CYTOKININS AND THE EXPANSION
OF RADISH COTYLEDONS

This thesis presents an account of research
carried out by myself in the Department of
Developmental Biology in the Australian National
University. Collaboration and assistance from
my colleagues are gratefully acknowledged.

MARGARET E. GORDON

June, 1973
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 is recorded under acknowledgements and in the text.

Margaret Gordon
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Theophylline was found to promote cotyledon expansion and appeared to act synergistically with cytokinin. However, cAMP was inactive in the presence of theophylline, a known inhibitor of cAMP phosphodiesterase.
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Chapter 5 - BAP had no significant effect on DNA or on RNA species during the lag period and hence all of the effects detected in this chapter could be indirect (i.e. consequences of BAP-induced growth. The total DNA and RNA contents per cotyledon were moderately stimulated but only after about 18 hours. Similarly, in cotyledons supplied with labelled uridine and thymidine, specific activity of the nucleic acids was not significantly stimulated by BAP until very late (i.e. at 30 hours for DNA and at 54 hours for RNA) when increases (< 20%) were observed. The substantial increase in RNA content prior to increase in specific activity suggested an inhibition of RNA degradation by cytokinin. Dual isotope labelling combined with RNA fractionation by polyacrylamide gel electrophoresis showed that within 18 hours, BAP caused a small promotion of uridine incorporation into RNA species with the electrophoretic mobility of cytoplasmic ribosomal RNA. When maturation of ribosomal RNA was inhibited by 5-FU-treatment of the cotyledons, this promotion was enhanced (relative to the control state), but not sufficiently markedly to suggest strongly that BAP stimulated incorporation of uridine into polydisperse RNA: BAP may have enhanced the accumulation of breakdown products of 5-FU-containing labelled ribosomal RNA.

Chapter 6 - Within 30 to 40 hours, the cytokinins
BAP and kinetin dramatically enhanced the total ribosome yield per unit weight of radish cotyledon tissue, the effect on 80S ribosomes being most marked. There was no obvious effect on the relative proportions of ribosomes and polysomes or of free and membrane-bound ribosomes. During the lag period, ribosome yields from both control and cytokinin-treated cotyledons were below detectable levels. In addition, mannitol depressed both growth and ribosome yield to the same degree. Hence, the enhanced ribosome yield appeared to be an indirect effect of BAP and probably a consequence of BAP-induced growth. Since the effect of BAP on ribosome yield was not reflected in enhanced levels of cytoplasmic ribosomal RNA, while recently labelled ribosomes were found to be more readily recovered from cytokinin-treated tissue than from control tissue, it is suggested that BAP merely enhances ribosome recovery from radish cotyledons. Ribosome yield is thus an unreliable indication of ribosome level in plant tissue.

Chapter 7 - The incorporation (\% uptake) of labelled amino acids into protein was enhanced by BAP during the lag period. The effect was marked within 12 hours in the case of both leucine and methionine incorporation and was not accompanied by any detectable change in protein content per cotyledon or in amino acid pool size. Hence, a stimulation of protein turnover is one likely interpretation. Inhibition of RNA synthesis (by about 50\%) by act. D had no effect on BAP-enhanced amino acid incorporation, although BAP-induced growth was completely eliminated by act. D. These results support the proposition that the effect of BAP on protein metabolism is apparently not a consequence of increased growth and may not depend on transcription.

Protein phosphorylation was not stimulated by BAP until after the lag period and the enhancement of amino acid incorporation into protein, and hence could merely reflect the increased supply of protein substrate. However, a stimulation of protein methylation accompanied and could have preceded BAP-induced methionine incorporation into protein within the lag period. A 25\% increase in methylation was observed after 5 hours.
It is suggested that the results presented in this part of the thesis support the view that cytokinins may act at the post-transcriptional level to induce expansion of radish cotyledons.

PART II : Chapter 8 - Metabolism and translocation of cytokinins

(a) Metabolism and translocation of zeatin - After the roots of intact radish seedlings had taken up \([G^{-3}H]\)zeatin for 1 hour, the seedlings were transferred to unlabelled nutrient and extracted at intervals. After 23 hours in the unlabelled medium, about 4\% of the extracted radioactivity was recovered from the cotyledon laminae and 6\% from the hypocotyls. Per unit weight of tissue, the radioactivity extracted from the root was about 40 times that recovered from any other region. Zeatin was rapidly metabolized by the root tissue, and, 9 hours after transfer of the seedlings to unlabelled medium, was not detectable. Zeatin was found in the cotyledons but zeatin riboside was the only significant source of radioactivity in the hypocotyl bleeding sap and therefore appeared to be the form in which the hormone was translocated. Of the other metabolites detected, zeatin riboside 5'-monophosphate and adenosine 5'-monophosphate were the principal ones in hypocotyl tissue, while raphanatin (7-glucosylzeatin) was dominant in the roots and cotyledons. The identity and possible inter-relationships of several of the zeatin metabolites are discussed.

(b) Metabolism of BAP - Excised cotyledons were labelled with \([G^{-3}H]\)BAP for up to 5 hours and then transferred to unlabelled medium for various intervals, after which they were extracted. BAP was rapidly metabolised by the tissue to two main products that were not the riboside and nucleotide of BAP, nor common purines. The major metabolite(s) accounted for 20\% of the extracted radioactivity after 1 hour of BAP incubation, and for 95\% when BAP had disappeared (within 9 hours after the labelling period). These products appeared to be very stable, and were chromatographically coincident with cytokinin activity detected with the radish cotyledon
bioassay. These metabolites were also formed when BAP was supplied to 9-day-old de-rooted seedlings in the transpiration stream. Mass spectrometry, u.v. spectroscopy and chemical degradation of the purified metabolites indicated that they were 6-benzylamino-7-glucosylpurine and 6-benzylamino-9-glucosylpurine. Although BAP riboside and ribotide are principal metabolites of BAP in some tissues, they were not detected in radish seedlings.
CHAPTER 1: INTRODUCTION

In higher organisms, the multicellular state creates a need for communication between cells for the purpose of co-ordinating growth and movement. In animals this function is performed by nerves and hormones. Plants have no nervous system, but they do have a well-developed system of hormones. These are now known to mediate a wide range of stimuli, including many that in animals are carried by nerves. Indeed, as our knowledge of plant hormone activities increases, it is becoming clear that all co-ordinating mechanisms in plants are hormone dependent.

The hormones of plants, like those of animals, are organic compounds which influence metabolism at sites remote from their site of production, and are active in minute amounts. But plant hormones, or "phytohormones," appear to be less specific than animal hormones both in the type of tissue upon which they can act and in the character of the response evoked in the target tissue. The effects may vary according to the relative amounts of other hormones present, for synergism and antagonism between different types of phytohormones are important aspects of a plant's control mechanism.

Five types are known at present. These are the auxins, gibberellins, cytokinins, abscisic acid (dormin) and ethylene. The first three are the principal growth promoters; auxins and gibberellins are distinguished by their ability to evoke certain growth responses that are due to the promotion of cell elongation, while cytokinins are characterized by their ability to induce cell division, in the presence of auxins, in certain tissue cultures (Chory et al., 1969). The spectra of responses to these three types show considerable overlap. Abscisic acid is a hormonal inhibitor which limits growth and is involved in the metabolic changes associated with senescence, abscission and dormancy. The effects of ethylene include the suppression of lateral bud growth, and the promotion of metabolic changes associated with ripening,
CHAPTER 1

In higher organisms, the multicellular state creates a need for communication between cells for the purpose of co-ordinating growth and movement. In animals this function is performed by nerves and hormones. Plants have no nervous system, but they do have a well developed system of hormones. These are now known to mediate a wide range of stimuli, including many that in animals are carried by nerves. Indeed, as our knowledge of plant hormone activities increases, it is becoming clear that all co-ordinating mechanisms in plants are hormone dependent.

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flowering, lateral cell expansion, and possibly abscission. (See Letham, 1969; Galston and Davies, 1969, 1970; Wilkins, 1969, for details of structure, effects and interactions of plant hormones).

The research to be discussed in this thesis is concerned with the cytokinins.

1. DISCOVERY OF CYTOKININS

As a recognized class of phytohormones the cytokinins have had a relatively brief history. The presence of cell division-inducing substances in plant extracts was first demonstrated by Haberlandt in 1913 and 1921 (see review by Fox, 1969), but prior to the discovery of cytokinins, instances of the induction of cell division tended to be attributed entirely to the action of auxin (see for example review by Thimann, 1969). The realization that auxin alone could not induce cell division came as a result of the tissue culture experiments of Skoog and co-workers at the University of Wisconsin. While investigating the chemical control of differentiation of plant tissues cultured heterotrophically, Skoog and Tsui (1948) observed that small segments of Nicotiana tabaccum cv. Wisconsin No. 38 required auxin for growth. If the pith of such segments was cultured in the absence of vascular and cortical tissue, the tissue responded to auxin by enormous cell enlargement (Jablonski and Skoog, 1954) but no cell division occurred unless the vascular portions were placed back in contact with the pith. The resulting search for a cell division factor in tobacco stems failed, but encouraged an extension of the search to other likely sources. Activity was detected in coconut milk, malt extract, yeast extract and autoclaved herring sperm DNA. From this DNA, the first cytokinin was isolated in crystalline form in 1954 (Miller et al., 1955b). This substance was shown to be 6-furfurylaminopurine (Miller et al., 1955a) which must have formed by the molecular re-arrangement of deoxyadenosine freed from DNA during autoclaving (Hall and deRopp, 1955).
The discovery of kinetin stimulated much new research: the physiological and biochemical effects of the compound were extensively studied, numerous analogues were synthesized, many of which proved to be highly active, and kinetin-like activity was demonstrated in diverse plant extracts, particularly rapidly dividing tissues such as embryos, fruitlets, cambium and tumors.

It was some years, however, before a naturally occurring cytokinin was purified, and this was first achieved from immature corn grains. From this source, Miller (1961a) partially purified a 6-(substituted amino)purine, and Letham (1963), independently and by use of different methods, isolated a crystalline cytokinin termed zeatin. This was identified as 6-(4-hydroxy-3-methylbut-trans-2-enyl)amino-purine (Letham et al., 1964, 1967). Subsequently Miller's factor was shown to be identical to zeatin (Letham and Miller, 1965). Corn extracts were soon shown to contain other cytokinins, possibly a nucleoside and a nucleotide (Miller, 1965), and zeatin riboside and zeatin riboside 5'-phosphate were isolated in crystalline form from this source (Letham, 1966a, 1966b, 1968). Zeatin and related compounds are now known to occur in plants of diverse genera both in the free form and also as components of tRNA. Naturally occurring cytokinins that have been isolated in a state of complete purity and unequivocally identified are listed in Table 1-1. Cytokinins with the chromatographic and u.v. spectral properties of zeatin have been purified from plum fruitlets (Letham, 1964, 1966c), immature sunflower fruits (Miller and Witham, 1964), sunflower leaves and root exudate (Klämbt, 1968) and pumpkin seeds (Gupta and Maheshwari, 1970), while zeatin or zeatin riboside have been found to co-chromatograph with the active compounds in numerous plant extracts. Gas-liquid chromatography, t.l.c. and u.v. spectra also indicate that 6-(3-methylbut-2-enylamino)purine occurs in culture filtrates of Agrobacterium tumefaciens, the bacterium causing crown gall tumors (Upper et al., 1970).

Although considerable information is thus available regarding the identity of naturally occurring
Table 1-1. Naturally occurring cytokinins of known structure

<table>
<thead>
<tr>
<th>Cytokinin</th>
<th>Source(s) from which isolated in state of purity</th>
<th>References</th>
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<tbody>
<tr>
<td>6-(4-hydroxy-3-methylbut-trans-2-enylamino)purine (zeatin)</td>
<td>Zea mays kernels</td>
<td>Letham (1966c)</td>
</tr>
<tr>
<td></td>
<td>Rhizopogon roseolus culture filtrates</td>
<td>Miller (1967)</td>
</tr>
<tr>
<td>9-β-D-ribofuranosylzeatin (zeatin riboside)</td>
<td>Zea mays kernels</td>
<td>Letham (1968)</td>
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<tr>
<td></td>
<td>coconut milk</td>
<td>Letham (1968)</td>
</tr>
<tr>
<td></td>
<td>Rhizopogon roseolus culture filtrates</td>
<td>Miller (1967)</td>
</tr>
<tr>
<td></td>
<td>Cichorium intybus roots</td>
<td>Bui-Dang-Ha and Nitsch (1970)</td>
</tr>
<tr>
<td></td>
<td>sRNA from pea shoots</td>
<td>Vreman et al. (1972)</td>
</tr>
<tr>
<td>zeatin riboside 5'-phosphate</td>
<td>Zea mays kernels</td>
<td>Letham (1968)</td>
</tr>
<tr>
<td>6-(3-methylbut-2-enylamino)purine</td>
<td>Corynebacterium fascians</td>
<td>Helgeson and Leonard (1966)</td>
</tr>
<tr>
<td>6-(3-methylbut-2-enylamino)-9-β-D-ribofuranosylpurine</td>
<td>tRNA of E. coli, yeast, animal tissues; sRNA of several higher plants</td>
<td>See Skoog and Armstrong (1970)</td>
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<tr>
<td></td>
<td>tobacco pith callus (autonomous strain)</td>
<td>Gauss et al. (1971)</td>
</tr>
<tr>
<td>6-(4-hydroxy-3-methylbutylamino)purine (dihydrozeatin)</td>
<td>immature lupin seeds</td>
<td>Dyson and Hall (1972)</td>
</tr>
<tr>
<td>cis-zeatin riboside</td>
<td>sRNA of sweet corn</td>
<td>Koshimizu et al. (1967)</td>
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<tr>
<td></td>
<td>sRNA of wheat germ</td>
<td>Hall et al. (1967)</td>
</tr>
<tr>
<td></td>
<td>sRNA of tobacco callus</td>
<td>Playtis and Leonard (1971)</td>
</tr>
<tr>
<td></td>
<td>sRNA of pea shoots</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>sRNA of pea roots</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>tRNA of E. coli</td>
<td>Gauss et al. (1971)</td>
</tr>
<tr>
<td></td>
<td>sRNA of S. epidermidis</td>
<td>Armstrong et al. (1970)</td>
</tr>
<tr>
<td>6-(4-hydroxy-3-methylbut-2-enylamino)-2-methylthio-9-β-D-ribofuranosylpurine</td>
<td>sRNA of wheat germ</td>
<td>Burrows et al. (1970)</td>
</tr>
<tr>
<td></td>
<td>sRNA of tobacco callus</td>
<td>Burrows et al. (1971)</td>
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<td>sRNA of pea shoots</td>
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cytokinins, our knowledge of the metabolism and translocation of exogenously supplied cytokinins (section 8A) is very limited.

2. STRUCTURAL REQUIREMENTS FOR CYTOKININ ACTIVITY

All known cytokinins of high activity, whether natural or synthetic, are 6-substituted adenine derivatives. But what determines whether such a derivative is active or inactive? The synthesis and subsequent bioassay of some hundreds of synthetic kinetin-like compounds has revealed a number of relationships between molecular structure and cytokinin activity. As these have been regularly reviewed (Strong, 1958; Kuraishi, 1959; Miller, 1961b; Letham, 1967; Skoog et al., 1967; Skoog and Armstrong, 1970), only the principal generalizations that have emerged will be listed here. These are as follows:

1. No mono-substituted adenines other than N6-derivatives are known to possess cytokinin activity.

2. A variety of different side chains confer activity on adenine. Side chain features favouring activity include planarity, unsaturation, a length of five carbons for aliphatic side chains, and the absence of strongly polar groups (e.g. -COOH).

3. Generally, modifications within the purine ring greatly depress or eliminate activity.

4. Substitutions at positions 1 and 3 of 6-(mono-substituted amino)purines eliminate activity. Position 2 substitutions have a less drastic effect, although they usually reduce activity, and naturally occurring 2-methylthio cytokinins are known (see Table 1-1). Information is lacking on second substitutions at positions 7 and 8. Substitution at the 9 position, however, usually reduces activity. Activity in 9-substituted compounds is not restricted to nucleosides and nucleotides.

While the above observations suggest that adenine
is a vital part of the cytokinin molecule, kinetin-like activity has been claimed for several non-purine compounds, notably phenyl derivatives of urea (Shantz and Steward, 1955; Bruce and Zwar, 1966) and the more purine-like 8-aza derivatives of kinetin, benzyladenine and isopentenyladenine. Until more is known of the metabolism of these substances it remains uncertain whether they are active per se, or whether they serve as side chain donors for adenine and thus as cytokinin precursors (Miller, 1961b, Skoog and Armstrong, 1970). The recent work of Dyson et al. (1972) suggests that urea cytokinins are not active per se, but are metabolised to active compounds.

3. GROWTH RESPONSES AND CERTAIN PHYSIOLOGICAL EFFECTS EVOKED BY CYTOKININS

When the biological activity of kinetin and its active synthetic analogues was explored, it soon became clear that these compounds produced a variety of physiological effects. These included the breaking of dormancy of seeds and buds, lateral bud development, the enlargement of leaves of dicotyledonous plants, including the cotyledons, the formation of parthenocarpic fruit, regulation of phloem transport and mobilization of metabolites, the delay of senescence in flowers and leaves, and the control of organogenesis in certain tissue cultures, such as tobacco pith callus (Skoog and Miller, 1957). Such effects have been reproduced using natural cytokinins. Hence synthetic and natural analogues of kinetin apparently fulfil the same biochemical role. The ability of cytokinins to restore meristematic activity to mature non-dividing cells, and to evoke cell differentiation, has indicated that cytokinins are involved in the regulation of gene expression.

Since this thesis is biochemically oriented, no attempt is made to discuss in detail the many growth responses evoked by cytokinins (refer, for example, to Srivastava, 1967; Fox, 1969; Kende, 1971). However, biochemical effects and possible mechanisms of action are
discussed in some detail below, following a review of the mechanisms which are currently thought to be involved in the control of gene expression.

4. MECHANISMS OF CONTROL OF ENZYME SYNTHESIS

Today gene expression is generally equated with protein formation and function. There are many possible points of control of protein synthesis and Varner (1971) has suggested 21. However points at which regulation (not necessarily hormonal) actually appear to operate in bacteria and eukaryotic cells are discussed below.

4-1 Availability of the DNA template

In bacteria, enzyme production is regulated at the level of mRNA synthesis by substrates upon which enzymes act. A highly probable mechanism for this control is the classic genetic regulation model of Jacob and Monod involving protein repressor molecules which bind to DNA to prevent transcription. In nucleated cells large portions of the chromosomal DNA are complexed with histones which appear to prevent transcription. According to Bonner and co-workers, chromosomal RNA, a species of RNA with unique properties, confers specificity on DNA histone interactions (Bonner et al., 1968). However such concepts are not generally accepted and the significance of chromosomal RNA has been seriously questioned (Heyden and Zachau, 1971; Szeszah and Pihl, 1972). Histones are acetylated, phosphorylated and methylated in vivo and in some cases such modifications can be induced by animal hormones and correlated with increased RNA synthesis. However the precise mechanisms of specific histone control of transcription are obscure at present (see De Lange and Smith, 1971).

4-2 Transcription of available DNA template

Several protein factors, notably sigma, psi, factor M and factor H markedly stimulate transcription by E. coli RNA polymerase in vitro. Sigma and factor M promote initiation at specific sites on the DNA template; psi factor
very markedly promotes ribosomal RNA synthesis. The bacterial rho factor regulates termination of RNA chains (for a discussion of these factors see Polya, 1973). Catabolite repression of genes involved in sugar metabolism, such as those of the galactose, lactose and arabinose operons, is associated with a decrease in the cAMP concentration in bacterial cells; repression of these operons is relieved by cAMP (Pastan and Perlman, 1971). A protein called catabolite gene-activating protein (CAP) binds cAMP, then attaches to lac DNA, stimulates β-galactosidase synthesis (Zubay et al., 1970) and is requisite for in vitro synthesis of lac-specific mRNA (De Crombrugghe et al., 1971a, b, c). cAMP-dependent protein kinases stimulate E. coli RNA polymerase activity on T4 DNA by phosphorylation of the sigma factor (Martelo et al., 1970). This raises the possibility of a more general control of transcription by cAMP in bacterial cells. The above observations indicate that cAMP and protein factors are intimately involved in control of prokaryote transcription.

Acidic nuclear proteins (ANPs), derived from chromatin of animal cells, enhance rates of transcription in vitro. Thus ANPs from rat kidney increase the rate of transcription of rat liver and rat kidney DNA by E. coli RNA polymerase by about 100% (Teng et al., 1970). It has been suggested that ANPs are eukaryote analogues of the bacterial sigma factors (Teng et al., 1971). Many ANPs are phosphorylated and phosphorylation increases at times of gene activation by drugs and hormones (Kleinsmith et al., 1966; Rudden and Rainey, 1971). These latter workers suggest that some animal hormones and inducing agents bind to ANPs thereby making them substrates for protein kinases. The phosphorylated ANPs bind to DNA and promote RNA polymerase binding and transcription. In the case of progesterone, it has recently been demonstrated that a hormone-cytoplasmic receptor protein complex binds to ANPs of oviduct cells (O'Malley et al., 1972). Protein factors which stimulate in vitro RNA synthesis by eukaryote RNA polymerase have now been purified from several sources, e.g. calf thymus (Stein and Hauson, 1970), and
coconut milk nuclei (Mondal et al., 1970). Hence protein factors which activate transcription appear to be of significance in both prokaryotes and eukaryotes.

Although the regulatory role of cAMP in prokaryote transcription is firmly established, evidence for a similar role in eukaryotes is indirect and fragmentary. However cAMP does appear to promote either transcription of specific messenger or its transport from nucleus to cytoplasm (Jost and Rickenberg, 1971).

4-3 mRNA release from nucleus and activation

Some RNA species are restricted to the nucleus in normal animal tissues but are transported to the cytoplasm in regenerating tissue and in tumour cells (Drews et al., 1968; Shearer and Smuckler, 1972). These observations indicate that selective transport of RNA to the cytoplasm may be associated with regulation of gene expression. It is relevant to note that poly A sequences in mRNA may be associated with transport across the nuclear membrane (see Naora, 1973 and references cited therein).

Some mRNA molecules of eukaryotes appear to exist in stable forms which may require activation before they can be translated. Acetabularia contains mRNA species which can remain in the cytoplasm of enucleated cells for at least 40 days before being translated (Harris, 1968). In wheat embryos, protein synthesis commences immediately after imbibition and appears to be directed by mRNA preserved in the dry embryo (Chen and Osborne, 1970). mRNA which is formed in the cytoplasm of sea urchin eggs during oogenesis is not translated until after fertilization. Evidence that such mRNA, which has been called "maternal" or "masked" messenger, can exist in the form of ribonucleoprotein particles ("informosomes") has been presented (Neyfakh, 1971; Glisin and Savic, 1971). There is evidence that some of these "masked" messengers code for microtubule proteins (Raff et al., 1972). Control of the unmasking process may prove to be an important regulatory mechanism during development in eukaryotes.
An intensive study of steroid-hormone induction of synthesis of tyrosine aminotransferase in hepatoma cell cultures, led Tomkins and co-workers to propose regulation by a labile post-transcriptional repressor. In their model, the repressor binds to the mRNA preventing translation but allowing mRNA degradation. The hormone functioned by blocking the action or synthesis of the repressor. (Tomkins et al., 1969; Levinson et al., 1971; Martin, 1971). In this system, actinomycin D can cause superinduction of the enzyme, a paradoxical effect. There are many other examples of such paradoxical effects of actinomycin D on protein synthesis (see references listed by Martin, 1971). Hence regulation by post-transcriptional repressors may be a general mechanism in eukaryotes. Very recently Gross and Rabinovitz (1972) have presented evidence that globin synthesis is controlled by a translational repressor inactivated by hemin.

4-4 Translational processes

To explain polar mutations in bacteria, Ames and Hartman (1963) proposed that certain tRNA species modulated translation by their availability or by their ability to dissociate the ribosome from the mRNA. Polar mutations can now be accounted for in other ways, but regulation of translation by tRNA isoacceptors remains a likely possibility. Anderson (1969) presented evidence that AGA and AGG are regulatory codons in *E. coli* because the corresponding tRNA is present in suboptimal amounts limiting the rate of translation. Changes in the relative amounts of isoaccepting tRNA species could influence mRNA translation.

tRNA molecules contain substantially modified bases (including cytokinins) adjacent to the 3' side of the anticodon and also many methylated bases. Methylation of tRNA and specific modification of the base adjacent to the anticodon can markedly affect the functional ability of tRNA *in vitro* (Letham, 1973). These structural modifications are likely translational regulatory mechanisms.

In eukaryotic cells, phosphorylation of ribosomal proteins now appears to have a regulatory role in ribosome
function (Li and Amos, 1971; Monier et al., 1972). Phosphorylation appears to inhibit incorporation of amino acids into protein. Evidence for a control mechanism in gene expression operating at the termination step in translation in neonatal liver has been obtained by Oliver and co-workers. In this mechanism cAMP induces release of enzyme from polysomes and a protein factor is also involved in the control (Oliver, 1972).

An important integral part of the regulatory mechanisms in prokaryotes is the rapid turnover of mRNA templates. In eukaryotic cells, mRNA molecules are frequently of considerable stability, but removal of an inducer can still cause rapid cessation of enzyme synthesis. Hence regulation of transcription cannot be entirely responsible for control of eukaryote gene expression. Post-transcriptional control is probably a general phenomenon of nucleated cells. A number of post-transcriptional control mechanisms have been mentioned above and some of these appear to be important in the action of animal hormones and cAMP.

It is also relevant to mention that both insulin and growth hormone have for some years been known to exert their promotion of protein synthesis in the absence of RNA synthesis (see Korner, 1970). Although animal hormones without doubt influence translational processes, in some cases probably through the medium cAMP, some steroid hormones probably do regulate transcription and bind to chromatin after first forming a complex with a cytoplasmic receptor protein (Steggles et al., 1971; O'Malley et al., 1972). It would not be surprising if phytohormones also regulated both transcriptional and translational processes. This is discussed below.
5. BIOCHEMICAL EFFECTS OF CYTOKININS, AND POSSIBLE MECHANISMS OF ACTION

5-1 Enzyme activities in tissues and the induction of certain metabolites

Hormonal effects on the activity of particular enzymes have been investigated in an attempt to define the biochemical basis of hormone action. There are now numerous reports that cytokinins modify enzyme activity in plant tissues. In some cases the action is inhibitory. Thus, in excised leaf tissues cytokinin application lowers the activity of certain hydrolytic enzymes, such as proteases (see review by Kende, 1971) and nucleases (see Kende, 1971; Wyen et al., 1972). The ribonuclease inhibition observed by Wyen and co-workers was detectable after only 2.5 hours of cytokinin treatment, but not all the nucleolytic enzymes were affected. Restriction of cytokinin-induced inhibition to certain nuclease species has also been observed by other workers (see for example Sodek and Wright, 1969; Birmingham and Maclachlan, 1972). Very recently cytokinins have been reported to inhibit the activity of protein kinases in chloroplast-nuclei preparations in vitro (Ralph et al., 1972).

Cytokinins have also been reported to stimulate the activity of a number of enzymes in vivo, for example tyramine methylpherase (Mann et al., 1963; Steinhart et al., 1964), β-1,3-glucan hydrolase (Moore and Stone, 1972), adenine phosphoribosyltransferase (Nicholls and Murray, 1968), IAA-oxidase (Gaspar et al., 1969) and certain of its isoenzymes (Lee, 1971), peroxidase (Gaspar and Xhaufflaire, 1968), amylase (Gepstan and Ilan, 1970), invertase (Rutherford and Bard, 1971), lipase and esterase (Sodek and Wright, 1969), and nitrate reductase (Lips and Roth-Bejerano, 1969; Rijven and Parkash, 1971; Parkash, 1972; Kende et al., 1971). Using excised embryos of Agrostemma githago, Kende and co-workers detected the cytokinin-enhanced nitrate reductase activity within one hour of BAP treatment, and obtained evidence that the cytokinin effect was not mediated through nitrate. Hirschberger et al. (1972) have now shown that the BAP
induction of nitrate reductase in *A. githago* embryos is caused by *de novo* synthesis of the enzyme. Their method, which involved labelling protein with H$_2$18O and subsequent isopycnic density gradient centrifugation, satisfied the criteria of Filner and Varner (1967) for *de novo* protein synthesis. In none of the other reports of stimulation of enzyme activity by cytokinins has an effect on *de novo* synthesis of the enzyme been rigorously proved. In such cases it is not clear, therefore, whether the cytokinins affected enzyme synthesis, enzyme degradation, the activity of the enzyme molecules themselves or, possibly, enzyme release from some lysosome-like body.

The accumulation of various metabolites in response to cytokinins has been reported, and probably reflects changes in enzyme activity. For example, chlorophyll accumulation was stimulated in a green strain of cultured tobacco pith callus (Kaul and Sabharwal, 1971) and pumpkin cotyledons (Narain and Laloraya, 1970) grown in the light. In cucumber cotyledons grown in the dark, the effect of cytokinin treatment on chlorophyll formation upon subsequent transfer to the light appeared to depend on the length of the dark pre-incubation time with cytokinin. A long (14-hour) pre-incubation enhanced chlorophyll levels within 30 minutes of exposure to light (Fletcher and McCullagh, 1971), while a short, half-hour pre-incubation was inhibitory (Narain and Laloraya, 1970). A cytokinin-induced promotion of amaranthin in *Amaranthus tricolor* seedlings (Giudici de Nicola et al., 1972) and of deoxyisoflavone in soybean callus (Miller, 1972) occurred only after a lag period of several hours. Both effects were inhibited by actinomycin D, suggesting dependence on RNA synthesis. The deoxyisoflavone accumulation was also sensitive to other inhibitors of RNA and protein synthesis (see Miller, 1972), but did not appear to occur before the cytokinin-induced growth response. Dravnieks et al. (1969) have reported that kinetin induces *de novo* formation of thiamine in tobacco callus cultures (as measured by following the isotope dilution of previously introduced 14C thiamine);
but, again, the accumulation was not observed before growth induction. Hence, these responses, and the enzymic changes which they reflect, could be indirect effects which occur after the initial action of the cytokinin.

It has now been established that cytokinins induce the \textit{de novo} synthesis of at least one enzyme, and probably other enzymes are synthesized in response to cytokinins in a \textit{de novo} manner. It should be emphasized, however, that even where cytokinin induction of \textit{de novo} synthesis has been established, the question still remains: are cytokinins acting on enzyme synthesis at the level of transcription, or is post-transcriptional control involved? Evidence for cytokinin action at transcription, translation and other levels of regulation is discussed below.

5-2 Cytokinins and RNA synthesis

Much attention has been paid to the possibility that phytohormones may act, like classical effectors, by regulating the transcription of DNA, and there are now numerous reports that cytokinins and other plant hormones affect RNA metabolism (see reviews by Oota, 1964; Letham, 1967; Trewavas, 1968; Key, 1969; Kende, 1971). The current status of this research is briefly assessed below.

\textit{Experiments with intact tissues} - There are many reports that cytokinins stimulate the incorporation of labelled precursors into RNA of plant tissues. When the present study was initiated, none of these experiments had assessed the effect in relation to all of the following parameters: (1) the lag period preceding the tissue's physiological response to the cytokinin (e.g. increased growth); (2) the rate of uptake of radioactive RNA precursors by the tissue; (3) the relative rates of synthesis and degradation of RNA species; (4) size or specific activity of precursor pools. Parameters (3) and (4) have proved very difficult to measure in most tissues, and have generally been ignored. Only recently have methods been devised to cope with these parameters. By studying the decline of label in \textit{Lemna minor} RNA, Trewavas (1970) showed conclusively that
cytokinin treatment stimulated both the synthesis and degradation of RNA. However, his data do not indicate whether the RNA turnover was stimulated before the end of the lag period preceding cytokinin-induced growth. By similar methods, and also by equilibrium labelling of precursor pools prior to cytokinin treatment, McCombs and Ralph (1972) have obtained evidence that kinetin increased RNA synthesis in Spirodela within 1 hour. This effect definitely preceded an increase in frond number, but information on frond expansion was not presented. An even more rapid effect has been reported in Lemna in which an increase in the level of RNA and protein in response to cytokinin was detected in 15 minutes (Fankhauser and Erismann, 1969).

In vitro experiments with nuclei and chromatin - In an attempt to show a more direct effect of cytokinins on RNA synthesis, several workers have investigated cytokinin effects on isolated nuclei incubated in the presence of radioactive RNA precursors. Of these studies, the most significant is that of Matthysse and Abrams (1970). These workers found that kinetin could increase the rate of RNA synthesis by isolated nuclei within 10 minutes, and could enhance transcription by purified chromatin and DNA in the presence of a certain protein factor derived from crude chromatin. It was suggested that kinetin might act on the DNA to increase RNA synthesis.

Treatment of cucumber embryos with kinetin for 12 hours increased the capacity of the purified chromatin to support RNA synthesis (Johnson and Purves, 1970).

5-3 Cytokinins and protein metabolism

(a) Incorporation of labelled amino acids into protein

Experiments with intact tissues - There have been many reports that cytokinins stimulate incorporation of labelled amino acids into protein in intact tissues. In most cases the direct relevance of this stimulation to the primary action of cytokinins is very doubtful, since the effect was not shown to be detectable during the lag period. Moreover, a stimulation of amino acid incorporation cannot
be assumed to indicate an increased rate of protein synthesis: the apparent rate of labelled amino acid incorporation is also dependent on the rate of protein degradation and the specific activity of the precursor pools. As in the studies on RNA synthesis in intact tissues, these variables have usually not been measured, and even corrections for uptake have frequently not been applied. The few studies to which these criticisms do not apply are briefly mentioned below.

By following the loss of radioactivity from labelled protein of leaf disks, Kuraishi (1968) and Tavares and Kende (1970) were able to show that cytokinins delayed the decline in the protein content by inhibiting protein degradation and not by promoting protein synthesis. The hypothesis that cytokinins delay senescence by inhibiting the breakdown of protein (and possibly RNA) is supported by the inhibitory action of cytokinins upon proteases and nucleases, and upon the increase in levels of free amino acids in detached leaves (see section 1.5-1 and Kende, 1971). By determining the specific radioactivity of methionine bound to tRNA and to protein, Trewavas (1972a,b) was able to calculate reliably the rates of protein synthesis and degradation in Lemna and to show that cytokinins can stimulate protein synthesis as well as inhibit protein breakdown. The relative rate of each process may depend on the metabolic state of the tissue.

Organelle experiments - In certain cell-free systems, cytokinins have been found to cause very rapid effects on protein metabolism. Davies and Cocking (1967) found that kinetin markedly stimulated incorporation of amino acids into protein in isolated chloroplasts of tomato-fruit locule tissue within 10 minutes. Similarly, Bhattacharyya and Roy (1969) reported an equally rapid effect of kinetin on protein synthesis in mitochondria from Vigna sinensis seedlings.

(b) Phosphorylation of ribosomes

The study of plant hormone effects on the modification of macromolecules is really only just beginning (see Chapter 7). Recently, Ralph et al. (1972) have found that cytokinins depress the phosphorylation of isolated
Chinese cabbage ribosomes. Since phosphorylation of animal ribosomes \textit{in vitro} seems to be associated with reduced protein synthetic activity (Monier \textit{et al.}, 1972), it seems possible that inhibition of ribosome phosphorylation by cytokinins might stimulate translation, possibly by preserving the integrity of ribosomes and/or promoting the initiation of protein synthesis.

\textbf{(c) Effect of cytokinins on isoaccepting tRNA species}

Through an effect on the relative amounts of isoaccepting tRNA species (see section 1.4-4), cytokinins could influence translation. While certain tRNA species do in fact contain cytokinins (see sections 1.4-4; 1.5-5), exogenous cytokinins do not appear to influence the synthesis of cytokinin-containing tRNAs (Chen and Hall, 1969; Rijven and Parkash, 1971). There is some evidence, however, that they do regulate the relative amounts of several isoaccepting tRNA species. Cytokinin treatment of soybean hypocotyls markedly altered the relative amounts of leucine-accepting tRNAs, the change being detectable after only three hours. The cytokinin also induced changes in serine and possibly tyrosine, but not valine or phenylalanine, isoacceptors. Cytokinin treatment appeared to result in changes in tRNA species containing 6-(3-methylbut-2-enylamino)purine (Anderson and Cherry, 1969; Cherry and Anderson, 1972). These workers have suggested that exogenous cytokinins may competitively inhibit specific nucleases that bind to cytokinin-containing tRNAs, thus having a sparing action on these forms. Hence the tissue treated with cytokinin would retain essential tRNA species, enabling it to synthesize protein required for the induction of growth.

\textbf{5-4 Effects on respiration and related processes}

The possibility that cytokinins might influence the synthesis of informational macromolecules has naturally attracted much attention. However, cytokinins might evoke some responses by affecting other regulatory areas of metabolism. One likely possibility is energy production.
When supplied to senescing tissues (e.g. tobacco and wheat leaves at concentrations effective in retarding senescence, cytokinins inhibit respiration (Sugiura, 1963; Shaw et al., 1965). The respiration of pea stem sections (Katsumi, 1963), and of suspensions from tobacco tissue cultures that were grown on a coconut milk medium (Bergmann, 1964), was also inhibited by kinetin. However, when kinetin and IAA were supplied together to carrot tissue, an increase in respiration rate was observed before induction of growth (Komamine et al., 1969). Kinetin also promoted respiration in excised tobacco pith tissue (Glaziou, 1957). The response, which preceded induction of cell division was observed within 3 to 8 hours and was usually accompanied by a promotion of cell enlargement. The very recent work of Moore and Miller (1972) provides further evidence that a stimulation of respiration is an early response to cytokinin in tissues requiring these compounds for growth. Moore and Miller demonstrated that kinetin and zeatin promoted respiration in cell suspensions derived from soybean tissue cultures, the effect being observed 3 hours after application of the cytokinin. Kinetin did not exert its control on respiration at the stage of oxidative phosphorylation, but appeared to regulate by increasing substrate entering the tricarboxylic acid cycle. A number of earlier investigations have indicated that cytokinins promote glycolysis and starch degradation. In carrot tissue, BAP caused a shift in the oxidative pathway of glucose from the pentose cycle to glycolysis (Lustinec et al., 1962). Kinetin was found to promote the growth of excised wheat coleoptiles and this response was accompanied by marked starch breakdown (Boothby and Wright, 1962). When excised coleoptiles were grown in glucose solution, the growth was as great as that which occurred in the presence of kinetin. When incubated with wheat seed halves lacking an embryo, kinetin also caused a large increase in the amount of reducing sugar released into the medium (Boothby and Wright, 1962). Kinetin has also been reported to enhance starch degradation in Chinese cabbage.
leaf disks (Berridge and Ralph, 1971) and to promote amylase activity in bean cotyledons (Gepstein and Ilan, 1970) and pea cotyledons (Varner et al., 1963).

Cytokinins have been reported to show marked effects on pyridine nucleotides. In leaves of spinach and tobacco, for example, kinetin reduced the NADH/NAD ratio (Yamamoto and Ohyama, 1962), and increased the level of total pyridine nucleotides. Both effects in spinach were detected within 3 hours. In view of these and previous results, Yamamoto and Ohyama (1962) have suggested that kinetin enhances glucose utilization. Brown and Cassels (1971) have obtained similar results with pea root tissue; likewise cytokinins increased the pyridine nucleotide content of detached wheat leaves (Mishra and Waygood, 1968). In the above studies, the contents of both NAD and NADP were increased by cytokinin.

Levels and turnover of ATP also appear to be influenced by cytokinin treatment. During retardation of senescence in bean leaves, BAP caused a slight decrease in the level of ATP, compared with the control level, but increased ATP turnover (Adedipe and Fletcher, 1970). When dormant axillary buds of tobacco were treated with this cytokinin, an increase in ATP level was observed after 6 hours (Schaeffer et al., 1972). Although this effect clearly preceded any marked synthesis of DNA and RNA, it is not clear from the data provided whether the ATP increase preceded growth. Since a similar result was obtained by releasing the buds from dormancy by topping the plants, the authors suggest that an increased ATP level is a biochemical characteristic of activated buds.

The literature concerning effects of cytokinins on processes related to the use and production of energy in plants is not without conflict (see review by Srivastava, 1967; Adedipe and Fletcher, 1970 and references therein). However, acceleration of metabolic processes associated with respiration seems to be an early effect of cytokinins.
5-5 Effects on Purine metabolism

To explain the synergistic action of kinetin and xanthine in induction of changes in tissue structure in *Planaria* (a primitive invertebrate), it was suggested that cytokinins affected enzymes involved in oxidative purine catabolism (Henderson *et al.*, 1962). These workers found that cytokinins were substrates for xanthine oxidase and their oxidation products were very effective inhibitors of this enzyme *in vitro*. However, Schlee *et al.* (1966) observed that kinetin did not inhibit the oxidative degradation of xanthine and hypoxanthine in detached leaves of *Pelargonium zonale*, and concluded that kinetin did not inhibit xanthine oxidase *in vivo*. The oxidative degradation of adenine, however, was suppressed in these leaves by kinetin, which was also shown to inhibit competitively adenosine deaminase *in vitro*. Nicholls and Murray (1967) have shown that kinetin also markedly increases the activity of adenine phosphoribosyl transferase, both in soybean callus and in barley leaf tissue.

5-6 A discussion of mechanism of cytokinin action

Cytokinins evoke a great many growth and physiological responses. It is of course not known whether this great diversity of responses is the result of one mechanism of action at the molecular level. It is relevant, however, that cytokinin structure-activity relationships are often very similar for different types of responses. Thus, a number of cytokinins have been tested in the radish cotyledon assay (Letham, 1971) and the Chinese cabbage leaf disk assay (Berridge *et al.*, 1970), responses due mainly to cell enlargement, and also in the tobacco pith assay (Skoog *et al.*, 1967), a response characterized by intensive cell division. The relative cytokinin activities in the three assays are strikingly similar. Hence it is possible that some diverse responses to cytokinins have an identical molecular basis.

In the previous sections, biochemical effects evoked by cytokinins have been outlined. What conclusions
can now be derived concerning the site and molecular mechanism of cytokinin action?

From labelling experiments with intact tissues no conclusive evidence has been obtained for direct transcriptional control by cytokinins. Such experiments would assume greater significance if the effect of cytokinin on incorporation into RNA was definitely shown to precede growth and protein synthesis. Experiments which apparently support the concept of transcriptional regulation by cytokinins involve in vitro studies of RNA synthesis in isolated nuclei (Roychoudhury et al., 1965; Maheshwari et al., 1966; Matthysse and Abrams, 1970), or in vitro transcription of chromatin or DNA (Matthysse and Abrams, 1970). Unfortunately, attempts to repeat these experiments have not been successful (Trewavas, 1968; Berridge et al., 1972) and hence they cannot be accepted as evidence for transcriptional control by cytokinin.

Further in vitro reports which suggest that cytokinins may be involved in transcriptional processes are based on evidence that these molecules can influence the thermal stability of purified pea DNA (Bamberger, 1971) and pea nucleoprotein (Fellenberg, 1971). The DNA stabilization by cytokinins implies binding of the hormone to the DNA helix, while the labilization of the nucleohistones was seen to support the hypothesis that cytokinins bind to nucleoproteins loosening the binding of histone to DNA and thus derepressing specific portions of the genome. However, the results of both of the above reports have been seriously questioned on the grounds that the effects observed were produced respectively by low pH (Penner and Early, 1972) and by aggregation-induced turbidity (Spiker and Chalkley, 1972).

Experiments which have precisely timed the latent period for cytokinin-evoked growth of pea seedlings provide strong evidence that the initial growth response does not depend on regulation of transcription (Warner and Leopold, 1971). The latent period for BAP was 12 minutes, while those for dinitrophenol, a metabolic poison, and actinomycin D,
an inhibitor of transcription, were 11 and 110 minutes respectively. These workers consider that 12 minutes is insufficient time for induction of RNA and protein synthesis and suggest action through direct enzymic control. This conclusion would seem justified. In bacteria, the time required for an inducer to cause gene activation, synthesis of mRNA and production of polypeptide is about 3 minutes (Kaempfer and Magasanik, 1967; Branscomb and Stuart, 1968). In eukaryotes this process would be expected to require a much longer period, and indeed the shortest lag period observed in substrate-induced enzyme formation in higher plants is 2 hours (Nissl and Zenk, 1969). An effect of cytokinins even more rapid than that reported by Warner and Leopold has been observed by Livne and Graziane (1972). Kinetin affected the rate of rehydration of partly dehydrated tobacco leaf tissue in less than 2 minutes and it was suggested that cytokinins might influence membrane permeability.

