# CYTOKININS IN LUPINUS ALBUS L.

*by*

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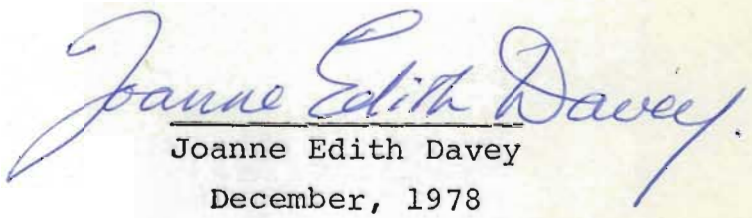
# CONTENTS

# PREFACE

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



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# ABSTRACT

A review of the literature pertaining to the sites of synthesis, distribution and metabolism of cytokinins, established that there was a need to investigate the quantitative and qualitative changes in cytokinin levels during the growth cycle of a whole plant. An annual legume, *Lupinus albus* L. (the white lupin), was selected as the experimental plant for such a study.

The levels of cytokinin activity in the root exudate, leaves and terminal (primary) apices of mature plants were examined between 5 and 17 weeks after planting. The results indicated that the total level of activity in the root exudate of a single plant is much greater than that which accumulates in the leaves and apices up to the time of flowering. This was considered to be indicative of rapid utilization of cytokinins in the actively growing shoot. It was also established that, after flowering, the level of activity in the terminal apices increases markedly, while that in the root exudate decreases.

The latter finding was examined more closely in the next experiment, during the course of which cytokinin levels in the root exudate, leaves, fruits and fruit sap were determined at 2 weekly intervals after flowering. As in the previous experiment, the results showed that, although there may be a slight increase in the amount of activity in the root exudate, this activity was

insufficiently high to account for the levels of cytokinin which accumulate in the fruiting apices. Cytokinin activity was however detected in the sap passing into the fruits (largely phloem sap), thereby indicating that at least some of the cytokinins detected in both pod wall and seed extracts could have originated in other parts of the plant.

Embryonic development was followed at the gross morphological, anatomical and ultrastructural levels, and estimates were made of the cytokinin levels in the embryos and adjacent tissues. The results indicated that the cytokinin levels in the flowers, and young fruits, were low, but gradually increased with time. At 2 weeks after anthesis the level of activity in the pod wall was higher than that in the individual seeds. By 6 weeks, however, the activity in each seed was in excess of that recorded in the pod wall. Decreasing levels of activity in the seeds, and increasing levels in the pod wall, reversed this trend at later developmental stages. The level of activity recorded in the maturing embryo was low, while in the adjacent tissues it was high. This was especially noticeable at 6 weeks after anthesis when the cytokinin activity in the endosperm reached a high level. It would appear that these cytokinins facilitate nutrient mobilization to the tissues surrounding the embryo, and that they are possibly utilized in order to promote certain growth processes taking place in the embryonic cells during development. The cytokinin activity in the mature embryo

was low, but increased slightly in the cotyledons, radicles and young shoots during germination and seedling establishment.

The cytokinins present in all white lupin extracts had chromatographic properties, on paper and/or Sephadex LH-20, similar to the naturally occurring cytokinins zeatin and zeatin riboside and their glucosylated or phosphorylated derivatives. Mass spectrometric-analysis of the compound co-eluting with zeatin showed that this cytokinin was present in white lupin extracts, while analysis of the peak co-eluting with zeatin riboside indicated that the cytokinin at this elution volume was an adenine derivative. The possible presence of dihydro-derivatives of these cytokinins was also indicated. The results did not exclude the possible presence of other cytokinin-like compounds. The significance of qualitative as well as quantitative changes in cytokinin activity were assessed.

It is concluded that cytokinins do not accumulate at excessively high levels in actively growing tissues, unless these tissues function as a transitory store for nutrients, as for example the endosperm, testa and pod wall. Cytokinins are probably involved in the control of a number of growth processes of which nutrient mobilization may be of considerable importance to the plant.

# LITERATURE REVIEW

## SOME ASPECTS OF THE INVOLVEMENT OF CYTOKININS IN PLANT GROWTH

### Introduction

Plant growth is a dynamic process involving an orderly series of successive changes, both in the plant as a whole and in the individual parts such as leaves, internodes and flowers (WAREING, 1971). These orderly changes taking place within the plant are controlled by factors both inside the plant body, and in the environment in which the plant is growing. Within the plant, hormones, or growth regulators, are factors which play a significant role in controlling certain aspects of growth. These compounds are active at the subcellular level and, although their mode of action is not clearly understood, they appear to exert their effects on plant metabolism as mediators, promoters or inhibitors of growth at a level close to, though not necessarily at, the genetic level. Falling within this category of growth regulators are a group of substances which have as one of their functions, the promotion of cell division.

The existence of substances which regulate cell division in plant tissues was recognised by early workers (HABERLANDT, 1913). Tissue culture experiments indicated the presence of these compounds in plant extracts (VAN OVERBEEK, CONKLIN and BLAKESLEE, 1941; CAPLIN and STEWARD, 1948; STEWARD and CAPLIN, 1952; JABLONSKI and SKOOG, 1954), and led eventually

to the discovery of 6-furfurylaminopurine (kinetin), an artifact resulting from the breakdown, during autoclaving, of herring sperm deoxyribonucleic acid (DNA). Kinetin was the first compound isolated, identified and synthesized which could induce cell division in callus tissue (MILLER, SKOOG, VON SALTZA and STRONG, 1955; MILLER, SKOOG, OKUMURA, VON SALTZA and STRONG, 1955; 1956). This compound does not occur naturally (SKOOG and ARMSTRONG, 1970), and it was only in 1963 that LETHAM isolated the first naturally occurring cell division promoter, 6-(4-hydroxy-3-methyl-*trans*-2-butenyl-amino) purine (zeatin), from maize seeds (LETHAM, 1963). Since these early discoveries many other compounds which elicit similar biological responses have been identified. These synthetic and naturally occurring cell division promoters were originally termed 'kinins', but later the term cytokinin was adopted (SKOOG, STRONG and MILLER, 1965).

Definitions of synthetic and naturally occurring cytokinins have favoured the comparison of these compounds with kinetin. This is especially evident in early definitions (MILLER, 1961; LETHAM, 1967a) and persists in some put forward more recently (SKOOG and ARMSTRONG, 1970; BURROWS, 1975). However, HALL (1973) designates the term cytokinin to a compound that promotes growth and differentiation in cultured callus cells. Although this definition accommodates both endogenous and synthetic cytokinins, it also allows for the possible inclusion of other plant growth regulators. SKOOG and ARMSTRONG (1970) point out that the structural requirements for high order cytokinin activity

include an adenine moiety with the purine ring intact and with an $N^6$-substituent of moderate molecular length. Although kinetin is an $N^6$-substituted adenine and provides a reference for comparison with other compounds which are not of plant origin, the response of plant tissues to kinetin cannot always be extrapolated to the endogenous cytokinins. It may be desirable, therefore, to define the endogenous cytokinins separately from the synthetic cytokinins. For the purposes of this project the endogenous cytokinins are defined as $N^6$-substituted adenines extracted from plants which promote growth in cultured callus cells, and which are responsible for particular biological responses within the plant.

The need to investigate the endogenous plant growth regulators has grown, and has led to the isolation and identification of numerous cytokinins from plant tissues. The cytokinins are, today, considered to be ubiquitous plant growth substances (KENDE, 1971). They have been studied in a variety of systems. Possibly the most thorough studies are those of HENSON and WAREING (1974; 1976; 1977a; 1977b) and HENSON and WHEELER (1976; 1977a; 1977b; 1977c), which make a valuable contribution to our understanding of their role in plant growth. In this latter research, and in studies where cytokinin levels have been related to a particular growth response such as germination (DIMALLA and VAN STADEN, 1977a) and tuberization (DIMALLA and VAN STADEN, 1977b; DIMALLA, VAN STADEN and SMITH, 1977; VAN STADEN and DIMALLA, 1977a; 1977b), support is given to the concept of the bound or storage form of cytokinin. This concept was

introduced by such workers as KENDE (1965), GAZIT and BLUMENFELD (1970), YOSHIDA and ORITANI (1971, 1972) and BANKO and BOE (1975). The glucosides, and riboside glucosides of zeatin, their dihydro-derivatives and the nucleotide cytokinins are considered to be storage cytokinins. While zeatin, 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosyl purine (zeatin riboside) and the dihydro-derivatives of these compounds tend to be regarded as active free base forms.

The studies described above were made possible by research which involved the application of naturally occurring and synthetic cytokinins to plant tissues. Such experiments facilitated the recognition of the properties common to the cytokinins, namely, the promotion of cell division (MILLER, 1961) and the retardation of leaf senescence (RICHMOND and LANG, 1957). Furthermore, they led not only to the establishment of a number of bioassay systems for the detection of cytokinins in plant extracts (MILLER, 1961; OSBORNE and McCALLA, 1961; MILLER, 1963; LINSMAIER and SKOOG, 1965; LE NOEL and JOUANNEAU, 1966; THIMANN and SACHS, 1966; LETHAM, 1967b; MILLER, 1969; SZWEYKOWSKA, 1969), but also to the assessment of the relative activities of different cytokinins within particular bioassay systems (LETHAM, 1967b; LEONARD, HECHT, SKOOG and SCHMITZ, 1969; VAN STADEN and PAPAPHILIPPOU, 1977). The use of bioassay systems for cytokinin research has permitted the investigation of the endogenous cytokinins to proceed, despite the low levels of these compounds present in plant tissues. When they are used in conjunction with gas-liquid chromatography

and mass spectrometry, they become a very sound research tool.

Although our knowledge of the endogenous cytokinins has increased considerably over the past decade, the cytokinins have not been studied in the whole plant throughout its entire growth cycle. This project was undertaken with two objectives. The first was to summarise both past and present knowledge of the involvement of cytokinins in plant growth in the form of a literature review. The second objective was to add, by experimentation, to our knowledge of the changes which take place in endogenous cytokinin levels during whole plant growth and development.

Issues of particular significance to such a study include the mechanisms and sites of cytokinin biosynthesis within the plant, the distribution of cytokinins throughout the plant body and the manner in which these compounds are metabolized during plant growth and development. Past and present research into the endogenous cytokinins will be reviewed in relation to these issues.

## Mechanisms and Sites of Cytokinin Biosynthesis

The term 'metabolism' conventionally refers to the chemical changes constructive and destructive, occurring in living organisms, while the term 'biosynthesis' is normally applied only to the building of compounds in plant tissues. Plant growth substances are thought to be produced at particular sites within the plant and utilized at others. The cytokinins are no exception, and the process of

cytokinin production has gradually acquired the term 'biosynthesis' while the process of utilization of cytokinins has become known as metabolism. The terms biosynthesis and metabolism will be interpreted in this manner throughout this thesis.

*Cytokinin biosynthesis*

The pathways by which cytokinins are synthesized in plant tissues are not well understood. The current lack of knowledge in this field is possibly due to the exceedingly low levels of these compounds actually present in plant tissues, and the lack of a suitable system in which to study this process. However, a number of ideas have been put forward to explain the origin of cytokinins in the plant. From these ideas it would appear that there are two possible methods by which cytokinins may be synthesized, namely:

(i) they are produced by breakdown of the tRNA, or

(ii) they are synthesized *de novo*.

As the present study involves a survey of the cytokinins in a particular plant during growth and development this aspect will not be discussed further. However, the proposed sites of cytokinin biosynthesis will be more carefully reviewed, as a knowledge of the possible sites of synthesis of these compounds is essential to an understanding of how the levels change during growth and development.

*Sites of cytokinin biosynthesis*

The characteristic property of cytokinins, namely, the

ability of these compounds to promote cell division, directed research workers to look to zones of meristematic activity as possible sites of cytokinin synthesis. The root tip, the developing seed, the vascular cambium and buds have been put forward as likely sites of synthesis.

The root was initially regarded as a possible site of cytokinin synthesis because substances originating in the roots, and translocated to the shoot, were shown to retard senescence in leaves in a manner similar to low concentrations of kinetin (KULAEVA, 1962). This discovery provided the link between cytokinins and the ideas put forward by earlier workers. WENT (1928) had postulated that substances, which were referred to as 'food factors', passed from the roots to the shoot where they were thought to maintain shoot growth. Later the 'food factor' was suggested to be a plant growth substance, and named caulocaline (WENT, 1943). Similar hypotheses were advanced by CHIBNALL (1939; 1954) who suggested that an hormonal factor produced by the roots might maintain protein synthesis in the shoot; and JACKSON (1956), who indicated that the root system had the ability to supply factors, other than water and mineral salts, required for optimal shoot growth. Soon after the isolation and identification of zeatin (LETHAM, 1963), it was demonstrated that the senescence delaying factors in root exudate had similar chromatographic properties to the newly identified cytokinin (KENDE, 1964). Further investigations established the presence of cytokinin-like compounds in the root exudate of a number of different plants (KENDE, 1965; CARR and BURROWS, 1966), and acceptance gradually grew for

the idea that cytokinins present in xylem sap were produced in the roots (KENDE and SITTON, 1967).

Indirect evidence which suggests that the root is the site of cytokinin synthesis was provided by BURROWS and CARR (1969) who showed that cytokinin activity in the root exudate decreased as the metabolism of the root tips was impaired by flooding, and by ENGELBRECHT (1972) who demonstrated that increased cytokinin levels in detached leaves were associated with the development of roots on the petioles of these leaves. Furthermore, adventitious roots have been shown to be able to maintain hypocotyl growth in a manner similar to cytokinins (WHEELER, 1971), and roots appeared to release cytokinins into the medium in which they were growing (WHEELER, 1971; VAN STADEN, 1976a).

More convincing evidence that the root synthesizes cytokinins, was provided by HENSON and WAREING (1976). They showed that cytokinin levels in the root exudate were in excess of the amount of cytokinin actually recorded in the roots. However, studies of the cytokinin content of excised roots of *Zea mays* L. (maize) and *Lycopersicon esculentum* MILL. (tomato) and the agar on which they were grown (VAN STADEN and SMITH, 1978), have yielded the most conclusive evidence for considering the root as a site of cytokinin synthesis. In sterile culture these roots not only accumulated cytokinins, but also either released excess cytokinin into the agar medium on which they were growing, or metabolized it to the glucoside form.

Within the root, the meristematic root tip has been

especially favoured as the possible site of synthesis. GOLDACRE's (1959) observations on the preferential formation of lateral roots adjacent to preformed root initials in isolated flax roots, led him to propose that root meristems synthesized cytokinins, and that cytokinin production may be a normal accompaniment of cell division. Cytokinins have been detected in root tip tissue of *Helianthus annuus* L. (sunflower) (WEISS and VAADIA, 1965), and studies of root tips of seedlings of *Pisum sativum* L. (pea) indicated that the majority of cytokinins in the root were located in the first millimetre of the root tip (SHORT and TORREY, 1972). Measurements made of cytokinin levels in the apical meristematic tissues of maize roots by FELDMAN (1975) endorsed the findings of SHORT and TORREY (1972), and indicated that cytokinins were produced in the root initials. Recently, it has been demonstrated that the removal of the root tip can result in an increase in the cytokinin levels of the root exudate (CARLSON and LARSON, 1977), thereby suggesting that the root tip is not the primary site of cytokinin synthesis. However, root initials have been shown to form within twenty four hours of tip removal in some species. In an experiment involving the removal of the root tip, some consideration should therefore have been given to this phenomenon. It may have influenced the cytokinin levels in the root exudate.

HEWETT and WAREING (1973a) showed that in *Populus* x *robusta* SCHNEID. (poplar), cytokinin levels in buds of excised twigs increased prior to, or at the same time as, bud burst. This finding led to the suggestion that the

roots are not an immediate, or necessary, source of cytokinins to the developing bud. The vascular cambium has therefore also been put forward as a possible site of cytokinin synthesis. However, the evidence for such a suggestion is indirect. SKENE (1972) demonstrated that high concentrations of cytokinin accumulated in the xylem sap of defoliated one-year-old canes of *Vitis vinifera* L. (grape) which were collected during the winter, and stored for six months at 1°C. This accumulation of cytokinins was independent of a root system, and SKENE (1972) therefore suggested that the cambium might provide significant quantities of cytokinin, especially during periods when the contributions from the roots are low. Increased cytokinin levels have also been noted in woody cuttings of *Malus* spp. (apple) during periods of overwintering (LUCKWILL and WHYTE, 1968). Pomological evidence favours the scion variety, and not the root stock variety, as the controller of bud burst, which is stimulated by increased cytokinin levels (LUCKWILL and WHYTE, 1968). Thus the cytokinins in the cuttings could have been synthesized in some part of the shoot, possibly in the cambium or adjacent tissues. However, it is also possible that cytokinins present in the spring sap could have been translocated from the leaves in autumn for storage in the stem tissues during the winter months, and then released in an active form in spring.

KANNANGARA and BOOTH (1974) put forward the idea that although cytokinins are present in the xylem exudate, and could be synthesized in the root, the presence of high concentrations of cytokinins in the apical buds of *Dahlia*

*variabilis* (WILLD) DESF. supported the view that the apical bud acts, not as a sink, where one would expect translocated substances to be utilized, but rather as a site of cytokinin synthesis. If it is possible for the apex to act as a site of cytokinin synthesis, then it is also possible that lateral buds may be responsible for cytokinin production. Although it is not clear whether or not lateral buds act as sites of cytokinin synthesis (VAN STADEN and BROWN, 1977), high cytokinin concentrations have been located in the buds of *Salix babylonica* L. (willow) (VAN STADEN and BROWN, 1977) and *Xanthium strumarium* L. (cocklebur) (HENSON and WAREING, 1976). HENSON and WAREING (1977a) demonstrated that cytokinin levels in buds decreased by as much as 80 percent during a 24 hour period after the buds had been detached from the parent plant. It was suggested that this observation was consistent with the view that the main source of cytokinins for these organs was the root.

