27G, 29G.</td>
</tr>
<tr>
<td>I</td>
<td>RCM 100 C-8 cartridge (Waters)</td>
<td>3.0</td>
<td>60% MeOH-0.2M HOAc</td>
<td>-</td>
</tr>
<tr>
<td>J</td>
<td>RCM 100 C-8 cartridge (Waters)</td>
<td>3.0</td>
<td>40% MeOH-0.2M HOAc</td>
<td>ZR, DZR.</td>
</tr>
<tr>
<td>K</td>
<td>RCM 100 C-8 cartridge (Waters)</td>
<td>3.0</td>
<td>10% MeOH-0.2M HOA</td>
<td>-</td>
</tr>
<tr>
<td>L</td>
<td>Bondapak C-18/Porasil B (7.8mm x 61cm)</td>
<td>4.0</td>
<td>(i) 5% MeOH-0.2M HOAc</td>
<td>bulk isolation of cytokinins.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(ii) 60% MeOH-0.2M HOAc</td>
</tr>
<tr>
<td>M</td>
<td>Partisil 10 C-8 H9 (9.4mm x 50cm; Whatman)</td>
<td>4.0</td>
<td>60% MeOH-0.2M HOAc</td>
<td>1P, 1PA.</td>
</tr>
<tr>
<td>N</td>
<td>Partisil 10 C-8 H9 (9.4mm x 50cm; Whatman)</td>
<td>4.0</td>
<td>20% MeOH-0.2M HOAc</td>
<td>Z, ZR, 29G, 27G.</td>
</tr>
<tr>
<td>P</td>
<td>Zorbax C-8 semi-prep. (9.4mm x 25cm; Du Pont)</td>
<td>5.0</td>
<td>30% MeOH-0.2M HOAc</td>
<td>Ado, Ade, ZR, DZR, Z, DZ.</td>
</tr>
<tr>
<td>Q</td>
<td>Zorbax C-8 semi-prep. (9.4mm x 25cm; Du Pont)</td>
<td>4.0</td>
<td>35% MeOH-0.2M HOAc</td>
<td>29G, 27G.</td>
</tr>
<tr>
<td>R</td>
<td>Zorbax C-8 semi-prep. (9.4mm x 25cm; Du Pont)</td>
<td>4.0</td>
<td>40% MeOH-0.2M HOAc</td>
<td>NPG, 31OH-Z, Z.</td>
</tr>
<tr>
<td>S</td>
<td>Zorbax C-8 semi-prep. (9.4mm x 25cm; Du Pont)</td>
<td>4.0</td>
<td>70% EtOH-0.2M HOAc</td>
<td>1P, 1PA.</td>
</tr>
<tr>
<td>T</td>
<td>Techsil 10 Cr (8.0mm x 25cm; HPLC Tech. Ltd)</td>
<td>4.0</td>
<td>10% MeOH-0.2M HOAc</td>
<td>27G, 29G.</td>
</tr>
</tbody>
</table>
0-glucoside hydrolysis. The cytokinin 0-glucoside residues were dissolved in 0.1M sodium acetate buffer, pH 5.4. β-D-Glucosidase (15 µl, EC 3.2.1.21, from sweet almonds, Boehringer, Mannheim, FRG) was added and the sample was incubated overnight at 28°C. The hydrolysis products were then extracted with water-saturated n-BuOH at pH 8-9.

N-glucoside hydrolysis. The sample residues containing Z9G and Z7G were dissolved in 1M HCl and heated at 80°C for 2-3 hours. Solvent was then evaporated in vacuo.

2.3.4 Derivatisation Procedures

Per-tBuDMS derivatives were made quantitatively by dissolving the sample in a mixture of dry pyridine, DMAP solution (1 mg/ml pyridine, EGA Chemie, Steinheim, FRG) and N-methyl-N-t-butyldimethylsilyl-trifluoroacetamide (MTBSTFA) containing 1% t-butylidimethylchlorosilane (Regis Chemical Co., Morton Grove, Illinois, USA) in the ratio 10:1:10 (v/v/v) and then heating the mixture at 90°C for 15 minutes. For TLC or HPLC, the samples were dried under nitrogen and the residue dissolved in the appropriate solvent. For GC-MS, the reaction solution was injected directly into the capillary column.

Per-methyl derivatives were prepared by a modification of the method of J. Eagles et al. (1974). The material to be methylated was dissolved in a solution of potassium t-butoxide (30 mg, EGA Chemie, Steinheim, FRG) in dry redistilled dimethylsulphoxide (DMSO, 1 ml, prepared by heating at 70°C for 30 minutes in a 1 ml reaction-vial). Methyl iodide (10 µl) was then added and the mixture allowed to stand at room temperature for 30 minutes when water (100 µl) was added and the mixture extracted with chloroform (3x50 µl). The chloroform solution was then washed with water (50 µl) and evaporated to dryness. The residues were then taken up in chloroform for GC-MS analysis.
Per-TMS derivatives were made by heating samples at 90°C for 15 minutes in N,0-bis-(trimethylsilyl)trifluoracetamide containing 1% trimethylchlorsilane (BSTFA-TMCS, 10 µl, Pierce Chemical Co., Illinois, USA) and pyridine (10 µl).

THP derivatives of iP and Z were prepared by dissolution in dioxan (200 µl, BDH Chemicals Ltd, Poole, England), 2,3-dihydropyran (50 µl, EGA Chemie) and formic acid (20 µl, May and Baker, Dagenham, England). The mixture was heated at 90°C for 2 hours, cooled, evaporated down and the residue dissolved in methanol.

Trifluoracetyl derivatives were made by heating the dried sample in a mixture of pyridine (10 µl) and N-methyl-bis(trifluoroacetamide) (MBTFA, 10 µl; Pierce Chemical Co.) at 90°C for 20 min. The reaction mixture was injected directly into the GC.

2',3'-O-Isopropylidene ethers of the cytokinin ribosides were made by dissolving samples in either 2,2-dimethoxypropane (30 µl, Fluka) or 2,2-diethoxypropane (30 µl, Aldrich), to which was added conc. HCl (1 µl; Ajax). Samples were allowed to stand for 5 minutes at 30°C, then rapidly evaporated under a stream of nitrogen, following which, either tBuDMS or TFA derivatives could be made by the procedures outlined above.

Cyclic n-butylboronic esters of the cytokinin ribosides were prepared by heating in a pyridine solution of 1-butaneboronic acid (20 µl, 1 µg/µl; Aldrich) at 90°C for 15 min. Addition of either BSTFA-TMCS (20 µl) or MSTFA (20 µl) to the mixture afforded the butaneboronate-TMS and the butaneboronate-tBuDMS derivatives, respectively.

2.3.5 Scintillation Counting

Aqueous samples were counted in a toluene-Triton X-100 scintillant (2:1, v/v) containing 2,5-diphenyloxazole (PPO, 2 g/l, Sigma). The
ratio of sample to scintillant was (1:10, v/v). Samples dissolved in ethanol or DMSO (200 µl) were counted in a toluene scintillant containing PPO (7 g/l, 5 ml), from which the radioactivity could then be recovered by extracting with water (5 ml). Samples were counted for five minutes in a 1215 Rackbeta II Liquid Scintillation Counter (LKB, Wallac, Finland). Disintegrations per minute (dpm) were automatically calculated using the external standard channels ratio and a memory­ stored quench correction curve derived from a series of [3H]-n­hexadecane (The Radiochemical Centre, Amersham, England) standards quenched with pyridine.

2.3.6 Mass Spectrometry

Mass spectra were obtained using a Finnigan 4500 GCMS. Samples were either introduced via an on-column injector to a SGE capillary column (vitreous silica BPI, 25 m, carrier gas helium) or via the direct inlet probe. Electron impact mass spectra (EIMS) were taken at 70 eV ionising energy and at a source temperature of 140°C. Chemical ionization mass spectra (CIMS) were recorded using either methane or ammonia reagent gas (140 eV, source temperature 120°C, 1 torr). High resolution mass measurements were carried out on an AEI MS-902 instrument.

2.3.7 UV Spectrometry

UV spectra were recorded in 1 cm quartz cells on a Shimadzu UV-240 UV-visible recording spectrophotometer using EtOH-H2O (3:1, v/v) containing ammonia (0.3M).

2.3.8 RIA

RIA was carried out according to procedures described by Badenoch-Jones et al., (1984).
2.3.9 Synthetic Cytokininins

Z and ZR were purchased (Sigma or Calbiochem) as mixtures of the *cis* and *trans* isomers which were purified by HPLC (C.W. Parker, unpublished). DZ was purchased (Sigma) as a racemic (±) mixture and was used as such. BAP was purchased from Sigma. All glucosides of cytokinins had been previously synthesised chemically by Cowley *et al.* (1975, 1978); Duke *et al.* (1975, 1978, 1979); Letham *et al.* (1975, 1977). The deuterium labelled cytokinins were synthesised by Summons *et al.* (1979b).

The radiolabelled cytokinins were synthesised as follows: [2,8-\(^3\)H]Z, 270 mCi/mmole, Letham and Young, 1971; [8-\(^3\)H]-ZR, 330 mCi/mmole, Summons *et al.*, 1980; [G-\(^3\)H]-BAP, 25 and 600 mCi/mmole, Wilson *et al.*, 1974; [\(^3\)H]-6GZ, 132 mCi/mmole, Letham and Gollnow, 1985; [\(^3\)H]-Z7G, 110 mCi/mmole, Letham and Gollnow, 1985.

DZR. ZR (50 mg, Sigma Chemical Co.) was dissolved in ethanol (20 ml) and stirred at room temperature under a hydrogen atmosphere (bubbled continuously through system) in the presence of platinum (IV) oxide-water (1 mg, 1/n, Type D, Johnson Matthey Chemicals Ltd. Herts., UK). The reaction was monitored by HPLC (system J). After 100 minutes, when essentially all the ZR had been consumed, the catalyst was filtered off and washed with MeOH-H\(_2\)O-HOAc (10:9:1, v/v). The combined filtrate and washings were evaporated down and the residue chromatographed on normal phase TLC system D. DZR (26 mg, 52%) was eluted from the silica gel with MeOH-H\(_2\)O-HOAc (10:9:1, v/v). A small amount of isopentanyladenosine was similarly isolated by TLC.

**NB** - The catalyst must be covered by the solvent at all times as an explosion may result if it is exposed to the hydrogen atmosphere in a dry state.
3. The Synthesis of Inhibitors of Cytokinin N-Glucosylation.

He that shoots oft
at last shall hit the mark

*Utopia*, Thomas More, 1478-1538

3.1 Introduction

Attempts to regulate the development and growth of plants often involves the supply of exogenous cytokinins. However, the effectiveness of exogenous cytokinins may be limited by rapid metabolic inactivation involving the formation of 7- and 9-glucosides or oxidative cleavage of the N6-sidechain (see discussion in Section 1.2). To enhance growth responses to cytokinins it is desirable to suppress this inactivation. This could be achieved by either selectively inhibiting the inactivating enzymes or by presenting the plant tissue with an active cytokinin derivative or analogue that is inert to the mechanisms of inactivation. Such compounds may also be useful in manipulating the levels of the endogenous cytokinins and thus varying the physiological status of the plant tissue.

In two of the systems we have chosen to study, sweet corn leaves and radish cotyledons, an important mode of inactivation is formation of the 7- and 9-glucosides. The synthesis of potential inhibitors of this glucosylation is reported in this chapter. In addition to 3-substituted xanthines (Section 3.2) and a 2-amino-BAP derivative (Section 3.4), these included a novel oxazolopyrimidine (Section 3.3), which
potentially could possess cytokinin activity itself, as well as inhibiting N-glucosylation of other cytokinins.

3.2. 3-Substituted Xanthines.

3.2.1 Introduction

It has been shown that the xanthine (1) derivative theophylline (2, 1,3-dimethylxanthine) will suppress, to a very limited extent, the formation of the 7- and 9-glucosides of exogenously supplied BAP in radish cotyledons (Letham and Tao, unpublished). In Amaranthus seedlings, 2 (at 5mM, as the ethylene diamine complex, aminophylline) has also been reported to partially suppress the conversion of BAP to metabolites which may be glucosides of BAP (Elliot and Murray, 1975). A more effective inhibitor was found to be 1-methyl-3-isobutylxanthine (3) (Letham and Tao, unpublished). Hence it was proposed that a series of 3-substituted xanthines be synthesised for testing as inhibitors of cytokinin N-glucosylation.

Three approaches to the construction of 3-alkylxanthines are available: (a) the synthesis from first principles utilising a mono-substituted urea to make a 1-alkyl-5,6-diaminouracil which may then be ring-closed after the method of Traube (1900); (b) a novel rearrangement of a suitably substituted oxazolopyrimidine has been
described by Ohtsuka (1973); and (c) the direct alkylation of an appropriate xanthine precursor. The first approach and modifications to it (Scheme 3.1) has been successfully exploited on many occasions (Traube, 1900; Papesch and Schroeder, 1951; Ukai et al., 1954; Ishidate et al., 1956; Wooldridge and Slack, 1962; Kishikawa and Yuki, 1966; Ohtsuka, 1973) to yield a variety of 3-alkylxanthine derivatives. The initial condensation with mono-substituted ureas occurs almost exclusively at the unsubstituted nitrogen atom, where steric hindrance is at a minimum. The subsequent ring-closure then leads to the 1-substituted 6-aminouracil (Papesch and Schroeder, 1951). This key intermediate could also be made by a simple stereoselective base alkylation of 6-aminouracil with one equivalent of an alkyl halide (Ukai, 1954). Nitrosation at position 5 and reduction of the nitroso group yielded the 5,6-diaminouracil derivative. Finally, ring annulation to produce the 3-alkylxanthine could then be achieved by use of reagents, such as formic acid or formamidine, that effectively add a one carbon fragment across the amino moieties of the pyrimidine ring.

Ohtsuka's (1973) new pathway to the 3-alkylxanthines is outlined in Scheme 3.2. The oxazole-imidazole conversion occurs under mild conditions and is thought to proceed by oxazole ring rupture followed by imidazole ring formation. The identity of the products of this reaction were confirmed by comparison of their spectroscopic properties with those of the 3-alkylxanthines unambiguously synthesised from substituted ureas through pyrimidine derivatives.

Of the three routes available for the synthesis of 3-alkylxanthines the most facile is the direct alkylation of 1,7-dimethylxanthine (4) at position 3, the only site available for alkylation (Blicke and Godt, 1954). Alternatively, 1-methylxanthine (5) may be alkylated.
Scheme 3.1: Synthesis of 3-alkylxanthines from 1-alkylurea.
Scheme 3.2: Formation of 3-substituted xanthines via 7-(6H)-iminoxazolopyrimidines.
Spectroscopic studies by Cavaleiri et al., (1954) have established that the sequence of ionisation of the hydrogen atoms of 5 is 3 followed by 7, suggesting that a controlled alkylation would give reasonable yields of the 1-methyl-3-alkylxanthine. However, it has been reported by Hu et al. (1980) that, in fact, the higher rate of alkylation of the less hindered 7-position outweighs the effect of the higher acidity of the 3 position. Nevertheless, these workers were able to obtain good overall yields of their required 1-methyl-3-alkylxanthines by the use of (pivaloyloxy)methyl to protect the more reactive 7-position.

A further advantage of these direct methods is that 1-methyl-3-substituted xanthines are produced readily and we were particularly interested in these methyl derivatives because of the observed activity of 2 and 3 as mentioned above.

3.2.2 Results and discussion

Alkylation of 1,7-dimethylxanthine (4) with the appropriate halide in DMF containing NaH yielded the 3-substituted 1,7-dimethylxanthines 6 to 13 and 16. Compounds 6 and 7 have been prepared previously by alkylation of 4 in aqueous ethanol containing KOH (Blicke and Godt, 1954). However, the yields obtained in the present study were markedly better (6, 89%, cf lit. 44%, 7, 63% cf lit. 35%). The remaining compounds have not been reported previously. The UV spectra of these compounds remained unaltered (λ max = 273 nm) with changes of pH, as would be expected for fully N-alkylated xanthines which can neither enolize or ionize. The identity of the alkylation products was confirmed by examination of the NMR spectra and by mass spectral measurements at both high and low resolution. The NMR spectra all featured singlet resonances for the proton at position 8 (av. δ 7.55), the N-methyl at position 7 (av. δ 4.03) and the N-methyl at position 1
<table>
<thead>
<tr>
<th>Compound</th>
<th>R'</th>
<th>R''</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>-CH₃</td>
<td>-H</td>
</tr>
<tr>
<td>5</td>
<td>-H</td>
<td>-H</td>
</tr>
<tr>
<td>6</td>
<td>-CH₃</td>
<td>-CH₂C₆H₅</td>
</tr>
<tr>
<td>7</td>
<td>-CH₃</td>
<td>-(CH₂)₂CHCH₃</td>
</tr>
<tr>
<td>8</td>
<td>-CH₃</td>
<td>-(CH₂)₂CH=CH₂</td>
</tr>
<tr>
<td>9</td>
<td>-CH₃</td>
<td>-CH₃CH=CH₂</td>
</tr>
<tr>
<td>10</td>
<td>-CH₃</td>
<td>-(CH₂)₄CH=CH₂</td>
</tr>
<tr>
<td>11</td>
<td>-CH₃</td>
<td>-CH₂C≡CH₂</td>
</tr>
<tr>
<td>12</td>
<td>-CH₃</td>
<td>-(CH₂)₄OH</td>
</tr>
<tr>
<td>13</td>
<td>-CH₃</td>
<td>-CH₂CH=CHCH₃</td>
</tr>
<tr>
<td>14</td>
<td>-CH₃</td>
<td>-CH₂CHOH·COH·CH₃</td>
</tr>
<tr>
<td>16</td>
<td>-CH₃</td>
<td>-CH₂CH=CH₂OH</td>
</tr>
<tr>
<td>17</td>
<td>-CH₂CH=CHCH₃</td>
<td>-H</td>
</tr>
<tr>
<td>18</td>
<td>-H</td>
<td>-CH₂CH=CHCH₃</td>
</tr>
<tr>
<td>19</td>
<td>-CH₂C₆H₅</td>
<td>-H</td>
</tr>
</tbody>
</table>
(av. δ 3.42). The methyl resonances were assigned by comparison with the methyl resonance observed in the NMR spectrum of 1-methylxanthine (δ 3.40). The remaining signals were due to the substituent at position 3.

The aromatic heterocyclic purine ring is very stable and not readily fragmented by electron impact. This property is reflected in the fact that the mass spectra of the 3-substituted xanthines generally have strong molecular ions (M⁺) and the major fragments are derived from cleavages of the alkyl sidechain. The ion, m/z 180, common to all the mass spectra of 1,7-dimethylxanthine derivatives, is derived from the loss of the alkyl substituent via a McLafferty rearrangement (I → II), and entails the transfer of a hydrogen to the purine moiety. Also common to these spectra are the ions resulting from the cleavage of the sidechain between C-1' and C-2'. The shorter butyl and butenyl substituents of 8 and 12 tend to be homolytically fragmented to produce m/z 193 (III). The longer and branched alkyl chains are cleaved between C-1' and C-2' with hydrogen transfer to the purine moiety and proceed via a four-membered (IV → Va) or a five-membered transition state (IV → Vb), yielding m/z 194. Fragmentation of the pentenyl derivative 9 yielded both m/z 193 and m/z 194 in almost equal abundance whereas the hexenyl (10) and branched chain derivatives (7, 13, 14, 16) yielded m/z 194 almost exclusively. These fragmentation pathways are identical with those previously described by Reiser (1969) and by Falch (1970) for the N-alkyluracils. In addition, the oxygenated sidechain of 12 may lose H₂O (M-18) and CHO₂H (M-29) or in the case of 16, H₂O and CH₂OH (M-31). The mass spectrum of 14 is dominated by the cleavage of the cis-diol in the alkyl substituent, affording m/z 223 (M-COH(CH₃)₂).
Trimethylsilylation of 14 produced a mixture of a mono-TMS and a di-TMS derivative which could be separated by GC. The base peak in the mass spectra of the mono-TMS derivative appeared at \( m/z \) 295 (M–COH(CH\(_3\))\(_2\)) indicating a preferential silylation of the 2° alcohol over the sterically crowded 3° alcohol.

\[
\text{m/z 295}
\]

Subsequent fragmentation of the xanthine moiety \( (m/z \) 180, 193, 194) then proceeds via a retro Diels Alder Reaction with expulsion of CH\(_3\)NCO involving the N-1 and C-2 atoms. These fragmentations parallel those described for caffeine and 1,7-dimethylxanthine by Spiteller and Friedmann-Spiteller (1962) and by Midha \textit{et al.} (1977). The formulae of all fragments were established by high resolution mass measurements.

Some aspects of the synthesis of the 3-substituted 1,7-dimethylxanthines merit comment. The production of 1,7-dimethyl-3-(3'-methyl-2'-buten-1'-yl)xanthine (13) via an alkylation of 4 required the production of a suitable alkyl halide. 3-Methyl-2-buten-1-ol was converted to the 1-iodide using a simple one-step treatment with iodonitrtrimethylsilane (Jung and Ornstein, 1977). The iodide was not isolated, the crude chloroform solution being used directly for the alkylation reaction. Iodination of 3-methyl-3-buten-1-ol resulted in the isomerisation of the double bond (as evidenced by NMR data) so that 13 was the only alkylation product isolated. The isomerisation was
probably catalysed by hydrogen iodide which is produced during the reaction. An attempt to synthesise this iodide via the trimethylsilyl ether (Jung and Ornstein, 1977), avoiding the production of hydrogen iodide, similarly resulted in isomerisation.

The presence and the position of hydroxyl groups in the aliphatic N⁶-substituents of the cytokinins markedly influences the biological activity (Matsubara, 1980 and references therein). Thus, it was decided to modify some of the synthetic xanthine derivatives by introducing hydroxyl groups to the sidechain. The diol 14 was produced by a permanganate oxidation of 13 using a modification of the method described by Wiberg and Saegebarth (1957). Selenium dioxide oxidations of 10 were performed according to the method of Olumucki et al. (1965). Very poor yields were obtained, probably due to the formation of organo-selenium complexes. The products isolated from SeO₂ oxidation of 10 were two isomeric alcohols, but due to poor yields, only mass spectral data were obtained. The possible structures of these alcohols may be inferred from previous studies of selenium dioxide oxidations by Wiberg and Nielsen (1964). Initially, selenous acid would be expected to add to the double bond with the selenium function at the least substituted end of the double bond. Proton abstraction would then restore the substituted alkene, following which, competing modes of nucleophilic displacement by the solvent, acetic acid, would produce the isomeric mixture of acetates observed in Scheme 3.3.

Selenium dioxide oxidation of 13 was also carried out, but poor yields of 16 were realised.