Although there is no definite evidence that cytokinins directly influence transcription some observations suggest that cytokinins may directly affect translational processes. Thus, cytokinins promote incorporation of amino acids into protein in less than 10 minutes in isolated plastids and mitochondria (section 1.5-3). This information is not sufficiently precise to eliminate a promotion through RNA synthesis. However, other observations also implicate cytokinins in translational control. These are (1) the binding of cytokinins to ribosomes, (2) the inhibition by cytokinins of the phosphorylation of ribosomes and (3) the presence of cytokinins in tRNA.

Berridge et al. (1970) showed that certain cytokinins could bind reversibly to purified Chinese cabbage leaf ribosomes, and detected a positive correlation between the binding affinity and the cytokinin activity of various substituted adenines. In attempts to determine the biological significance of the cytokinin binding, these workers tested the effects of cytokinins on *in vitro* protein synthesis (Berridge et al., 1972). Since no effect was obtained in several *in vitro* systems, it was concluded that the cytokinins
probably did not affect the rate or extent of polypeptide elongation; however, a possible effect on polypeptide initiation could not be assessed in these systems. The possibility that cytokinins may stimulate translation by inhibiting phosphorylation of ribosomes is an attractive concept, but at present there is no evidence that this occurs in vivo. However, the binding of cytokinins to ribosomes, and the cytokinin-induced inhibition of phosphorylation of ribosomes strongly implicate cytokinins in the control of ribosomal processes. It is relevant to note that other small molecules (e.g. chloramphenicol, puromycin) also bind to ribosomes and affect protein synthesis (see Berridge et al., 1970).

Natural cytokinins have been found in sRNA preparations from a wide spectrum of organisms (see Table 1-1). In sequenced cytokinin-containing tRNA species, the single cytokinin molecule is adjacent to the 3' end of the anticodon, where it appears to play an important part in the binding of tRNA to the mRNA-ribosome complex, and may modify codon recognition (see Fittler and Hall, 1966; Gefter and Russel, 1969; Ghosh and Ghosh, 1970). Exogenously supplied cytokinins appear to be incorporated into sRNA. Fox and Chen (1967) reported that when soybean callus tissue was treated with [methylene-\(^{14}\)C]BAP, a small proportion of this labelled cytokinin could be recovered, as a nucleotide, from certain sRNA species purified from the tissue.

Additional evidence for the incorporation of BAP into sRNA was provided by Burrows et al. (1971), who extracted purified sRNA from tobacco pith callus grown in the presence of BAP. Enzymic hydrolysis yielded benzyladenosine in addition to three natural cytokinin ribosides. It is uncertain whether the BAP moiety in tRNA is incorporated in toto or is formed by benzyl group transfer.

Some investigators considered that exogenous cytokinins, as a consequence of incorporation into tRNA affected protein synthesis and hence regulated growth. However, the following evidence indicates that the incorporation of cytokinins into tRNA is not associated with
mechanism of action.

1. Although E. coli contains cytokinins in tRNA, no cytokinin auxotroph mutants could be found by Kende and Tavares (1968) who examined over 10,000 colonies. This suggests that cytokinins are not precursors in tRNA biosynthesis.

2. Cytokinins retard senescence of tobacco leaves but do not appear to be incorporated into tRNA in this tissue (Richmond et al., 1970).

3. 9-Methyl BAP was not incorporated into soybean callus tRNA (Kende and Tavares, 1968), although active in inducing callus growth.

4. Certain 8-aza-9-deaza adenines are weakly active as cytokinins, although it is highly unlikely that they are incorporated into tRNA, since they contain a carbon atom in place of a nitrogen atom at the position normally engaged in nucleotide linkage (Hecht et al., 1971). Similarly the urea cytokinins are unlikely to be incorporated into tRNA (see Kende, 1971).

In addition, there is evidence that 6-(3-methylbut-2-enylamino)purine, which occurs naturally in tRNA as the nucleotide, is not incorporated in toto but is formed by the addition of an isopentenyl group to the appropriate adenine moiety (Chen and Hall, 1969).

Although it thus seems unlikely that the mode of action of cytokinins involves their incorporation into tRNA, there may be some functional relationship between free cytokinins and those found in tRNA. Indeed, a likely possibility has already been discussed, namely, that cytokinins may have an effect on translational processes by altering the relative amounts of isoaccepting tRNA species [see section 1.5-3(c)]. This hypothesis is attractive in that changes in the proportions of isoaccepting tRNA species are known to accompany growth induction and differentiation in animal and bacterial systems (see Sueoka

So far the possible effects of cytokinins on transcription and translation have been stressed. However, important regulatory mechanisms could operate at other levels, and these are now discussed. The demonstration by Moore and Miller (1972) that cytokinins can markedly stimulate respiration in soybean tissue, before the induction of growth (c.f. Miller, 1972), emphasizes the importance of further investigation into the relationships between respiratory processes and cytokinin induction of growth. The stimulation appeared to be due to an increase in substrate entering the tricarboxylic acid cycle. For many years it has been realized that cytokinins can markedly affect glycolytic processes (see section 1.5-4) and this may be an important aspect of cytokinin action. The effects on respiration and glycolysis may be associated with the observed changes in total pyridine nucleotide content and NADH/NAD ratio in treated tissue. Reports that cytokinins can markedly change NADP content have also been mentioned. It should be noted that generally enzymes responsible for oxidations that supply energy use NAD, while those that catalyze reductive biosynthesis employ NADP. NADP has been found in various plant tissues at such a low concentration that it could be rate-limiting for specific enzyme reaction sequences (Yamamoto, 1963). NADP appears to play a key role in metabolic control (see Tezuka and Yamamoto, 1972). Hence cytokinin-induced changes in NADP level may have important metabolic repercussions.

The ability of cytokinin to affect the activity of enzymes involved in purine metabolism, suggests that cytokinins could regulate purine pools and thus influence growth. It should be noted that adenine itself appears to play an important role in certain phases of plant growth, notably in bud development. In some tissues its activity cannot be attributed to conversion to a cytokinin, since adenine and cytokinin are both required to induce bud development (Nitsch et al., 1967; Nitsch and Nitsch, 1967).

In the preceding discussion reference has been
made to biochemical events that may be important in mechanism of cytokinin action and merit intensive investigation. Clearly, however, the precise site of cytokinin action is not yet known. To initiate a response, cytokinins must interact with some cellular component or receptor. For several reasons this receptor seems most likely to be a protein. Firstly, only proteins are so far known to recognize and bind small molecules with high specificity, and to be able to undergo a conformational change as a result. Secondly, evidence already exists for the reversible binding, by purified proteins, of small regulatory molecules (see for example Gilbert and Müller-Hill, 1966; Changeux et al., 1968), and in the case of steroid hormones, protein-hormone complexes have been detected (Toft et al., 1967; O'Malley et al., 1972; section 1.4-2).

The first unequivocal evidence for cytokinin binding to a macromolecule was the demonstration that these compounds bind reversibly to purified ribosomes (Berridge et al., 1970). The correlation between binding affinity and cytokinin activity suggests that this binding is of functional significance. One site of cytokinin action may, therefore, be the ribosome. However, because of the recent findings of very rapid effects of cytokinins, gibberellins and especially auxins, it is unlikely that the initial action of these hormones involves transcriptional and/or translational control. Rapid responses caused possibly by effects on enzymes or membranes, and slower responses involving transcription and translation, are of course not mutually exclusive. In the case of auxin, there is considerable evidence for the existence of auxin-binding proteins which modify transcription (Venis, 1971; Mondal et al., 1972; c.f. Hardin et al., 1972). Other binding proteins no doubt mediate in the rapid responses due to auxin. Similarly there is probably more than one molecular mechanism of cytokinin action, each involving a different binding protein. Mechanisms of action involving translational control and other mechanisms based on preformed enzymes both appear to be important in cytokinin-induced growth. In the latter category may well be effects
on protein kinases and enzymes dependent on NADP.

6 MAJOR UNSOLVED PROBLEMS IN CYTOKININ BIOCHEMISTRY AND THE PURPOSE OF THE INVESTIGATIONS DETAILED IN THIS THESIS

Our present knowledge of cytokinin biochemistry is particularly deficient in two areas - mechanism of action and the metabolism of cytokinins.

Elucidation of the mechanism of action of cytokinins and other phytohormones undoubtedly ranks among the major unsolved problems in modern cell biology. Before the mechanism of cytokinin action can be fully understood, a number of questions must be answered.

1. To what proteins do cytokinins bind to evoke a growth response?

2. Does cytokinin binding give rise to the hormone action directly, or does it first result in the production of a mediator?

3. Cytokinins are repeatedly claimed to stimulate the synthesis of DNA, RNA and protein. Are any such effects the primary reaction evoked, or are they merely the consequences?

4. Is the presence of cytokinins in tRNA related to their role as growth promoting hormones?

To appreciate the significance of cytokinin metabolism within plants we must find answers to the following. To what compounds are naturally occurring cytokinins metabolized? Are some metabolites translocaional forms while others are storage forms? Are cytokinins (such as zeatin and the active synthetic cytokinin 6-benzylaminopurine) active per se or are metabolites the functional forms?

The aim of the research described in this thesis was to contribute new evidence that might help to answer some of the questions listed above. This purpose required the investigation, from several different approaches, of a
cytokinin evoked response with an onset of cytokinin-induced growth sufficiently delayed to allow biochemical examination of the lag phase.

The expansion of excised immature radish cotyledons in response to cytokinin was chosen for examination for the reasons given below.

1. This system had already been developed as a cytokinin bioassay by Letham (1968) who established its specificity and its sensitivity to a wide variety of cytokinins (Letham, 1971). The small response to gibberellin can be distinguished by including hypocotyl segments in the assay as a cell elongation test.

2. Although the response is very rapid, there is a suitable lag period of several hours (see Chapter 2).

3. The speed and ease of handling of the radish cotyledon assay are attractive. The cotyledons can easily be cultured in Petrie dishes or flasks with a simple medium of dilute phosphate buffer as a vehicle for exogenous cytokinin. The germination of radish seeds takes only 30 hours and cytokinin-induced enlargement of the excised cotyledons is essentially complete within a further three days.

4. The assay may be carried out under sterile conditions.

5. Another feature of the radish cotyledon system that seemed desirable for the type of study proposed was its close resemblance to a natural situation. There is evidence in the literature that cytokinins are produced in the roots of plants and migrate to the aerial parts. Certainly excised radish cotyledons grow very little, compared with those still attached to the seedling, unless exogenous cytokinin is present. The exogenous cytokinin is probably substituting for the endogenous hormone produced in the root. It seemed likely, therefore, that the role played by exogenous cytokinin in excised radish cotyledons would closely resemble its natural function.
in the intact plant.

Throughout the study three cytokinins have been used: zeatin - a naturally occurring cytokinin which is strongly active in the radish cotyledon assay; kinetin - a synthetic cytokinin widely used by research workers, probably because it was the first cytokinin discovered; and, principally, 6-benzylaminopurine (BAP) - a synthetic cytokinin readily available commercially and almost as active as zeatin in the assay.

The thesis is divided into two parts. Part I examines the physiological and biochemical aspects of the radish cotyledon growth response evoked by cytokinins. After an initial examination of visible and some physiological parameters of the response (Chapter 2) the possible role of a hormone mediator is considered (Chapter 3). Chapters 4 to 7 examine effects of cytokinins on macromolecules: Chapter 4 describes the effects of inhibitors of macromolecular synthesis, and also examines the effect of BAP on respiration; a more detailed examination follows of cytokinin effects on DNA and RNA (Chapter 5), ribosomes and polyribosomes (Chapter 6) and proteins and their modification (Chapter 7). Part II (Chapter 8) is concerned with the translocation of cytokinins in intact radish seedlings and the metabolism of these compounds in the various organs of this plant.
PART I

PHYSIOLOGICAL AND BIOCHEMICAL ASPECTS OF CYTOKININ-INDUCED RADISH COTYLEDON GROWTH

CHAPTER 2 : THE KINETICS AND CELLULAR BASIS OF THE GROWTH RESPONSE

A. INTRODUCTION

The ability of cotyledons to enlarge in response to cytokinin was discovered by Ikuma and Thinann (1963), who treated lettuce seeds with kinetin. The expansion promoted by this cytokinin was observed not only with cotyledons in intact seeds but also with isolated cotyledons, and the response now appears to be widespread in epigeal dicotyledonous plants in which the cotyledons undergo extensive growth during germination. Thus, marked cytokinin-induced expansion has been found with excised cotyledons of pumpkin (Edwards et al., 1969), radish (Leopold, 1961), watermelon (Nishizono and Aoki, 1969), cucumber (Kasai and Letham, 1966), flax and poppy (Syzhnikova and Khokhlova, 1969), corn (Seto and Letham, 1970), mustard (Uyei and Moore, 1970) and fennel (Lowen and Parkash, 1971).

At the time these experiments were initiated, the stimulation of expansion of lettuce cotyledons (Kasai and Leopold, 1961) and radish cotyledons (Letham, 1961) had been developed as rapid and convenient cytokinin bioassays. Except where the assays were conducted in the presence of mannitol, the radish assay was the more specific cytokinin test, in that it was considerably less sensitive to gibberellin (see Letham, 1971, for details of specificity tests). In addition, the ease of manipulations involved in the radish assay was particularly attractive. Radishes germinate to the point of cotyledon separation within 40 hours, and at this stage the cotyledons can be quickly and easily excised. Thus it was practicable to collect an appreciable quantity of tissue for any one experiment.

The above features of the radish cotyledon assay prompted its selection for use in investigating the biochemistry of cytokinin action. In preparation for this work, experiments were carried out to establish, firstly, the conditions necessary for optimum growth stimulation by a suitable cytokinin, and secondly, the important physiological
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At the time the present study was initiated, the stimulation of expansion of Xanthium cotyledons (Esahi and Leopold, 1968) and radish cotyledons (Letham, 1968) had been developed as rapid and convenient cytokinin bioassays. Except when the assays were conducted in the presence of mannitol, the radish assay was the more specific cytokinin test, in that it was considerably less sensitive to gibberellin (see Letham, 1971, for details of specificity tests). In addition, the ease of manipulations involved in the radish assay was particularly attractive. Radishes germinate to the point of cotyledon separation within 40 hours, and at this stage the cotyledons can be quickly and easily excised. Thus it was practicable to collect an appreciable quantity of tissue for any one experiment.

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parameters of the hormone-induced growth, namely its cellular basis, its time of onset and the period of requirement for exogenous cytokinin. These experiments are described in this chapter. The cytokinin chosen for study was 6-benzylaminopurine (BAP). This compound was readily available and highly active in the assay, being nearly as effective as zeatin (Letham, personal communication, now published: 1971).

B. MATERIALS AND METHODS

1. Germination of radish seed and culture of excised radish cotyledons

The method of radish cotyledon culture was similar to that developed by Letham (1968, 1971). Briefly, seeds of radish (*Raphanus sativus* cv. Long Scarlet) were washed with distilled water, surface sterilized with 1% "zephiran" (Winthrop Laboratories) followed by sodium hypochlorite solution (0.6% available chlorine), rinsed in several changes of sterile water and finally sown in trays on sterile wet filter paper. The trays were covered with aluminium foil and left in darkness at 23°C (± 1°C). About 40 hours later, the smaller cotyledon (i.e. that in the inner position of each folded pair) was excised from each seedling of average size and development. In one experiment the larger cotyledon was excised for comparison. Cotyledons were excised in the light (60 to 100 ft. candles) except in one experiment in which they were excised under dim green safelight [total energy at bench height, 1.7 µW/cm²/sec/nm; maximum transmission, 510 nm at which wavelength the energy at bench height was about 10⁻⁴ µW/cm²/sec/nm (Carr et al., 1972)]. The cotyledons were collected on moist filter paper in a Petrie dish, and then those of uniform size (about 7 mg) were selected and placed, in batches of 8 to 10, in 9-cm Petrie dishes lined with Whatman No. 1 filter paper. The dishes each contained 3 ml of 2 mM potassium phosphate (pH 6) henceforth referred to as "phosphate buffer". In cytokinin treatments, the phosphate buffer contained BAP
2.1 (Calbiochem), at concentrations of 6 µg/ml unless otherwise specified. The purity of this product was confirmed by t.l.c. and u.v. spectra. (In one experiment kinetin was used). The control and BAP treatments, and the cotyledons so treated, are henceforth frequently referred to as (-BAP) and (+BAP) respectively. For each solution, two to five replicate batches of cotyledons were prepared. Incubation was at 23° (± 1°) for 3 to 4 days, either under continuous fluorescent light (65 ft. candles unless otherwise stated) or in total darkness. (Radish cotyledons grow only very slowly after 4 days of incubation). At the end of the incubation period (and in some cases also at intervals throughout this time), each batch of cotyledons was blotted between filter papers and then weighed.

2. Estimation of cell number in cotyledons incubated under various conditions

Batches of 8 to 10 radish cotyledons were dropped into sodium EDTA (0.03M, pH 10.4) and heated at 50°, according to the method of Letham (1960). Five hours later the tubes were vigorously shaken to break up the tissue as much as possible. After a further 12 hours, each mixture was again shaken and then blended at low speed for 1 minute in a Virtis homogenizer with the blades blunted by a covering of masking tape. Each suspension was washed into a volumetric flask and made up to a final volume of 25 to 75 ml with distilled water. The number of cells in each suspension was estimated using a haemocytometer slide. For each suspension at least five different aliquots were used for cell counting. All cell types were counted except xylem elements.

3. Statistical treatment of results

Data were tested for homogeneity of variance by the method of Leslie and Brown (1966), which involves a comparison of sample ranges. The significance of the difference between means was assessed by analysis of variance using the Studentized range Q method (Snedecor and Cochran, 1967). In some cases the difference required for significance at the 5% level [D(5%), i.e. Q x s_x] was simply calculated.
Where a more refined statistical treatment was required the sequential testing procedure described by Snedecor and Cochran (1967) was employed. When more than two means are compared, \( D(5\%) \) is greater than the "least significant difference" (LSD), a commonly used criterion for significance. The latter \( \left( \frac{2S}{\sqrt{n}} \times t \right) \) was not used herein because it can lead to erroneous conclusions regarding significance in multiple comparisons (Snedecor and Cochran, 1967).

C. RESULTS

4. Determination of conditions for an optimal growth response to BAP

4-1. Effect of BAP concentration on radish cotyledon growth

Batches of smaller cotyledons were exposed to various concentrations of BAP (0 to 100 \( \mu \text{M} \)), five batches of 10 cotyledons being used at each concentration. The effect of BAP concentration on the fresh weight attained by these cotyledons after incubation for 3 days is shown in Figure 2-1. A promotion of growth was detected at all tested BAP concentrations, the maximum response corresponded to a BAP concentration of 25 \( \mu \text{M} \) or 5.7 \( \mu \text{g/ml} \).

In another experiment similar to the above, it was found that the larger cotyledons were not as sensitive as the smaller cotyledons to low concentrations of BAP, the lowest detectable concentration being 1 \( \mu \text{M} \), while for a maximum growth response BAP concentrations of 25 \( \mu \text{M} \) and 50 \( \mu \text{M} \) were equally effective and optimal. Expressed as a percentage of the final weight of the control cotyledons, the maximum BAP-induced weight increment was 50\% for the larger cotyledons compared with 81\% for the smaller cotyledons.

These tests showed that both the sensitivity and the magnitude of the response to BAP were greater in the smaller cotyledons than in the larger. Accordingly, smaller cotyledons were generally used in subsequent experiments, and the growth response studied was that evoked
Figure 2-1. Mean fresh weights of radish cotyledons cultured at various concentrations of BAP

Batches of smaller radish cotyledons were incubated for 3 days in the presence of BAP at various concentrations (0 - 100 \mu M). The batches were then weighed and the mean cotyledon weight calculated for each treatment.
by BAP at 6 µg/ml, the approximate equivalent of the optimum concentration (25 µM). A typical growth response is illustrated in Plate 2-1.

4-2. Effect of light intensity on BAP-induced cotyledon growth

Letham (1971) observed that the growth response of radish cotyledons to cytokinins was similar in light or in darkness, although the absolute size attained by light-grown cotyledons was larger. In these experiments, cotyledons were excised in daylight. An experiment was carried out in the present study to test whether the growth response to cytokinin could be evoked from cotyledons excised in the complete absence of red light. From radish seeds imbibed and germinated in the dark, cotyledons were excised under dim green safelight and incubated for 3 days in the dark in (+BAP) or (-BAP) media. Further cotyledons were excised and incubated (+ or -BAP) in the light. The three batches of nine cotyledons incubated in each treatment were weighed after 3 days, and mean cotyledon weights compared. The results (Table 2-1) are similar to those obtained when light and dark treatments were initiated after cotyledon excision in the light. The cytokinin-induced response can thus occur in the complete absence of any stimulus from red light during germination, cotyledon excision or incubation.

Table 2-1. BAP-induced increment in the fresh weight of radish cotyledons grown in light and in darkness

<table>
<thead>
<tr>
<th></th>
<th>(-BAP)</th>
<th>(+BAP)</th>
<th>BAP-induced increment (% control weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light</td>
<td>18.0</td>
<td>34.2</td>
<td>90.0</td>
</tr>
<tr>
<td>Dark</td>
<td>9.9</td>
<td>18.1</td>
<td>82.8</td>
</tr>
</tbody>
</table>

For light treatments a light intensity of 65 ft. candles was used. In dark treatments cotyledons received only dim green safelight at time of excision. Cotyledons were weighed after incubation for three days. D(5%) in cotyledon weight = 6.04
Plate 2-1. Radish cotyledons grown in continuous light for 3 days on phosphate buffer (upper row) and on phosphate buffer containing 25 𝜇M BAP (lower row).
Next examined was the effect on the response of a light intensity much higher than 65 ft. candles. The growth of cotyledons incubated at a light intensity of 65 ft. candles as previously described (section 2.1), was compared with that of cotyledons incubated at the same temperature in a growth cabinet providing a light intensity of 1,000 ft. candles. The results of this comparison appear in Table 2-2 below. Under the higher light intensity cotyledons exhibited greater growth. However, the per cent increment induced by BAP was smaller. It was also observed that the higher light intensity caused severe rolling of many cotyledon laminae. Hence cotyledons grown for further studies were incubated at the lower light intensity of 65 ft. candles.

5. Effect of variation in length of the period of contact with BAP

Groups of four Petri dishes, each containing 10 (smaller) radish cotyledons, were assigned to five different incubation treatments as follows:

1 : (-BAP) throughout 4-day incubation.
2 : (+BAP) for 5 hours, (-BAP) thereafter.
3 : (+BAP) for 14 hours, (-BAP) thereafter.
4 : (+BAP) for 24 hours, (-BAP) thereafter.
5 : (+BAP) throughout 4-day incubation.

During transfers from a (+BAP) to a (-BAP) medium, cotyledons were rinsed with distilled water and blotted with filter paper; fresh weights of all cotyledon batches were

---

Table 2-2. BAP-induced increment in the fresh weight of radish cotyledons grown at two different light intensities

<table>
<thead>
<tr>
<th>Light intensity (ft. candles)</th>
<th>Mean cotyledon fresh weight (mg)</th>
<th>BAP-induced increment (% control weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-BAP)</td>
<td>(+BAP)</td>
</tr>
<tr>
<td>65</td>
<td>14.3</td>
<td>29.4</td>
</tr>
<tr>
<td>1,000</td>
<td>21.0</td>
<td>37.0</td>
</tr>
</tbody>
</table>
measured at intervals (Figure 2-2). The growth curves shown indicate that removal of the supply of BAP from the cotyledons, even after 24 hours of incubation in the presence of the cytokinin, resulted in a decline in the rate of growth compared with that shown by cotyledons still being supplied with cytokinin. Although an initial exposure time to BAP of only 5 hours produced a measurable stimulation of cotyledon growth, BAP was clearly required for more than 24 hours to evoke a maximal growth response. The mean weights of cotyledons in the five different treatments were all significantly different by the end of the incubation period.

6. Length of the lag period preceding BAP-induced growth

The data presented in Figure 2-2 show that the time required for onset of the BAP-induced growth was clearly less than 20 hours. To define this period more precisely, cotyledons in a similar experiment (see Figure 4-1 in section 4.6-1) were weighed at earlier times. After 5 hours of incubation there was no difference in cotyledon fresh weight between (+BAP) and (-BAP) treatments, while at 10 hours there was only a very small difference (8.4%) which was not significant at the 5% level (P = 0.09). By 21 hours, however, a significant (P < 0.01) and marked stimulation of growth was evident, a result similar to that obtained at 20 hours in the experiment concerned with BAP contact time. Hence the lag period preceding BAP-induction of growth was probably less than 10 hours. This is supported by the following observation. Between 5 and 10 hours the percentage increment in the weight of (+BAP) cotyledons (16.8%) was significantly greater (P < 0.01) than the weight increment of the controls (11.5%).

7. Effect of BAP on cell number

To determine the relative degrees of stimulation of cell division and cell expansion in the growth response, batches of 10 cotyledons were treated as follows immediately after excision. Three batches were weighed and then dropped into sodium EDTA solution for determination of cell number (Table 2-3). All other batches were incubated in Petrie
Figure 2-2. Mean fresh weights of radish cotyledons incubated with BAP for various periods before transfer to (-BAP) medium

Batches of cotyledons were assigned to five different treatments as indicated below. Fresh weights of all batches were measured at intervals and the mean cotyledon weight calculated.

Treatments were:
A : (-BAP) throughout 4-day incubation.
B : (+BAP) for 5 hours, (-BAP) thereafter.
C : (+BAP) for 14 hours, (-BAP) thereafter.
D : (+BAP) for 24 hours, (-BAP) thereafter.
E : (+BAP) throughout 4-day incubation.

D(5%) at last sampling time = 4.53; sequential testing showed that all means were significantly different at the 5% level.

D(5%) at 20 hours = 1.79.
dishes for three days under one of the following four treatments: (-BAP) or (+BAP) in continuous light (three replicates each); (-BAP) or (+BAP) in total darkness (five replicates each). The estimates of total cell number per cotyledon and mean cell weight at the end of the 3 days are presented in Table 2-3.

Table 2-3. Mean total cell number, mean cell weight and their BAP-induced increments, in radish cotyledons incubated in the presence or absence of BAP

Cotyledons were incubated either in continuous light or in continuous darkness. BAP-induced increments are expressed as a percentage of the mean cell number or mean cell weight of cotyledons incubated in the absence of BAP. D(5%) in cell number = 0.265 for time zero and light-incubated cotyledons and 0.154 for dark-incubated cotyledons. The corresponding values of D(5%) in cell weight are 3.14 and 2.18 respectively.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean cotyledon weight (mg)</th>
<th>Cells per cotyledon x 10^{-5}</th>
<th>BAP-induced increment in cell number (%)</th>
<th>Mean cell weight (ng)</th>
<th>BAP-induced increment in cell weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none (time zero)</td>
<td>7.0</td>
<td>0.793</td>
<td></td>
<td>8.86</td>
<td></td>
</tr>
<tr>
<td>(-BAP) lt.</td>
<td>15.9</td>
<td>1.077</td>
<td></td>
<td>14.82</td>
<td></td>
</tr>
<tr>
<td>(+BAP) lt.</td>
<td>30.0</td>
<td>1.374</td>
<td>27.6</td>
<td>21.91</td>
<td>47.8</td>
</tr>
<tr>
<td>(-BAP) dk.</td>
<td>12.5</td>
<td>1.014</td>
<td></td>
<td>12.39</td>
<td></td>
</tr>
<tr>
<td>(+BAP) dk.</td>
<td>19.6</td>
<td>1.227</td>
<td>21.0</td>
<td>16.08</td>
<td>29.8</td>
</tr>
</tbody>
</table>

Both in light and in darkness, BAP induced a moderate increase in cell number (about 28 and 21% respectively) and a more marked increase in cell weight. Light enhanced the BAP-induced increment in both cell number and cell weight but the effect on cell weight was more pronounced. Some cell division and cell expansion also occurred in the absence of BAP, both in light and in darkness.
8. The orientation of cytokinin-induced expansion of radish cotyledons

Various degrees of cytokinin-induced cotyledon expansion were produced by incubating seven batches of radish cotyledons each in the presence of a different concentration of kinetin. After incubation for 3 days, all batches of cotyledons were blotted dry and weighed. The mean cotyledon surface area was determined by placing the cotyledons between two sheets of glass and measuring their shadow areas using a photographic enlarger. Cotyledon volume was determined by submerging replicate batches of cotyledons in a volumetric flask containing a known volume of water and measuring the volume of water needed to reach the graduation mark. From the data obtained it was possible to compute cotyledon density and the weight of each cotyledon per unit area. In Table 2-4 the weight/area ratio is shown for each kinetin concentration, together with the data from which the ratio was derived. With

<table>
<thead>
<tr>
<th>Kinetin concentration (μg/ml)</th>
<th>Mean cotyledon weight (mg)</th>
<th>Kinetin-induced increment in weight (%)</th>
<th>Mean cotyledon area (sq. mm)</th>
<th>Kinetin-induced increment in area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>22.5</td>
<td>72</td>
<td>6.5</td>
<td>0.312</td>
</tr>
<tr>
<td>0.05</td>
<td>24.0</td>
<td>6.7</td>
<td>77</td>
<td>6.9</td>
</tr>
<tr>
<td>0.10</td>
<td>25.1</td>
<td>11.6</td>
<td>80</td>
<td>11.1</td>
</tr>
<tr>
<td>0.20</td>
<td>26.5</td>
<td>17.8</td>
<td>85</td>
<td>18.1</td>
</tr>
<tr>
<td>1.0</td>
<td>35.0</td>
<td>55.6</td>
<td>113</td>
<td>57.0</td>
</tr>
<tr>
<td>5.0</td>
<td>38.6</td>
<td>71.5</td>
<td>121</td>
<td>68.1</td>
</tr>
<tr>
<td>25.0</td>
<td>47.0</td>
<td>109</td>
<td>147</td>
<td>104</td>
</tr>
</tbody>
</table>

Density: \((\text{Kinetin}) = 1.01\)

: \((\text{+Kinetin}) = 5 \text{ μg/ml} = 0.98\)
increasing kinetin concentration, there was an increase in cotyledon weight and in cotyledon surface area. The increase in surface area was proportional to the weight increase at all kinetin concentrations and this is reflected in the constancy of the weight/area ratio. Since cotyledon density showed negligible change in response to kinetin (refer to Table 2-4, footnote), cotyledon volume per unit area was also constant. Thus, kinetin treatment increased the surface area of the cotyledons without increasing their thickness, and it may therefore be concluded that the direction of cytokinin-induced cotyledon expansion is entirely lateral.

This experiment was not repeated with BAP after the latter was selected for detailed study of cytokinin effects on radish cotyledons; however, observations made during light microscopy of hand cut sections indicated that the direction of growth induced by BAP was the same as that induced by kinetin.

D. DISCUSSION

Excised radish cotyledons showed a marked stimulation of expansion in response to BAP. The more responsive smaller cotyledons, from the inner position of each folded pair, responded to an optimum concentration of BAP (25 μM) with an 81% increase in fresh weight. The sensitivity of the smaller cotyledons, which could detect $5 \times 10^{-5}$ M BAP, was similar to that of other cotyledon bioassays currently available, namely, the xanthium cotyledon assay of Esahi and Leopold (1968), which can detect $1 \times 10^{-5}$ M kinetin, and the recently developed fenugreek cotyledon assay (Rijven and Parkash, 1970), which can detect $5 \times 10^{-5}$ M BAP.

The growth of the radish cotyledons was stimulated by light as well as by BAP, and especially by both (compare mean cotyledon weights in Table 2-1). The BAP-induced increase in cotyledon weight, as a percentage of the control weight, was nearly the same in weak light and in darkness. However, weak light and BAP acted synergistically to promote cotyledon expansion. Similarly Rijven and Parkash (1970) observed a marked synergism between light and cytokinins in the promotion of fenugreek cotyledon growth; in this case,
however, the percentage increments induced by cytokinins in darkness were considerably less than those evoked in light. Although the experiments reported in this chapter have eliminated the possibility that the cytokinin-induced growth of radish cotyledons is dependent on red light, and hence probably on phytochrome mediated processes, possible dependence on light of other wavelengths was not entirely eliminated. A weak green safelight was employed in the excision of the cotyledons. It is relevant to note that the germination of light-sensitive lettuce seeds is more markedly stimulated by kinetin if the seeds receive a small amount of dim green light than if they are kept in total darkness (Miller, 1956, 1958).

Estimates of cell number in BAP-treated and control radish cotyledons indicated that some cell division was induced by BAP, both in the light and in the dark, and that some cell division continued to occur in the control cotyledons. However, when the BAP-induced increments in cell number and in cell weight were compared (as percentages of the corresponding control cell number and cell weight - see Table 2-3), the increase in cell weight was greater. Hence, BAP stimulates both cell division and cell expansion in radish cotyledons, and the stimulation of expansion is the major response. Data presented in section 2.8 showed that the cell expansion was confined to the lateral direction. A marked stimulation of cell expansion and moderate stimulation of cell division was also observed when radish cotyledons were treated with zeatin in the light (Letham, 1971); kinetin, however, was reported to promote cell division only slightly. Marked stimulation of cell expansion appears to be a general response of leaf tissue to cytokinin treatment. A cell enlargement response has been reported from bean leaf disks grown in the dark (Miller, 1956; Powell and Griffith, 1960), radish leaf disks grown in the light (Kuraishi, 1959) and flax cotyledons grown in the light (Sveshnikova and Khokhlova, 1969). The expansion of etiolated bean leaf disks, and flax cotyledons, appears to be due to cell enlargement entirely (Humphries and Wheeler, 1960; Sveshnikova and Khokhlova, 1969). Cytokinins
are also known to evoke the unrolling of etiolated wheat and barley leaves (Beever sl et al., 1970; Loveys and Wareing, 1971; Kang, 1971; Carr et al., 1972). The unrolling response has been attributed to expansion of the upper mesophyll cells (Burström, 1942).

To exhibit a maximum growth response to BAP, radish cotyledons appear to require the presence of the cytokinin for at least 24 hours, i.e. at least 14 hours after induction of growth. Thus BAP does not seem to act by merely triggering some process. This conclusion is in agreement with observations made by Brandes and Kende on cytokinin induction of bud development in protonema filaments of the moss Funaria hygrometrica. Induced bud formation was detectable within 10 hours of cytokinin treatment (Brandes, 1967). On removal of the cytokinin, however, the induced buds tended to dedifferentiate to an extent which decreased with progressing duration of the cytokinin treatment. Bud induction was completely irreversible only after 72 hours of cytokinin treatment (Brandes and Kende, 1968). By contrast Nitsch (1968) has reported that when cytokinins are supplied to tobacco pith explants, as a 24-hour pretreatment, they are able to trigger proliferation of growth when the pith explants are transferred to a medium containing auxin (but no cytokinin). Nitsch suggests that these results cannot be adequately explained in terms of cytokinin storage, since the cytokinin effect is abolished by the presence of inhibitors of DNA and RNA synthesis (FUDR and actinomycin D) during the pretreatment.

Information on the effect of variation in cytokinin contact time is lacking in the other cotyledon studies, and data on lag periods are also scanty. However, an onset of cytokinin-induced growth 6 hours after commencement of incubation is reported for flax cotyledons (Sveshnikova and Khokhlova, 1969), while the results of Kursanov et al. (1969) indicate that onset of BAP-induced growth in pumpkin cotyledons occurs within 2 days. In radish cotyledons, the lag period was clearly longer than 5 hours, and appeared to be less than 10 hours as judged by mean per
cent increments in cotyledon weight. The response is thus considerably more rapid than those observed in most tissue cultures, but is sufficiently delayed to allow biochemical investigation of the events occurring in the lag phase.
CHAPTER 3: THE POSSIBLE ROLE OF HORMONE MEDIATORS IN CYTOKININ-INDUCED GROWTH

In animal systems it is now well established that a variety of hormonal responses are mediated within the target cells by a "secondary messenger" whose production is stimulated as a result of the initial hormone-cell interaction. Evidence is also increasing for mediation in phytohormone action. Hence, before investigating apparent cytokinin effects on the biochemistry of radish cotyledon tissue, it was clearly desirable to test the possibility that the growth response to cytokinin was mediated by some other compound.

In plants, hormones themselves seem to function as mediators in some instances. One type of hormone may act by affecting the level of another. Thus endogenous auxins have been shown to affect the application of cytokinins to the shoot apical bud and root, 1962; Vaidovics et al., 1966. Including cytokinins (see, for example, Rasmus, 1972; Lee, 1974; Gaspar and Knopf, 1965). However, auxins have previously been shown to be inhibitory in the radish cotyledon assay (Letham, 1971) and were therefore considered unlikely to mediate the action of cytokinins, at least in this tissue.

Several groups of workers have shown that treatment of plants with cytokinins leads to increased gibberellin levels, or to the maintenance of gibberellin levels under conditions that would otherwise lead to decline (Hormes and Vade, 1969; Sabato, 1956; Chin and Beavers, 1970). In addition, Lovesey and Wareing (1971) have reported that kinetin stimulates wheat test unrolling by increasing levels of endogenous gibberellins-like substances. In this case, however, both exogenous kinetin and exogenous GA were effective in evoking the unrolling response, and the effects of the two hormones were not additive. Similarly GA and kinetin were non-additive in promoting cell division in somatic cells of Agar pseudosideratus (Blythe and Wareing, 1968). Several gibberellins, including GA, show weak root-promoting activity in the radish cotyledon bioassay.
A. INTRODUCTION

In animal systems it is now well established that a variety of hormonal responses are mediated within the target cells by a "secondary messenger" whose production is stimulated as a result of the initial hormone-cell interaction. Evidence is also increasing for mediation in phytohormone action. Hence, before investigating apparent cytokinin effects on the biochemistry of radish cotyledon tissue, it was clearly desirable to test the possibility that the growth response to cytokinin was mediated by some other compound.

In plants, hormones themselves seem to function as mediators in some instances. One type of hormone may act by affecting the level of another. Thus endogenous auxin levels in plant tissues appear to be influenced by the application of other phytohormones (Kuraishi and Muir, 1962; Valdovinos et al., 1966) including cytokinins (see, for example, Hemberg, 1972; Lee, 1971; Gaspar and Xaufflaire, 1967). However, auxins have previously been shown to be inactive in the radish cotyledon assay (Letham, 1971) and were therefore considered unlikely to mediate the action of cytokinins, at least in this tissue.

Several groups of workers have shown that treatment of plants with cytokinins leads to increased gibberellin levels, or to the maintenance of gibberellin levels under conditions that would otherwise lead to decline (Karanov and Vassilev, 1969; Sebanek, 1966; Chin and Beevers, 1970). In addition, Loveys and Wareing (1971) have reported that kinetin stimulates wheat leaf unrolling by increasing levels of endogenous gibberellin-like substances. In this response both exogenous kinetin and exogenous GA3 were effective in evoking the unrolling response, and the effects of the two hormones were not additive. Similarly GA and kinetin were non-additive in promoting cell division in cambial cells of *Acer pseudoplatanus* (Digby and Wareing, 1966). Several gibberellins, including GA3, show weak growth-promoting activity in the radish cotyledon bioassay.
(Letham, 1971). Since gibberellins show considerable species specificity, the endogenous gibberellins could be more effective. The effect of cytokinins on cotyledon expansion could be a consequence of an increased gibberellin level caused by enhanced synthesis or suppressed degradation. If cytokinins promote gibberellin synthesis, inhibitors of gibberellin biosynthesis should inhibit the growth response evoked by exogenous cytokinin. The results of such a test appear in this chapter.

It has been known for some time that ethylene production can be stimulated by auxins (Zimmermann and Wilcoxon, 1935), and the effect has since been observed in a wide variety of angiosperm genera (see Abeles, 1966; Burg and Burg, 1968; Fuchs, 1968; Fuchs and Lieberman, 1968; Gamborg and La Rue, 1971; Loveys and Wareing, 1971; Sakai and Imaseki, 1971). Gibberellins and ethylene are often antagonistic in their action (see for example Scott and Leopold, 1967), but stimulation of ethylene production by gibberellic acid has been reported (Abeles and Rubenstein, 1964; Lewis et al., 1967; Loveys and Wareing, 1971). The effect of cytokinins on ethylene production has been studied very little, but a few positive relationships have been reported. Thus, Abeles et al. (1967) reported that kinetin and BAP stimulated ethylene production in bean explants, while Fuchs and Lieberman (1968) detected a stimulation of ethylene, in response to $10^{-8}$ to $10^{-4}$ M kinetin, in seedlings of Alaska pea, French bean, cucumber, corn, barley and wheat. Similar results have been obtained with wheat leaf sections (Loveys and Wareing, 1971). The ethylene production of slices of green banana fruit is also hastened and enhanced by kinetin (20 - 100 uM) even though the cytokinin delays ethylene-induced de-greening of the peel (Wade and Bradey, 1971). In view of these reports of a positive effect of cytokinins on ethylene production, and of the common ability of these two types of phytohormone to induce lateral cell expansion, the possibility was examined that cytokinin-induced expansion of radish cotyledons might be a consequence of cytokinin-evoked ethylene production. The results are presented herein.
Recent evidence suggests that certain hormonal responses in plants could possibly be mediated by polyamines. Certain polyamines appear to be essential for the growth of some micro-organisms in culture. Polyamines have also been found to stimulate *in vitro* protein synthesis, to increase the activity of certain purified enzymes and, especially in the case of long chain polyamines such as spermidine and spermine, to form complexes with DNA *in vitro* (see review by Tabor and Tabor, 1964). It has been suggested that polyamines may function as physiological stabilizers in structural units containing nucleic acids (see Bagni *et al.*, 1971, and references cited therein); however, their role *in vivo* is still speculative. Polyamines such as spermidine and spermine have been found in numerous species of animals and micro-organisms, but relatively little work has been reported on the distribution of these compounds in plant tissues. Nevertheless, spermine, spermidine and putrescine are known to occur in several plants of diverse phyla and genera (see Smith, 1970), and evidence is accumulating which suggests that such polyamines may be as important in plants as they appear to be in animals and micro-organisms. Thus, spermine, spermidine and putrescine have been shown to stimulate the growth of Jerusalem artichoke tuber explants (Bertossi *et al.*, 1965; Bagni, 1966). A combination of spermidine and putrescine stimulated *in vitro* protein synthesis by ribosomes from tuber slices (Cocucci and Bagni, 1968), and spermidine was found to increase RNA levels both in this tissue (Bagni *et al.*, 1971) and in *Petunia* pollen (Linskens *et al.*, 1968). Bagni and co-workers reported that the increase in RNA content induced in the tuber tissue by spermidine was similar to that induced by IAA. In addition, activation of dormant tubers by IAA treatment resulted in a rapid rise in the polyamine content of this tissue (Cocucci and Bagni, 1968). These reports raise the possibility that a growth effect of a plant hormone (auxin) might be mediated by a polyamine. It was therefore of interest to examine the effect of polyamines on the expansion of radish cotyledons, and the results obtained are included in this chapter.

The action of adenosine 3', 5'-cyclic phosphate,
or "cyclic AMP" (cAMP), as the mediator of several mammalian hormones has been established for some years (Jost and Rickenberg, 1971). It is also known to be an important intermediary in the regulation of gene expression in certain micro-organisms (see section 1.4-2), and in the differentiation of slime moulds (see references cited by Jost and Rickenberg, 1971).

Hormonal activation of cAMP synthesis appears to involve an interaction, at the outer cell membrane, between the hormone and either adenylyl cyclase or the membrane with which this enzyme is associated (see Rall et al. (1969) for details of suggested models). Adenylyl cyclase then catalyzes the formation of cAMP from ATP. There is more than one hypothesis that attempts to account for the multiple effects of cAMP in terms of a common molecular basis, but the activation of specific protein kinases by this cyclic nucleotide seems to be the basis of many of its effects (see Robison and Sutherland, 1971; Greengard, 1971; Jost and Rickenberg, 1971 for discussion of current hypotheses). cAMP is degraded by a specific phosphodiesterase to AMP. However, this reaction has not been established as an important site of hormone action (Robison and Sutherland, 1971).