Another possible site of cytokinin synthesis located in the shoot system is the seed[1]. Although cytokinins have been extracted from a wide variety of fruits and seeds, and are present at high concentrations in immature seed and fruit tissues (LETHAM and BOLLARD, 1961; BURROWS and CARR, 1970) it is often difficult to establish whether this reflects the ability of these tissues to produce, or to

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[1]The term 'fruit' is used to refer to the fertilised and developed ovary of a plant, while the term 'seed' refers to the fertilised ovule. However, the one seeded fruits of *Zea mays*, *Protea compacta*, *Carya illinoensis* and *Leucadendron daphnoides* are commonly also referred to as seeds. This convention will be used throughout this project.

accumulate, cytokinins. LETHAM and WILLIAMS (1969) suggested that in apples the seed is probably a site of active cytokinin biosynthesis, and that cytokinins could possibly be translocated from the seed to the receptacle. BLUMENFELD and GAZIT (1971) also concluded that the seed, and more specifically the embryo, was a site of cytokinin synthesis. They were able to demonstrate that the embryo of *Persea americana* MILL. (avocado) not only contained cytokinin, but that callus derived from the avocado cotyledons was able to synthesize cytokinins and did not require an exogenous cytokinin source to maintain callus growth. HAHN, DE ZACKS and KENDE (1974) showed that pea seeds, grown in their pods in sterile culture, and thus independent of a root system, could increase their cytokinin content. However, the cytokinins accumulated in the seeds might have originated in the pod tissues. Recent publications (KRECHTING, VARGA and BRUINSMA, 1978; VAN STADEN and BUTTON, 1978) have indicated that cytokinin biosynthesis did not take place in pea seeds cultured *in vitro*.

Caution should be exercised when comparisons are made, with respect to cytokinin synthesis, between events taking place in detached organs and the situation in the intact plant. MIURA and MILLER (1969) suggested that zeatin is synthesized in a cytokinin-independent strain of soybean callus as a result of a zeatin synthesizing mechanism being 'switched on'. Such a suggestion implies that all cells have the ability to synthesize cytokinins at certain times during their growth cycle. Indeed, as WAREING, HORGAN,

HENSON and DAVIS (1976) point out, if cytokinin biosynthesis takes place merely as a result of tRNA breakdown, all organs of the plant could synthesize cytokinin.

## The Distribution of Cytokinins in Plant Tissue

The foregoing discussion has illustrated that cytokinins are generally believed to be synthesized in one part of the plant. However, it also shows that these compounds have been isolated from a variety of different plant tissues, not all of which have been shown, with any certainty, to synthesize cytokinins. Indeed, the roots alone have been shown conclusively to be sites of synthesis. The presence of cytokinins within a plant organ at any one time thus depends on the transport of these compounds from organ to organ.

### *Cytokinin translocation*

In the plant, cytokinins may be transported in the non-living xylem or in the living phloem tissues. Cytokinins, present in the translocation stream which have been extensively studied, are those that occur in the xylem. The quantitative and qualitative estimates of the cytokinins in the root exudate[1] are of considerable significance as they have gradually become accepted as being indicative of the contribution made by the roots to the cytokinin complement of the shoot.

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[1]Throughout this project the term 'root exudate' will be used to refer to the sap exuding from the surface of the stem and the term 'xylem sap' will be used to refer to sap removed under vacuum from the xylem tissues of plants, respectively.

The presence of cytokinin-like compounds in the xylem sap of *Nicotiana rustica* L. (tobacco) was first reported by KULAEVA (1962) and further substantiated by KENDE (1964; 1965). Subsequently cytokinins were shown to be present in the root exudates of a wide variety of herbaceous plants (ITAI and VAADIA, 1965; CARR and BURROWS, 1966; KLAMBT, 1968; VAADIA and ITAI, 1969; YOSHIDA, ORITANI and NISHI, 1971; BEEVER and WOOLHOUSE, 1973; 1974; BANKO and BOE, 1975). The xylem saps of a number of woody plants have also been shown to contain compounds which exhibit cytokinin activity (LOEFFLER and VAN OVERBEEK, 1964; NITSCH and NITSCH, 1965; LUCKWILL and WHYTE, 1968; REID and BURROWS, 1968; HEWETT and WAREING, 1973b; ALVIM, HEWETT and SAUNDERS, 1976; VAN STADEN and DAVEY, 1976). In the majority of instances cytokinins with chromatographic properties similar to zeatin and zeatin riboside have been reported to occur in xylem sap and root exudate after separation of extracts by means of either paper and/or column chromatography. Gas-liquid chromatography and mass spectrometry have been used in only a few instances to identify these cytokinins more conclusively. HORGAN, HEWETT, PURSE, HORGAN and WAREING (1973) identified *trans*-zeatin riboside from a partially purified extract of the bleeding sap of *Acer pseudoplatanus* L. (sycamore) by combined gas-chromatography and mass-spectrometry, and PURSE, HORGAN, HORGAN and WAREING (1976) working with the spring sap of the same species and using similar techniques identified zeatin riboside, *trans*-zeatin and dihydro-zeatin. Mass spectrometry has also been used to identify zeatin

riboside in tomato root exudate (VAN STADEN and MENARY, 1976). Although zeatin and dihydro-zeatin have been shown to occur in xylem sap, zeatin riboside has been considered to be the major translocational form of cytokinin in the xylem sap (GORDON, LETHAM and PARKER, 1974; HEWETT and WAREING, 1974; LETHAM, 1974). Indeed, in time course studies of the composition of xylem sap and root exudate, cytokinins co-eluting with zeatin riboside appeared to be the predominant forms present (DAVEY and VAN STADEN, 1976; HENSON and WAREING, 1976) at almost all developmental stages.

Other forms of cytokinin have also been reported to occur in xylem sap and root exudate of woody and herbaceous plants. These cytokinins have often been referred to as 'bound' forms of the hormone. KENDE (1965) was the first to draw attention to a compound which can be hydrolyzed to yield an active cytokinin. He was able to show that there were two compounds in xylem sap with different chromatographic properties, which were able to delay chlorophyll breakdown in *Hordeum vulgare* L. (barley) leaf sections. He reported that the slower-moving compound on paper chromatograms ($R_f$ 0,0-0,3 using an *n*-butanol:acetic acid:water (4:1:1 v/v) solvent system), although it delayed senescence, was not active in inducing cell division in callus. Hydrolysis of this compound in 3 millilitres of N HCl at 90°C for one hour and subsequent chromatography resulted in the detection of a cytokinin-like compound at $R_f$ 0,5-0,8 on paper chromatograms run in a similar solvent system.

Further reports of the presence of a bound form of

cytokinin in root exudate have been made by other workers (CARR and BURROWS, 1966; MENARY and VAN STADEN, 1976) and recent research has revealed the presence of two kinds of 'bound' cytokinin in extracts of plant tissues. These may be either nucleotides or glucosides of zeatin and zeatin riboside. The nature of the bound forms of cytokinin in root exudates has not been conclusively established, although it would appear as if it is often a zeatin nucleotide. The possible presence of a nucleotide cytokinin was recorded in the root exudate of *Impatiens glandulifera* ROYLE., *Lupinus angustifolius* L., *Pisum arvense* L. (CARR and BURROWS, 1966), the cocklebur (CARR and BURROWS, 1966; VAN STADEN and WAREING, 1972) and the tomato (DAVEY and VAN STADEN, 1976). MENARY and VAN STADEN (1976) demonstrated that alkaline phosphatase treatment of this bound form of cytokinin in tomato root exudate resulted in an increase in cytokinin activity at regions co-eluting with zeatin and zeatin riboside, after column chromatography of the treated exudates on Sephadex LH-20. Furthermore, CARNES, BRENNER and ANDERSEN (1975) reported that, after high pressure liquid chromatography, eight different cytokinins could be detected in tomato root exudate, indicating that, although zeatin riboside may be the major translocational form of cytokinin, other cytokinins may also be translocated in the xylem.

The amount of cytokinin translocated in the xylem sap and root exudate has been shown to be influenced by a number of environmental factors. Flooding the root system of sunflower plants reduced the level of cytokinin in the root exudate (ITAI and VAADIA, 1965; BURROWS and CARR, 1969),

while stress produced by reducing the availability of water also reduced the cytokinin output of the roots of tobacco plants (ITAI and VAADIA, 1971). Both low (BANKO and BOE, 1975) and high temperatures (ITAI, BEN-ZIONI and ORDIN, 1973) have been shown to reduce the cytokinin activity of root exudates. Low pH of the growing medium, poor nutrition (BANKO and BOE, 1975), high soil salinity (ITAI, RICHMOND and VAADIA, 1968) and the application of growth regulators such as ethephon and chlormequat to the root systems (BANKO and BOE, 1975) also adversely affect the amount of cytokinin in the translocation stream.

Changing cytokinin levels in the root exudate may be seasonal or may depend on the stage of development reached by the plant. REID and BURROWS (1968) observed that cytokinins and gibberellins were present in spring sap of sycamore and *Betula pubescens* EHRH. BELTR. and suggested that these growth regulators may be involved in the control or stimulation of bud burst. Later LUCKWILL and WHYTE (1968) reported that cytokinins were at their maximum levels in the spring sap of apple trees in full bloom and again it was postulated that these substances stimulated bud break. It was also suggested that cytokinins could be involved in the control of extension growth of both roots and shoots. HEWETT and WAREING (1973a; 1973b) reported that high cytokinin levels in the xylem sap coincided with bud burst in poplar. The abscisic acid (ABA) and cytokinin activity in the xylem sap of *Salix viminalis* L. was estimated throughout two growth cycles. It was established that the onset of dormancy was associated with low levels of

cytokinins and a high ABA content, while growth in spring was preceded by decreasing ABA levels and an increase in cytokinin levels. It was suggested that bud burst was facilitated by decreasing ABA and increasing cytokinin levels (ALVIM *et al.*, 1976). Thus in the deciduous woody perennial plant cytokinin levels appeared to rise at a time when there was a flush of new growth. The situation in an herbaceous annual appeared to be slightly different and decreasing levels of cytokinins in the root exudate have been interpreted as contributing to the senescence of the shoot (KENDE and SITTON, 1967; SITTON, ITAI and KENDE, 1967). Root produced cytokinins, in this instance, were considered essential for the control of protein metabolism and related processes in the leaves but not directly for the regulation of growth. SITTON *et al.* (1967) observed that as flower buds developed on the sunflower there was a reduction in the amount of cytokinin in the root exudate which apparently contributed ultimately to whole plant senescence. Subsequent studies have shown, however, that floral induction (short day treatments) in such plants as cocklebur also resulted in a reduction in the cytokinin levels present in the root exudate (VAN STADEN and WAREING, 1972a; HENSON and WAREING, 1974), and it was postulated that low cytokinin levels were a necessary prerequisite for some part of the sequence of events leading to flowering. In the tomato, flower bud development was also associated with decreasing cytokinin levels. However, after flowering there was an increase in the level of cytokinin present in the root exudate, although this level did not appear to be as high

as that in the root exudate of young herbaceous plants (DAVEY and VAN STADEN, 1976). There thus appears to be some evidence that cytokinin levels change when plants enter the reproductive phase of growth.

Recently cytokinins have been shown to occur in the phloem sap of both woody and herbaceous plants (HALL and BAKER, 1972; PHILLIPS and CLELAND, 1972; HOAD, 1973; VONK, 1974; VAN STADEN, 1976b). Although the precise identity of these cytokinins has not been established, current evidence suggests that a number of different cytokinins are present. PHILLIPS and CLELAND (1972) were the first to report the presence of cytokinins in aphid honey-dew. These cytokinins co-eluted with zeatin and zeatin riboside on paper chromatograms separated in *n*-butanol:acetic acid:water (12:3:5 v/v). Cytokinin activity was detected in both the fast- and slow-moving regions of paper chromatograms of extracts of phloem sap collected from *Ricinus communis* L. and it was suggested that there were both free base and bound forms of cytokinin present in this sap (HALL and BAKER, 1972). Later VONK (1974) reported the occurrence of cytokinins in the phloem sap collected from young inflorescence stalks of *Yucca flaccida* HAW. He found that only a small amount of cytokinin activity could be attributed to the free base and nucleoside cytokinins. The majority of the activity was due to the presence of cytokinin-like compounds which could be hydrolyzed by alkaline phosphatase. It was suggested that these cytokinins were nucleotides of zeatin. VAN STADEN (1976b) detected the presence of zeatin, zeatin

riboside and a cytokinin glucoside in aphid honey-dew collected from willow trees. Further work by VONK (1976) demonstrated the presence of $5^1$-mono-di- and tri-phosphates of zeatin in the phloem sap of *Y. flaccida*, as well as the presence of enzymes in the flower parts which could convert the nucleotide cytokinins to nucleoside cytokinins. Furthermore, he established that in the phloem tissues zeatin could be converted by enzymes to a mono-ribonucleotide.

Translocation in the phloem is bidirectional and the presence of cytokinins in phloem sap implies that these compounds can move not only from organ to organ within the aerial parts of the plant but also from the shoot to the root. Indeed VAN STADEN (1976b) suggested that, in woody plants, a redistribution or recycling of cytokinins occurred in autumn. Cytokinins (zeatin and zeatin riboside) transported through the xylem apparently accumulated in the leaves of deciduous trees during spring and early summer. They were metabolized during late summer and autumn and appeared to be exported, principally in the form of glucosides, via the phloem to storage organs (VAN STADEN, 1976b). These storage organs may be fruits and/or roots in deciduous trees and, if the same situation prevails in annuals, fruits in these latter plants. HENSON and WAREING (1976) reported that in cocklebur the cytokinins present in the root exudate did not merely represent the recirculating products of shoot metabolism. In woody deciduous plants the presence of cytokinin glucosides in the phloem in autumn may indicate the possibility that cytokinins are stored in this tissue.

This hypothesis would explain the rise in cytokinin levels in the xylem sap of stem cuttings stored at low temperatures. Lateral translocation from the phloem to the xylem could take place without necessitating the proposed synthesis of cytokinins by the cambium. It would merely require the release of active free base cytokinins from the tissue in which they were stored.

From the foregoing discussion it is evident that what initially appeared to be a simple root to shoot translocation of cytokinins has been complicated by the knowledge that cytokinins are also present in the phloem and can therefore be translocated bidirectionally. Indeed, WAREING *et al.* (1976) suggest that the assumption that the transport of cytokinins in the plant is confined to the movement of zeatin and zeatin riboside in the transpiration stream, is probably not valid. The cytokinins present in the translocation stream reflect the cytokinin status and cytokinin needs of the organs of the rest of the plant, and are often influenced by changing growth patterns. One must turn to look at what is known of the cytokinin status of different parts of the plant.

*Cytokinins in roots*

Cytokinins were first isolated from sunflower roots by WEISS and VAADIA (1965). Extracts of the root tip region exhibited cytokinin activity in two zones of paper chromatograms developed with an *n*-butanol:acetic acid: water (4:1:1 v/v) solvent system. Extracts of physiologically older tissue exhibited little or no cytokinin

activity. In an attempt to identify the root factor responsible for shoot growth, SETH and WAREING (1965) isolated two senescence delaying factors from the roots of *Phaseolus vulgaris* L. (bean). These were considered to be purine derivatives, one an adenine derivative, the other a derivative of guanine. BUI-DANG-HA and NITSCH (1970), in order to identify the compound responsible for bud initiation on roots of *Cichorium intybus* L. (chicory), extracted quantities of these roots and isolated zeatin riboside in crystalline form. Later, cytokinins considered to be zeatin, zeatin riboside and zeatin ribotide were found to occur in pea seedling roots (SHORT and TORREY, 1972). In other studies of the cytokinins in extracts of *Oryza sativa* L. roots zeatin, zeatin riboside and zeatin ribotide (YOSHIDA and ORITANI, 1971) and later zeatin glucoside were identified (YOSHIDA and ORITANI, 1972). In these studies gas chromatography and infra red spectrophotometry were used for cytokinin identification. HENSON and WHEELER (1976; 1977a) reported the presence of cytokinin activity in the roots and root nodules of a number of plant species. The cytokinins in the roots had similar chromatographic properties to zeatin, zeatin riboside and their dihydro-derivatives on paper chromatograms developed in an *iso*-propanol:ammonia:water (10:1:1 v/v) solvent system (HENSON and WHEELER, 1977b). Cytokinins co-eluting with zeatin, zeatin riboside and zeatin glucoside were detected in extracts of *Vicia faba* L. roots after elution through Sephadex LH-20 columns with 35% ethanol (HENSON and WHEELER, 1976). Similar cytokinins were shown to occur in maize

and tomato roots (SMITH, 1977; VAN STADEN and SMITH, 1978).

In 1971 RADIN and LOOMIS, working with extracts of developing roots of *Raphanus sativus* L. (radish), reported that a number of different cytokinins were present. These workers suggested that although some of these cytokinins originated in the roots, the remainder were transported from the shoot to the root tissues where they stimulated cambial activity. As the radish root is a swollen storage organ the situation with respect to cytokinins is atypical; nevertheless it is of interest that certain cytokinins were associated with the development of such a storage organ. VAN STADEN and SMITH (1978) reported that extracts of roots of tomato and maize, detached from the shoot and grown aseptically, accumulated a cytokinin glucoside, whereas roots of a similar age from which the shoot was not removed contained less cytokinin and did not appear to exhibit a build-up of cytokinin glucosides. Roots in such plants may only accumulate the cytokinin glucosides when excess cytokinin is being produced. They therefore have the ability to metabolize cytokinins, and the presence of bound forms of cytokinin in the roots should not necessarily imply that these compounds originate in the shoot. A further point of interest with respect to the importance of understanding the kinds of cytokinins in the roots and their influence on the remainder of the plant, is the detection of high cytokinin concentrations in root nodules. Although $^{14}$C-labelled zeatin may be metabolized within the root nodule tissue, or become distributed throughout the shoot

(HENSON and WHEELER, 1977c), nodules do not appear to contribute significantly to the cytokinin content of this region (WHEELER and HENSON, 1978).

*Cytokinins in shoots*

Although for some time, it has been suggested that the cytokinins necessary for the growth of the shoot are synthesized in the root and transported in the transpiration stream, two relatively recent observations have altered our concept of the relationship between root and shoot cytokinins. Firstly, certain of the above ground parts have been put forward as possible sites of cytokinin synthesis. (In this context the possibility of cytokinin production in the seeds and cambium has been mentioned). Secondly, the detection of cytokinins in phloem sap implies that the cytokinin distribution in the above ground parts of the plant may be controlled, and may not necessarily represent the end products of cytokinins originally translocated in the transpiration stream. As these considerations must influence an assessment of the involvement of cytokinins in shoot growth, they are emphasized prior to the discussion of current literature on the cytokinin content of leaves and seeds.