Alkylations of 1-methylxanthine (5) yielded the 3- or 7-substituted 1-methylxanthines, 17 to 19. 1-Methylxanthine is poorly soluble in DMF and so the alkylations were carried out in DMSO. A mixture of di-
Scheme 3.3: Proposed course of the selenium dioxide oxidation of 10.
substituted and mono-substituted derivatives was extracted from the reaction mixture together with unreacted 5. Structural assignments for these xanthine derivatives (17, 18, 19) were based primarily on UV spectra which are diagnostic of the position of substitution on the xanthine ring (Cavalleiri et al., 1954; see also Table 3.1). A marked bathochromic shift caused by basification is characteristic of a 1,7-disubstituted xanthine, while alkylation with 4-iodo-2-methyl-2-butene yielded a mixture of the 3- and 7-substituted products (18 and 17, respectively). Reaction with benzyl bromide gave only the 7-benzyl derivative (19). The mass spectra of 17 and 18 were identical and did not provide information relevant to the positions of substitution. Alkylations of 5 in the 3-position may be more profitably carried out after first protecting the 7-position as described recently by Hu et al. (1980), using a (pivaloyloxy)methyl derivative. However, this information was not available when the present research was initiated.

Table 3.1: The bathochromic shift of UV maxima is characteristic of the substitution pattern of disubstituted xanthines.

<table>
<thead>
<tr>
<th>Substituted xanthine</th>
<th>UV λ max (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>neutral pH</td>
</tr>
<tr>
<td>1,7-dimethylxanthine</td>
<td>270</td>
</tr>
<tr>
<td>1-methyl-3-isobutylxanthine</td>
<td>273</td>
</tr>
<tr>
<td>1,9-dimethylxanthine</td>
<td>239, 271</td>
</tr>
</tbody>
</table>
3.3 Oxazolopyrimidines

3.3.1 Introduction

An alternative approach to preventing 7- and 9-glucosylation is to modify the purine ring of the exogenously supplied cytokinin so that glucosylation becomes impossible. Alkylation of BAP at the N-9 position would at first sight seem to be a suitable modification. However, some such derivatives, for example, the 4-chlorobutyl derivative (Letham et al., 1978), have been reported to be metabolically unstable as they are slowly dealkylated to BAP. Usually 9-alkyl derivatives of BAP exhibited very weak cytokinin activity (Letham, 1978). The above problem could, perhaps, be avoided by a formal replacement of the nitrogen at position 9 with an oxygen. The resulting BAP analogue would be 7-benzylaminooxazolo[5,4-d]pyrimidine (24).

![Compound 24](image)

Although such structural modification of the imidazole ring is likely to result in a reduction of cytokinin activity (Matsubara, 1980 and references therein), the degree of reduction is not predictable. Compound 24 may prove to be a very stable, active cytokinin. Alternatively, if supplied to plant tissues together with BAP, the oxazolopyrimidine may act as a competitive inhibitor of the glucosylating enzymes.

A series of 7-alkylaminooxazolopyrimidines were synthesised by ring-closure of 4-cyano-5-ethoxymethylenaminooxazole (22) with a variety
of alkylamines (Ohtsuka, 1970b). However, as the size of the alkyl groups increased, progressively more vigorous reaction conditions were required, and the product yields decreased. An attempt to carry out this ring-closure using either aniline or β-naphthylamine required prolonged heating at high temperature and the only products reported were the corresponding 9-substituted hypoxanthines (Scheme 3.4). Consequently, it was decided to attempt the synthesis of 24 starting with 7-aminooxazolo[5,4-d]pyrimidine, 23.

The oxazolo[5,4-d]pyrimidines have generally been prepared by the ring closure of either suitably substituted pyrimidine derivatives or the appropriate oxazole precursors (Senga, et al., 1977 and references therein). However, the synthesis of 23 has only been reported by Ferris and Orgel (1966) and by Ohtsuka (1970a). Ohtsuka’s procedure was reported to have the better overall yield, and was therefore the method of choice. In a relevant preliminary study, methods for the N6-substitution of adenine were reviewed. Substitution of the exocyclic nitrogen of adenine has generally been achieved by five different approaches. Firstly, adenine may be N-alkylated at position 1, which when followed by an alkali-induced Dimroth rearrangement, produced the required 6-substituted aminopurine (Leonard et al., 1966 and references therein; Robins et al., 1967; Grimm and Leonard, 1967; Martin and Reese, 1968; Robins and Trip, 1973). Secondly, the primary amine of adenine may be exchange-aminated by heating in the presence of a secondary amine and the amine hydrochloride (Whitehead and Traverso, 1960; Leonard and Deyrup, 1962). It is also possible to acylate the primary amino group of adenine and to subsequently reduce this intermediate with lithium aluminium hydride to yield a 6-substituted aminopurine (Baizer et al., 1956; Bullock et al., 1957; Lettre and Ballweg, 1958). Fourthly,
Scheme 3.4: Synthesis of 7-benzylaminooxazolo[5,4-d]pyrimidine
nucleophilic substitution (aminolysis) of 6-chloropurine (prepared from adenine via hypoxanthine), by refluxing with the appropriate amine in α-BuOH, will also yield the required adenine derivative (Daly and Christensen, 1956; Robins et al., 1967; Fleysher et al., 1968; Corse and Kuhnle, 1972; Summons et al., 1979b; Scott and Horgan, 1980). Finally, 9-(tetrahydropyran-2-yl)adenine can be directly alkylated at N6 by reaction with an alkyl bromide in an aprotic solvent in the presence of NaH or potassium t-butoxide (Young and Letham, 1969). The 9-blocking group can then be cleaved with acid. Consideration of these procedures suggested that direct benzylation of 23 in the presence of NaH was probably the procedure to try initially.

3.3.2 Results and discussion

Compound 23 was synthesised according to the method prescribed by Ohtsuka (1970a). Benzylation of 23 in DMF with NaH produced a mixture of di-substituted compounds, as evidenced by mass spectral data. Even when the calculated amount of NaH was used, this disubstitution resulted and the conversion of the mono- to the di-substituted derivative appeared to occur at a rate which greatly exceeded that of the initial alkylation. Transamination reactions also yielded only di-benzyl derivatives. Alkylation of 23 in a DMF solution of NaH with 1-bromo-3-methylbutane similarly yielded di-substituted derivatives. A Sandmeyer reaction (Vogel, 1964) and a modified Sandmeyer reaction (Talik et al., 1974) with adenine both produced 6-chloropurine. However, when applied to 23, no 7-chlorooxazolopyrimidine was detected. Acetylation of 23 produced a mixture of both the mono- and di-acetyl derivatives. As the diacetyl derivative was the major component of the mixture, such a procedure was also considered inadequate as a pathway to 24.
Hexamethylphosphoramide (HMPA) has been reported by Juaristi and Reyna (1984) to be a solvent capable of facilitating the direct alkylation of aromatic amines by alkyl halides. When the N-arylation reaction was repeated using HMPA plus a trace of DMAP in the reaction mixture a small amount of the required compound, 24, was isolated. The structure of 24 was confirmed by analysis of the spectroscopic data. High resolution mass measurements confirmed the formulae of the molecular ion \((m/z \ 226)\) and of the strong benzylimine ion \((m/z \ 106)\) in the mass spectrum. The proton NMR data proved to be consistent with the structure of 24, the resonances assigned to the exocyclic amino proton (δ 5.95) being readily exchangeable with \(D_2O\). These spectra eliminated the possibility that the product was 9-benzyl hypoxanthine (cf. Scheme 3.4).

3.4 Benzyaminopurine derivatives

3.4.1 Introduction

An enzyme in radish cotyledons that is responsible for the formation of Z7G (and trace quantities of Z9G) has been purified and studies of its kinetic behaviour and substrate specificity have been described (Entsch et al., 1979). Subsequent studies included a search for potential enzyme inhibitors (Parker et al., 1985, in press), the most effective of which was found to be 2-(2-hydroxyethylamino)-9-methyl-6-benzylaminopurine (27).
Entsch et al. (1979) claimed that the 9-methyl substituent blocked glucosylation of the imidazole ring and that this effect was enhanced by the 2-hydroxyethylamino group at position 2, binding to the enzyme. In the radish cotyledon expansion bioassay, 27 was found to be inactive as a cytokinin but did not suppress the activity of the endogenous cytokinins. When supplied together with suboptimal quantities of BAP, 27 enhanced the growth response to BAP, and the level of free BAP, in the tissue was also increased (Letham et al., 1982). Subsequent studies have suggested that the inhibitory role of 27 may be neutralized, in the cotyledons, by conversion to an O-glucoside (see Section 5.3). However, the effectiveness of 27 as an inhibitor of N-glucosylation may be improved by sterically hindering the O-glucosylation, utilising a 3° alcohol instead of a 1° alcohol in the substituent at C-2 of the purine ring. Thus, we proposed to synthesise and test 2-(2-hydroxy-2-methylpropylamino)-9-methyl-6-benzylaminopurine (29).

Compound 27 was originally synthesised as outlined in Scheme 3.5 (Parker et al., 1985 in press). It involved the production of both the 7- and 9-methyl derivatives which were separated by TLC. As the 9-methylation had been found to produce a more effective inhibitor of cytokinin N-glucosylation when compared with the 7-methyl derivative, we decided to modify this synthetic scheme to minimise the production of the 7-methyl isomer. Adenine and N6-substituted adenines can be alkylated almost exclusively at N-9 (Robins et al., 1961; Nagasawa et al., 1966). Thus, by reversing the order of methylation and benzylation of 2,6-dichloropurine one would expect to favour the production of 2-chloro-9-methyl-6-benzylaminopurine (26) rather than the 7-methyl isomer. The synthesis of 27 and 29 was then carried out as outlined in Scheme 3.6.
Scheme 3.5: Synthesis of 27 from 2,6-dichloropurine as described by Parker et al (1985, in press).
Scheme 3.6: Synthesis of 27-29 from 2-chloro-6-benzylaminopurine, optimising methylation at N-9.
3.4.2 Results and discussion

The methylation of 2-chloro-6-benzylaminopurine, in dry DMF containing NaH, yielded the 9-methyl derivative (26) with only a trace of the 7-methyl isomer. The use of excess methylating reagent resulted in the methylation of the exocyclic nitrogen (25). Reaction of 26 with ethanolamine in DMF yielded 27. An attempt to synthesise 29 as per the synthesis of 27, but including traces of the hydrochloride salt of 2-aminomethyl-2-propanol, resulted in the exclusive production of 28. The aminomethyl function is presumed to be derived from methylamine, a decomposition product of the solvent, DMF. 29 was eventually synthesised following the use of the free base derived from the passage of the hydrochloride salt though an anion-exchange resin in the hydroxide form. When compared with the original synthesis of 27, the preparation according to Scheme 3.6 was much more convenient and TLC steps were not essential in the preparation of the intermediate 26.

3.5 Experimental

3.5.1 General

The equipment and procedures for mass spectrometry, high resolution mass spectrometry (HRMS), HPLC, TLC, UV spectrophotometry and trimethylsilylation have been described previously in Section 2.3.

Low resolution electron impact mass spectra were also obtained using a Du Pont 21-491B instrument (70 eV, source temperature 240°C) (Wilmington, Ind., USA) interfaced to a VG 2025 data system (Altrincham, Cheshire, UK). Underivatised compounds were introduced via the direct probe inlet. TMS derivatives were introduced via a gas chromatograph (GCMS) (130V-101 on 80-100 Gaschrom Q, Applied Science Laboratories, State College, Pa, USA; temperature 200-300°C at 10°C/min).
Nuclear magnetic resonance (NMR) spectra were recorded with a Varian CFT-20 100 MHz spectrometer, a Joel JNM-MH-100 spectrometer or a Joel JNM-FX-200 spectrometer, using tetramethylsilane as an internal standard. Chemical shifts are quoted on the δ scale and the signals are described in terms of chemical shift, assignment, multiplicity and, where applicable, coupling constants.

Melting points were determined on a Kofler microheating stage and are uncorrected.

UV spectra were obtained in both basic (75% ethanol/0.4M NH₃ (aq) and neutral solvents (70% ethanol).

3.5.2 Alkyl derivatives of 1,7-dimethylxanthine and 1-methylxanthine

Alkylation of 1,7-dimethylxanthine (4)

4 (Sigma Chemical Co., St. Louis, Mo., USA) was dissolved in DMF, together with a 20% excess of sodium hydride (55-60% oil suspension Fluka AG, Buchs, Switzerland). The mixture was then stirred at 60-70°C for 20 minutes when the alkyl halide was added. The progress of the reaction was monitored by TLC (System A). At completion, the reaction mixture was diluted with water and extracted (x 3) with an equal volume of ethyl acetate. The extract was washed with dilute hydrochloric acid, a saturated sodium bicarbonate solution and water before drying over anhydrous sodium sulphate. The solvent was then evaporated and the residue recrystallised.

1,7-dimethyl-3-benzylxanthine (6)

4 was alkylated with benzyl bromide (Fluka) as described above to give 6 after recrystallisation from chloroform-η-hexane, yield 40%, mp 162-164°C, λ max 273.5 nm (base, neutral)
MS m/z (%) 270 (M⁺, base) 184 (13), 165 (20), 150 (13), 136 (6).
NMR (CDCl₃, 100 MHz) δ 7.51 (s, 1-H, C-8 proton),
7.40 (m, 5-H, aromatic), 5.28 (s, 2-H, benzyl)
3.97 (s, 3-H, N-7 methyl), 3.40 (s, 3-H, N-1 methyl)
HRMS for C₁₄H₁₄N₄O₂; calc. m/z 270.1118, found 270.1100.

1,7-dimethyl-3-(3'-methylbutyl)xanthine (7)

4 was alkylated with 1-bromo-3-methylbutane (Aldrich Chemical Co.,
Milwaukee, Wis. USA) to yield 7 after recrystallisation from chloroform-
n-hexane, yield 63%, mp 116-118°C, λ max 275 nm (neutral, base)
MS m/z (%) 250 (M⁺,53), 194 (base), 180 (72), 165 (15), 150 (15),
136 (87), 123 (32).
NMR (CDCl₃, 100 MHz) δ 7.62 (s, 1-H, C-8 proton)
4.18 (m, 2-H, C-1' methylene), 4.05 (s, 3-H, N-7 methyl),
3.45 (s, 3-H, N-1 methyl), 1.68 (m, 3-H, C-2' methylene and
C-3' methine), 1.00 (d, 6-H, J=7 Hz, terminal methyls).
HRMS for C₁₂H₁₈N₄O₂; calc. m/z 250.1431, found 250.1415.

1,7-dimethyl-3-(3'-butenyl)xanthine (8)

4 was alkylated with 4-bromo-1-butene (Fluka) to yield 8 after
recrystallisation from chloroform-n-hexane, yield 60%, m.p. 70-71°C,λ
max 274 nm (base, neutral)
MS m/z (%), 234 (M⁺,19), 193 (30), 180 (22), 136 (base).
NMR (CDCl₃, 100 MHz) δ 7.65 (s, 1-H, C-8 proton), 5.98 (m, 1-H,
C-3' vinyl), 5.20 (m, 2-H, C-4' vinyls), 4.26 (m, 2-H, C-1'
methylene), 4.08 (s, 3-H, N-7 methyl), 3.48 (s, 3-H, N-1 methyl)
2.60 (m, 2-H, C-2' methylene).
HRMS for C₁₁H₁₄N₄O₂; calc. 234.11165, found 234.11151.
1,7-dimethyl-3-(4'-pentenyl)xanthine (9)

4 was alkylated with 5-bromo-1-pentene (Fluka) to yield 9 after recrystallisation from chloroform-n-hexane, yield 82%, mp 53-54°C, $\lambda_{\text{max}}$ 274 nm (base, neutral)

MS $m/z$ (%) 248 (M+$^+$, 25), 194 (35), 193 (30), 180 (base), 136 (98), 123 (30)

NMR (CDCl$_3$, 100 MHz) δ 7.60 (s, 1-H, C-8 proton), 5.96 (m, 1-H, C-4' vinyl), 5.12 (m, 2-H, C-5' vinyls)
4.10 (m, 2-H, C-1' methylene), 4.04 (s, 3-H, N-7 methyl)
3.44 (s, 3-H, N-1 methyl), 2.10 (m, 3-H, C-2' and C-3'methylene)

HRMS for $\text{C}_{12}\text{H}_{16}\text{N}_4\text{O}_2$; calc. 248.12730, found 248.12712.

1,7-dimethyl-3-(5'-hexenyl)xanthine (10)

4 was alkylated with 6-bromohexane (Fluka) to yield 10 after recrystallisation from chloroform-n-hexane, yield 93%, mp 65-67°C.

MS $m/z$ (%) 262 (M+$^+$, base), 247 (10), 245 (6), 232 (2), 221 (8), 208 (3), 207 (3), 194 (22), 180 (28), 136 (5).

NMR (CDCl$_3$, 100 MHz) δ 7.57 (s, 1-H, C-8 proton), 5.86 (m, 1-H, C-5' vinyl), 5.04 (m, 2-H, C-6' vinyls), 4.10 (m, 2-H, C-1' methylene), 4.02 (s, 3-H, N-7 methyl), 3.42 (s, 3-H, N-1 methyl), 2.14 (m, 2-H, C-4' methylene), 1.80 (m, 2-H, C-2' methylene), 1.50 (m, 2-H, C-3' methylene)

HRMS for $\text{C}_{13}\text{H}_{18}\text{N}_4\text{O}_2$; calc. 262.1430, found 262.1429.

1,7-dimethyl-3-(2'-methyl-2'-propenyl)xanthine (11)

4 was alkylated with 3-chloro-2-methylpropene (Aldrich) to yield 11 after recrystallisation from methylene chloride-n-hexane, yield 63%, mp 118-119°C.

MS $m/z$ (%) 234 (M+$^+$, base), 219 (11), 177 (45), 165 (16), 162 (18), 149 (48), 136 (29), 123 (12), 108 (11).
NMR (CDCl$_3$, 100 MHz)  δ 7.51 (s, 1-H, C-8 proton), 4.78 (d, 2-H, J = 19Hz, vinyls), 4.62 (s, 2-H, C-1' methylene), 4.00 (s, 3-H, N-7 methyl), 3.40 (s, 3-H, N-1 methyl), 1.80 (s, 3-H, vinyl methyl).

HRMS for C$_{11}$H$_{14}$N$_4$O$_2$; calc. 234.11165, found 234.11195

l,7-dimethyl-3(4'-hydroxybutyl)xanthine (12)

4 was alkylated with 4-chloro-1-butanol (Fluka) to yield 12 after recrystallisation from ethanol-γ-hexane, yield 48%, mp 109-110°C, $\lambda_{max}$ 273.5 nm (base, neutral).

MS m/z (%) 252 (M$^+$, 12), 234 (11), 222 (18), 193 (33), 180 (base).

HRMS for C$_{11}$H$_{16}$N$_4$O$_3$; calc. 252.1222, found 252.12321.

1,7-dimethyl-3-(3'-methyl-2'-butenyl)xanthine (13)

3-Methyl-2-buten-1-ol (Aldrich) or 3-methyl-3-buten-1-ol (Aldrich) together with a slight excess of iodotrimethylsilane (Aldrich), were dissolved in dry chloroform under nitrogen in a reacti-vial containing 3x4 A Linde molecular sieves. The mixture was heated at 70°C for 30-45 min, cooled and the chloroform solution washed with an aqueous solution of sodium metabisulphite and water before drying over anhydrous sodium sulphate. The crude chloroform solution of 4-iodo-2-methyl-2-butene was then used to alkylate 4 (as detailed above) to yield 13 after recrystallisation from chloroform-γ-hexane, yield 57%, mp 113-114°C, $\lambda_{max}$ 274.5 nm (base, neutral).

MS m/z (%) 248 (M$^+$, 21), 180 (base), 151 (3), 136 (2), 123 (45)

NMR (CDCl$_3$, 100 MHz)  δ 7.56 (s, 1-H, C-8 proton), 5.40 (m, 1-H, C-2' vinyl), 4.72 (d of d, 2-H, J=8 Hz, C-1' methylene) 4.02 (s, 3-H, N-7 methyl), 3.43 (s, 3-H, N-1 methyl), 1.90 (s, 3-H, vinyl methyl), 1.74 (s, 3-H, vinyl methyl).

HRMS for C$_{12}$H$_{16}$N$_4$O$_2$; calc. 248.1273, found 248.1273.
1,7-dimethyl-3-(3'-methyl-2',3'-dihydroxybutyl)xanthine (14)

A solution of 13 in t-butanol-H₂O (3:1, v/v) at -10°C was stirred vigorously and a cold alkaline solution of potassium permanganate (1.5 equivalents in 60 mM sodium hydroxide) was added dropwise. The permanganate colour was discharged after 5 minutes and the manganese dioxide was then solubilized by adding a solution of sodium metabisulphate. The solution was extracted with an equal volume of ethyl acetate (x 3) which was then dried and the residue redissolved in 10% methanol-water (0.2M acetic acid). 14 was then purified by reverse phase HPLC, system K. Yield 57%, mp. 129-131°C, λ_max 274 nm (base, neutral).

MS m/z (%) 282 (M⁺, 4), 267 (3), 252 (1), 237 (1), 223 (base), 193 (22).

GC-MS di-TMS derivative m/z (%) 426 (M⁺, 1), 425 (2), 411 (6), 409(4), 368 (11), 295 (22), 252 (base), 237 (20), 193 (7).

GC-MS mono-TMS derivative m/z (%) 354 (M⁺, 1), 339(9), 295 (base) 279 (9), 252 (59), 237 (26), 206 (11), 193 (16).

HRMS for C₁₂H₁₈N₄O₄; calc. 282.1328, found 282.13153.

1,7-dimethyl-3-(6'-hydroxy-4'-hexenyl)xanthine (15)

10 (11.9 mg) was dissolved in a heated (105-110°C) and stirred solution of acetic acid/acetic anhydride (2:1, v/v, 5ml), together with selenium dioxide (5 mg, freshly sublimed, Ajax Chemicals, Sydney, Australia). Heating and stirring continued for 1 hour when the mixture was cooled and decanted from the black selenium precipitate before diluting with water and extracting into ethyl acetate. The extract was evaporated and the acetate isolated by preparative TLC (system A).

MS m/z (%) 320(M⁺, 9) 278 (10), 262 (16), 260 (10), 194 (24), 193 (25), 181 (40), 180 (base).
The acetate was hydrolysed by heating in a solution of 2M HCl (0.5 ml) and methanol (5 ml) at 70°C for 1 hour. The solvent was evaporated and the products isolated by preparative TLC (system A).

MS m/z (%) 278 (M⁺, 18), 260 (12), 212 (13), 207 (10), 206 (12), 194 (30), 193 (27), 181 (82), 180 (base), 165 (13), 150 (13).

GC-MS analysis of 15 as the TMS derivative showed it to consist of two isomers which were subsequently preparatively separated using reverse phase HPLC, system I. λ_max 15a and 15b 273 nm (base neutral), yield < 5%.

GC-MS TMS derivative 15a m/z (%) 350 (M⁺, 22), 335 (28), 253 (52), 237 (27), 206 (43), 181 (53), 180 (32), 170 (base).
GC-MS TMS derivative 15b m/z (%) 350 (M⁺, 7), 335 (12), 260 (23), 237 (13), 234 (7), 193 (28), 181 (55), 180 (base), 170 (20).