Recently there has been increasing interest in the possibility that cAMP might play an important role in the metabolism of higher plants. The role of cAMP in catabolite repression in bacteria has previously been mentioned (section 1.4-2). Inhibition by sugar of duckweed flowering has been explained in terms of catabolite repression, and cAMP alleviates this repression (Oota, 1972). Evidence for the occurrence of cAMP in plants is now substantial. Wood et al. (1972) have extracted an adenylyl cyclase from tumor cells of Vinca rosea, and very recently cAMP has been detected in sieve tube sap of Robinia pseudoacacia using a highly specific assay (Becker and Ziegler, 1973). Previous work (especially that of Pollard, 1970) had strongly suggested the occurrence of cAMP in plants.

cAMP has been shown to mimic a number of effects
of auxins and gibberellins. Thus, in barley aleurone layers, both cAMP and gibberellic acid induced α-amylase (Duffus and Duffus, 1969; Galsky and Lippincott, 1969), protease, acid phosphatase and ATPase activity (Nickells et al., 1971; Gilbert and Galsky, 1972; Earle and Galsky, 1971). In lettuce, cAMP promoted hypocotyl elongation, a response also stimulated by gibberellic acid (Kamisaka et al., 1972), and synergistically enhanced the gibberellin-promoted germination of light-sensitive lettuce seeds (Kamisaka and Masuda, 1971). In barley aleurone layers, the amount of radioactivity found in cAMP of gibberellic acid-treated tissue was twice that of control tissue (Pollard, 1970). It was in this study that Pollard identified the cyclic nucleotide by chromatography and electrophoresis (10 systems in all), hydrolysis and deamination. Further work (Pollard, 1971) indicated that both cAMP and gibberellic acid could stimulate α-amylase activity in aleurone layers within 15 minutes, as evidenced by increased secretion of soluble sugar (Pollard, 1971). Auxin effects that are mimicked by cAMP include the stimulation of tryptophan oxygenase activity in chick pea seedlings (Azhar and Murti, 1971) and the delay of petiole abscission in Coleus (Salomon and Mascarenhas, 1971). In addition cAMP synergistically enhanced the effect of auxin on cell expansion in Jerusalem artichoke tuber tissue, as did gibberellic acid (Kamisaka and Masuda, 1970a; Kamisaka, 1972). Auxin-induced incorporation of label into cAMP has been reported by two groups who used methods similar to those of Pollard (1970). Azhar and Murti (1971) detected the effect in chick pea seedlings while Salomon and Mascarenhas (1971) used Avena coleoptiles and detected the effect within 30 minutes.

The above results indicate that gibberellic acid and auxin can promote incorporation of label into cAMP. However, the recent work of Kessler and Kaplan (1972) suggests that the relationship between cAMP and gibberellic acid may be complex, since in barley endosperm, cAMP appears to induce the synthesis of gibberellin.

No cytokinin effects are yet known to be mimicked by cAMP, despite the many similarities between the effects
of cytokinins on plant cells and the effects of cAMP on bacterial and animal cells (see Ralph et al., 1972, and references therein). However, in view of these similarities and the increasing evidence that cAMP may be of considerable importance in the co-ordination of plant growth, the effect of this cyclic nucleotide on the expansion of radish cotyledons was examined in some detail during the study reported here. Also included were preliminary examinations of the effects of adenosine 2', 3'-cyclic phosphate and guanosine 3', 5'-cyclic phosphate (cGMP). Like cAMP, adenosine 2', 3'-cyclic phosphate has been reported to enhance auxin-induced cell expansion in Jerusalem artichoke tuber tissue (Kamisaka and Masuda, 1970). Although cGMP has not been shown to occur in plants, there is some evidence that it may have a regulatory role in animal systems (Greengard, 1971; Jost and Rickenberg, 1971) where it appears to activate a spectrum of kinases distinct from that associated with cAMP.

B. MATERIALS AND METHODS

1. Compounds tested

<table>
<thead>
<tr>
<th>Chemical*</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP (adenosine 3', 5'-cyclic phosphate)</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>adenosine 2', 3'-cyclic phosphate</td>
<td>Sigma Chemical Co.</td>
</tr>
<tr>
<td>AMO 1618 [2'-isopropyl-4'-(trimethylammonium chloride)-5'-methylphenylpiperidine-1-carboxylate]</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>N₆-butryladenine</td>
<td>Synthesized by Dr. D.S. Letham of this laboratory</td>
</tr>
<tr>
<td>CCC (2-chloroethyltrimethylammonium chloride)</td>
<td>British Drug Houses</td>
</tr>
<tr>
<td>DBcAMP (N₆,O-dibutyryladenosine 3', 5'-cyclic phosphate)</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>ethrel (2-chloroethylphosphonic acid), purity &gt; 98%</td>
<td>Amchem Products, Ambler, U.S.A.</td>
</tr>
<tr>
<td>cGMP (guanosine 3', 5'-cyclic phosphate)</td>
<td>Sigma Chemical Co.</td>
</tr>
<tr>
<td>theophylline (1, 3-dimethylxanthine)</td>
<td>Calbiochem</td>
</tr>
</tbody>
</table>

* Chemicals were used as solutions (pH 6) in 2 mM phosphate buffer.
2. Methods

Radish cotyledons were cultured on test solutions as previously described (section 2.1). In each experiment, (unless otherwise stated), three batches of eight cotyledons were cultured for 3 days on each test solution.

Statistical treatment of results was as described in section 2.3.

C. RESULTS

3. Effect of inhibitors of gibberellin biosynthesis

The possibility that BAP-induced expansion of radish cotyledons might be the result of gibberellin synthesis was tested by examining the effects, on the growth response, of two inhibitors of gibberellin biosynthesis, namely AMO 1618 and CCC (see Barnes et al., 1969 and references cited therein, for details of the points of action of these inhibitors). Details of the solutions tested and the mean fresh weights attained by the cotyledons are shown in Table 3-1 below.

Table 3-1 Mean fresh weights of radish cotyledons grown for 3 days in (+BAP) or (-BAP) media containing one of the following compounds: GA, AMO 1618, CCC.

<table>
<thead>
<tr>
<th>Additive to medium</th>
<th>Mean cotyledon weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-BAP) medium</td>
</tr>
<tr>
<td>none</td>
<td>21.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>AMO 1618 (0.3 mg/ml)</td>
<td>21.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CCC (3.0 mg/ml)</td>
<td>24.7&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>GA&lt;sub&gt;3&lt;/sub&gt; (30 μg/ml)</td>
<td>27.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means lacking a common letter are significantly different at the 5% level.

These results confirmed that the effects of BAP and GA<sub>3</sub> were approximately additive, as observed by Letham (1971). However, neither inhibitor of gibberellin biosynthesis had any significant effect on BAP-induced cotyledon
growth or on the growth of the controls, although CCC did inhibit greening of the cotyledons. In previous tests, not detailed here, lower concentrations of these inhibitors were also without effect.

4. **Effect of ethylene**

The effect of ethylene on the expansion of radish cotyledons was assessed by culturing the cotyledons in solutions containing ethrel. In the presence of water this compound liberates ethylene as shown in the following scheme:

\[
\begin{align*}
\text{Cl-CH}_2\text{-CH}_2\text{-POH} & \quad \text{pH} > 4 \\
\text{CH}_2\text{-CH}_2 + \text{HO-POH} + \text{Cl}^- & \\
\text{ethrel} & \quad \text{ethylene} \\
\text{phosphate} & 
\end{align*}
\]

The mean fresh weights of radish cotyledons grown in test solutions containing various initial concentrations of ethrel are shown in Table 3-2. Depending on concentration ethylene either inhibited growth or had no effect.

**Table 3-2 Mean fresh weights of radish cotyledons grown in the presence of various concentrations of ethrel**

D(5%) for significance = 4.13 (experiment 1); 1.57 (experiment 2).

<table>
<thead>
<tr>
<th>Additive to medium</th>
<th>Mean cotyledon weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>none</td>
<td>17.3</td>
</tr>
<tr>
<td>ethrel (0.002 µg/ml)</td>
<td>17.1</td>
</tr>
<tr>
<td>&quot; (0.02)&quot;</td>
<td>18.6</td>
</tr>
<tr>
<td>&quot; (0.16)&quot;</td>
<td>10.4</td>
</tr>
<tr>
<td>&quot; (0.80)&quot;</td>
<td>10.1</td>
</tr>
<tr>
<td>&quot; (4.00)&quot;</td>
<td>10.7</td>
</tr>
<tr>
<td>&quot; (20.0)&quot;</td>
<td></td>
</tr>
<tr>
<td>&quot; (100)&quot;</td>
<td></td>
</tr>
<tr>
<td>BAP</td>
<td>31.2</td>
</tr>
</tbody>
</table>

5. **Effects of the polyamines spermine and spermidine**

At concentrations of 100 µg/ml or less, spermine had no effect on radish cotyledon growth, while a concent-
A spermidine concentration of 300 µg/ml caused slight growth inhibition, but concentrations of 100 µg/ml or less were without effect.

6. Effect of cAMP, adenosine 2', 3'-cyclic phosphate and cGMP

The possibility that BAP-induced radish cotyledon expansion might be mediated by cAMP, adenosine 2', 3'-cyclic phosphate or cGMP was tested by examining the effect of each compound on cotyledon expansion. The range of concentrations tested was 0 to 120 µg/ml for cAMP and adenosine 2', 3'-cyclic phosphate, and 0 to 40 µg/ml for cGMP. No effect of these compounds on radish cotyledon fresh weight could be detected at any concentration tested. Thus, it seemed unlikely that cytokinin-induced cotyledon growth could be due to increased levels of these compounds. However, the following explanations could account for the lack of response.

(1) The tissue had degraded the cyclic nucleotide before it could have any effect.

(2) The cyclic nucleotide had failed to penetrate the tissue in an amount sufficient to elicit a growth response.

In view of the reports in the literature which implicate cAMP as a mediator of certain plant hormone effects, experiments were performed to determine whether either of these explanations might apply to cAMP. The results are described in the following two sections.

7. Effect of cAMP in the presence of theophylline

Theophylline inhibits the enzymic degradation of cAMP. Preliminary experiments with this compound had indicated that a concentration of 300 µg/ml had no significant effect on cotyledon growth in the absence of cytokinin; however, a very slight stimulation was possibly indicated at 100 µg/ml. The results obtained when cotyledons were incubated with cAMP in the presence of theophylline (100 µg/ml) are shown in Table 3-3.
Table 3-3  Mean fresh weights of radish cotyledons grown in the presence of theophylline (100 μg/ml) and various concentrations of cAMP (Weights were compared with those attained by cotyledons grown on (+BAP) or (-BAP) media without theophylline). Means lacking a common letter are significantly different at the 5% level.

<table>
<thead>
<tr>
<th>Additive to medium</th>
<th>Mean cotyledon weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-BAP) medium (±BAP) medium</td>
</tr>
<tr>
<td>none</td>
<td>17.4*a</td>
</tr>
<tr>
<td>theophylline</td>
<td>19.4*a</td>
</tr>
<tr>
<td>theophylline + cAMP (1.2 μg/ml)</td>
<td>18.9*a</td>
</tr>
<tr>
<td>&quot;</td>
<td>+ cAMP (6.0 μg/ml)</td>
</tr>
<tr>
<td>&quot;</td>
<td>+ cAMP (24 μg/ml)</td>
</tr>
<tr>
<td>&quot;</td>
<td>+ cAMP (120 μg/ml)</td>
</tr>
</tbody>
</table>

The small stimulatory effect of theophylline was again detected, and was found to be more marked in the presence of BAP. cAMP, however, had no effect on cotyledon growth in the presence of theophylline. Thus it appeared unlikely that the apparent inactivity of cAMP in this system was due to rapid enzymic degradation.

Experiments relevant to the question of cAMP penetration are described below.

8. Effect of N₆, O-dibutryladenosine 3', 5'-cyclic phosphate

In many animal tissues, N₆, O-dibutryladenosine 3', 5'-cyclic phosphate (DBcAMP) exhibits greater activity than cAMP, and appears to enter cells more readily than cAMP (Henion et al., 1967). Hence, radish cotyledons were grown in the presence of various concentrations of this compound. At a concentration of 58 μg/ml, the highest concentration tested, DBcAMP significantly stimulated growth of radish cotyledons. The activity of this compound could have been due to the cyclic phosphate moiety, or to the presence of a substituent on the exocyclic nitrogen atom. The latter
possibility seemed unlikely since Skoog et al. (1967) had reported that amides of adenine were inactive as cytokinins. However, so that these alternative possibilities could be assessed, Dr. D.S. Letham of this laboratory synthesized 6-butyrylaminopurine. The results obtained when this compound was tested for activity are now reported.

9. The relative activities of DBCAMP and 6-butyrylaminopurine

The activities of DBCAMP and 6-butyrylaminopurine are compared in Table 3-4.

Table 3-4 Mean fresh weights of radish cotyledons grown in the presence of various concentrations of DBCAMP or 6-butyrylaminopurine (6BuAP). D(5%) for significance = 4.34.

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Mean cotyledon weight (mg)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DBCAMP medium</td>
</tr>
<tr>
<td>0</td>
<td>18.2</td>
</tr>
<tr>
<td>20</td>
<td>17.5</td>
</tr>
<tr>
<td>100</td>
<td>27.8</td>
</tr>
<tr>
<td>200</td>
<td>30.5</td>
</tr>
</tbody>
</table>

* c.f. BAP medium (25 μM): 32.4

When the mean cotyledon weights at each concentration of these two purine derivatives were compared, it was clear that 6-butyrylaminopurine was as active as DBCAMP at the higher concentrations tested, and indeed appreciably more active than the cyclic compound at the lowest concentration (20 μM). Thus cytokinin activity appeared to reside in the Nα-butyrylaminopurine of DBCAMP.

D. DISCUSSION

In the experiments described above, some seven possible mediators of cytokinin action were tested for their ability to promote expansion of excised radish cotyledons, namely, gibberellins, ethylene, spermine, spermidine, cGMP, adenosine 2', 3'-cyclic phosphate and cAMP. The cAMP
derivative, \( N_6O\)-dibutyryl adenosine 3', 5'-cyclic phosphate (DBcAMP) was also tested.

\( \text{GA}_3 \) showed some promotion of radish cotyledon growth. However, the failure of inhibitors of gibberellin biosynthesis to inhibit cytokinin-induced cotyledon expansion strongly suggested that the cytokinin effect was not the result of an increase in the synthesis of endogenous gibberellin. The possibility that the inhibitors had not entered the tissue was not tested directly, but characteristic inhibition of chlorophyll formation (c.f. Knypl and Chylinska, 1972) was observed in cotyledons treated with CCC, indicating successful entry of the inhibitor. It is also noteworthy that the concentrations of CCC and AMO 1618 used in this study were effective in inhibiting unrolling and gibberellin synthesis in wheat leaves (Loveys and Wareing, 1971). The possibility was not eliminated that cytokinin treatment inhibited gibberellin degradation or resulted in a release of bound gibberellins. However, it is relevant that Loveys and Wareing (1971) detected the release of bound gibberellins of wheat leaf sections in the presence of AMO 1618, but no gibberellin response of the tissue (i.e. unrolling). Thus, the release of bound gibberellin did not appear to be directly responsible for leaf unrolling. Unlike cytokinin-induced unrolling of wheat leaves, cytokinin-evoked expansion of radish cotyledons does not appear to be due to a promotion of gibberellin synthesis. These differing mechanisms of action are probably reflected in the different relationships between the gibberellin and cytokinin responses in the two systems. In radish cotyledons the effects of the two hormones are additive, while in wheat leaves they are not.

The effect of ethylene on radish cotyledon growth was determined using ethrel as the ethylene source. Depending on concentration, ethylene either inhibited growth or had no effect. Since the inhibition was significant at ethrel concentrations of 0.8 \( \mu \text{g/ml} \) or higher, it may be concluded that ethylene did penetrate the cotyledons. Thus ethylene does not seem to be a mediator of cytokinin action in this system. Similarly, ethylene does not appear to mediate the unrolling of barley leaves (Menhenett, 1972) or
wheat leaves (Kang, 1971; Loveys and Wareing, 1971). Spermine and spermidine likewise are apparently not mediators of cytokinin-induced growth of radish cotyledons.

CAMP appeared to have no effect on cotyledon growth at concentrations up to 120 µg/ml. cGMP and adenosine 2', 3'-cyclic phosphate were also ineffective. The lack of response of radish cotyledons to CAMP is in agreement with the recent report of Ralph et al. (1972) that in contrast to cytokinins CAMP had no significant effect on the expansion of Chinese-cabbage leaf disks, on the growth of Spirodela or on phosphorylation of plant proteins in vitro. In the present study, results of experiments using theophylline indicated that the inactivity of the cyclic nucleotide was not due to rapid enzymic degradation. However, theophylline itself had a slight growth-promoting effect on radish cotyledons, when supplied at a concentration of 100 µg/ml. In tobacco callus, theophylline (300 µg/ml) has been observed to promote growth slightly (Wood et al., 1972). At the high concentration of theophylline used, cytokinin impurities in this compound could possibly account for the effect observed by these workers. However, the stimulation of radish cotyledon growth was very probably not due to cytokinin impurities since the effect was also observed in the presence of an optimal concentration of cytokinin. Indeed cytokinin and theophylline appeared to act synergistically. The reason for the growth response associated with theophylline is not known. Theophylline possibly acted directly as a weak growth stimulant. Alternatively, growth stimulation could have resulted from the preservation of endogenous CAMP. In the latter case, the failure of exogenous CAMP to induce further cotyledon growth would, presumably, be due to non-penetration.

DBcAMP, the N₆,O-dibutyryl derivative of CAMP, is known to be more active than CAMP in evoking certain responses in animal systems (see Hilz and Tarnowski, 1970). It has generally been assumed that DBcAMP penetrates cells more easily than does CAMP, and there is evidence that the dibutyryl derivative is more resistant to enzymic degradation.
The difference in activity between cAMP and DBcAMP has recently been observed with plant tissues also. Thus DBcAMP promotes amylase production in aleurone layers (Pollard, 1971) and cell expansion in artichoke tissue (Kamisaka and Masuda, 1970; Kamisaka, 1972) to a greater degree than cAMP. Hence, in the present study, it was relevant to test the ability of DBcAMP to promote expansion of radish cotyledons. The possibility was also of interest that this compound might exhibit activity distinct from that of cAMP. The spectrum of effects of DBcAMP is by no means identical to that of cAMP, and indeed in some instances the two compounds exert opposite effects. In such cases the activity of DBcAMP appears to be due to the dibutyryl compound per se or to N\textsubscript{6}-butyryl cAMP (Szabo and Burke, 1972).

When its effect on radish cotyledon growth was tested, DBcAMP was found to be active. At the time the experiments described herein were carried out, it seemed unlikely that the activity of DBcAMP might be due to the presence of a butyryl substituent on the exocyclic nitrogen atom, since Skoog et al. (1967) had reported that amides of adenine were inactive in the tobacco pith cytokinin bioassay. 6-Butyrylaminopurine, however, stimulated cotyledon growth considerably more strongly than did DBcAMP. Hence, the activity of the cyclic nucleotide appeared to be due entirely to its 6-butyrylaminopurine moiety. This result indicated that the cytokinin activity of 6-acylaminopurines (amides of adenine) should be re-assessed. Subsequently, the cytokinin activity of several amides of adenine, including DBcAMP and 6-butyrylaminopurine, was confirmed in several bioassay systems including soybean tissue culture. These results, which are not detailed in this thesis, have appeared elsewhere (Letham et al., 1972). Similar results have been reported by Dekhuijzen and Overeem (1972). Letham et al. (1972) showed that the amide 6-benzoylaminopurine was almost as active as its alkyl analogue BAP, but was not metabolized to this cytokinin \textit{in vivo}; indeed the amide appeared to be remarkably stable compared with BAP, a feature of possible horticultural significance. The demonstration that certain amides of adenine, including 6-butyrylaminopurine, possess
cytokinin activity in plants raises interesting questions concerning the role of the 6-butyrylaminopurine moiety of DBCAMP in animal systems. On the other hand these findings do not support the possibility that cAMP acts as a mediator of cytokinin action in plants.

In view of the results presented in this chapter, it appeared unlikely that the growth response evoked by BAP in radish cotyledons was mediated by compounds which effect certain other phytohormonal responses. The remainder of Part I of this thesis records attempts to define some of the biochemical events evoked by BAP.
CHAPTER 4: THE EFFECT, ON THE GROWTH RESPONSE, OF INHIBITORS OF MACROMOLECULAR BIOSYNTHESIS AND MITOCHONDRIAL FUNCTION

A. INTRODUCTION

Two important aspects of cellular metabolism which cytokinins are widely acknowledged to influence are the synthesis of macromolecules and respiration. The possibility that cytokinins may regulate growth by affecting these processes was discussed in the introductory chapter. It was noted there was the lack of convincing evidence for effects of cytokinins upon metabolism of macromolecules before the onset of a growth response. In addition, an examination of the literature on the influence of cytokinins on respiratory processes revealed considerable diversity in the response of different tissues to cytokinins. In some tissues respiration was stimulated, in others inhibited, and in a few instances respiration was independent upon cytokinins. Experimentation using inhibitor studies were appropriate initially because of their rapidity and convenience. It was, of course, realized that the significance of the results obtained with inhibitors of the above processes would be limited by two considerations. Firstly, when a hormonally induced physiological response is blocked by an inhibitor of, for example, mRNA synthesis, all that is shown is a requirement for continued RNA synthesis for the accomplishment of the event. Other evidence is required to establish that the hormonal response is due to a direct effect of the hormone on transcription. Secondly, unless inhibitory actions other than the intended one have been disproved, results obtained using an inhibitor should be interpreted with caution. Current information concerning the mode of action and the specificity of inhibitors used in this chapter is outlined below.

5-fluoro-2'-deoxyuridine (FUDR) inhibits the synthesis of DNA. When converted in vivo to the 5'-monophosphate derivative, FUDR is a potent inhibitor of thymidylate synthetase which catalyses the conversion of deoxyuridylic acid to thymidylic acid. FUDR binds specifically and irreversibly to
A. INTRODUCTION

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the enzyme, thus blocking the biosynthesis of thymidylate (Cohen et al., 1958; Hartmann and Heidelberger, 1961).

Specificity of the action of FUDR is strongly suggested by the ability of exogenous thymidine to alleviate the inhibitory effects (see e.g. Cohen et al., 1958; Nitsan and Lang, 1965; Bopp et al., 1972). In addition, results of other specificity tests with plant tissues have now been reported. Thus, FUDR completely inhibited DNA synthesis in normal wheat seedlings when applied at concentrations that had no effect on the growth of gamma-irradiated wheat seedlings, which lack the capacity for DNA synthesis (Haber and Schwarz, 1972). Hence, in this concentration range (10^-3 M to 10^-1 M), FUDR had no side effects in wheat seedlings. Similar results were obtained with lettuce seed germination.

Actinomycin D is widely used as an inhibitor of DNA-dependent RNA synthesis. This chromopeptide antibiotic interacts with the double-stranded DNA helix to form stable but reversible complexes. The formation of the complex inhibits the synthesis of all types of RNA on the DNA template, but particularly ribosomal RNA. The exact nature of the structure of the complex is not yet certain, but guanine appears to play an important role in creating a binding site for the antibiotic (see Goldberg and Friedman, 1971; Sobell et al., 1971). The action of actinomycin D (act. D) in animals and micro-organisms is generally accepted as being specific in its action and is not observed to bind to RNA. Although act. D has been found to inhibit DNA synthesis by intact cells or by isolated DNA polymerase, these effects require considerably higher levels of the antibiotic than does RNA inhibition (Goldberg and Friedman, 1971). However, it has been emphasized by Harris (1968) that act. D may in some cases produce drastic secondary effects. Thus, Loewenstein and Penn (1967) observed that act. D caused a decline in protein synthesis that can be reversed by glucose. Clearly this effect is unlikely to be associated with an inhibition of RNA synthesis.

In plant systems, little is known about possible effects of act. D other than the inhibition of RNA synthesis, but there are several reasons for assuming specificity of
1. Act. D suppresses the growth of DNA viruses but permits normal growth and multiplication of most RNA viruses (Reich and Goldberg, 1964), including certain species in plant tissues in which normal RNA synthesis is inhibited by the antibiotic (see Key, 1969).

2. Protein synthesis continues in plant tissues for several hours after RNA synthesis is inhibited, and the gradual decline in protein synthesis parallels the decay in functional polyribosomes (see Key, 1969). Thus, in barley aleurone layers in which the incorporation of uracil and uridine into RNA was inhibited by act. D, the antibiotic had no effect on leucine incorporation into protein or on leucine pool size (Goodwin and Carr, 1972); in addition, uptake of leucine and uridine or uracil were not affected.

3. Act. D did not alter the respiration rate of barley aleurone layers (Goodwin and Carr, 1972) or potato disks (Click and Hackett, 1963).


Cycloheximide (actidione) is an effective inhibitor of protein synthesis in the cells of higher plants and other eukaryotes, but does not influence protein synthesis in prokaryotes or in mitochondria and chloroplasts (Ennis and Lubin, 1964; Sisler and Siegel, 1967; Stewart, 1973). The antibiotic acts on 80S (cytoplasmic) ribosomes inhibiting both initiation and translocation (see Stewart, 1973). The inhibition is reversible and, unlike the action of puromycin, is not accompanied by premature release of polypeptide chains (Ennis and Lubin, 1964). The specificity of cycloheximide has been questioned, however. Thus, MacDonald and Ellis (1969) reported that it inhibited the uptake of chloride in storage tissue disks of beet, carrot and potato, and in roots of wheat and pea plants. It was also found that oxygen uptake was markedly stimulated by the antibiotic in beet and carrot disks, slightly stimulated in potato disks, and not affected in wheat or pea roots. These
findings were considered to suggest that cycloheximide can affect cellular metabolism in other ways than by the inhibition of protein synthesis, for example, by interfering with the energy supply (Ellis and MacDonald, 1970). However, Kirk (1970) has reported that these workers could find no effect of cycloheximide (100 µg/ml) on respiration or chloride uptake in *Lemna gibba* plants and in leaf disks of lettuce, leek and cabbage. In addition, Kirk himself found that cycloheximide had no effect on respiration or motility of *Euglena gracilis*. He concluded that cycloheximide can still be used, in green cells at least, as a selective inhibitor of protein synthesis on 80S ribosomes. Other reports indicate a specific effect of cycloheximide on protein synthesis in higher plants, even in etiolated tissues (Abeles and Holm, 1966; Walton, 1966; Kang and Ray, 1969). It should be mentioned that in some animal and plant systems, cycloheximide has been reported to inhibit DNA synthesis (Bennett et al., 1964) and formation of 18S RNA (Mayo et al., 1968), and to cause accumulation of nascent RNA in the nucleus, presumably as a result of either increased RNA synthesis or inhibition of RNA transport to the cytoplasm (Waters and Dure, 1966). Although each of these effects may well be due to the absence of specific proteins, the synthesis of which is inhibited by cycloheximide, this has not yet been demonstrated.

The antibiotic action of D-threo chloramphenicol (CAP) in prokaryotic organisms results from inhibition of protein synthesis through specific association of the compound with the 70S ribosomes of these organisms. CAP does not bind to the 80S cytoplasmic ribosomes of eukaryotic cells (Vazquez et al., 1969), and Ellis (1964) has questioned claims that it can inhibit general protein synthesis in plants. The antibiotic appears able to associate with the prokaryotic-like ribosomes of plastids (Smillie et al., 1968), and stereospecific inhibition of protein synthesis has been demonstrated in isolated chloroplasts (Ellis, 1969) and plant and animal mitochondria (Ellis and MacDonald, 1968; Freeman, 1970). *In vivo*, CAP inhibited formation of a number of chloroplast enzymes in *Euglena gracilis* (Smillie et al., 1968) and specifically
blocked formation of cytochrome oxidase in regenerating rat liver (de Vries and Kroon, 1970). CAP may, therefore, indirectly affect many metabolic processes of eukaryotic cells through a direct effect on protein synthesis in the energy-convert ing organelles. Plant tissues vary considerably in their sensitivity to CAP. Thus, Masuda (1966) and Setterfield (1970) found that auxin-induced cell expansion of Jerusalem artichoke tuber tissue was inhibited by very low CAP concentrations, of the order required for marked inhibition of protein synthesis in prokaryotes. In general, however, most plant and animal tissues are insensitive to these low concentrations of the antibiotic (see Setterfield, 1970, for a compilation of some reported inhibitory actions of CAP in plants, and the concentrations of CAP that were used). Especially in cases where high CAP concentrations are required to inhibit protein synthesis or inhibit some physiological event, possible side effects of CAP must be considered. There is evidence that the L-isomer of CAP, which is not an antibiotic and has little effect on protein synthesis, is nevertheless metabolically active in plants. Like CAP itself, L-threo CAP inhibits elongation of lupin roots (Ronnike, 1958), salt uptake by root tissues of several higher plants (Ellis, 1963) and oxidative phosphorylation by maize mitochondria (Ellis and MacDonald, 1970). The lack of stereospecificity of these effects strongly suggests that they are not caused by CAP-inhibited protein synthesis.

To examine the relevance of mitochondrial function to BAP-induced radish cotyledon growth by use of an inhibitor, the antibiotic ethidium bromide was employed. This phenanthridine intercalates with DNA thereby impairing template function (Waring, 1968). RNA synthesis in mitochondria appears to be particularly sensitive to the inhibitor and as a result mitochondrial development and function are impaired. Thus, in HeLa cells, ethidium bromide selectively inhibits synthesis of mitochondrial RNA at concentrations which have little or no effect on synthesis of all other species of cellular RNA (Zylber et al., 1969). Binding of ethidium bromide to the circular DNA of mitochondria
probably causes marked changes in tertiary structure, while binding to linear nuclear DNA does not (see references cited by Zylber et al., 1969). In *Acetabularia*, the inhibitor caused cessation of synthesis of all cytoplasmic DNA but did not inhibit nuclear DNA synthesis. A marked decrease in respiratory and cytochrome oxidase activity indicated that the inhibitor impaired mitochondrial function. The activity of chloroplasts was not affected however (Heilporn and Limbosh, 1971). In *Euglena* ethidium bromide did not affect chloroplast development but did inhibit very effectively the development of mitochondrial function. Lack of side effects with ethidium bromide cannot be assumed, however, since the antibiotic can bind to RNA molecules with base pairing in their secondary structure, and is known to inhibit aminoacylation of tRNA (see Lurquin and Buchet-Mahieu, 1971). Moreover, Bottomly et al. (1971) have reported some inhibition of RNA synthesis by ethidium bromide, in both nuclei and chloroplasts.

It was found that the respiration rate of radish cotyledons could be simply assessed directly, using an oxygraph, and the experimental results obtained with this equipment are also presented in this chapter because of their relevance to the experiments with ethidium bromide.

B. MATERIALS AND METHODS

1. Inhibitors

<table>
<thead>
<tr>
<th>Chemical*</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>act. D (Actinomycin D)</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>CAP (D-threo chloramphenicol)</td>
<td>Sigma</td>
</tr>
<tr>
<td>cycloheximide</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>ethidium bromide</td>
<td>Boots Pure Drug Co.</td>
</tr>
<tr>
<td>FUDR (5-fluoro-2'-deoxyuridine)</td>
<td>Hoffman-La Roche</td>
</tr>
</tbody>
</table>

* Chemicals were used as solutions (pH 6) in 2 mM phosphate buffer with or without BAP.
2. Radish cotyledon culture and statistical analysis of data

Radish cotyledons were cultured as previously described (section 2.1). In each experiment, unless otherwise stated, cotyledons were incubated for 3 days in continuous light. Mean cotyledon weights were determined from four batches of 10 cotyledons. Results were statistically analysed as indicated in section 2.3.

3. Determination of chlorophyll in cotyledons

Cotyledons were weighed and then dropped into 80% ethanol at 75°C. After 10 minutes at this temperature the solutions were cooled and centrifuged, and adjusted to a volume of 1 ml per cotyledon. The absorbance was then measured at 663 nm.

4. Determination of respiration rates

Batches of 20 radish cotyledons were grown in Petrie dishes in (+BAP) or (-BAP) phosphate buffer (three replicate batches per treatment). At the start of the incubation, and at subsequent intervals, each batch in turn was placed in the chamber of an oxygraph (Yellow Springs Instruments, U.S.A.). For each recording, the chamber was filled with 3 ml of fresh phosphate buffer (with or without BAP as appropriate) which had previously been equilibrated to the operating temperature of 25°C. The oxygraph recorder was then adjusted to give an oxygen tension reading of 100%. The rate of oxygen uptake (i.e. the % reduction in oxygen tension per ml per minute either per cotyledon or per gram fresh weight of tissue) was estimated by calculating the time each batch took to reduce the oxygen tension from 80% to 40%. Rate of oxygen uptake was approximately linear between 90% and 30% oxygen tension at 25°C. Batches of cotyledons were weighed after each recording.

C. RESULTS

5. Effect of FUDR

BAP-induced radish cotyledon expansion was found to be strongly inhibited by FUDR. The results of an experiment
in which the cotyledons were cultured in light are presented in Table 4-1. In the presence of 50 µg/ml of FUDR, BAP did not evoke a significant growth response; at 10 µg/ml of FUDR, the BAP-induced growth increment was 28% of the increment induced in the absence of the inhibitor. In darkness a similar inhibition was observed.

Table 4-1 Mean fresh weights of radish cotyledons grown in light on (+BAP) or (-BAP) media and in the presence of various concentrations of FUDR. Means lacking a common letter are significantly different at the 5% level.

<table>
<thead>
<tr>
<th>FUDR concentration (µg/ml)</th>
<th>Mean cotyledon weight (mg)</th>
<th>(-BAP) medium</th>
<th>(+BAP) medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>19.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>13.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td>13.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

5-1. Reversal of FUDR inhibition by thymidine

To ascertain whether the FUDR inhibition of cytokinin-induced growth was indeed due to an inhibition of thymidylate synthesis, the effect of exogenous thymidine on the inhibition was examined (Table 4-2).

Table 4-2 Mean fresh weights of radish cotyledons grown on (+BAP) medium, with or without FUDR (10 µg/ml), and in the presence of various concentrations of thymidine. D(5%) for significance = 5.2

<table>
<thead>
<tr>
<th>Thymidine concentration (µg/ml)</th>
<th>Mean cotyledon weight (mg)</th>
<th>(-BAP) medium</th>
<th>(+BAP) medium</th>
<th>(+BAP) + FUDR medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>18.1</td>
<td>31.0</td>
<td>14.9</td>
</tr>
<tr>
<td>120</td>
<td></td>
<td>-</td>
<td>-</td>
<td>29.5</td>
</tr>
<tr>
<td>240</td>
<td></td>
<td>-</td>
<td>31.1</td>
<td>34.7</td>
</tr>
</tbody>
</table>

A thymidine concentration of 240 µg/ml completely alleviated the inhibition of BAP-induced growth caused by FUDR. Thymidine itself had no effect on BAP-induced growth.
6. **Effect of act. D**

The BAP-induced growth of radish cotyledons was found to be sensitive to act. D both in light and in darkness. The lowest concentration to inhibit the response almost completely (> 93%) was 20 µg/ml. At 20 µg/ml, however, act. D appeared to have little effect on control cotyledon growth. Increasing the concentration of act. D to 100 µg/ml caused further reduction in growth of both (+BAP) and (-BAP) cotyledons, and retarded greening of cotyledons grown in light. The lower concentration (20 µg/ml) was therefore used for subsequent experiments.

6-1. **Time of onset of inhibition induced by act. D**

(+BAP) and (-BAP) cotyledons were cultured in the presence and in the absence of act. D (20 µg/ml) and the time course of their growth was studied by weighing the batches of cotyledons at intervals (Figure 4-1). Although cotyledon growth in the absence of BAP was not appreciably inhibited by act. D, cytokinin-induced growth was markedly inhibited. The antibiotic strongly inhibited the initial cytokinin-induced growth (i.e. that occurring between 10 and 20 hours). However, throughout the incubation period, and especially during the first 2 days, the rate of growth of (+BAP) cotyledons was considerably greater in the absence of act. D than in its presence. Hence the antibiotic appeared to inhibit the later stages of BAP-induced growth as well as the initial growth response. To confirm this, cotyledons were grown for 24 hours in the presence of BAP alone, and then transferred to media containing act. D (Figure 4-2). Subsequent growth in the presence of BAP was found to be markedly inhibited by the antibiotic.

6-2. **The effect of increasing BAP concentration on the inhibition by act. D**

The results obtained above indicated a requirement for DNA-dependent RNA synthesis for the BAP-induced growth of radish cotyledons. Since the results of other workers have suggested that cytokinins could compete with act. D for binding sites on DNA (see Roychoudhury et al.,
Figure 4-1  Mean fresh weights of (+BAP) and (-BAP) cotyledons cultured in the presence or absence of act. D

The batches of cotyledons were weighed at intervals throughout the culture period.

Treatments were as follows:

- ••• (+BAP)
- •• (+BAP) + act. D
- o---o (-BAP)
- o--o (-BAP) + act. D

D(5%) for significance =
1.12 (10 hours)
1.39 (21 hours)
1.86 (29.5 hours)
3.84 (51.5 hours)
3.70 (74 hours)

Sequential testing showed that:

At 5 and 10 hours, no means differed significantly.
At 21 hours and at subsequent times, (+BAP) mean > all other means.
At 29 hours and at subsequent times, [(+BAP) + act. D] > [(-BAP) + act. D]
At 74 hours [(+BAP) + act. D] > (-BAP)

Throughout the incubation period,

(-BAP) + [(-BAP) + act. D]
(LSD at 74 hours = 2.72).
The graph shows the relationship between cotyledon weight (mg) and time (hours). The x-axis represents time in hours ranging from 0 to 70, while the y-axis represents cotyledon weight in mg ranging from 0 to 35. Three distinct lines are plotted, each representing different intervals, with the solid line indicating the progression over time.
Figure 4-2  Mean fresh weights of radish cotyledons cultured in the presence of act. D after a 24-hour pre-incubation in its absence

After 24 hours of (+BAP) or (-BAP) culture, batches of radish cotyledons were transferred to media containing act. D, as indicated below, and cultured for a further 4 days, during which they were weighed at intervals.

Treatments were as follows:

- ••• transferred from (+BAP) to (+BAP).
- •--• " " " (+BAP) + act. D.
- o--o transferred from (-BAP) to (-BAP).
- o--o " " " (-BAP) + act. D.

D(5%) for significance =

- 2.01 (24 hours)
- 3.13 (72 hours)
- 5.27 (120 hours)

Sequential testing did not reveal any additional significant differences.
1965), it was of interest to compare the growth of cotyledons on media containing increasing concentrations of BAP in the absence and presence of a constant concentration of act. D. The mean fresh weights of the cotyledons after 3 days are shown in Figure 4-3. Increasing the BAP concentration above 25 μM, in the absence of act. D, suppressed growth (c.f. section 2.4-1 in which the same effect was noted). In the presence of act. D, however, this suppression was not observed. At high BAP concentrations, the absolute inhibition caused by act. D was less than that observed at low BAP concentrations.

7. **Effect of cycloheximide**

Growth of both (+BAP) and (-BAP) cotyledons was markedly inhibited by low concentrations of cycloheximide: the lowest concentration to cause detectable growth inhibition was 0.5 μg/ml for (+BAP) cotyledons, and 1 μg/ml for -BAP cotyledons. At a concentration of 20 μg/ml, the antibiotic almost completely arrested all growth and caused death of about 25% of both (+BAP) and (-BAP) cotyledons during the 3-day incubation period. A cycloheximide concentration of 1 μg/ml caused marked (> 90%) inhibition of BAP-induced growth without appreciably inhibiting control growth and without apparently killing any cotyledons. Hence this concentration of cycloheximide was used in the experiment described below.

7-1. **Effect of cycloheximide on the initial and later phases of BAP-induced growth**

Cotyledons were cultured on (+BAP) and (-BAP) media containing cycloheximide (1 μg/ml), while other cotyledons were grown on (+BAP) and (-BAP) media for 18 hours before transfer to media containing cycloheximide. At 18 hours and at subsequent intervals, all batches of cotyledons were weighed to compare their growth rates (Figure 4-4). Both the initial BAP-induced growth, and that evoked by the cytokinin after 18 hours, appeared to be completely abolished by cycloheximide. It was of interest that the addition of cycloheximide after 18 hours had a toxic effect on (+BAP) cotyledons, 86% of which died between 1 and 2 days after the addition of the antibiotic. (+BAP) cotyledons
Figure 4-3  Mean fresh weights of radish cotyledons cultured at various concentrations of BAP in the presence or absence of act. D (20 µg/ml)

The batches of cotyledons were weighed after 3 days' culture.

Treatments were as follows:

- - - - act. D.
- - - act. D.

Mean fresh weight of (-BAP) cotyledons (grown in absence of act. D) was 17.4 mg.

D(5%) = 6.76

Sequential testing showed that (+BAP) + act. D > (-BAP) + act. D at all tested concentrations of BAP.

(+BAP) - act. D > (+BAP) + act. D at highest tested concentration of BAP (i.e. 100 µM).
Figure 4-4  Mean fresh weights of (+BAP) and (-BAP) cotyledons grown in the presence or absence of cycloheximide (1 μg/ml).

The batches of cotyledons were weighed at intervals throughout the culture period.

Treatments were as follows:

- ••• (+BAP) throughout.
- ••• (+BAP) + cycloheximide, throughout.
- ▲..▲ (+BAP) for 18 hours, (+BAP) + cycloheximide thereafter.
- o--o (-BAP) throughout.
- o--o (-BAP) + cycloheximide, throughout.
- △..△ (-BAP) for 18 hours, (-BAP) + cycloheximide thereafter.

D(5%) for significance =

1.91 (18 hours)
2.76 (45 hours)
2.16 (70 hours)

Sequential testing did not reveal any additional significant differences.
exposed to cycloheximide from the time of excision were not thus affected although BAP-induced growth was strongly inhibited throughout the entire incubation period.

8. Effect of CAP

CAP (40 µg/ml) was observed to inhibit greening of radish cotyledons cultured in continuous light. It was therefore of interest to find out if the aspect of chloroplast function inhibited by the antibiotic (i.e. probably chloroplast protein synthesis) was involved in cytokinin-induced cotyledon expansion. Therefore cotyledons were cultured on (+BAP) and (-BAP) media with and without CAP (40 µg/ml). Batches of cotyledons were sampled at intervals, weighed and then extracted for chlorophyll determination. The results (Figure 4-5) indicated that CAP (40 µg/ml) inhibited both the onset and extent of greening of all radish cotyledons treated, but had no effect on growth until 72 hours when a small inhibition of both (+BAP) and control growth was observed. In two other experiments no significant effect on growth was observed at 72 hours, and even at a concentration of 100 µg/ml, CAP had no consistent effect on growth although greening was very markedly inhibited. The growth of cotyledons in darkness was also not affected by CAP (40 to 100 µg/ml).

9. Respiration

9-1. Effect of ethidium bromide

Ethidium bromide (0.1 mM or 39 µg/ml, in culture medium renewed daily) had no significant effect on the growth of either (+BAP) or (-BAP) radish cotyledons either in light or in darkness. A slight inhibition of growth was possibly indicated during the third day of incubation in the light, but was not consistently observed. The concentration of ethidium bromide used was slightly greater than that (25 µg/ml) which markedly reduced respiration rate in Acetabularia, and was 10 times the concentration which impaired mitochondrial function in Euglena [see Heilporn and Limbosch (1971) and Stewart and Gregory (1969) respectively]. These observations suggest that growth-induction by BAP was not dependent upon a
Figure 4-5 Mean fresh weights (A) and chlorophyll content (B) of (+BAP) and (-BAP) radish cotyledons grown in the presence or absence of CAP (40 µg/ml)

Batches of cotyledons were sampled at intervals. Each batch was weighed for calculation of mean cotyledon weight (A). Mean chlorophyll content per cotyledon (B) was estimated by extracting each batch of cotyledons in 80% ethanol at 75° for 10 minutes. Absorbance of the extracts was read at 663 nm.