(i) Cytokinins in leaves

Until 1971 few efforts were made to characterize the cytokinins present in leaf tissues or to assess the significance of endogenous cytokinin levels in the growth of the leaf, or in the metabolism of the whole plant. Prior to this, however, the application of synthetic cytokinins to leaf tissue in order to understand how cytokinins

applied to detached leaves prevented chlorophyll degradation and thus senescence in the dark (RICHMOND and LANG, 1957), yielded three interesting observations. They were firstly, that cytokinin applications to detached leaf tissue affected the protein synthesizing mechanism (RICHMOND and LANG, 1957; OSBORNE, 1962; TAVARES and KENDE, 1970); secondly, that cytokinin application could result in the mobilization of nutrients from untreated to treated leaves or parts of leaves (MOTHES, ENGELBRECHT and KULAJEWA, 1959; MOTHES and ENGELBRECHT, 1961; MOTHES, ENGELBRECHT and SCHUTTE, 1961); and thirdly, that leaves of different ages responded in different ways to applied cytokinins (RICHMOND and LANG, 1957; OSBORNE, 1962). These observations are the very broadly interpreted results of applied hormone studies. As this present study involves endogenous hormones, they will not be pursued further. However, the significance of these observations lies in the contribution they have made to our attempts to understand the importance of quantitative and qualitative fluctuations in the cytokinins both in leaf tissues and in other parts of the plant.

Interest in endogenous leaf cytokinins was initially stimulated by research on the phenomenon of 'green islands' caused by fungal infestations in mature or ageing leaves. The 'green islands' were shown to be regions of stimulated nitrogen metabolism with increased RNA and protein synthesis, of enhanced chlorophyll retention and synthesis, and of active nutrient accumulation (ENGELBRECHT, 1971b). As mentioned previously, these same effects resulted from the application of cytokinins to leaf tissues. Subsequently,

these fungi were shown to produce compounds active as cytokinins (ENGELBRECHT, 1971a) and the 'green island' effect was linked with cytokinin-like compounds. It was also demonstrated that 'green islands' caused by insect larval infection contained considerable amounts of these growth substances, and that certain insect larvae were able to synthesize cytokinins (ENGELBRECHT, ORBAN and HEESE, 1969). These results led ENGELBRECHT (1971a) to investigate cytokinin levels throughout the growing season in leaves infected with insect larvae. During the course of this investigation it became evident that increased cytokinin levels were not only associated with the infected leaf tissues, but that both quantitative and qualitative changes also took place in the cytokinin levels in leaves during the growth cycle.

Following up this research, ENGELBRECHT (1971b) published the first quantitative and qualitative study of leaf cytokinins in *Populus tremula* L., *Acer platanoides* L. and *Betula verrucosa* EHRH. This study established that during periods of active growth there were low levels of cytokinins present in the leaf tissues but that as the leaves matured the cytokinin levels in these organs increased. Two distinct peaks of cytokinin activity were present on paper chromatograms separated in *n*-butanol:25% ammonium hydroxide (4:1) and assayed using the tobacco callus bioassay. These were a slow-moving fraction ($R_f$ 0,1-0,25) suggested to be zeatin mono-nucleotide, and a fast-moving fraction ($R_f$ 0,5-0,7) which co-chromatographed with zeatin and zeatin riboside. It was demonstrated that the level of the

slow-moving fraction increased with increasing age of the leaves.

Subsequent research has shown that the quantitative and qualitative fluctuations outlined by ENGELBRECHT (1971b) for these two species occur in slightly modified forms in other woody deciduous angiosperms (ENGELBRECHT, 1971b; HEWETT and WAREING, 1973b) and the deciduous gymnosperm *Ginkgo biloba* (VAN STADEN, 1976d).

HEWETT and WAREING (1973b) identified at least seven cytokinins in extracts of mature poplar leaves after chromatography on Sephadex LH-20. Two of these had similar elution volumes to zeatin and zeatin riboside. A third appeared to be a cytokinin glucoside while a fourth was described as a new cytokinin susceptible to mild oxidation and yielding two cytokinin-active products after acid hydrolysis. It was also demonstrated (HEWETT and WAREING, 1973b) that these cytokinins underwent qualitative and quantitative changes during development in a manner similar to those described for *Populus tremula* (ENGELBRECHT, 1971b). It was suggested that once apical sink activity ceases, cytokinins in the xylem sap were diverted into leaves and converted to a cytokinin glucoside, possibly as a storage form of the hormone. LORENZI, HORGAN and WAREING (1975) investigated the cytokinin content of the needles of *Picea sitchensis* CARRIERE and were able to show that, during active growth after bud burst in spring, zeatin riboside was the major cytokinin present, while during the winter a cytokinin with a similar elution volume to zeatin-9-glucoside was present.

The latter cytokinin did not, however, appear to be either a 9- or 7-glucoside of zeatin upon mass spectrometry. Using a deciduous gymnosperm, *Ginkgo biloba* L., VAN STADEN (1976d) demonstrated that in extracts of young expanding leaves the major cytokinins co-eluted with zeatin and zeatin riboside after fractionation on a Sephadex LH-20 column. In mature and ageing leaves most of the cytokinin activity was due to compounds which could be hydrolyzed by β-glucosidase, to yield zeatin and zeatin riboside. These latter cytokinins were taken to represent O-glucosides of zeatin and its riboside.

A trend similar to that described for *Ginkgo biloba* was also demonstrated for the willow (VAN STADEN, 1977) and *Erythrina latissima* E. MEY. (VAN STADEN and DAVEY, 1978). Similar cytokinins were identified in extracts of these leaves which had been separated on Sephadex LH-20 columns. Cytokinins co-chromatographing with zeatin and its derivatives on Sephadex LH-20 were detected in extracts of the leaves of three other gymnospermous species, *Podocarpus henkelii* STAPF., *Welwitschia mirabilis* HOOK. and *Encephalartos natalensis* DYER and VERDOORN (VAN STADEN, 1978). A cytokinin co-chromatographing with *iso*-pentenyl adenine was detected in the leaves of *W. mirabilis* and *E. natalensis* (VAN STADEN, 1978).

It would appear, from our present knowledge, that the cytokinin originally described by ENGELBRECHT (1971b) as zeatin-mono-nucleotide was possibly a cytokinin glucoside. It is interesting, however, that the glucoside cytokinins

present in ageing leaves apparently do not have the same effect on leaves as applied zeatin. Although these cytokinins were present in yellow senescing leaves in relatively high concentrations, they did not appear to retard senescence, or to participate in cell division (it no longer took place in these leaves), or participate in nutrient mobilization (the leaves at that stage exported rather than imported nutrients). ENGELBRECHT (1971b) suggested that the slow-moving cytokinins on paper chromatograms were storage forms of cytokinin which the leaves were unable to utilise, as the enzymes necessary for their activation were not present. However, the attachment of a glucose moiety to zeatin and to zeatin riboside has also been regarded as a means of inactivating these compounds and thus enabling them to be transported through the phloem (VAN STADEN, 1976b). Indeed, the presence of high levels of zeatin and zeatin riboside in actively growing leaf tissue of *Picea sitchensis* CARRIERE alternating with high levels of a bound/glucoside form during dormancy, strongly suggested that zeatin riboside was the active cytokinin in this tissue and that the glucoside was an inactivation or storage product (LORENZI *et al.*, 1975). Experiments initially conducted by ENGELBRECHT (1972) and later repeated by WAREING *et al.* (1976) on bean leaf cuttings yielded results which also support the view that the glucoside cytokinins are storage forms. ENGELBRECHT (1972) originally observed that bean leaves allowed to root on the petiole accumulated large quantities of cytokinin in the laminae, and that the appearance of extractable cytokinins in the laminae coincided with the appearance of roots

on the petioles. Using a modified system WAREING *et al.* (1976) confirmed these observations and positively identified the cytokinin that accumulated in the leaves as dihydro-zeatin-O-β-D-glucoside. Extrapolating from their results to the situation in the normal plant, these workers suggested that the glucosylating capacity of the leaf may cause it to act as an effective sink for root produced cytokinins.

From the above discussion it is apparent that leaf cytokinins originate in the roots, and that compounds such as zeatin and zeatin riboside, which are present in the transpiration stream, may be metabolized in the leaves. Indeed, studies by LETHAM, PARKER, DUKE, SUMMONS and MACLEOD (1976) using $^{14}$C-zeatin and $^{14}$C-zeatin riboside indicated that the leaves were able to convert these compounds to glucosylated forms when they were supplied in the transpiration stream. HENSON (1978) suggested that the capacity for glycosylation of cytokinins increases during expansion and maturation of *Alnus glutinosa* (L.) GAERTN. leaves. He also indicated that there was evidence that a greater overall metabolism of cytokinins took place in younger leaves.

Further, more conclusive evidence for the origin of leaf cytokinins has resulted from studies using radioactive labels. HENSON and WHEELER (1977c) demonstrated that $^{14}$C-zeatin supplied to root nodules was translocated to the leaf tissues. It was initially present in the leaves as zeatin, but the gradual disappearance of zeatin and its riboside from the shoot was accompanied by an increase in glucoside cytokinins.

Up to this point, the influence of age or growing season and root produced cytokinins on the cytokinin status of the leaf has been considered. However, other factors have also been shown to influence the cytokinin status of leaf tissue. In 1972 VAN STADEN and WAREING (1972a) showed that floral induction of cocklebur plants (by means of a single short day or long dark period) caused an irreversible fall in cytokinin levels in both the leaves and root exudate. HENSON and WAREING (1974) were able to repeat this work and postulated that low cytokinin levels in the plant were a necessary prerequisite for some part of the sequence of events leading to flowering. In this way cytokinin fluxes were linked with a change from vegetative to reproductive development. Although further investigations in this area showed a dependence on the leaves and buds for root produced cytokinins, it was also apparent that cytokinins underwent a rapid turnover in both the leaves and buds of the cocklebur. The possible involvement of a shoot-to-root signal capable of moderating cytokinin production in the root system was indicated (HENSON and WAREING, 1976). A direct effect of day length on cytokinin metabolism therefore was not postulated and changes in cytokinin levels were considered to be the result of some interaction within the plant as a whole.

Despite the obvious effect of light on cytokinins as described above, the root-to-leaf theory for leaf cytokinin origin was not challenged. However, a further observation by WAREING *et al.* (1976) that removal of apical buds of the bean resulted in an accumulation of glucoside cytokinins in

the leaf tissues, indicated that other parts of the shoot also influenced the cytokinin status of the leaf. Indeed, as cytokinin translocation can take place in the phloem, the relationship may well be reciprocal at different developmental stages.

(ii) Cytokinins in fruits and seeds

A further substantial contribution to a greater understanding of the involvement of cytokinins in the developmental process is the outcome of research into cytokinins in fruits and seeds. This work has added considerably to our knowledge of the different kinds of cytokinins present in plant tissues. Cytokinin changes during seed development, and also during seed germination, are gradually receiving more attention in the literature.

Cell division promoters were first detected in seed extracts by physiologists working in the field of tissue culture. It was noticed that high levels of these compounds were present in immature fruits (LETHAM and BOLLARD, 1961), an observation which culminated in the isolation of zeatin from immature maize seeds (LETHAM, 1963). It soon became apparent that other cytokinins besides zeatin were present in plant extracts, and many of these cytokinins were subsequently identified in maize seeds (LETHAM, 1973) and coconut milk (LETHAM, 1974; VAN STADEN and DREWES, 1975; VAN STADEN, 1976c).

Cytokinins have been extracted from the seeds and fruits of a variety of different plants (GOLDACRE and BOTTOMLEY, 1959; STEWARD and SHANTZ, 1959; LETHAM and

BOLLARD, 1961; MILLER, 1961; LETHAM, 1963; ROGOZINSKA, HELGESON, SKOOG, LIPTON and STRONG, 1965; KOSHIMIZU, MATSUBARA, KUSAKI and MITSUI, 1967; BLUMENFELD and GAZIT, 1970; 1971; BURROWS and CARR, 1970; GAZIT and BLUMENFELD, 1970; GUPTA and MAHESHWARI, 1970; PRAKASH and MAHESHWARI, 1970; BROWN and VAN STADEN, 1973; SANDSTEDT, 1974; VARGA and BRUINSMA, 1974; SCHULMAN and LAVEE, 1976; VAN STADEN and BUTTON, 1978). Although seeds have often been put forward as possible sites for cytokinin synthesis, this has not been conclusively proven, and the origin of cytokinins present in seed tissues is debatable. Attempts to assess the relative importance of seed cytokinins in seed and fruit growth at different developmental stages has been neglected because the isolation and identification of individual cytokinins has only recently come to be considered a routine procedure. However, a number of studies have monitored gross changes in cytokinin levels both in the seeds and in the fruit tissues, and some more recent studies have attempted to relate these changes to fluctuations in cytokinin levels in the whole plant. LETHAM and WILLIAMS (1969) reported that the cytokinin levels in apple fruitlets changed during development. Different parts of the fruit were extracted and assayed for cytokinins and it was evident that extracts of developing seeds were much more active than extracts of the fruitlet flesh. Furthermore, extracts of receptacles and ovaries, derived from unfertilized flowers, yielded appreciable cytokinin activity. It thus appeared that although the ovule was a rich source of cytokinins, the cytokinins could accumulate in the

receptacle without the presence of fertilized ovules.

GUPTA and MAHESHWARI (1970) demonstrated the presence of cytokinins in seeds of *Cucurbita pepo* L. (pumpkin). Three chromatographically distinguishable cytokinins, which were retained on Dowex 50-W, were extracted by ethanol and *n*-butanol. Two active factors were precipitable by silver nitrate at acidic pH values. One of these cytokinins had chromatographic characteristics similar to those of zeatin. The other two cytokinins were not identified. An investigation of the cytokinin content of immature seeds of *Citrullus lanatus* (THUNB.) HANS. (watermelon) yielded similar results (PRAKASH and MAHESHWARI, 1970). In both studies the cytokinin activity in the seed tissues changed with development. In the pumpkin the cytokinin activity was at its lowest in very young and in mature seeds. In the watermelon, however, it was demonstrated that the cytokinin level in the seeds reached a maximum at about eleven days after pollination. In contrast the fresh and dry weights, as well as the size of the seed, increased most rapidly between four and nine days after pollination. From these results it was suggested that the growth of the seed does not result from increased cytokinin levels, but that the increase in cytokinin activity is an accompaniment of growth in the seed.

Morphological and anatomical changes take place within the seed during its growth, and there is considerable interaction between the developing seed and its surrounding tissues. Recent research has indicated that cytokinin

levels in different parts of the seed and in the external covering of the seeds change during development. BLUMENFELD and GAZIT (1970) studied the cytokinin levels in the seed coat, endosperm, and embryo of avocado seeds during development. Cytokinin levels in the endosperm were very high as long as this tissue was present in the seed. In the seed coat, cytokinin levels were initially high, but fell as maturity was attained, while in the embryo high levels were recorded at early stages of development, but showed a gradual decrease as maturation proceeded.

Bound cytokinins were detected in the mesocarp of the avocado fruit (BLUMENFELD and GAZIT, 1970) and it appeared that a reduction in fruit growth paralleled a reduction in the activity of bound cytokinins. Cytokinins have been extracted from the flesh of other fruits. PRAKASH and MAHESHWARI (1970) reported the presence of cytokinin-like substances in the pulp of watermelons. They were able to demonstrate that the levels of activity in this tissue were low when compared to those present in the immature seed. CHACKO, SAIDHA, SWAMY, REDDY and KOHLI (1977), working with *Vitis vinifera* L. x *V. labrusca* L. showed that the cytokinin-like substances present in the berries were at a maximum at anthesis and during the first period of rapid growth. The cytokinin levels however, declined during the lag period and a subsequent period of rapid growth. Cytokinin levels have also been examined in tomato fruits (VARGA and BRUINSMA, 1974; KOVÁCS and VÖRÖS, 1975) and in Manzanilo olives (SCHULMAN and LAVEE, 1976). VARGA and BRUINSMA (1974) detected the presence of cytokinin-like

compounds in tomato fruits, and later KOVÁCS and VÖRÖS (1975) demonstrated that the level of these compounds in the green fruit was significantly higher than that of the ripe fruits. In Manzanilo olives however, cytokinins were at appreciably higher levels in the green fruits than they were in the black fruits and a relationship was postulated between the amount of cytokinin present and the accumulation of anthocyanin pigments (SCHULMAN and LAVEE, 1976). While this may well be the situation in olives, in tomatoes a non-ripening mutant was found to have higher cytokinin content than the ripe fruit (DAVEY and VAN STADEN, 1978).

VARGA and BRUINSMA (1974) drew attention to the relationship between cytokinin levels in fruits, and the remainder of the plant. The observation of these workers that the cytokinin content of fruits in clusters of four was always lower than that of fruits in clusters of two, led to the suggestion that there could be competition between fruits for cytokinins derived from other parts of the plant. In addition, a large difference in cytokinin content between the seeded fruits of intact and partially defoliated plants was suggested to be indicative of strong competition between fruits and foliage for cytokinins derived from the roots. It has been demonstrated that the removal of fruit from rooted cuttings increased the concentration of a cytokinin glucoside in leaf tissue extracts and it was suggested that the developing fruits became major sites for cytokinins originally derived from the root, and that fruit removal resulted in an accumulation

of these compounds in the leaves (HOAD, LOVEYS and SKENE, 1977).

The significance of the cytokinins present in fruit pulp is unclear and the levels of cytokinin may well affect fruit ripening. On the basis of the fact that parthenocarpic tomatoes have less cytokinins than seeded fruits, it has been suggested that the cytokinins in the pulp originate in the seeds (VARGA and BRUINSMA, 1974). A similar origin was envisaged for auxins, gibberellins and ABA in seed pods (EEUWENS and SCHAWBE, 1975). Indeed, the high levels of various hormones found in seed and fruit tissues has led to the hypothesis that elevated levels of hormone are necessary in order to create a strong physiological sink capable of competing with the stem and root apices for metabolites and mineral elements (LUCKWILL, 1977). Competition between the seed and the fruit wall tissues for available nutrients may also be dependent on a balance of hormones between the seed and the surrounding fruit tissues.