(2'E)-1,7-dimethyl-3-(4'-hydroxy-3'-methyl-2'-butenyl)xanthine (16)

Tri-n-octylphosphine (1.06 g, Aldrich) was added dropwise to a stirred solution of (2E)-4-hydroxy-2-methylbut-2-en-1-yl tetrahydropyranyl ether (0.5 g, synthesised by J. Eichholzer and J.K. MacLeod according to the procedure of H. Young and D.S. Letham, unpublished), together with carbon tetrabromide (1.06 g, BDH Ltd, Poole, England) in benzene and petroleum ether (3 ml and 12 ml respectively, distilled and stored over sodium) cooled to -20°C, under a dry nitrogen atmosphere. Stirring continued for a further 45 min. The petroleum ether layer was removed and the residue washed with petroleum ether (4 x 2 ml) at -20°C. The combined extract was partially evaporated and the crude unstable bromide was used without further purification in the alkylation of 4 (as described above) to yield 16 following preparative TLC (system A), hydrolysis of the THP ether by dissolution in 50% 0.1M
HCl-MeOH at room temperature for 6 hours and recrystallisation from ethanol-water, yield 32%, $\lambda_{\text{max}}$ 273 nm (base, neutral).

MS $m/z$ (%): 264 (M$^+$, 18), 246 (20), 233 (25), 194 (5), 181 (base), 180 (59), 163 (7), 161 (13), 151 (10), 136 (11), 123 (60).

HRMS for C$_{12}$H$_{16}$N$_4$O$_3$; calc. 264.1222, found 264.1220.

l-methyl-7-(3'-methyl-2'-butenyl)xanthine (17) and l-methyl-3-(3'-methyl-2'-butenyl)xanthine (18)

5 (Sigma) was alkylated (as per alkylation of 4) in DMSO using one equivalent of sodium hydride and 4-iodo-2-methyl-2-butene (prepared as described in the synthesis of 13). The product was preparatively chromatographed on TLC (system A) and the mono-alkylated products separated by use of HPLC system M.

MS 17 and 18 $m/z$ (%): 234 (M$^+$, 21), 166 (base), 109 (37).

UV 17 $\lambda_{\text{max}}$ 293.5 nm (base) 269.4 nm (neutral).

UV 18 $\lambda_{\text{max}}$ 276.5 nm (base) 273 nm (neutral).

l-methyl-7-benzylxanthine (19)

5 was alkylated with benzyl bromide (Fluka) and the mono-alkylated product isolated as per the synthesis of 17 and 18.

MS $m/z$ (%): 256 (M$^+$, 70), 91 (base).

UV $\lambda_{\text{max}}$ 294.5 nm (base), 270 nm (neutral).

3.5.3. Synthesis of 7-benzylaminooxazolo[5,4-d]pyrimidine

phenylazomalononitrile (20)

Aniline (Ajax, 19.6 g) was dissolved in a solution of conc. HCl (64 ml) and water (64 ml). Sodium nitrite (16.0 g) was dissolved in water (80 ml) and both solutions cooled to 0-5°C. The sodium nitrite solution was then slowly added to the aniline solution, the temperature being maintained below 5°C. This phenyldiazonium chloride solution was then slowly added to a cooled (0-5°C) solution of malononitrile (13.2 g),
sodium acetate (80 g) and water (400 ml) which was vigorously stirred. The resulting thick yellow precipitate was filtered, dried and recrystallised from chloroform, yield 34.0 g (95%).

MS m/z (%), 170 (M⁺, base), 105 (22), 92 (36), 91 (29).

NMR (CDCl₃, 100 MHz) δ 10.1 (br.s., 1-H, C-H) 7.36 (m, 5-H, aromatic).

5-amino-4-cyanooxazole (21)

Zinc powder (BDH, 30 g) was suspended in formic acid (99%, 200 ml), stirred and warmed to 40°C under dry N₂. 20 (20 g) was added slowly so that the temperature did not rise above 50°C. After the addition, the suspension was stirred for 30 minutes, when it was filtered and the residue washed with formic acid. The filtrate and washings were then concentrated to near dryness, taken up in water (50 ml) and evaporated again. After standing overnight in a refrigerator, the crystals of 21 were filtered off and recrystallized from water, yield 6.2 g (48%).

MS m/z (%), 109 (M⁺, base), 81 (31), 66 (94), 54 (81), 44 (39), 39 (39), 38 (35), 28 (60).

Heating 21 with BSTFA in pyridine produced a mixture of the mono- and di-TMS derivatives (1:10).

GC-MS di-TMS-21 m/z (%) 253 (M⁺, 25) 238 (19), 75 (10, 73 (base)

GC-MS mono-TMS-21 m/z (%) 181 (M⁺, 17), 166 (8), 75 (10), 73 (base).

N-phenylformamide was isolated following preparative TLC (system A) of the mother liquor.

MS m/z (%), 121 (M⁺, base), 93 (75), 92 (12), 77 (6), 66 (65), 65 (24).

5-ethoxymethylenamino-4-cyanooxazole (22)

21 (1.8 g) was dissolved in dry acetic anhydride (10 ml) and
triethylorthoformate (10 ml) was refluxed for 8 hours and left at room temperature overnight. The reaction mixture was concentrated and distilled under reduced pressure (50°C/0.7 mm Hg).

CIMS (CH₄) m/z (%) 166 ([MH]⁺), 194 ([MC₂H₅]⁺).

7-aminooxazolo[5,4-d]pyrimidine (23)

22 (0.81 g) was dissolved in a slight excess of 14% ammonia and stirred at room temperature for 30 minutes. The precipitate of 23 was filtered, dried and recrystallized from ethanol-water. (0.54 g, 81%). \( \lambda_{\text{max}} \) 204 nm, 252 nm (base, neutral).

MS m/z (%) 136 (M⁺, base), 109 (45), 81 (14), 66 (10), 54 (57), 28 (60).

NMR (D₆-DMSO, 100 MHz) 8.60 (s, 1-H, C-2 proton), 8.23 (s, 1-H, C-5 proton), 7.68 (br, 2-H, amino).

HRMS for C₅H₄N₄O; calc. 136.0385, found 136.0385.

7-benzylaminooxazolo[5,4-d]pyrimidine (24)

23 (50 µg mg) was dissolved in hexamethylphosphoramide (500 µl, distilled, stored over 4A sieves), together with sodium hydride (16 mg) and 15 µg DMAP (1.5 µg/ µl, pyridine solution). Benzylbromide (45 µl) was added and the mixture was maintained at 80°C for two days. The mixture was diluted with water and extracted with ethyl acetate. The extract was then subject to preparative TLC (system A) and three UV absorbing zones, (1-B, 2-B, 3-B) of higher Rf than 23, eluted. These products were further purified by reverse phase HPLC. The two zones of lower Rf (2-B, 3-B) were eluted from HPLC system D and the higher Rf zone (1-B) eluted from HPLC system E.
24 (2-B) was recrystallized from ethanol-water, \( \lambda_{\text{max}} \) 209, 259 nm (base, neutral).

\[
\begin{align*}
\text{MS } m/z (\%) & \quad 226 (\text{M}^+, 72), 210 (2), 198 (4), 170 (3), 149 (6), 121 (14), 106 (92), 91 (\text{base}). \\
\text{NMR (CDCl}_3, 200 \text{ MHz}) & \quad 6.84 (\text{s}, 1-H, C-5 proton), 7.94 (\text{s}, 1-H, C-2 proton), 7.37 (\text{m}, 5-H, aromatic H), 5.94 (\text{br.m.}, 1-H, C-7 amino H), 4.89 (\text{br. m.}, 2-H, benzylic H). \\
\text{HRMS for } & \quad \text{C}_{12}\text{H}_{10}\text{N}_4; \text{calc. } 226.0855, \text{found } 226.0848; \text{ for } \text{C}_7\text{H}_8\text{N}, \text{calc. } 106.0657, \text{found } 106.0654.
\end{align*}
\]

1-B, 3-B were shown to be di-benzyl derivatives

\[
\begin{align*}
\text{MS (1-B) } m/z (\%) & \quad 316 (\text{M}^+, 5), 225 (\text{base}), 198 (2), 147 (5), 132 (3), 120 (8), 106 (20), 104 (9), 91 (80). \\
\text{MS (3-B) } m/z (\%) & \quad 316 (\text{M}^+, 34), 225 (47), 198 (1), 178 (2), 147 (1), 135 (9), 120 (5), 119 (5), 106 (5), 91 (\text{base}).
\end{align*}
\]

Reactions of 23

(a) Transamination

23 (25.4 mg) dissolved in benzylamine (52 µl) together with benzylamine hydrochloride (25.7 mg), was sealed in an evacuated tube and heated for 5 hours at 180-185°C. The mixture was then diluted with water and extracted with ethylacetate. The extract was then subject to preparative TLC (system A), the products eluted and shown to be identical (Rf, MS) to the di-benzyl derivatives, 1-B and 3-B.

(b) Sandmeyer

23 (50 mg) was dissolved in water (10 ml) together with concentrated hydrochloric acid (80 µl, 2.5 equivalents) and the solution cooled to 0-5°C. A cold aqueous solution (5ml) of sodium nitrite (28 mg, 1.1 equivalents) was slowly added and the solution left to stir at 0-5°C for 15 minutes.
Cuprous chloride was freshly prepared according to standard procedures (Vogel, 1964) and stored in a dessicator. A solution of cuprous chloride (37 mg, 1 equivalent) in conc. HCl (50 µl) and water (5 ml) was slowly added, with stirring, to the diazotised amine and a deep brown precipitate formed. The temperature of the reaction solution was allowed to rise slowly to room temperature and the solution was then warmed on a water bath to about 60°C. The solution was then neutralized with sodium hydroxide and extracted with ethyl acetate. CIMS indicated that no 7-chlorooxazolopyrimidine had been formed.

(c) Acetylation

23 (10 mg) was heated at 80°C for 90 min in acetic anhydride (10 l) and pyridine (10 µl). GC-EIMS analysis of the mixture showed it to contain 23, the mono-acetyl and the di-acetyl derivatives in the ratio (peak height) of 1:2.8:8.3.

MS mono-acetyl m/z (%) 178(M⁺, 8) 163 (1), 150 (4), 136 (base), 109 (42), 93 (2), 81 (11).

MS di-acetyl m/z (%) 220 (M⁺, 1), 192 (2), 178 (35), 163 (base), 150 (4), 136 (57), 120 (1), 109 (18), 93 (4), 81 (4).

(d) Trimethylsilylation

23 (10 mg) was heated at 80°C for 10 min in BSTFA-TMCS (10 µl) and pyridine (10 µl). GC-EIMS analysis of the silylation solution showed the major product (99%) to be the mono-TMS derivative of 23.

MS m/z (%) 208 (M⁺, 64), 193 (base), 181 (3), 176 (2), 166 (8), 150 (1), 139 (10), 126 (12), 123 (7), 111 (2), 99 (11), 84 (8).

(e) Benzylation

23 (15 mg) was dissolved in DMF (3 ml) together with NaH (4.8 mg) and benzyl bromide (13 µl). The mixture was stirred and heated at 55-60°C for 90 minutes. The reaction mixture was diluted with water and
extracted with ethyl acetate before preparative TLC, system A. Mass
spectral analysis of the products showed them to be dibenzyl
derivatives, MS as per 1-B and 3-B described above.

3.5.4 Derivatives of benzylaminopurine

2-chloro-9-methyl-6-benzylmethylaminopurine (25)

2-Chloro-6-benzylaminopurine (26 mg; synthesised by D.S. Letham)
was dissolved in DMF (1 ml) together with NaH (15 mg) and the mixture
stirred at 70° C. Methyl iodide (15 µl, Aldrich, 2.4 equivalents) was
added. After 2 hours the reaction mixture was diluted with water and
extracted into ethyl acetate. The extract was then subject to
preparative TLC, system A and the zone corresponding to 25 was eluted
with 20% MeOH-CHCl₃, yield 83%, \( \lambda_{\text{max}} \) 281 (base, neutral).

MS \( m/z \) (%) 289 (M⁺ Cl³⁷, 22), 287 (M⁺ Cl³⁵, 64), 274 (Cl³⁷, 22),
272 (Cl³⁵, 67), 260 (Cl³⁷, 9), 258 (Cl³⁵, 27), 252 (2), 236 (3),
210 (5), 198 (Cl³⁷, 10), 196 (Cl³⁵, 30), 169 (8), 167 (4), 120
(10), 106 (2).

HRMS for \( c_{14}H_{14}N_{5}Cl \), calc. 287.0938, found 287.0928;
for \( c_{8}H_{10}N \), calc. 120.0813, found 120.0813.

2-chloro-9-methyl-6-benzylaminopurine (26)

2-Chloro-6-benzylaminopurine (24 mg) was methylated with methyl
iodide (6 µl, 1 equivalent) in the presence of sodium hydride (3 mg) in
DMF as described in the synthesis of 25. Yield 21 mg (82%), \( \lambda_{\text{max}} \) 274
nm, shoulder 266 nm (base, neutral).

MS \( m/z \) (%) 275 (M⁺ Cl³⁷, 17), 273 (M⁺ Cl³⁵, 51), 238 (7),
237 (3), 198 (Cl³⁷, 2), 196 (Cl³⁵, 7), 168 (7), 147 (3), 133
(21), 119 (4) 106 (base), 91 (64), 79 (14), 77 (8), 65 (26).

NMR (D₆-DMSO, 200 MHz) 8 9.29 (s, 1-H, C-8 proton), 8.49
(m, 5-H, aromatic H), (br m, 1-H, C-6 amino H), 5.83 (br. m,
2-H, benzyllic H), 3.68 (s, 3-H, N-9 methyl).

HRMS for C_{13}H_{12}N_{5}Cl{^{35}}; calc. 273.0781, found 273.0775.

2-(2'-hydroxyethylamino)-9-methyl-6-benzylaminopurine (27)

26 (19.2 mg) was dissolved in DMF (2 ml) under N\_2 together with an excess of ethanolamine. The mixture was heated and stirred at 160°C for 2.5 hours, then left overnight at room temperature. The mixture was then diluted with water and extracted with n-butanol (sat. water). The n-butanol extract was then washed with water and the pH adjusted to 7.8 with 2M HCl. After a further water wash the extract was subject to preparative TLC (system A) and the zone corresponding to 27 eluted with 50% methanol-chloroform, yield 18.1 mg (89%), mp 125-126°C, \( \lambda_{max} \) 231, 288 nm (base, neutral).

MS \( m/z \) (%): 298 (M\(^+\), base), 281 (2), 280 (4), 279 (5), 268 (34), 267 (72), 254 (14), 163 (2), 149 (6), 106 (1).

NMR (D\(_6\)-DMSO, 200MHz) \( \delta \): 7.80 (s, 1-H, C-8 proton) 7.25 - 7.48 m, 5-H, aromatic H), 6.09 (br. m, 1-H, C-6 amino H), 4.75 (br. m., 2-H, benzyllic H), 3.65 (s, 3-H, N-9 methyl), 3.60 (d of t, 2-H, C-1' methylene), 3.44 (d of t, 2-H, C-2' methylene)

HRMS for C_{15}H_{18}N_{6}O; calc. 298.1542, found 298.1529.

2-Aminomethyl-9-methyl-6-benzylaminopurine (28)

2-Aminomethyl-2-propanol hydrochloride (Aldrich) was dissolved in water and the pH adjusted to 7-8 with 4M sodium hydroxide. The solution was extracted with n-butanol (sat. water) and the extract evaporated to obtain the free base plus traces of the hydrochloride salt. 26 (20 mg) was dissolved in DMF (2 ml) under N\_2 together with an excess of the dried extract. The mixture was heated and stirred at 150°C for 2.5 hours, then extracted and chromatographed as per the synthesis of 27.
2-(2'-hydroxy-2'-methylpropylamino)-9-methyl-6-benzylaminopurine (29)

2-Aminomethyl-2-propanol hydrochloride (Aldrich) was converted to the free base by passage through a column of Dowex 1X8-200 in the hydroxide form and elution with deionised water as the free base. 28 (20 mg) was dissolved in 2-aminomethyl-2-propanol (2 ml) under N₂ and the mixture heated and stirred overnight at 255-260°C. 29 was extracted and isolated as per the synthesis of 27 and recrystallized from acetone-ethylacetate-n-hexane, 20 mg (84%) λ max· 232, 291 (base, neutral).

MS m/z (%) 326 (M⁺, 22), 311 (3), 309 (3), 268 (91), 267 (71), 251 (7), 240 (2), 191 (2), 177 (7), 163 (24), 148 (9), 133 (4), 121 (5), 105 (12), 91 (base).

NMR (CDCl₃, 200 MHz) δ 7.40 (s, 1-H, C-8 proton), 7.25-7.39 (m, 5-H aromatic proton), 5.91 (br. m., 1-H, C-6 amino proton), 5.23 (br. m., 1-H, C-2 amino proton), 4.77 (br. d, 2-H, benzylic H), 3.64 (s, 3-H, N-9 methyl), 3.40 (d, J = 6.2 Hz, 2-H, C-1' methylene), 1.66 (br. s, 1-H, hydroxyl), 1.25 (s, 6-H, C-2' methyl and C-3' methyl).

HRMS for C₁₇H₂₂N₆O; calc. 326.1855, found 326.1847.
4: The Identification, Quantification, Biosynthesis and Metabolism of Cytokinins in Germinating *Zea mays* Caryopses and Metabolism of Cytokinins in Seedling Leaves.

We all place a great deal of reliance, on the theory and practice of science, But the hopeful intentions, Of so many inventions, Can be quite buggered up in appliance.


4.1 Introduction

While the morphological (Toole, 1924; Haywood, 1938; Tucker, 1957) and biochemical changes (Ingle *et al.*, 1964; Ingle and Hageman, 1965a) in *Zea mays* kernels during germination have been well described, there have been no unequivocal studies to identify the endogenous cytokinins that may be involved in the control of germination and the subsequent seedling development.

The cytokinins in the dry seed have been examined by Smith and van Staden (1978) and by Julin-Tegelman (1979) using simple extraction and purification procedures combined with a bioassay. (The inadequacies and problems associated with the use of bioassays have been discussed in Section 2.1.1). The relative changes in the levels of the cytokinins during imbibition and germination were also observed. In neither study
was any attempt made to minimise the degradation of the cytokinin nucleotides by the action of non-specific phosphatases during the extraction procedure (see Section 2.2.1 for further discussion). The cytokinins in the dry kernels of *Zea mays* were tentatively identified by Smith and van Staden (1978) as Z, ZR, OGZ, OGZR and their respective dihydro derivatives, but these workers discarded the fraction not retained by a cation-exchange resin which would have contained the cytokinin nucleotides. However, Julin-Tegelman (1979) reported the presence of cytokinin activity in a nucleotide fraction. Over a 3 day period from imbibition, Smith and van Staden (1978) proposed, on the basis of decreasing levels of the cytokinin glucosides in the endosperm and an increase in $\beta$-glucosidase activity in the embryo, that the glucosides were transported from the endosperm to the embryo. As the endogenous cytokinins have been implicated as primary factors in the initiation of radicle elongation (Pinfield and Stobart, 1972; Dodd and van Staden, 1982), this could be a significant observation. In a complementary study, Smith and van Staden (1979) found that a partially purified plant extract, putatively containing cytokinin glucosides, partially replaced the effect of the endosperm on the growth of excised maize embryos. However, in feeding experiments with $[8-^{14}C]$-zeatin supplied selectively to the embryo and endosperm of germinating kernels, van Staden (1981a, 1981b) found no evidence for an embryo-endosperm interaction for up to 3 days following imbibition. This labelled zeatin was oxidised rapidly in both the embryo and endosperm tissues, to a stable compound with the implication that cytokinins with a saturated sidechain may be more likely to be involved in the germination process as they are resistant to oxidation by cytokinin oxidase (Whitty and Hall, 1974). The major metabolite detected by van Staden (1981a) was
tentatively identified as 6-(2,3,4-trihydroxy-3-methylbutylamino)purine (di-OH-Z) (van Staden and Drewes, 1982).

Cytokinin-like activity was detected by van Staden and Forsyth (1984) in the excised roots of 10 day-old maize seedlings, suggesting that the tissue may be capable of de novo biosynthesis of the free cytokinins. When supplied with either \([U^{-14}C]\)-Ado or \([8^{-14}C]\)-Ade no incorporation of radioactivity into the free cytokinins was detected at the end of a 42 day period of incubation. However, such a result is not unexpected, given the long incubation time and the rapid turnover of purine derivatives in such metabolically active tissue.

Di-OH-Z and N-(purin-6-yl)glycine (NPG) have been proposed by van Staden (1983) as alternative precursors to the cytokinins instead of, or in addition to, the normally accepted AMP, Ado or Ade, but there is no evidence for this in *Zea mays*.

Although the regulation of the hydrolysis of endosperm starch and protein in germinating *Zea mays* caryopses has been investigated, conflicting reports appear in the literature. In 1924, Toole indicated that the endosperm was capable of independently digesting its own starch and protein reserves but Dure (1960) and Ingle and Hageman (1965b) found that the hydrolysis of starch reserves in excised endosperm required some scutellum or embryo derived factor. Harvey and Oaks (1974a, 1974b) observed that the presence of neither the embryo nor supplied GA3 was necessary for the hydrolysis of endosperm starch and protein in maize. These results were confirmed by Goldstein and Jennings (1975). Although the levels of endogenous GA3 were not determined, a central role for GA in the production of hydrolases was claimed by Harvey and Oaks (1974a, 1974b) on the basis of two observations. Firstly, that the ABA-imposed inhibition of the development of \(\alpha\)-amylase and protease activity in
maize endosperm could be specifically overcome by addition of GA$_3$ and not IAA or kinetin, and secondly, that dwarf maize, which is naturally deficient in GA, showed a marked increase in hydrolase activity in response to added GA$_3$ (Harvey and Oaks, 1974b). Thus, the variability in response to added GA$_3$ observed in the two sets of experiments may be attributed to differing levels of endogenous GA in the different cultivars of maize. The appearance of both $\alpha$-amylase and protease in the maize endosperm was found to be dependent on protein synthesis (Harvey and Oaks, 1974a; Goldstein and Jennings, 1975).

At present the information available on the presence, biosynthesis, metabolism and transport of phytohormones in germinating *Zea mays* kernels is inadequate to confidently establish a role for them in the initiation of germination, radicle growth and the correlation of reserve mobilisation and seedling development. Studies carried out to date have provided very little unequivocal information in this regard. However, it seems that there may be some similarity to the aleurone layer system of cereals (described in Section 1.7.2) in that the production of $\alpha$-amylase may be dependent on GA, in which case, it is possible that an initial stimulation by cytokinin from the endosperm is required before the synthesis of $\alpha$-amylase can occur (Eastwood et al., 1969; Section 1.7.2).