Treatments were as follows:

- ••• (+BAP)
- •--• (+BAP) + CAP
- o--o (-BAP)
- o--o (-BAP) + CAP

At 72 hours,

D(5%) in cotyledon weight = 1.09
D(5%) in absorbance = 0.53
A

COTYLEDON WEIGHT mg

B

ABSORBANCE 663 nm

0 20 30 40 50 60 70
Figure 4-6 (A) Respiration rates of radish cotyledons cultured in darkness in the presence or absence of BAP

An oxygraph (section 4.3) was used to record the respiration rates of the batches of radish cotyledons at intervals during the incubation. During recording the cotyledons were kept in darkness. Respiration rates are expressed as the % reduction of oxygen tension per ml per minute, either per cotyledon or per g tissue fresh weight. Treatments and computation of respiration rate (R) were as follows:

- - - - (+BAP), R per cotyledon.
- - - - (+BAP), R per g fresh wt.
- - - - (-BAP), R per cotyledon.
- - - - (-BAP), R per g fresh wt.

In R / cotyledon,

(+BAP) > (-BAP) significantly at 45 hours (P = 0.01).
(+BAP) > (-BAP) almost significantly at 71 hours (P = 0.058).

In R / g fresh wt.,

(+BAP) < (-BAP) significantly at 71 hours (P = 0.012).

(B) Mean fresh weights of radish cotyledons cultured in the presence and absence of BAP

The batches of radish cotyledons referred to above (Figure 4-6, A) were weighed immediately after each recording of respiration rate. Treatments were as follows:

- - - - (+BAP)
- - - - (-BAP)
stimulation in respiration rate (see discussion of ethidium bromide in the introduction). The effect of BAP on respiration rate was studied more directly as described below.

9-2. **Effect of BAP on respiration rate**

Measurements of respiration rate, derived from oxygraph recordings, showed that there was no consistent early effect of BAP on the respiration rate of radish cotyledons grown in continuous light. Of three similar experiments only one showed a small significant increase in the respiration rates of (+BAP) cotyledons at 3.5 hours after the start of incubation. On a per cotyledon basis, but not on a per unit tissue weight basis, the respiration rate in the presence of BAP was consistently higher than the control rate between 1 and 2 days. At 3 days, the respiration rate of (+BAP) cotyledons per g of tissue weight was significantly lower than the control rate.

To eliminate possible effects of photosynthesis on the apparent rate of oxygen consumption, further radish cotyledons were incubated (with or without BAP) in the dark, and were also kept in darkness during recording of their rate of oxygen consumption. The results obtained (Figure 4-6) were very similar to those derived from light-grown cotyledons. Thus, there was no significant effect of BAP on respiration rate until 45 hours when the rate per cotyledon in (+BAP) cotyledons was 44% higher than the control rate. As in light-grown cotyledons, however, there was no significant difference between the respiration rates per unit tissue fresh weight at this time, while at 71 hours the rate per g of (+BAP) cotyledons was significantly (35%) lower than that of the controls.

D. **DISCUSSION**

The results reported in this chapter show that the BAP-induced growth of radish cotyledons is markedly depressed by inhibitors of DNA, RNA and protein synthesis. FUDR (50 μg/ml) caused 89% inhibition of BAP-induced growth, and control growth was inhibited by about 30%.
This concentration of FUDR is well within the range that is known to produce no side effects in wheat seedlings and lettuce seeds (Haber and Schwarz, 1972). Hence the effect of FUDR on radish cotyledon growth is very probably due to inhibition of DNA synthesis. This conclusion is further supported by the ability of thymidine to alleviate the inhibition completely. Hence BAP-induced expansion of radish cotyledons, a growth response which in the light is due principally to cell expansion, appeared to be almost completely suppressed by inhibition of DNA synthesis. In contrast, FUDR did not inhibit cytokinin-induced expansion of fenugreek cotyledons (Rijven and Parkash, 1971).

DNA synthesis could be required for BAP-induced radish cotyledon cell expansion for several reasons. The growth response involves some stimulation of cell division and the cell enlargement observed could be dependent on this division. In certain tissues, cell enlargement appears to be associated with DNA synthesis, even when the elongating cells will not divide any further. To explain the ability of both FUDR and the mitotic inhibitor colchicine to suppress cell elongation in seedling stems, Atsmon (1972) suggested that certain cell expansion responses might depend on meristematic activity within and around the expanding region (c.f. Holm and Key, 1969). The reason that expansion of some cells should require the division of others is not clear. Mechanical considerations could be important; for example, an inhibition of cell division in epidermal and vascular portions of radish cotyledons could restrict expansion of mesophyll cells. Conceivably, cell enlargement could depend on the secretion of some stimulatory substance by dividing cells.

On the other hand it has been suggested that the expansion of a particular cell may require DNA synthesis within that cell. This appeared to be the case in seedling stem tissue of lentil and Sinapsis alba, for FUDR imposed a thymidine-reversible inhibition on cell elongation in these tissues in the absence of the apical meristem (Nitsan and Lang, 1965, 1966; Bopp et al., 1972). In these experiments there was possibly a small amount of cell division occurring...
in provascular tissue. However, the specific cell expansion response evoked by auxin in Jerusalem artichoke tuber tissue (Kamisaka and Masuda, 1970b) and tobacco pith tissue (Maheshwari and Noodén, 1971) is also inhibited by FUDR in a thymidine-reversible manner, and cambial tissue would have been eliminated from the latter tissue at least. Observations of \[^{3}H\]thymidine incorporation into nuclei of differentiated, nondividing cells in animal tissues have also been reported (Pelc, 1964; Wessells, 1964) and in some dipteran species a highly localized DNA replication in single or a few bands of salivary gland chromosomes was observed (Picq and Pavan, 1957; Rudkin and Corlette, 1957). This might suggest that, for the proper operation of at least some genes, DNA synthesis has to take place. Metabolic turnover of "low-molecular-weight DNA" has been shown to occur in growing plant tissues (Sampson et al., 1963; Stern, 1964). Whether this turnover occurred in the cell nucleus or in other cell organelles was not known. However, Degani and Atsmon (1970) reported that in cucumber hypocotyls, the synthesis of metabolically labile DNA, which accompanied normal or hormonally (IAA or GA) accelerated cell elongation, was mostly extranuclear and was concentrated in the chloroplasts. Inhibition of cell elongation, by mannitol or by decapitation, reduced incorporation of labelled thymidine into this DNA, indicating a close correlation between elongation and DNA synthesis, but not necessarily a causal relationship between the two processes (Atsmon, 1972). In the radish cotyledon system, synthesis of extranuclear DNA seems unlikely to be an important requirement for BAP-induced cell expansion: ethidium bromide did not inhibit the growth response, and there was also no significant effect of CAP, which interferes with chloroplast function at least at the level of translation, and possibly also at the level of DNA synthesis (c.f. Kern and Lang, cited by Atsmon, 1972).

The relationship between DNA synthesis and cell enlargement is still obscure. Substantial increases of the DNA content have long been known to occur in elongating and differentiating plant cells during the establishment of polyploidy and polyteny. However, there is presently no
evidence that these phenomena are essential for the growth and differentiation of plant cells. Certainly in cotyledon tissue, the available evidence indicates that the increase in DNA level induced by cytokinins is not sufficiently marked to suggest that either polyploidy or polyteny occurs during the growth response (Sveshnikova and Khokhlova, 1969; Rijven and Parkash, 1971); moreover, results presented in Chapter 5 indicate that this conclusion may also be applied to radish cotyledons, since BAP-induced growth is neither sufficiently marked nor sufficiently early. Further work is needed to determine conclusively whether synthesis of particular fractions of either nuclear or cytoplasmic DNA is necessary for cell expansion, or whether such synthesis is simply a normal result of this expansion.

The kinetics of the inhibition of BAP-induced cotyledon expansion by act. D indicated a requirement for RNA synthesis both in the initial and in the later (after 24 hours) stages of the growth response. In contrast to the BAP-induced growth, however, control growth was not significantly inhibited by act. D. This result was of interest since it suggested that the control growth may not be due to endogenous cytokinin remaining in the cotyledons after excision. The result also suggested that growth in the control tissue depended on pre-existing and probably stable RNA while BAP-induced growth was dependent on new synthesis of RNA species, probably of relatively short half life. In relation to the growth of control cotyledons it is relevant to note that inhibition of RNA synthesis by act. D failed to inhibit much of the protein synthesis that takes place in the cotyledons of cotton seeds during the first 3 days of germination (Ihle and Dure, 1972). The probable occurrence of stored messenger RNA in seeds is well known (see references cited by Ihle and Dure, 1972, and Chen and Osborne, 1970).

The act. D inhibition of BAP-induced growth contrasts with the effect of act. D on auxin-induced growth of pea stem segments (Penny and Galston, 1966). Thus, in segments in which RNA synthesis was inhibited by act. D, IAA induced appreciable growth stimulation for 2 to 3 hours before the inhibitory effect of act. D was observed. Such a result
is in agreement with the conclusion of Nissl and Zenk (1969) and of Warner and Leopold (1971) that the initial growth response of auxins and cytokinins is too rapid, at least in some tissues, to depend on RNA and protein synthesis. If there was any such rapid growth response in the radish cotyledon system, it was too minor to detect. The observed inhibition by act. D of the later growth of cotyledons on (+BAP) medium (Figure 4-2) was not unexpected, since BAP does not seem to act as a trigger in this system, but rather to be required for at least 24 hours to effect a maximal growth response (see section 2.5). It is interesting that high concentrations of BAP depressed cytokinin-induced growth in the absence of actinomycin D, but did not have this effect in the presence of the antibiotic. This result is consistent with an alleviation by BAP of act. D-imposed growth inhibition, and hence is also consistent with a mode of action of the cytokinin which involves an interaction with the genetic material, a possibility discussed in section 1.5-2. However, the result could be interpreted in other ways.

Using methods described in Chapter 5, it was shown that act. D (20 µg/ml) depressed by 60% the specific activity of RNA in (+BAP) cotyledons supplied with [H]-uridine between 6 and 10 hours after the start of the incubation period. Similarly, Rijven (1972) has reported that in isolated fenugreek cotyledons, act. D inhibited both the growth response and the net RNA synthesis promoted by cytokinins.

A requirement for RNA synthesis implies also a requirement for protein synthesis, and indeed the kinetics of the inhibition of BAP-induced growth by cycloheximide resembled those of the act. D inhibition. Thus the effect of cycloheximide was apparent both as soon as BAP-induced growth was detectable in cotyledons not exposed to the antibiotic and also in the later stages of BAP-induced growth. Prolonged exposure to cycloheximide also inhibited growth of control cotyledons that were treated with the antibiotic from the time of excision although, as with act. D, control growth was inhibited far less than cytokinin-induced growth.
At the same concentration as was used herein, cycloheximide likewise strongly inhibited fenugreek cotyledon growth (Rijven and Parkash, 1971). It is of interest that cycloheximide does not inhibit the initial auxin-induced growth of lupin hypocotyls (Penny, 1971) or wheat coleoptile sections (Pope and Black, 1972).

While protein synthesis on cytoplasmic ribosomes thus appears to be essential for BAP-induced growth of radish cotyledons, chloroplast function, including synthesis of protein by 70S ribosomes, does not seem to be necessary for this growth response. Concentrations of chloramphenicol which markedly inhibit greening of the cotyledons, have no significant effect on cotyledon expansion earlier than 72 hours, a result in harmony with those obtained by Rijven and Parkash (1971) using fenugreek cotyledons. Since certain tissues lacking chlorophyll (e.g. albino barley leaves) do not respond to cytokinin (Mothes, 1964; Srivastava, 1963) it has been suggested that cytokinins may exert their primary influence on the chloroplasts. However, the results reported above with cotyledon tissue do not support this idea.

Studies on the effect of BAP on the respiration rate of radish cotyledons corroborated the observation that ethidium bromide had no significant effect on BAP-induced (or control) growth. BAP had no consistent early effect on respiration (i.e. within 5 hours) whether the cotyledons were incubated in light or in darkness; a slight, significant early stimulation of the respiration rate was observed only once (with light-grown cotyledons) in a total of four experiments. The only consistent effect of BAP on cotyledon respiration in equal weights of tissue was a significant depression of the rate after about 70 hours of incubation. Such a late effect could well be consistent with reports that the delay of senescence of detached leaves by cytokinins is accompanied by a depressed respiration rate (see section 1.5-4), but this depression is clearly not required for cytokinin-induced cotyledon growth.

The inhibitor studies described in this chapter suggested that the cytokinin-induced growth of radish cotyledons was unlikely to require metabolic changes that
result in alterations of respiration rate, but appeared to be dependent on the synthesis of macromolecules. The marked and early requirements for synthesis of nuclear DNA, RNA and protein translated in the cytoplasm clearly merited further investigation. The results of such studies are presented in the remaining chapters of this part of the thesis.
CHAPTER 5  :  NUCLEIC ACID METABOLISM DURING CYTOKININ TREATMENT

A. INTRODUCTION

All five types of phytohormones are known to influence nucleic acid metabolism (Key, 1969). Evidence for effects of cytokinins on these macromolecules has been briefly outlined in Chapter 1, which discussed the relevance of these effects to the molecular basis of cytokinin action. There appears to be little doubt that cytokinins can markedly influence the metabolism of RNA, in some cases very rapidly, but no compelling evidence that such changes precede cytokinin-induced physiological responses and therefore be essential for their initiation. This lack of decisive information is apparent in Table 5-A, which summarizes a selection of published observations on cytokinin effects on RNA metabolism. The examples are taken from systems in which the cytokinin influences growth as it does in redish brown meristematic tissue and some are, of course, not confined to systems in which growth is promoted. There has been considerable interest, for example, in the reputed ability of cytokinins to enhance transcription in isolated nuclei within minutes (1.5-2) and it has been realized for some time that the delay of leaf senescence by cytokinins is associated with their ability to maintain or increase the levels of RNA and proteins in these organs (Ker, 1961; 1967; Osborn, 1962; Key, 1969; Nemé, 1971).

There is little information on effects of cytokinins on DNA, and, as with RNA, it is uncertain whether increased synthesis of DNA is required for the initiation of cytokinin-induced growth. Several workers (e.g. Osborn, 1964; Jensen et al., 1964) have reported that kinetin does not stimulate DNA synthesis. It is relevant, however, that in some growing tissues stimulation has been reported. Thus, in tobacco pith, cytokinin markedly promoted DNA synthesis and was required for mitosis and cytokinesis (Hata, 1957). However, a proportion of the tetraploid cells present were stimulated by kinetin to enter mitosis and undergo cytokinesis without thymine incorporation.
A. INTRODUCTION

All five types of phytohormones are known to influence nucleic acid metabolism (Key, 1969). Evidence for effects of cytokinins on these macromolecules has been briefly outlined in Chapter 1, which discussed the relevance of these effects to the molecular basis of cytokinin action. There appears to be little doubt that cytokinins can markedly influence the metabolism of RNA, in some cases very rapidly, but no compelling evidence that such changes precede cytokinin-induced physiological responses and may hence be essential for their initiation. This lack of decisive information is apparent in Table 5A, which summarizes a selection of published observations on cytokinin effects on RNA metabolism. The examples are taken from systems in which the cytokinin influences growth as it does in radish cotyledons. However, cytokinin effects on RNA are, of course, not confined to systems in which growth is promoted. There has been considerable interest, for example, in the reputed ability of cytokinins to enhance transcription in isolated nuclei within minutes (1.5-6) and it has been realized for some time that the delay of leaf senescence by cytokinins is associated with their ability to maintain or enhance the levels of RNA and proteins in these organs (see Wollgiehn, 1961, 1967; Osborne, 1962; Key, 1969; Kende, 1971).

There is little information on effects of cytokinins on DNA, and, as with RNA, it is uncertain whether increased synthesis of DNA is required for the initiation of cytokinin-induced growth. Several workers (e.g. Osborne, 1962; Jensen et al., 1964) have reported that kinetin does not stimulate DNA synthesis. It is relevant, however, that in some growing tissues stimulation has been reported. Thus, in tobacco pith, cytokinin markedly promoted DNA doubling and was required for mitosis and cytokinesis (Patau et al., 1957). However, a proportion of the tetraploid cells present were stimulated by kinetin to enter mitosis and undergo cytokinesis without thymine incorporation.
Table 5A - Some effects of cytokinins on RNA metabolism

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Concentration</th>
<th>Effect</th>
<th>Plant Material</th>
<th>Timing of effect</th>
<th>Correction(s) applied</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kinetin</td>
<td>4.6 μM</td>
<td>Induces RNA accumulation in dividing and interphase nuclei.</td>
<td>onion roots</td>
<td>N (&lt; 1 hr)</td>
<td>-</td>
<td>Guttman, 1957</td>
</tr>
<tr>
<td>Kinetin</td>
<td>4.6 μM; 23 μM for kinetin.</td>
<td>Above effect not observed.</td>
<td>onion roots</td>
<td>N (&lt; 1 hr)</td>
<td>-</td>
<td>Chaly and Setterfield, 1972</td>
</tr>
<tr>
<td>Kinetin</td>
<td>4.3 μM; 21.5 μM for BAP.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kinetin</td>
<td>10 μM</td>
<td>Inhibits abscisic acid effects on 25S and 18S RNA, namely the promotion of (^{32}P) labelling and the increase in (\frac{A+U}{C+G}) and (\frac{U}{G}) ratios.</td>
<td>Lens culinaris root tips</td>
<td>N (5 hr)</td>
<td>-</td>
<td>Khan et al., 1970</td>
</tr>
<tr>
<td>Kinetin</td>
<td>20 μM</td>
<td>Reverses inhibition, by abscisic acid, of (^{32}P) incorporation into total rRNA, tRNA, sRNA and DNA-RNA hybrid; promotes labelling of tRNA.</td>
<td>pear embryos</td>
<td>N (&lt; 5 hr)</td>
<td>-</td>
<td>Khan and Heit, 1969</td>
</tr>
<tr>
<td>Kinetin</td>
<td>120 μM</td>
<td>Inhibits synthesis of rRNA completely and of tRNA partially. Inhibition stronger in tissue treated with auxin, growth induction by which is also inhibited.</td>
<td>elongating region of soybean hypocotyl</td>
<td>A (probably); (about 8 hr)</td>
<td>-</td>
<td>Vanderhoef and Key, 1968</td>
</tr>
<tr>
<td>Kinetin</td>
<td>1 μM</td>
<td>Stimulates incorporation of labelled uracil into nuclear RNA.</td>
<td>de-differentiating isolated moss leaves</td>
<td>N (2 hr)</td>
<td>Upt.</td>
<td>Giles, 1971</td>
</tr>
<tr>
<td>Kinetin</td>
<td>4.65 μM</td>
<td>Causes 15-fold increase in RNA with change in base composition to one like DNA.</td>
<td>Funaria protonema</td>
<td>N7(14 days)</td>
<td>c.f. induced buds visible at 21 days</td>
<td>Schneider et al., 1969</td>
</tr>
</tbody>
</table>
Table 5A continued

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Concentration</th>
<th>Effect</th>
<th>Plant Material</th>
<th>Timing of effect</th>
<th>Correction(s) applied</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Kinetin | 10 µg/ml      | Increases levels of all RNA species and markedly increases the $^{32}$P specific activity of a species thought to be mRNA. 
|         |               |        | tobacco pith tissue | N (probably A) | -                      | Srivastava, 1967a. |
| BAP     | 1 mM in 2 µl  | Increases RNA level and incorporation of labelled uridine into RNA. | the inhibited auxiliary buds of *Cicer arietinum* | N (3 to 6 hr) | -                      | Usciati et al., 1972. |
| Kinetin | 1.15 µM       | Increased rate of RNA synthesis by over 100% and decreased the rate of RNA degradation by 60%. | *Spirodela aligorthiza* (dormant plants in darkness). | N (probably B) | Upt. and sp. McCombs and Ralph, 1972 act. of prec. pool accounted for. |
| BAP     | 43 µM         | Increased RNA level and incorporation of $[^{14}C]$adenine into RNA. | pumpkin cotyledons | N (< 24 hr) | -                      | Mikulovich et al., 1971. |
| Kinetin | 50 µM         | Increased RNA level by 25%. | fenugreek cotyledons | A (< 24 hr) | -                      | Rijven and Parkash, 1971. |

1. These are selected from systems in which cytokinins could influence (i.e. stimulate or inhibit) growth. For stimulatory effects of cytokinins on RNA of mature and/or senescing leaf tissue see, for example, section 1.5-3, Carpenter and Cherry (1966), Von Abrams and Pratt (1968), Burdett and Wareing (1968), Paranjothy and Wareing (1971), and references cited therein.
2. In this column, B = "effect detectable before growth response"; A = "effect detectable after growth response. If paper does not make this clear ("N"), the shortest cytokinin-contact time observed to produce the effect is stated, if known.
3. This column is for corrections other than a simple comparison with a control or a weight correction. Included are corrections or allowances for uptake (Upt) and for precursor pool size or its specific activity (size/sp. act. of prec. pool); a minus sign means "not indicated/measured".
4. Abbreviations: rRNA - ribosomal RNA; mRNA - light ribosomal RNA; hrRNA - heavy ribosomal RNA; tRNA - tenaciously bound RNA; mRNA - messenger RNA.
Thus, the effect of kinetin on cell division was not always associated with DNA synthesis (Patau et al., 1957; Patau and Das, 1961). In *Lemna minor* cultures inhibited by abscisic acid, BAP markedly promoted DNA synthesis (Van Overbeek et al., 1967). Kinetin stimulated precursor incorporation into DNA, RNA and protein of *Spirodea* within an hour after the addition of the cytokinin (McCombs and Ralph, 1972). Similarly, during BAP-induced axillary bud development in *Cicer arietinum*, a significant BAP-induced increase in DNA level was observed within 3 hours of cytokinin treatment (Usciati et al., 1972).

Since the inhibitor studies described in Chapter 4 implicated both DNA and RNA in the cytokinin-induced growth of radish cotyledons, a more direct examination of these nucleic acids was undertaken to determine whether there was any measurable effect of BAP on their levels or their rates of precursor incorporation before the onset of cytokinin-induced growth. The results of this investigation are detailed in this chapter.

**B. MATERIALS AND METHODS**

1. **Estimation of RNA**

Total RNA was estimated by measuring the u.v. absorption of its nucleotides, essentially according to the method of Matthews (1958). Briefly, the method involved the following steps. To kill the tissue and remove u.v.-absorbing substances of low molecular weight, radish cotyledons (in batches of 0.5 g) were dropped into boiling 70% ethanol (0.1 N with respect to acetic acid) and re-extracted with boiling absolute ethanol. After an acetone extraction the tissue was left to dry and then hydrolysed in 2 N potassium hydroxide overnight at room temperature. The alkali was neutralized with 2 N perchloric acid and ethanol equal in volume to the suspension was added prior to centrifugation. The nucleotides in the supernatant were isolated by paper chromatography, with 70% isopropanol as solvent, in an atmosphere saturated with ammonia. The
chromatograms were over-run from a serrated edge and the nucleotides were located under u.v. light (250 nm) as two absorbing zones which were just separated. The paper containing the nucleotide zones and a corresponding blank were cut from each chromatogram and eluted in 0.1 N HCl; the absorbance of each eluate was measured at 260 nm. Finally, the amount of RNA in each original tissue sample was calculated from the absorbance reading (A) using the relationship: $A_{260} = 32.3$ for nucleotides from 1 mg RNA in 1 ml of 0.1 N HCl. This was determined as described below.

Three lines of evidence for the validity of this method of estimating RNA were reported in the original paper. Since these applied to turnip leaf tissue, similar tests were made to check that in radish cotyledons also the absorbance measured was due solely to ribonucleotides. The results obtained are summarized as follows.

1. Although some fluorescent compounds were not cleanly separated from the nucleotide zones on the chromatogram, their absorbance at 260 nm was negligible relative to that of the nucleotides.

2. When the nucleotide zone was eluted with 0.1 N NH$_4$OH and the eluate subjected to paper electrophoresis (ammonium formate, pH 3.5), only four u.v.-absorbing compounds were detected; these possessed the mobilities of adenylic, guanylic, cytidylic and uridylic acids.

3. The eluate of the nucleotide zone was rechromatographed on sodium phosphate-impregnated paper, using isopropanol—14 N ammonia—water (7:1:2 v/v) as solvent (see Nestle and Roberts, 1968). This system completely separates all four common ribonucleotides. Only four u.v.-absorbing compounds were revealed by u.v. light. Their spectral characteristics (Table 5-1) and $R_f$ values were identical to those of adenylic, guanylic, cytidylic and uridylic acids. Elution of the remainder of the chromatogram and measurement of the absorbance of eluates also failed to reveal other u.v.-absorbing compounds.
Table 5-1  Spectral location (nm) of maxima and minima of ribonucleotides derived from radish cotyledon RNA

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>nm observed (pH 1.0)</th>
<th>nm expected (Beaven et al., 1955) (pH 1.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenylic acid, max.</td>
<td>257.5</td>
<td>257.5</td>
</tr>
<tr>
<td>min.</td>
<td>230.0</td>
<td>230.0</td>
</tr>
<tr>
<td>Cytidylic acid, max.</td>
<td>278.5</td>
<td>279.5</td>
</tr>
<tr>
<td>min.</td>
<td>241.0</td>
<td>241.0</td>
</tr>
<tr>
<td>Guanylic acid, max.</td>
<td>257.5</td>
<td>256.5</td>
</tr>
<tr>
<td>min.</td>
<td>228.0</td>
<td>228.0</td>
</tr>
<tr>
<td>Uridylic acid, max.</td>
<td>262.0</td>
<td>262.0</td>
</tr>
<tr>
<td>min.</td>
<td>235.0</td>
<td>230.5</td>
</tr>
</tbody>
</table>

From the maximum u.v. absorbance of the four nucleotide eluates and the known specific extinctions, the nucleotide composition of radish cotyledon RNA was calculated and the relationship between the absorbance reading of the mixed nucleotides and amount of RNA established. Thus, nucleotides from 1 mg of radish RNA per ml of 0.1 N HCl was calculated to give an absorbance of 32.3.

2. Estimation of DNA

A method was required which would allow DNA to be estimated in tissue also used for RNA determination. An approach that seemed suitable was to remove and estimate RNA by the method of Matthews (1958) with modifications, and then to extract DNA with hot perchloric acid and estimate it colorimetrically by the method of Burton (1956). After removal of pigments and low molecular weight compounds, DNA is frequently extracted by 0.5 N perchloric acid at 70° for about 20 minutes (Kupila et al., 1961; Lavee, 1968; Ogur and Rosen, 1950) and the extracted DNA is usually determined by Burton's method. Such a procedure seemed likely to be successful and more reliable than methods based on u.v. absorption.
To establish precisely the experimental procedure required, the following preliminary extraction was undertaken. Radish cotyledons (40 g) were firstly added to absolute ethanol (800 ml) at 75°, held at this temperature for 6 minutes, and then stirred with 800 ml of 70% ethanol, 0.1 N with respect to acetic acid, at room temperature overnight. This procedure avoided exposing the tissue to hot acid conditions, which might cause some hydrolysis of purine bases and render the DNA labile to alkali, while still providing the extraction steps necessary for the removal of pigments and other low molecular weight materials. The tissue was further extracted by stirring for two hours with 70% ethanol (600 ml). The residue was then stirred with acetone, dried and finally hydrolysed with KOH as already described. The neutralized hydrolysate (approximately 50 ml) was stirred with acetic acid—ethanol (1:20 v/v, 80 ml) and left overnight at 2 to 4°. After centrifugation, the pellet was given two more overnight extractions in 70% ethanol (0.1 N with respect to acetic acid) at 2 to 4°. The three supernatants (constituting the ribonucleotide fraction) were evaporated and assayed by Burton's determination and found to be free of DNA, while the pellet was dried and used to test various methods of DNA extraction. Pellet equivalent to 3 g of cotyledons was incubated with 8 ml of 0.5 N perchloric acid at 70° for 0.5, 1.0, 2.0 and 3.0 hours, and the amount of DNA extracted was estimated by Burton's method. The DNA yield increased at each extension of the extraction time; hence a single extraction for about 20 minutes was obviously inadequate for this tissue. Prolonged extraction times would result in destruction of deoxyribose (Burton, 1956). Repeated 1-hour extractions were therefore carried out. Aliquots of the pellet (the equivalent of 2 g of tissue) were mixed with 5 ml of 0.5 N perchloric acid, left at 30° for 18 hours and then heated at 70° for 1 hour with occasional stirring. The mixture was centrifuged and the resulting supernatant decanted. The residue was suspended in 5 ml of fresh 0.5 N perchloric acid and heated at 70° for 1 hour as before. The process was repeated yielding five extracts in all. Aliquots
(1 ml) of each solution were removed for DNA estimation which was performed according to Burton's method with the following two modifications. (1) An equal, not a double, volume of diphenylamine reagent was added to the extract. This yielded a higher absorbance at 595 nm. (2) As suggested by Giles and Meyers (1965), the absorbance at 700 nm was subtracted from the absorbance at 595 nm. This corrects for faint turbidity which sometimes appears in the solutions. The DNA recovered during the five successive extractions was 184.8, 49.5, 15.8, 4.8 and 2.6 µg respectively. Hence, three extractions were considered necessary. The 30° incubation of the tissue residue with perchloric acid increased the amount of DNA obtained in the first extract. Omission of this step necessitated four extractions. The entire experiment was repeated with very similar results.

The absorption spectra of the blue solutions obtained in the DNA estimation of the first two perchloric acid extracts were identical to the spectrum of a corresponding solution derived from pure DNA. The $\lambda_{\text{max}}$ in all spectra was 597 nm.

The u.v. spectra of the perchloric acid extracts, both of the tissue residue and of pure DNA, were also determined, before the addition of Burton's reagent. The spectral characteristics found are listed in Table 5-2.

Table 5-2. Characteristics of u.v. spectra of DNA samples heated in 0.5 N perchloric acid at 70° for 1 hour.

<table>
<thead>
<tr>
<th>Spectral Characteristics (nm)</th>
<th>Radish cotyledon</th>
<th>Pure DNA from calf thymus</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{\text{max}}$</td>
<td>267</td>
<td>267</td>
</tr>
<tr>
<td>$\lambda_{\text{min}}$</td>
<td>243</td>
<td>232</td>
</tr>
<tr>
<td>$E_{\text{max}}$</td>
<td>1.29</td>
<td>3.0</td>
</tr>
<tr>
<td>$E_{\text{min}}$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The u.v. spectrum of the tissue extract was divergent from that of the hydrolysed DNA and was hence an unreliable measure of DNA content.
Figure 5-1 Flowsheet for extraction and estimation of DNA and RNA from radish cotyledons

Cotyledon sample (0.5 g)

- Drop into 50 ml absolute ethanol at 75-80°. Leave 6 min. Decant. → Supernatant
- Add 50 ml 70% ethanol, 0.1 N w.r.t. acetic acid. Leave stirring overnight at room temperature. Decant. → Supernatant
- Add 40 ml 70% ethanol, leave 2 hr. stirring occasionally. Decant. → Supernatant
- Add 40 ml acetone, leave 2 hr. stirring occasionally. Decant. → Supernatant

Air dry cotyledons and add 0.5 ml 2 N KOH. Leave at room temperature overnight. Add 0.5 ml water and stir occasionally for 4 hours. Neutralize with 2 N perchloric acid to pH 5-7. Add 0.1 ml acetic acid and 2 ml ethanol and stir. Leave at 2-4° overnight. Centrifuge. → Supernatant

- Stir residue with 2 ml 70% ethanol, 1 N w.r.t. acetic acid. Stir occasionally for 1 day at 2-4°. Centrifuge and repeat step. → Supernatant

Dry pellet

- Add 1.6 ml 0.5 N perchloric acid. Stir. Leave 18 hr. at 30°. Heat at 70° for 1 hr. stirring occasionally. Centrifuge. → Supernatant

DNA fraction
Combine and make volume up to 5 ml with 0.5 N perchloric acid. Take two 1-ml aliquots for Burton estimation.

- Add 1 ml diphenylamine reagent to each aliquot and standard solution. Mix well and keep at 30° overnight. Read absorbance at 595 nm and 700 nm; subtract latter reading from former.

- Calculate amount of DNA by preparing standard curve from absorbance readings of DNA standards

Pigments and low molecular weight compounds. discards

Ribonucleotide fraction for determination of RNA
Combine and evaporate at 30-35° in vacuum oven. Take up in 1 ml 0.1 N NH₄OH and remove 50 µl aliquots for chromatography.

Apply aliquots to 2.5-cm origin on Whatmann 3 MM chromatography paper. Develop in 70% isopropanol for 21 hr. or preferably overrun. Elute samples and blanks in 0.1 N HCl, read absorbance at 260 nm.

DNA standards
During third heating of samples of extracted DNA also heat solutions of DNA standards with perchloric acid (total volume 1 ml)
The method of DNA extractions and estimation finally adopted is summarized in Figure 5-1, which also incorporates the procedure for simultaneous determination of RNA.

3. **Preparation of nucleic acids**

To determine a suitable procedure for the extraction of undegraded nucleic acids from radish cotyledon tissue, the following methods were tested:

1. The phenol method as described by Loening and Ingle (1967, method "B"), except that for uniformity of tissue grinding methods, a glass rather than a Virtis homogenizer was used.

2. A phenol method using the tissue extraction medium recommended by Burdett and Wareing (1968).

3. The diethyl pyrocarbonate (DEP) method as described by Solymosy *et al.* (1968), with minor modifications. Dialysis of the crude precipitated RNA was omitted to minimize possible degradation by RNase. Figure 5-2 supplies details of the procedure used. For testing the method two tissue extractions were carried out, while in the subsequent use of the method the tissue was extracted only once unless otherwise stated.

The two phenol methods were each compared with the DEP method in two separate experiments both of which were repeated. Nucleic acids were extracted from 1-g samples of radish cotyledons excised from 7-day-old seedlings that were grown in trays of sand in a glasshouse under continuous light. All final nucleic acid preparations were dissolved in 2 ml of electrophoresis buffer (formula as in Figure 5-2, but without sucrose added) and in each experiment identical aliquots (25 to 75 µl per g fresh weight of cotyledons) were diluted in 2 ml of distilled water for measurement of u.v. spectrum. The u.v. spectra indicated that the nucleic acid prepared by all three methods was of high purity. The spectra were almost coincident with a spectrum of highly purified yeast RNA. The nucleic acid extracts prepared by the DEP method and phenol method 1 (that based on the method of Loening and Ingle, 1967) were further examined by gel
Homogenise 1 g tissue (briefly chilled) in a mortar and pestle at 4°C in 3 ml Tris buffer (0.05 M, pH 7.6) containing:

- 1% sodium dodecyl sulphate,
- 5 mM magnesium chloride, and
- 0.1 ml DEP

Add 3 ml Tris buffer and 0.1 ml DEP to the slurry, grind briefly and rinse into centrifuge tube. Rinse mortar with 4 ml Tris buffer containing 1.33 ml DEP. Add to centrifuge tube.

Incubate homogenate at 37°C for 5 minutes, stirring occasionally. Centrifuge at 8,000 x g at 4°C for 5 minutes.

Transfer supernatant(s) (10 ml) to further centrifuge tube(s). Add 1 g NaCl; Stir vigorously to dissolve. Incubate at 37°C for 5 minutes stirring occasionally. Centrifuge at 10,000 x g at 4°C for 20 minutes.

Pour supernatant into 25 ml of 96% ethanol (50 ml if extracted twice) at 0°C and stir. Leave overnight, or at least 5 hours, at -16°C. Stir gently to release any air bubbles.

Centrifuge at 10,000 x g at 4°C for 10 minutes.

Resuspend pellet (or combine 2 pellets) in 10 ml ethanol. Stir; recentrifuge.

Discard Supernatants. Dry pellet in vacuum desiccator = Purified nucleic acids

Dissolve pellet in 2 ml electrophoresis buffer (pH 7.8) containing
- 12 mM Tris
- 0.13 mM Na-EDTA
- 10 mM NaI, PO4

Add and dissolve 20% sucrose (RNase free) to facilitate loading of aliquots on to gels. Centrifuge at 25,000 x g at 4°C for 10 minutes, and retain supernatant.

Use 10-25 µl aliquots for gel electrophoresis

For analysis of u.v. absorption spectrum in Unicam SP-800 spectrophotometer, dilute aliquot (20-100 µl) to 2 ml with distilled water.

For accurate determination of RNA, hydrolyse aliquot (100 µl) with 0.4 ml 0.2 N KOH at 30°C for 24 hours. Dilute to 2 ml with 0.1 N HCl and read absorbance at 260 nm.

* DEP was added to the Tris buffer just before homogenizing. The following brands of DEP were tested in the above method and found to give satisfactory results: Bayer 'Baycovin' (the brand usually used), Pfluger, Calbiochem and TCI, Japan.
electrophoresis (as described below) to assess the integrity and relative amounts of the constituent nucleic acid species. U.v. scanning and staining of the gels revealed that both methods of extraction yielded good nucleic acid preparations, since in all gels the total number, position and relative amounts of the constituent species were identical to those obtained by Loening and Ingle (1967) in electrophoresed preparations of undegraded nucleic acids prepared from cotyledons of light-grown 6-day-old radish seedlings.

Absorbance readings of diluted aliquots of the purified nucleic acid solutions obtained by the DEP method were considerably higher than those obtained by either phenol method and there was a close agreement between duplicate experiments. Estimated from mean absorbance, the yields of nucleic acid obtained by phenol methods 1 and 2 were 66% and 51% respectively of the yield obtained by the DEP method. Hence, the DEP method was used for all subsequent nucleic acid preparation.

4. Gel electrophoresis methods

Nucleic acid species were separated by polyacrylamide gel electrophoresis based on the method used by Loening and Ingle (1967). Acrylamide gels (2.4%) were prepared in 12-cm 'Perspex' tubes (of 0.63 cm internal diameter) and held in place by dialysis tubing tied to the lower ends of the tubes. Each batch of 6 gels (10 to 11 cm) was prepared from a mixture containing 3.78 ml of acrylamide stock solution, 7.49 ml of gel buffer solution and 11.02 ml of water. The acrylamide stock solution contained 15% acrylamide recrystallized from chloroform and 0.75% bisacrylamide recrystallized from acetone. The gel buffer consisted of electrophoresis buffer at three times the concentration specified in Figure 5-2. The gel mixture was de-aerated under vacuum and then 0.18 ml of 10% ammonium persulphate solution (freshly prepared) and 18 µl of TEMED (N,N,N',N'-tetramethylethylenediamine) were stirred into the solution which was immediately pipetted into the tubes. Freshly prepared gels were inserted into the electrophoresis apparatus which was kept in a cold room at 4°C. Pre-cooled
electrophoresis buffer was added to the buffer compartments and the equipment was left for 30 minutes to allow the gels to cool. Impurities and interfering materials were removed from the gels during pre-electrophoresis for 20 minutes at 3 V/cm, 5 m. amp for each tube. Aliquots (10-25 µl) of nucleic acid solutions in electrophoresis buffer containing 20% RNase free sucrose (refer Figure 5-3) were then layered on to the gels, and electrophoresis was continued. For recovery of all nucleic acid species gels were run for about 1 hour, while for recovery of ribosomal RNA with improved resolution of the component species gels were run for 2 to 3 hours.

After electrophoresis gels were removed from the tubes under gentle air pressure and rinsed for 30 minutes in test tubes of distilled water. To locate and estimate the nucleic acid species, the gels were then scanned at 265 nm in a Joyce Loebl Chromoscan. When staining of the nucleic acids was desirable, gels were emersed for 1 hour in 0.025% Gurr's Azur 1 dye dissolved in a buffer (pH 4) containing disodium monohydrogen orthophosphate (10.9 g/l) and citric acid (12.9 g/l). Differentiation was accomplished by de-staining the gels in distilled water overnight. The bands obtained by staining with Azur 1 were found to be more pronounced than those obtained by staining with either acridine orange or lanthanum acetate.

5. Labelling experiments

\[ ^{5-\text{H}}\text{uridine (5 Ci/mmol) and } ^{2-\text{C}}\text{uridine} \]
(50 to 60 mCi/mmol) were used to label RNA; \[ ^{2-\text{C}}\text{thymidine} \]
(57 mCi/mmol) was used to label DNA (Amersham Chemicals).

Experiments concerned with the incorporation of radioactive precursors into nucleic acids of excised radish cotyledons involved dual isotope labelling.

The methods of incubation, labelling and sampling in the two types of experiment employed are outlined below. In single labelling experiments these manipulations were performed as in the latter of the double labelling experiments (see 5.5-2), the only basic differences being in the nature of the radioactivity supplied and in the replication of treatments.
5-1 Dual isotope labelling of cotyledons for RNA and DNA extraction

Some 640 radish cotyledons were excised aseptically into sterile Petrie dishes lined with wet filter paper. Two batches (0.5 g) of cotyledons were removed for immediate alcoholic extraction to determine nucleic acids, while the rest of the cotyledons were distributed in batches of 20 to 12.5-cm Petrie dishes lined with Whatman No. 1 filter paper which was wetted with 6 ml of phosphate buffer with or without BAP. The Petrie dishes were kept under weak fluorescent light at 23°C (c.f. section 2.1). After 18, 30 and 54 hours, the media in some Petrie dishes were replaced with media containing 16.7 μM $[^{14}C]$thymidine and 1.67 μM $[^{3}H]$uridine. The cotyledons were bathed thoroughly in this solution and then spread out evenly over the Petrie dish. Their incubation was continued in the presence of the radioactive solution for 6 hours. After the labelling period, the cotyledons were washed into a strainer, rinsed with 1 l of distilled water, blotted on filter paper and then weighed. Three 0.5-g batches of these cotyledons were each dropped into boiling ethanol. RNA and DNA extraction of all batches, including those extracted immediately after excision, was continued as described in Figure 5-1. Aliquots of each fraction obtained during the extraction were removed for scintillation counting of both $^1H$ and $^{14}C$ radioactivity in order to calculate the total uptake of each precursor. Incorporation into nucleic acids was calculated by measuring the radioactivity in chromatographed RNA nucleotides and in DNA hydrolysates. All aliquots taken are listed in Table 5-3(A) which also describes how each one was prepared for the addition of scintillant for counting.

5-2 Dual isotope labelling of cotyledons for RNA extraction by the DEP method

From cotyledons excised after germination as described above, four batches of 30 cotyledons of equal weight were selected. The incubation media prepared for the batches comprised four 7-ml volumes of phosphate buffer autoclaved in
Steward flasks one of which contained BAP. The cotton wool bung was briefly removed from each flask while chloramphenicol (15 µg/ml) was dissolved in each solution and then one batch of cotyledons was added. The bungs were then replaced and the flasks clamped to a vertical wheel, where they were left rotating at 1 r.p.m. at 23° in the dark.

After the required period of preincubation, each flask received an equimolar amount (0.44 µmol) of \(^{3}H\)uridine (usually 125 µCi) or \(^{14}C\)uridine (usually 25 µCi) delivered from a sterile syringe in 1 ml of phosphate buffer, with or without BAP as appropriate to the flask. The concentration of \(^{3}H\)uridine was increased to the molarity of the \(^{14}C\)uridine by the addition of unlabelled uridine. The isotope added to the flasks was as follows:

(+BAP) medium (1 flask), \(^{14}C\)uridine
(-BAP) " (1 flask), \(^{14}C\)uridine
(-BAP) " (2 flasks), \(^{3}H\)uridine

After the appropriate labelling time, the batches of cotyledons were poured separately into a strainer and washed with distilled water (500 ml), before being blotted and weighed. For extraction, batches of the cotyledons were mixed as follows:

(a) (+BAP) with \(^{3}H\)-labelled (-BAP)
(b) \(^{14}C\)-labelled (-BAP) with \(^{3}H\)-labelled (-BAP)

The nucleic acids extracted from these two mixtures are termed (+BAP) and (=BAP) RNA respectively. The cotyledon mixtures are also referred to by this symbolism.