A consideration of cytokinin levels in the mature seed has indicated that in some species there is a relatively low level of cytokinin present (THIMANN, SHIBAOKA and MARTIN, 1970; VAN STADEN and BROWN, 1973). Applied cytokinins have been shown to promote germination in many species and KHAN (1975) has suggested that cytokinins play a permissive role in seed germination and interact with inhibitors. However, evidence for such a suggestion is based largely on applied hormone studies, and endogenous hormone work does not necessarily support such an hypothesis

(WEBB, VAN STADEN and WAREING, 1973; DIMALLA and VAN STADEN, 1977a).

Initial investigations of cytokinin involvement in seed germination were concerned mainly with the breaking of dormancy. In both *Acer saccharum* MARSH. (VAN STADEN, WEBB and WAREING, 1972) and *Protea compacta* R. BR. (VAN STADEN and BROWN, 1973) it appeared that *de novo* synthesis of cytokinins takes place in the embryos of stratified seed. It was suggested that in *P. compacta* the failure of seed to germinate was due to insufficient amounts of promoters being available rather than the presence of inhibitors (VAN STADEN and BROWN, 1973). A similar situation was apparent in the seed of *Carya illinoensis* (WANG.) K. KOCH. When this seed was imbibed at 30°C a rise in the level of endogenous cytokinins was noted prior to germination. The increase in cytokinin level was not as marked when the seed was incubated at 20°C, and germination also proceeded at a slower rate (DIMALLA and VAN STADEN, 1977a). In this species, as in *A. saccharum* and *P. compacta*, increasing cytokinin levels were noted during stratification. These results suggested that a certain level of endogenous cytokinin was required for germination to take place.

Although in the seeds mentioned above *de novo* synthesis of cytokinins appeared to take place, in other species there appeared to be an interconversion of bound forms of cytokinin to free base forms as was found in seed of *Leucadendron daphnoides* MEISN. stratified at 5°C (BROWN

and VAN STADEN, 1973). THOMAS and KHAN (1976) reported that there was a reduction in the level of a fairly immobile cytokinin fraction ($R_f$ 0,2 on paper chromatograms run in borate buffer) during barley seed germination. In maize seed SMITH (1977) demonstrated that there was a reduction in the level of the slow-moving fraction ($R_f$ 0,2 on paper chromatograms run in *iso*-propanol:25% ammonium hydroxide: water (10:1:1)) during germination. These cytokinins in germinating maize seed were shown to include the glucosides of zeatin, its riboside and the dihydro-derivatives of these compounds (SMITH, 1977). In lettuce seed it was demonstrated by VAN STADEN (1973) that the total cytokinin activity was affected by red light. It is interesting to note here, however, that the butanol-soluble cytokinins increased while those in the aqueous fraction decreased. In lettuce, exposure to red light appeared to promote the conversion of bound cytokinin to free base forms. In seed of *Rumex obtusifolius* L., exposure to red light resulted in a reversible increase in cytokinins (VAN STADEN and WAREING, 1972b). The role of cytokinins during germination is not yet clear, though it would appear as if the primary effect of these hormones is related to energy mobilization (DIMALLA and VAN STADEN, 1977a). Experimental data from applied hormone studies would appear to support this idea. It has been shown in the seeds of *Cucurbita maxima* DUCHESNE and *Arachis hypogea* L. that cytokinins, but not gibberellins, can partially substitute for the embryo in promoting the formation of *iso*-citrate lyase, one of the enzymes of the glyoxylate cycle (PENNER and ASHTON, 1967).

In *Protea compacta* increases in cytokinins and gibberellins appear to be closely correlated with the development of glyoxysomes and the breakdown of lipids (VAN STADEN, DAVEY and DU PLESSIS, 1976), while in *Triticum* spp. (wheat) catabolism of part of the triglyceride reserves is induced by cytokinin (TAVENER and LAIDMAN, 1972). The promotion of cotyledon expansion and the acceleration of lipid digestion by benzyladenine has also been reported (LONGO, LONGO, ROSSI and SERVETTAZ, 1976).

Cytokinin Metabolism

The examination of cytokinin levels in different plant organs has shown that these compounds change both quantitatively and qualitatively during growth. Furthermore, it is evident that these fluctuations are considered to represent the extent to which the compounds are utilized within the plant tissues. Therefore, although localized synthesis of cytokinin may take place in plant tissues, the metabolism of the free base cytokinins does not appear to be restricted to certain tissues and probably occurs throughout the plant.

The metabolism of cytokinins can follow at least three routes in plant tissues:

(i) Cytokinins can be metabolized to storage forms which are then metabolically inactive (i.e. inactivation products are formed). Reversible sequestration may ensure that these compounds can then be converted to free base forms.

(ii) Cytokinins can be metabolized by utilization, i.e. in order to promote or to maintain growth these compounds are attached to or incorporated into other molecules.

(iii) Cytokinins can be broken down by catabolic processes and thus destroyed.

Of these three possible routes for cytokinin metabolism only the first will be discussed in depth.

The naturally occurring cytokinins, zeatin and zeatin riboside, when supplied to plant tissues, are metabolized in a manner similar to the synthetic cytokinins. SONDHEIMER and TZOU (1971) reported that 8-$^{14}$C-zeatin was taken up rapidly and extensively metabolized by excised bean axes during a 12 hour incubation period at 26$^{o}$C. Most of the activity found in the 80% ethanolic soluble fraction consisted of zeatin, zeatin riboside, zeatin-5-ribotide as well as the corresponding dihydro-derivatives of these compounds. When {$^{3}$H}-zeatin was supplied to rootless radish seedlings a number of compounds including adenine, adenosine, adenosine-5-monophosphate, zeatin riboside and zeatin riboside-5-monophosphate were detected. Dihydro-zeatin was not detected (SONDHEIMER and TZOU, 1971). However, by far the most significant metabolite in these tissues was a 7-glucoside of zeatin, 6-(4-hydroxy-3-methyl-2-butenylamino)-7-glucopyranosyl-adenine (raphanatin) which was responsible for 25% of the radioactivity in the seedlings (PARKER and LETHAM, 1973; GORDON *et al.*, 1974) and it was suggested that this was either a storage form, or an active form, of zeatin. HORGAN (1975) reported that

callus, to which a drop of zeatin was added, rapidly metabolized this cytokinin to 6-(4-O-β-D-glucosyl-3-methyl-*trans*-2-butenylamino) purine (zeatin glucoside).

Glucoside cytokinins have been extracted from a variety of plant tissues and are present at especially high levels in senescent leaves and in seeds. In leaves, glucoside cytokinins accumulate with age, and appear to be the result of the metabolism of zeatin and zeatin riboside entering the leaves in the transpiration stream (LETHAM *et al.*, 1976). It has, however, been shown that the metabolism of these compounds in leaves changes with time (LETHAM *et al.*, 1976). Whereas mature leaves are able to metabolise zeatin chiefly to 6-(4-O-β-D-glucopyranosyl-3-methyl-*trans*-2-butenylamino-9-β-D-ribofuranosyl-purine) (zeatin riboside glucoside) and its dihydro-derivative, in senescent leaves the ribosyl derivatives are fewer and zeatin glucoside and dihydro-zeatin glucoside are the predominant metabolites. It was suggested that it is in senescing, and not in mature, leaves, that the 9-ribosyl moiety is readily cleaved. This observation is of interest in the light of experiments conducted on cytokinin-dependent strains of callus of *Glycine max* L. (soybean) grown on medium containing kinetin (VAN STADEN and DAVEY, 1977). These workers demonstrated that zeatin and its immediate derivatives were not present in callus tissue grown on medium containing kinetin. However, when the callus was grown on medium containing zeatin or zeatin riboside, five cell-division-inducing compounds were found in the callus irrespective of whether it was grown on medium containing zeatin or zeatin riboside.

The following metabolic pathway was proposed:

zeatin riboside glucoside ← zeatin riboside ⇄ zeatin →
zeatin phosphate → zeatin glucoside

It is possible that in senescent leaves the enzymatic conversion of zeatin through zeatin riboside to zeatin riboside glucoside was not as active as it had been in mature leaves.

WAREING *et al.* (1976) have studied the metabolism of cytokinins in bean seedlings. When $^{14}$C-zeatin was fed to rooted, de-budded bean seedlings, the major root-produced metabolite was zeatin ribotide. The presence of small quantities of di- and tri-phosphates was also detected. The major metabolite in the stem and petiole of the rooted system was dihydro-zeatin riboside. Other minor metabolites were present in the leaves including dihydro-zeatin-O-glucoside and glucosides of zeatin riboside and dihydro-zeatin riboside. From these results it was suggested that the ability of the leaf to form glucoside cytokinins allowed it to act as an effective sink for these compounds. The significance of dihydro-derivatives as metabolites should also be considered, and it was suggested that these compounds are resistant to attack by cytokinin oxidase type enzymes. Cytokinin oxidases have been purified from corn endosperm, and will readily cleave cytokinins with the *iso*-pentenyl type of side chain, while the saturated side chains are not attacked (WHITTY and HALL, 1974).

Although the above discussion indicates that cytokinins

supplied to plant tissues are metabolized to a variety of different end products, careful work still remains to be done on the actual pathways involved in cytokinin degradation. It is possible that greater attention to the minor metabolites and to the extraction and purification of different enzyme systems will make a valuable contribution to our understanding of how cytokinins are converted to storage forms or degraded completely within plant tissues.

## Concluding Remarks

In the preceding pages the sites of synthesis, distribution and metabolism of endogenous cytokinins have been discussed, and it is evident that considerable knowledge of the distribution of cytokinins in plant tissues has been gained in recent years. Cytokinins have been shown to occur in the phloem sap, thereby altering the original concepts of cytokinin distribution in the plant. The root-to-shoot type translocation of cytokinins in the xylem tissues, originally thought to occur in plants, no longer accounts for the cytokinin complement of the shoot. Thus, although the roots are the only proven sites of cytokinin synthesis, this added information must be considered when attempts are made to assess the significance of fluctuating cytokinin levels in the plant. Indeed, in order to understand the fluctuations in cytokinin levels in different parts of the plant, the cytokinin status of the plant should be viewed in totality. This outlook should encompass a knowledge not only of gross changes in cytokinin

levels in individual tissues during growth but also attempt to achieve an understanding of the qualitative changes in these cytokinins. It is noticeable that, wherever a study of changing cytokinin levels has been accompanied by the characterisation of the cell division promotors present in the plant, our knowledge of the possible role of the cytokinins in plant growth has increased. This is an especially important consideration when it is realised that the only mechanism of cytokinin metabolism currently being investigated with success, is the aspect that involves the conversion of cytokinins to glucosylated and phosphorylated derivatives.

The experiments which follow were conducted with these considerations foremost in mind. They attempt to establish how the cytokinin levels change both quantitatively and qualitatively throughout the growth cycle of an annual plant, and to assess the importance of these changes, in different organs, in terms of the involvement of cytokinins in whole plant growth and development. In addition to surveying cytokinin levels in the mature plant, changes during embryonic growth, germination and seedling development have also been followed. These latter phases of growth have not received sufficient attention in the past.

# MATERIALS AND METHODS

In order to study the cytokinins in a plant during its growth and development, one has to establish the kinds of cytokinins present and monitor their levels of activity throughout the growth cycle. An annual legume, the white lupin, *Lupinus albus* L., was chosen for study as it is a plant with a life cycle of relatively short duration (approximately 6 months from germination to complete senescence). Furthermore, the white lupin grows well under field conditions during the winter months in Pietermaritzburg. This attribute was of importance as adequate growth room facilities were not available.

For the purposes of experimental convenience, the life cycle of this plant was divided into two phases. The first phase, that of mature plant growth, covers vegetative, flowering and fruiting growth, and it is dealt with in Experiments 1 and 2. The second phase, that of embryonic growth, includes early fruit development, seed development, germination and early seedling growth. It is the subject of Experiments 3 and 4.

In Experiments 1 and 2 cytokinin changes were monitored in the root exudate, leaves, apices, pod walls and seeds. In Experiment 3 the activity in the flowers and young fruits was investigated. The cytokinin levels in different parts of the seed were also examined. Anatomical and ultrastructural changes in the developing embryo were followed in this section. In Experiment 4 the cytokinin changes in

the cotyledons, radicles and young shoots of the embryo were studied during the first 12 days of seedling growth. Although attempts were made in Experiments 1 - 4 to assess the qualitative as well as quantitative differences in cytokinins in the white lupin during these growth phases, precise identification of these compounds on such a scale was not possible. However, the identification of the cytokinins present in the white lupin was not neglected, and Experiment 5 deals with this aspect of the project.

Plant Material

The white lupin seed used in all experiments was a generous gift from Mr. Landman of de Rust in the Cape Province. The white lupin plants were grown in a similar manner for all experiments. The seeds were germinated in trays containing vermiculite moistened with tap water. The seeds germinated within 3 days of planting, and five days after germination the seedlings were transplanted into individual black polythene bags (20 centimetre diameter x 25 centimetre in depth) containing a sand:loam (3:1) potting mixture. All seeds were put in to germinate on 14th April each year and the plants were grown under field conditions in the winter (April to September) in Pietermaritzburg. The experiments were conducted over three growing seasons.

*Harvesting of Plant Material*

(i) Collection of root exudate (Experiments 1 and 2)

Although different techniques have been employed for the collection of xylem sap or root exudate, when sap is collected from herbaceous plants care must be taken to avoid crushing the soft plant material, and thereby contaminating the root exudate with the contents of adjacent cells. The technique described below has been used fairly extensively and successfully for the collection of root exudate from herbaceous plants in other laboratories (KENDE, 1964; 1965; CARR and BURROWS, 1966; BEEVER and WOOLHOUSE, 1973; 1974), and in the laboratory in Pietermaritzburg (MENARY and VAN STADEN, 1976; VAN STADEN and MENARY, 1976). This technique causes minimal damage to the stem tissues, and allows the amount of sap passing into the shoot tissues over a given time to be estimated.

At each sampling time 20 plants were placed in the greenhouse 24 hours before sampling in order to facilitate the collection of the root exudate. At noon the plants were de-topped 3 centimetres above soil level (below the cotyledonary scars) and sterile rubber tubes were attached to the stumps. The sap exuding from the cut surface of the stem was collected in sterile flasks over a 24 hour period. The amount of sap produced by twenty plants was recorded and it was then stored at -20$^{o}$C in a deep freeze.

(ii) Collection of remaining plant material

All plant material was harvested at noon. Immediately

after harvest, the tissues being harvested for analysis were divided into the desired fractions, weighed, packed into polythene bags and stored at -20°C. The endosperm material was collected from seeds at 4 and at 6 weeks after anthesis by puncturing the testa and collecting the liquid and semi-cellular endosperm on a wick of filter paper. The weight of the endosperm was obtained by weighing each seed before and after the removal of the endosperm. The weight lost by the seed represented the weight of the endosperm. Endosperm from 8-week-old seeds was obtained by carefully removing the residual layer of endosperm immediately below the testa. Fruit sap was collected in 5 microlitre pipettes after the stalk had been severed. The fruit sap was collected over a half hour period by repeatedly cutting the stalk and collecting the resultant exudate.

Extraction and Purification of Plant Material for Cytokinins (Experiments 1 and 2)

*Root exudate*

Crude root exudate collected from 20 plants was filtered through Whatman No. 1 filter paper, concentrated under vacuum at 40°C and the residue taken up in 1 millilitre of 35 percent (v/v) ethanol and separated by means of column chromatography (ARMSTRONG, BURROWS, EVANS and SKOOG, 1969). In Experiment 2 a slightly different procedure was adopted. After the root exudate had been concentrated to dryness it was taken up in 10 millilitres 80 percent ethanol and then halved. Half of each extract was then strip loaded onto

Whatman No. 1 chromatography paper and separated as described later. The remaining half was fractionated by means of column chromatography on Sephadex LH-20.

*Leaf material, shoot apices, seeds and pod walls*

The cytokinins were extracted from leaves, shoot apices, pods and seed tissues for Experiments 1 and 2 by homogenizing the appropriate quantities of material (on a fresh weight basis) in 80 percent ethanol (15 millilitres of 80 percent ethanol was used per gramme of material) and allowing them to stand overnight at 5°C. These extracts were then filtered through Whatman No. 1 filter paper and concentrated to dryness under vacuum at 40°C. The residues were re-dissolved in 70 millilitres 70 percent ethanol and the pH of the ethanolic extracts adjusted to 2,5 with hydrochloric acid. The acidified extracts were passed through Dowex 50W-X8 cation exchange resin (J.T. Baker Chemical Co. Phillipsburg, N.J.; $H^+$ form; 200-400 mesh 2,5 x 25 centimetres) at a flow rate of 20 millilitres per hour. The columns were washed with 100 millilitres of distilled water followed by 100 millilitres of 80 percent ethanol. The aqueous and ethanolic eluates were discarded. The cytokinins were then eluted from the column with 200 millilitres of 5N ammonium hydroxide. The ammonia eluates were concentrated to dryness under vacuum, the residue taken up in 5 millilitres of 80 percent ethanol and strip loaded onto Whatman No. 1 chromatography paper.

Cation exchange resins have been widely used for the purification of ethanolic extracts of cytokinins

(ENGELBRECHT, 1971b; HEWETT and WAREING, 1973c; LORENZI *et al.*, 1975; VAN STADEN, 1976a; 1976b; WANG, THOMPSON and HORGAN, 1977). VAN STADEN (1976e) demonstrated that in addition to cytokinins co-eluting with zeatin and zeatin riboside, cytokinin glucosides could be recovered from the cation exchange resin, Dowex 50W-X8 (J.T. Baker Chemical Co. Phillipsburg, N.J.; $H^+$ form; 200-400 mesh). Although DEKHUIZEN and GEVERS (1975) had implied that these glucosides might be hydrolyzed by a strongly acidic ion exchange resin, VAN STADEN (1976e) showed that glucosides of zeatin and zeatin riboside were not hydrolyzed when such a resin was used.

Extraction and Purification of Plant Material for Cytokinins (Experiments 3 and 4)

*Flower parts and developing fruits*

Flower parts excluding the young developing fruits, were extracted for cytokinins as described in the previous section. The developing fruits were extracted by homogenising in a ground glass mortar and pestle with 80 percent ethanol (15 millilitres per gramme of plant material). Both sets of extracts were then filtered through Whatman No. 1 filter paper and concentrated under vacuum. The residues were taken up in 5 millilitres 80 percent ethanol and strip loaded directly onto Whatman No. 1 chromatography paper.