In this chapter I intend to describe the use of methods, outlined in Chapter 2, to identify and quantify the cytokinins in dry maize kernels. The metabolism and translocation of high specific activity $[^3H]$-ZR (synthesised by a new method) in germinating kernels is also described and this metabolism has been compared with that in the primary leaves of maize. Further insights into the biochemistry and physiology of the cytokinins may also be gained by the use of compounds designed to
inhibit cytokinin glucosylation (synthesis described in Chapter 3). In this way, it may be possible to manipulate the level of endogenous cytokinin and ultimately, to modify seedling development.

The experimental work described in Sections 4.3 and 4.5 was carried out in collaboration with D.S. Letham and C.W. Parker. All RIA were performed by C.W. Parker and J. Badenoch-Jones and MS by R.E. Summons. It should be noted that the term 'shoot', used with respect to the maize seedling experiments described in this thesis, includes the coleoptile, the enclosed shoot apex and first leaves, the coleoptilar node and the mesocotyl.

4.2 Cytokinin identification and quantification

4.2.1 Stable isotope dilution assay

Whole dry kernels of Zea mays L. (F1 hybrid, Iochief) were extracted by the method of Bieleski (1964) (Scheme 2.1, discussion Section 2.2.1). Deuterated analogues of the cytokinins to be assayed were added to the extracting solvent. As no deuterated analogues of iPA-5'-P or DZR-5'-P were available, deuterium labelled DZR and iPA were added to the extract following nucleotide hydrolysis with alkaline phosphatase. The extract was purified and fractionated as outlined in Scheme 4.1 (for discussion see Section 2.2.2). The cytokinins were located in the HPLC eluates by RIA.

The cytokinin bases were converted to the tBuDMS derivatives (see Section 2.2.3) and the cytokinin ribosides to the TMS derivatives (see Section 2.2.4) prior to GC-MS.

GC-MS analyses were carried out using a VG 70E instrument interfaced to a VG 11/250 data system. The GC was fitted with a 25 m x 0.3 mm id bonded phase (BP-1) fused silica column (SGE), temperature
Scheme 4.1: Purification of the endogenous cytokinins of dry Zea mays kernels for GC-MS analysis.

**CRUDE EXTRACT**

- **cellulose phosphate column**
  - acidic wash (nucleotides)
  - basic eluate (bases, ribosides, glucosides)
  - + $^3$H-AMP

**DEAE cellulose column**

- wash (neutrals) (discard)
- $\text{HCO}_3^-$ eluate (nucleotides)
- alkaline phosphatase hydrolysis
- n-BuOH partition

**cellulose phosphate column**

- acidic wash (discard)
- basic eluate (nucleotide-derived ribosides)
- purified as per ribosides
  - $\text{iPA, iP}$
  - $\text{Z, ZR, DZ, DZR}$
  - $\text{Z, ZR, DZ, DZR}$
  - $\text{Z, ZR, DZ, DZR}$
  - $\text{Z, ZR, DZ, DZR}$
  - $\text{Z, ZR, DZ, DZR}$
  - $\text{Z, ZR, DZ, DZR}$

**HPLC**

- Zorbax C-8
- Zorbax C-8
- Zorbax C-8
- Zorbax C-8
- Zorbax C-8

**TLC**

- cellulose (iPrOH/HOAc)
- glucosides
- $\beta$-glucosidase hydrolysis
- acid hydrolysis

**GC-MS**

- iPA, ZR, DZ, DZR
- Z, ZR, DZ, DZR
- Z, ZR, DZ, DZR
- Z, ZR, DZ, DZR
- Z, ZR, DZ, DZR
- Z, ZR, DZ, DZR
- Z, ZR, DZ, DZR
- Z, ZR, DZ, DZR
programmed from 200-300°C at 8°C/min, using helium (0.6 kg/cm²) as carried gas. Ionisation was by electron impact (70 eV) and selective ion recording was carried out using magnet peak switching with a sample time of 100 ms and delay of 10 ms per peak. Samples were separately analysed as mixtures of the bases or ribosides with the ion currents for the two most intense high mass ions for each compound and its labelled analogue being monitored over a sample cycle time of 1.0 to 1.5 s. Quantification was then based on the relative intensities of these ions (Table 4.1) as observed at the elution time characteristic for each compound (Fig. 4.1). Standard curves were constructed for each ion pair using the second and third injection of each sample to minimise memory effects. The recorded results are based on calculations utilizing the most intense ion pair and these were confirmed by comparison with the second pair of ions.

4.2.2 RIA

Dry Zea mays kernels (same batch and cultivar as used in the GC-MS assay) were dissected into embryo (20.5 g) and endosperm (59.0 g) fractions. The difference in texture between the soft embryo and the hard endosperm was used as a criterion that no residual endosperm was included with the embryo fraction. These fractions were then extracted by the method of Bieleski (1964) (Scheme 2.1). The crude extract was purified and fractionated as described in Scheme 4.2 (for discussion see Section 2.2.2).

RIA was performed according to the procedures outlined by Badenoch-Jones et al. (1984). Antisera elicited against IPA, ZR and DZR were used. The ZR antiserum has been described previously (Badenoch-Jones et al., 1984). The cross-reactivity characteristics of the various antisera enabled detection and quantification of iP and IPA with the anti-iPA serum, of Z and ZR with the anti-ZR serum, and of DZ and DZR
Table 4.1: Ion pairs monitored for GC-MS analysis of the endogenous cytokinins in dry *Zea mays* kernels; M, molecular ion; M-15, loss of methyl radicle from M; M-57, loss of t-butyl radicle from M; a, loss of tBuDMS-OH from M; b, loss of TMS-OCH$_3$ from M; B, cleavage of ribosyl moiety with charge retention on the base.

<table>
<thead>
<tr>
<th>Cytokinin derivative</th>
<th>Ion pairs (labelled/non-labelled ion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMS-iPA</td>
<td>557/551 (M) *, 542/536 (M-15)</td>
</tr>
<tr>
<td>TMS-ZR</td>
<td>629/624 (M-15), 539/536 (b) *</td>
</tr>
<tr>
<td>TMS-DZR</td>
<td>631/626 (M-15), 327/322 (B+CH$_2$O) *</td>
</tr>
<tr>
<td>tBuDMS-iP</td>
<td>319/317 (M), 304/302 (M-15) *</td>
</tr>
<tr>
<td>tBuDMS-Z</td>
<td>359/390 (M-57), 320/315 (a) *</td>
</tr>
<tr>
<td>tBuDMS-DZ</td>
<td>436/434 (M-15), 394/392 (M-57) *</td>
</tr>
</tbody>
</table>

* most intense pair
Figure 4.1: Selected ion profiles obtained during the mass spectrometric quantification of the levels of endogenous iP (a), DZ (b) and Z (c) in dry Zea mays kernels by the stable isotope dilution technique. The tBuDMS derivatives of iP, DZ and Z eluted from the GC at 9:47, 12:10 and 13:05 min. respectively.
Scheme 4.2: Purification of the endogenous cytokinins, present in excised embryo or endosperm tissue of dry *Zea mays* kernels, for RIA.

**CRUDE EXTRACT**

- **cellulose phosphate column**
  - acidic wash
    - (nucleotides)
    - + ^3_H-AMP
  - DEAE cellulose column
    - + acidic wash
      - (nucleotides)
      - (discard)
    - HCO_3^- eluate
      - (nucleotides)
    - alkaline phosphatase
    - n-BuOH partition
    - cellulose phosphate column
      - acidic wash
        - (discard)
      - basic eluate
        - (ribosides)
        - (nucleotide-derived ribosides)
      - purified as per ribosides
        - iPA, ZR, DZR
        - RIA
    - water wash
      - (discard)
  - paraffin silica gel column
    - cytokinin eluate
    - TLC silica gel
      - (n-BuOH/HOAc)
      - iPA, IP, Z, ZR, DZ, DZR
      - Glucosides
      - 8-glucosidase hydrolysis
      - TLC silica gel
        - (n-BuOH/HOAc)
        - Z, ZR, DZ, DZR
        - Z9G, DZ9G
        - Z7G, DZ7G
        - acid hydrolysis
          - Z, ZR, DZ, DZR
          - RIA
          - Z, ZR, DZ
with the anti-DZR serum.

A small aliquot of [3H]-ZR was used as an internal standard to account for losses during purification and to validate the chromatographic procedures.

4.2.3 Results and discussion

Eleven cytokinins were unequivocally identified and quantified by GC-MS in an extract of dry whole Zea mays caryopses (see Table 4.2). The most prominent of these were Z, DZ, DZR, OGZ, OGDZ and OGDZR. iP and iPA were identified for the first time in sweet corn tissue. RIA was used to determine the identity and the distribution of the cytokinins between the endosperm and embryo of the dry Zea mays kernels. These data suggested that the levels of Z, DZR, OGZ, DZ7G and Z9G were much higher in the embryo than in the endosperm, however, iP and Z nucleotide were detected only in the endosperm.

The cytokinin levels in dry caryopses are substantially lower than those determined in immature (milk stage) sweet corn kernels (Table 4.3). In particular, zeatin nucleotide, the major cytokinin present in immature kernels (Letham, 1973), is only just detectable in the mature tissue. The level of ZR in the immature kernels is about 350 times that in dry seed.

Quantitatively, the RIA data corroborated those which were obtained using GC-MS. However, apparent quantitative discrepancies were found between the data from the two assays. This may be due to the lack of internal standards for use in the RIA to assess the losses of each cytokinin assayed. In the cases of the O-glucosides and the nucleotides, which were subject to enzymic hydrolysis during the purification procedures, there is no information as to the recovery of the hydrolysis products. In RIA, the recovery of all cytokinins was
Table 4.2: Cytokinin content of dry *Zea mays* caryopses as measured by RIA and by GC-MS.

<table>
<thead>
<tr>
<th>Cytokinin</th>
<th>RIA estimation</th>
<th>GC-MS estimation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Embryo (ng/g)</td>
<td>Endosperm (ng/g)</td>
</tr>
<tr>
<td>iP</td>
<td>nd c</td>
<td>3.00</td>
</tr>
<tr>
<td>Z</td>
<td>0.68</td>
<td>0.12</td>
</tr>
<tr>
<td>DZ</td>
<td>0.28</td>
<td>0.48</td>
</tr>
<tr>
<td>IPA</td>
<td>8.00</td>
<td>2.80</td>
</tr>
<tr>
<td>ZR</td>
<td>1.48</td>
<td>1.44</td>
</tr>
<tr>
<td>DZR</td>
<td>0.60</td>
<td>0.10</td>
</tr>
<tr>
<td>OGZ</td>
<td>0.64</td>
<td>0.20</td>
</tr>
<tr>
<td>OGDZ</td>
<td>nd c</td>
<td>nd d</td>
</tr>
<tr>
<td>OGZR</td>
<td>0.35</td>
<td>0.76</td>
</tr>
<tr>
<td>OGDZR</td>
<td>0.28</td>
<td>0.16</td>
</tr>
<tr>
<td>Z7G</td>
<td>nd c</td>
<td>nd d</td>
</tr>
<tr>
<td>Z79G</td>
<td>15.2</td>
<td>2.48</td>
</tr>
<tr>
<td>DZ7G</td>
<td>2.64</td>
<td>2.96</td>
</tr>
<tr>
<td>DZ7G</td>
<td>2.52</td>
<td>0.44</td>
</tr>
<tr>
<td>iP-5'-P</td>
<td>nd c</td>
<td>nd d</td>
</tr>
<tr>
<td>ZR-5'-P</td>
<td>0.28</td>
<td>-</td>
</tr>
<tr>
<td>DZR-5'-P</td>
<td>nd c</td>
<td>nd d</td>
</tr>
</tbody>
</table>

a. ratio of cytokinins in embryo/endosperm

b. calculated from embryo and endosperm estimations but some tissue discarded in dissection

c. not detected, lower limit of detection is 0.12ng/g

d. not detected, lower limit of detection is 0.10ng/g

e. not assayed.
Table 4.3: Comparison of cytokinin concentrations in sweet corn kernels at commercial maturity and in the dry, quiescent state.

<table>
<thead>
<tr>
<th>Cytokinin</th>
<th>Commercial maturity kernels</th>
<th>Dry kernels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Summons et al. (1979b)</td>
<td>present study</td>
</tr>
<tr>
<td></td>
<td>(ng/g)</td>
<td>(ng/g)</td>
</tr>
<tr>
<td>Z</td>
<td>220&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ZR</td>
<td>530&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DZR</td>
<td>-</td>
<td>9.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>OGDZR</td>
<td>-</td>
<td>10.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>OGDZ</td>
<td>-</td>
<td>6.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Z9G</td>
<td>15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>stable isotope dilution  
<sup>b</sup>UV absorbance in HPLC eluate  
<sup>c</sup>RIA
assumed to be the same as the recovery of ZR (25%). It is therefore likely that values obtained for cytokinins by RIA are underestimates. Furthermore, the summation of the data obtained by RIA for the embryo and endosperm cannot be directly equated with the data obtained by the GC-MS assay in the whole seed because, in the process of dissecting out pure embryo, some of the scutellum was discarded. Hence, the possibility exists that some cytokinins, for example Z, may be concentrated in the scutellum and/or the adjacent endosperm and this would result in the summed RIA value being less than the GC-MS value for the whole seed. In contrast, the RIA estimate for iPA was considerably higher than that obtained by GC-MS. The RIA estimation was made initially after passage of the extract through reverse phase HPLC (system M) and confirmed following further chromatography using a different column (HPLC system G). It should be noted that the dissected embryos remained capable of germination following imbibition despite the loss of scutellum.

A considerable amount of activity was detected by RIA, using all three antisera, in the wash from the DEAE cellulose column, in the aqueous fraction remaining after n-BuOH partitioning of the products from alkaline phosphatase hydrolysis, and in the acid wash from the second cellulose phosphate column (see Scheme 4.2). This activity could not be extracted by ethyl acetate or by alkaline n-BuOH and was therefore not attributable to any known cytokinin. The presence of such potentially interfering substances serves to underline the importance of some form of purification of samples prior to RIA, if meaningful results are to be realized.

RIA proved to have a useful application in locating the cytokinin fractions eluted from HPLC prior to GC-MS. The usual methods for doing
this in the presence of a high UV background are based on either co-chromatography with authentic radiolabelled compounds or the use of bioassay. Both these alternatives suffer severe disadvantages, the former because of a general non-availability of high specific activity radiolabelled standards and the latter because of its consumption of a substantial part of the sample and difficulty in detecting cytokinins that are very weakly active in bioassays.

4.3 Biosynthesis of cytokinins

Dry Zea mays kernels (same batch and cultivar as used in GC-MS and RIA assay, Section 4.2) were surface sterilised by immersion in a dilute sodium hypochlorite solution (Ajax Chemicals, 30 g/l available chlorine diluted 10 times), together with 3 drops of detergent (Zephiran, Winthrop Lab., Sydney, NSW), for 5 minutes and then rinsed thoroughly with sterile water. The kernels were then incubated for 3 days, embryo uppermost, on moist filter paper in the dark at room temperature before dissecting the embryo out of the kernels in two batches of 15 seeds. One batch of embryos was then soaked in an aqueous solution (20 ml) of [8-14C]-Ade (Amersham International, Bucks., England; 250 μCi, specific activity 62 mCi/m mole) and the second batch was soaked in an aqueous solution (20 ml) of [G-3H]-hypoxanthine (Amersham; 473 μCi, specific activity 2.3 Ci/m mole). Over a 10.5 hour period, 96% of the [8-14C]-Ade and 24% of the [G-3H]-hypoxanthine was taken up by the respective batches of embryos.

The embryos were then extracted as per Scheme 2.1 (Bieleski, 1964) and the extract subject to ion exchange chromatography on cellulose phosphate and DEAE cellulose to yield a nucleotide fraction (Scheme 4.1). In a preliminary experiment in which [14C]-adenine was supplied,
the basic fraction was chromatographed on TLC, but no $^{14}$C appeared to be associated with the cytokinin markers. However, the phosphatase hydrolysate of the nucleotide fraction appeared to contain $^{14}$C which co-chromatographed with ZR and/or DZR. Accordingly, in the present experiment with supplied adenine and hypoxanthine, only the nucleotide fraction was examined.

The nucleotide fractions were chromatographed on cellulose (TLC system C) and the zones corresponding to the $R_f$ of Z nucleotide (located immediately below and overlapping with 6-(5-hydroxypentylamino)-9-β-D-(5'-monophosphate) ribofuranosylpurine used as a co-chromatographed marker) were eluted (30% MeOH) and hydrolysed by treatment with alkaline phosphatase. The hydrolysis products were extracted into alkaline $n$-BuOH and chromatographed on cellulose (TLC system H). The zone corresponding to DZR and ZR was eluted (60% MeOH). These compounds were resolved by chromatographing on reversed phase TLC (system L) and eluted (60% EtOH with 4% HOAc). A significant amount of radioactivity was detected as co-chromatographing with the ZR derived from the presumed Z nucleotide (ca. 2100 dpm) biosynthesised from $[^{14}$C]-Ado. Less than 200 dpm could be attributed to ZR derived from the $[^{3}$H]-hypoxanthine feed.

The putative $[^{14}$C]-ZR was chromatographed on silica gel (TLC system D). Approximately 20% of the chromatogram was eluted with EtOH and taken up in a scintillation cocktail (7 g PPO/1 toluene) from which the radioactive material could be recovered by extraction with 0.25M HOAc after counting. The radioactivity co-chromatographed with ZR as shown in Fig. 4.2a. The recovered radioactivity and the remainder of the radioactivity were separately chromatographed on silica gel (TLC system E); the ZR zones which corresponded to a peak of $^{14}$C were eluted (50% MeOH) and the eluates combined. Colloidal silica present in the eluates
Figure 4.2: Chromatography of radioactivity derived from the nucleotide fraction, with authentic (a) ZR (TLC system D); (b) 2',3'-O-isopropylidene-ZR (TLC system A); (c) mono-tBuDMS-2'-3'-O-isopropylidene-ZR (TLC system A).
was removed by passage through a column of cellulose phosphate.

The isopropylidene derivative was then made (Section 2.3.4) and one half of the sample was further derivatised to form the tBuDMS ether. Radioactivity coeluted with 2',3'-O-isopropylidene-ZR (TLC system A) and with mono-tBuDMS-2',3'-O-isopropylidene-ZR (TLC system B) (Figs. 4.2b, c).

These data provided strong evidence for the biosynthesis of Z nucleotide from [14C]-Ado (0.00038% of the radioactivity taken up by the tissue). Little if any radioactivity from [3H]-hypoxanthine was incorporated into a cytokinin nucleotide. Such incorporation would have required a conversion of the hypoxanthine to Ado via the purine salvage pathway (Ashihara, 1983 and references therein).

The site of this biosynthesis is unknown but the radicle tip is a likely possibility (Section 1.4.3).

4.4 Metabolism and translocation of [3H]-ZR in germinating seeds.

4.4.1 Synthesis of [3H]-ZR

Tri-n-octylphosphine (2.13 g, Aldrich) was added dropwise to a stirred solution of (2E)-4-hydroxy-2-methylbut-2-en-1-yl tetrahydropyranyl ether (1.0 g, synthesised by J. Eichholzer and J.K. MacLeod according to the method of H. Young and D.S. Letham, unpublished) together with carbon tetrabromide (2.13 g, BDH) in benzene and petroleum ether (3 ml and 12 ml respectively, distilled and stored over sodium) cooled to -20°C, under a dry nitrogen atmosphere. Stirring continued for a further 45 min. The petroleum ether layer was removed and the residue washed with petroleum ether (4 x 2 ml) at -20°C. The combined extracts were partially evaporated and the crude, unstable bromide used without further purification.
[2-^3H]-Adenosine (24.0 Ci/m mole, approximately 100 µg; Amersham), adenosine (200 µg, Sigma) and the crude bromide were dissolved in dry DMF (5 ml) in the presence of 4 x 4 A molecular sieves (non-nucleophilic acid acceptor). The solution was then left to stand for 3 days at room temperature in the dark. The solvent was evaporated under nitrogen and the residue taken up in dimethylamine-methanol (1:1, v/v) and allowed to stand at room temperature for 6 hours, when the solvent was again removed under nitrogen. The tetrahydropyranyl group was hydrolysed by dissolution of the residue in 0.1M HCl at room temperature overnight. The pH of the solution was adjusted to 8-9 with NaOH and the [^3H]-ZR extracted with water-saturated n-BuOH. The extract was then chromatographed on TLC (system H). The [^3H]-ZR was eluted with MeOH-HOAc-H_2O (10:1:10, v/v), the solvent evaporated and the residue redissolved and stored in EtOH-H_2O (1:1, v/v) at -20°C (see Scheme 4.3). Yield (UV), 54 µg; specific activity 4.8 Ci/m mole.

4.4.2 Metabolism of [^3H]-ZR

Dry Zea mays kernels (as analysed in Section 4.2) were surface sterilized, incubated and extracted as described in Section 4.3.

The soluble ^3H-labelled cytokinin metabolites were analysed by the TLC and HPLC systems outlined in Tables 2.6 and 2.7 respectively. TLC system C (using dye markers A, B, C and D) was used to fractionate the metabolites into four zones: 1, iP, iPA; 2, Z, DZ, ZR, DZR; 3, Ade, Ado, Z9G; 4, Z7G, OGZ, OGDZ, OGZR, OGDZR (see Fig. 2.1). A two-dimensional separation of the metabolites could be achieved on silica gel using the 2D-TLC with the following added markers, iP, iPA, Z, ZR, Ade, Ado and dyes A and D. Before developing in the second dimension, dyes J and K were added to the dye D zone. In the second dimension, OGZ, OGDZ, OGZR, OGDZR and Z7G ran as a partly resolved band (the 'glucoside band')
Scheme 4.3: Synthesis of $[^3H]^{-ZR}$. 
between dyes J and K. The 2D-TLC did not resolve the cytokinins with unsaturated side chains from their corresponding dihydro analogues. These mixtures of Z/DZ and ZR/DZR could be separated after elution (MeOH–HOAc–H₂O, 10:1:10, v/v) by reverse phase TLC on DMPS-5X or paraffin impregnated silica gel layers (TLC systems M, N and P). Alternatively, the cytokinins eluted from silica gel TLC (system C or 2D-TLC) could be separated by reverse phase HPLC (system P).

Whole seed. Whole seeds were surface sterilized (6 batches of 20 seeds), and incubated for 2 hrs in sterile water containing 9 µCi [³H]-ZR. More than 99% of the radioactivity was taken up by the seed which was then thoroughly washed with sterile water and incubated as described above (Section 4.3). Batches of seed were then extracted at 0, 12, 24, 36 and 48 hours following the cessation of feeding [³H]-ZR. The total radioactivity extracted declined with time (Table 4.4). An aliquot of each extract was then analysed by normal phase TLC (system C) using dyes A, B, C and D. The major band of radioactivity in each chromatogram corresponded to the Rf values of Z, ZR, DZ, DZR, Ade and Ado. No significant levels of radioactivity were noted in the glucoside zone. The distribution of radioactivity between these metabolites was determined by 2D-TLC (Table 4.5).