Total uptake of uridine was measured by removing an aliquot from the initial homogenate, counting it for \(^{3}H\) and \(^{14}C\), and finally calculating the ratio of \(^{14}C\) c.p.m./\(^{3}H\) c.p.m. The (=BAP) treatment gave the control ratio; comparison of this with the (+BAP) uptake ratio showed whether BAP had affected uptake. To measure incorporation of uridine into total RNA and into individual RNA species, similar counting procedures were applied to aliquots of the purified nucleic acid solution and to slices of scanned polyacrylamide gels respectively. The preparation of the aliquots and gel slices for scintillation counting is described in Table 5-3(B).
### Table 5-3. Preparation of samples for scintillation counting

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Parameter(s) assessed</th>
<th>Sampling procedure and processing of aliquots for scintillation counting</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Extraction of labelled RNA and DNA (distinguished by dual isotope labelling with $^{3}H$uridine and $^{14}C$thymidine).</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Ethanolic and acetone extracts</td>
<td>uptake (partial)</td>
<td>Combine extracts, evaporate 1 ml aliquot in a scintillation vial at 50°C or in vacuum desiccator. Dissolve residue in 0.5 ml water.</td>
</tr>
<tr>
<td>2. Ribonucleotide fraction</td>
<td>uptake (partial)</td>
<td>From extract dissolved in 0.1 N ammonium hydroxide remove 50 μl aliquot. Allow to dry in scintillation vial. Dissolve residue in 0.5 ml water.</td>
</tr>
<tr>
<td>3. Chromatographed ribonucleotides</td>
<td>incorporation (RNA)</td>
<td>From eluted sample in 0.1 N HCl remove 0.2 ml aliquot. Neutralize in scintillation vial with dilute ammonium hydroxide. Make volume up to 0.5 ml with distilled water.</td>
</tr>
<tr>
<td>4. DNA fraction</td>
<td>uptake incorporation (partial) (DNA)</td>
<td>From extracts dissolved in 0.5 N perchloric acid remove 100 μl aliquot. Neutralize in scintillation vial with dilute ammonium hydroxide. Make volume up to 0.5 ml with distilled water.</td>
</tr>
<tr>
<td>5. Insoluble residue</td>
<td>uptake (partial)</td>
<td>To residue pelleted after perchloric acid extractions add 0.1 N sodium hydroxide dropwise, while resuspending pellet, until the suspension is neutral. Dry at 50°C or in vacuum desiccator. Add 1 ml Soluene; stir. Leave 48 hours at 30°C. Centrifuge. Remove 0.5 ml aliquot to scintillation vial.</td>
</tr>
<tr>
<td>B. Extraction of labelled nucleic acids by DEP method.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Total homogenate</td>
<td>uptake (total)</td>
<td>Remove 100 μl aliquot from thoroughly stirred homogenate. Hydrolyse in small centrifuge tube with 0.9 ml 0.2 N potassium hydroxide at 30°C for 24 hours. Mix, centrifuge and remove 100 μl of the supernatant to scintillation vial. Neutralize with 1% acetic acid and make volume up to 0.5 ml with distilled water.</td>
</tr>
<tr>
<td>2. Total purified nucleic acids</td>
<td>incorporation (total)</td>
<td>From solution of nucleic acids in electrophoresis buffer remove 100 μl aliquot and hydrolyse in small tube with 0.4 ml 0.2 N potassium hydroxide at 30°C for 24 hours. Stir, remove 100 μl to scintillation vial. Neutralize with 1% acetic acid and make volume up to 0.5 ml with distilled water.</td>
</tr>
<tr>
<td>3. Polyacrylamide gels containing electrophoresed nucleic acids</td>
<td>incorporation (individual nucleic acid species)</td>
<td>After scanning gel to locate nucleic acids, freeze on dry ice and cut into 1-mm slices with a Mickell gel slicer. Place each slice in a scintillation vial. Add 0.5 ml of 10% piperidine to each vial and leave 1 to 2 hours. Place capped vials in oven at 50°C to 55°C for 1.5 hours. Remove caps and evaporate rapidly in air current at 50°C. Add 0.5 ml distilled water to each vial and leave 4 hours to allow gel slices to swell before adding scintillant.</td>
</tr>
</tbody>
</table>
5-3 Scintillation counting of radioactive samples

All aliquots were prepared and counted in duplicate. Those in aqueous solution were each mixed with 10 volumes of Toluene/Triton scintillant (toluene—Triton X-100, 2:1 v/v, containing PPO, 2 g/l and dimethyl POPOP, 0.2 g/l). Aliquots dissolved in 'Soluene' (Packard) were mixed with toluene scintillant (toluene containing PPO, 3 g/l and dimethyl POPOP, 0.3 g/l). Use of this scintillant avoided the persistent high background count produced by the mixing of toluene/Triton scintillant with the alkaline Soluene. The vials were kept in the dark at room temperature for 24 hours and then counted in a Beckman LS-250 liquid scintillation spectrometer. For each series of vials counted, levels of background radioactivity were determined from at least two blank vials. These were prepared from aliquots of equivalent solutions of unlabelled extract or from aliquots of the solvent alone if this was found to give an equivalent background. All counts were appropriately corrected for quenching.

The steps required for correcting each doubly labelled sample for spillover and counting efficiency were incorporated into a computer programme which was adjustable for use with different window settings. Efficiency curves were checked before each experiment in case it was necessary to modify the equations by which they were represented in the computer programme. One version of the programme, written in BASIC computer language, is shown in Appendix 1, together with an explanation of its implementation and a sample print-out using arbitrary figures. The version of the programme shown is that used for other labelling experiments described in Chapter 7, which required expression of the channels ratio in the form \( \frac{3^\text{H}}{1^\text{H}} \) rather than \( \frac{1^\text{H}}{3^\text{H}} \).

6. Statistical treatment of results

Analyses of variance and associated calculations were performed as described in section 2.3. All values analysed were the means of three replicates.
C. RESULTS

7. Effect of BAP on the contents and specific activities of total DNA and RNA

Radish cotyledons were incubated in the light for 54 hours on (+BAP) or (-BAP) media. At intervals during the incubation, batches of cotyledons were labelled for 6 hours with \(^{3}H\)uridine and \(^{14}C\)thymidine, as described in section 5.5-1, and then weighed and extracted for RNA and DNA as outlined in Figure 5-1. The mean cotyledon weights at each extraction time are recorded in Figure 5-3 so that the results described in this section may be related to BAP-induced growth.

7-1 DNA content and specific activity

The effect of BAP on DNA content is shown in Figure 5-4. In both (-BAP) and (+BAP) treatments, the DNA content per cotyledon increased throughout the period of growth studied. After 18 hours of incubation, the DNA extracted per (+BAP) cotyledon was significantly greater than that extracted per control cotyledon, and the rate of increase in DNA per cotyledon between 18 and 54 hours was enhanced by BAP. At 54 hours, the final sampling time, the per cent increment in DNA content induced by BAP was 42% of the control increment.

The effect of BAP on \(^{14}C\)thymidine incorporation into DNA was assessed by determining the radioactivity in aliquots of the DNA extract (Figure 5-1) as described in Table 5-3(A). Total thymidine uptake per µg fresh weight of cotyledons was determined by assaying all fractions of each extraction for \(^{14}C\) [refer Table 5-3(A)]. Figure 5-5 shows the specific activities of DNA before and after their correction for differences in thymidine uptake. Before correction for uptake, the specific activities of DNA from (+BAP) cotyledons at both 18 hours and 30 hours were significantly higher than control values. There was a small effect of BAP on the specific activity of DNA corrected for differences in thymidine uptake, but this was not significant.
Figure 5-3  Mean fresh weights of radish cotyledons incubated in (+BAP) or (-BAP) media for various periods

The cotyledons were cultured in Petrie dishes in continuous light. Samples were removed at intervals and were counted and weighed (prior to extraction of aliquots for estimation of DNA and RNA).
Graph showing the changes in cotyledon weight over time. The x-axis represents time in hours (0 to 60), and the y-axis represents cotyledon weight in mg (0 to 35). Two lines are plotted, one with solid dots and another with open circles, indicating different conditions or groups over the 60-hour period.
Figure 5-4 DNA content of radish cotyledons grown on (+BAP) and (-BAP) media

The cotyledons were cultured in Petrie dishes in continuous light. At intervals, 0.5-g batches of cotyledons were extracted for estimation of nucleic acids.

- DNA content per (+BAP) cotyledon.
- DNA content per (-BAP) cotyledon.
- DNA content per mg fresh wt. of (+BAP) cotyledons.
- DNA content per mg fresh wt. of (-BAP) cotyledons.

D(5%) in µg of DNA per cotyledon = 0.516.
D(5%) in µg of DNA per mg fresh wt. of cotyledons = 0.025.

Sequential testing revealed no additional significant differences.
Specific activity of DNA extracted from radish cotyledons grown on (+BAP) and (-BAP) media

The cotyledons were cultured in Petrie dishes in continuous light. At intervals, batches of cotyledons were labelled with [2-14C] thymidine for 6 hours, and then extracted for estimation of nucleic acids. After estimation of DNA, the 14C radioactivity in aliquots of each DNA fraction was counted. Total uptake per sample (c.p.m./µg fresh weight of cotyledon tissue) was also calculated.

- DNA, specific activity in (+BAP) cotyledons
- DNA, specific activity in (-BAP) cotyledons
- DNA, specific activity uptake (c.p.m./µg fresh wt)
- DNA, specific activity uptake (c.p.m./µg fresh wt)

D(5%) in specific activity of DNA = 0.656 (c.p.m. x 10⁻³ per µg DNA).

D(5%) in specific activity of DNA corrected for uptake = 0.351 (c.p.m. x 10⁻³ per µg DNA/c.p.m. per µg fresh wt. of tissue). Sequential testing showed that at 30 hours the (+BAP) value was significantly higher than the (-BAP) value.
SPECIFIC ACTIVITY (CPM × 10^{-3} PER μg DNA)

TIME (HOURS)

SPECIFIC ACTIVITY / UPTAKE

(CPM × 10^{-3} PER μg DNA / CPM PER μg FRESH WT OF TISSUE)
until 30 hours.

7-2 RNA content and specific activity

Figure 5-6 shows the total RNA content of radish cotyledons extracted after various periods of (+BAP) or (-BAP) incubation. The results closely resembled those obtained for DNA. Thus, in both (+BAP) and (-BAP) cotyledons, there was an increase in the RNA content per cotyledon throughout the observed growth period, and a significant BAP-induced increase in RNA per cotyledon occurred after 18 hours of incubation. At the final sampling time (i.e. 54 hours), the per cent increment in RNA content induced by BAP was 37% of the control increment. Per unit fresh weight of tissue, RNA levels of (+BAP) cotyledons and (-BAP) cotyledons were not significantly different at any sampling time except the last, when the RNA level in (+BAP) tissue was 17% lower than the control level.

To determine the specific activities of the RNA samples, aliquots of the nucleotide solutions eluted from chromatographed RNA hydrolysates were counted, after reading of u.v. absorbance (Figure 5-7). Only at 54 hours was the specific activity of the RNA from (+BAP) cotyledons significantly higher than that of the controls, and this difference was almost significant at the 5% level when specific activities were corrected for differences in uridine uptake per unit weight of tissue. Although there were no earlier differences in specific activity, the incorporation/uptake ratios did show a difference at 30 hours, when the ratio for (+BAP) tissue was significantly greater than the control ratio at the 5% level.

From the results described above, it appeared unlikely that BAP had any marked effect on total RNA synthesis during the lag period. However, BAP could have affected one or more specific RNA species without apparently changing total RNA level. This possibility was examined as detailed below.

8. Effect of BAP on specific RNA species

An attempt was made to assess the levels and specific activity of ribosomal and soluble RNA after separation
Radish cotyledons were cultured in Petrie dishes in continuous light. At intervals, 0.5 g batches of cotyledons were extracted for estimation of nucleic acids.

- RNA content per (+BAP) cotyledon
- RNA content per (-BAP) cotyledon
- RNA content per mg fresh wt. of (+BAP) cotyledons
- RNA content per mg fresh wt. of (-BAP) cotyledons

$D(5\%)$ in $\mu g$ of RNA per cotyledon = 8.43
$D(5\%)$ in $\mu g$ of RNA per mg fresh wt. of cotyledons = 0.450
Specific activity of RNA extracted from radish cotyledons grown on (+BAP) and (-BAP) media

The cotyledons were cultured in Petrie dishes in continuous light. At intervals, batches of cotyledons were labelled with [5-3H]uridine for 6 hours, and then extracted for estimation of nucleic acids. After estimation of RNA, the 3H radioactivity in aliquots of each RNA fraction (refer Table 5-3) was counted. Total uptake per sample (c.p.m./µg fresh weight of cotyledon tissue) was also calculated.

- - - RNA, specific activity in (+BAP) cotyledons
○--○ RNA, specific activity in (-BAP) cotyledons
●--● RNA, specific activity
uptake (c.p.m./µg fresh wt)
○--○ RNA, specific activity
uptake (c.p.m./µg fresh wt)

D(5%) in specific activity of RNA = 0.55 (c.p.m. x 10^{-3} per µg RNA). Sequential testing showed that at 54 hours the (+BAP) value was significantly higher than the (-BAP) value.

D(5%) in specific activity of RNA corrected for uptake = 0.22 (c.p.m. x 10^{-3} per µg RNA/c.p.m. per µg fresh wt. of tissue). No significant differences were revealed by sequential testing, but at 54 hours the difference between (+BAP) and (-BAP) was almost significant (difference observed = 0.116; difference required for significance at 5% level = 0.17).
by polyacrylamide gel electrophoresis. However, it was felt
that assessment of RNA concentration by measuring areas under
the peaks in absorbance tracings of gels was not sufficiently
accurate. Hence the double labelling experiments described
below were carried out.

Several experiments were performed in which
(+BAP) cotyledons labelled with \[^{14}C\]uridine were mixed with
an equal number of cotyledons labelled with \[^{3}H\]uridine and
the mixture was then extracted to yield a doubly labelled
preparation of undegraded RNA (see section 5.5-2). This
mixed RNA product was designated (+BAP). The control
preparation (no BAP supplied to the cotyledons labelled with
\(^{14}C\) was designated (=BAP). Since chloroplast RNA did not
appear to be essential for BAP-induced growth, the cotyledons
used in these experiments were incubated in the dark. This
simplified the gel profiles, since chloroplast RNA was almost
completely eliminated.

In the first experiment, cotyledons were pre-
incubated (with or without BAP) for 6 hours and then labelled
for 4 hours, while in the second experiment they were pre-
incubated for 12 hours and then labelled for 6 hours. Both
experiments were repeated using cotyledons from a different
batch of radish seedlings. The \(^{14}C/^{3}H\) ratios in total RNA
preparations and in individual RNA species in polyacrylamide
gel slices were then determined (see section 5.5-2). Table
5-4 shows the \(^{14}C/^{3}H\) ratios of the total RNA of all
preparations, both before and after corrections for differences
in uptake ratio. Since cotyledons treated with BAP were
labelled with \[^{14}C\]uridine, a positive effect of BAP on
incorporation of uridine into RNA is indicated by an increase
in the \(^{14}C/^{3}H\) ratio.
Table 5-4  $^{14}$C/$^3$H ratios of total undegraded RNA prepared from (+BAP) and (=BAP) cotyledon mixtures.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Period of contact with BAP (hr)</th>
<th>Period of labelling (hr)</th>
<th>(=BAP) mixture $^{14}$C/$^3$H RNA</th>
<th>(=BAP) mixture $^{14}$C/$^3$H RNA corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(a)</td>
<td>10</td>
<td>4</td>
<td>0.495</td>
<td>0.439</td>
</tr>
<tr>
<td>1(b)</td>
<td>10</td>
<td>4</td>
<td>0.611</td>
<td>0.536</td>
</tr>
<tr>
<td>2(a)</td>
<td>18</td>
<td>6</td>
<td>0.509</td>
<td>0.548</td>
</tr>
<tr>
<td>2(b)</td>
<td>18</td>
<td>6</td>
<td>0.378</td>
<td>0.332</td>
</tr>
</tbody>
</table>

1. Ratios obtained when equal weights of tissue were mixed and RNA prepared (where tissue weights were not quite equal the ratio has been corrected). Preparations hydrolysed with piperidine in the manner used for gel slices, gave ratios almost identical to those obtained after hydrolysis with KOH.

2. Corrected for difference in uptake ratio compared with (=BAP) control.

In each experiment, the $^{14}$C/$^3$H ratio of the (+BAP) mixture was slightly greater than the control ratio, after correction for uptake; however, all differences were very small. The results were thus in agreement with those described in the previous section (5.7-2) involving the extraction of total RNA from cotyledons incubated in the light.

In RNA preparations from the first double labelling experiment (designated 1(a) in Table 5-4) in which the period of contact with BAP was 10 hours, polyacrylamide gel electrophoresis did not reveal any BAP-induced increase in the $^{14}$C/$^3$H ratio of any RNA species. However, in both experiments in which the period of contact with BAP was 18 hours, a small positive effect on this ratio was observed over the ribosomal region of the gel. The results of the first 18-hour incubation experiment [2(a) in Table 5-4] are shown in Figure 5-8, and the results of the second such experiment [2(b)] in Figure 5-9. In the latter case, the
Figure 5-8: Absorbance (E$_{265}$) traces of radish cotyledon RNA fractionated on acrylamide gels, and $^{14}$C/$^3$H c.p.m. ratios of labelled RNA contained in the gel slices.

Figure 5-9: Cotyledons were labelled with [5-$^3$H]uridine or with [2-$^{14}$C]uridine for the final 6 hours of an 18-hour incubation in Steward flasks in darkness, and doubly labelled cotyledon mixtures were extracted to prepare RNA. In (=BAP) RNA preparations, neither the $^3$H- nor the $^{14}$C-labelled cotyledons received BAP. In (±BAP) RNA preparations, the $^{14}$C-labelled cotyledons received BAP throughout the incubation. The (±BAP) $^{14}$C/$^3$H ratio shown has been corrected to the (=BAP) uptake ratio. (0.435 in Figure 5-8 and 0.218 in Figure 5-9). The total radioactivity in each gel slice (dashed trace) is that of the (±BAP) gel. Electrophoresis was at 40° for 1 hour (Figure 5-8) or 2 hours (Figure 5-9).

- 25S: heavy cytoplasmic ribosomal RNA
- 18S: light cytoplasmic ribosomal RNA
- 5S + 4S: soluble RNA
or an

labelled

received

$\text{C}^{3}\text{H}$

in

gel

e1.

5-8

ELECTROPHORETIC MOBILITY

TOTAL RADIOACTIVITY (C.P.M. x 10$^3$)

 Electrophoretic Mobility

E 265

$^{14}\text{C}/^{3}\text{H}$ RATIO

(±BAP)

(=BAP)

25S

18S

5S+4S

RNA
c.p.m.
ELECTROPHORETIC MOBILITY
region of slower moving RNA species was expanded by extending the period of electrophoresis. Replicate gels in the first experiment gave almost identical ratio profiles.

To obtain further information on the species of RNA affected by BAP, the experiment involving a total incubation time of 18 hours was repeated, using 5-fluorouracil (5-FU; 0.3 mg/ml in the incubation medium) as an inhibitor of mature ribosomal RNA formation (see discussion). In the presence of 5-FU, BAP-induced growth was still observed (see Table 5-5, footnote 3). However, polyacrylamide gel electrophoresis (Figure 5-10) showed that 5-FU had strongly inhibited formation of ribosomal RNA: in both (=BAP) and (±BAP) gels, counts in the ribosomal RNA region were very low and there were no distinct radioactive peaks. Calculation of $^{13}$C/$^3$H ratios of gel slices in this region (Figure 5-10) showed, however, that the stimulation of uridine incorporation caused by BAP was proportionally greater than it was in the absence of any inhibition of ribosomal RNA formation. This was also clear from the $^{13}$C/$^3$H ratio of total RNA (see Table 5-5 below and compare with Table 5-4). These results are consistent with a preferential stimulation by BAP of the incorporation of precursors into polydisperse RNA.

Table 5-5 $^{13}$C/$^3$H ratios in extracts of total RNA prepared from (±BAP) and (=BAP) cotyledon mixtures incubated in the presence of 5-FU (0.3 mg/ml).

<table>
<thead>
<tr>
<th>Period of contact with BAP (hr)</th>
<th>Period of labelling (hr)</th>
<th>(=BAP) mixture $^{13}$C/$^3$H RNA$^1$</th>
<th>(±BAP) mixture $^{13}$C/$^3$H RNA$^1$ corrected$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>6</td>
<td>0.665</td>
<td>0.880</td>
</tr>
</tbody>
</table>

1: 2. See footnotes, Table 5-4.

3. Mean cotyledon weights were as follows:
   (±BAP) cotyledons ($^3$H-labelled) : 12.2 mg
   ($^3$H-labelled) : 12.0 mg
   ($^{13}$C-labelled) : 12.7 mg
   (=BAP) cotyledons ($^{13}$C-labelled) : 13.2 mg

The effect of BAP on growth was confirmed in another experiment.
Cotyledons, cultured in Steward flasks in the dark, were labelled with $[5-\text{H}]$uridine or with $[2-\text{C}]$uridine for the final 6 hours of an 18-hour incubation in the presence of 5-FU (0.3 mg/ml); doubly labelled cotyledon mixtures were then extracted to prepare RNA. In (=BAP) RNA preparations, neither the $\text{H}$- nor the $\text{C}$-labelled cotyledons received BAP. In (+BAP) RNA preparations, the $\text{C}$-labelled cotyledons received BAP throughout the incubation. The (+BAP) $\text{C}/\text{H}$ ratio shown has been corrected to the (=BAP) uptake ratio (0.66). The total radioactivity in each gel slice (dashed trace) is that of the (+BAP) gel. Electrophoresis was for 2 hours at 4°C.

25S: heavy cytoplasmic ribosomal RNA
18S: light cytoplasmic ribosomal RNA
ELECTROPHORETIC MOBILITY

\[ \frac{^{14}C}{^{3}H} \text{ RATIO} \]

\( (\pm \text{BAP}) \)
\( (= \text{BAP}) \)

25S

18S

TOTAL RADIOACTIVITY (CPM × 10³)

E_{265}
D. DISCUSSION

(a) Methods

In this chapter, experiments were presented which described the effect of BAP on aspects of nucleic acid metabolism. Interpretation of the results of such experiments depends on several assumptions. Firstly it must be assumed that the nucleic acid extracted was representative of the total nucleic acid in the tissue. However, in methods for the preparation of undegraded nucleic acid species, there appears to be some variation in the efficiency of the extraction and in the integrity and the proportional yields of the constituent RNA species (Ingle and Burns, 1968; Solymosy et al., 1970). Hence two principal methods were compared, one based on phenol extraction, and the other on the use of DEP as an inhibitor of ribonuclease. Both methods were found to give nucleic acid preparations that showed no evidence of degradation or preferential loss of any RNA species (c.f. Loening and Ingle, 1967), but the DEP method was selected for use in the experiments described herein because it produced consistently higher yields. A subsequent experiment (described in Chapter 6) confirmed that the DEP method gave a thorough extraction of RNA from both (+BAP) and (-BAP) cotyledons; the total RNA yields obtained by DEP extraction were comparable to those estimated by Matthews' method for quantitative determination of RNA.

Accurate methods for estimating the tissue content of nucleic acids are also important. For quantitative extraction of RNA, and for its determination, a modification of Matthews' procedure was employed. The specificity of the method was first confirmed for radish cotyledons. Methods commonly used for RNA estimation based on u.v. absorption or pentose content of crude extracts are very unreliable, but are still often employed by plant physiologists. For estimation of DNA, methods based on u.v. absorption of the DNA bases have commonly been employed (see Hutchison and Munro, 1961). These methods are likely to be inaccurate, however, since u.v.-absorbing substances other than DNA are likely to be present (e.g. protein degradation products and
traces of RNA); this certainly appeared to be the case in radish cotyledon tissue (see Table 5-2). The method of Burton (1956) was considered likely to be reliable, since it depends on a highly specific colorimetric reaction between diphenylamine and the deoxyribose residues combined with purines in the DNA. However, with respect to the duration of the 70° perchloric acid extraction that is employed in this method, the commonly used time of 10 to 20 minutes (c.f. Burton, 1956 and references cited in section 5.2) was found to be inadequate for the thorough extraction of DNA from radish cotyledon tissue: 1-hour extractions were employed and at least three were necessary for a high (> 95%) recovery of DNA. It is very relevant to note that Lyttleton and Hole (1967) found the extractability of DNA from tobacco leaves by perchloric acid at 70° varied with leaf maturity. Mature leaves required much longer extraction times than young leaves. It would appear that the perchloric acid extraction conditions must be critically assessed for particular plant tissues.

A second assumption required for the interpretation of the results presented herein is that the observed incorporation of radioactive precursors into nucleic acids was by the tissue studied, and not by contaminating bacteria. All radish seeds used in the experiments detailed in this thesis were surface sterilized, and in all labelling experiments particular care was taken to preserve sterility throughout the incubations. The problem of persistent bacterial contamination after surface "sterilization" of seeds has been stressed by Lonberg-Holm (1967). Both he and Hock (1967), noted that penicillin and streptomycin treatment does not necessarily prevent this contamination. In the present study, CAP was used to prevent bacterial contamination. Use of CAP for this purpose has been criticized because of the possibility that the antibiotic might alter the metabolism of the system studied. At the concentration used in the present study, however, CAP has no effect on BAP-induced growth (see section 4.8). While it is possible that there was a small amount of bacterial contamination in the experiments described herein, it is relevant that in the
RNA preparations that were subjected to polyacrylamide gel electrophoresis, there was no distortion of the labelling pattern relative to the absorbance trace in the gels (c.f. Hock, 1967). In these experiments, moreover, the formation of chloroplast RNA was inhibited because cotyledons were incubated in the dark, and in the presence of CAP (c.f. Ingle, 1968). Hence, even a small degree of labelling of bacterial RNA species would have been clearly distinguishable in these gels.

Ideally, for accurate assessment of the effect of BAP on nucleic acid metabolism in radish cotyledons, the specific activity of the precursor pool should be known. An attempt was made to isolate UTP from 5 g of radish cotyledons by a phosphate ester extraction procedure based on the method of Bieleski (1968). However, this amount of tissue yielded insufficient UTP to detect as a u.v.-absorbing spot on a chromatogram, and hence, determination of the specific activity of UTP was not practicable with radish cotyledon tissue. Since the growth of radish cotyledons is finite and rapid, this system is unsuitable for experiments based on procedures adopted by Trewavas (1970) and McCombs and Ralph (1972) to avoid the complication of variation in pool size (see section 1.5-2). The study could be extended since sensitive methods of ATP determination using the enzyme firefly luciferase have been developed (Emerson and Humphries, 1971). However, evidence presented below indicates that an effect of BAP on pool size was unlikely in the double labelling experiments.

(b) Results

When radish cotyledons were incubated for two days in the light, BAP induced a 36% increase in DNA content per cotyledon. Since there was a lag period of about 18 hours before this effect was observed, it appears that an appreciable acceleration in the normal rate of DNA increase is not a pre-requisite for BAP-induced growth, but rather a result of this growth. The BAP-induced increase in DNA level per cotyledon was associated with a higher specific activity of DNA. This strongly suggests that the increased DNA level observed in
(+BAP) cotyledons was a real effect and not an apparent one arising from increased extractability. When specific activities were corrected for differences in thymidine uptake per unit fresh weight of cotyledon tissue, a small increase in specific activity was still observed in (+BAP) cotyledons, but it was not significant before 30 hours. This result is consistent with the conclusion reached above that the increased DNA synthesis was a consequence of BAP-induced growth. This conclusion is further supported by the observation that when DNA content was measured on a per unit fresh weight basis, there was no significant difference in DNA level between (-BAP) and (+BAP) cotyledons. Reasons why BAP-induced growth of radish cotyledons should require DNA synthesis have been discussed in Chapter 4. The most obvious use for at least the majority of the DNA synthesised in response to cytokinin treatment is in the formation of new chromosomes during the BAP-induced increase in cell number (c.f. 2.7) which accompanies cell enlargement. The data presented herein are consistent with this suggestion.

An examination of the effect of BAP on total RNA produced results very similar to those discussed above. Thus, BAP caused an increase (23%) in RNA content per cotyledon, but this was not observed until after the onset of BAP-induced growth and was not accompanied by a significant increase in specific activity when this was corrected for differences in uridine uptake. Hence, BAP-induced growth of radish cotyledons clearly did not depend on any marked effect on total RNA synthesis during the lag period. Between 18 and 30 hours, BAP caused a marked increase in RNA content per cotyledon but no change in specific activity. This suggests that BAP retarded RNA degradation in these expanding cotyledons. The ability of cytokinins to suppress ribonuclease activity in leaf tissue is well known (see section 1.5-1).

BAP could have had a substantial early effect on the proportions of individual RNA species, or on their rates of incorporation of precursors, without significantly changing the amount or specific activity of total RNA. Accordingly, experiments were undertaken to label, extract and separate the individual RNA species.
Double labelling experiments revealed that BAP caused a small stimulation of incorporation of uridine into RNA species that were located in the ribosomal RNA region of the polyacrylamide gels. The lack of an increased ratio over the 4S-5S peak (Figure 5-8) indicates that the observed stimulation of incorporation was probably not a consequence of an increased specific activity of UTP. If this had occurred, an increased $^{14}C/^{1}H$ ratio would be expected for all RNA species. Although the effect was not observed before 18 hours of BAP treatment, it was of interest to determine whether the RNA involved was ribosomal, or minor polydisperse species. When the formation of mature ribosomal RNA was inhibited with 5-FU, BAP appeared to induce a proportionally greater stimulation of uridine incorporation into the RNA in the ribosomal region of the gel. The most interesting interpretation of this result is that BAP preferentially stimulated the incorporation of precursors into polydisperse RNA, for this effect could well reflect a stimulation of the synthesis of messenger RNA, a constituent of polydisperse RNA. This hypothesis is supported by observations that while 5-FU strongly inhibits ribosomal RNA production in higher plants, it has little effect on RNA species thought to be messenger RNA (Cherry and van Huystee, 1965; Key and Ingle, 1964, 1968; Key, 1966). There is, however, another possible explanation for the result presented herein. There is now considerable evidence that 5-FU does not inhibit the synthesis of ribosomal RNA precursors; rather it appears that the incorporation of 5-FU into these forms results in their instability and premature non-specific breakdown, and hence in a lack of functional mature ribosomal RNA species and ribosomes (Paranjothy and Wareing, 1971; Rose and Setterfield, 1971; Bex, 1972; King and Chapman, 1972; Rose et al., 1970). Whether breakdown occurs before or after ribosome formation is not clear (see Willen and Stenram, 1967; Rose and Setterfield, 1971; King and Chapman, 1972). Gel electrophoresis data presented by Paranjothy and Wareing (1971) and Bex (1972) have shown that preparations from 5-FU-treated plant tissue contain a broad spread of radioactivity in the region of ribosomal RNA. Between the absorbance peaks of
ribosomal RNA, the level of this radioactivity is higher than in preparations derived from untreated tissue or from tissue in which ribosomal RNA synthesis was preferentially inhibited with abscisic acid (Bex, 1972). These results suggest that the apparent polydisperse RNA from 5-FU-treated tissue may in fact be the rapidly accumulated breakdown products of newly synthesized labelled ribosomal RNA. If this were the case in radish cotyledons, the increased $^{14}$C/$^3$H ratio in Figure 5-10 could be due to a positive effect of BAP on the accumulation of such breakdown products, possibly through inhibition of their degradation. (This inhibition could be readily explained by the known ability of cytokinins to suppress nuclease activity, which has already been mentioned in another context in this discussion). The similar results of Paranjothy and Wareing (1971) with 5-FU treated radish leaf tissue could be interpreted in the same way. These workers claimed without justification, however, that a cytokinin-induced increase in radioactivity in polydisperse RNA species in the ribosomal region of gels was the result of cytokinin stimulation of the synthesis of polydisperse RNA.

From the results of the present study it is not possible to decide definitely whether incorporation of uridine into polydisperse RNA was promoted. The data presented in Figures 8 to 10 can be explained entirely by an effect on ribosomal RNA but probably not by an effect on polydisperse RNA solely. If incorporation into polydisperse RNA was promoted, the effect was certainly not marked relative to incorporation into ribosomal RNA.

No evidence was obtained for an effect of BAP on the RNA synthesis during the lag period. However, the earliest detectable growth induced by BAP is suppressed by actinomycin D. Hence the synthesis of minor RNA species may be essential for cytokinin-induced growth, but their formation was not detectable by the methods employed in this chapter. If messenger RNA was synthesized in response to cytokinin, an effect on polysome levels might be detectable. This possibility is considered in the next chapter.
CHAPTER 6: AN INVESTIGATION OF THE EFFECT OF CYTOKININS ON RIBOSOME AND POLYSOME YIELDS

INTRODUCTION

Changes in plant metabolism associated with development, senescence and environmental responses are known to be accompanied by modifications in the levels of ribosomes and polysomes, and in the proportions of these organelles that are free and membrane-bound. Studies with developing cells suggest that cells undergoing rapid cell division have predominantly free ribosomes, and differentiating cells have mainly membrane-bound ribosomes (Javot, 1964; Malling, 1968). Thus, although the RNA content of developing cells in coleoptiles remained approximately constant, the quantity of membrane-bound ribosomes increased (Payne and625; Malling, 1969). In early phases of seed germination, initiation of protein synthesis is correlated with the rapid increase in free ribosomes to polysomes in coleoptiles and roots (Malling, 1969). On the other hand, the senescence of leafy barley leaves is accompanied by a marked decline in the level of both ribosomes and polysomes (Hiratake, 1968). It has been reported that the exposure of plants to sunlight results within minutes in a rise in RNA levels and a decrease in 80S ribosome levels (Clark, 1964). Water stress also decreases the proportion of polysomes to 80S ribosomes (Hiratake, 1970).

In animal tissues, ribosomes bound to rough endoplasmic reticulum appear to be concerned with the synthesis of secretory proteins; ribosomes free in the cytoplasm appear to be involved in the synthesis of intracellular proteins. Whether a distinction in function of free and membrane-bound ribosomes applies to developing tissues is not clear, although in some instances it has been suggested that the two ribosomal forms may synthesize different groups of proteins (see references cited by Payne and Malling, 1969).

In view of the above observations, it is not surprising that plant and animal responses markedly affect...
CHAPTER 6

A. INTRODUCTION

Changes in plant metabolism associated with development, senescence and environmental responses are known to be accompanied by modifications in the levels of ribosomes and polysomes, and in the proportions of these that are free and membrane-bound. Studies with developing tissues suggest that cells undergoing rapid cell division have predominantly free ribosomes and differentiating cells have mainly membrane-bound ribosomes (Buvat, 1963; Loening, 1961). Thus, although the RNA content of developing *Vicia faba* cotyledons remained approximately constant, the quantity of membrane-bound ribosomes increased (Payne and Boulter, 1969). In early phases of seed germination, initiation of protein synthesis is correlated with the rapid conversion of a considerable part of the monoribosome population to polysomes (see references cited by Weeks and Marcus, 1971). On the other hand, the senescence of detached barley leaves is accompanied by a marked decline in the level of both ribosomes and polysomes (Srivastava and Arglebe, 1967). It has been reported that the exposure of plants to sunlight results within minutes in a rise in polysome levels and a decrease in 83S ribosome level (Clark et al., 1964). Water stress also decreases the proportion of polysomes to 80S ribosomes (Hsaio, 1970).

In animal tissues, ribosomes bound to membranes of the endoplasmic reticulum appear to be concerned with the synthesis of secretory proteins; ribosomes free in the cytoplasm appear to be involved in the synthesis of intracellular proteins. Whether a distinction in function of free and membrane-bound ribosomes applies to developing plants is not clear, although in some instances it has been suggested that the two ribosomal forms may synthesize different groups of proteins (see references cited by Payne and Boulter, 1969).

In view of the above observations, it is not surprising that plant and animal hormones markedly affect
the ribosome population of target tissues. Auxins have been reported to increase the levels of ribosomes and polysomes in a number of tissues (see, for example, Penon et al., 1970; Fowke and Setterfield, 1968). In etiolated pea tissue and in soybean hypocotyl, the effect involves an increase in the proportion of polysomes relative to ribosomes (Trewavas, 1968a; Fites et al., 1969). Similarly, gibberellic acid increases ribosome levels and the formation of polyribosomes in barley aleurone layers, effects suppressed by abscisic acid (Evins and Varner, 1972). There is little information on effects of cytokinins on ribosome populations. However, cytokinins are known to maintain ribosome levels in excised leaf tissue (Srivastava and Arglebe, 1968; Berridge and Ralph, 1969), and to increase the proportion of free ribosomes in Spirodela (McCombs and Ralph, 1972). A recent significant observation concerning animal hormones is the ability of certain steroids to promote binding of polysomes to smooth microsomal membranes of rat liver in vitro (Sunshine et al., 1971).

In this chapter, an attempt was made to assess the effect of cytokinins on the proportions of free and membrane-bound ribosomes and on ribosome and polysome levels in radish cotyledons.

B. MATERIALS AND METHODS

1. Chemicals

<table>
<thead>
<tr>
<th>Chemical*</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bentonite</td>
<td>Sigma</td>
</tr>
<tr>
<td>DOC (sodium deoxycholate)</td>
<td>BDH</td>
</tr>
<tr>
<td>DTT (dithiothreitol)</td>
<td>Sigma</td>
</tr>
<tr>
<td>HEPES (N-2-hydroxyethylpiperazine-N(^\prime)-2-ethanesulphonic acid)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Lipase</td>
<td>Serva</td>
</tr>
<tr>
<td>PVS (polyvinyl sulphate)</td>
<td>Dr. P.R. Whitfeld, C.S.I.R.O., Canberra</td>
</tr>
</tbody>
</table>

* This list includes only those special reagents for which a source has not previously been given.
2. Extraction and analysis of ribosomes

The procedure adopted for the extraction of ribosomes from radish cotyledons was based on the method of Berridge et al., 1970. This method is designed to yield ribosomes of high purity; however, in the present study, a higher priority was placed on quantitative recovery. Hence, the number of purification steps was slightly reduced, while several compounds reputed to enhance ribosome or polyribosome yields during extraction were tested, using radish cotyledons, and those found to be effective were incorporated into the extraction buffer. Details of these tests appear in results (6.5). The method of ribosome extraction finally adopted is outlined below.

Batches of cotyledons (2.5 g) were each treated as follows. The cotyledons were chilled for 5 minutes at 4°, and then ground in a mortar at this temperature with 2 ml of Nirenberg buffer (60 mM KCl, 6 mM mercaptoethanol, 16 mM magnesium acetate and 10 mM Tris acetate, pH 7.6) to which had been added Triton-X-100 (0.5%) and DOC (0.5%) (see 6.5). The resulting smooth paste was rinsed from the mortar with 11 ml of grinding solution and then centrifuged at 600 x g for 5 minutes to remove cell debris. Smaller fragments and mitochondria were sedimented at 18,000 x g for 15 minutes, followed by 10 minutes at 27,000 x g. The supernatant was then transferred to a preparative ultracentrifuge tube, and centrifuged at 225,000 x g for 60 minutes to sediment the ribosomes.

For examination of ribosome sedimentation profiles and assessment of the concentration of individual ribosome species, each ribosome pellet was resuspended in 0.5 ml of Nirenberg buffer and centrifuged at 40,000 x g in a Spinco model E analytical ultracentrifuge equilibrated to 20°. Since the presence of protein in the ribosomal preparations made assessment of sedimentation velocity unreliable, the sedimentation coefficient of radish cotyledon cytoplasmic ribosomes was established by co-sedimentation with pure yeast 80S ribosomes (gift of Dr. R.S.T. Yu of this Department).

To confirm quantitative information obtained by analytical ultracentrifugation, ribose determinations were
carried out by the orcinol method (Markham, 1955).

3. **Electron microscopy**

Radish cotyledons were fixed in glutaraldehyde (3% in sodium phosphate buffer, pH 7.2) and post-fixed in 2% osmium tetroxide (also in phosphate buffer). The cotyledons were then dehydrated in a graded alcohol series and embedded in Spurrs ERL 4206 resin. Sections were cut with a Reichert OMU2 ultramicrotome and were then stained with Reynolds lead citrate stain and with uranyl acetate. Preparations were examined in a Hitachi HU11E electron microscope.

4. **Preparation of RNA and polyacrylamide gel electrophoresis**

The procedures followed are detailed in Table 5-2 and section 5.4 respectively.

C. **RESULTS**

5. **The effect, on ribosome and polysome yield, of various additives or substitutions in the grinding buffer**

In each of the tests summarized below, the cotyledons used for extraction of ribosomes were incubated, as described in section 2.1, in Petrie dishes for 3 days in continuous light. BAP was added to the incubation medium to increase the growth of the cotyledons. The effect of each alteration to the grinding buffer was assessed by grinding 2.5 g of cotyledons in modified buffer and 2.5 g of cotyledons in unmodified Nirenberg buffer and comparing the yield of ribosomes and polyribosomes by analytical ultracentrifugation. Unless otherwise stated, resuspension of ribosomal pellets for analysis was in unmodified Nirenberg buffer. The results of these tests (Table 6-1) confirmed that 16 mM Mg\(^{2+}\), which was appropriate for extraction of Chinese cabbage ribosomes when 10 mM Tris was used (Berridge et al., 1970), was also appropriate for extraction of radish cotyledon ribosomes. Lower concentrations of Mg\(^{2+}\) resulted in lower yields of all ribosome species. DOC and Triton-X-100 each appreciably improved
Table 6-1  Relative ribosome + polyribosome yields estimated by analytical ultracentrifugation of ribosome fractions prepared using various modifications of Nirenberg Buffer

<table>
<thead>
<tr>
<th>chemical</th>
<th>effect for which chemical is known¹</th>
<th>medium to which added</th>
<th>concentration</th>
<th>relative yield²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg²⁺</td>
<td>concentration (particularly relative to monocationic concentration) affects ribosome stability (Ghysen et al., 1970).</td>
<td>NB - Mg²⁺</td>
<td>1 mM</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NB - Mg²⁺</td>
<td>5 mM</td>
<td>-</td>
</tr>
<tr>
<td>DOC</td>
<td>releases ribosomes from membranes.</td>
<td>NB</td>
<td>0.05% (w/v)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NB</td>
<td>0.50% (w/v)</td>
<td>+++</td>
</tr>
<tr>
<td>HEPES</td>
<td>a buffer base like Tris; recommended by some workers for increased yields (Arglebe and Hall, 1969).</td>
<td>NB + DOC (0.5%)</td>
<td>20 mM</td>
<td>-</td>
</tr>
<tr>
<td>Triton-X-100 releases ribosomes from membranes.</td>
<td>NB</td>
<td>0.5% (w/v)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NB</td>
<td>1.0% (w/v)</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NB + DOC (0.5%)</td>
<td>0.5% (w/v)</td>
<td>+++</td>
</tr>
<tr>
<td>Lipase⁵</td>
<td>removes ribosomes from membranes (van Dijk-Salkinoja et al., 1970).</td>
<td>NB + DOC (0.5%)</td>
<td>100 µg/ml</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ TX' (0.5%)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>DEP⁴</td>
<td>inhibits RNase³ (Weeks and Marcus, 1969)</td>
<td>NB</td>
<td>1% (v/v)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NB + DOC (0.5%)</td>
<td>1% (v/v)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NB + DOC (0.5%)</td>
<td>0.5% (v/v)</td>
<td>-</td>
</tr>
<tr>
<td>PVS</td>
<td>inhibits RNase (Clark et al., 1963).</td>
<td>NB + DOC (0.5%)</td>
<td>5 mg/ml</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TX' (0.5%)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NB + DOC (0.5%)</td>
<td>10 mg/ml</td>
<td>-</td>
</tr>
<tr>
<td>DTT</td>
<td>inhibits RNase Breen et al., 1971.</td>
<td>NB</td>
<td>4 mM</td>
<td>-</td>
</tr>
<tr>
<td>In⁶⁺</td>
<td>inhibits RNase (Payne and Loening, 1970).</td>
<td>NB</td>
<td>5 mM</td>
<td>-</td>
</tr>
<tr>
<td>Bentonite</td>
<td>binds RNase (Watts and Mathias, 1967).</td>
<td>NB (twice normal conc.) + DOC (0.5%)</td>
<td>0.25 mg/ml</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NB + DOC (0.5%)</td>
<td>0.25 mg/ml</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NB + DOC (0.5%)</td>
<td>1 mg/ml</td>
<td>-</td>
</tr>
</tbody>
</table>

1. For general reference, see Loening, 1968.
2. Each yield is compared with that obtained using Nirenberg buffer. The column refers to total yield since in each case the effects (if any) on ribosomes and polyribosomes were similar.
   = : no effect (± 5%).
   + : ± 5% to 25% difference (positive or negative respectively).
   ++ : ± 25% to 50% difference.
   +++: ± >50% difference.
3. NB = Nirenberg buffer.
4. TX = Triton-X-100.
5. Lipase was added after initial grinding.
6. DEP was added to the grinding buffer immediately before use.
7. The additional effect of Triton-X-100 was usually but not consistently observed.
8. RNase = ribonuclease.
the yield of both ribosomes and polyribosomes, and the presence of both in the buffer was usually slightly more effective. This result suggested that a considerable proportion of ribosomes and polyribosomes of the radish cotyledons were membrane bound. It seemed likely that grinding the tissue in the presence of both DOC and Triton-X-100 extracted a high proportion of the membrane-bound ribosomal species, since addition of lipase (100 µg/ml) to the homogenate did not further increase the ribosome yield. At the concentrations tested, no other compound listed in Table 6-1 further improved the yield of either ribosomes or polyribosomes. In view of these results, the grinding buffer defined in section 6-1 was employed for most further studies. To confirm the positive effects of Mg\(^{2+}\), DOC and Triton-X-100 on ribosome yield, aliquots of the appropriate ribosome preparations were also assayed for ribose (Table 6-2). The relative area under the 80S ribosomal peak and the relative ribose content of each preparation were in approximate agreement.