*Embryos, endosperms, testas, cotyledons, radicles and young shoots*

Although the basic extraction and purification

procedures remained the same as described on page 55, due to the unavailability of Dowex 50W-X8 (200-400 mesh) cation exchange resin, Dowex 50W-X8 (J.T. Baker Chemical Co. Phillipsburg, N.J.; $H^+$ form; 20-50 U.S. mesh) had to be used as a substitute. This resin was used in the proportion of 2 grammes of resin per gramme of plant material. Slightly different procedures were, however, adopted wherever small quantities of material obviated the necessity for the use of the cation exchange resins. Thus 2-week-old seeds, 4- and 6-week-old embryos and suspensors removed from 4-week-old seeds were ground in a glass homogeniser with 80 percent ethanol. These extracts were then filtered, concentrated taken up in 1 millilitre 80 percent ethanol and either strip loaded directly onto Whatman No. 1 chromatography paper (embryos and suspensors from 4-week-old seeds) or fractionated by means of column chromatography on Sephadex LH-20 (2-week-old seeds and 6-week-old embryos). The cytokinins present in the endosperm were extracted by eluting the thin strips of filter paper, on which the endosperm was collected, with 200 millilitres of 80 percent ethanol. The extracts were filtered, concentrated to dryness and strip loaded onto Whatman No. 1 chromatography paper.

## Chromatographic Techniques

### *Paper chromatography*

Extracts were applied in a 1 centimetre strip to sheets of Whatman No. 1 chromatography paper. The chromatograms were run with *iso*-propanol: 25 percent ammonium hydroxide:

water (10:1:1 v/v) (PAW) in a descending manner until the solvent front was approximately 30 centimetres from the origin. The chromatograms were then dried in a drying oven at 25°C for 24 hours. The dry chromatograms were divided into 10 $R_f$ zones. If, at this point, the chromatographed extracts were to be analyzed for cytokinin activity, the strip of paper corresponding to each $R_f$ zone was cut up and placed into an erlenmeyer flask and subsequently assayed for cell division-promoting activity using the soybean callus bioassay (MILLER, 1965). The chromatograms were stored at -20°C, if further analysis was necessary.

*Column chromatography*

Column chromatography was used as an aid in the determination of the possible kinds of cytokinins present in white lupin extracts. The technique used here was based on that described by ARMSTRONG *et al.* (1969). The columns (90 x 2,5 centimetres) were packed with Sephadex LH-20 which had been swollen in 35 percent ethanol. They were eluted with 35 percent ethanol at a flow rate of 15 millilitres per hour. Forty millilitre fractions were collected. Cytokinins co-eluting with zeatin and zeatin riboside were separated efficiently using this technique. However, there appeared to be at least two different kinds of cytokinin glucosides present in the lupin extracts which did not separate into discrete peaks using this system. A similar column was therefore packed with Sephadex LH-20 which had been swollen in 20 percent ethanol. This column

was eluted with 20 percent ethanol. The flow rate and volume of the sample collected remained the same. Samples of authentic zeatin (mixed isomers) and zeatin riboside (mixed isomers) (Calbiochem A grade) were eluted through the columns and assayed for cell division activity, in order to obtain the elution volumes of these compounds in each individual system. The zone marked zeatin glucoside (ZG) on the histograms indicates the elution volume of the O-glucoside of zeatin described by VAN STADEN and PAPAPHILIPPOU (1977).

For the majority of extracts prior analysis of paper chromatograms had indicated the presence of cell division-promoting compounds in different zones of the chromatograms. The active regions of these chromatograms were therefore eluted with 500 millilitres of 80 percent ethanol, concentrated and taken up in 1 millilitre of 35 percent ethanol and 20 percent ethanol, depending on the nature of the column being used. However, if availability of material at a particular stage was restricted to small quantities, or if very clean extracts were obtained, without the necessity for paper chromatography, they were applied directly to the appropriate columns. In Experiments 1 and 2 unless otherwise stated, the paper chromatograms were divided into two fractions: $R_f$ 0,15-0,5 (Fraction A) and $R_f$ 0,5-0,9 (Fraction B). In Experiments 3, 4 and 5, however, Fraction A represented $R_f$ 0,05-0,5 and Fraction B, $R_f$ 0,5-1,0.

After elution through the columns the 40 millilitre

fractions were transferred to erlenmeyer flasks, dried on a hot plate set at 30°C in a stream of air, and assayed for cell division activity using the soybean callus bioassay.

Treatment with β-glucosidase

The more polar cytokinins present on paper chromatograms have, in several instances, been shown to be readily hydrolyzed by β-glucosidase and are considered to be glucoside cytokinins (YOSHIDA and ORITANI, 1972; HEWETT and WAREING, 1974; PARKER, LETHAM, WILSON, JENKINS, MACLEOD and SUMMONS, 1975; VAN STADEN, 1976a; 1976b; 1976c; 1976e). In order to establish whether such compounds were present in white lupin extracts, cytokinins present at the $R_f$ zones corresponding to the A fractions of paper chromatograms were treated with β-glucosidase (Sigma - from almonds) (VAN STADEN, 1976c). The active fractions were eluted from the paper chromatograms with 500 millilitres of 80 percent ethanol, and concentrated to dryness. They were subsequently taken up in 2 millilitres of 0,02 Molar Tris buffer at pH 5,2 and incubated with 1,5 milligrammes of β-glucosidase for 24 hours at 22°C. The reaction was terminated by adding 4 millilitres of 80 percent ethanol to the reaction mixture. This fraction was then concentrated, and either taken up in 5 millilitres of 80 percent ethanol and strip loaded onto sheets of Whatman No. 1 chromatography paper and separated using PAW, or taken up in 1 millilitre of 35 percent ethanol and applied directly to the Sephadex LH-20 columns and eluted with 35 percent ethanol. The fractions obtained by these techniques and control fractions

which had been similarly treated, except for the addition of β-glucosidase, were assayed simultaneously for cell division-promoting activity.

Alkaline Phosphatase Treatment

White lupin extracts were treated with alkaline phosphatase enzyme according to procedures outlined by MILLER (1965). The extract was concentrated to dryness and taken up in 2 millilitres of buffer (0,01 M $MgCl_2$, 0,1 M *tris*-hydroxymethylaminoethane at pH 8,2) to which 2 milligrammes of alkaline phosphatase (Sigma; from calf intestine mucosa) was then added. This mixture was incubated at 32°C for 24 hours. The reaction was then terminated by the addition of 80 percent ethanol.

Treatment with alkaline phosphatase has been shown to cause hydrolysis of certain fractions active as cytokinins and to yield active compounds which co-chromatograph with zeatin and its riboside (VAN STADEN and MENARY, 1976; VAN STADEN and DAVEY, 1977), when separated on Sephadex LH-20 columns eluted with 35 percent ethanol. This result was considered to indicate the presence of a nucleotide of zeatin (VAN STADEN and MENARY, 1976) or phosphorylated zeatin (VAN STADEN and DAVEY, 1977).

Potassium Permanganate Oxidation

The technique of potassium permanganate ($KMnO_4$) oxidation of cytokinins was utilised in order to establish whether or not a particular fraction contained zeatin and

zeatin derivatives or another cytokinin not oxidised by $KMnO_4$. If the fraction does contain zeatin or an unsaturated zeatin derivative, treatment with potassium permanganate will result in a complete loss of activity in a bioassay. The double bond in the allyl group of the *iso*-pentenyl chain of zeatin is broken by $KMnO_4$ treatment and the inactive N-(purin-6-yl)-glycine is formed (MILLER, 1965). The efficiency of $KMnO_4$ oxidation was tested using authentic zeatin and zeatin riboside (mixed isomers, Calbiochem, A. grade). This test showed that treatment with potassium permanganate resulted in complete absence of cell division. Thus cytokinin activity remaining in a sample after such treatment, must be due to other compounds which co-chromatograph with zeatin or unsaturated zeatin derivatives. Extracts to be treated with $KMnO_4$ were concentrated to dryness. A few drops of neutral aqueous 0,01 $KMnO_4$ solution were added to the residue until the permanganate colour began to persist for a few seconds. An excess of 80 percent ethanol was then added to complete the decomposition of the permanganate. After evaporation to dryness, the residues of the treated extracts were either taken up in 5 millilitres of 80 percent ethanol and separated by means of paper chromatography, or redissolved in 1 millilitre of 35 percent ethanol and fractionated by means of column chromatography. The fractions obtained by either technique together with control fractions which had been treated similarly except for the omission of $KMnO_4$ addition, were simultaneously assayed for cell division promoting activity. Potassium permanganate oxidation was

used in conjunction with β-glucosidase treatment in Experiment 5, in order to determine whether the compounds hydrolysed by β-glucosidase could also be oxidised by $KMnO_4$.

Soybean Callus Bioassay

The fractions obtained as a result of paper and column chromatography were assayed for cytokinin activity using the soybean callus bioassay (MILLER, 1963; 1965). The soybean callus bioassay has been used extensively, as the range of concentration over which a linear response is obtained is greater in this assay than in the tobacco pith callus bioassay (FOX, 1969; SKOOG and ARMSTRONG, 1970). Furthermore, the cell division assays are considered to be superior to the chlorophyll retention bioassays (KENDE, 1971). Callus was obtained from the cotyledons of *Glycine max* L. var. Acme according to the procedures described by MILLER (1963; 1965) and was maintained by 3-weekly subculture.

Four stock solutions were prepared and the nutrient medium was made up as outlined in Table 1. Either 30 millilitres of medium or 15 millilitres of medium were added to 50 millilitre flasks or 25 millilitre flasks which contained 0,3 grammes and 0,15 grammes of agar, respectively. The flasks were stoppered with non-absorbant cotton wool bungs, which were then covered with aluminium foil. The flasks were then autoclaved at a pressure of 1,05 bars for 20 minutes before being transferred to a 'sterile transfer'

chamber. Once the agar had solidified, three pieces of soybean callus of approximately 20 milligrammes were placed on the basal medium. The flasks were then incubated in a growth room where conditions of constant temperature ($26^{o}C \pm 2^{o}C$) and continuous light of low intensity (cool white fluorescent tubes) were maintained. After 28 days the three pieces of callus in each flask were weighed simultaneously. The amount of callus growth in each fraction was plotted on a histogram relative to the control value. The significance limit at the 0,01 percent level was calculated and indicated on the histograms as a broken line. Standards were included with each bioassay. Initially (Experiments 1 and 2) kinetin standards were used. However, as zeatin is a naturally occurring cytokinin, zeatin standards were later adopted (Experiments 3, 4 and 5). In order to estimate gross levels of cytokinin activity at a given time the results were expressed in kinetin or zeatin equivalents. However, the inherent difficulties in making such an estimate were recognised.

TABLE 1

BASAL MEDIUM FOR SOYBEAN CALLUS BIOASSAY
(adapted from Miller, 1963, 1965)

| Stock Solution | Chemical | $gl^{-1}$ stock solution | ml stock solution for litre medium |
|---|---|---|---|
| STOCK 1 | $KH_2PO_4$ | 3,0 | 100 |
| | $KNO_3$ | 10,0 | |
| | $NH_4NO_3$ | 10,0 | |
| | $Ca(NO_3)_2 \cdot 4H_2O$ | 5,0 | |
| | $MgSO_4 \cdot 7H_2O$ | 0,715 | |
| | KCl | 0,65 | |
| | $MnSO_4 \cdot 4H_2O$ | 0,14 | |
| STOCK 2 | NaFeEDTA | 1,32 | 10 |
| | $ZnSO_4 \cdot 7H_2O$ | 0,38 | |
| | $H_3BO_3$ | 0,16 | |
| | KI | 0,08 | |
| | $Cu(NO_3)_2 \cdot 3H_2O$ | 0,035 | |
| | $(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$ | 0,01 | |
| STOCK 3 | myo-inositol | 10,0 | 10 |
| | nicotinic acid | 0,2 | |
| | pyridoxine HCl | 0,08 | |
| | thyamine | 0,08 | |
| STOCK 4 | NAA | 0,02 | 10 |
| ADDITIONAL | Sucrose | | 30 $gl^{-1}$ medium |
| | Agar | | 10 $gl^{-1}$ medium |
| pH adjusted to 5,8 with NaOH | | | |

Microscopy Techniques

Seed material for microscopy was harvested at 4, 6, 8, 10 and 12 weekly intervals after anthesis of the individual flowers. After harvest the embryos were dissected out and 3 millimetre pieces of cotyledon, shoot and root apex were fixed at 4°C in 6 percent glutaraldehyde, buffered at pH 7,2 with 0,05 Molar sodium cacodylate, for 6 hours. At 4 and 6 weeks after anthesis the embryos were small, fragile and difficult to fix. It was found that fixation of these embryos was enhanced by allowing small portions of the seed coat and endosperm to remain around the embryo. The fixed material was washed 3 times for periods of 30 minutes each in 0,05 Molar sodium cacodylate buffer. It was then post fixed in 2 percent osmium tetroxide, buffered as above and again washed 3 times in 0,05 Molar sodium cacodylate buffer. The material was dehydrated in an alcohol series, followed by propylene oxide and embedded in araldite resin. Polymerisation lasted 48 hours at 70°C. Sections for both light and electron microscopy were cut with a glass knife (light microscopy) and a diamond knife (electron microscopy) on an LKB microtome. The sections were stained for electron microscopy with uranyl acetate and lead citrate as described by Reynolds (1963). These sections were examined using an Hitachi HU 11E electron microscope at an accelerating voltage of 50 KV and photographed.

Monitor sections were cut at each developmental stage for light microscopy. These sections were stained using 1 percent Toluidine blue in 1 percent borax:1 percent pyronin Y

(1;1 v/v). Carbohydrate was detected using the periodic acid Schiff's reagent (FEDDER and O'BRIEN, 1968). The slide was placed in 1 percent aqueous periodic acid for 5-10 minutes. It was then washed in running water for 10 minutes and placed in Schiff's reagent (0,5 grammes basic Fuchsin in 100 millilitres of 1 percent HCl containing 1 gramme of potassium metabisulfite) and washed in 3 baths of 5 percent sodium metabisulfite (2 minutes each) followed by running water (5 minutes). Protein was detected by staining fresh sections for 2 hours with mecuric bromphenol blue (1 gramme $HgCl_2$ and 0,05 grammes bromphenol blue were dissolved in 100 millilitres 2 percent acetic acid). Fresh sections were stained for lipid using a saturated solution of Sudan III in 70 percent ethanol (GURR, 1956) and the phloroglucinol test was used in order to detect the presence of lignin in the cell walls (GURR, 1956). Sections were flooded with phloroglucinol solution (1 percent in 70 percent ethanol) for 5 minutes. Excess phloroglucinol was then poured off and a few drops of concentrated HCl were added.

# PART I

## CYTOKININS IN THE MATURE PLANT

### EXPERIMENT 1

### Cytokinins in Vegetative, Flowering and Fruiting White Lupin Plants

#### Introduction

The occurrence of cytokinin-like compounds in a variety of plants and plant organs was discussed in the literature review, and the fluctuations of individual cytokinins within plant organs was emphasised. However, it was also stressed that if the involvement of cytokinins in plant growth and development is to be more fully understood, the quantitative and qualitative changes taking place in plant organs must be related to whole plant growth and development.

An annual legume, *Lupinus albus* L., was selected as the experimental plant for such a study. However, although GRIFFANT (1975) indicated that under certain conditions excised white lupin roots were able to synthesize cytokinins, little information was available in respect of the kinds of cytokinins present in this plant, or of the distribution of these cytokinins within the plant body. The first experiment was conducted in order to acquire an understanding of the quantitative and qualitative changes in cytokinins during the vegetative, flowering, and fruiting stages of growth. Three parameters were selected for investigation, namely: the root exudate, leaves and the terminal shoot apices (primary apex).


a
b
c
d
e
10 cm

Experimental Procedure

The root exudate, leaves and terminal shoot apices were harvested at 3 weekly intervals between 5 and 17 weeks after planting. The material was collected, weighed, extracted and purified according to procedures outlined in the materials and methods. For the analysis of cytokinins in root exudate, the root exudate produced by 20 plants over 24 hours was used. The leaf material was extracted and purified in 25 gramme (fresh weight) lots. As there were considerable weight differences between samples of the terminal shoot apices, all the material collected from 20 plants was extracted and purified at 5, 8 and 11 weeks after planting, and 75 grammes (fresh weight) of material at 14 and 17 weeks after planting. Twenty five grammes of the extract of the material collected at 14 and 17 weeks after planting was assayed after paper chromatography. The remaining material was utilized for column chromatography. Extracts applied to Sephadex LH-20 columns were eluted with 35 percent ethanol.