Whole seed dissected before extraction. Batches of whole seed were surface sterilized, fed [³H]-ZR and incubated as described above. At 0, 6, 12, 24, 51 and 72 hours following the cessation of feeding, each seed was washed with sterile distilled water then dissected into an embryo fraction (including radicles and shoots) and an endosperm fraction before extraction. The extracts were then subject to cellulose phosphate chromatography to yield an acidic fraction (containing nucleotides) and a basic fraction (containing cytokinin bases, ribosides
Table 4.4: The radioactivity extracted from *Zea mays* seed following uptake of \([^3H]-ZR\).

<table>
<thead>
<tr>
<th>time (^a) (hr)</th>
<th>(^3H) extracted total (x10^6 dpm) (^b)</th>
<th>% total uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.66</td>
<td>51</td>
</tr>
<tr>
<td>12</td>
<td>1.13</td>
<td>35</td>
</tr>
<tr>
<td>24</td>
<td>0.85</td>
<td>26</td>
</tr>
<tr>
<td>36</td>
<td>0.73</td>
<td>22</td>
</tr>
<tr>
<td>48</td>
<td>0.75</td>
<td>23</td>
</tr>
</tbody>
</table>

\(^a\) Time elapsed from cessation of \([^3H]-ZR\) feed

\(^b\) Total radioactivity extracted from each batch of 20 seeds.
Table 4.5: Distribution of radioactivity between cytokinin metabolites as determined by 2D-TLC. The radioactivity co-chromatographing with each marker is expressed as a percentage of that eluted from the plate. The entire plate was cut for counting.

<table>
<thead>
<tr>
<th>time (^{a}) (hr)</th>
<th>TLC markers</th>
<th>nucleotide zone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ado</td>
<td>Ade</td>
</tr>
<tr>
<td>0</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>12</td>
<td>37</td>
<td>24</td>
</tr>
<tr>
<td>48</td>
<td>25</td>
<td>20</td>
</tr>
</tbody>
</table>

\(^{a}\) time elapsed from cessation of \([^{3}H]\)-ZR feed

\(^{b}\) region containing both AMP and ZR-5'-P.
and glucosides) (Table 4.6).

Aliquots of the basic fractions and the seed washings were then examined by 2D-TLC and the radioactivity co-chromatographing with Ado, Ade, Z/DZ and ZR/DZR was tabulated as a percentage of that applied to the TLC plate (Table 4.7). As the 2D-TLC does not resolve Z and ZR from their dihydro analogues, DZ and DZR, respectively, several samples were also examined using HPLC (system P), however, no significant levels of radioactivity were co-eluted with either DZ or DZR (Table 4.8).

Aliquots of the acidic fractions from cellulose phosphate chromatography were treated with alkaline phosphatase and the radioactivity was analysed by HPLC (system P) (Table 4.9).

**Excised embryo and endosperm.** Dry *Zea mays* kernels were dissected into embryo and endosperm. A single piece of endosperm was cut from each kernel by a single transverse section. The tissue was surface sterilized (4 batches of 5 embryos or 5 endosperm pieces), and imbibed with an aqueous solution of $[^{3}H]$-ZR for 2 hrs such that 0.22 µCi and 0.13 µCi were absorbed by each batch of embryo and endosperm tissue, respectively. The tissue was then thoroughly washed and incubated as above. At 0, 6, 12 and 48 hours following the cessation of the $[^{3}H]$-ZR feed (all 5 embryos had germinated after 48 hrs incubation), the tissue was extracted and subject to cellulose phosphate chromatography (Table 4.10). The acidic fraction was treated with alkaline phosphatase and then both it and the basic fraction were analysed by TLC (Table 4.11).

4.4.3 Translocation of $[^{3}H]$-ZR

In each series of experiments, *Zea mays* kernels (same batch and cultivar as assayed, Section 4.2; 3 batches of 5 seeds per experiment) were surface sterilized, incubated and extracted as described in Section 4.3.
Table 4.6: Radioactivity extracted from dissected *Zea mays* kernels fed with [³H]-ZR and the radioactivity in the incubation solution. The total radioactivities in the fractions derived from cellulose phosphate chromatography of extracts are also given.

<table>
<thead>
<tr>
<th>time (hr)</th>
<th>embryo</th>
<th>endosperm</th>
<th>incubation solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cell. Po⁴ fr</td>
<td>cell. Po⁴ fr</td>
</tr>
<tr>
<td>0</td>
<td>60,582</td>
<td>174,173</td>
<td>45,614</td>
</tr>
<tr>
<td>6</td>
<td>8,120</td>
<td>1,944</td>
<td>68,270</td>
</tr>
<tr>
<td>12</td>
<td>29,153</td>
<td>22,032</td>
<td>19,948</td>
</tr>
<tr>
<td>24</td>
<td>12,662</td>
<td>82,980</td>
<td>16,889</td>
</tr>
<tr>
<td>51</td>
<td>6,559</td>
<td>12,482</td>
<td>8,587</td>
</tr>
<tr>
<td>72</td>
<td>8,847</td>
<td>15,647</td>
<td>4,547</td>
</tr>
</tbody>
</table>

a time elapsed from cessation of [³H]-ZR feed.
Table 4.7: Analysis of radioactivity by 2D-TLC, in the basic fraction from cellulose phosphate chromatography (A) and the incubation solution (B). The radioactivity is expressed as a percentage of that eluted from the plate. The entire plate was cut for counting. Radioactivity expressed as a percentage of the total extract is given in parenthesis.

<table>
<thead>
<tr>
<th>A</th>
<th>Embryo</th>
<th>Endosperm</th>
</tr>
</thead>
<tbody>
<tr>
<td>time&lt;sup&gt;a&lt;/sup&gt; (hr)</td>
<td>Ado</td>
<td>Ade</td>
</tr>
<tr>
<td>0</td>
<td>29 (29)</td>
<td>14 (14)</td>
</tr>
<tr>
<td>6</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40 (37)</td>
</tr>
<tr>
<td>12</td>
<td>30 (29)</td>
<td>38 (36)</td>
</tr>
<tr>
<td>24</td>
<td>28 (20)</td>
<td>33 (24)</td>
</tr>
<tr>
<td>51</td>
<td>23 (13)</td>
<td>44 (25)</td>
</tr>
<tr>
<td>72</td>
<td>24 (13)</td>
<td>25 (14)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B</th>
<th>Incubation solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>time&lt;sup&gt;a&lt;/sup&gt; (hr)</td>
<td>Ado</td>
</tr>
<tr>
<td>0</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>26</td>
</tr>
<tr>
<td>12</td>
<td>33</td>
</tr>
<tr>
<td>24</td>
<td>38</td>
</tr>
<tr>
<td>51</td>
<td>44</td>
</tr>
<tr>
<td>72</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>time elapsed following cessation of feeding [³H]-ZR<br><sup>b</sup>not determined.
Table 4.8: Radioactivity in basic fraction from cellulose phosphate chromatography was analysed by HPLC (system P). The radioactivity co-eluting with the markers was expressed as a percentage of that eluted from the column.

<table>
<thead>
<tr>
<th>time (^a) (hr)</th>
<th>Embryo</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Endosperm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ado</td>
<td>Ade</td>
<td>ZR</td>
<td>DZR</td>
<td>Z</td>
<td>DZ</td>
<td></td>
<td>Ado</td>
</tr>
<tr>
<td>12</td>
<td>32</td>
<td>42</td>
<td>18</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>72</td>
<td>18</td>
<td>31</td>
<td>13</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>34</td>
</tr>
</tbody>
</table>

\(^a\) time elapsed following cessation of \([^{3}H]\)ZR feed.

Table 4.9: Radioactivity in acidic fraction from cellulose phosphate chromatography following treatment with alkaline phosphatase was analysed by HPLC (system P). The radioactivity co-eluting with the markers was expressed as a percentage of that eluted from the column and in parenthesis as a percentage of the total extract.

<table>
<thead>
<tr>
<th>time (^a) (hr)</th>
<th>Embryo</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Endosperm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ado</td>
<td>Ade</td>
<td>ZR</td>
<td>DZR</td>
<td>Z</td>
<td>DZ</td>
<td></td>
<td>Ado</td>
</tr>
<tr>
<td>24</td>
<td>89 (25)</td>
<td>-</td>
<td>3 (1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>45 (1)</td>
</tr>
<tr>
<td>72</td>
<td>63 (29)</td>
<td>-</td>
<td>2 (1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>46 (12)</td>
</tr>
</tbody>
</table>

\(^a\) time elapsed following cessation of \([^{3}H]\)ZR feed.
Table 4.10: Radioactivity extracted from embryo and endosperm, fed with $[^3H]-\text{ZR}$ and fractionated by cellulose phosphate chromatography. Radioactivity in the incubation solution is also given as dpm/g tissue.

<table>
<thead>
<tr>
<th>time $^a$ (hr)</th>
<th>embryo</th>
<th></th>
<th>endosperm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cell.P0$_4$ fr</td>
<td>incubation solution</td>
<td>cell.P0$_4$ fr</td>
</tr>
<tr>
<td></td>
<td>acidic (dpm/g)</td>
<td>basic (dpm/g)</td>
<td>(dpm/g)</td>
</tr>
<tr>
<td>0</td>
<td>32,550</td>
<td>128,643</td>
<td>4,600</td>
</tr>
<tr>
<td>6</td>
<td>46,360</td>
<td>18,865</td>
<td>31,889</td>
</tr>
<tr>
<td>12</td>
<td>49,604</td>
<td>14,379</td>
<td>45,548</td>
</tr>
<tr>
<td>48</td>
<td>20,859</td>
<td>9,643</td>
<td>16,374</td>
</tr>
</tbody>
</table>

$^a$ time elapsed following cessation of feeding.
Table 4.11: Radioactivity co-chromatographing with markers in reverse phase TLC (systems N and K) and 2D-TLC. The radioactivity is expressed as a percentage of that eluted from the plate and in parenthesis as a percentage of the total extraction. No radioactivity was detected as co-chromatographing with Z, DZ or DZR.

<table>
<thead>
<tr>
<th>time (hr)</th>
<th>embryo basic fr. cell PO$_4$</th>
<th>embryo TLC system</th>
<th>embryo acidic fr. cell PO$_4$-TLC system</th>
<th>embryo incubation solution</th>
<th>endosperm</th>
<th>endosperm incubation solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ado</td>
<td>Ade</td>
<td>ZR</td>
<td>Ado</td>
<td>ZR</td>
<td>N</td>
</tr>
<tr>
<td>0</td>
<td>44 (35)</td>
<td>13 (10)</td>
<td>26 (21)</td>
<td>N</td>
<td>70 (50)</td>
<td>16 (11)</td>
</tr>
<tr>
<td>6</td>
<td>39 (11)</td>
<td>30 (9)</td>
<td>14 (4)</td>
<td>N</td>
<td>70 (50)</td>
<td>16 (11)</td>
</tr>
<tr>
<td>12</td>
<td>24 (5)</td>
<td>40 (9)</td>
<td>5 (1)</td>
<td>N</td>
<td>70 (50)</td>
<td>16 (11)</td>
</tr>
<tr>
<td>48</td>
<td>14 (4)</td>
<td>56 (18)</td>
<td>3 (1)</td>
<td>N</td>
<td>70 (50)</td>
<td>16 (11)</td>
</tr>
<tr>
<td>0</td>
<td>7</td>
<td>1</td>
<td>82</td>
<td>N</td>
<td>70 (50)</td>
<td>16 (11)</td>
</tr>
<tr>
<td>6</td>
<td>38</td>
<td>16</td>
<td>27</td>
<td>N</td>
<td>70 (50)</td>
<td>16 (11)</td>
</tr>
<tr>
<td>12</td>
<td>52</td>
<td>26</td>
<td>7</td>
<td>N</td>
<td>70 (50)</td>
<td>16 (11)</td>
</tr>
<tr>
<td>48</td>
<td>50</td>
<td>22</td>
<td>5</td>
<td>N</td>
<td>70 (50)</td>
<td>16 (11)</td>
</tr>
<tr>
<td></td>
<td>41 (39)</td>
<td>4 (4)</td>
<td>34 (33)</td>
<td>K</td>
<td>94 (7)</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>61 (56)</td>
<td>14 (13)</td>
<td>14 (13)</td>
<td>N</td>
<td>94 (7)</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>54 (44)</td>
<td>14 (11)</td>
<td>7 (6)</td>
<td>N</td>
<td>94 (7)</td>
<td>-</td>
</tr>
<tr>
<td>48</td>
<td>48 (29)</td>
<td>17 (11)</td>
<td>4 (3)</td>
<td>N</td>
<td>94 (7)</td>
<td>-</td>
</tr>
<tr>
<td>0</td>
<td>7</td>
<td>1</td>
<td>82</td>
<td>N</td>
<td>83 (30)</td>
<td>2 (1)</td>
</tr>
<tr>
<td>6</td>
<td>65</td>
<td>8</td>
<td>23</td>
<td>N</td>
<td>83 (30)</td>
<td>2 (1)</td>
</tr>
<tr>
<td>12</td>
<td>75</td>
<td>5</td>
<td>16</td>
<td>N</td>
<td>83 (30)</td>
<td>2 (1)</td>
</tr>
<tr>
<td>48</td>
<td>75</td>
<td>13</td>
<td>3</td>
<td>N</td>
<td>83 (30)</td>
<td>2 (1)</td>
</tr>
</tbody>
</table>

The soluble $^3$H-labelled cytokinin metabolites were initially analysed on silica gel TLC (system D) using Ade, Ado, Z, ZR, AMP, inosine and dye L as markers. The glucoside zone was delineated by dye L and inosine, while zeatin nucleotide co-chromatographed with AMP. Radioactivity was eluted from TLC with MeOH-HOAC-H$_2$O (10:1:10, v/v) and re-chromatographed on reverse phase TLC (system N, resolving Z, DZ, ZR, DZR, Ado and Ade), or in the case of the nucleotides, eluted, treated with alkaline phosphatase (Section 2.3.3), and then re-chromatographed. The glucoside zone was re-chromatographed on reverse phase HPLC (system A: resolving Z7G, Z9G, OGZ, OGDZ, OCGZ, OGDZR; Fig. 4.3).

**ZR applied to endosperm after imbibition for 2 hr.** Zea mays kernels were allowed to imbibe water for 2 hours. A small portion of the testa, covering the endosperm surface on the side opposite the embryo, was removed and the area surrounded by a smear of silicon grease. Into this well was placed an aqueous solution of $[^3H] $ZR (2 µl, 0.114 µCi/seed). The seeds were then incubated, embryos down, for 24, 48 or 72 hrs, when they were washed, dissected into endosperm and embryo fractions and then extracted (Table 4.12). The extracts were then analysed by TLC (system D) (Table 4.13). The presence of ZR in the 72 hr embryo extract was confirmed by re-chromatography on reverse phase TLC (system N). No significant levels of radioactivity were found to co-chromatograph with DZR. Re-chromatography of the nucleotide zone (72 hr) after hydrolysis showed it to consist of Ado (92%) and ZR (5%).

**ZR applied to embryo after imbibition for 2 hr.** Zea mays kernels were allowed to imbibe water for 2 hrs. A small portion of the testa covering the embryo was removed and the area surrounded by a smear of silicon grease. Into this well was placed an aqueous solution of $[^3H]$-
Figure 4.3: HPLC of the cytokinin glucosides on HPLC system A. 1, Z7G; 2, Z9G; 3, OGZ; 4, OGZR; 5, OGDZ; 6, OGDZR.
Table 4.12: Distribution of radioactivity following application of \([3^\text{H}]-\text{ZR}\) to endosperm of whole seed which had imbibed for 2 hr.

<table>
<thead>
<tr>
<th>time (\text{(hr)})</th>
<th>tissue</th>
<th>total (\times 10^4 \text{ dpm})</th>
<th>extracted (3^\text{H})</th>
<th>% applied</th>
<th>% recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>endosperm</td>
<td>101 (84.2)(^b)</td>
<td>80.8</td>
<td>98.66</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>embryo</td>
<td>1.37 (2.2)(^b)</td>
<td>1.1</td>
<td>1.34</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>endosperm</td>
<td>36.0 (24.0)(^b)</td>
<td>28.8</td>
<td>96.5</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>embryo</td>
<td>1.31 ( 2.0)(^b)</td>
<td>1.04</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>endosperm</td>
<td>15.3 ( 9.9)(^b)</td>
<td>12.2</td>
<td>80.1</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>embryo</td>
<td>3.80 ( 5.7)(^b)</td>
<td>3.04</td>
<td>19.9</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) time elapsed following application of \([3^\text{H}]-\text{ZR}\)

\(^b\) dpm/g tissue.

Table 4.13: TLC analysis (TLC system D) of radioactivity in tissue extracts following application of \([3^\text{H}]-\text{ZR}\) to the endosperm. The radioactivity is expressed as a percentage of that eluted from the plate.

<table>
<thead>
<tr>
<th>time (\text{(hr)})</th>
<th>tissue</th>
<th>Ado</th>
<th>Ade</th>
<th>ZR</th>
<th>(Z) glucoside</th>
<th>nucleotide (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>endosperm</td>
<td>28</td>
<td>6</td>
<td>20</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>48</td>
<td>endosperm</td>
<td>25</td>
<td>13</td>
<td>11</td>
<td>-</td>
<td>21</td>
</tr>
<tr>
<td>72</td>
<td>endosperm</td>
<td>16</td>
<td>14</td>
<td>6</td>
<td>-</td>
<td>39</td>
</tr>
<tr>
<td>72</td>
<td>embryo</td>
<td>7</td>
<td>9</td>
<td>8</td>
<td>-</td>
<td>49</td>
</tr>
</tbody>
</table>

\(^a\) time elapsed (hr) following application of \([3^\text{H}]-\text{ZR}\).

\(^b\) zone containing AMP and ZR-5'P.
ZR (2 μl, 0.114 μCi/seed). The seeds were then incubated, embryo uppermost, for 24, 48 or 72 hrs when they were washed, dissected into endosperm and embryo fractions and then extracted (Table 4.14). The extracts were then analysed by TLC (system D) (Table 4.15). The presence of ZR in the three endosperm extracts was confirmed by re-chromatography on reverse phase TLC (system N). No significant levels of radioactivity was found to co-chromatograph with DZR.

ZR applied to radicle of 3 day-old seedling. Zea mays kernels were allowed to imbibe water for 3 days and a solution of [3H]-ZR (1 μl, 0.114 μCi/seed) was then placed on the tip of the radicle. The seeds were then incubated for 24, 48 or 72 hrs when they were washed, dissected into endosperm, shoot, radicle, adventitious roots and embryo-remnant fractions, which were then extracted (Table 4.16). The extracts were then analysed by TLC (system D) (Table 4.17). The presence of ZR in the 24 hr extracts of embryo, plumule and radicle was confirmed by re-chromatography on reverse phase TLC (system N). The nucleotide zone was re-chromatographed, after hydrolysis, on reverse phase TLC (system N) (Table 4.18). The glucoside zone was also eluted and re-chromatographed on reverse phase HPLC (system A) and significant levels of radioactivity were found to co-elute only with Z9G.

ZR applied to endosperm of 3 day-old seedling. Zea mays kernels were allowed to imbibe water for 3 days. A solution of [3H]-ZR (2 μl, 0.114 μCi/seed) was placed in wells similar to those described above for ZR application to endosperm after imbibition for 2 hr. The seeds were incubated for a further 24, 48 or 72 hours when they were washed, dissected into endosperm, shoot, radicle, adventitious roots and embryo-remnant fractions, and then extracted (Table 4.19). The extracts were then analysed by TLC (system D) (Table 4.20). Z-nucleotide was found in
Table 4.14: Distribution of radioactivity following application of $[^3\text{H}]-\text{ZR}$ to embryo of whole seed which had imbibed for 2 hr.

<table>
<thead>
<tr>
<th>time (hr)</th>
<th>tissue</th>
<th>total $\times 10^4$ dpm</th>
<th>extracted $^3\text{H}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>% applied</td>
</tr>
<tr>
<td>24</td>
<td>endosperm</td>
<td>11.8 (10.2)$^b$</td>
<td>9.4</td>
</tr>
<tr>
<td>24</td>
<td>embryo</td>
<td>82.4 (137)$^b$</td>
<td>65.9</td>
</tr>
<tr>
<td>48</td>
<td>endosperm</td>
<td>14.5 (10.3)$^b$</td>
<td>11.6</td>
</tr>
<tr>
<td>48</td>
<td>embryo</td>
<td>42.0 (66.7)$^b$</td>
<td>33.6</td>
</tr>
<tr>
<td>72</td>
<td>endosperm</td>
<td>20.2 (14.2)$^b$</td>
<td>16.1</td>
</tr>
<tr>
<td>72</td>
<td>embryo</td>
<td>19.6 (30.6)$^b$</td>
<td>15.7</td>
</tr>
</tbody>
</table>

$^a$Time elapsed following application of $[^3\text{H}]-\text{ZR}$

$^b$DPM/g tissue.
Table 4.15: TLC analysis (system D) of radioactivity in tissue extracts following application of [\textsuperscript{3}H]-ZR to the embryo. The radioactivity is expressed as a percentage of that eluted from the plate.

<table>
<thead>
<tr>
<th>time(^a)</th>
<th>tissue</th>
<th>Ado</th>
<th>Ade</th>
<th>ZR</th>
<th>Z</th>
<th>glucoside(^b)</th>
<th>nucleotide(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>endosperm</td>
<td>20</td>
<td>4</td>
<td>50</td>
<td>1</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>48</td>
<td>endosperm</td>
<td>24</td>
<td>4</td>
<td>36</td>
<td>9</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>72</td>
<td>endosperm</td>
<td>19</td>
<td>6</td>
<td>48</td>
<td>-</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>24</td>
<td>embryo</td>
<td>16</td>
<td>1</td>
<td>39</td>
<td>1</td>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td>48</td>
<td>embryo</td>
<td>10</td>
<td>3</td>
<td>10</td>
<td>-</td>
<td>3</td>
<td>63</td>
</tr>
<tr>
<td>72</td>
<td>embryo</td>
<td>9</td>
<td>4</td>
<td>13</td>
<td>-</td>
<td>3</td>
<td>53</td>
</tr>
</tbody>
</table>

\(^a\)time elapsed (hr) following application of [\textsuperscript{3}H]-ZR

\(^b\)zone delineated by dye L and inosine

\(^c\)zone containing AMP and ZR-5'-P.
Table 4.16: Distribution of radioactivity following application of $[^3\text{H}]$-ZR to the radicle tip of 3 day-old seedlings.