Table 6-2  Relative ribosome yields estimated by ribose content of ribosome pellets prepared from radish cotyledon tissue ground in various modifications of Nirenberg buffer

<table>
<thead>
<tr>
<th>Modification to Nirenberg buffer</th>
<th>Ribose content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100.0</td>
</tr>
<tr>
<td>1 mM Mg(^{2+})</td>
<td>66.7</td>
</tr>
<tr>
<td>5 mM Mg(^{2+})</td>
<td>82.2</td>
</tr>
<tr>
<td>+ 0.05% DOC</td>
<td>159.3</td>
</tr>
<tr>
<td>+ 0.5% DOC</td>
<td>192.0</td>
</tr>
<tr>
<td>+ 0.5% Triton-X-100</td>
<td>177.4</td>
</tr>
<tr>
<td>+ 0.5% DOC + 0.5% Triton-X-100</td>
<td>195.5</td>
</tr>
</tbody>
</table>

During tests concerning the effect of [Mg\(^{2+}\)], it was found that between replicates within an experiment, the variation in ribosome yield, as determined from Schlieren profiles, was less than 5%. Rather more variation was detected between equivalent treatments in different
experiments, possibly because of slight differences in incubation conditions which depended on the number of cotyledons required for each experiment, and were subject to small fluctuations in temperature. However, this variation did not significantly alter the relative effects of various treatments within a particular experiment.

6. **The effect of BAP and kinetin on ribosome and polyribosome yields**

6-1. **Total ribosomal species**

Several experiments were performed in which radish cotyledons were cultured aseptically in Petrie dishes in continuous light (section 2.1) on (+BAP) medium, on medium containing kinetin, 10 μg/ml [(+KIN) medium] and on control medium (i.e. phosphate buffer). At intervals, a 2.5-g sample of cotyledons from each treatment was extracted as described in section 6.2 and the resulting ribosome preparations were examined by analytical ultracentrifugation.

Schlieren profiles illustrating the results of the first experiment are presented in Figure 6-1. In this experiment the earliest time at which ribosomes were detected in cotyledon extracts was at 40 hours after the commencement of incubation [Figure 6-1(A)]. An 80S ribosomal peak was detected at this time in extracts of (+KIN) and (+BAP) cotyledons but not of control cotyledons. The ribosome yield from cytokinin-treated tissue thereafter increased rapidly [Figure 6-1(B) and (C)], and between 50 and 72 hours there was also a marked increase in polysome yield. (-BAP) cotyledons, however, yielded ribosomes only at the 72-hour sampling time and in this experiment no distinct polysomes were observed. In a second similar experiment in which (+BAP) and (-BAP) cotyledons only were employed, ribosomes were detected in (-BAP) cotyledons at 50 hours, and a more substantial 80S peak and a dimer peak were seen at 72 hours. However, the major trends observed in the experiment illustrated in Figure 6-1 were confirmed. Thus, at each sampling time when ribosomal species were detected, yields of 80S ribosomes and polysomes from tissue treated with cytokinin were markedly higher than those from control tissue.
Figure 6-1  Analytical sedimentations of ribosomal preparations from radish cotyledons that were cultured on (+KIN), (+BAP) and control media

Batches (2.5 g) of cotyledons were sampled after 24, 40, 50 and 72 hours of incubation and extracted as described in section 6.2. For each sampling time, ribosome preparations derived from (+BAP) and control cotyledons were sedimented during the same centrifugation, a 1° positive wedge window being used to displace the profile designated "(+BAP)"; the profile designated "(+KIN)" is superimposed. Line drawings were traced from Schlieren photographs taken at an analyzer angle of 60°. At 24 hours (not illustrated) no ribosomal peaks were observed.

Note (Figures 6-1 to 6-4): The 18S component in the patterns [e.g. (A)] is Fraction I protein of chloroplasts, which, because of its size, is often a contaminant of ribosomal preparations of leaves (see Kawashima and Wildman, 1970; Whitfeld, 1973). In this study, cytokinins did not appear to affect the levels of this protein.
(A) 40 HOURS

(B) 50 HOURS

(C) 72 HOURS
In addition, no ribosomes were detectable in tissue from either treatment earlier than 40 hours. Similarly, in a third experiment cotyledons incubated for 24 and for 36 hours yielded no ribosomes. Moreover, when ribosomes were not detected by analytical ultracentrifugation, u.v. and ribose analysis of the 225,000 x g pellet also indicated an absence of RNA. It appeared therefore that in (+BAP)- and control-treated tissue incubated for less than 40 hours, ribosomes were either very scarce or were not being extracted.

To determine whether radish cotyledon tissue contained ribosomes by the time incubations were initiated, radish cotyledons were fixed immediately after excision and prepared for electron microscopy (see section 6.3). Examination of numerous sections indicated that most cells were heavily packed with storage material, and no ribosomes could be distinguished (Plate 6-1). However, in the corners of a few sectioned cells, cytoplasm was observed which appeared to contain endoplasmic reticulum and ribosomes (Plate 6-2). Since it was not possible to assess the ribosome content of a typical cell by electron microscopy, this approach contributed little to the problem.

6-2. Free and membrane-bound ribosomal species

In the experiments reported above, BAP and kinetin markedly enhanced the total (i.e. free + membrane-bound) ribosome yield of radish cotyledon tissue, the earliest observed effect being on 80S ribosomes. To determine whether cytokinin also affected the yield of free ribosomes, batches of cotyledons cultured on (+KIN) and control media for 30, 48 and 72 hours were homogenized for ribosome extraction using Nirenberg buffer without added DOC or Triton-X-100. While all ribosome yields were low, Schlieren photographs of these extracts (Figure 6-2) showed trends similar to those observed when total ribosomes were extracted. Thus, a peak of 80S ribosomes was first observed with kinetin-treated tissue after 48 hours of incubation [Figure 6-2(A)], while in control tissue 80S ribosomes were first detected at 72 hours as a very small Schlieren peak. At the latter time the yield of 80S ribosomes from kinetin-treated tissue was considerably higher and 70S and polysome species were
Plate 6-1  Electron micrograph of a section through a typical mesophyll cell in radish cotyledon tissue fixed after 40 hours of germination in darkness.

1 μ
Plate 6-2  Electron micrograph of a section through a cell of unidentified type in radish cotyledon tissue fixed after 40 hours of germination in darkness

1 μ
Figure 6-2 Analytical sedimentations of ribosomal preparations from radish cotyledons that were cultured on (+KIN) and control media

Batches (2.5 g) of cotyledons were sampled after 30, 48 and 72 hours of incubation and homogenized in Nirenberg buffer. Ribosome extraction was continued as described in section 6.2. At each sampling time, ribosome preparations derived from (+KIN) and control cotyledons were sedimented during the same centrifugation, a 1° positive wedge window being used to displace the profile designated "(+KIN)". Line drawings were traced from Schlieren photographs taken at an analyzer angle of 55° (A) and 60° (B). At 30 hours (not illustrated) no ribosomal peaks were observed.
(A) 48 HOURS

(B) 72 HOURS
also detected [Figure 6-2(B)]. These results were consistent with previous observations (Table 6-1) that most of the ribosomes in developing radish cotyledons are membrane-bound. The results also indicated that kinetin had a similar effect on the yield of both free and membrane-bound ribosomes.

To obtain a more direct comparison between the kinetin-enhanced yields of bound and unbound ribosomes, a further experiment was performed in which duplicate samples of both (+KIN) and control cotyledons were taken at each sampling time. Of each duplicate pair, one sample was homogenized in Nirenberg buffer and the other in Nirenberg buffer + 0.5% DOC. All centrifugation profiles containing ribosomal peaks are illustrated in Figure 6-3. When the results are compared with those of the previous experiment, the appearance of free ribosomes in extracts of both (+KIN)-treated and control tissue was somewhat delayed: in (+KIN)-tissue extracts, free 80S ribosomes were first observed at 72 hours, while in control preparations ribosomes were not observed at all. In contrast, 80S ribosomes were observed as early as 30 hours in preparations of (+KIN) tissue that had been homogenized in the presence of DOC. However, the major effects observed in the previous experiments were confirmed. An enhancement of both free and membrane-bound ribosomes by kinetin was clear, as was the high proportion (>90%) of membrane-bound ribosomes and polysomes [compare Figures 6-3(C) and (D)].

7. The effect of mannitol on cytokinin-enhanced ribosome yield

To examine the possibility that the enhancement of ribosome yield was dependent upon cytokinin-induced growth, ribosomal fractions were prepared from batches of (+BAP) and (-BAP) cotyledons incubated for 3 days in the presence and in the absence of mannitol (0.3 M). This concentration of mannitol eliminated cytokinin-induced growth. Schlieren profiles of the ribosomal fractions prepared from these batches of cotyledons (Figure 6-4) showed that the ribosome yield from (+BAP) cotyledons grown in the presence of mannitol was reduced to the control level, i.e. the yield from (-BAP) cotyledons grown in the absence of mannitol
Figure 6-3  Analytical sedimentations of ribosomal preparations from radish cotyledons that were cultured on (+KIN) and control media and homogenized in the presence or absence of DOC (0.5%) 

Duplicate batches (2.5 g) of (+KIN) and control cotyledons were sampled after incubation periods of 30, 48 and 72 hours. Of each duplicate pair, one sample was homogenized in Nirenberg buffer and the other in Nirenberg buffer + 0.5% DOC. At each sampling time, ribosome preparations derived from (+KIN) and control cotyledons were sedimented during the same centrifugation, a 1° positive wedge window being used to displace the profile designated "(+KIN)". Line drawings were traced from Schlieren photographs taken at an analyzer angle of 55° [(A) and (D)] and 60° [(B) and (C)]. The profiles of the 30-hour and 48-hour preparations that were homogenized in the absence of DOC (not illustrated) showed no ribosomal peaks.
(A) 30 HOURS
+DOC

(B) 48 HOURS
+DOC

(C) 72 HOURS
+DOC

(D) 72 HOURS
-DOC
Figure 6-4  Analytical sedimentations of ribosomal preparations from radish cotyledons cultured on (+BAP) and (-BAP) media in the presence and absence of mannitol (0.3 M)

After incubation for 3 days on the media designated in the figure, batches (2.5 g) of cotyledons were extracted as described in section 6.2. Line drawings were traced from Schlieren photographs taken at an analyzer angle of 60°. Profiles of preparations sedimented during the same centrifugation appear in a common frame. A 1° positive window was used to displace the upper profile in each frame.
[Figure 6-4(A)]. Thus, in BAP-treated tissue, mannitol had a similar effect on both growth and ribosome yield. The growth and ribosome yield of control tissue was also depressed by mannitol; moreover, in a similar experiment in which a higher concentration of mannitol was used (0.5 M), both growth and ribosome yield were further depressed.

In view of these results, it was possible that the effect on ribosome yield was a consequence of cytokinin-induced growth, and not a direct effect of cytokinin. In reaching this conclusion, it has of course been assumed that mannitol had no side effects, that is, it functioned simply as a regulator of osmotic pressure and did not directly affect basic metabolism.

8. **Effect of BAP on ribosomal RNA levels**

Results reported in section 5.7-2 indicated that BAP did not markedly affect total RNA per unit fresh weight of cotyledon tissue. Since most of the total RNA was ribosomal RNA, as confirmed by gel electrophoresis, it seemed unlikely that the dramatic effect of cytokinins on ribosome yield could be due to an enhancement of ribosome levels in this tissue. However, to obtain more exact information on the levels of ribosomal RNA in (+BAP) and (-BAP) radish cotyledons, extractions of undegraded RNA were carried out by the DEP method and the efficiency of the method with (+BAP) and (-BAP) cotyledons was tested.

Cotyledons were cultured in Petrie dishes, in continuous light, on (+BAP) and (-BAP) media. After 24, 48, 72 and 96 hours, a 1-g sample of cotyledons was removed from each treatment and nucleic acid species extracted according to Figure 5-2, using twice the stated quantities, and a second extraction of the tissue. For each nucleic acid preparation, three 1%-aliquots were subjected to polyacrylamide gel electrophoresis, which was continued for 3 hours to improve the clarity of the base line in absorbance traces and the resolution of RNA species. The areas under the 25S and 18S cytoplasmic ribosomal RNA peaks in absorbance traces were measured, and the mean combined area per aliquot calculated for each nucleic acid preparation (Figure 6-5). The results indicated that per unit fresh weight of tissue, the amount of
Cotyledons were cultured in Petrie dishes in continuous light. RNA extraction was by the DEP method. The amounts of cytoplasmic RNA (25S + 18S) per 100 mg fresh weight of cotyledon tissue are expressed in terms of the total area (cm²) under the cytoplasmic RNA peaks in gel absorbance tracings of the nucleic acid preparations. (For total RNA content in mg/g fresh weight of tissue, see Table 6-4).

Culture treatments were as follows:

- (BAP)
- (-BAP)
ribosomal RNA extracted from (+BAP) cotyledons was higher than the amount extracted from (-BAP) cotyledons only at the 24-hour sampling time; thereafter, (-BAP) cotyledons yielded more RNA than (+BAP) cotyledons.

To test the efficiency of the DEP method, Matthews' method was used to determine the following:

1. total RNA in the original tissues.
2. RNA in the purified nucleic acid fraction.
3. RNA in the cell debris and denatured protein pelleted during nucleic acid extraction by the DEP method.

Results of these estimations revealed that the DEP method had extracted RNA equally thoroughly from both (+BAP) and (-BAP) cotyledons (Table 6-3).

Table 6-3 Total RNA extracted from radish cotyledons by the DEP method, and estimates of recovery

<table>
<thead>
<tr>
<th>Period of incubation (hours)</th>
<th>Total RNA extracted by DEP method* (mg/g fresh wt.)</th>
<th>RNA in debris and denatured protein pellets as % of RNA extracted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(+BAP) medium</td>
<td>(-BAP) medium</td>
</tr>
<tr>
<td>24</td>
<td>0.414</td>
<td>0.378</td>
</tr>
<tr>
<td>48</td>
<td>0.606</td>
<td>0.829</td>
</tr>
<tr>
<td>72</td>
<td>0.488</td>
<td>0.719</td>
</tr>
<tr>
<td>96</td>
<td>0.319</td>
<td>0.544</td>
</tr>
</tbody>
</table>

* The yields were estimated by Matthews' method. They are about 92% of the total cell RNA of both (+BAP) and (-BAP) tissue.

Examined as a whole, the results presented in Figure 6-5 and Table 6-3 corroborate the results obtained during the examination of BAP effects on total RNA which were described in Chapter 5, and suggest very strongly that the effect of BAP on ribosome yield is due to a cytokinin-enhanced extractability of ribosomes.
9. Distribution of labelled 25S RNA between ribosome pellets and cell debris fractions

To test the possibility that BAP affected the extractability of recently synthesized ribosomes, the distributions of labelled 25S RNA between the cell debris pellets and ribosome pellets derived from (+BAP) and (-BAP) cotyledons were compared. The experimentation outlined in Figure 6-6 was followed. The cell debris pellet was re-extracted with Nirenberg buffer containing DOC and Triton-X-100. In this respect, the procedure for ribosome extraction differs from that previously employed (section 6.2). The yield of cell debris nucleic acid (CDNA) from (+BAP) cotyledons was 57% of the yield from (-BAP) cotyledons. CDNA was subjected to gel electrophoresis which revealed DNA, 25S, 23S, 18S, 17S, 16S and 13S RNA (gels stained Azur I). The 25S zones were excised and ^3H determined; the total radioactivity in 25S RNA of CDNA was then calculated. rRNA-1 and rRNA-2 were also electrophoresed and the % of the applied radioactivity in 25S RNA determined. The radioactivities in the ribosome pellets 1 and 2 [this radioactivity was entirely precipitated by 2 volumes of ethanol—acetic acid (20:1 v/v)] were multiplied by the corresponding % to give the radioactivity contained in the pellets as 25S RNA. The results obtained appear as Table 6-3. Radioactivity

Table 6-4 The radioactivity in ribosome pellets and cell debris fractions derived from (+BAP) and (-BAP) cotyledons

<table>
<thead>
<tr>
<th></th>
<th>Radioactivity in 25S RNA (c.p.m. x 10^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-BAP)</td>
</tr>
<tr>
<td>ribosome pellet 1</td>
<td>35.9</td>
</tr>
<tr>
<td>ribosome pellet 2</td>
<td>23.8</td>
</tr>
<tr>
<td>CDNA</td>
<td>17.6</td>
</tr>
</tbody>
</table>

in the first ribosome pellet, expressed as a % of the total in 25S RNA, was 46% for (-BAP) cotyledons and 72% for (+BAP) cotyledons. Hence, growth in the presence of BAP enhanced either extractability or recovery of recently synthesized
Figure 6-6 Procedure for preparation of RNA from cell debris and ribosome pellets

Cotyledons cultured (+BAP) and (-BAP) media for 2 days in light, labelled with $[^{3}H]$uridine (2.5 uCi/ml) for 4 hours, washed and then stirred with media containing unlabelled uridine (0.03 mg/ml) for 2.5 hours; 2.5 g tissue ground with NB (10 ml) containing DOC and TX; centrifuged:

- Cell debris pellet: 600-27,000 x g
- Stirred NB (10 ml) containing DOC and TX for 7 minutes; centrifuged as above.
- Supernatant 1
  - 225,000 x g
  - Ribosome pellet 1
    - Stirred NB (10 ml) and repelleted; aliquot hydrolysed (0.1 N NaOH) for counting.
- Cell debris pellet
  - RNA preparation
    - (DEP method, 2 extractions)
  - Supernatant 2
    - As for supernatant 1
    - RNA preparation (DEP method)
- CD NA
- rRNA-2
- rRNA-1

Note: abbreviations as in Table 6-1.
ribosomes. In another experiment in which the RNA was extracted by a phenol method (Burdett and Wareing, 1968), a similar result was obtained.

D. DISCUSSION

The results presented clearly establish that cytokinins markedly increase the yield of free ribosomes and ribosomes released by detergent which are presumably membrane-bound. Enhanced yields of polysomes were similarly observed. These observations could be accounted for in the following ways, which are, of course, not mutually exclusive.

1. Cytokinins enhance levels of both free and membrane-bound ribosomes.
2. Cytokinin treatment enhances the recovery of free and membrane-bound ribosomes from radish cotyledon tissue.
3. There are three classes of ribosomes, namely, free, membrane-bound and a further class that is not readily extractable, or non-extractable, by methods used in this study (n.e. ribosomes). Cytokinin treatment decreases the ratio of n.e. ribosomes to free and membrane-bound ribosomes.

In relation to the third possibility it is relevant that ribosomes which are not extracted by detergent (DOC) can be released from highly purified preparations of cell walls of barley shoots by passing the suspension of cell walls through a French press (Jervis and Hallaway, 1970). It seemed probable that the ribosomes thus isolated had been incorporated into cell wall structure.

Consideration of the results obtained indicates that the first possibility can be eliminated. By the time the cytokinin-enhanced ribosome yield was detectable, levels of ribosomal RNA in (+BAP) tissue were in fact lower than those of (-BAP) tissue. The ribosome yield results obtained could, however, be adequately explained by either of the two remaining possibilities, namely the effects on ribosome recovery and extractability. The labelling experiment is in agreement with this proposal, but does suggest that an effect
on recovery is the more likely explanation, at least with respect to recently synthesized ribosomes. This is evidenced by the substantial proportion of radioactivity obtained in the second extraction of the debris pellet derived from (-BAP) tissue (see Table 6-3).

How BAP could affect ribosome recovery is not clear. It is possible, however, that the high density of storage granules in the cytoplasm of radish cotyledons, especially at the time of excision before significant cell expansion has occurred (c.f. Plate 6-1), might interfere with ribosome recovery. Cytokinins could alleviate this effect by enhancing cell expansion and consequent vacuole enlargement, or by increasing the rate of degradation of storage material.

If the cytokinin effect on ribosome yield is indeed merely a consequence of enhanced recovery (i.e. possibility 2), the phenomenon is clearly of no significance to the mechanism of cytokinin action. Even if the effect is a consequence of possibility 3, two observations suggest that the effect is an indirect result of cytokinin action:

1. The effect is not detected until well after the induction of growth.
2. The effect is eliminated in the presence of concentrations of mannitol which abolish cytokinin-induced growth. Ribosome yield from, and the growth of, (-BAP) cotyledons are also suppressed by mannitol. Thus, ribosome yield and growth seem to be closely associated.

Even in the presence of a number of inhibitors of ribonuclease, polysome yields from (+BAP) cotyledons were very low relative to ribosomes; the yield from (-BAP) cotyledons was frequently too low for detection. Hence, it was not possible to determine whether cytokinins influenced the relative proportions of ribosomes and polysomes. It is relevant that Pearson (1969) was unable to improve polysome yields from radish leaves by using various polysome "protectants".

The results presented in this chapter indicate
that ribosome yield is not a reliable indication of the tissue content of ribosomes. Numerous reports in the literature which consider these parameters to be equivalent require re-assessment.
CHAPTER 7

A. INTRODUCTION

Although some observations suggest that cytokinins may directly affect translational processes (see section 1.5-1), there is little convincing evidence that an effect on protein metabolism precedes cytokinin-induced growth. The only observations which would support this possibility are those of Raskin and Erigam (1999) and of McCool and Ralph (1972). In the former study, kinetin was found to increase the quantity of protein in tobacco micro cultures within 15 minutes without influencing the levels of free amino acids. McCool and Ralph detected a stimulation in the rate of incorporation of \([^{14}S]\) sulphate into protein in etiolated 1 hour after the addition of kinetin to the growth medium. A number of other references in the literature report the labelling of amino acids into protein, but in cases where the cytokinin influences growth, there is insufficient information to determine whether the effect on protein precedes the growth response to cytokinin (see e.g. Neeraj and Lautrey, 1967; Hung, 1971). Such effects on cellular metabolism may be indirect responses to cytokinin. Effects observed in many labelling studies may merely reflect changes in specific activity of precursor pools.

This chapter describes experiments performed in an attempt to ascertain whether cytokinin-induced amino acid incorporation into protein precedes growth induction in tobacco cotyledons. Effects of cytokinin on amino acid uptake and pool sizes were also assessed.

While the structure of a protein molecule is determined primarily by the sequence of amino acids, certain proteins are modified after translation by alterations to specific amino acid residues. These modifications are usually the result of phosphorylation, acetylation, methylation or thiolation. The activities of a number of proteins (e.g. glycogen phosphorylase) are now known to be readily changed by phosphorylation. Protein phosphorylation
CHAPTER 7

A. INTRODUCTION

Although some observations suggest that cytokinins may directly affect translational processes (see section 1.5-3), there is little convincing evidence that an effect on protein metabolism precedes cytokinin-induced growth. The only observations which would support this possibility are those of Fankhauser and Erismann (1969) and of McCombs and Ralph (1972). In the former study, kinetin was found to increase the quantity of protein in *Lemna minor* cultures within 15 minutes without influencing the levels of free amino acids. McCombs and Ralph detected a stimulation in the rate of incorporation of $[^{35}\text{S}]$sulphate into protein in *Spirodea* 1 hour after the addition of kinetin to the growth medium. A number of other references in the literature report that cytokinins induce the incorporation of labelled amino acids into protein or enhance the levels of protein, but in cases where the cytokinin influences growth, there is insufficient information to determine whether the effect on protein precedes the growth response to cytokinin (see e.g. Banerji and Laloraya, 1967; Kang, 1971). Such effects on protein metabolism may be indirect responses to cytokinin. Effects observed in many labelling studies may merely reflect changes in specific activity of precursor pools.

This chapter describes experiments performed in an attempt to ascertain whether cytokinin-induced amino acid incorporation into protein precedes growth induction in radish cotyledons. Effects of cytokinin on amino acid uptake and pool sizes were also assessed.

While the structure of a protein molecule is determined primarily by the sequence of amino acids, certain proteins are modified after translation by alterations to specific amino acid residues. These modifications are usually the result of phosphorylation, acetylation, methylation or thiolation. The activities of a number of enzymes (e.g. glycogen phosphorylase) are now known to be markedly changed by phosphorylation. Protein phosphorylation
by cAMP-stimulated protein kinases is now regarded as a regulatory mechanism of great significance in animal tissues (see Jost and Rickenberg, 1971; Holzer and Duntze, 1971). Phosphorylation, methylation and acetylation of histones has been shown to occur and may be involved in regulation of transcription (see Holzer and Duntze, 1971; Paik and Kim, 1971; Benjamin, 1971). Methylated amino acids are particularly abundant in histones. There are no reports on effects of plant hormones on protein methylation. However, cytokinins have recently been reported to stimulate phosphorylation of protein in Chinese cabbage leaf disks, but to inhibit phosphorylation of nuclei plus chloroplast preparations and of purified ribosomes (Ralph et al., 1972). The activity of protein kinases partially purified from soybean tissue was not affected by BAP or cAMP (Elliott, 1973).

In this thesis, the effect of BAP on protein phosphorylation and methylation in radish cotyledons has been assessed.

B. MATERIALS AND METHODS

1. Radiochemicals

The following chemicals were obtained from the Radiochemical Centre, Amersham.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Abbreviation used in text</th>
</tr>
</thead>
<tbody>
<tr>
<td>[l-14C]L-leucine</td>
<td>[14C]leucine</td>
</tr>
<tr>
<td>[l-14C]DL-methionine</td>
<td>[14C]methionine</td>
</tr>
<tr>
<td>[methyl-3H]L-methionine</td>
<td>[3H]methionine</td>
</tr>
<tr>
<td>[methyl-8H]S-adenosyl-L-methionine</td>
<td>[8H]S-adenosylmethionine</td>
</tr>
<tr>
<td>[32P]orthophosphate</td>
<td>32P</td>
</tr>
</tbody>
</table>

2. Labelling of radish cotyledons

Excised radish cotyledons (section 1.2) were collected in a sterile Petrie dish, and batches of 10 cotyledons were then selected for uniformity of size, and assigned to various treatments (3 replicate batches per treatment unless otherwise stated). All batches of
cotyledons were incubated in darkness in Steward flasks (c.f. section 5.5-2) in 7 ml of (+BAP) or (-BAP) phosphate buffer. For labelling of the cotyledons, a solution of the radioactive compound in sterile phosphate buffer (pH 6, 0.5 ml) was delivered to each Steward flask. After the appropriate period of labelling, each batch of cotyledons was then given three 5-minute rinses at 0°C in a 1 mM solution of the appropriate unlabelled compound before being blotted and weighed.

3. Protein and RNA extractions, analyses and scintillation counting

After being weighed, cotyledon tissue (section 7.2) was killed and extracted. The extracts were then prepared for scintillation counting and protein estimation, all methods being outlined in Figure 7-1. The figure also describes the preparation of "zero time control" material used to assess the percentage of counts adsorbed to protein, RNA or lipid and not truly incorporated. The sequence of steps used for washing of filter disks is based on that of Mans and Novelli (1961); in the present studies, however, filtration rather than disk emersion was appropriate for the size of aliquot loaded on to each filter paper disk. Tests with zero time control tissue, and with tissue labelled as described in section 7.2, indicated that negligible acid-soluble radioactivity remained in the filter disks washed as described in Figure 7-1; it was noted, however, that the second washing cycle was essential for accurate results, particularly in the processing of 32P- or [3H]methyl-labelled protein, which had a low specific activity. Filtration tests with two Whatman 3 MM filter disks per sample indicated that the loss of acid-insoluble radioactivity from the upper disk during filtration as described in Figure 7-1 was less than 5%.

For independent estimation of RNA content and incorporation (corrected for uptake) of radioactivity into RNA in radish cotyledons, the methods outlined in Figure 5-1 and Table 5-3 were followed.
Figure 7-1  Extraction and millipore filtration of protein and nucleic acids from radish cotyledon tissue, and preparation of samples for scintillation counting

(A) Extraction of protein only or of protein plus lipid

Drop 10 cotyledons into 1 ml 10% TCA, 1 mM w.r.t. precursor at 90° in graduated centrifuge tube. Maintain at 90° for 15 min. Check volume.

Rinse (1 ml 10% TCA) into mortar; grind.

Remove 2 x 200 µl aliquots to assess protein content and c.p.m. incorporated.

Filter each aliquot in millipore apparatus using a Whatman 3 MM filter paper disk.

Wash with 5% TCA (1 mM w.r.t. appropriate unlabelled compound) . . . . . . . . . 3 x 1 ml ethanol—ether (1:1 v/v) . . . . . . . . . 2 x 1 ml ether—chloroform (1:1 v/v) . . . . . . . . . 3 x 1 ml

Dry filter paper disks in scintillation vials at 100° for 15 minutes.

Dry uptake samples in scintillation vials.

Add 0.5 ml 0.1 N NaOH to uptake, protein-plus-RNA and protein-plus-lipid samples; add 0.7 ml 0.1 N NaOH to protein-only samples. Shake gently at 37° overnight in sealed vials.

Neutralize contents of each vial with 0.5 ml 0.1 N acetic acid.

Add 10 ml toluene/Triton-X-100 scintillant (section 5.5-3). Shake vials in darkness overnight. Keep in darkness a further 24 hours before scintillation counting.

For lipid + protein estimation, rinse 5 more times with TCA. Dry filter paper disks in scintillation vials at 100° for 15 minutes.

To 32P-labelled samples, add 16 ml Toluene scintillant (section 5.5-3) for scintillation counting.

(B) Extraction of protein plus RNA

Kill 10 cotyledons with appropriate TCA to which was added 10 µl of radioactive soln.

Rinse (1 ml 10% TCA) into mortar; grind.

Remove 4 x 200 µl aliquots to assess protein and RNA content and c.p.m. incorporated.

Heat 2 aliquots in small tubes at 90° for 15 min. (yields protein only).

Add 400 µl ethanol to 3rd and to 4th aliquots; shake overnight (yields protein plus RNA).

For preparation of 32P-labelled protein the TCA contained pyrophosphate (0.05 mM) as well as phosphate (0.05 mM). For material labelled with S-adenosylmethionine, the TCA contained methionine (1 mM) and adenine (1 mM).
4. Estimation of amino acid pool size

Batches of 10 radish cotyledons were incubated for 12 hours in (+BAP) and (-BAP) media as described in section 2 and then dropped into methanol—chloroform—water (12:5:3 v/v; MCW) at -20° and left overnight at this temperature. Tissue homogenization and quantitative recovery of free amino acids from the samples followed the method of Bieleski and Turner (1966), which involved two MCW extractions of each homogenate followed by four extractions of the residue with 80% ethanol. Following phase separation of the two combined MCW supernatants by addition of water and chloroform, the aqueous phase was added to the four combined 80% ethanol supernatants and the total extract then evaporated under vacuum in a rotary evaporator at 25°.

For amino acid analysis, each dried extract was taken up in 7.5 ml of buffer [0.2 N sodium citrate in deionized water containing HCl (1.65% v/v), thiodiglycol (0.5% v/v), Brij 35 (0.27% v/v) and caprylic acid (0.01% v/v), pH 2.2]. Aliquots (1 ml) of each solution were analyzed in a Beckman 120C Amino Acid Analyzer. The amount of leucine per cotyledon and per mg fresh weight of tissue was calculated by triangulation assessment of peak areas in the absorbance patterns imprinted on the analyzer chart paper.

5. Statistical treatment of results

Analyses of variance and associated calculations were performed as described in section 2.3. Unless otherwise stated, all values analyzed were the means of three replicates.

C. RESULTS

6. Effect of BAP on the incorporation of leucine into protein

6-1. Incorporation of leucine into total protein

At intervals during incubation on (+BAP) and (-BAP) media, batches of radish cotyledons were labelled for 2 hours with $[^{14}C]$leucine 0.2 µCi/ml before being washed, blotted and weighed as described in section 7.1. Extraction and estimation of protein, and scintillation counting to assess $[^{14}C]$leucine incorporation into protein and total


14C uptake followed the procedure described in Figure 7-1(A).

The mean cotyledon weights and protein contents at each sampling time are presented in Figure 7-2. These results show that the mean cotyledon weights of (+BAP) and (-BAP) cotyledons differed significantly at 24 hours and at subsequent sampling times, while there was no significant change in the protein content per cotyledon. The significant difference at 3.5 hours was not consistently observed: in later experiments, there were no significant differences at all in protein content per cotyledon.

BAP was found to have a positive effect on [14C] leucine uptake. Measured on a per unit weight basis, this effect was significant by 8 hours, and was particularly marked at the final sampling time of 48 hours [Figure 7-3(B)].

In another experiment, (section 7.7-2) a similar early effect was observed on methionine uptake. After correction for uptake, however, there was at 12 hours and 24 hours a significant enhancement of [14C] leucine incorporation into protein in response to BAP [Figure 7-3(A)]. Similarly, at these times, and also at the final sampling time of 48 hours, BAP had a marked effect on the specific activity of protein, even after correction for uptake (Figure 7-4). Thus, at 12 hours and at 24 hours, the percent increases in the corrected specific activity caused by BAP were 38.3 and 37.6 respectively. Since this effect of BAP was not accompanied by any detectable increase in total protein level, a stimulation of protein turnover was implied.

The effects of BAP on [14C] leucine uptake and incorporation (corrected for uptake) into protein were examined in a second such experiment with similar results: BAP had a marked positive effect on these parameters but significant differences were not observed before 12 hours.

In a further experiment, radish cotyledons were preincubated in phosphate buffer for 21 hours before the addition of BAP for 3 hours, [14C] leucine being supplied during the final two. In this case BAP evoked a significant increase in the leucine incorporation/uptake ratio within this time, while no significant growth stimulation or alteration in protein content was detected.
Figure 7-2  Mean fresh weights and protein content of radish cotyledons cultured in (+BAP) and (-BAP) media for various periods.

Batches of cotyledons were sampled at intervals and weighed prior to extraction and protein estimation as outlined in Figure 7-1.

A cotyledon weight (+BAP)
B cotyledon weight (-BAP)
C protein (+BAP)
D protein (-BAP)

D(5%) in cotyledon weight = 1.48 mg.
Sequential testing showed that (+BAP) > (-BAP) at 24 hours but not at earlier sampling times.

D(5%) in protein content = 0.130 mg per cotyledon.
Sequential testing showed that (+BAP) < (-BAP) only at 3 hours.
Figure 7-3 \([^{14}C]\)leucine incorporation/uptake ratios of (+BAP) and (-BAP) cotyledons, and \([^{14}C]\)leucine uptake of (+BAP) cotyledons as percentages of control (-BAP) values

Batches of cotyledons were labelled for 2 hours with \([^{14}C]\)leucine after various periods of incubation. After labelling, cotyledons were weighed and processed as described in Figure 7-1(A).

A, B: c.p.m. incorporated into total protein
c.p.m. in total uptake

\[ \bullet - \bullet \text{ (+BAP)} \]
\[ \circ - \circ \text{ (-BAP)} \]

C: total uptake (c.p.m.) per cotyledon
\[ \left( (+BAP) \%ight. \text{(-BAP)} \] \]

D: total uptake (c.p.m.) per mg fresh wt.
\[ \left( (+BAP) \%ight. \text{(-BAP)} \] \]

D(5%) in incorporation/uptake ratio = 0.128.

Sequential testing revealed that (+BAP) > (-BAP) at 12.5 hours and at 24 hours.

Comparison of mean (+BAP) and (-BAP) uptake values and sequential testing showed that in uptake per cotyledon (+BAP) > (-BAP) at 5.5 hours and thereafter; in uptake per mg fresh weight (+BAP) > (-BAP) at 8 hours and thereafter.
Figure 7-4  **Specific activities of $[^{14}\text{C}]$leucine-labelled protein extracted from (+BAP) and (-BAP) radish cotyledons**

The cotyledons were labelled for 2 hours after various periods of incubation, and then extracted as described in Figure 7-1 (A).

- - protein, specific activity in (+BAP) cotyledons.

o--o protein, specific activity in (-BAP) cotyledons.

- - protein, specific activity/uptake in (+BAP) cotyledons.

o--o protein, specific activity/uptake in (-BAP) cotyledons.


\[
D(5\%) \text{ in specific activity} = 12.29 \text{ c.p.m.} \times 10^{-3} \text{ per mg protein. Sequential testing revealed that at 8 hours (+BAP) > (-BAP)}
\]

\[
D(5\%) \text{ in specific activity/uptake} = 4.65 \text{ c.p.m.} \times 10^{-3} \text{ per mg protein per mg fresh wt. of tissue}
\]

Sequential testing did not reveal any additional significant differences; at 12 hours, however, the difference approached significance at the 5% level (difference required = 3.88; difference observed = 3.23).
6-2. Leucine incorporation in the presence of act. D

The nature of the earliest significant effect of BAP on leucine incorporation into protein of freshly excised cotyledons was examined in more detail to determine whether the increased incorporation reflected an effect on amino acid pool size, and whether protein synthesis, if involved, was dependent upon transcription. Batches of 10 radish cotyledons were pre-incubated for 6 hours in phosphate buffer (7 ml in Steward flasks) in the presence and in the absence of act. D. The replicate flasks containing each of these two media were then assigned to various treatments as follows.

<table>
<thead>
<tr>
<th>Replicates and presence or absence of act. D</th>
<th>Treatment</th>
<th>Time in (+BAP) or (-BAP) media</th>
<th>Precursor for 2-hour labelling</th>
<th>Parameter assessed on extraction of the tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>4+; 4-</td>
<td></td>
<td>none</td>
<td>[3H] uridine (70 µCi/ml)</td>
<td>Total RNA: incorporation/uptake</td>
</tr>
<tr>
<td>3+; 3-</td>
<td>12 hours (+BAP)</td>
<td>[14C] leucine (0.2 µCi/ml)</td>
<td>Total protein: incorporation/uptake</td>
<td></td>
</tr>
<tr>
<td>4-; 4-</td>
<td>12 hours (+BAP)</td>
<td>none</td>
<td>Total free amino acids, especially leucine</td>
<td></td>
</tr>
<tr>
<td>4-; 4-</td>
<td>12 hours (-BAP)</td>
<td>(incubation continued 2 hours)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* A+ indicates that act. D (20 µg/ml) was supplied throughout the incubation until sampling time.

The effect of Act. D on the specific activity of RNA labelled for 2 hours after the 6-hour pre-incubation in (+ act. D) and (- act. D) media is shown in Table 7-1 below.
Table 7-1  Specific activity of total RNA extracted from radish cotyledons incubated on (+ act. D) and (- act. D) media for 8 hours

During the last 2 hours of incubation the cotyledons were labelled with [\(^3\)H]uridine. In each column, means lacking a common letter are significantly different at the 5% level.

<table>
<thead>
<tr>
<th>medium</th>
<th>sp. act.(^1)</th>
<th>sp. act. corr. uptake(^2)</th>
<th>incorporation /uptake(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>- act. D</td>
<td>295(^a)</td>
<td>7.16(^a)</td>
<td>0.477(^a)</td>
</tr>
<tr>
<td>+ act. D</td>
<td>100(^b)</td>
<td>3.79(^b)</td>
<td>0.249(^b)</td>
</tr>
</tbody>
</table>

1. specific activity = c.p.m./µg RNA
2. specific activity corrected for uptake
   \[ \frac{\text{c.p.m.}}{\text{mg RNA}} = \frac{\text{c.p.m. in total uptake}}{\text{per mg fresh weight of tissue}} \]
3. c.p.m. incorporated into RNA/c.p.m. in total uptake.

After correction for uptake, the reduction in specific activity of RNA by act. D was 47%. Thus, RNA synthesis was severely inhibited before commencement of BAP treatment.

Results presented in Table 7-2 show the effects of pre-incubation plus continued treatment with act. D upon BAP-induced cotyledon growth and BAP-enhanced [\(^4\)C]leucine incorporation into protein. Comparison of mean fresh weights of these cotyledons at the end of incubation revealed that act. D completely inhibited early BAP-induced growth. This inhibition was preceded by an inhibition of precursor incorporation into RNA (Table 7-1) and accompanied by an inhibition of precursor incorporation into protein (Figure 7-2), both parameters being corrected for uptake. However, despite these absolute effects of act. D, the antibiotic did not inhibit the stimulation, by BAP, of precursor incorporation into protein, an effect evoked in the absence of BAP-induced growth. It thus appeared that the earliest detectable stimulation of protein metabolism by BAP was not
Table 7-2 Mean values for fresh weight, protein content and protein incorporation/uptake of radish cotyledons cultured in (+BAP) and (-BAP) media in the presence and absence of act. D (20 µg/ml)

The cotyledons were labelled with \[^{14}C\]leucine during the final 2 hours of a 14-hour incubation (see text for details). In each column, means lacking a common letter are significantly different at the 5% level. In the lower section of the table the (+BAP)/(-BAP) ratio of each parameter in the absence of act. D may be compared with this ratio in the presence of act. D.

<table>
<thead>
<tr>
<th>medium</th>
<th>mean cotyledon fresh weight (mg)</th>
<th>incorporation(^1) uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+BAP) - act. D</td>
<td>10.66(^a)</td>
<td>0.720(^a)</td>
</tr>
<tr>
<td>(+BAP) + act. D</td>
<td>8.74(^b)</td>
<td>0.508(^c)</td>
</tr>
<tr>
<td>(-BAP) - act. D</td>
<td>9.30(^b)</td>
<td>0.674(^b)</td>
</tr>
<tr>
<td>(-BAP) + act. D</td>
<td>8.74(^b)</td>
<td>0.469(^d)</td>
</tr>
</tbody>
</table>

BAP-induced increment as % of (-BAP) value for the parameters listed above.

<table>
<thead>
<tr>
<th></th>
<th>incorporation(^1) uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>- act. D</td>
<td>14.62</td>
</tr>
<tr>
<td>+ act. D</td>
<td>0.00</td>
</tr>
</tbody>
</table>

\(^1\) = c.p.m. incorporated into protein/c.p.m. in total uptake

a consequence of BAP-induced growth and may not have been dependent on RNA synthesis.

6-3 Leucine pool size

Results of amino acid analyses of unlabelled cotyledon extracts (Table 7-3) indicated that after 14 hours of incubation, BAP had no effect on total free leucine content of radish cotyledons. As in earlier experiments, however, uptake-corrected leucine incorporation into protein was significantly stimulated by BAP within this time (Table 7-2).
Table 7-3 Amounts of free leucine in radish cotyledons incubated in (+BAP) and in (-BAP) media for 14 hours

In both rows the two means did not differ significantly (P > 0.4).

<table>
<thead>
<tr>
<th></th>
<th>leucine content (M x 10^-8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-BAP) medium</td>
</tr>
<tr>
<td>per cotyledon</td>
<td>0.973</td>
</tr>
<tr>
<td>per mg tissue fresh weight</td>
<td>0.1032</td>
</tr>
</tbody>
</table>

An examination of the peaks corresponding to the other (non-basic) amino acids separated during these analyses did not reveal any effect of BAP on their levels. This generalization applies also to methionine (precursor used in section 7.7-2). The levels of methionine in the extracts were very low (< 10% of the leucine level); however, since the extracts were not oxidized to convert -SH groups to sulphones, a proportion of methionine was probably lost, and hence the levels of this amino acid could not be determined exactly.

7. Effect of BAP on modification of protein RNA and lipid

7-1. Phosphorylation of protein and lipid

During incubation on (+BAP) and (-BAP) media, radish cotyledons were labelled for 3 hours with $^{32}$P supplied as inorganic phosphate at a final radioactivity level of 16.5 µCi/ml. Tissue extraction and estimation of the percentage of the $^{32}$P uptake incorporated into protein and into protein plus lipid was carried out as described in Figure 7-1(A). Counts in phospholipid were assessed from the difference between incorporation values for protein plus lipid and protein only. These calculations are summarized in Figure 7-5 which shows the incorporation (% uptake) values determined for protein and for phospholipid at each sampling time. Phosphorylation of both molecular types was enhanced by BAP, but not until after the induction of BAP-induced growth, which was detected at 12 hours.