Results

During the 12 week period over which this experiment was conducted, the white lupin plants increased in fresh weight (Table 1:1). Although the average number of mature fully expanded leaves per plant increased from 8,3 leaves per plant on 5-week-old plants to 37,4 leaves per plant on 17-week-old plants, the increase in fresh weight of the plants was due to development of the terminal shoot apices (Table 1:1). At 5 weeks after planting the apical region

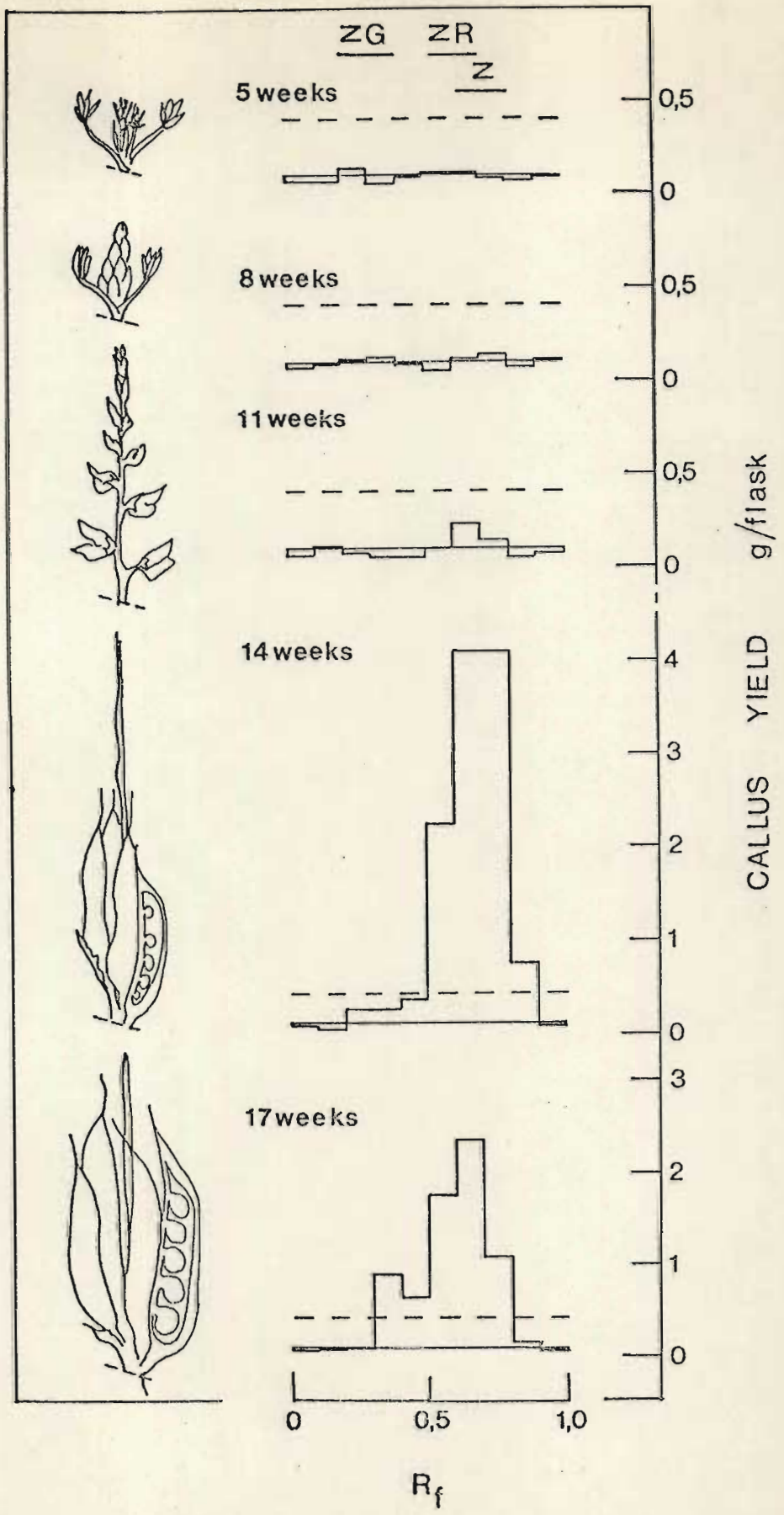



Figure 1:1. The distribution of cytokinin activity in apex material at 5, 8, 11, 14 and 17 weeks after planting. Extracts were passed through Dowex 50 cation exchange resin and the ammonia eluates chromatographed in *iso*-propanol:25 percent ammonium hydroxide:water (10:1:1 v/v). Drawings accompanying the graphs are an indication of the developmental stage reached by the apices. Callus grown on 10 microgrammes per litre kinetin yielded 0,65 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. Broken line indicates the confidence limit at the level P = 0,01.

of the plant was vegetative. However, by 8 weeks after planting flower buds had begun to develop and by 11 weeks the inflorescence was in full flower. Developing fruits were present on the terminal shoot apices of 14- and 17-week-old plants (Plate 1:1). At 5 weeks after planting the apical region weighed 0,13 grammes and contributed 3,2 percent of the fresh weight. However, the average weight of the apex of 17-week-old plants was 20,02 grammes and made up 47,5 percent of the fresh weight of the whole plant. The amount of root exudate produced per plant increased up to 14 weeks after planting. At 17 weeks there was a reduction in the amount of root exudate produced (Table 1:1).

TABLE 1:1. Growth of white lupins. Table showing trends in fresh weight, leaf production, root exudate production and development of the terminal apex between 5 and 17 weeks after planting.

| Age of plants, weeks | 5 | 8 | 11 | 14 | 17 |
|---|---|---|---|---|---|
| Average shoot weight, g | 4,03 | 10,89 | 13,45 | 20,77 | 42,12 |
| Average weight apices, g | 0,13 | 0,59 | 1,15 | 4,37 | 20,02 |
| Root exudate production, ml plant$^{-1}$ 24$^{-1}$ hours | 0,41 | 0,56 | 1,20 | 1,75 | 0,38 |
| Average number of leaves, plant$^{-1}$ | 8,3 | 13,3 | 21,2 | 31,3 | 37,4 |
| Developmental state of apex | vege-tative | flower buds | flower-ing | fruit-ing | fruit-ing |

*Terminal shoot apices (including young unexpanded leaves)*

The increase in fresh weight and the developmental changes taking place at the shoot apex were accompanied by significant changes in the detectable levels of cytokinins in this region. Figure 1;1 shows the results after paper chromatography of extracts of the terminal apex. Low levels of activity were

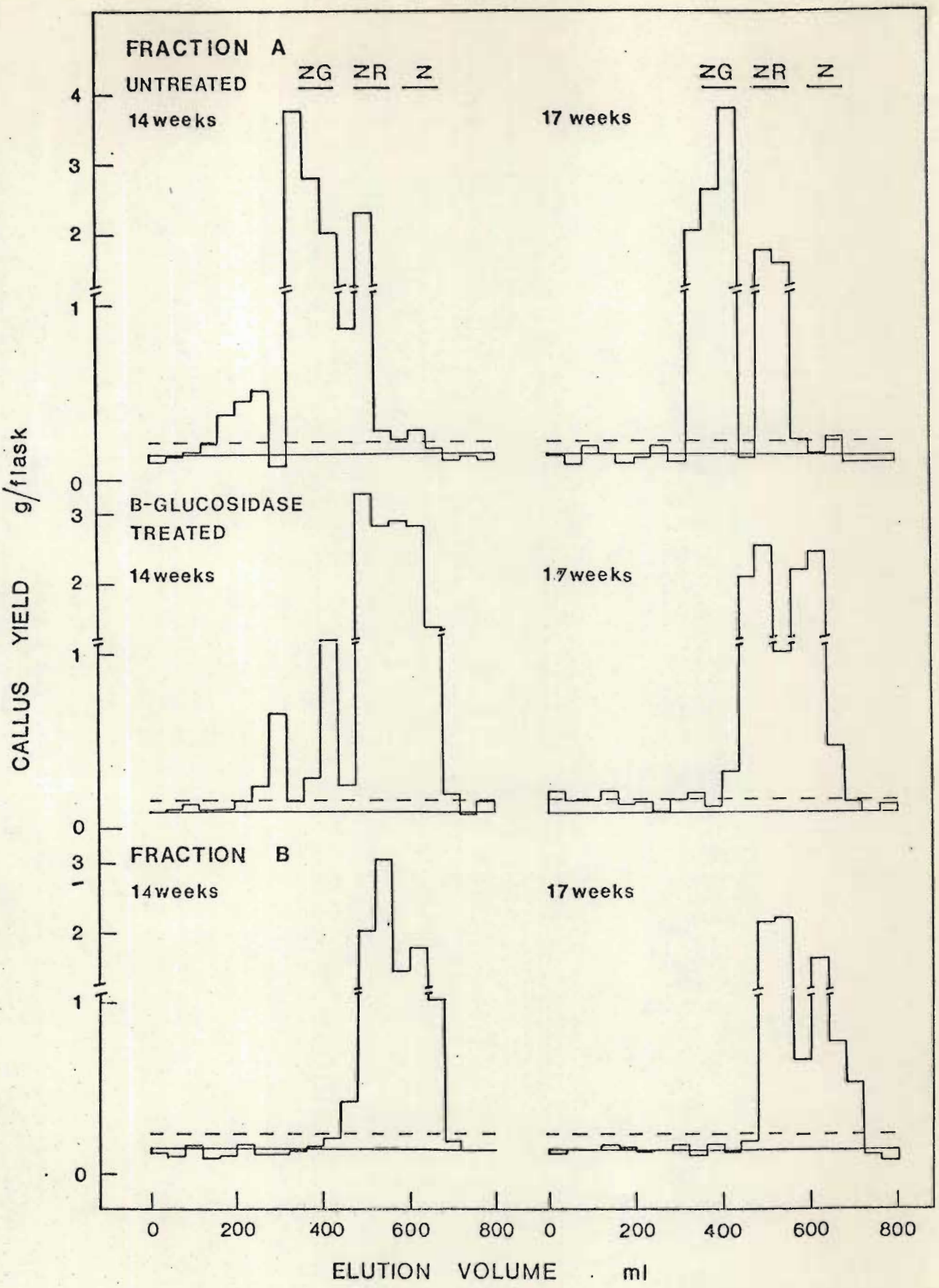



Figure 1:2. Fractionation on Sephadex LH-20 of the activity at $R_f$ 0,2-0,5 (Fraction A) and $R_f$ 0,5-0,9 (Fraction B) from paper chromatograms of 25 grammes of the apical region of 14 and 17 week old plants. Note that, after fractions A were treated with β-glucosidase, activity at the elution volume 360-440 millilitres was reduced. Activity co-eluting with zeatin and zeatin riboside increased. Callus grown on 10 microgrammes per litre kinetin yielded 1,33 grammes of fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. Broken line indicates the confidence limit at the level P = 0,01.

detected in 5 and 8 week old apices when the apical region was vegetative and in bud respectively. However, a slight increase in activity was noted at 11 weeks after planting.

Extracts of fruiting apices harvested at 14 weeks and 17 weeks after planting exhibited very high levels of cytokinin activity. Activity was detected mainly at $R_f$ 0,5-0,9. However, extracts of apical material harvested at 17 weeks indicated the presence of some cytokinin-like compounds at $R_f$ 0,2-0,5. Because of activity in this zone on paper chromatograms, and because high levels of activity were present, apical material was utilised in order to investigate the nature of cytokinins in white lupins.

To this end paper chromatograms of extracts of apical material of 14 and 17 week old plants were divided into two fractions, an A fraction ($R_f$ 0,15-0,5) and a B fraction ($R_f$ 0,5-0,9). Fractionation of the B fractions on columns packed with Sephadex LH-20 and eluted with 35 percent ethanol revealed the presence of cytokinin activity co-eluting with zeatin and zeatin riboside (Figure 1:2). Activity in the A fraction following column chromatography was present in three peaks. One peak co-eluted with zeatin riboside, a second had an elution volume of 320-440 millilitres, and a third eluted at 160-280 millilitres. Treatment of the A fractions with β-glucosidase resulted in a reduction in the activity at the elution volume 320-440 millilitres and an increase in the activity co-eluting with zeatin and zeatin riboside. The callus yield at the elution volume corresponding to zeatin increased from 0,68 grammes to 6,91

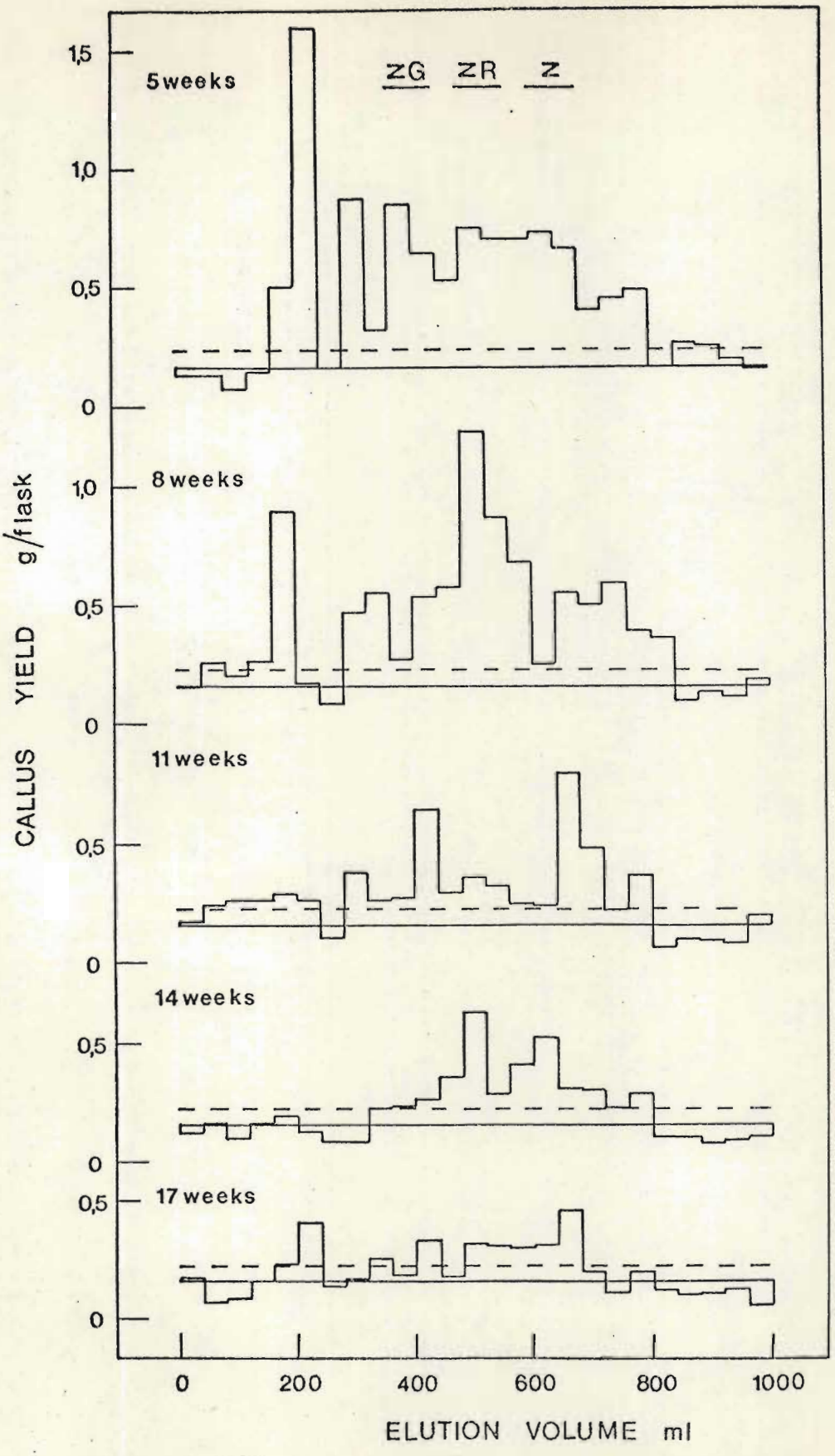



Figure 1:3. Cytokinin activity in the crude root exudate of white lupin plants at 5, 8, 11, 14 and 17 weeks after planting. The root exudate was collected at each stage from 20 plants over a 24 hour period and fractionated on Sephadex LH-20. Callus grown on 10 microgrammes per litre kinetin yielded 1,73 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. Broken line indicates the confidence limit at the level P = 0,01.

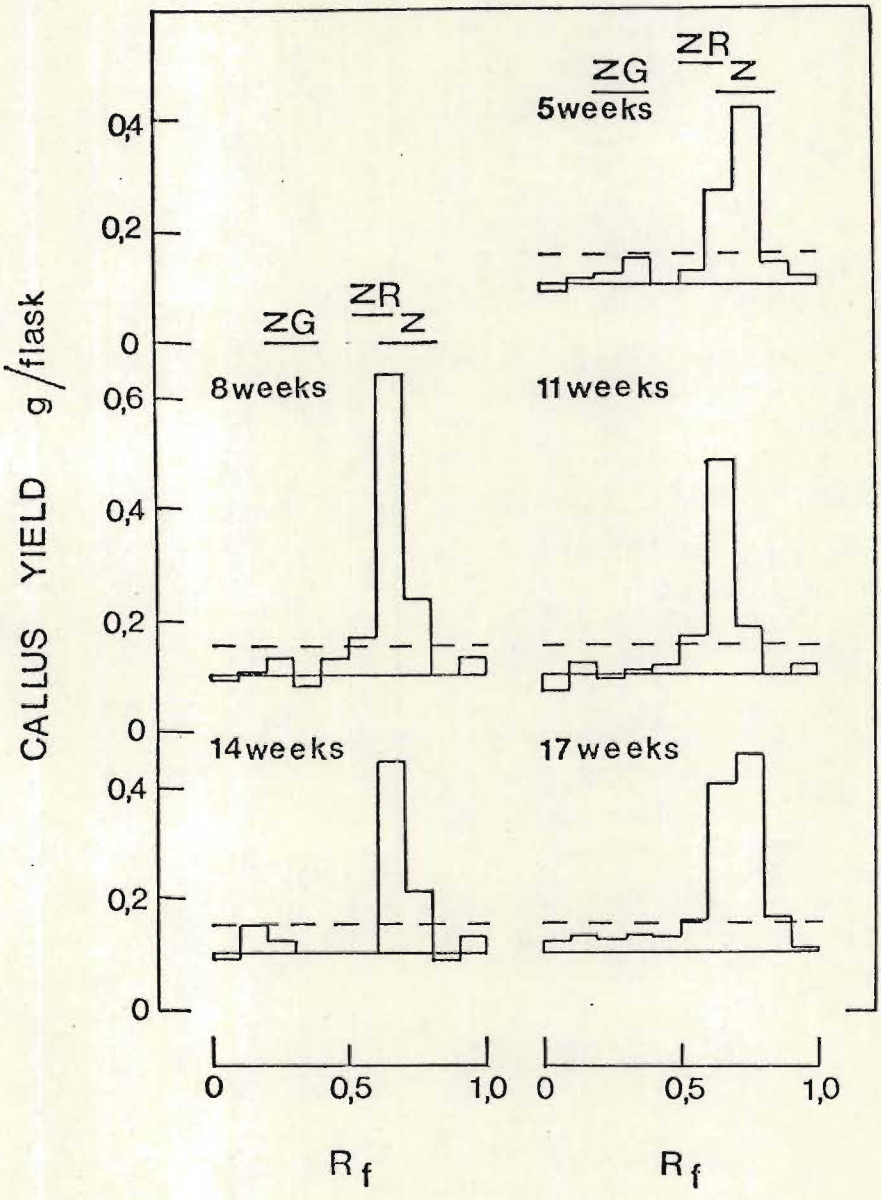



Figure 1:4. Cytokinin activity in extracts of 25 grammes of mature leaf material collected from 5-, 8-, 11-, 14- and 17-week-old plants. The extracts were purified on Dowex 50 cation exchange resin and the ammonia eluates separated on paper with *iso*-propanol:25 percent ammonium hydroxide:water (10:1:1 v/v). Callus grown on 10 microgrammes per litre kinetin yielded 0,68 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. Broken lines indicate the confidence limit at the level P = 0,01.

grammes and from 0,19 grammes to 5,86 grammes in the material from 14 and 17 week old plants respectively. The activity co-eluting with zeatin riboside increased from 2,29 grammes to 6,03 grammes and from 3,08 grammes to 5,91 grammes, respectively. The activity with the elution volume 160-280 millilitres was not reduced by treatment with β-glucosidase (Figure 1:2).