<table>
<thead>
<tr>
<th>time(^a) (hr)</th>
<th>tissue</th>
<th>extracted $[^3\text{H}]$ % applied</th>
<th>% recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(x10(^4) dpm)</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>endosperm</td>
<td>0.21 (0.13)(^c)</td>
<td>0.2</td>
</tr>
<tr>
<td>24</td>
<td>embryo(^b)</td>
<td>2.46 (3.46)(^c)</td>
<td>2.0</td>
</tr>
<tr>
<td>24</td>
<td>shoot</td>
<td>0.74 (10.6)(^c)</td>
<td>0.6</td>
</tr>
<tr>
<td>24</td>
<td>radicle</td>
<td>79.5 (530)(^c)</td>
<td>63.6</td>
</tr>
<tr>
<td>24</td>
<td>adv. roots</td>
<td>0.18 (3.6)(^c)</td>
<td>0.1</td>
</tr>
<tr>
<td>48</td>
<td>endosperm</td>
<td>1.09 (0.70)(^c)</td>
<td>0.9</td>
</tr>
<tr>
<td>48</td>
<td>embryo(^b)</td>
<td>5.53 (8.37)(^c)</td>
<td>4.4</td>
</tr>
<tr>
<td>48</td>
<td>shoot</td>
<td>4.40 (22.0)(^c)</td>
<td>3.5</td>
</tr>
<tr>
<td>48</td>
<td>radicle</td>
<td>56.5 (282)(^c)</td>
<td>45.2</td>
</tr>
<tr>
<td>48</td>
<td>adv. roots</td>
<td>0.65 (9.28)(^c)</td>
<td>0.5</td>
</tr>
<tr>
<td>72</td>
<td>endosperm</td>
<td>0.70 (0.42)</td>
<td>0.6</td>
</tr>
<tr>
<td>72</td>
<td>embryo(^b)</td>
<td>5.65 (6.89)</td>
<td>4.5</td>
</tr>
<tr>
<td>72</td>
<td>shoot</td>
<td>2.70 (5.51)</td>
<td>2.2</td>
</tr>
<tr>
<td>72</td>
<td>radicle</td>
<td>43.7 (146)</td>
<td>35.0</td>
</tr>
<tr>
<td>72</td>
<td>adv. roots</td>
<td>0.32 (2.67)</td>
<td>0.3</td>
</tr>
</tbody>
</table>

\(^a\) time elapsed following application of $[^3\text{H}]$-ZR

\(^b\) this represents the remainder of the seedling following dissection

\(^c\) dpm/g tissue.
Table 4.17: TLC analysis (system D) of radioactivity in tissue extracts following application of $[^3\text{H}]$-ZR to a 3 day-old radicle. The radioactivity is expressed as a percentage of that eluted from the plate.

<table>
<thead>
<tr>
<th>time $^a$ (hr)</th>
<th>tissue</th>
<th>Ado</th>
<th>Ade</th>
<th>ZR</th>
<th>Z</th>
<th>glucoside $^b$</th>
<th>nucleotide $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>embryo</td>
<td>6</td>
<td>19</td>
<td>15</td>
<td>3</td>
<td>-</td>
<td>46</td>
</tr>
<tr>
<td>24</td>
<td>shoot</td>
<td>11</td>
<td>8</td>
<td>18</td>
<td>6</td>
<td>-</td>
<td>35</td>
</tr>
<tr>
<td>24</td>
<td>radicle</td>
<td>15</td>
<td>4</td>
<td>12</td>
<td>4</td>
<td>14</td>
<td>28</td>
</tr>
<tr>
<td>48</td>
<td>embryo</td>
<td>8</td>
<td>35</td>
<td>4</td>
<td>-</td>
<td>10</td>
<td>35</td>
</tr>
<tr>
<td>48</td>
<td>shoot</td>
<td>21</td>
<td>18</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>40</td>
</tr>
<tr>
<td>48</td>
<td>radicle</td>
<td>12</td>
<td>6</td>
<td>4</td>
<td>-</td>
<td>37</td>
<td>13</td>
</tr>
<tr>
<td>72</td>
<td>embryo</td>
<td>10</td>
<td>26</td>
<td>2</td>
<td>-</td>
<td>11</td>
<td>27</td>
</tr>
<tr>
<td>72</td>
<td>shoot</td>
<td>20</td>
<td>14</td>
<td>4</td>
<td>3</td>
<td>-</td>
<td>25</td>
</tr>
<tr>
<td>72</td>
<td>radicle</td>
<td>10</td>
<td>6</td>
<td>2</td>
<td>-</td>
<td>36</td>
<td>14</td>
</tr>
</tbody>
</table>

$^a$ time (hr) from application of $[^3\text{H}]$-ZR to radicle

$^b$ zone delineated by dye L and inosine

$^c$ zone containing AMP and ZR-5'-P.
Table 4.18: Reverse phase TLC (system N) analysis of the eluate of the nucleotide zone (Table 4.17) following treatment with alkaline phosphatase. Radioactivity is expressed as a percentage of that eluted from the plate.

<table>
<thead>
<tr>
<th>time (hr)</th>
<th>tissue</th>
<th>Ado</th>
<th>ZR</th>
<th>DZR</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>embryo</td>
<td>40 (18) \textsuperscript{b}</td>
<td>55 (25) \textsuperscript{b}</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>shoot</td>
<td>50 (17) \textsuperscript{b}</td>
<td>45 (16) \textsuperscript{b}</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>radicle</td>
<td>63 (18) \textsuperscript{b}</td>
<td>25 (7) \textsuperscript{b}</td>
<td>-</td>
</tr>
<tr>
<td>72</td>
<td>shoot</td>
<td>67 (17)</td>
<td>20 (5)</td>
<td>-</td>
</tr>
</tbody>
</table>

\textsuperscript{a} time (hr) from application of \([\textsuperscript{3}H]\)-ZR to radicle

\textsuperscript{b} percentage of total extract eluted from TLC (system D) (Table 4.17).
Table 4.19: Distribution of radioactivity following application of $[^{3}\text{H}]-\text{ZR}$ to the endosperm of 3 day-old seedlings.

<table>
<thead>
<tr>
<th>time (hr)</th>
<th>tissue</th>
<th>total (x10^4 dpm)</th>
<th>% applied</th>
<th>% recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>endosperm</td>
<td>80.0 (53.3)c</td>
<td>64.0</td>
<td>91.2</td>
</tr>
<tr>
<td>24</td>
<td>embryo b</td>
<td>5.90 (10.2)c</td>
<td>4.7</td>
<td>6.7</td>
</tr>
<tr>
<td>24</td>
<td>shoot</td>
<td>0.98 (16.3)c</td>
<td>0.8</td>
<td>1.1</td>
</tr>
<tr>
<td>24</td>
<td>radicle</td>
<td>0.25 (2.1)c</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>24</td>
<td>adv. roots</td>
<td>0.54 (13.5)c</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>48</td>
<td>endosperm</td>
<td>23.0 (14.8)c</td>
<td>18.4</td>
<td>86.4</td>
</tr>
<tr>
<td>48</td>
<td>embryo b</td>
<td>2.21 (3.7)c</td>
<td>1.8</td>
<td>8.3</td>
</tr>
<tr>
<td>48</td>
<td>shoot</td>
<td>0.53 (2.5)c</td>
<td>0.4</td>
<td>2.0</td>
</tr>
<tr>
<td>48</td>
<td>radicle</td>
<td>0.36 (1.56)c</td>
<td>0.3</td>
<td>1.4</td>
</tr>
<tr>
<td>48</td>
<td>adv. roots</td>
<td>0.53 (10.6)c</td>
<td>0.4</td>
<td>2.0</td>
</tr>
<tr>
<td>72</td>
<td>endosperm</td>
<td>17.66 (11.2)c</td>
<td>14.1</td>
<td>67.5</td>
</tr>
<tr>
<td>72</td>
<td>embryo b</td>
<td>4.46 (6.2)c</td>
<td>3.6</td>
<td>17.0</td>
</tr>
<tr>
<td>72</td>
<td>shoot</td>
<td>1.73 (3.5)c</td>
<td>1.4</td>
<td>6.6</td>
</tr>
<tr>
<td>72</td>
<td>radicle</td>
<td>1.40 (4.4)c</td>
<td>1.1</td>
<td>5.3</td>
</tr>
<tr>
<td>72</td>
<td>adv. roots</td>
<td>0.93 (9.3)c</td>
<td>0.7</td>
<td>3.6</td>
</tr>
</tbody>
</table>

*a time elapsed following application of $[^{3}\text{H}]-\text{ZR}$

*b this represents the remainder of the seedling following dissection

c dpm/g tissue.
Table 4.20: TLC analysis (system D) of radioactivity in tissue extracts following application of $[^{3}\text{H}]-\text{ZR}$ to 3 day-old endosperm. The radioactivity is expressed as a percentage of that eluted from the plate.

<table>
<thead>
<tr>
<th>time (hr)</th>
<th>tissue</th>
<th>Ado</th>
<th>Ade</th>
<th>ZR</th>
<th>Z</th>
<th>glucoside</th>
<th>nucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>endosperm</td>
<td>24</td>
<td>3</td>
<td>26</td>
<td>16</td>
<td>1</td>
<td>22</td>
</tr>
<tr>
<td>24</td>
<td>embryo</td>
<td>5</td>
<td>3</td>
<td>9</td>
<td>4</td>
<td>4</td>
<td>67</td>
</tr>
<tr>
<td>48</td>
<td>endosperm</td>
<td>10</td>
<td>4</td>
<td>7</td>
<td>14</td>
<td>3</td>
<td>55</td>
</tr>
<tr>
<td>48</td>
<td>embryo</td>
<td>13</td>
<td>5</td>
<td>7</td>
<td>4</td>
<td>8</td>
<td>54</td>
</tr>
<tr>
<td>72</td>
<td>endosperm</td>
<td>6</td>
<td>7</td>
<td>7</td>
<td>6</td>
<td>2</td>
<td>63</td>
</tr>
<tr>
<td>72</td>
<td>embryo</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>3</td>
<td>10</td>
<td>53</td>
</tr>
<tr>
<td>72</td>
<td>shoot</td>
<td>20</td>
<td>5</td>
<td>6</td>
<td>1</td>
<td>9</td>
<td>44</td>
</tr>
<tr>
<td>72</td>
<td>radicle</td>
<td>14</td>
<td>15</td>
<td>9</td>
<td>3</td>
<td>15</td>
<td>5</td>
</tr>
</tbody>
</table>

a time (hr) from application of $[^{3}\text{H}]-\text{ZR}$ to endosperm

b zone delineated by dye L and inosine

c zone containing AMP and ZR-5'-P.
the 72 hr extracts of embryo and shoot (Table 4.21) and HPLC analysis of the 72 hr extract of radicle confirmed the presence of Z9G.

4.4.4. Results and discussion

Zeatin riboside was unequivocally identified as one of the endogenous cytokinins in dry *Zea mays* kernels (Section 4.2). Hence, it was decided to synthesise $[^3H]$-ZR of high specific activity, so metabolism and translocation studies could be conducted with near-physiological amounts of exogenous ZR. The synthesis was based on the procedure of H. Young and D.S. Letham (unpublished) and involved the selective alkylation of the N-1 position in the presence of a non-nucleophilic acid acceptor, followed by a Dimroth rearrangement under alkaline conditions (see Robins and Trip, 1973, and references therein) and finally hydrolysis of the tetrahydropyranyl protecting group.

The metabolism of exogenous $[^3H]$-ZR in germinating *Zea mays* kernels is unusually simple. The sidechain is cleaved to produce Ados and the ribofuranosyl moiety is then removed to yield Ade. This is in accord with the observation that only trace quantities of Z, or no Z are produced. No reduction of the double bond in the isoprenoid sidechain was observed. Ade. and Ados. are prominent metabolites in both embryo and endosperm at all sampling times and adenosine is the dominant metabolite at the end of the uptake period. $[^3H]$-ZR and adenosine derived from $[^3H]$-ZR are also subject to phosphorylation and adenine nucleotides are the major metabolites in the embryo at 72 hr. No significant levels of radioactivity was found to co-chromatograph with the glucosides, 6-(2,3,4-trihydroxy-3-methylbutylamino)purine or N-(purin-6-yl)glycine. The total amount of radioactivity recovered in the extracts declined with time (e.g. Table 4.4) suggesting the incorporation of the $^3$H-label into insoluble material such as DNA and
Table 4.21: Reverse phase TLC (system N) analysis of the eluate of the nucleotide zone (Table 4.20) following treatment with alkaline phosphatase. Radioactivity is expressed as a percentage of the total eluted from the TLC plate.

<table>
<thead>
<tr>
<th>time (^a) (hr)</th>
<th>tissue</th>
<th>Ado (^b)</th>
<th>ZR (^b)</th>
<th>DZR</th>
</tr>
</thead>
<tbody>
<tr>
<td>72</td>
<td>embryo</td>
<td>62 (33)</td>
<td>5 (3)</td>
<td>-</td>
</tr>
<tr>
<td>72</td>
<td>shoot</td>
<td>66 (29)</td>
<td>3 (1)</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) time (hr) from application of \(^3\)H-ZR to endosperm

\(^b\) percentage of total extract eluted from TLC (system D) (Table 4.20).
RNA (see Nooden and Letham, 1984). Radioactivity in the form of Ado, Ade and ZR was found to leach out of intact tissue into the surroundings (Tables 4.6 and 4.7).

When \[^{3}\text{H}\]-ZR was supplied to excised endosperm and embryos, clear differences in metabolism were evident (Tables 4.10 and 4.11). Thus, in the embryo at 6hr, nucleotides of Ade and Z \text{(in toto 61\% of the extracted radioactivity)} predominated over the ribosides, Ado and ZR \text{(17\% extracted radioactivity)}, and free Ade \text{(9\% extracted radioactivity)}. In contrast, in the endosperm at 6 hr, Ado, Ade and ZR collectively accounted for about 82\% of the extracted \[^{3}\text{H}\], while Ade nucleotides contributed only 7\% and Z nucleotides were not detected. The relative amounts of Ade and Ado differed greatly between embryo and endosperm throughout the experiment. In the embryo at 12 and 48hr, Ade predominated, but in the endosperm the radioactivity co-chromatographing with Ado was 3-4 times that of Ade.

During the initial stages of germination, a polar movement of ZR was found to occur. When \[^{3}\text{H}\]-ZR was supplied to the endosperm, 3.5 and 20\% of the recovered radioactivity was found in the embryo after 48 and 72 hr respectively (Table 4.12). Of this radioactivity at 72h, approximately 8\% was ZR and 5\% Z nucleotide. However, when \[^{3}\text{H}\]-ZR was fed to the embryo, some 26 and 51\% of the recovered radioactivity was found in the endosperm after 48 and 72 hr respectively (Table 4.14), mostly as ZR (Table 4.15). This polarised movement of cytokinins may have important implications in determining a possible role for cytokinins in directing the development of the germinating seed, and especially in control of endosperm reserve mobilisation by the embryo.

When \[^{3}\text{H}\]-ZR was applied to the radicle of 3 day-old seedlings, a large proportion of the radioactivity remaining in the radicle at the
end of 3 days (Table 4.16) had been metabolised to Z9G (36% of the extracted radioactivity). Over the same period, some 8% of the recovered radioactivity had migrated from the radicle to the embryo-remnant and 4% to the shoot. The translocated radioactivity included Z9G, ZR and Z nucleotide, and at 24 hr, 15% and 25% of the $^3$H in the embryo was due to ZR and Z nucleotide respectively. The above results indicate that cytokinin produced in the radicle tip would tend to remain localised in the radicle, and the proportion exported would move principally to the embryo-remnant and shoot. This observation was confirmed by an analysis of the concentration of exported radioactivity in the various tissues (Table 4.16) which indicated that a significant amount of radioactivity was also exported to the adventitious roots. The concentrations of radioactivity exported from the radicle seemed to peak at 24 hours in the embryo-remnant ($8 \times 10^4$ dpm/g), the shoot ($22 \times 10^4$ dpm/g) and the adventitious roots ($9 \times 10^4$ dpm/g). The significance of these results lies in the fact that the radicle tip is a likely site of biosynthesis (see Section 1.4.3) and biosynthesis has now been demonstrated to take place in germinating seed (Section 4.3).

The application of $[\text{3}^\text{H}]$-ZR to the endosperm of 3 day-old seedlings resulted in the translocation, over a 3 day period, of significant quantities of radioactivity (Table 4.19) to the embryo (17%), shoot (7%) and radicle (5%). The major cytokinins in the embryo were ZR (7%) and Z9G (10%); in the shoot, ZR (6%) and Z9G (7%); and in the radicle, ZR (9%) and Z9G (15%) (Tables 4.20 and 4.21). This movement of radioactivity appears to coincide with the mobilisation of the food reserves in the endosperm (see Toole, 1924) and may be part of a general translocation of metabolites rather than a specific movement of hormone.
4.5 Metabolism of $[^{3}\text{H}]$-ZR and $[^{3}\text{H}]$-BAP in Zea mays seedling leaves.

4.5.1 Metabolism of $[^{3}\text{H}]$-ZR

The first leaf of 10 day-old Zea mays seedlings (grown from the same batch and cultivar of seed as analysed in Secton 4.2) was excised and 4 x 1 cm segments were cut from the centre of each leaf. The segments were placed, lower surface upwards on a filter paper (Whatman No. 1, 8.5 cm) in a petri dish (i.e. adaxial surface of leaf was in contact with filter paper). A solution of $[^{3}\text{H}]$-ZR (0.20 µM in water, 2.5 ml) was then applied to the filter paper. The segments were incubated at 23°C under light (700 lux) for 3 days and then blotted dry, rinsed thoroughly with distilled water, and again blotted before being weighed (1.5 g) and extracted as per Scheme 2.1.

An aliquot of the crude extract was analysed by silica gel TLC (system D) using Ade, Ado, Z, ZR, Z9G and inosine as markers. The nucleotide zone (sited at and just above origin) was found to contain 41.1% of the eluted radioactivity; the glucoside zone (area of TLC between and including inosine and Z9G), 27.2%; Ado 7.3%; ZR, 2.9%; Ade, 10.5%. Because of the complexity of the metabolism, the extract was then fractionated by cellulose phosphate chromatography, yielding a basic fraction ($1.03 \times 10^6$ dpm) and an acidic fraction ($1.26 \times 10^6$ dpm). The acidic fraction (containing nucleotides) was hydrolysed by treatment with alkaline phosphatase (Section 2.3.3). Aliquots of the basic fraction and the hydrolysed acidic fraction were then analysed on silica gel TLC (system D) as described above. Of the radioactivity in the hydrolysed acidic fraction, 73% co-chromatographed with Ado, compared with an insignificant amount of radioactivity co-chromatographing with ZR. In the TLC of the basic fraction, clear peaks of radioactivity co-chromatographed with Ade, Ado and Z9G (see Fig.
Figure 4.4: Chromatography of radioactivity (TLC system D), in the basic eluate from cellulose phosphate, extracted from maize leaf segments supplied with [\(^{3}\)H]-ZR. The radioactivity co-chromatographing with each zone is expressed as a percentage of that eluted from the plate.
The basic fraction was then preparatively chromatographed (TLC system D) and the zones corresponding to Ade, Ado, Z9G and the area between inosine and Z9G were eluted with MeOH-HOAc-H2O (10:1:10, v/v). The eluates from these zones and the hydrolysed acidic fraction were then further examined.

Re-chromatography on silica gel (system C) resulted in single peaks of radioactivity co-chromatographing with Ado (95% of the eluted radioactivity in the case of Ado from the basic fraction and 94% for the nucleotide-derived Ado) and Ade (94% of eluted radioactivity). However, only 38% of the radioactivity from the Z9G zone co-chromatographed with Z9G and no single peak of radioactivity was observed when the inosine-Z9G zone was re-chromatographed. Similar results were obtained using reverse phase TLC (system P).

The Z9G zone and inosine-Z9G zone were examined using reverse phase HPLC systems H and T. In both cases, approximately 40% of the eluted radioactivity from the Z9G zone coeluted with Z9G and the radioactivity from the inosine-Z9G zone was evenly distributed over the collected fractions.

In summary, the radioactivity extracted after 3 days incubation with [3H]-ZR was largely distributed between adenine nucleotides (38%), Ado (6%), Ade (12%) and Z9G (2%). Thus only 58% of the extracted radioactivity can be accounted for in terms of identified metabolites. Hence the metabolism of exogenous ZR is much more complex in the leaf than in the germinating seed.
4.5.2 Metabolism of $[^3H]$-BAP

The study of BAP metabolism in maize leaves was carried out as described in Section 4.5.1 using $[^3H]$-BAP (5 µM; 20 mCi/mmmole). The extract was chromatographed on TLC system (system D) using BAP, BAP-9-G, Ado and Ade as markers (Fig. 4.5). The BAP, BAP-9-G and Ado zones were eluted with MeOH-HOAc-H$_2$O (10:1:10, v/v) and subject to further analysis.

Aliquots of the BAP-9-G eluate were re-chromatographed on cellulose TLC (system H) and also on reverse phase TLC (system Q) and it was determined that 90% of radioactivity co-chromatographed with authentic BAP-9-G. An aliquot of the BAP-9-G eluate was hydrolysed in 0.5 M HCl at 95°C for 1 hr. The hydrolysis product co-chromatographed with BAP on silica gel TLC (system D).

The eluate from the BAP zone and the Ado zone were also re-chromatographed using TLC systems H and Q and it was found that 80% and 70% of the radioactivity co-chromatographed with BAP and Ado, respectively.

Hence the principal metabolite of BAP was identified as BAP-9-G, while Ado was a minor metabolite.

4.5.3 Metabolism of $[^3H]$-BAP in the presence of potential inhibitors of N-glucosylation

The centres of 6 leaves from 15 day-old Zea mays seedlings (grown from same batch and cultivar of seed as assayed in Section 4.2) were cut into 5 mm segments which were bisected longitudinally; 6 halved segments, one from each of the 6 leaves, were used in each assay. The segments were each placed on a 20 µl drop of test solution in plastic petri dishes with the abaxial surface downwards. The sides of the petri dishes and the edges of the lids were lined with wetted filter paper and
Figure 4.5: Chromatography of radioactivity (TLC system D), extracted from maize leaf segments supplied with [\(^3\)H]-BAP. The radioactivity in each TLC zone is expressed as a percentage of that eluted from the plate.
the dishes were wrapped in clear plastic film. The test solution used was Tween 80 (0.05%) containing $7.5 \mu M$ $[^3H]$-BAP plus test compound. The segments were left for 2.5 - 3 days in light (700 lux) at 23°C when the segments were washed sequentially with Tween 80 (0.05%), a solution of Tween 80 (0.05%) containing unlabelled BAP (40 $\mu M$), and water. The segments were then blotted dry and extracted as per Scheme 2.1. The extracts were then analysed by silica gel TLC (system D) using BAP and BAP-9-G as markers and the radioactivity co-chromatographing with these markers was then assessed (Table 4.22).

4.5.4 Results and discussion

The metabolism of $[^3H]$-ZR in maize seedling leaves is dominated by the activity of cytokinin oxidase. Over a 3 day period, approximately 56% of the extracted radioactivity could be accounted for as Ado, Ade or the Ade-nucleotides. The only metabolite with an intact side chain that was conclusively identified was Z9G (2% extracted radioactivity). In contrast, the major metabolite produced, when BAP was supplied to maize leaves, was found to be BAP-9-G (44% of the extracted radioactivity). In studies with purified cytokinin oxidase isolated from *Zea mays* kernels, Whitty and Hall (1974) found that the BAP side chain was not susceptible to cleavage. However, the side chain is slowly cleaved *in vivo* by maize leaf segments but the resultant Ado is only a minor metabolite (< 11% of the extracted radioactivity). Thus, it was decided to test the effectiveness of the compounds previously synthesised (Chapter 3), in inhibiting the N-glucosylation of BAP, rather than ZR, in the maize leaf segments.