As shown in Figure 7-6, BAP also
Figure 7-5  $^{32}$P incorporation & uptake ratios for protein and phospholipid of (+BAP) and (-BAP) radish cotyledons

Batches of cotyledons were labelled for 3 hours with $[^{32}P]$phosphate after various periods of incubation. After labelling, cotyledons were processed as described in Figure 7-1 (A).

- protein, (+BAP)
- protein, (-BAP)
- phospholipid, (+BAP)
- phospholipid, (-BAP)

D(5%) in protein incorporation % uptake = 0.086. Sequential testing did not reveal any additional significant differences.

D(5%) in phospholipid incorporation % uptake = 0.090.
Sequential testing showed that (+BAP) > (-BAP) at 18 hours.

Significant BAP-induced growth was first detected at 12 hours.
Figure 7-6 Specific activities of $[^{32}P]$phosphate-labelled protein extracted from (+BAP) and (-BAP) radish cotyledons

The cotyledons were labelled for 3 hours after various periods of incubation. After labelling, cotyledons were weighed and processed as described in Figure 7-1 (A).

- - - (+BAP) specific activity
o--o (-BAP) specific activity
- - (-BAP) specific activity % uptake
o--o (-BAP) specific activity % uptake

$D(5\%)$ in specific activity $= 0.79 \text{ c.p.m.}/\mu\text{g protein.}$
Sequential testing did not reveal any additional significant differences.

$D(5\%)$ in specific activity% uptake $= \frac{1.70 \text{ c.p.m. per mg protein} \times 100}{\text{c.p.m. per mg fresh wt. of tissue}}$
Sequential testing showed that at 18 hours, (+BAP) > (-BAP).
SPECIFIC ACTIVITY (CPM /µg PROTEIN)

TIME (HOURS)

SPECIFIC ACTIVITY % UPTAKE

(CPM PER mg PROTEIN %CPM PER mg FRESH WT OF TISSUE)
increased the specific activity of total protein, but when uptake corrections were applied, no stimulation was detected before the onset of cytokinin-induced growth. In a second such experiment, in which only protein phosphorylation was examined, a similar small increase in $^{32}$P incorporation into protein and in protein specific activity was observed after the initiation of the growth response to BAP.

7-2. Methylation of protein and RNA

An attempt was made to assess the possible effects of BAP on methylation of protein and RNA by doubly labelling radish cotyledons with [methyl-$^3$H]methionine and [2-$^{14}$C]methionine (0.8 µCi/ml and 4.0 µCi/ml respectively). These were supplied as a mixed solution to cotyledons incubated for various periods on (+BAP) and (-BAP) media. After uptake of methionine for 4 hours, cotyledons were extracted for RNA, protein, and protein plus lipid [see Figure 7-1 (A), (B)].

Incorporation of $^3$H due to methylation of protein was calculated thus:

$$^3\text{H-c.p.m. due to methylation} = ^3\text{H c.p.m.} - (^{14}\text{C c.p.m.} \times R)$$

where $R = ^3\text{H} / ^{14}\text{C}$ supplied in the medium.

This calculation is based on the fact that $^3$H counts incorporated due to peptide bond formation would be proportional to $^{14}$C counts so incorporated, as defined by R (see Appendix for calculation of R).

BAP stimulated methylation at each sampling time [Figure 7-7(D)] the difference between (+BAP) and (-BAP) cotyledons being significant at 15 hours, and almost significant at 5 hours. Moreover, there was a significant difference ($P= 0.02$) between (+BAP) and (-BAP) cotyledons, in the overall means of the c.p.m. due to methylation at 5 hours and 10 hours. This indicates that the stimulation of protein methylation by BAP is initiated within the lag period preceding cytokinin-induced growth.

To determine whether the protein methylation evoked by BAP was accompanied by a stimulation of incorporation of methionine into protein in peptide linkage, protein incorporation values were calculated as percentages of uptake for $^{14}$C alone [Figure 7-7(C)] BAP significantly
Figure 7-7 Protein and RNA methylation and associated parameters, in (+BAP) and (-BAP) radish cotyledons

After various periods of incubation the cotyledons were doubly labelled for 4 hours with $[1-^{14}C]$ methionine and [methyl-$^3$H]methionine. After labelling, the cotyledons were sampled and weighed prior to extraction as outlined in Figure 7-1(B). Incorporation of $^3$H due to methylation of protein was calculated thus:

$$^3H - \text{c.p.m. due to methylation} = \frac{^3H \text{ c.p.m.} - (^{14}C \text{ c.p.m.} \times R)}{^3H/^{14}C \text{ supplied in the medium.}}$$

Incorporation of $^3$H due to methylation of RNA was calculated as the difference between the $^3$H c.p.m. incorporated into protein plus RNA, and the $^3$H c.p.m. incorporated into protein only.

In each frame,

- $\bullet\bullet = (+BAP)$
- $\circ\circ = (-BAP)$

A Mean cotyledon weights

D(5%) = 1.33 mg; thus BAP had induced significant growth by the final (15-hour) sampling time. Sequential testing revealed no additional significant differences.

B Protein content (mg per cotyledon)

D(5%) = 0.11 mg. Sequential testing revealed no significant differences.

C $[1-^{14}C]$methionine incorporation into protein (c.p.m. % uptake)

D(5%) = 2.22

Thus (+BAP) > (-BAP) at each sampling time.

D Methylation of protein (c.p.m. % uptake)

D(5%) = 5.32

Sequential testing revealed that at 15 hours (+BAP) > (-BAP). In a comparison of the overall mean values for 5 hours and 10 hours, (+BAP) > (-BAP) ($P = 0.02$).

E Methylation of RNA (c.p.m. % uptake)

D(5%) = 4.84

Sequential testing revealed that at 10 hours and at 15 hours, (+BAP) < (-BAP).
increased these ratios at all three sampling times. Thus, a significant effect of BAP on methionine incorporation into protein accompanied BAP-induced methylation. The effect of BAP on methionine incorporation appeared to occur earlier than the previously observed stimulation of leucine incorporation (c.f. Figure 7-3).

Since methylation of RNA is known to be important for RNA function, the apparent depression of this process by BAP within 10 hours [Figure 7-7(E)] was questioned. This result was obtained by subtraction. To examine the effect of BAP on RNA methylation by a more direct method, [methyl-\(^3\)H]S-adenosylmethionine (11 μCi/ml) was supplied for 4 hours to radish cotyledons incubated on (+BAP) and (-BAP) media for 5 or 11 hours. In this experiment, BAP was found to have no effect on methylation of RNA at either sampling time.

There did not appear to be any effect of BAP on lipid methylation. However, the proportion of c.p.m. in lipid relative to the c.p.m. in protein was too small at all sampling times to make a reliable estimation, by difference, of lipid \(^3\)H radioactivity.

D. DISCUSSION

The results presented in this chapter clearly show that BAP promotes the incorporation of labelled amino acid into protein in excised radish cotyledons. This effect was observed after correction for uptake. No change in free amino acid (leucine and probably methionine) content was detectable at 12 hours when incorporation of both amino acids was promoted by BAP. The response is thus probably not a consequence of differing specific activity of amino acids in vivo.

When \([^{14}\text{C}]\text{leucine}\) was used as precursor, the effect on incorporation was significant only about the end of the lag period. When \([^{14}\text{C}]\text{methionine}\) was used, however, the effect was significant within 5 hours, while significant BAP-induced growth did not occur before 15 hours (see Figure 7-7). This suggests that the promotion of amino acid incorporation was not merely a consequence of BAP-induced growth. In the case of leucine, this conclusion was confirmed by two further experiments, namely, that in which growth, but
not BAP-promoted leucine incorporation, was inhibited by act. D, and the experiment in which older (21-hour pre-incubated) cotyledons showed a stimulation of leucine incorporation in response to BAP before significant growth was induced by the cytokinin.

The basis for the differential effect on leucine and methionine incorporation into protein in freshly excised cotyledons is uncertain. The effect may indicate a preferential synthesis of methionine-rich proteins in response to BAP, or that the effect of BAP on peptide chain initiation was more marked than its promotion of chain elongation.

Since the effect of BAP on protein specific activity was quite marked (38% at 12 hours in the case of leucine) and occurred in the absence of any detectable change in protein content, BAP may stimulate protein turnover generally. However, promotion of the synthesis, or inhibition of the degradation, of a minor protein fraction is an alternative possibility. If the majority of cotyledon protein is storage protein, which would not be subject to turnover (i.e. degradation plus resynthesis), enhanced synthesis of a minor protein fraction could markedly increase specific activity of total protein. Such an effect need not influence total protein content to a detectable degree.

The effect of BAP on amino acid incorporation was not suppressed by act. D at a concentration that inhibited RNA synthesis by about 50%. This result is consistent with, but does not unequivocally establish, the possibility that the effect of BAP on protein metabolism is a direct one and independent of RNA synthesis. However, it is relevant to note that approximately 50% inhibition of total RNA synthesis by a 16-hour incubation in the presence of act. D completely eliminates gibberellin-induced synthesis of α-amylase in barley aleurone layers (Zwar and Jacobsen, 1972).

Protein phosphorylation, and the incorporation of phosphate into lipids, were not significantly stimulated by BAP until after both BAP-induced growth and BAP-enhanced amino acid incorporation (compare Figures 7-5, 7-3 and 7-7). Hence, it is not clear whether the observed increase in $^{32}$P-specific activity of protein, in response to BAP, was a consequence of BAP-induced protein synthesis, or
reflected an activation of protein kinases or suppression of phosphoprotein phosphatase. Both protein kinase activity and phosphoprotein phosphatase activity have been detected in plant tissues (McCombs and Ralph, 1972; Elliott, 1973), and Elliott (1973) reports that increased protein phosphorylation is an early event after the addition of BAP to cytokinin-starved soybean tissue; however, it is not yet known whether this stimulation precedes BAP-induced protein synthesis. Moreover, the activity of callus protein phosphokinase was not stimulated by BAP. Similarly Ralph et al. (1972) have detected a promotion by kinetin of protein phosphorylation in Chinese cabbage leaf disks, but an inhibition of this process in nuclei plus chloroplast preparations from Chinese cabbage and tobacco leaves, and an inhibition of protein kinase activity of purified Chinese cabbage leaf ribosomes. For this discrepancy they have no unequivocal explanation. However, the results presented herein raise the strong possibility that the effect obtained with leaf disks may be a consequence of cytokinin-enhanced protein synthesis, which would increase the amount of substrate available for phosphorylation.

In contrast to phosphorylation of protein in radish cotyledons, methylation appears to accompany enhanced amino acid incorporation into protein. The results presented indicate that there is a statistically significant effect of BAP on protein methylation during the lag period. At the time of conclusion of work for this thesis, however, the significance of the observed BAP effects on protein methylation have yet to be clarified.

An extension of the study with experiments involving protein fractionation according to cellular location and electrophoretic mobility in polyacrylamide gels might provide answers to the principal questions remaining open, namely:

1. Is general protein turnover stimulated by BAP, or are its effects confined to specific protein species?
2. If specific protein species are affected, what is their cellular location?
3. Could protein fractionation reveal BAP effects on phosphorylation in the lag period?
4. Is the effect of BAP on protein methylation of regulatory significance, and what proteins are affected?
5. Are other types of modification, namely acetylation, adenylation and thiolation also early responses?

Experimentation to answer these questions is likely to be more sensitive if dual isotope techniques are employed (c.f. section 5.5-2). These would permit a more precise comparison of fractionated labelled protein species from control and BAP-treated tissue. Such techniques have been of great value in determining the effects of gibberellic acid on the incorporation of uridine and adenosine into RNA of barley aleurone layers (Zwar and Jacobsen, 1972).

It is relevant, in concluding this discussion, to compare the results presented in this chapter with those which dealt with nucleic acid metabolism during BAP treatment. No promotion of transcription of any RNA species could be detected during the lag period preceding cytokinin-induced growth, but effects on post-transcriptional process have been detected during the lag period, namely, the promotion of methionine and leucine incorporation into protein and the promotion of protein methylation. Such results support the view that cytokinins act at the post-transcriptional level. Reports in the literature that have provided evidence for post-transcriptional control by cytokinins have been discussed (section 1.5-3). Evidence for the action of certain animal hormones (steroids) at this level was also mentioned. In addition, it may be noted here that data are now accumulating which suggest that gibberellic acid acts at a post-transcriptional control point. Thus, Chen and Osborne (1970) have obtained evidence that gibberellic acid enhances the expression of pre-formed messenger RNA in germinating wheat embryos, an event detected as enhanced amino acid incorporation in the lag phase preceding synthesis of new RNA in the embryos. On the basis of their experimental results, they suggest that the GA effect could be achieved by unmasking the messages ("informosomes"), by facilitating the interaction of message with the ribosomes or by
regulating the rate of message read out. Similarly Carlson (1972) has proposed that gibberellic acid causes the induction of de novo synthesis of α-amylase in barley aleurone tissue by modifying post-transcriptional processes. His experiments are based on the use of 5-FU to produce abnormal messenger RNA. α-Amylase synthesized from such RNA is distinguishable from normal α-amylase in being thermostable. The results indicated that after a 5-FU pulse followed by essentially complete removal of this compound from RNA precursor pools, the application of gibberellic acid to the tissue promoted synthesis of thermostable α-amylase, indicating an effect of gibberellic acid on translation of pre-existing message. The results of these gibberellin studies raise the possibility that similar hormonal response mechanisms occur in widely divergent organisms, for the results of both studies could be accounted for in terms of the model proposed by Tomkins and co-workers for steroid hormonal control by the regulation of a post-transcriptional repressor (see section 1.4-3).

The above comments do not, of course, exclude the possibility that transcription may be important for the expression of cytokinin action. Results reported in this thesis have not eliminated the possibility that BAP may directly affect the synthesis of minor RNA species not detected by gel electrophoresis. Moreover, continuing transcription is highly likely to be an essential requirement for cytokinin-induced growth, and indeed such a requirement is strongly suggested by the results of experiments with act. D that were presented in Chapter 4. However, the experimental evidence presented for effects of BAP on post-transcriptional processes, and a consideration of their timing, do suggest that these effects are important events in the molecular mechanism of action of this cytokinin.
A. INTRODUCTION

Although zeatin and related compounds are now known to occur in a wide variety of plant species (see Letham and Williams (1969), and review by Baskin (1970)), little is known of the metabolism of zeatin. The only information on the metabolites formed when zeatin is supplied exogenously to plant tissues is that of Schelkeimer and Tsou (1971). These workers supplied isotopically labelled zeatin to bean axes and on extracting the tissues 12 hours later detected radioactivity in zeatin, dihydrozeatin and their ribosides and 5’-ribosides. More is known of metabolites formed from BAP, and the information available when this investigation was initiated is outlined below.

McCalla et al. (1967) provided the first report on BAP metabolism. They supplied [8-14C]BAP to leaf discs of bean and noted zeatin and its riboside as the major product. Other metabolites produced were 8-azahypoxanthine, 8-azahypoxanthine, adenosine, guanosine, guanine, purine and x-uracil.

Conversion of BAP to its riboside has since been detected in other plant tissues (Quern et al., 1968; Dyon et al., 1972; Woolley and Margolin, 1972) and further evidence of BAP metabolism to its nucleotide BAP riboside 5’-monoethylphosphate has also been obtained (Hertzer, Hyman and Veldstra, 1972; Dyon et al., 1972). Several reports have mentioned major unidentified metabolites. McCalla et al. (1967) reported the conversion of BAP to a single metabolite by leaves of French beans (Phaseolus vulgaris); this product appeared to be different from the compounds produced by rooting leaves.

Fox et al. (1972) detected a very stable BAP metabolite in soybean tissue cultures which appeared to be active as a cytokinin (Dyon et al., 1972). Preliminary observations suggested that the compound was a phosphate ester.

It has for some time been thought very probable that cytokinins are synthesized in the root apex and translocated to the shoot (Kende, 1964, 1965; Wain and Noddia, 1965; Kende and Sutton, 1967; Short and Torrey,
A. INTRODUCTION

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McCalla et al. (1962) provided the first report on BAP metabolism. They supplied [8-14C]BAP to leaf disks of Xanthium pennsylvanicum and detected N6-benzyladenosine as the major product. Other metabolites produced were N6-benzyladenylic acid, adenylic acid, guanylic acid, inosinic acid, adenine, guanine, urea and a ureide. Conversion of BAP to its riboside has since been detected in other plant tissues (Guern et al., 1968; Dyson et al., 1972; Woolley and Wareing, 1972) and further evidence of BAP metabolism to its nucleotide BAP riboside 5'-monophosphate has also been obtained (Bezem er-Sybrandy and Veldstra, 1971; Dyson et al., 1972). Several reports have mentioned major unidentified metabolites. McCalla et al. (1962) reported the conversion of BAP to a single metabolite by leaves of French bean (Phaseolus vulgaris); this product appeared to be different from the compounds produced by Xanthium leaves. Fox et al. (1972) detected a very stable BAP metabolite in soybean tissue cultures which appeared to be active as a cytokinin (Dyson et al., 1972). Preliminary observations suggested that the compound was a phosphate ester.

It has for some time been thought very probable that cytokinins are synthesized in the root apex and translocated to the shoot (Kende, 1964, 1965; Weiss and Vaadia, 1965; Kende and Sitton, 1967; Short and Torrey,
1972). The presence in various root sap exudates of cytokinins that co-chromatograph with zeatin or zeatin riboside (e.g. Klämbt, 1968; Skene, 1972) has suggested that zeatin probably moves in the transpiration stream; however there is no information on the movement of exogenously supplied zeatin. By contrast there are numerous reports on the translocation of exogenous synthetic cytokinins such as BAP and kinetin.

At first, little evidence was obtained for the movement of exogenous cytokinins away from the site of application. Thus, when kinetin was applied to the surface of leaves (Mothes, 1964) and stems (Sachs and Thimann, 1964; Thimann and Laloraya, 1960) its effects were extremely localized. Such experiments suggested that cytokinins were immobile. Most early evidence to the contrary was derived from experiments involving the application of cytokinin to the cut ends of stems and petioles (see Fox, 1969). Thus, Osborne and co-workers obtained evidence for the movement of BAP with predominantly basipetal polarity in petiole segments of Phaseolus vulgaris and observed that this movement was enhanced by the auxin IAA (Osborne and Black, 1964; Black and Osborne, 1965; Osborne and McCready, 1965). Other instances of basipetal cytokinin movement have been reported. Seth, Davies and Wareing (1966) detected basipetal movement of kinetin applied to the stumps of decapitated Phaseolus plants and demonstrated that the movement was stimulated by IAA. Kinetin also moved with basipetal polarity when applied to segments of Zea mays roots, whether or not the root apex was present (El-Saidi, 1971). Lagerstedt and Langston (1966) were able to confirm the results of Osborne and Black (1964), although they did find that the polar movement of kinetin was dependent upon the age of the tissue and the kinetin concentration as well as upon the concentration of IAA. Basipetal movement does not appear to be a general phenomenon however: Fox and Weis (1964) obtained no evidence for polar transport in Phaseolus petioles, with or without IAA, and obtained similarly negative results using petioles of Coleus blumei (a result confirmed by Veen and Jacobs, 1969),
epicotyls of *Pisum sativum* and coleoptiles of *Avena sativa*. Lack of polarity was also possibly indicated in experiments with petioles of *Xanthium pennsylvanicum* (Osborne and McCready, 1965), and clearly shown in experiments with stems and petioles of *Gossypium hirsutum* (Lagerstedt and Langston, 1966), stem sections of *Lens* (Pilet *et al*., 1967) and mesocotyl and coleoptile segments of *Zea mays* (El-Saidi, 1972).

The significance of the reported basipetal transport of cytokinin is uncertain. In experiments where this was observed, the cytokinin concentrations were about 500 times greater than optimal levels for callus growth, were certainly unphysiological and may even have been toxic. Furthermore, in excised tissues, the cytokinins may come into direct contact with tissues to which they do not normally have access. IAA is known to influence cytokinin translocation - a point discussed further on - and abnormal auxin levels and hormone balance may well exist in the experiments discussed above. Hence the artificiality of the experiments in which basipetal translocation has been reported, casts doubt on the existence of the phenomenon in the intact plant.

Using autoradiography, Lagerstedt and Langston (1967) confirmed that kinetin remained localized when applied to laminar areas of several types of excised leaves, but they also demonstrated that when the kinetin was applied to the leaf surface directly over a vein, it moved freely in the vascular system with acropetal polarity, suggesting entry into the transpiration stream. (The leaves were supplied with water at the petiole). In view of the evidence for the production of cytokinins in root apices and for their presence in ascending xylem sap, acropetal translocation of cytokinins would seem more likely than basipetal, and indeed, in the few experiments involving intact or nearly intact plants, acropetal movement of labelled cytokinins has been observed. When $[^{14}\text{C}]$BAP was applied to the stems of intact seedlings in a superficial paste (Pilet, 1968) or by injection (Guern *et al*., 1968), the radioactivity moved acropetally, and in the latter case a stimulation of growth
was observed in the axillary buds above the injection sites. Similarly 6-(benzylamino)-9-(tetrahydropyran-2-yl)purine, supplied to the roots of pea seedlings, stimulated lateral growth on the stems (Kende and Sitton, 1967). Lagerstedt and Langston (1967) found that \( [8-^{14}C] \) kinetin was readily absorbed by the roots of intact tobacco seedlings and that \( ^{14}C \) radioactivity moved throughout the plants. In addition, agar blocks that had been inserted into petiole notches produced positive results in kinetin bioassays, but it was not established that kinetin itself was the active substance. Detection of the original cytokinin after translocation is also lacking in the other studies of cytokinin movement within intact plants.

Evidence has been accumulating that a major factor determining the direction of cytokinin translocation in intact plants is the location of actively growing buds. Pilet (1968) reported that acropetal movement of BAP in \textit{Lens} seedlings is suppressed by decapitation. The work of Guern and Sadorge (1967) indicated that the polarity of BAP movement in young plants of \textit{Cicer arietinum} depended on the relative positions of the site of cytokinin application and the growing shoots on the plant. Thus, in decapitated seedlings with a developing axillary shoot, injected BAP moved preferentially into the axillary shoot whether the site of injection on the main stem was above or below the axillary shoot. Morris and Winfield (1972) obtained a similar result with \( [^{14}C] \) kinetin applied to decapitated dwarf pea seedlings. Whether kinetin was applied to the root or upper stem, appreciable radioactivity was transported to the axillary buds. Treatment of the apical stump with IAA inhibited this transport, and instead label moved as it did in intact plants, accumulating in the stem and apical region. This suggests that the effect of an active apex on cytokinin transport can be mimicked by exogenous auxin. Although transport of label from intact root to shoot was demonstrated in this experiment, the chemical nature of the translocated activity was not determined. Another recent study concerning relationships between cytokinins and apical dominance is that of Woolley.
and Wareing (1972) who used decapitated plants of *Solanum andigena*. Lateral buds of decapitated plants tended to develop into stolons, rather than leafy shoots, if an IAA/GA$_3$ mixture was applied to the apical stump. When [$^{14}$C]BAP was supplied to such plants with roots excised, BAP and BAP riboside did accumulate to some extent in the tips of the induced stolons, but showed an increased accumulation there after removal of the IAA/GA$_3$ mixture from the apex. This increased cytokinin content appeared to induce transformation of the stolon into a leafy shoot. These results differ from those of Morris and Winfield in that the IAA/GA$_3$ mixture did not appear to promote acropetal movement of BAP towards the apex. These two experiments indicate that auxin affects the distribution of exogenously supplied cytokinin. Auxin may also influence cytokinin metabolism (Woolley and Wareing, 1972). The mechanisms by which such auxin effects would operate is quite unknown. Certainly the control would appear to be subject to other physiological variables related, for example, to species and age.

Cytokinins are now known to occur naturally in phloem sap (Hall and Baker, 1972; Phillips and Cleland, 1972), and evidence for the movement of cytokinins in the transpiration stream has already been discussed. However, cytokinins presumably move out of the vascular tissue to induce their effects. Lateral movements of cytokinins have not been extensively studied, but Bowen and Wareing (1969) have demonstrated a bidirectional interchange of [8-$^{14}$C]kinetin between the xylem and sieve tube sap of willow stems. Chromatographic evidence indicated that movement in either direction was accompanied by extensive metabolism of the cytokinin, but in each case some kinetin was recovered. Tangential movement of kinetin has also been observed. Wareing (1970) showed that if [$^{14}$C]kinetin was applied to lateral abrasions on horizontal willow stem sections, radioactivity accumulated in the buds on the upper surfaces. That this movement was not entirely bud directed was shown by Lepp and Peel (1971), who found that accumulation in the upper portion of the bark still occurred when buds had been removed.
Clearly it has been established that when labelled cytokinins are supplied to plant tissues, the radioactivity is extensively translocated. In some cases movement of the cytokinin itself or of an active metabolite has been demonstrated; frequently, however, the identity of the translocated labelled compounds is not known.

Further studies of the metabolism of cytokinins by plant tissues are important for at least two reasons. Firstly, from a more precise knowledge of the enzyme modifications which occur in vivo and lead to inactivation, it may be possible to synthesize cytokinins that are less susceptible to enzymic degradation. Such compounds might be more effective in evoking growth responses and of possible significance in agriculture and horticulture. Secondly, studies of metabolites may lead to recognition of the active forms of cytokinins. Are cytokinins such as BAP and zeatin active per se or are they converted by plant tissues to derivatives which are the true functional molecular species? This is clearly a vital question. When it has been answered, it should be possible to attack the problem of mechanism of cytokinin action in a new way, namely, by affinity chromatography. By this procedure, involving chemical bonding of the active molecule to an inert chromatographic matrix (e.g. cellulose, or Sepharose; c.f. Venis, 1971), it may be possible to purify protein species to which the hormone binds to exert a physiological response. In view of the above, metabolites formed from BAP by excised radish cotyledons were investigated and the results obtained are described in this chapter.

Important questions closely related to these metabolic studies concern the translocation and possible storage of cytokinins. Are some metabolites translocational forms of cytokinins, while others are storage forms - i.e. forms which are relatively insensitive to degradative enzymes, but which can be converted in vivo to active compounds? The root is recognised as a very probable site of active cytokinin biosynthesis, and the cotyledons of seedlings appear dependent on exogenous cytokinin for development. Are root cytokinins
translocated to the cotyledon and, if these compounds reach this organ, do they exist in an active form there, or are they inactivated enzymically? To provide information relevant to these questions, labelled zeatin was supplied to the roots of intact radish seedlings. The metabolites formed and their translocation were studied, and the results obtained are presented herein.

B. MATERIALS AND METHODS

1. Uptake of [\(^3\text{H}\)]zeatin by radish seedlings and extraction of tissue

[G-\(^3\text{H}\)]Zeatin (194 mCi/mmole), prepared according to Letham and Young (1971), was obtained from Dr. D.S. Letham. Seedlings of radish (Raphanus sativus cv. Long Scarlet) were grown at 23° in a glasshouse (lit artificially at night) and were supported upon a wire mesh that rested on a Petrie dish filled with Hoagland's nutrient medium. After five days, the mesh was transferred to nutrient medium containing 8.4 μM [\(^3\text{H}\)]zeatin. The roots were allowed to imbibe this solution for one hour before being returned to plain nutrient medium which was immediately replaced five times to eliminate free zeatin.

At subsequent intervals batches of 20 intact seedlings were removed from the mesh and prepared for extraction. The plants were rinsed in distilled water, blotted between filter papers and dissected into regions which were weighed. Then, since conversion of zeatin to nucleotides was likely, the fresh tissue was dropped into methanol—chloroform—formic acid—water (12:5:1:2 v/v, MCF) at -20° to inactivate phosphatase (Bieleski, 1968). After 18 hours at this temperature, the tissue and solvent were homogenized and then centrifuged. The pellet was re-extracted with methanol—water—formic acid (60:40:1 v/v). The combined extracts were evaporated in a rotary film evaporator at 25° and the residue extracted with 2 ml of
absolute ethanol then with 2 ml of water. Usually both fractions contained similar levels of radioactivity and were combined for chromatography.

Further radish seedlings required for extraction of xylem sap were grown in a shallow bed of moist sand. \([^3H]\) zeatin (12 μM in 2 mM phosphate buffer pH 6) was applied to the sand until this was saturated, care being taken to avoid wetting the aerial portions of the plants. Two and a half hours later the hypocotyls were excised with a sharp razor blade just above ground level and the cut ends of the decapitated plants were blotted to remove products of broken cells. The bleeding sap was collected at intervals on strips of filter paper which were immediately dropped into MCF at -20°. After 18 hours the paper was removed and the solvent evaporated at 25° yielding a residue for chromatography.

2. Uptake of \([^3H]\) 6-benzylaminopurine by radish cotyledons

\([G-^3H]BAP\) (27 mCi/mmole) was obtained from Dr. D.S. Letham. Before use the preparation was tested for radiochemical purity by t.l.c. using silica gel and solvent D (section 8.3). Of the total radioactivity chromatographed 98.5% moved with marker BAP; the remaining 1.5% of the counts co-chromatographed with adenine.

From 500 40-hour old radish seedlings, the inner cotyledons were excised as previously described (section 2.1), and collected in a sterile Petrie dish. They were then transferred to a sterile 250-ml conical flask containing 50 ml of \([^3H]\) BAP solution (6 mg/l in phosphate buffer) and the flask was left on a slow speed (120 r.p.m.) shaker at 23° under weak fluorescent light (65 ft. candles). Five hours later the cotyledons were washed in a strainer with 1 l of sterile distilled water, given three 5-minute rinses in 50 ml of sterile phosphate buffer, and finally returned to the shaker in a conical flask containing a further 50 ml of sterile phosphate buffer. Incubation was continued as before for 4 days.

At intervals, both before and after the transfer to
unlabelled medium, cotyledons (minimum sample 50) were
removed from the flask for extraction. Each sample was washed
with 1 l of distilled water and those cotyledons that were
removed during the labelling period were given 3 additional
rinses of 5 minutes each. At each sampling time after the
labelling period, remaining cotyledons were transferred to
fresh sterile phosphate buffer.

Each washed cotyledon sample was blotted between
filter papers, weighed and then dropped into MCF at -20° for
18 hours. Extracts were prepared and evaporated by the
method followed for [3H]zeatin labelled plant parts. The
residues were taken up in 1 ml of 50% ethanol per 100
cotyledons.

Another 500 cotyledons were incubated, sampled
and extracted as described above, except that no BAP was
included in the phosphate buffer and samples were washed
only once with distilled water. The extracts derived from
these unlabelled cotyledons provided control material in
bioassays for cytokinin activity on chromatograms of [3H]BAP
labelled extracts.

3. Chromatographic methods

Schleicher and Schull 598L paper was used for all
paper chromatography. The materials used for t.l.c. were
Serva DEAE-cellulose with 0.7% Woelm green fluorescent
indicator added, and Merck PF254 silica gel.

Chromatographic solvents used were: A, n-butanol—
14 N aqueous ammonia—water (6:1:2 v/v, upper phase); B, n-
butanol—acetic acid—water (12:3:5 v/v); C, 50% methanol;
D, methyl ethyl ketone, water saturated; E, ethanol—water
(17:8 v/v) containing sodium borate (0.6% w/v); F, 0.03 N
hydrochloric acid containing potassium chloride (0.03 M).

Marker compounds for co-chromatography were added
to aliquots of plant extract before application to the paper
or thin layer.

The solvents used to elute the zones of
chromatograms were water for paper chromatograms, 1% acetic
acid for silica gel, and 0.1N hydrochloric acid containing
2.4% sodium chloride for DEAE cellulose.

4. Determination of radioactivity

Chromatogram zones for counting were eluted in scintillation vials either at room temperature overnight or at 50°C for three hours. The eluates were mixed with 10 volumes of scintillant (toluene—Triton X-100, 2:1 v/v, containing PPO, 2g/l and dimethyl POPOP, 0.2g/l) and counted in a Beckman LS-250 liquid scintillation spectrometer. Levels of background radioactivity were determined for each chromatogram from at least two blank zones taken from the unlabelled margins of the paper or layer. Vials from chromatograms of low activity were counted repeatedly and the mean count calculated for each zone.

5. Phosphatase hydrolysis and periodate oxidation of nucleotide fractions

Paper chromatogram eluates containing nucleotides were hydrolysed at 35°C for 3.5 hours with either Sigma type III-S *E. coli* alkaline phosphatase (1.5 mg/ml, pH 9.5) or Sigma *Crotalus adamanteus* venom (5 mg/ml, pH 9.5). The venom hydrolyzed adenosine 5'-phosphate rapidly, but did not dephosphorylate adenosine 2'- or 3'-phosphate. Digests containing materials likely to interfere with subsequent chromatography were shaken with butanol to extract the products of phosphatase digestion. Impurities remained in the aqueous phase and were eliminated.

Eluates (50 µl) were also oxidized with an excess of periodate (0.2 mg) at 35°C for 12 hours. Cyclohexylamine (10 µl) was then added and the mixture left at 35°C for 3 hours before being co-chromatographed with zeatin. Adenosine 2'– and 3'–phosphates were not degraded under these conditions, but adenosine 5'–phosphate yielded adenine. This procedure is based on that of Yu and Zamecnik (1960) for degrading nucleosides to bases.

6. Bioassay methods

The radish cotyledon bioassay was used to test the cytokinin activity of BAP metabolites in extracts of radish...
cotyledons. The extract used was that prepared from cotyledons sampled 19 hours after the end of the labelling period with $[^3H]BAP$. From this labelled extract and the corresponding control, aliquots equivalent to 15 cotyledons were applied to separate 5.1-cm origins on 598L paper. After development in solvent A, each chromatogram was marked into 11 3.8-cm zones, and divided vertically into two strips 3.8 cm and 1.3 cm wide. Zones from the narrower strip of the radioactive chromatogram were eluted for scintillation counting to locate the radioactive compounds. Zones from the wider strips were placed in 5-cm Petrie dishes and wetted with 1-ml volumes of phosphate buffer. Seven radish cotyledons (section 2.1) were then placed on each paper square, and the dishes were left under fluorescent light (65 ft. candles) at 23°. Cytokinin activity in the chromatogram zones was determined by weighing the batches of cotyledons 3 days later. From the c.p.m. recorded for each counted zone, the equivalent amount (in ug) of $[^3H]BAP$ in the bioassayed zones was calculated.

C. RESULTS

7. Distribution throughout the seedlings of radioactivity derived from zeatin

Samples of 20 intact seedlings grown on liquid medium and labelled with $[^3H]zeatin$ for one hour were removed from the wire mesh support at 1, 2, 5, 10, and 24 hours after the roots were first immersed in the $[^3H]zeatin$ solution. So that the distribution of radioactivity at each sampling time could later be assessed, plants were cut into five parts. These were the root, hypocotyl, apex (a 4-mm length of tissue centred on the apical meristem), petioles with excised midribs of the cotyledons, and the de-ribbed cotyledon laminae (henceforth referred to as the cotyledons). Corresponding plant regions in each sample were pooled and weighed before phosphatase inactivation and extraction.
Aliquots of all extracts were counted to determine the total radioactivity extracted per plant and per plant region at each sampling time. Figure 8-1 displays the results obtained for the roots, hypocotyls and cotyledons.

Throughout the experiment over 87% of the total extracted radioactivity was derived from the roots. Between 1 and 5 hours after zeatin application the amount of radioactivity recovered from the roots decreased about 50% and then remained approximately constant. Since this decrease could not be accounted for by translocation to the shoot (see Figure 8-1, B and C), there was probably a marked diffusion of radioactivity into the nutrient solution. After 5 hours the levels of radioactivity in all five plant regions remained approximately constant. Per unit weight of tissue the radioactivity in the root was approximately 40 times that in either the hypocotyl or the cotyledons which were similar in content of radioactivity. The radioactivity per unit weight in the apical and petiolar zones (not presented in Figure 8-1) was similar to that of the cotyledons.

The amount of zeatin, or its equivalent, extracted per seedling was calculated from the known specific activity of the $[^3H]$zeatin supplied to the seedlings and the counting efficiency of $^3$H. In order of sampling time these quantities were 0.191, 0.176, 0.098, 0.091 and 0.083 µg respectively.

8. Zeatin metabolites in the roots

Aliquots of the root extracts were chromatographed on paper using solvent A. Figure 8-2, shows the distribution of radioactivity over these chromatograms and the positions of co-chromatographed marked compounds. Within this group of chromatograms there were three major peaks of radioactivity, one at $R_f$ 0.70 which co-chromatographed with zeatin, another at and just ahead of the origin ($R_f$ 0.03) and the third at $R_f$ 0.39 just ahead of adenosine. A minor peak occurred at $R_f$ 0.19 and there was a suggestion of a fifth peak at $R_f$ 0.09. The peak that co-chromatographed with zeatin was initially the most prominent, accounting for 44% of the total radioactivity (Figure 8-2, A) but by 5 hours had almost disappeared (Figure 8-2, C). At two hours the peak at the origin was the
Figure 8-1. Radioactivity extracted from the roots (A), hypocotyls (B) and cotyledons (C) of radish seedlings sampled at various intervals after the application of $[^3\text{H}]$zeatin to the roots.
Figure 8-2. The distribution of radioactivity over paper chromatograms of extracts prepared from radish roots that had received $[^3H]$zeatin.

Roots were sampled at the following intervals (hours) after the application of zeatin: 1 (A), 2 (B), 5 (C), 10 (D) and 24 (E). The location of marker compounds was as follows: AMP, adenosine 5'-monophosphate; A, adenosine; Z, zeatin. An unidentified u.v.-absorbing zone derived from the extract is marked U. An arrow locates the origin. The chromatograms were developed in solvent A.
most conspicuous representing 37% of the total radioactivity, but thereafter this proportion declined gradually and by 24 hours was only 13%. Meanwhile the radioactivity associated with the peak at R_f 0.39 rose from an initial 10% of the total to 48% at 10 hours, after which it changed only slightly reaching 55% at 24 hours. The minor peak at R_f 0.19 followed a similar trend.

To obtain further information on the compounds associated with the peaks of radioactivity, the remaining extract from each sample was firstly chromatographed using the same system. The major radioactive zones, located with the aid of marker compounds, were then eluted and subjected to further chromatography.

8-1. The zone of R_f 0.70

Eluates corresponding to three sampling times were chromatographed on thin layer plates spread with a 1-mm layer of silica gel and developed in solvent A (Figure 8-3, A-C). This system separates zeatin and zeatin riboside which are not resolved by the paper system. At one hour most of the radioactivity in the eluate co-chromatographed with zeatin but a small proportion co-chromatographed with zeatin riboside. During t.l.c. on DEAE-cellulose using solvent C, similar proportions of the radioactivity in the eluate moved with zeatin and zeatin riboside. At 10 hours although the total radioactivity in the eluate was extremely low, label that moved with zeatin and zeatin riboside was still detectable (Figure 8-3, B) and that associated with zeatin riboside was dominant. By 24 hours, however, the total radioactivity in the eluate was so low that the presence of either compound was doubtful.

No paper or t.l.c. system is known that will separate dihydrozeatin from zeatin. These compounds can be distinguished, however, by the difference in their susceptibility to oxidation by dilute permanganate solution: dihydrozeatin resists oxidation while zeatin is readily degraded to oxidation products with distinct chromatographic properties, namely adenine, N-(purin-6-yl)glycine and a vicinal glycol, probably 6-(2,3,4-trihydroxy-3-methylbutylamino)
Figure 8-3. The distribution of radioactivity over silica gel thin-layer chromatograms of the zeatin zone eluates derived from chromatographed extracts of roots that had received $[^3]H$ zeatin. The distributions correspond to sampling times of 2 hours (A), 10 hours (B) and 24 hours (C) after zeatin application. The location of marker compounds was as follows: ZR, zeatin riboside; Z, zeatin. An arrow locates the origin. The chromatograms were developed in solvent A.
purine (Letham et al., 1967).

To determine whether any dihydrozeatin was present in the zeatin zone an aliquot of eluate of the 2-hour sample was treated as follows. Zeatin riboside and interfering materials were eliminated by preparative t.l.c. on 1-mm silica gel using solvent A. The zone occupied by the zeatin marker was scraped from the plate, packed into a column and eluted with 5 ml of 80% methanol. The eluate collected was concentrated in vacuo for final chromatography involving the oxidation procedure. Before application to a 0.25-mm silica gel plate, an aliquot of this solution was mixed with a small volume of \( ^{[8-14C]} \) zeatin solution measured to contain a similar total radioactivity count. This procedure enabled the oxidation products of the \(^3\)H compounds in the eluate to be directly compared with those of authentic zeatin. Dihydrozeatin was applied to a separate origin to indicate the R\(_f\) of unoxidized zeatin. The plate was developed briefly in solvent D to move zeatin and dihydrozeatin off the origin which retained materials that interfere with permanganate oxidation. The origin region was then scraped from the plate and discarded, while the region containing zeatin and dihydrozeatin was sprayed with 0.5% potassium permanganate, the remainder of the plate being shielded with cardboard. When the spray had dried the plate was developed in solvent A and divided into ten zones which were eluted for counting.

Both the \(^3\)H and the \(^{14}\)C radioactivity coincided with three u.v.-absorbing zones derived from marker zeatin, and the \(^3\)H/\(^{14}\)C ratios of these zones were very similar (2.27, 2.09 and 2.11 in order of increasing R\(_f\)) indicating that the oxidation products of the \(^3\)H-labelled material were the same as those of \(^{14}\)C-labelled authentic zeatin. The zone in the position of zeatin and dihydrozeatin showed no u.v. absorption and very low radioactivity, accounting for only 3.4% of the \(^{14}\)C counts and 1.7% of the \(^3\)H counts. These results indicated that \(^3\)H radioactivity in the purified zeatin zone eluate was due entirely to zeatin; dihydrozeatin, if present at all, could occur in trace amounts only.
8-2. The zone of $R_f$ 0.03

The radioactive zone that remained close to the origin in the paper chromatograms of root extract included the marker compound adenosine 5'-monophosphate and was therefore considered likely to contain nucleotides derived from zeatin. To examine this possibility aliquots of origin zone eluates were hydrolysed with alkaline phosphatase and chromatographed on paper using solvent A. Figure 8-4 shows the resulting distribution of radioactivity in preparations corresponding to sampling times of 2, 10 and 24 hours after zeatin application. The phosphatase digest yielded three radioactive peaks, one of which co-chromatographed with adenosine and another with zeatin riboside (Figure 8-4, A). The peak corresponding to zeatin riboside accounted for 70% of the total radioactivity at two hours, but by 10 hours had almost disappeared, while the peak that moved with adenosine was still very pronounced at 24 hours.

Although radioactivity co-chromatographed with zeatin riboside, it could be due to dihydrozeatin riboside, or possibly to the cis isomer of zeatin riboside. All these compounds would possess very similar $R_f$ values in the solvent system used. To clarify this point the eluate of the zeatin riboside zone was subjected to t.l.c. on silica gel using as solvent chloroform—methanol (9:1 v/v) which separates zeatin riboside from the cis isomer (Playtis and Leonard, 1971). All of the radioactivity co-chromatographed with zeatin riboside. Permanganate oxidation studies, similar to those detailed in section 8.8-1, established that the eluate did not contain significant amounts of dihydrozeatin riboside.

Some radioactivity remained at and near the origin; the contribution of this peak to the radioactivity of the hydrolysates increased with time. The compounds responsible for the peak were not identified, but may be metabolites that are not degraded by phosphatase.

The results of the phosphatase digestion indicated that the root extract contained a zeatin nucleotide and an adenosine phosphate. To characterize these nucleotides more precisely, further eluate derived from the 2-hour sample was
Figure 8-4. The distribution of radioactivity over paper chromatograms of the phosphatase hydrolysed origin zone eluates derived from chromatographed extracts of roots that had received $[^3\text{H}]$zeatin.

The distributions correspond to sampling times of 2 hours (A), 10 hours (B) and 24 hours (C) after zeatin application. The location of marker compounds was as follows: A, adenosine; ZR, zeatin riboside. An arrow locates the origin. The chromatograms were developed in solvent A.
incubated with venom of *Crotalus adamanteus* which hydrolyses only 5'-nucleotides. After subsequent paper chromatography in solvent A, the distribution of radioactivity was identical to that obtained after alkaline phosphatase digestion. This indicated that the nucleotides in the origin zone eluate were zeatin riboside 5'-phosphate and adenosine 5'-phosphate. Confirmation was provided by oxidation of the eluate with periodate followed by reaction with cyclohexylamine, a procedure which cleaves ribonucleosides and ribonucleoside 5'-phosphates to bases. After this treatment, the majority of the radioactivity in the eluate co-chromatographed with zeatin during t.l.c. on silica gel (solvent A). When subjected to t.l.c. on DEAE-cellulose (solvent F), the majority of the radioactivity in the origin zone eluate co-chromatographed with authentic zeatin riboside 5'-monophosphate. These results establish that in the 2-hour sample, the principal nucleotide was zeatin riboside 5'-monophosphate.