*Root exudate*

Over the 12 week sampling period there was a reduction in the level of cytokinin activity in the root exudate produced by 20 plants over a 24 hour period (Figure 1:3). Four major peaks of cytokinin activity were detected in the root exudate following column chromatography on a Sephadex LH-20 column eluted with 35 percent ethanol. Two peaks co-eluted with zeatin and zeatin riboside. The remaining peaks eluted at 160-320 millilitres and 320-480 millilitres respectively.

*Mature fully expanded leaves*

Cytokinin activity, co-chromatographing with zeatin and zeatin riboside on paper chromatograms ($R_f$ 0,5-0,9) was detected in the leaf tissue at all developmental stages (Figure 1:4). The level of cytokinin activity in 25 grammes of leaf material fluctuated slightly over the experimental period. Column chromatography (using Sephadex LH-20 eluted with 35 percent ethanol) confirmed the presence of compounds co-eluting with zeatin and zeatin riboside in extracts of leaves harvested at 8 and 17 weeks after planting (Figure 1:5). Cytokinin activity was also shown to be present in

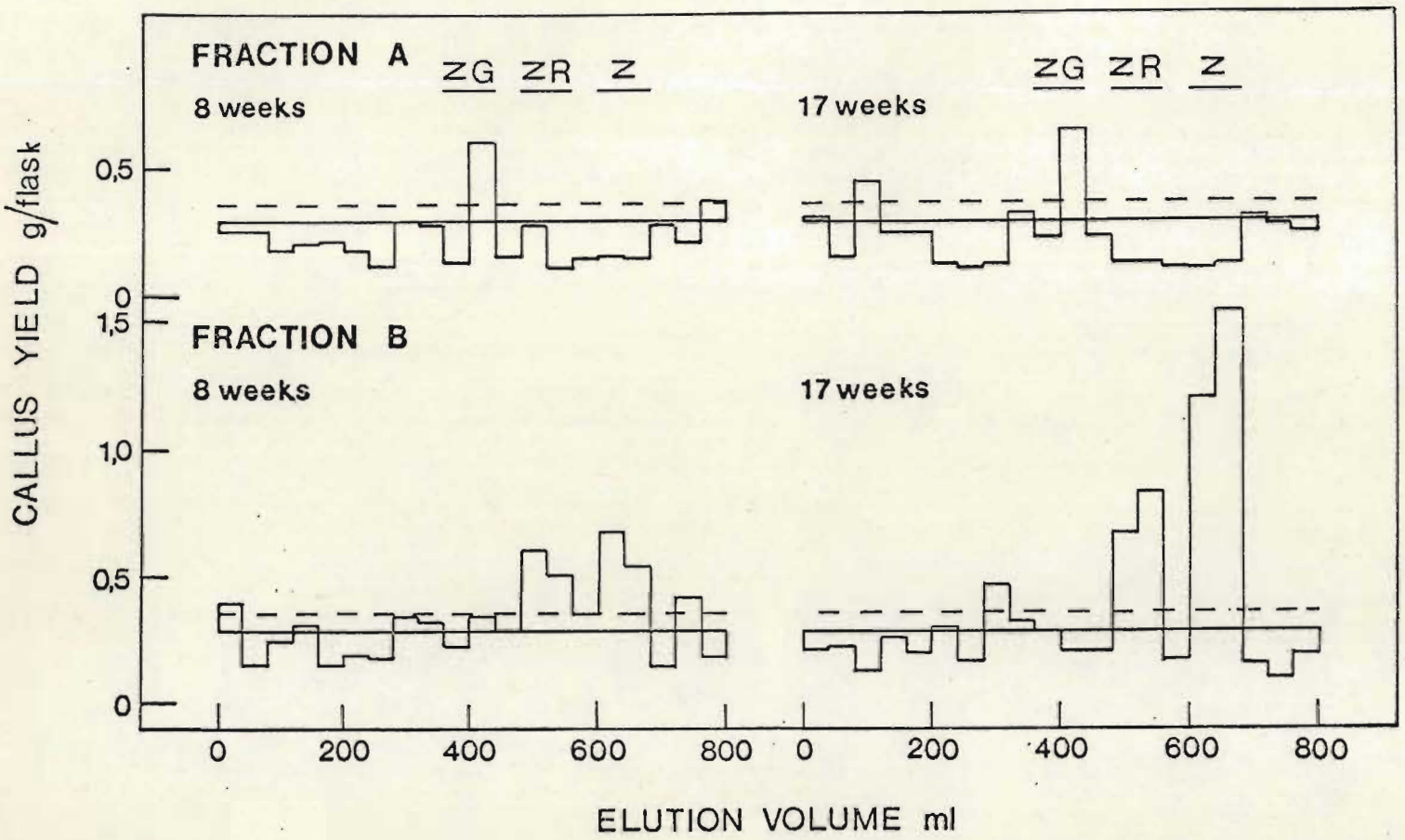



Figure 1:5. Cytokinin activity in 25 grammes of mature leaf material fractionated on a Sephadex LH-20 column. The leaf material collected from 8- and 17-week-old plants was purified using Dowex 50 cation exchange resin and the ammonia eluates separated on paper with *iso*-propanol:25 percent ammonium hydroxide: water (10:1:1 v/v). The cytokinins were eluted from $R_f$ 0,15-0,5 (Fraction A) and $R_f$ 0,5-0,9 (Fraction B) of paper chromatograms and then fractionated on Sephadex LH-20 columns. Callus grown on 10 microgrammes per litre kinetin yielded 1,73 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. Broken lines indicate the confidence limit at the level P = 0,01.

eluates from $R_f$ 0,15-0,5 of paper chromatograms (Figure 1:5). This activity had an elution volume of 400-440 millilitres.

Discussion

During the period between 5 and 17 weeks after planting there was a reduction in the level of cytokinin activity in the root exudate of the white lupin. Similar quantitative changes have been shown to occur in the root exudate of sunflowers. In this latter species the decrease in the level of cytokinin activity in the root exudate during floral development was considered to contribute to whole plant senescence which typically takes place after flowering in annuals (SITTON *et al.*, 1967). However, in tomatoes, although there was an overall decrease in the level of cytokinin activity in the root exudate during floral development, there was an increase in cytokinin activity in the root exudate during fruiting (DAVEY and VAN STADEN, 1976). In the present experiment only two samples were taken during the course of fruit development and more frequent sampling might have indicated a similar trend.

It has been proposed that the cytokinins present in the root exudate are translocated to the shoot where they contribute to the maintenance of shoot growth (BURROWS and CARR, 1969; HENSON and WAREING, 1976). When the level of cytokinin activity in the root exudate is examined in relation to that present in the leaves and terminal shoot apices, it is evident that although there was a high level of activity in the root exudate of vegetative plants (5 weeks old) the level of activity in the leaves was low (11,0 microgramme

kinetin equivalents (KE) 20 plants$^{-1}$). Cytokinin activity in the apices was too low to be estimated at this time. While the cytokinin activity in the root exudate produced by 20 plants decreased at 8 weeks, there was an increase in the level of activity associated with the leaves. Cytokinin activity in the apex remained very low.

If it is assumed that the level of cytokinin activity in the root exudate produced by 20 plants over a 24 hour period remained constant and at a level intermediate between those recorded at 5 weeks and at 8 weeks after anthesis, then the total amount of activity translocated to the shoot over the 21 day period would amount to 2652,3 KE. Over this same period however, the level of activity in the leaves of 20 plants increased from 11,0 KE per 20 plants to 48,5 KE. Thus, there was a gain of only 37,5 KE by the leaves. This situation appeared to continue between 8 and 11 weeks after planting. There, therefore, seemed to be more cytokinin entering the shoot than was accumulated in the leaves and the apices. Metabolism of these cytokinins by the leaf tissues to glucosylated derivatives did not appear to account for this build up as relatively low levels of activity co-eluting with the glucoside cytokinins were recorded in the leaves at 8 weeks (Figure 1:5). These cytokinins may therefore be utilised in the rapidly growing young shoot, retranslocated to the roots in the phloem or stored in the stem tissues. The low levels of activity recorded in the terminal shoot apices of 5- and 8-week-old plants suggested that rapid utilisation of cytokinins probably took place during growth of the young leaves and during inflorescence

development. Cytokinins, produced in the roots of the grape, are reportedly involved in the control of inflorescence development (MULLINS, 1967). They also appeared to be involved in flower development in *Bougainvillea* spp. (TSE, RAMINA, HACKETT and SACHS, 1974), and the tomato (MENARY and VAN STADEN, 1976).

The appearance of a slight peak of activity in the flowering apices of 11-week-old plants may have been indicative of the onset of cytokinin accumulation in the maturing ovaries and young fruits already present when the inflorescences were in full flower. At later developmental stages it would appear as if cytokinins accumulated at high levels in the leaves and fruiting apices while the level of activity in the root exudate was considerably reduced. As cytokinins are known to stimulate nutrient mobilization to regions of their high concentration (MOTHES and ENGELBRECHT, 1961), the presence of high cytokinin levels in the fruit tissues may have created an active sink for translocated nutrients. The occurrence of high cytokinin levels in the apices of white lupins at a time when the cytokinin levels in the root exudate were reduced, detracted from the idea that cytokinins in the fruit tissues were translocated from the roots in the xylem sap. However, although it has been suggested that the seeds are a possible site of cytokinin synthesis, there is also evidence in favour of the idea that young legume fruits are unable to synthesize cytokinins (KRECHTING *et al.*, 1978; VAN STADEN and BUTTON, 1978). Cytokinins have been shown to occur in the phloem sap of a number of plants (PHILLIPS and CLELAND, 1972; VONK, 1974; 1976; VAN STADEN, 1976b) and should cytokinin activity in the fruits originate in other

parts of the plant (e.g. stems and leaves) it is possible that it is translocated to the fruit via the phloem.

Cytokinin levels in the fruiting apices were sufficiently high to permit a cursory investigation of the nature of these compounds in lupin plants. Column chromatography of these cytokinins yielded four peaks of cytokinin activity. The co-elution of these peaks with zeatin and zeatin riboside on a column packed with Sephadex LH-20 and eluted with 35 percent ethanol indicated the presence of zeatin and zeatin riboside-like compounds in white lupins. A third peak of activity broke down after β-glucosidase treatment to yield compounds co-eluting with zeatin and zeatin riboside. This suggested that activity occurring at the elution volume 320-440 millilitres was due to compounds which were probably zeatin glucosides and zeatin riboside glucosides. The fourth peak of activity at the elution volume 160-320 millilitres failed to break down after β-glucosidase treatment indicating that it was apparently not a glucosylated derivative. A peak of activity with a similar elution volume was recorded from tomato root exudate, and it was suggested to be zeatin ribotide. However, according to MILLER (1965) it would be unusual for such a compound to remain attached to the Dowex 50 cation exchange resin under the acidic conditions used in this experiment.

Peaks of cytokinin activity with similar elution volumes described above were detected in root exudate. The detection of compounds co-eluting with zeatin and zeatin riboside in the root exudate was consistent with the results of other

workers (HORGAN *et al.*, 1973; PURSE *et al.*, 1976; VAN STADEN and MENARY, 1976). The remaining peaks of activity co-eluted with the nucleotide (160-240 millilitres) and the glucoside (280-360 millilitres) cytokinins. Although cytokinin glucosides have not previously been reported to occur in root exudates, the presence of such compounds is not unexpected as CARNES *et al.* (1975) reported the presence of no less than eight compounds with cytokinin activity in root exudates following high pressure liquid chromatography. Furthermore, cytokinin glucosides have also been shown to occur in root tissues (YOSHIDA and ORITANI, 1972). However, it has been reported that bleeding, apparently from the phloem, may occur at the cut surface of the stem of young plants, whereas in older plants it does not occur (PATE, SHARKEY and LEWIS, 1974). As glucoside cytokinins have been shown to occur in phloem sap (VAN STADEN, 1976b) it is possible that in the young plants xylem sap was contaminated with phloem sap. Activity associated with the faster and slower moving compounds decreased during fruit development.

Active compounds were detected in the leaves at all developmental stages. Column chromatography indicated that these cytokinins co-eluted with zeatin, zeatin riboside and the glucoside cytokinins (Figure 1:5). Zeatin and zeatin riboside are considered to be the free base and nucleoside cytokinins and probably the active forms (ENGELBRECHT, 1972). The glucoside cytokinins, which occur in mature leaves, are considered to be storage forms of cytokinin (HEWETT and WAREING, 1973b). They may also represent inactivation products of excess cytokinin arriving with the transpiration

stream. It is possible that the presence of glucoside cytokinins at both developmental stages is indicative of the ability of mature fully expanded leaves to metabolize cytokinins.

EXPERIMENT 2

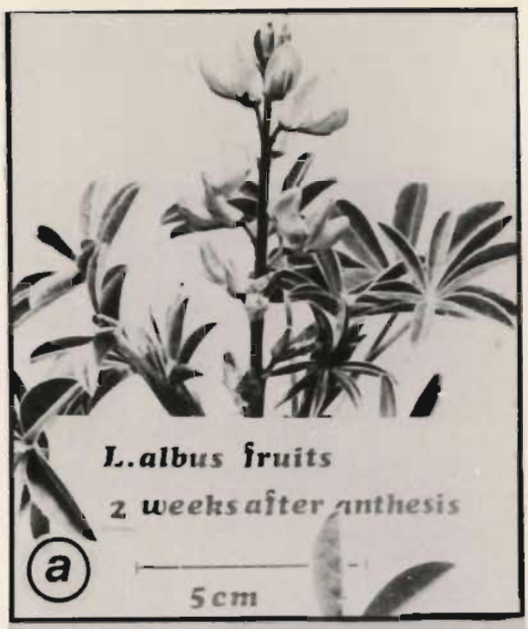
## Cytokinins in White Lupin Plants During Fruit Development at the Terminal Shoot Apex

### Introduction

In the preceding experiment, fluctuating levels of cytokinin activity were recorded in the root exudate, leaves and primary shoot apices of white lupin plants. It was established that the level of cytokinin activity in the developing apices increased markedly after flowering, despite a reduced level of cytokinin activity in the root exudate. Although the level of cytokinin activity in the leaves increased, the level of cytokinin glucoside did not increase markedly.

These findings raised a number of questions: Where did the cytokinins in the fruit tissues originate? Did the level of cytokinin activity in the root exudate increase at any stage during fruit development as apparently takes place in other plants, and, do the cytokinin glucoside levels in the leaves increase during the course of the growth cycle as in other annual plants?

In an attempt to answer some of these questions the cytokinin levels in the white lupin plant were examined during the period of fruit development. The nature and levels of cytokinin activity in the root exudate, leaves, fruits and fruit sap were therefore examined at two-weekly intervals after anthesis of the first flowers on the terminal inflorescences.


L. albus fruits
2 weeks after anthesis
a
5 cm


b


c


d


e

Experimental Procedure

The root exudate, leaves, fruits and fruit sap of *Lupinus albus* L. were harvested at 2, 4, 6, 8 and 10 weekly intervals after anthesis of the first flower on the primary inflorescence, according to procedures outlined in the materials and methods. At 2 weeks after anthesis, the fruits were small and were not divided into seed and pod fractions. At all the remaining developmental stages seeds and pods were separated. Procedures, detailed in the materials and methods section, were used for the extraction, purification, and analysis of this material. Leaves, pod walls and seed were extracted in 50 gramme (fresh weight) batches except at 4 and 6 weeks when 8 and 25 grammes (fresh weight) of seed material, respectively, were used. A sample of mature seed (6 grammes) was also extracted and analysed for cytokinin activity. At 2 weeks 16 grammes (fresh weight) of fruits were analysed for cytokinin activity. The 2-week-old fruits and 4-week-old seeds were assayed for cytokinin activity after extracts had been separated by means of paper chromatography. The equivalent of 25 grammes (fresh weight) of leaf, and pod material and the equivalent of 12,5 grammes (fresh weight) of seed material was assayed after paper chromatography. Twenty five grammes (fresh weight) of leaf material was also used for column chromatography. However, as the levels of activity recorded on paper chromatograms was exceedingly high, the equivalent of 12,5 grammes (fresh weight) of pod material and 6,25 grammes (fresh weight) of seed material was used for column chromatography. The activity at $R_f$ 0,15-0,5 of paper chromatograms

TABLE 2:1. Shoot and fruit growth. The average length of the lateral shoots was calculated from measurements made of the length of the three lateral branches closest to the terminal apex.

| Time, weeks after anthesis | 2 | 4 | 6 | 8 | 10 |
|---|---|---|---|---|---|
| Average fresh weight of the shoot (without fruits at terminal apex), g | 31,60±14,59 | 24,29± 8,75 | 32,27±12,75 | 65,88±22,13 | 66,95±25,50 |
| Average fresh weight of the fruits at the terminal apex, g | 0,85± 0,05 | 3,29± 1,24 | 14,97± 6,45 | 39,58±13,13 | 38,77±11,3 |
| Average fresh weight of the fruits at the terminal apex as a % of the total shoot weight | 2,6 | 12,0 | 31,6 | 37,5 | 36,7 |
| Average length of the lateral shoots, cm | 14,2 ± 3,7 | 17,0 ± 5,9 | 21,7 ± 7,9 | 26,9 ± 8,6 | 27,1 ± 7,1 |

of leaf, pod and seed material was eluted from the paper, treated with β-glucosidase and then passed through a Sephadex LH-20 column. The activity at $R_f$ 0,5-0,9 of paper chromatograms of the equivalent of 2 grammes (fresh weight) of seed material was treated with potassium permanganate, and passed through a column packed with Sephadex LH-20, and eluted with 35 percent ethanol.