A number of compounds appreciably inhibited the formation of BAP-9-G, namely, the xanthine derivatives 7, 10, 17, and 18, and the BAP analogue 24, and these inhibitors also elevated the level of free BAP.
Table 4.22: Distribution of radioactivity, as assessed by TLC (system D), extracted from Zea mays leaf segments following incubation with [3H]-BAP in the presence of potential inhibitors of N-glucosylation

<table>
<thead>
<tr>
<th>Substituent</th>
<th>Concentration (mM)</th>
<th>% Eluted Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BAP-9-G</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>43</td>
</tr>
<tr>
<td>Derivatives of 1-methylxanthine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3-CH₂CH(CH₃)₂</td>
<td>5</td>
</tr>
<tr>
<td>17</td>
<td>7-CH₂CH=CH(CH₃)₂</td>
<td>3</td>
</tr>
<tr>
<td>17</td>
<td>7-CH₂CH=C(CH₃)₂</td>
<td>1</td>
</tr>
<tr>
<td>18</td>
<td>3-CH₂CH=C(CH₃)₂</td>
<td>5</td>
</tr>
<tr>
<td>18</td>
<td>3-CH₂CH=C(CH₃)₂</td>
<td>1</td>
</tr>
<tr>
<td>19</td>
<td>7-CH₂C₆H₅</td>
<td>2.5</td>
</tr>
<tr>
<td>Derivatives of 1,7-dimethylxanthine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>3-CH₂C₆H₅</td>
<td>2.5</td>
</tr>
<tr>
<td>7</td>
<td>3-[CH₂]₂CH(CH₃)₂</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>3-[CH₂]₂CH(CH₃)₂</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>3-[CH₂]₂CH=CH₂</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>3-[CH₂]₂CH=CH₂</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>3-[CH₂]₂CH=CH₂</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>3-[CH₂]₂CH=CH₂</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>3-CH₂C(CH₃)=CH₂</td>
<td>3</td>
</tr>
<tr>
<td>12</td>
<td>3-[CH₂]₄OH</td>
<td>5</td>
</tr>
<tr>
<td>12</td>
<td>3-[CH₂]₄OH</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>3-CH₂CH=C(CH₃)₂</td>
<td>5</td>
</tr>
<tr>
<td>13</td>
<td>3-CH₂CH=C(CH₃)₂</td>
<td>1</td>
</tr>
<tr>
<td>14</td>
<td>3-CH₂CHOHCH₂CH₃</td>
<td>5</td>
</tr>
<tr>
<td>16</td>
<td>3-CH₂CH=C(CH₂OH)CH₃</td>
<td>5</td>
</tr>
<tr>
<td>16</td>
<td>3-CH₂CH=C(CH₂OH)CH₃</td>
<td>1</td>
</tr>
<tr>
<td>Derivatives of 9-methyl-BAP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>2-NH(CH₂)₂OH</td>
<td>1</td>
</tr>
<tr>
<td>29</td>
<td>2-NHCH₂C(CH₃)₂OH</td>
<td>0.8</td>
</tr>
<tr>
<td>29</td>
<td>2-NHCH₂C(CH₃)₂OH</td>
<td>0.2</td>
</tr>
<tr>
<td>Derivatives of oxazolo[5,4-d]pyrimidine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>7-NHCH₂C₆H₅</td>
<td>1</td>
</tr>
<tr>
<td>24</td>
<td>7-NHCH₂C₆H₅</td>
<td>0.2</td>
</tr>
</tbody>
</table>
However, inhibition by the first four compounds was exhibited only at the highest concentration tested, usually 5mM. In the case of 10, the inhibition was very pronounced (87% reduction in the formation of BAP as compared with the control), but may have been associated with a toxic effect, since the incubation solution had become yellow after 3 days. Compound 24, 7-benzylaminoazolo[5,4-d]pyrimidine was the most effective inhibitor tested, in that it suppressed BAP-9-G formation and elevated the free BAP level appreciably even at 0.2mM. Some inhibitors, especially 17 at 1mM, elevated the level of free BAP but did not inhibit BAP-9-G formation and hence appeared to suppress BAP metabolism other than N-glucosylation.
5.1 Introduction

The physiological effect of exogenous cytokinin on excised immature radish cotyledons is to markedly stimulate the lateral expansion of the cotyledons. This response is largely due to cell enlargement rather than cell division which also occurs (Letham, 1971; Gordon and Letham, 1975). Applied to discs excised from mature cotyledons and to leaves, cytokinin can also delay the onset of senescence (Letham et al., 1983; Letham and Gollnow, 1985). The BAP-induced expansion of cotyledons has been related to the tissue levels of BAP in the base, riboside and nucleotide forms and not the 7- and 9-glucosides which are only weakly active, or the 3-glucoside which exhibits activity similar to that of BAP (Letham et al., 1983; Letham and Gollnow, 1985). However, BAP-9-G was found to be at least as effective as BAP in delaying the senescence of radish leaves (Letham et al., 1983).
The metabolism of exogenous cytokinin has been studied extensively in the radish system (see Section 1.5, and Table 1.2 and references therein). While BAP and DZ are converted to the 3-, 7- and 9-glucosides, zeatin appears to be metabolised primarily to Z7G which is essentially ineffective both in stimulating cotyledon expansion and in delaying senescence. Unfortunately, there is no information about the endogenous cytokinins in radish cotyledons or leaves. Hence it is difficult to relate the physiological effects and metabolism of exogenous cytokinin to the development of the radish seedling while the endogenous cytokinins and their movement is unknown.

As the predominant fate of exogenous cytokinin is N-glucosylation, it was thought that inhibitors of this process may prove useful in potentiating the effect of cytokinin, whether applied or exogenous, and thereby modify plant development. Potential inhibitors have been synthesised (Chapter 3) and their activity has now been assessed using both radish leaves and cotyledons. One compound that has previously been found to inhibit N-glucosylation in vivo was 2-(2-hydroxyethylamino)-9-methyl-6-benzylaminopurine (27; Letham et al., 1982). However, this compound is itself metabolised in radish cotyledons (Tao and Letham, unpublished) and since the identity of the resulting metabolite may assist in the design of more effective inhibitors, the structure of this derivative has been determined.

The experimental work described in this chapter was carried out in collaboration with D.S. Letham and G.-Q Tao.
5.2 Identification of the endogenous cytokinins in radish cotyledons

Radish seedlings (*Raphanus sativus* cv. Long Scarlet) were germinated asceptically on wetted filter paper. Immature cotyledons (2 batches, 2 separate plantings) and mature cotyledons (2 batches, 2 separate plantings) were excised from 12 and 20 day-old radish seedlings, respectively. The cotyledons were extracted as per Scheme 2.1 with the addition of deuterated cytokinins (D_5-Z7G, -Z9G, -ZR and -Z; 7 µg per sample, each 80-120 g fresh weight tissue) to the extracting solvent. The extract was purified and fractionated by passage through cellulose phosphate, HPLC (system L) and then TLC on cellulose (system F) using marker dyes E, F, G and H (see scheme 4.1). The cellulose zones containing Z plus ZR and Z9G plus Z7G were eluted with MeOH-HOAc-H_2O (10:1:10, v/v). The two eluates were then chromatographed on HPLC (system N) to yield individual fractions containing Z, ZR, Z9G and Z7G.

Z9G. The Z9G fraction was further chromatographed on HPLC (system H) and the fraction containing the UV peak at the retention volume of Z9G was collected. The fraction was dried and the residue methylated (Section 2.3.4) before chromatographing on silica gel HPTLC (system I; plates pre-washed with MeOH and EtOAc). The UV spot at the R_f (0.68) of hexamethyl-Z9G was eluted with redistilled EtOAc.

The sample was introduced into the mass spectrometer via the direct insertion probe and ionized by EI (instrumentation as described in Section 2.6.6). Mass spectral scans were obtained over the range m/z 200-230 and m/z 430-480. No ions were detected at m/z 465(M^+), 434 (M-CH_3O) or 216 (M-glucosyl-CH_3O) to correspond with ions from D_5-Z9G at m/z 470, 439 and 221 respectively.
The Z7G fraction was further chromatographed on HPLC (system H) and the fraction containing the UV peak at the retention volume of Z7G was collected and trimethylsilylated (Section 2.3.4).

GC-MS analysis was carried out as described previously (Section 2.6.6). The TMS-Z7G from one batch of mature cotyledons was analysed using CI (ammonia), scanning $m/z$ 720-750 for the $[\text{MH}]^+$ ion pair, $m/z$ 742 and 747. The TMS-Z7G from the other batch of mature cotyledons was analysed using El ionisation, scanning $m/z$ 600-680 for ion pairs $m/z$ 652 and 657 (M-TMSOH) and $m/z$ 638 and 641 (M-TMSOCH$_2$). Standard curves were constructed for each ion pair and the Z7G content of the two batches of mature radish cotyledons determined as 83 and 120 ng/g fresh tissue, respectively.

The Z7G from two batches of immature cotyledons was similarly isolated and the first batch methylated by the usual method (Section 2.3.4) before chromatographing and eluting from silica gel HPTLC (system I) ($R_f$ 0.57) as per HPTLC of hexamethyl-Z9G above. The major product isolated was trimethyl-Z. The concentration of endogenous Z7G was calculated from the EIMS (direct insertion probe, mass range scanned $m/z$ 200-280) using the ion pair $m/z$ 230 and 235 (M-CH$_3$O), to be 63 ng/g fresh weight tissue. The second batch of Z7G was methylated using a reaction time of 3 minutes instead of 30 minutes to yield both hexamethyl-Z7G (major product) and trimethyl-Z after elution from HPTLC as above, $R_f$ 0.51 and 0.56 respectively. The concentration of endogenous Z7G in the second batch of immature cotyledons was calculated from the EIMS of hexamethyl-Z7G (direct insertion probe, mass ranges scanned $m/z$ 200-230 and $m/z$ 430-480) using the ion pairs $m/z$ 434 and 439 (M-CH$_3$O) and $m/z$ 216 and 221 (M-glucosyl-CH$_3$O), to be 52 ng/g fresh weight tissue.
ZR. The ZR fraction was dried and converted to the tBuDMS ether of 2',3'-O-isopropylidene-ZR (Section 2.3.4) which was chromatographed and eluted from HPTLC (system J) as per hexamethyl-Z9G above. The concentration of endogenous ZR in mature cotyledons was calculated from the EIMS of the above derivative (direct insertion probe), by MID of ion pairs m/z 604 and 609 (M-15) and m/z 562 and 567 (M-57), to be 1.4 ng/g fresh weight tissue. This result was confirmed using CIMS (ammonia) and MID of m/z 620 and 625, [MH]+.

Z. The Z fraction was dried and converted to the di-tBuDMS ether. The derivatising solution was evaporated and the residue taken up in EtOAc, evaporated, dissolved in 80% EtOH, evaporated and finally dissolved in EtOH-EtOAc (1:1, v/v) prior to HPTLC (system I) on pre-washed plates. The UV spot at the Rf of mono-tBuDMS-Z was eluted with EtOH for direct insertion probe CIMS (ammonia). The ion pair m/z 334 and 339, [MH]+, was monitored by MID but no endogenous Z was detected (if present, < 0.2 ng/g fresh weight tissue).

5.3 The identification of a metabolite of 2-(2-hydroxyethylamino)-9-methyl-6-benzylaminopurine (27).

Seedlings of radish (Raphanus sativus L. cv Long Scarlet) were grown on nutrient agar under asceptic conditions. After 10 days exposure to light at room temperature, the cotyledons were excised and placed in petri dishes containing one circle of Whatman No. 1 filter paper (diam. 9 cm) wetted with a 1mM sterile solution of 27 (3 ml). The cotyledons were incubated for 3 days under light when they were washed and extracted as per Scheme 2.1. The extract was then subject to preparative 2D-TLC on silica gel. Two spots which were not normal constituents of cotyledon extract could be visualised under UV light and
these were eluted (50% MeOH), following which a final purification was obtained by reverse phase HPLC (System J).

The identity of the less polar compound, which co-chromatographed with 27 on TLC, was confirmed as unmetabolised 27 by EIMS. The molecular weight of the polar compound was determined by CIMS (methane) to be 460. The EIMS of the underivatised metabolite exhibited ions only at \( m/z \) 298, 280, 279, 267 and 254, which are indicative of an intact moiety of 27 (cf MS of 27, Section 3.5.4). The EIMS of the TMS derivative of the polar compound is shown in Fig. 5.1. The ions \( m/z \) 297, 281, 280, 267 and 254 are also attributable to an intact moiety of 27 and cleavage within the substituent at the 2-position of the purine ring. The molecular ion, \( m/z \) 748, and the ions \( m/z \) 451, 450, 361, 319, 305, 217 and 204 are indicative of a silylated hexose residue. The high ratio of fragment ions \( m/z \) 204:217 suggests that this is a hexopyranose ring (see Morris, 1977 and references therein).

Treatment of the polar compound with \( \beta \)-glucosidase followed by 2D-TLC and elution, as above, afforded 27 as identified by \( R_f \) and MS data. Thus the polar metabolite of 27 was assigned structure 30, the \( \beta \)-D-glucoside of 27.
Figure 5.1: EIMS (solid probe) of the TMS derivative of 30, a metabolite of 27, produced \textit{in vivo} by excised 10 day-old radish cotyledons.
Fig. 5.1

![Mass Spectrum Image]

- **b-C₂H₅O**: 254
- **b-CH₂O**: 267
- **b-O**: 297
- **b**: 309
- **326**: 339
- **353**: 369
- **M**: 748
- **M-15**: 451, 733
- **450**: 450
5.4 Metabolism of $[^3H]$-BAP in the presence of potential inhibitors of N-glucosylation

5.4.1 Radish leaves

Leaf discs were excised from fully expanded radish leaves (*Raphanus sativus* L. cv Long Scarlet) cut from 40-50 day-old radish seedlings grown asceptically on nutrient agar. Each assay was conducted as described previously for maize leaf segments (Section 4.5.3) using 6 discs (diam. 6.5 mm) per petri dish, each set being excised from the same 6 leaves, 1 disc per leaf. Each disc was placed on a 20 µl solution of $[^3H]$-BAP (7.5 µM) in Tween 80 (0.05%). After a 3 day incubation, the tissue was washed (as per Section 4.5.3), extracted and the extracts analysed by silica gel TLC (system D) using BAP, BAP-3-G, -7-G, -9-G and BAP nucleotide as markers. The radioactivity co-chromatographing with these markers was then assessed (Table 5.1).

5.4.2 Radish cotyledons

Cotyledons were excised from 15 day-old radish seedlings (*Raphanus sativus* L. cv Long Scarlet) and 4 discs (diam. 4.5 mm) cut from each cytoledon. The assay was conducted in essentially the same manner as when using radish leaves (Section 5.4.1), with the exception that only 10 µl of $[^3H]$-BAP solution was applied to each disc. The tissue extract was analysed both by TLC system D and by 2D-TLC which effected the resolution of the BAP N-glucosides (Table 5.2).

5.5 Results and discussion

Endogenous Z7G and ZR were unequivocally identified and quantified in radish cotyledons. Increasing amounts of Z7G were found in the older cotyledons suggesting that Z7G may be accumulated over time (52 and 63 ng/g in immature cotyledons compared with 83 and 120 ng/g in mature
Table 5.1: Distribution of radioactivity as assessed by TLC (system D), extracted from radish leaf discs following incubation with $^{3}H$-BAP in the presence of potential inhibitors of N-glucosylation.

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>% eluted radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BAP-5'-P</td>
</tr>
<tr>
<td>control</td>
<td>8.8</td>
</tr>
</tbody>
</table>

**derivatives of 1-methylxanthine**

<table>
<thead>
<tr>
<th>substituent</th>
<th>concentration (mM)</th>
<th>% eluted radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3-CH&lt;sub&gt;3&lt;/sub&gt;CH(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>3-CH&lt;sub&gt;3&lt;/sub&gt;CH(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1</td>
</tr>
<tr>
<td>17</td>
<td>7-CH&lt;sub&gt;3&lt;/sub&gt;CH=C(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>3</td>
</tr>
<tr>
<td>17</td>
<td>7-CH&lt;sub&gt;3&lt;/sub&gt;CH=C(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1</td>
</tr>
<tr>
<td>18</td>
<td>3-CH&lt;sub&gt;3&lt;/sub&gt;CH=C(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>5</td>
</tr>
<tr>
<td>18</td>
<td>3-CH&lt;sub&gt;3&lt;/sub&gt;CH=C(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1</td>
</tr>
<tr>
<td>19</td>
<td>7-CH&lt;sub&gt;3&lt;/sub&gt;C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;</td>
<td>2.5</td>
</tr>
</tbody>
</table>

**derivatives of 1,7-dimethylxanthine**

<table>
<thead>
<tr>
<th>substituent</th>
<th>concentration (mM)</th>
<th>% eluted radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>3-CH&lt;sub&gt;3&lt;/sub&gt;C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;</td>
<td>2.5</td>
</tr>
<tr>
<td>7</td>
<td>3-CH&lt;sub&gt;3&lt;/sub&gt;C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>3-(CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;CH(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>3-(CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;CH(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>3-(CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;CH(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.2</td>
</tr>
<tr>
<td>8</td>
<td>3-(CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;CH=CH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>3-(CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;CH=CH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>3-(CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;CH=CH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>3-(CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;CH=CH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>3-(CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;CH=CH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>3-(CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;CH=CH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>3-(CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;CH=CH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>5</td>
</tr>
<tr>
<td>12</td>
<td>3-(CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;4&lt;/sub&gt;SH</td>
<td>5</td>
</tr>
<tr>
<td>12</td>
<td>3-(CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;4&lt;/sub&gt;SH</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>3-CH&lt;sub&gt;3&lt;/sub&gt;C=CH(C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>5</td>
</tr>
<tr>
<td>14</td>
<td>3-CH&lt;sub&gt;3&lt;/sub&gt;C=CH(C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1</td>
</tr>
<tr>
<td>14</td>
<td>3-CH&lt;sub&gt;3&lt;/sub&gt;C=CH(C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.2</td>
</tr>
<tr>
<td>14</td>
<td>3-CH&lt;sub&gt;3&lt;/sub&gt;C=CH(C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>3-CH&lt;sub&gt;3&lt;/sub&gt;C=CH(C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.2</td>
</tr>
<tr>
<td>15</td>
<td>3-CH&lt;sub&gt;3&lt;/sub&gt;C=CH(C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>5</td>
</tr>
<tr>
<td>16</td>
<td>3-CH&lt;sub&gt;3&lt;/sub&gt;C=CH(C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>5</td>
</tr>
<tr>
<td>16</td>
<td>3-CH&lt;sub&gt;3&lt;/sub&gt;C=CH(C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>5</td>
</tr>
<tr>
<td>16</td>
<td>3-CH&lt;sub&gt;3&lt;/sub&gt;C=CH(C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.2</td>
</tr>
</tbody>
</table>

**derivatives of 9-methyl-BAP**

<table>
<thead>
<tr>
<th>substituent</th>
<th>concentration (mM)</th>
<th>% eluted radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>2-NH(CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;4&lt;/sub&gt;OH</td>
<td>1</td>
</tr>
<tr>
<td>27</td>
<td>2-NH(CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;4&lt;/sub&gt;OH</td>
<td>0.2</td>
</tr>
<tr>
<td>29</td>
<td>2-NHCH&lt;sub&gt;2&lt;/sub&gt;C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;</td>
<td>0.8</td>
</tr>
</tbody>
</table>

**derivative of oxazolo[5,4-d]pyrimidine**

<table>
<thead>
<tr>
<th>substituent</th>
<th>concentration (mM)</th>
<th>% eluted radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>7-NHCH&lt;sub&gt;2&lt;/sub&gt;C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;</td>
<td>1</td>
</tr>
</tbody>
</table>

---

$^a$Radioactivity corresponding to BAP-9-G, -7-G and -3-G.
Table 5.2: Distribution of radioactivity, as assessed by 2D-TLC, extracted from radish cotyledons following incubation with \([^3H]\)-BAP in the presence of potential inhibitors of N-glucosylation.

<table>
<thead>
<tr>
<th>test compound</th>
<th>BAP</th>
<th>BAP-5'-P</th>
<th>BAP-9-G</th>
<th>BAP-7-G</th>
<th>BAP-3-G</th>
<th>Total G</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.9 (1.5)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.2 (6.5)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.9</td>
<td>48.0</td>
<td>3.8</td>
<td>80.7 (84.8)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>7 (1mM)</td>
<td>11.4 (11.5)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34.1 (35.2)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.3</td>
<td>14.3</td>
<td>0.6</td>
<td>26.2 (30.3)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10 (1mM)</td>
<td>11.5 (13.5)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34.9 (36.0)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.6</td>
<td>16.4</td>
<td>1.8</td>
<td>29.8 (34.5)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>27 (1mM)</td>
<td>12.2 (12.5)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.0 (30.8)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.3</td>
<td>20.7</td>
<td>4.1</td>
<td>29.1 (35.1)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values in parenthesis represent % radioactivity co-eluting in the one dimensional TLC system D.
cotyledons). No Z or Z9G was detected. These results confirm the observations, made with exogenous $[^3H]^{-}Z$ (Table 1.2), that 7-glucosylation is the dominant form of metabolism of zeatin in radish cotyledons. Exposure of Z7G to the dimethylsulphinyl anion during methylation was found to result in the hydrolysis of the glucosyl residue. This could be minimised, however, by using a shorter reaction time (see also MacLeod et al., 1976). No such hydrolysis was found when methylating 9-substituted zeatins, Z9G and ZR.

As a result of the identification of 30 as a metabolite of the N-glucosylation inhibitor, 27, in radish cotyledons, a new BAP derivative 29, with a $^3O$ alcohol at the terminus of the 2-substituent was synthesised. It was hoped that the $^3O$ alcohol would sterically hinder O-glucosylation at this position and perhaps result in a more stable and effective inhibitor of N-glucosylation. However, when tested in the radish leaf assay, 29 was found to be essentially ineffective in preventing the N-glucosylation of exogenous BAP.

With the exception of 29, 18 and 24, all compounds inhibited N-glucosylation of BAP appreciably in radish leaf discs, at least at the highest concentration tested. In the case of the 1-methylxanthines, 3 and 17, this inhibition was accompanied by a marked elevation in the level of free BAP, while the level of BAP nucleotide was not appreciably altered. However, in the cases of all 1,7-dimethylxanthines and also 27, the levels of both free BAP and BAP nucleotide were elevated markedly when N-glucosylation was suppressed. The most effective inhibitors of N-glucosylation in radish leaves were found to be three derivatives of 1,7-dimethylxanthine, 7, 10 and 13, which all showed significant activity at 0.2mM (Table 5.1). These compounds were more effective than the BAP analogue 27 and the 1-methylxanthine 3, the most
effective inhibitors of this type known prior to the present study. At 5mM, 10 elevated the free BAP level 40-fold.