8-3. The zone of $R_f$ 0.39

The behaviour of the radioactive component of root extract that moved just ahead of adenosine during paper chromatography was examined in three systems of t.l.c. Eluates of this component were prepared from preparative paper chromatograms corresponding to seedling sampling times of 10 and 24 hours after zeatin application. When eluate from the 10-hour sample was chromatographed on DEAE-cellulose in solvent C (Figure 8-5), less than 9% of the total radioactivity in the eluate co-chromatographed with adenine and adenosine, most of the counts being associated with a peak at $R_f$ 0.74 that closely followed zeatin riboside ($R_f$ 0.77). On 1-mm silica gel developed in solvent A, the radioactivity of the eluate was concentrated at $R_f$ 0.19 compared with $R_f$ values of 0.37 for zeatin riboside, and 0.31 for adenosine which overlapped a small proportion (about 7%) of the radioactive peak. During this chromatography adenine travelled ($R_f$ 0.54) well beyond all radioactivity, allowing the minor radioactive component of Figure 8-5 to be attributed to adenosine. In both of these systems, and also during the
Figure 8-5. The distribution of radioactivity over a DEAE-
cellulose thin-layer chromatogram of R_f 0.39
or "X" zone eluate derived from the chromatographed extract of roots sampled 10 hours after
they had received [\textsuperscript{3}H]zeatin.
The location of marker compounds was as follows:
A, adenosine; Ad, adenine; ZR, zeatin riboside.
An arrow locates the origin. The chromatogram
was developed in solvent C.
paper chromatography, the major radioactive component did not appear to be a common purine derivative and is referred to henceforth as compound X. In both of the above thin-layer systems and also during paper chromatography, X had the same relative mobilities as a new zeatin metabolite termed raphanatin which was detected almost simultaneously in this laboratory by C.W. Parker. Raphanatin was a metabolite which accumulated in the cotyledons of derooted radish seedlings when these were supplied with zeatin through the transpiration stream. To confirm that the root metabolite X and the cotyledon metabolite raphanatin were identical, a mixture of the two $^3$H-labelled compounds was chromatographed on silica gel (solvent B). The counts due to X and raphanatin in the mixture were approximately equal. Elution of the developed chromatogram indicated that the two compounds co-chromatographed ($R_f$ 0.23). Hence X and raphanatin appeared to be identical.

8-4. The zone of $R_f$ 0.19

The minor metabolite at $R_f$ 0.19 in the paper chromatograms of root extract resembled hypoxanthine in its mobility in this system, but the radioactivity eluted from the $R_f$ 0.19 region (of the 24-hour sample) did not move with marker hypoxanthine during t.l.c. on silica gel, either in solvent A or in solvent B. The t.l.c. in solvent B also eliminated xanthine, xanthosine and inosine as possible identities of the metabolite.

9. Zeatin metabolites in the hypocotyls

Paper chromatograms of hypocotyl extracts were prepared similarly to those of the root extracts, but only the aqueous fractions were used since these contained over 90% of the extracted radioactivity. In all chromatograms most of the radioactivity remained at the origin, as illustrated in Figure 8-6 (A-C). (The distributions of counts at 1 hour and 10 hours, which are not shown, were very similar to those at 2 and 5 hours respectively).

At the two earliest sampling times, some 15% of the total radioactivity co-chromatographed with zeatin (Figure 8-6, A), but few of these counts (derived from the
Figure 8-6. The distribution of radioactivity over paper chromatograms of extracts prepared from the hypocotyls of radish seedlings supplied with $[^{3}\text{H}]$zeatin through the roots. The distributions correspond to sampling times of 2 hours (A), 5 hours (B) and 24 hours (C) after the application of zeatin to the roots. The location of marker compounds was as follows: AMP, adenosine 5'-monophosphate; A, adenosine; Z, zeatin. An arrow locates the origin. The chromatograms were developed in solvent A.
2 hour sample) remained with the zeatin marker during t.l.c. on 1-mm silica gel in solvent A (Figure 8-7): most of the radioactivity occurred in two peaks of similar prominence, one coincident with zeatin riboside and the other associated with an unidentified component at the solvent front. No radioactive peaks were revealed by similar t.l.c. of zeatin zone eluate corresponding to the sampling time of 24 hours.

The origin zones (four sampling times) were eluted, hydrolysed with phosphatase, extracted into butanol and chromatographed on paper in solvent A. In the resulting chromatograms (three of which are illustrated in Figure 8-8) the distributions of radioactivity at each sampling time resembled those of the chromatographed origin zone hydrolysates from root extracts in that the labelled products of hydrolysis co-chromatographed with adenosine and zeatin riboside. The occurrence of radioactivity in these forms was confirmed by paper chromatography using solvent B. The initially high proportion of radioactivity associated with zeatin riboside declined and disappeared within 24 hours, while the radioactive peak accompanying adenosine persisted throughout this period. There was, however, no indication of labelled components at the origin, apart from a low plateau of counts in that region at 24 hours. Such material could have remained in the aqueous phase during butanol extraction since not all of the counts eluted for hydrolysis were extracted into butanol after phosphatase digestion: at 1, 5 and 24 hours the percentage recoveries were 93, 90 and 61 respectively.

To determine the form in which zeatin was trans-located in the hypocotyls, chromatograms of bleeding sap were prepared. The sap was collected from decapitated radish seedlings after their roots had been irrigated with dilute $[^3H]$zeatin solution for 3, 5 and 9 hours. When chromatographed on silica gel in solvent A, each sap sample yielded only one distinct radioactive zone which co-chromatographed with zeatin riboside. This result was also obtained when the sap collected after 5 hours of zeatin uptake was chromatographed on silica gel in solvent E and on DEAE cellulose in
Figure 8-7. The distribution of radioactivity over a silica gel thin-layer chromatogram of the zeatin zone eluate derived from the chromatographed extract of hypocotyls of radish seedlings sampled 2 hours after the application of $[^3\text{H}]$zeatin to the roots.

The location of marker compounds was as follows: ZR, zeatin riboside; Z, zeatin. An arrow locates the origin. The chromatogram was developed in solvent A.
Figure 8-8. The distribution of radioactivity over paper chromatograms of the phosphatase hydrolysed origin zone eluates derived from chromato-graphed extracts of hypocotyls of radish seedlings supplied with \(^{3}\text{H}\)zeatin through the roots. The distributions correspond to sampling times of 1 hour (A), 5 hours (B) and 24 hours (C) after the application of zeatin to the roots. The location of marker compounds was as follows: A, adenosine; ZR, zeatin riboside. An arrow locates the origin. The chromatograms were developed in solvent A.
solvent C. In all chromatograms the radioactivity associated with zeatin riboside represented 60 to 70% of the total (Figure 8-9), while over the rest of the chromatogram counts were at very low levels and radioactivity in the zones occupied by zeatin and adenosine 5'-monophosphate did not exceed 4% and 5% respectively.

10. Zeatin metabolites in the cotyledons

Since little radioactivity reached the cotyledons until 5 hours after \[^{3}H\]zeatin was supplied to the roots, only those extracts prepared from cotyledons sampled at 5, 10 and 24 hours after zeatin application were chromatographed on paper in solvent A. The resulting chromatograms (Figure 8-10) closely resembled those prepared from root extract (Figure 8-2, C-E) in the number of radioactive peaks, their R\(_f\) values and the changes, with time, in their relative proportions.

The radioactivity that co-chromatographed with zeatin on paper was eluted from each chromatogram and subjected to t.l.c. on 1-mm silica gel. Eluates from the 5- and 10-hour samples were chromatographed in solvent A, while further eluate from the 5-hour sample and all of the eluate from the 24-hour sample were chromatographed in solvent B. At 5 and 10 hours, there was only one distinct radioactive zone (see Figure 8-11, A and B) which co-chromatographed with the zeatin marker. At 24 hours a trace of zeatin was detected although all zones of the chromatogram produced less than 10 c.p.m. above background. The t.l.c. in solvent A indicated the absence of zeatin riboside.

Origin zone eluates from the paper chromatograms of the 5-hour and 24-hour samples were digested with phosphatase and extracted into butanol. When chromatographed on paper in solvent A, both digests yielded one major peak of radioactivity which co-chromatographed with adenosine (Figure 8-12). At 5 hours some 20% of the counts moved with zeatin riboside but no radioactivity accompanied this marker at 24 hours. Neither butanol extract yielded more than a trace of radioactivity that remained at the origin.
Figure 8-9. The distribution of radioactivity over a silica gel thin-layer chromatogram of the bleeding sap collected from radish hypocotyls 5 hours after the application of $[^3H]$zeatin to the roots.
The location of marker compounds was as follows: AMP, adenosine 5'-monophosphate; ZR, zeatin riboside; Z, zeatin. An arrow locates the origin. The chromatogram was developed in solvent A.
Figure 8-10. The distribution of radioactivity over paper chromatograms of extracts prepared from the cotyledons of radish seedlings supplied with \(^3\text{H}\) zeatin through the roots. The distributions correspond to sampling times of 5 hours (A), 10 hours (B) and 24 hours (C) after the application of zeatin to the roots. The location of marker compounds was as follows: AMP, adenosine 5'-monophosphate; A, adenosine; Z, zeatin. An arrow locates the origin. The chromatograms were developed in solvent A.
Figure 8-11. The distribution of radioactivity over silica gel thin-layer chromatograms of the zeatin zone eluates derived from chromatographed extracts of cotyledons of radish seedlings supplied with $[^3H]$zeatin through the roots. The distributions correspond to sampling times of 5 hours (A), 10 hours (B) and 24 hours (C) after the application of zeatin to the roots. The location of marker compounds was as follows: ZR, zeatin riboside; Z, zeatin. An arrow locates the origin. The chromatograms were developed in solvent A (A and B) or in solvent B (C).
Figure 8-12. The distribution of radioactivity over paper chromatograms of the phosphatase hydrolysed origin zone eluates derived from chromatographed extracts of cotyledons of radish seedlings supplied with $[^{3}\text{H}]{\text{zeatin}}$ through the roots.

The distributions correspond to sampling times of 5 hours (A) and 24 hours (B) after the application of zeatin to the roots.

The location of marker compounds was as follows: A, adenosine; ZR, zeatin riboside. An arrow locates the origin. The chromatograms were developed in solvent A.
The major cotyledon metabolite moved just ahead of adenosine during paper chromatography, and therefore appeared to be due to compound X. When eluates of this zone (from sampling times of 5 and 24 hours) were chromatographed on DEAE-cellulose in solvent C (Figure 8-13), over 80% of the radioactivity had a mobility characteristic of X, namely just less than that of zeatin riboside. Only 6% of the counts co-chromatographed with adenosine.

11. **Zeatin metabolites in the apical region**

Because little tissue was available from apical regions, analysis of the metabolites was limited. Although there was no evidence for a concentration of radioactivity in this tissue, very recent experiments (not detailed in this thesis) have indicated the presence of a labelled compound which co-chromatographed with zeatin and zeatin riboside during paper chromatography of the 5-hour extract in solvent A. Per unit weight of tissue, the radioactivity associated with this compound (10.2% of the total) was higher than that due to zeatin plus zeatin riboside in the corresponding hypocotyl and cotyledon extracts (3% and 5% of the total respectively). In the chromatographed extract of the apical region, three other major radioactive zones were detected: 23% of the total counts co-chromatographed with adenosine, 19.5% were at or near the origin, and at Rf 0.52 there occurred a peak not detected in the other seedling regions examined. This latter component accounted for 20.3% of the total radioactivity in the apical extract. Compound X was not detected.

12. **Metabolites of BAP in excised radish cotyledons**

Cotyledons were sampled for extraction at 1, 5, 13.5, 24 and 96 hours after the beginning of a 5-hour labelling period with \([3H]BAP\) solution. Aliquots of each extract were chromatographed on paper using solvent A. Figure 8-14 shows the distribution of radioactivity over these chromatograms. At 1 hour appreciable (at least 32%) metabolism of BAP was already apparent (Figure 8-14, A). Of the extracted radioactivity no longer associated with BAP,
Figure 8-13. The distribution of radioactivity over a DEAE-cellulose thin-layer chromatogram of the Rf 0.39 or "X" zone eluate derived from the chromatographed extract of cotyledons of radish seedlings sampled 5 hours after the application of \([^3H]zeatin\) to the roots. The location of marker compounds was as follows: A, adenosine; ZR, zeatin riboside. An arrow locates the origin. The chromatogram was developed in solvent C.
Figure 8-14. The distribution of radioactivity over paper chromatograms of extracts prepared from excised radish cotyledons that had received $[^3H]$BAP. Cotyledons were sampled at the following intervals (hours) after initial contact with BAP: 1(A), 5(B), 13.5(C), 24(D) and 96(E). The labelling period, for all samples except the first, was 5 hours. The location of co-chromatographed marker compounds was as follows: AMP, adenosine 5'-monophosphate; A, adenosine; BAP, BAP. BAP riboside was developed beside chromatogram A, and its position is indicated by an arrow denoted BAPR. An unidentified u.v.-absorbing zone derived from the extract is marked U. An arrow locates the origin. The chromatograms were developed in solvent A.
most moved as a broad peak at $R_f$ 0.64. The proportion of radioactivity in this region increased during a corresponding decline in the radioactivity associated with BAP. Within 9 hours after the labelling period (Figure 8-14, C), the BAP peak had disappeared, while the peak at $R_f$ 0.64 accounted for over 90% of the total extracted radioactivity, and maintained this high proportion throughout the remaining experimental period (3.5 days). The only other distinct radioactive peak was that of a minor metabolite at $R_f$ 0.19 (coinciding with a zone of endogenous u.v.-absorbing material). None of the chromatograms contained a radioactive peak at or near the origin; thus there were no significant amounts of nucleotide derivatives of BAP. Counts were very low in the region of adenosine and no peak of radioactivity was associated with this compound.

From the specific activity of the $[^3H]$BAP and the counting efficiency of $^3H$, it was possible to calculate the amount of BAP, or its equivalent, extracted per cotyledon. In order of sampling time these quantities were 0.106, 0.193, 0.205, 0.185 and 0.164 µg respectively. Between 1 and 4 days after labelling the cotyledons (fourth and fifth sampling times) over 95% of the radioactive material was in the form of the metabolite(s) at $R_f$ 0.64. The decline in total extracted radioactivity during this period amounted to only 11%.

12-1. The zone of $R_f$ 0.64

The labelled BAP metabolite(s) at $R_f$ 0.64 appeared to be active. When the zones of a further paper chromatogram of the 24-hour sample of cotyledon extract were bioassayed using radish cotyledons (section 8.6), cytokinin activity coincided with this radioactive zone (Figure 8-15). No cytokinin activity was detected in the chromatogram of the control extract. The assay was repeated with similar results. Further, when the zone at $R_f$ 0.64 was eluted from another chromatogram of the same extract and chromatographed on silica gel using solvent B, cytokinin activity again coincided with the radioactive zone. In this system, the $R_f$
Figure 8-15. The distribution of (A) cytokinin activity and 
(B) BAP metabolites over a paper chromatogram 
of extract prepared from radish cotyledons 
sampled 19 hours after a 5-hour incubation with 
[^3H]BAP.

After development in solvent A, the chromatogram 
was divided vertically into two strips. The 
zones of one strip (equivalent to 7.5 cotyledons) 
were bioassayed using radish cotyledons. A 
chromatogram prepared using extract of unlabelled 
control cotyledons (section 8.2) was similarly 
bioassayed, and the mean cotyledon weight for 
each zone subtracted from that for the corres­
ponding zone of the labelled chromatogram. The 
differences, i.e. the mean increments in cotyledon 
weight (mg) due to substances in the labelled 
zones, are shown in histogram A.

The zones of the other strip were counted, and 
from their c.p.m. were calculated the equivalent 
amount of[^3H]BAP in each bioassayed zone 
(histogram B). The location of marker compounds 
was as follows: A, adenosine. An arrow locates 
the origin.
of the radioactivity was 0.55. However, when another chromatogram with a much lower loading was divided into narrower zones for counting, two peaks of radioactivity were revealed (Figure 8-16), one at $R_f$ 0.41 (metabolite A) and the other at $R_f$ 0.55 (metabolite B). The latter peak contained approximately half as much radioactivity as the slower moving peak. Thus, at least two labelled metabolites were present in the zone at $R_f$ 0.64 on the paper chromatograms. The chromatographic behaviour of this labelled material was further characterized by t.l.c. on silica gel using solvent A. The radioactivity was located at $R_f$ 0.21, but in this case no heterogeneity was revealed.

When labelled BAP was supplied to de-rooted radish seedlings through the transpiration stream, two major metabolites were formed. Chromatographic studies indicated these were identical to the two labelled compounds revealed in Figure 8-16.

13. **Purification of the metabolites of BAP**

The method devised for the isolation of the two principal metabolites is now outlined. About 2,000 de-rooted, 9-day-old radish seedlings (200 g) were allowed to take up BAP solution (0.12 mM) for 18 hours and were then dropped into boiling 80% methanol. This was maintained at 65° for 5 minutes before being cooled rapidly. Homogenization and filtration yielded an extract which was evaporated to dryness under vacuum to give a residue which was suspended in water (150 ml) and centrifuged. The resulting solution was extracted with four 150-ml volumes of water-saturated n-butanol. The extracts were evaporated and an aqueous solution (150 ml, pH 3.0) of the residue was percolated through a column of cellulose phosphate (40 g, NH$_4^+$ form equilibrated to pH 3.0) which was washed with 0.05 N acetic acid and then with water (1 and 3 column volumes respectively). The column was then eluted with 0.3 N ammonia (2.5 litres). Evaporation of the eluate under vacuum yielded a residue which was dissolved in 50% ethanol (20 ml).

Purification was continued by preparative t.l.c.; at each chromatographic step, the location of the desired
Figure 8-16. The distribution of radioactivity over a silica gel thin-layer chromatogram of the R$_f$ 0.64 zone eluate derived from a chromatographed extract of radish cotyledons that had received [$^3$H]BAP.

The distribution corresponds to a sampling time of 19 hours after the 5-hour incubation with [$^3$H]BAP. The location of marker BAP is indicated. An arrow locates the origin. The chromatogram was developed in solvent B.
metabolites was determined from a preliminary running of a corresponding labelled fraction. Preparative t.l.c. on seven 20 x 20 x 1-mm layers of silica gel (solvent B) yielded a zone (R_f 0.61) containing metabolite A and a second fraction (R_f 0.70) with metabolite B. All preparative t.l.c. zones were eluted with 80% methanol containing 1% acetic acid. The eluate with metabolite A was further purified by two additional preparative t.l.c. steps on silica gel using as solvents first n-butanol—14N ammonia—water—ethanol (6:1:2:1 v/v) and then ethanol—water—14N ammonia (18:2:1 v/v). The resulting

Table 8-1 Structures for fragment ions in the mass spectra of metabolites A and B (numbers in parentheses are mass measurements from high resolution - spectra)

<table>
<thead>
<tr>
<th>m/e</th>
<th>Metabolite A</th>
<th>Metabolite B</th>
<th>Structure assigned</th>
</tr>
</thead>
<tbody>
<tr>
<td>387</td>
<td>+</td>
<td>+</td>
<td>M^+ + C_{18}H_{19}N_5O_5 (calc. 387.1543)</td>
</tr>
<tr>
<td>370</td>
<td>+</td>
<td>M - OH</td>
<td></td>
</tr>
<tr>
<td>312</td>
<td>+</td>
<td>M - OH</td>
<td></td>
</tr>
<tr>
<td>296</td>
<td>+</td>
<td>C_{15}H_{14}N_4O_2 (calc. 296.1148); M - C_3H_4O_3</td>
<td></td>
</tr>
<tr>
<td>282</td>
<td>+</td>
<td>M = (C_4H_5-CH=NH)</td>
<td></td>
</tr>
<tr>
<td>268</td>
<td>+</td>
<td>M - C_3H_4O_4</td>
<td></td>
</tr>
<tr>
<td>254</td>
<td>+</td>
<td>M - C_3H_4O_4</td>
<td></td>
</tr>
<tr>
<td>225</td>
<td>+</td>
<td>C_{12}H_{11}N_5 (calc. 225.1014); BAP ion</td>
<td></td>
</tr>
<tr>
<td>209</td>
<td>+</td>
<td>225 - NH^*</td>
<td></td>
</tr>
<tr>
<td>197</td>
<td>+</td>
<td>225 - (H + HCN)^*</td>
<td></td>
</tr>
<tr>
<td>148</td>
<td>+</td>
<td>6-methylenearminopurine ion^*</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>+</td>
<td>purinyl ion</td>
<td></td>
</tr>
<tr>
<td>106</td>
<td>+</td>
<td>C_8H_8N (calc. 106.0657), C_6H_8-CH_2-\text{NH}</td>
<td></td>
</tr>
<tr>
<td>91</td>
<td>+</td>
<td>benzyl or tropylium ion</td>
<td></td>
</tr>
</tbody>
</table>

* For structure of these fragment ions, which also occur in the mass spectrum of 6-benzylaminopure, see Shannon and Letham (1966).

zone was eluted and subjected to chromatography on paper (exhaustively washed with 20% ethanol) using water—saturated n-butanol (carefully redistilled) as solvent. The u.v.-absorbing component at R_f 0.55 was pure metabolite A which
Figure 8-17. The mass spectrum of metabolite A.
The spectrum was obtained with an AEI MS9 mass spectrometer.
Figure 8-18. The mass spectrum of metabolite B.

The spectrum was obtained with an AEI MS9 mass spectrometer.
was eluted with purified ethanol for mass spectrometry. The yield was about 100 μg. The metabolite B was purified by essentially the same sequence of steps yielding about 100 μg of compound (Rf 0.63 on paper, solvent water—saturated n-butanol).

The mass spectra for metabolites A and B are presented in Figure 8-17 and 8-18; in Table 8-1, accurate mass measurements from high resolution spectra and probable structures for fragment ions are listed. Both metabolites exhibited a molecular ion at m/e 387 and the high resolution spectra unequivocally established the formula C_{18}H_{21}N_{6}O_{5}. Below m/e 226, the spectra were almost identical to that of 6-benzylaminopurine (see Shannon and Letham, 1966). Consideration of the spectra strongly suggested the metabolites were 6-benzylaminopurine—hexose sugar conjugates.

D. DISCUSSION

Isotopically labelled zeatin was supplied to the roots of intact radish seedlings. About 90% of the radioactivity extracted from the seedlings was recovered from the root tissue. Similar evidence for the localization of exogenous cytokinins at the site of application has already been discussed. However, in the experiments reported here with radish seedlings, some translocation of zeatin from the root to the cotyledons was detected. Zeatin moved in the transpiration stream in the form of the riboside which was converted in the cotyledons to zeatin and a metabolite termed X. While radioactivity equivalent to 95.6 x 10^{-3} μg of zeatin was extracted from the root system of each seedling at 5 hours, less than 0.6 x 10^{-3} μg of zeatin was recovered from the cotyledons which also yielded X equivalent to 2.4 x 10^{-3} μg of zeatin. The radioactivity per unit of tissue weight was approximately constant in the various regions of the shoot.
There was no evidence for a concentration of radioactivity in the apical meristem region, but the concentration of zeatin or zeatin riboside in this tissue may be higher than in the hypocotyls and cotyledons. A metabolite not present in other tissues also occurred in extracts of the apical region. Hence the radioactive compounds in the meristematic tissue of the apical region merit a more detailed investigation.

Five hours after the application of zeatin, X was the principal metabolite in the root. However, at the end of the 1-hour period of supply of zeatin, the majority of the radioactivity due to metabolites chromatographed on paper (solvent A) near the location of adenosine 5'-monophosphate (R_f 0.03), and zeatin riboside 5'-phosphate was the principal labelled compound in this region. The radioactivity peak near the origin and that attributable to X (R_f slightly greater than that of adenosine) appeared to be related; as the X peak increased with time, the origin peak decreased. This is consistent with, but does not establish, the existence of a precursor-product relationship between X and compounds of R_f 0.03.

When zeatin was supplied to the cut ends of de-rooted radish seedlings, a metabolite termed raphanatin accumulated in the cotyledons (work of C.W. Parker in this laboratory). Evidence has been presented that the root metabolite X and the cotyledon metabolite raphanatin are identical. The isolation of raphanatin was undertaken in this laboratory by C.W. Parker and D.S. Letham, who obtained 60 µg of the pure compound from about 2,000 seedlings. Mass spectrometry, u.v. spectroscopy and chemical degradation indicated that raphanatin is 7-glucosylzeatin (Parker et al., 1972). However, the ring form of the sugar and the configuration of the glycoside link are not known at present. Naturally occurring compounds with a sugar moiety attached to a purine ring at positions other than 9 are extremely rare. The only known natural purine compounds with a sugar at position 7 are certain vitamin B_{12}-like substances (pseudovitamin B_{12} and Factor A). In these compounds the sugar is ribose. Very recently Deleuze et al.
(1972) presented evidence that the stable metabolite of BAP detected by Fox et al. (1972) in soybean tissue cultures was the 7-glucoside of BAP. Glucosylation at position 7 therefore appears to occur with both natural and synthetic cytokinins. In addition a naturally occurring cytokinin which appears to be a glucoside of zeatin has been isolated from the roots of rice plants (Yoshida and Oritani, 1972), but evidence concerning the site of attachment of the sugar (\(?\) glucose) has not been presented.

In radish cotyledons raphanatin is a very stable metabolite and it is probably a storage form of zeatin (Parker and Letham, unpublished results). Hence zeatin may be stored in the radish seedling as raphanatin both in the cotyledon and in the root. Raphanatin possesses cytokinin activity. Further investigation is required to determine whether it is active \textit{per se} and the functional form of zeatin. It is uncertain if raphanatin occurs normally in radish plants. The only investigation of the naturally occurring cytokinins in radish is that of Radin and Loomis (1971) who detected marked cytokinin activity in extracts of mature tap roots but no activity in extracts of 9-day-old radish roots. Although the active extracts were chromatographed, and evidence thereby obtained for the presence of zeatin, zeatin nucleotide and zeatin riboside, it is not clear from the results presented whether raphanatin was present. However, this compound could have contributed to the cytokinin activity observed by Radin and Loomis at \(R_f^0 0.0 - 0.2\) on silica gel (solvent: water-saturated n-butanol); in this chromatographic system raphanatin exhibits an \(R_f\) of approximately 0.23.

All main peaks of radioactivity observed on chromatograms of the seedling extracts could be accounted for by one (or two) of the following compounds: zeatin, zeatin riboside, adenosine \(5'\) phosphate, zeatin riboside \(5'\) phosphate, or raphanatin. However, certain minor metabolite peaks could not be attributed to known compounds. At \(R_f\) 0.19 on paper chromatograms (solvent A) of root extract, and of cotyledon extract, a minor unidentified metabolite was
located. This is also present in cotyledon extracts of de-rooted seedlings supplied with zeatin and is derived from raphanatin (Parker and Letham, unpublished). In the roots, labelled compounds were detected that co-chromatographed with adenosine 5'-phosphate on paper (solvent A) and did not change in R_f when treated with phosphatase. These increased in amount relative to zeatin nucleotide and adenosine 5'-phosphate as the period in unlabelled nutrient medium was extended following the uptake of zeatin by the roots.

Although both zeatin riboside 5'-phosphate and zeatin riboside were present in extracts of hypocotyls, only zeatin riboside occurred in the xylem sap. The zeatin nucleotide was possibly located in cells adjacent to xylem elements, where it may have formed from zeatin riboside which moved laterally from the transpiration stream. Such lateral movement of other compounds, including auxin (see review by Bollard, 1960), gibberellic acid and kinetin (Bowen and Wareing, 1969) has been established previously. Alternatively the nucleotide may have formed in the cotyledons and been transported in the phloem of the hypocotyl. In further very recent experiments not detailed in this thesis, [3H]zeatin was supplied to the roots of decapitated radish seedlings. Labelled zeatin nucleotide was a major metabolite in the hypocotyls. Hence the labelled nucleotide in hypocotyls of intact seedlings is probably at least partly derived from zeatin riboside in the transpiration stream.

Endogenous cytokinins have been detected in the xylem sap exuded by numerous plant species following decapitation. Certain of these have been characterized chromatographically and evidence for the occurrence of zeatin in Helianthus annuus (Klämbt, 1968; Kende and Sitton, 1967) and zeatin riboside in Vitis vinifera (Skene, 1972) has been presented. In other species, nucleotide cytokinins appear to occur in addition to other active compounds, probably bases and nucleosides (Carr and Burrows, 1966; Carr and Reid, 1968; Yoshida et al., 1971). It has been suggested that xylem sap cytokinins are synthesized in the root apex (Weiss
and Vaadia, 1965), but they could also be products of the autolysing, differentiating xylem elements and phloem cells in roots (Sheldrake and Northcote, 1968 a and b), or they might be derived from the phloem and circulated through the root into the xylem. Regulatory functions have been proposed for cytokinins in xylem exudate (e.g. Sitton, Itai and Kende, 1967). Because of the occurrence of cytokinins in xylem sap collected after decapitation, it has been generally assumed that these hormones also occur in the xylem sap of intact plants. This is not necessarily true, however: the presence of endogenous cytokinins in sap exuded in response to decapitation could be an artifact, a consequence of the removal of the shoot. In this connection, the experimental results presented herein are relevant. Labelled zeatin and raphanatin were detected in radish cotyledons following uptake of labelled zeatin by the roots of intact seedlings. Hence zeatin does appear to be translocated in the xylem of the intact radish seedling and moves from root to cotyledons as the riboside. The radioactivity of the cotyledons did not increase after 5 hours (see Figure 1). This may be a consequence of the depletion of zeatin riboside, the translocational form of the hormone, in the root at this time. Cytokinins previously detected in xylem exudates of decapitated plants are probably normal constituents of the transpiration stream in the intact plant. Excised immature radish cotyledons enlarge in response to exogenously supplied cytokinins. These may act as a substitute for endogenous cytokinins translocated from the root to the cotyledons. The metabolism of BAP by excised, immature radish cotyledons was also investigated. This synthetic cytokinin was rapidly converted to compounds which exhibited an Rf of about 0.64 on paper chromatograms (solvent A), and which could be resolved into two fractions by t.l.c. on silica gel. After the metabolism of BAP was complete, the amount of these compounds extracted per cotyledon showed little change. Hence these metabolites appeared to be stable. Surprisingly there was no appreciable conversion to nucleotides or other compounds. This contrasts with the
metabolism of zeatin in radish cotyledons, and with the metabolism of BAP in soybean tissue which has been investigated by Fox et al. (1972) and Dyson et al. (1972). These workers found that conversion to nucleotides was initially the dominant metabolic change. In soybean, formation of BAP riboside was also marked. These workers also observed that BAP was converted to a very stable metabolite, which probably possessed cytokinin activity, but this product appeared to differ in Rf from the metabolites observed in the present experiments. Results presented by Dyson et al. (1972) indicated that BAP riboside had an Rf of 0.73 when chromatographed on Whatman 3MM paper using as solvent 1-butanol—14 N ammonia—water (85:5:9 v/v), while the stable metabolite (termed "fraction C") had an Rf of 0.17. To compare the radish cotyledon metabolites with fraction C, they were chromatographed using the system just described. Although the Rf of the BAP riboside marker (0.76) was very similar to that obtained by Dyson and co-workers, the major BAP metabolites had a much higher mobility (Rf 0.52) than fraction C.

Since these metabolites appeared to be active as cytokinins and probably differed from the BAP metabolite detected by Fox, Dyson and co-workers, isolation was attempted. From about 2,000 seedlings, 100 µg of both compounds were obtained in a state of purity. The mass spectra indicated that the metabolites were BAP—hexose sugar conjugates. Further structural studies were carried on by D.S. Letham. On hydrolysis both metabolites yielded glucose which was identified with the specific enzyme glucose oxidase. U.v. spectroscopy established that metabolite A was 6-benzylamino-7-glucosylpurine, and that B was 6-benzylamino-9-glucosylpurine. The former compound was therefore possibly identical to Fox's metabolite, but, of course, the compounds could differ in the ring structure of the sugar moieties.

If zeatin is converted to the 9-glucoside at all in radish seedlings, the amount formed must be very small relative to the 7-glucoside. This conclusion can be reached from knowledge of the chromatographic behaviour of the
synthetic 9-glucoside relative to raphanatin. The conversion of zeatin almost exclusively to the 7-glucoside, and the metabolism of BAP to both the 7- and 9-glucosides is surprising. Are these unusual purine derivatives the functional forms of BAP, or are they storage forms? Only further experiments will resolve these issues.
APPENDIX

N.B.E.S. BASIC PROGRAMME LIBRARY

"DOUBLE LABELLING CALCULATIONS"
including

counting efficiency corrections

Contributed by M.E. Gordon and A.J. Gibbons

1. Function

This programme is used to determine the number of c.p.m. due to \(^3\text{H}\) and due to \(^{14}\text{C}\) in a radioactive sample labelled with both isotopes. The programme requires the
print-out information from a scintillation counter with more
than one channel capability, and it completes the resolution
of \(^3\text{H}\) and \(^{14}\text{C}\) radioactivity which the counter has partially

distinguished.

Variation in counting efficiency is removed
by the programme, which requires all resolved \(^3\text{H}\) and \(^{14}\text{C}\)
c.p.m. to their values at the maximal counting efficiency
appropriate to the scintillant. Thus the programme may also
be used for efficiency corrections to \(^3\text{H}\) or \(^{14}\text{C}\) c.p.m. in
singly labelled samples.

2. Limitations of applicability (without programme
   modification)

The calculations in the programme were designed to
modify the print-out from a Beckman LS-250 3-channel liquid
scintillation spectrometer operating with automatic quench
compensation (AQC). (AQC automatically compensates for
spectral shift in the energy range of \(^3\text{H}\) and \(^{14}\text{C}\) which is
caused by sample quenching. Thus, with AQC, counting
efficiency still varies with quench, but the proportion of
\(^{14}\text{C}\) spillover into the \(^3\text{H}\) channel is kept constant.) The
programme is appropriate for 1.9 ml aqueous samples mixed
with 16 ml of toluene/Triton X-100 scintillant (toluene/
Triton X-100, 2:1 v/v, containing PPO, 2 g/l and dimethyl
DOPPOP, 0.3 g/l). The external standard ratio ("X") of all
samples should be within the range of 0.30 - 0.35. (The
programme may be used with half the above volume of sample
and scintillant, provided that a correction factor of 0.65
is added to each external standard ratio).
APPENDIX

R.S.B.S. BASIC PROGRAMME LIBRARY

"DOUBLE LABELLING CALCULATIONS"
including
counting efficiency corrections
Contributed by M.E. Gordon and A.J. Gibbs

1. Function

This programme is used to determine the number of c.p.m. due to $^3$H and due to $^{14}$C in a radioactive sample labelled with both isotopes. The programme requires the print-out information from a scintillation counter with more than one channel capability, and it completes the resolution of $^3$H and $^{14}$C radioactivity which the counter has partially distinguished.

Variation in sample counting efficiency is removed by the programme, which corrects all resolved $^3$H and $^{14}$C c.p.m. to their values at the maximal counting efficiency appropriate to the scintillant. Thus the programme may also be used for efficiency corrections to $^3$H or $^{14}$C c.p.m. in singly labelled samples.

2. Limitations of applicability (without programme modification)

The calculations in the programme were designed to modify the print-out from a Beckman LS-250 3-channel liquid scintillation spectrometer operating with automatic quench compensation (AQC). (AQC automatically compensates for spectral shift in the energy ranges of $^3$H and $^{14}$C which is caused by sample quenching. Thus, with AQC, counting efficiency still varies with quench, but the proportion of $^{14}$C spillover into the $^3$H channel is kept constant). The programme is appropriate for 1.0 ml aqueous samples mixed with 10 ml of toluene/Triton X-100 scintillant (toluene/Triton X-100, 2:1 v/v, containing PPO, 2 g/l and dimethyl POPOP, 0.2 g/l). The external standard ratios ("X") of all samples should be within the range of 0.50 - 0.55. (The programme may be used with half the above volume of sample and scintillant, provided that a correction factor of 0.05 is added to each external standard ratio).
3. The programme calculates and prints:

(a) The "true" $^3$H count (i.e. $^3$H c.p.m. corrected for $^{14}$C spillover and for differences in counting efficiency).

(b) The "true" $^{14}$C count (i.e. $^{14}$C c.p.m. corrected for $^{14}$C spillover into $^3$H and for differences in counting efficiency).

(c) The $^3$H/$^{14}$C ratio (i.e. (a)/(b)).

(d) The $^3$H/$^{14}$C ratio multiplied by an arbitrary correction factor, "C".

The correction of the $^3$H/$^{14}$C ratio [supplied by (d)] is an optional calculation which may be repeated as often as required, using different correction factors. Uses of this step include: the transformation of $^3$H/$^{14}$C c.p.m. ratios to $^3$H/$^{14}$C d.p.m. ratios; correction (e.g. to an equal precursor uptake ratio) of the $^3$H/$^{14}$C ratios in particular doubly labelled macromolecules, such as DNA, RNA and protein; correction of $^3$H/$^{14}$C ratios for unequal supply of the two isotopes in the incubation medium - an adjustment that facilitates comparison of the results of different experiments and may improve the clarity of graphed or tabular information.

4. Implementation

The programme asks firstly for the proportion of the $^{14}$C count that has spilled into $^3$H. (This ratio is determined by preparing a vial containing only $^{14}$C radioactivity and counting it in both the $^3$H and $^{14}$C channels. The width settings of both channels must be the same as the settings to be used in counting the doubly labelled samples).

The programme then asks for the number of factors required for correcting the final $^3$H/$^{14}$C ratio and, if appropriate, asks for these factors, one at a time.

Counting details of the first sample can now be entered: the programme requires the apparent $^3$H c.p.m., apparent $^{14}$C c.p.m., and the external standard or X ratio. The return button is pressed after each entry.
After dealing with these print-out data, the programme provides an opportunity to change factors (or stop) if desired.

A sample run is illustrated in Figure 1 following the listing of the whole programme.
Appendix

Figure 1  Computer programme for double labelling calculations and example of a run
DOUBL E LABELLING CALCULATIONS

WHAT PROPORTION OF THE 14C COUNT HAS SPILLED INTO 3H
7.3
NUMER OF FACTORS FOR CORRECTING THE FINAL 3H/14C RATIO
2
WHAT IS FACTOR 1
7.9
WHAT IS FACTOR 2
11.1
WHAT ARE THE APPARENT 3H,14C, AND X RATIO
7.18
7.18
5.5
THE TRUE 3H COUNT IS 69.4929
THE TRUE 14C COUNT IS 129.9896
THE 3H/14C RATIO IS 0.534638
3H/14C * C 1
IS .4811434
3H/14C * C 2
IS .5996642

DO YOU WANT TO STOP(0),GO ON(1),CHANGE FACTORS(2)
? 1
WHAT ARE THE APPARENT 3H,14C, AND X RATIO
?
5. Selection of window settings and amount of $^{14}\text{C}$ spillover

The energy distribution of $^{3}\text{H}$ and $^{14}\text{C}$ radiation can be easily determined experimentally by counting a sample of each type of radioactivity, using a variable window as follows. The lower energy limit or "discriminator setting" is set at zero. The upper limit is set close to this value and the c.p.m. recorded during a short count. The upper limit is increased gradually, c.p.m. being determined at arbitrary intervals until no increase in c.p.m. is observed when the upper limit is further extended. All c.p.m. values are expressed as a percentage of the maximum c.p.m. obtained, and graphed with respect to upper discriminator setting. Figure 2 illustrates the graph obtained using a Beckman LS-250 scintillation spectrometer and toluene/Triton X-100 scintillant (Department of Developmental Biology, August 1972).

From such a graph one can readily assess the effect of any combination of window settings on the percentage of total $^{3}\text{H}$ or $^{14}\text{C}$ c.p.m. that will be counted and on the proportion of $^{14}\text{C}$ spilled over into $^{3}\text{H}$. The lower limit of $^{14}\text{C}$ detection should if possible be set clear of $^{3}\text{H}$ counts (i.e. at discriminator setting 1.9 to 2.0) especially if $^{3}\text{H}$ counts are high relative to $^{14}\text{C}$. Similarly, $^{14}\text{C}$ spillover corrections can be reduced (although never eliminated) by counting a lower percentage of $^{3}\text{H}$ counts. Reduction of the spillover correction is desirable if $^{14}\text{C}$ counts are high relative to $^{3}\text{H}$. Generally, therefore, it is best to count only the lower energy region of $^{3}\text{H}$ and the higher energy region of $^{14}\text{C}$. However, when counts are low, as they often are in gel slices, a consideration of this sort of graph in relation to the relative amounts of $^{3}\text{H}$ and $^{14}\text{C}$ may help select window settings that provide a reasonable compromise between high counting efficiency and low proportion of spillover.
Appendix

Figure 2

The energy distribution of $^3$H and $^{14}$C radiation as measured in a Beckman LS-250 scintillation spectrometer.

The curves show the percentages of the total c.p.m. of $^3$H and of $^{14}$C that are detected at various settings of the upper energy limit of a variable window, the lower energy limit of which is set at zero.

Note that all $^3$H radioactivity falls within the range 0.00 - 1.90 (approx.); all $^{14}$C radioactivity falls within the range 0.00 - 4.30.

If $^{14}$C is counted with lower energy limit at 2.0, no $^3$H spillover into $^{14}$C will be detected.
6. Efficiency curve calibrations

The counting efficiencies of $^3$H and $^{14}$C with respect to quench (as indicated by the external standard ratio "X") vary according to the volume and type of scintillation cocktail used. Fluctuations in ambient temperature may also influence counting efficiency. It is desirable that $^3$H and $^{14}$C efficiency curves are prepared prior to use of this programme and checked against those curves used in its preparation. To facilitate this comparison these efficiency curves are reproduced in Figure 3 below.

The curves were prepared as follows. Two dilute aqueous solutions were prepared, one containing $^3$H and the other $^{14}$C. Six 1.0-ml aliquots were taken from each solution and put in separate scintillation vials. In each set of six vials the first two received only toluene/Triton scintillant (10 volumes) and were thus "unquenched". The contents of vials 2 to 6 were quenched to varying degrees by the addition of 10, 20, 30 and 40 μl of pyridine respectively, before being mixed with scintillant. The required subtraction of background radioactivity was determined from two blanks consisting of scintillant mixed with distilled water only. The windows of the $^3$H and $^{14}$C channels were set for appropriate spillover by considering (as previously described) the quality and quantity of radioactivity in the doubly labelled samples, and then the two series of singly labelled samples were counted in both $^3$H and $^{14}$C channels. For each quenched series, the total c.p.m. of each sample were expressed as a proportion of the mean unquenched value. These relative efficiency values were then plotted against external standard ratio. The curves were thus designed for correcting c.p.m. in quenched samples to the c.p.m. expected at the external standard ratio characteristic of unquenched aqueous samples in toluene/Triton scintillant (10 ml).

To change the efficiency curves at present in the programme, data points of new efficiency curves are fitted to new equations by means of regression programmes available in the R.S.B.S. computer library. The new equation for $^3$H
Appendix

Figure 3

The counting efficiencies of $^3$H and $^{14}$C radioactivity at various degrees of quenching as indicated by the external standard ratio "X". Samples were prepared as described in the text and mixed with 10 ml of toluene/Triton X-100 scintillant for counting.
efficiency is inserted at line 160 of the programme, while the corresponding equation for $^{14}\text{C}$ is inserted at line 180.

$(X = \text{external standard ratio}; \ Y = \text{relative counting efficiency of } ^3\text{H}; \ Z = \text{relative counting of } ^{14}\text{C})$.

7. Other modifications

For repeated use of the programme when factors are infrequently changed, the programme can be modified to bypass the provision for change of factors.

By appropriate alteration of equations this programme format could be used for double labelling involving $^{32}\text{P}$.

With minor alteration to the programme the reciprocal ratio can be determined (i.e. $^{14}\text{C}/^3\text{H}$).
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