In the above studies, screening for inhibition was carried out using leaf discs rather than cotyledon discs, primarily because of the convenience of cutting many discs from the one leaf rather than only 4 discs from any one cotyledon, given that the metabolism of exogenous BAP is essentially the same in the two tissues. However, the inhibitory activity of 7, 10 and 27 were also compared using radish cotyledons (Table 5.2). In this study, the radioactivity in BAP-9-G, BAP-7-G and BAP-3-G were determined in addition to that in free BAP and BAP nucleotide. It is evident that 27 preferentially inhibited formation of BAP-9-G which has been reported previously (Letham et al., 1982) and explained in terms of effective inhibition of one of the two cytokinin glucosylating enzymes in radish cotyledons. These two enzymes have been extracted from radish cotyledons and have been shown to form the 7- and 9-glucosides of BAP in markedly different proportions, the ratios for 7-G/9-G being 10-11 for one and 1.5 for the major enzyme (Entsch and Letham, 1979). The latter enzyme has been characterised and 27 was designed to inhibit its action (Letham et al., 1982; Parker et al., 1985 in press). In contrast to 27, the new inhibitors 7 and 10 suppressed formation of BAP-7-G and BAP-9-G about equally and also reduced formation of BAP-3-G. Thus 7 and 10 seem to inhibit both glucosylation enzymes.
6. Conclusions and Final Discussion

'Would you tell me, please, which way I ought to go from here?'
'That depends a good deal on where you want to get to,' said the Cat.
'I don't much care where,' said Alice.
'Then it doesn't matter which way you go,' said the Cat.
'So long as I get somewhere,' Alice added as an explanation.
'Oh, you're sure to do that,' said the Cat, 'If you only walk long enough.'

Alice's Adventures in Wonderland
Lewis Carroll 1832-1898

The major objectives of this thesis have been three-fold. Firstly, to improve the analytical techniques of cytokinin quantitation so that unequivocal data regarding the identity and quantity of the cytokinins in tissues are more readily obtained (i.e., faster and more sensitive). Secondly, to use these techniques to study the role of cytokinins in seed germination and seedling development. Thirdly, to attempt to manipulate cytokinin metabolism, and ultimately plant development, by selectively inhibiting a major form of cytokinin inactivation, namely 7- and 9-glucosylation.
6.1 Purification and quantification of cytokinins

Several new chromatographic methods were developed in the present study with the aim of devising purification procedures with general applicability to plant tissues. In developing these procedures, priority was given to methods that were relatively rapid, and low cost if possible, so that ultimately large numbers of samples could be processed. This would overcome a major difficulty that has been generally experienced in attempting to conduct physiologically relevant experiments using physico-chemical methods.

A paraffin-impregnated silica gel column was found to give a bulk separation of cytokinins similar to that achieved by HPLC (system L) with the obvious advantage that many such columns can be run at one time at very low cost. Silica gel TLC also has advantages of speed and low cost over HPLC, however, when eluted with polar solvents, colloidal silica is also eluted and must be removed prior to MS analysis. This problem was avoided by the development of an equivalent TLC system using cellulose as the stationary phase (TLC system F). However, the disadvantages of this new TLC system were that the loading capacity per plate was much reduced compared with silica gel and the cytokinin zones were less compact. A boric acid gel column was used to recover cytokinin ribosides from silica gel TLC eluates and although this procedure was not retained as a purification step in preparing samples for MS, it was found useful in other cytokinin studies in this laboratory. In particular it was used to purify iPA from water-chestnut extracts as described by Tsui et al. (1983). Reverse phase TLC systems using silica gel impregnated with silicone oil and paraffin were also developed, primarily for the analysis of crude extracts in the metabolism studies. Following a water wash, complete resolution of Ado,
Ade, ZR, DZR, Z and DZ could be achieved, a separation previously accomplished only on HPLC and which saved much time and money.

New derivatives of the cytokinin bases and ribosides have been developed and applied to studies of cytokinins in *Zea mays* and *Raphanus sativus*. The cytokinins occur as free purine bases, ribosides, nucleotides, glucosides and alanine conjugates. However, it is likely that the free base is the functional or active form of the cytokinins (Laloue and Pethe, 1982, 1985), and hence quantification of the cytokinin bases is particularly important. The analysis of the bases by GC-MS has proved more difficult than the determination of the ribosides. The TMS derivatives of the cytokinin ribosides have generally proved satisfactory for GC-MS studies, however, when zeatin and related bases are subject to GC as TMS derivatives, considerable losses and multiple peak formation have been observed. These problems do not arise with the tBuDMS derivatives which were prepared and characterised in the present study.

When derivatisation is carried out under conditions often used for other silylations, mixtures of mono- and di-tBuDMS derivatives are produced from Z and DZ. However, dimethylaminopyridine was found to catalyse the derivatisation reaction effectively and thus enabled the di-tBuDMS derivatives to be prepared quantitatively, for the first time, from Z, DZ and *cis*-Z. These derivatives proved to have good GC properties and their MS fragmentation was rationalised and a number of unusual ion reactions identified. Although hydrolysis of the tBuDMS derivative does occur, the hydrolysis is exclusive to the 9-tBuDMS group and is complete. TLC and HPLC may be performed with the mono-0-tBuDMS derivatives of Z, DZ and *cis*-Z which can be eluted and run on GC without rederivatising. These mono-derivatives have shorter retention times.
than their fully derivatised counterparts and give very distinctive probe spectra. If used to quantify the cytokinin bases, using the stable isotope dilution assay, they permit the isotope ratio measurements to be made in two different mass regions and at two different retention times, providing an additional insurance against possible interfering ions.

Complete and quantitative conversion of the cytokinin ribosides to tBuDMS derivatives proved impossible, even after prolonged heating at high temperatures. This is perhaps not surprising when one considers, firstly, the bulky nature of the trialkylsilyl group and, secondly, the steric crowding around the cis-2',3'-diol of the ribosyl moiety. This problem was circumvented by selectively converting the diol to the 2',3'-0-isopropylidene derivative and then forming the tBuDMS ethers of the remaining primary alcohol groups. Although stable enough for TLC and HPLC to be performed, the di-tBuDMS-2'-3'-0-isopropylidene derivatives of ZR, DZR and cis-ZR were found to be too involatile for satisfactory GC and so MS analysis was carried out via the direct insertion probe. Very recently, new reagents have been developed specifically for the silylation of 1,2- and 1,3-diols, for example, the cyclic di-tert-butylsilylene (DTBS) derivatives (Brooks, Cole and Barrett, 1984; Brooks and Cole, 1985). These DTBS derivatives have been prepared quantitatively with a variety of compounds, such as acyclic and alicyclic diols and catechols, and have been shown to be stable in addition to having good GC properties.

Although the mass increment for this group is much greater than for the isopropylidene ethers (140 amu cf 40 amu) there may be advantages of increased volatility, improved yields and convenience of preparation. However, an additional silylation step would be required to silylate the
remaining hydroxyl groups in, for example, the sidechain and the 5'-position of ZR. In this connection analogues of the reagent used by Miyazaki et al. (1984), in which the silicon monohydride, N,O-bis(diethylsilyl)trifluoroacetamide, was used to form concurrently, diethylsilylene and diethlysilyl ether derivatives of various mono-, di- and trihydroxysteroids, would be of interest.

Significant increases in sensitivity have also been obtained by the use of CI, limiting molecular fragmentation and concentrating the ion current into only a few ions of interest (e.g., [MH]+). The use of ammonia as a reagent gas was found to give greater sensitivity than either CI using methane or EI ionisation.

The development of physico-chemical methods, outlined in this thesis, was complemented by the use of RIA, both in qualitative and quantitative senses. RIA enabled quantitation to be carried out using small samples. In samples to be examined by GC-MS it also enabled the detection of deuterium-labelled standards plus endogenous cytokinins in the HPLC eluates when UV absorbing compounds masked the UV absorbance of the cytokinins. However, RIA may be subject to non-specific interferences, so it is important that samples be purified to limit this possibility and to quantify individual cytokinins. In the present study, using extracts of dry sweet corn kernels, a considerable amount of activity in RIA was detected in the fractions which are usually discarded following ion exchange chromatography and enzymic hydrolysis. Such findings indicate that RIA could not provide any meaningful data on crude extracts, although there are several reports in the literature of data obtained in this way (e.g., Weiler, 1980; Weiller and Ziegler, 1981).
The physico-chemical methods and techniques outlined above were used to unequivocally identify and quantify, for the first time, the endogenous cytokinins in both dry sweet corn seed and in radish cotyledons (Sections 4.2 and 5.2). The identification and quantification of ZR in sweet corn meant that the metabolic studies which were subsequently conducted could be performed using near-physiological levels of a cytokinin that was known to occur naturally. Estimates of the endogenous cytokinins in maize seed by GC-MS were compared with estimates made by RIA. In a qualitative sense the assays produced comparable results, however, quantitatively the cytokinins were, with the exception of ZR and iPA, underestimated by RIA. These differences may be due to the fact that, firstly, losses accrued during the purification procedures prior to RIA were corrected for, only on the basis of the recovery of [3H]-ZR, and secondly, the RIA was carried out on dissected embryos and endosperm, so that any cytokinin concentrated in the discarded fragments (scutellum plus endosperm) resulting from the dissection, would be also lost.

The identification of Z7G and ZR as endogenous cytokinins in radish cotyledons, complements the extensive studies that have been carried out on the metabolism and translocation of exogenous cytokinins in this plant (Table 1.2). Both mature and immature cotyledons were analysed for Z7G which was found to occur at quite high levels and which seemed to be accumulated in the cotyledon over time (52 and 63 ng/g tissue in 12 day-old seedlings cf. 83 and 120 ng/g tissue in 20 day-old seedlings). The absence of Z9G was also quite noteworthy and is in accord with the observation that the cotyledons convert Z only to the 7-glucoside. In recent studies in this laboratory it has been established
that \(^{3}\text{H}\)-Z7G is transported to the root from fully developed cotyledons and that conversion to free zeatin occurs in the lateral roots (D.S. Letham, personal communication). It is reasonable to propose that the endogenous Z7G identified and quantified in this present study would also be subject to this movement.

In conclusion, the unequivocal identification of the endogenous cytokinins in both radish cotyledons and dry maize seeds provides a basis for subsequent studies on the physiological role of cytokinins in these tissues.

6.2 Cytokinin metabolism in germinating maize seed and seedling leaves

The metabolism of exogenous \(^{3}\text{H}\)-ZR in germinating \(\text{Zea mays}\) caryopses is unusually simple but very rapid. The \(N^6\)-sidechain is cleaved oxidatively to produce adenosine and the ribofuranosyl moiety is then removed to yield adenine. Consequently, only trace amounts of zeatin were ever observed. No reduction of the sidechain double bond to yield either DZ or DZR was detected. Radioactivity was also found in the adenine and zeatin nucleotides, indicating that phosphorylation of the ribofuranosyl moiety also occurred. When \(^{3}\text{H}\)-ZR was fed to the radicles of 3 day-old seedlings, N-glucosylation occurred, yielding Z9G, in significant quantities. In the first leaf of \(\text{Zea mays}\) seedling, sidechain cleavage was dominant with Z9G accounting for only 2% of the extracted radioactivity. However, metabolism in the leaf was much more complex than in the seed. Similar patterns of metabolism involving sidechain cleavage by cytokinin oxidase and 9-glucosylation have been reported in other \(\text{Zea mays}\) tissues. Parker and Letham (1974) supplied \(^{3}\text{H}\)-Z to the roots of intact 10 day-old seedlings and isolated the nucleotides of Ado and ZR as well as ZR, Ado, Ade and Z9G as the major
metabolites. When \(^{3}\text{H}\)-Z was fed to derooted \textit{Zea mays} seedlings the principal metabolites were Ado-5\'-P, Ado and Ade (Parker and Letham, 1974). In studies with kernels at commercial maturity, Summons \textit{et al.} (1980), using \(^{3}\text{H}\)-Z and \(^{3}\text{H}\)-ZR, also found that the metabolism of exogenous cytokinin consisted mainly of sidechain cleavage, though small amounts of the O-glucosides, DZ and DZR were also observed. On the basis of these studies and on the results of the above experiments, it would seem that 9-glucosylation is a principal form of Z and ZR metabolism only in the root tissue in \textit{Zea mays} whereas sidechain cleavage by cytokinin oxidase appears to occur actively throughout the sweet corn plant.

Over a period of 3 days from imbibition, a significant amount of \(^{3}\text{H}\)-ZR has been shown to move from the embryo (site of application) to the endosperm. In a complementary experiment, comparatively little movement from the endosperm (site of application) to the embryo occurred and hence the translocation is strongly polarised. There is evidence (Section 1.7.2) that cytokinin moves from the embryo (a probable site of biosynthesis) of dicotyledonous seeds to the cotyledon to promote mobilisation of storage reserves. The present study seems to indicate that an analogous movement from embryo to endosperm may also occur in the monocotyledonous seeds. The application of \(^{3}\text{H}\)-ZR to the tip of 3 day-old radicles (also a likely site of cytokinin biosynthesis, see Sections 1.4.3 and 4.3) also resulted in transportation of \(^{3}\text{H}\)-ZR, this time largely to the embryo-remnant, shoot and adventitious roots, but much of the applied radioactivity remained localised in the radicle. It may be significant that on a fresh weight basis the total radioactivity, and that due to cytokinins (mainly ZR and Z nucleotide) in the shoot, exceeded that in all other regions, the radicle excepted. Like the oat
coleoptile (see Section 1.7.4), the maize shoot may have a requirement for root-derived cytokinin. If the movement and metabolism of exogenous ZR reflects that of the endogenous cytokinin, then this suggests that cytokinins may play a role in the germination of maize seed and in the early development of the seeding.

Significant levels of OGZ, OGDZ and OGDZR were found in the dry maize kernels, and in the light of Smith and van Staden's (1978, 1979) postulated movement of the 0-glucosides and their reported effects on excised embryos, it would be of interest to study the movement and hydrolysis of these compounds in the germinating seed. Do they also move between the embryo and endosperm? DZR also occurred at significant levels in the dry seed and given the fact that it occurred at much higher levels than ZR and also that it is resistant to the sidechain cleaving activities of cytokinin oxidase, its presence may be of much more significance to the germinating seed than ZR. Thus, a similar series of studies on its translocation and metabolism, as has been described in this thesis for ZR, would be warranted.

An analogous series of experiments in which $^{14}$C-Z was supplied to germinating maize kernels has been reported by van Staden (1981a, 1981b). However, the nucleotide fractions from ion-exchange chromatography were discarded and no rigorous identification of the metabolites was carried out. Despite this, van Staden claimed that the applied Z was rapidly metabolised to a compound that co-eluted with a KMnO$_4$ oxidation product of zeatin, later identified as 6-(2,3,4-trihydroxy-3-methylbutylamino)purine (di-OH-Z) (van Staden and Drewes, 1982). In addition, van Staden concluded that there was no clear evidence for cytokinin interactions between the embryo and endosperm during the early stages of germination. This is in stark contrast with
the results reported here for applications of $[^{3}\text{H}]-\text{ZR}$. These discrepancies might be explainable as a function of feeding ZR rather than Z, but in a brief experiment in which $[^{14}\text{C}]-\text{Z}$ was supplied to germinating seed neither di-0H-Z or NPG were identified as metabolites. It should be pointed out here that these compounds can be readily separated from other known metabolites of Z and ZR by TLC and HPLC. The di-0H-Z isolated by van Staden may be an artifact of the extraction and Dowex 50 chromatography that was utilized (see discussion in Section 2.2.2).

6.3 Inhibition of N-glucosylation in maize and radish tissue

The formation of the 7- or 9-glucosides is an important form of cytokinin inactivation in both radish and maize tissues, respectively. Hence inhibitors of the responsible enzymes are of considerable interest, since they may provide a means of elevating the levels of both endogenous and exogenous cytokinins and thus influence plant development. Based on the observations that theophylline, 1-methyl-3-isobutylxanthine and 2-(2-hydroxyethylamino)-9-methyl-6-benzylaminopurine had all been demonstrated to inhibit N-glucosylation to varying degrees in radish cotyledons, it was decided to synthesise a series of 3-substituted xanthines, a 2-substituted BAP, and a BAP analogue (24, 7-benzylaminooxazolo[5,4-d]pyrimidine) with the objective of finding new more potent inhibitors.

Major difficulties were experienced with the synthesis of 24 and a number of routes were attempted before 24 was finally made, albeit in poor yield, by direct alkylation of 7-aminoazolo[5,4-d]pyrimidine in HMPA with benzyl bromide. An alternative approach to the synthesis of 24 might be based on work described by Ishidate and Yuki (1960) and by
Patil and Townsend (1970) in which 7-hydroxy-2-methyloxazolo[5,4-d]-pyrimidine was directly synthesised by ring annulation of 5-amino-4,6-dihydroxypyrimidine. This compound was then converted to the 7-chloro derivative, and in turn, subjected to a nucleophilic displacement of the 7-chloro group with either piperidine or morpholine to produce the N-substituted 7-amino-2-methyloxazolopyrimidine (Scheme 6.1).

$$\text{Scheme 6.1: Synthesis of } N^7\text{-substituted 7-amino-2-oxazolo[5,4-d]pyrimidine.}$$

However, Patil and Townsend claimed that the 2-substituent did not significantly increase the stability of the oxazole ring, so it is possible that the use of other reagents (e.g., formamidine or formic acid) in the initial ring closure would produce 7-hydroxyoxazolo[5,4-d]pyrimidine which could then be converted to 24 and other derivatives.

Alkylations of 1-methylxanthine yielded mixtures of products with substituents at either 3- or 7-positions. It is possible to direct alkylation to the 3-position by means of a protecting group at the 7-position (Hu et al., 1980), however, the mixtures of products were readily separated by HPLC and it was thought possible that inhibitory activity might be obtained from both 3- and 7-substituted 1-methylxanthine.
The 3-substituted 1,7-dimethylxanthines could be prepared readily using the improved alkylation conditions devised in Chapter 3.

The metabolism of BAP in both maize and radish leaves and radish cotyledons is very simple as it consists principally of N-glucosylation and to a much lesser degree, the interconversion of the base, riboside and nucleotide forms. Consequently, the potency of the synthetic compounds in preventing the N-glucosylation of BAP was tested in these tissues. It was apparent from the test results that the structural requirements for inhibitors in the two species were quite different. The xanthine derivative 3 and the BAP derivative 27, which are known to inhibit N-glucosylation in radish cotyledons, had little effect in the maize leaf system. Similarly, compounds 7, 10 and 13 which were observed in the present study to suppress N-glucosylation markedly in radish, were ineffective inhibitors in maize. Conversely, compound 24 is an effective inhibitor of 9-glucosylation in maize and oat leaves (S. Singh, personal communication), but has no activity in radish.

In addition to being an inhibitor of 9-glucosylation, 24 could have anticytokinin activity or it may be a cytokinin itself. If 24 is active as a cytokinin, then this would mean that 9-ribosylation is not a prerequisite for cytokinin action (see Laloue and Pethe, 1982, 1985).

While the inhibitors synthesised in this study were intended primarily as inhibitors of glucosylation in plant tissues, it is likely that they also inhibit other enzymes of cytokinin metabolism. This view is substantiated by the observation that 17 and 18 elevated the level of BAP in maize and radish leaves, respectively but did not inhibit glucosylation. Consequently, the inhibitors have now also been tested for their ability to inhibit the conversion of BAP to the inactive alanine conjugate, the principal form of metabolism, in soybean
leaves. Compound 10 was found to be particularly effective, and elevated the level of free BAP markedly at 0.2 mM (Zhang Ren, personal communication). BAP retards the senescence of intact soybean leaves and 10 was found to enhance this effect at 0.1 mM, presumably by suppressing alanine conjugation (see Fig. 6.1). By itself, 10 had negligible senescence-retarding activity.

Much effort has been directed to the synthesis of plant hormone analogues with enhanced growth promoting activity. It may now be more profitable to devote effort to designing and synthesising inhibitors of hormone inactivation. Such inhibitors would potentiate the activity of known hormones and may eventually contribute to our ability to modify plant development in desirable ways.

It is possible that the compounds synthesised above may have, in common with the methylxanthines, cytokinins and related compounds, the ability to inhibit the action of mammalian cyclic adenosine-3',5'-monophosphate (cAMP) phosphodiesterase (Chasin and Harris, 1976; Amer and Kreighbaum, 1975; Johnson et al., 1974; Hecht et al., 1974; Appleman et al., 1973). This enzyme is responsible for the conversion of cAMP to adenosine 5'-monophosphate (5'-AMP) so that any inhibition of its action leads to an accumulation of cAMP. This cyclic nucleotide is reported to be an intracellular mediator for the action of a variety of animal hormones (see for example, Garst et al., 1977; Peytreman et al., 1973; Ashcroft et al., 1972; and Beavo et al., 1970, 1971). The hormone, via its extracellular receptor site, stimulates the activity of the membrane bound enzyme, adenylate cyclase, and thus raises the level of intracellular cAMP. The cAMP is subsequently removed by the action of a phosphodiesterase, lowering the concentration of cAMP. The metabolic action of a given quantity of hormone can thus be multiplied several
Figure 6.1: The effect of BAP and 10 on the senescence of soybean leaf laminae. Areas within lanolin rings (diameter 1.4 cm) on the surface of an intact leaf of a soybean plant (Glycine max L. cv. Bragg) were painted with BAP and 10 as follows:—

1: an aqueous solution (5 µl) of BAP (0.2mM) and 10 (0.01mM) in 0.05% Tween and 0.5% DMSO was applied.
2 and 3: an aqueous solution of BAP alone (as per zone 1) was applied.
4: an aqueous solution of 10 alone (as per zone 1) was applied.

The plants were at the stage of late podfill.

(Photo courtesy of Mr Zhang Ren.)
fold in the presence of a phosphodiesterase inhibitor due to the non-
removal of the accumulated intracellular cAMP.

Caffeine and other related xanthine bases containing a 3-
methylpyrimidine-2,4-dione moiety, constitute a well known group of
inhibitors of cytokinesis, rapidly inducing cell plate breakdown in the
final stages of mitosis, although high concentrations are required
(Gunning, 1982). The mode of action has not been elucidated, though a
role for intercellular calcium seems to be implicated by the
demonstrated antagonism of calcium with caffeine (Becerra and Lopez-
Saez, 1978). Hence, the derivatives of 1,7-dimethylxanthine (6 - 16)
and 1-methylxanthine (17 - 19) merit testing as inhibitors of cell plate
formation.

In summary, the xanthine, BAP and oxazolopyrimidine derivatives may
ultimately prove to be useful biochemical and histological tools with
applications in both plant and animal studies.
REFERENCES


Summons, R.E., MacLeod, J.K., Parker, C.W. and Letham, D.S. (1977) FEBS Letters 82, 211.