Results

Between 2 and 8 weeks after anthesis of the first flowers on the primary inflorescences the white lupin plants increased in fresh weight (Table 2:1). As in Experiment 1 a large proportion of the fresh weight increase was attributable to the growth of fruits on the primary inflorescence, which at 8 weeks after anthesis comprised 37,5 percent of the fresh weight of the whole plant. Lateral branch growth took place between 2 and 10 weeks after anthesis. Inflorescences which developed on the lateral branches flowered approximately 4-6 weeks after anthesis of the first flowers on the primary inflorescences. By 8 weeks the plants had ceased rapid growth and the lower leaves on the plants were beginning to turn yellow, by 10 weeks these leaves were abscising.

*Root exudate*

Over the 10-week growth period root exudate production showed an initial increase between 2 and 4 weeks after anthesis but thereafter decreased to a low level at 10 weeks (Table 2:2). Chromatographic separation of root exudates on paper revealed that during the course of fruit development

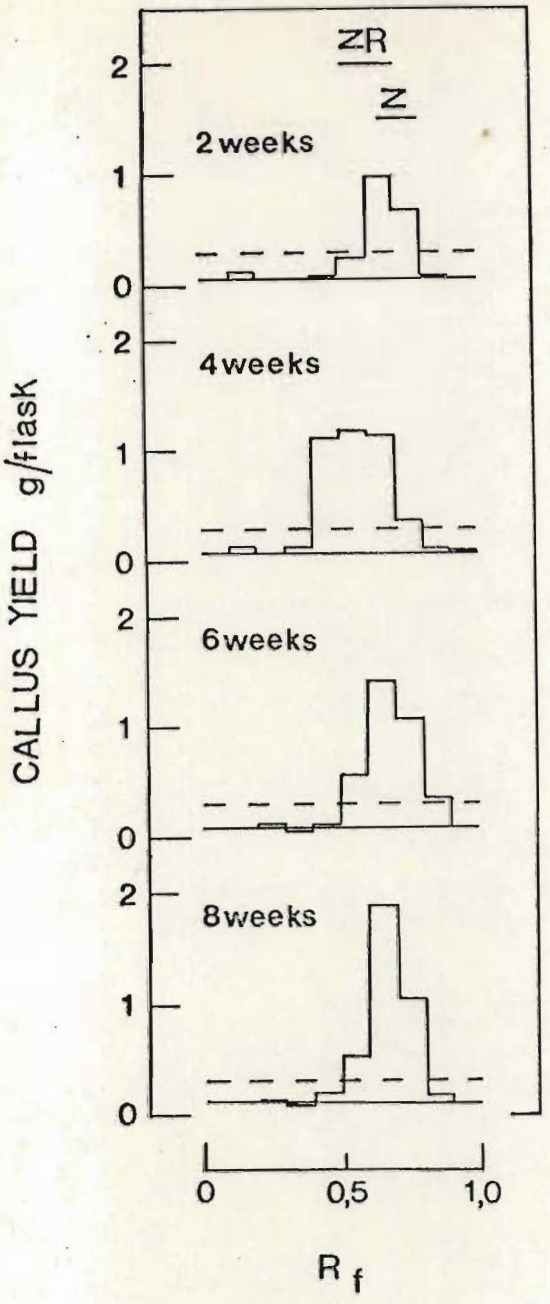



Figure 2:1. Cytokinin activity in half of the crude root exudate collected at 2, 4, 6, and 8 weeks after anthesis from 20 plants over a 24 hour period. The cytokinins in the exudate were separated on paper with *iso*-propanol:25 percent ammonium hydroxide:water (10:1:1 v/v). Callus grown on 10 microgrammes per litre kinetin yielded 0,80 grammes fresh weight. Z = zeatin; ZR = zeatin riboside. Broken line indicates confidence limit at the level P = 0,01.

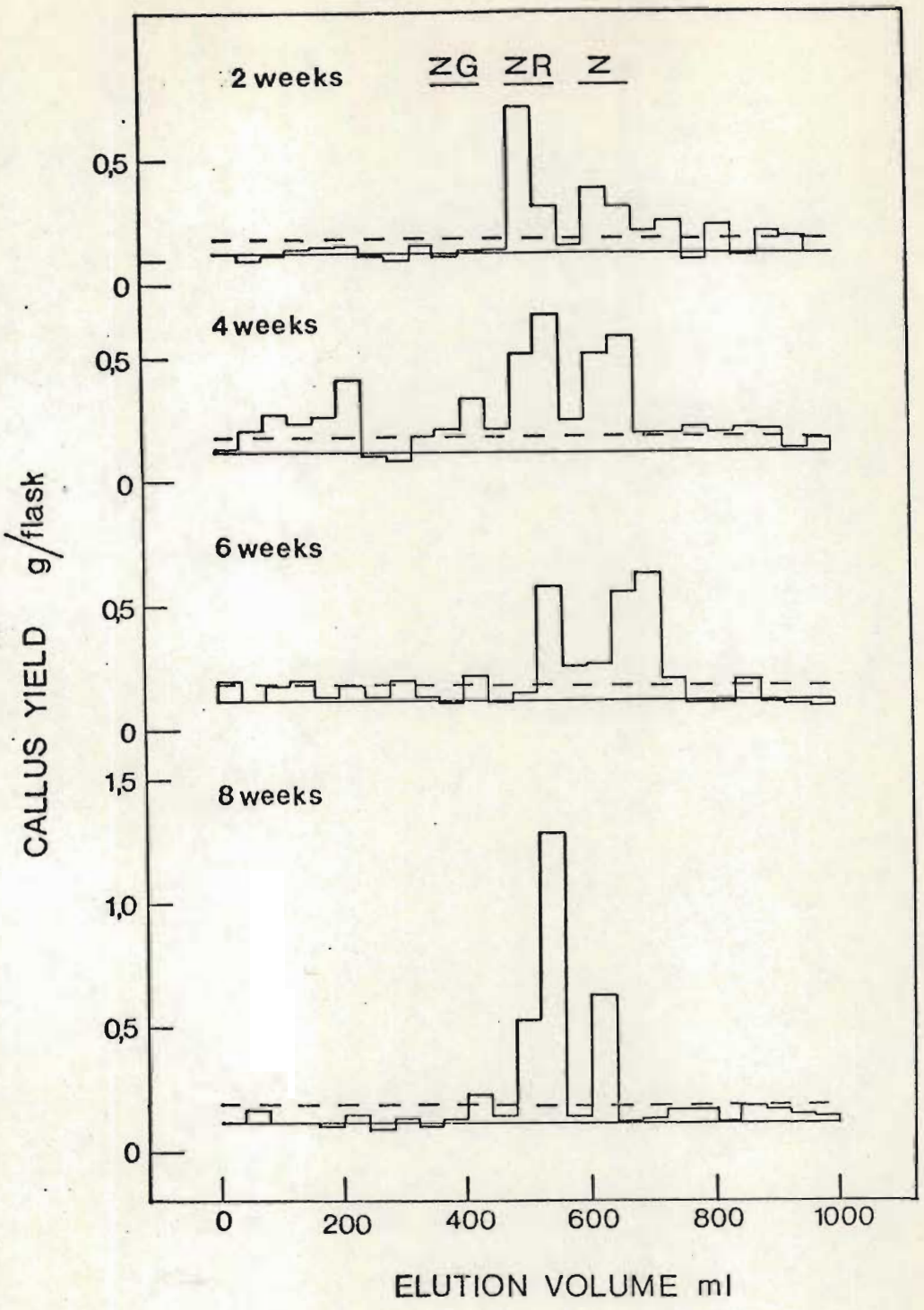



Figure 2:2. Cytokinin activity in half of the crude root exudate collected from 20 plants at 2, 4, 6 and 8 weeks after anthesis following fractionation on Sephadex LH-20. Callus grown on 10 microgrammes per litre kinetin yielded 0,29 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. Broken line indicates the confidence limit at the level P = 0,01.

cytokinins, co-chromatographing with zeatin and zeatin riboside were present in the transpiration stream (Figure 2:1). These results also indicate that the amount of cytokinin in the root exudate increased between 2 and 8 weeks after anthesis. This trend was confirmed by assaying fractions after column chromatography (Figure 2:2). Compounds co-eluting with zeatin and zeatin riboside appeared to be the major cytokinins present in the root exudate between 2 and 8 weeks after anthesis.

TABLE 2:2. Root exudate production and cytokinin translocation by white lupin plants during fruit development. The results of paper chromatography were expressed as microgramme kinetin equivalents (KE). In order to determine an approximate value for the total amount of cytokinin activity in the root exudate over the 14 day period between harvests, the activity in the root exudate at consecutive sampling times was averaged and multiplied by 14.

| Time, weeks after anthesis | 2 | 4 | 6 | 8 | 10 |
|---|---|---|---|---|---|
| Root exudate production, ml plant$^{-1}$ 24$^{-1}$ hours | 2,4 | 3,2 | 3,2 | 3,1 | 0,1 |
| Cytokinin activity in root exudate KE plant$^{-1}$ 24$^{-1}$ hours | 2,05 | 5,85 | 4,55 | 6,85 | |
| Cytokinin activity in root exudate KE plant$^{-1}$ 14$^{-1}$ days | | 55,3 | 72,8 | 79,8 | |

*Leaves*

Figure 2:3 and Table 2:3 show that the cytokinin activity in the leaf extracts after paper chromatography increased between 2 and 8 weeks after anthesis. Cytokinin activity was present on the paper chromatograms at $R_f$ 0,5-0,9.

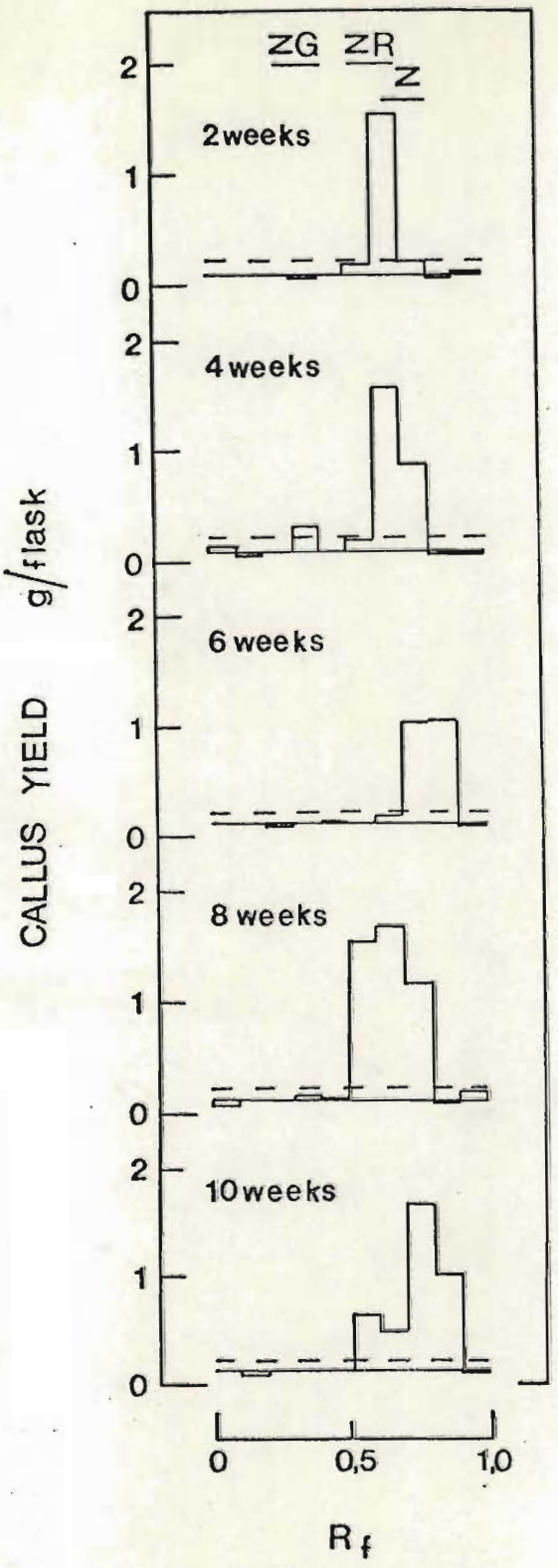



Figure 2:3. Cytokinin activity in extracts of 25 grammes of mature leaf material collected at 2, 4, 6, 8 and 10 week intervals after anthesis. The extracts were purified on Dowex 50 cation exchange resin and ammonia eluates separated on paper with *iso*-propanol:25 percent ammonium hydroxide:water (10:1:1 v/v). Callus grown on 10 microgrammes per litre kinetin yielded 1,09 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. Broken line indicates the confidence limit at the level P = 0,01.

TABLE 2:3. The cytokinin activity in 25 grammes of mature leaf material collected at 2 weekly intervals after anthesis. Extracts were separated on paper and the activity co-chromatographing with zeatin and zeatin riboside ($R_f$ 0,5-0,9) expressed as microgramme kinetin equivalents (KE).

| Time, weeks after anthesis | 2 | 4 | 6 | 8 | 10 |
|---|---|---|---|---|---|
| Cytokinin activity co-chromatographing with zeatin and zeatin riboside, KE | 13,5 | 20,0 | 18,0 | 39,5 | 31,5 |

However, as the presence of compounds co-eluting with the glucoside cytokinins appeared to be masked in previous work on cytokinins in leaf extracts (Experiment 1), paper chromatograms were eluted from $R_f$ 0,15-0,9 and fractionated on a Sephadex LH-20 column. After column chromatography cytokinins co-eluting with zeatin, zeatin riboside and the glucoside cytokinins were detected in leaf extracts (Figure 2:4). The levels of cytokinin activity co-eluting with the glucoside cytokinins increased from very low or undetectable levels at 2, 4 and 6 weeks to high levels at 8 weeks after anthesis. This activity subsequently decreased (Table 2:4).

TABLE 2:4. The cytokinin activity in extracts obtained from 25 grammes (fresh weight) mature leaf material collected at 2 weekly intervals after anthesis. Cytokinins were eluted from the paper and fractionated on Sephadex LH-20. The peaks co-eluting with the cytokinin glucosides (320-440 millilitres) are expressed as microgramme kinetin equivalents (KE).

| Time, weeks after anthesis | 2 | 4 | 6 | 8 | 10 |
|---|---|---|---|---|---|
| Cytokinin activity at elution volume 320-440 ml, KE | 3,5 | - | 1,0 | 60,5 | 24,0 |

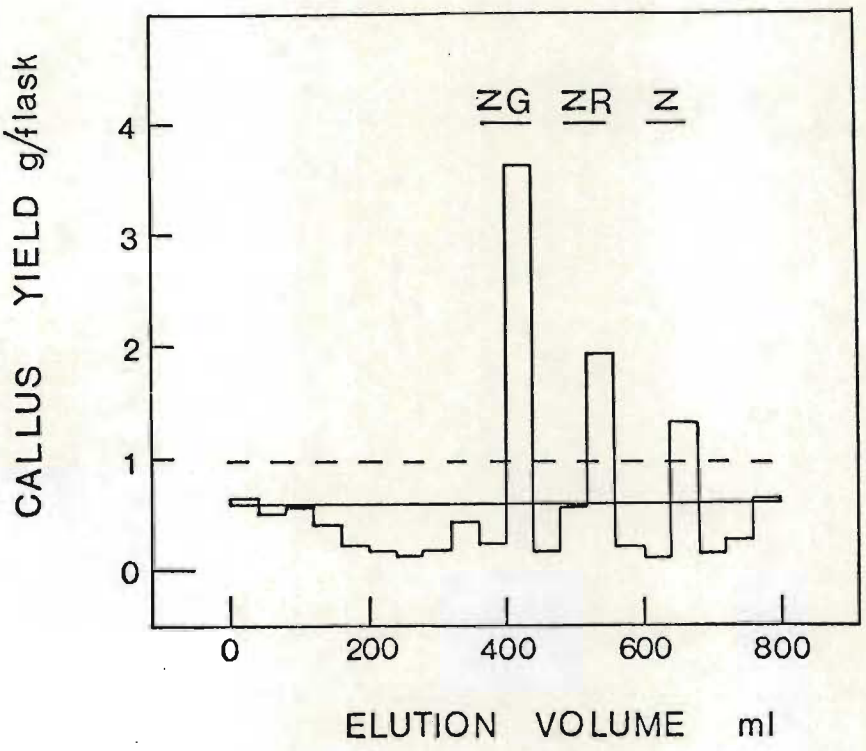



Figure 2:4. Cytokinin activity in an extract of 25 grammes of mature leaf material after fractionation on a Sephadex LH-20 column. The leaf material, collected at 8 weeks after anthesis, was purified using Dowex 50 cation exchange resin and the ammonia eluates separated on paper with *iso*-propanol:25 percent ammonium hydroxide:water (10:1:1 v/v). The cytokinins were eluted from $R_f$ 0,15-0,9 of paper chromatograms and then fractionated on the column eluted with 35 percent ethanol. Callus grown on 10 microgrammes per litre kinetin yielded 1,73 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. Broken line indicates the confidence limit at the level P = 0,01.

*Fruits at the terminal shoot apex*

Figure 2:5 shows that between 2 and 8 weeks after anthesis of the first flower on the primary inflorescence, the white lupin fruits increased in fresh weight. The pod wall increased in fresh weight between 2 and 8 weeks after anthesis. However, dehydration resulted in a loss of fresh weight at 10 weeks. Seed growth was rapid between 4 and 8 weeks. Over this period the embryo increased in size within the testa, displacing the endosperm. At two weeks after anthesis cytokinin activity was recorded in the whole fruits (Figure 2:6).

(i) Fruit sap

Cytokinin activity was detected in the sap collected from the severed fruit stalk (Figure 2:7). At 6 weeks the activity co-chromatographed on paper with zeatin, zeatin riboside and the glucoside cytokinins.

(ii) Pod wall

The assay of paper chromatograms of extracts of pod wall material indicated that the level of cytokinin activity associated with these tissues increases between 4 and 8 weeks after anthesis (Figure 2:8). By 10 weeks the level of cytokinins in the pod tissues was declining (Figure 2:8). The cytokinin activity in pod wall extracts of 4 and 6 week old material co-chromatographed predominantly with zeatin and zeatin riboside. At 8 and 10 weeks cytokinin activity was present in both fast- ($R_f$ 0,5-0,9) and slow-moving ($R_f$ 0,15-0,5) fractions. Column chromatography of activity at $R_f$ 0,15-0,5 (Fraction A) indicated that low levels of cytokinins

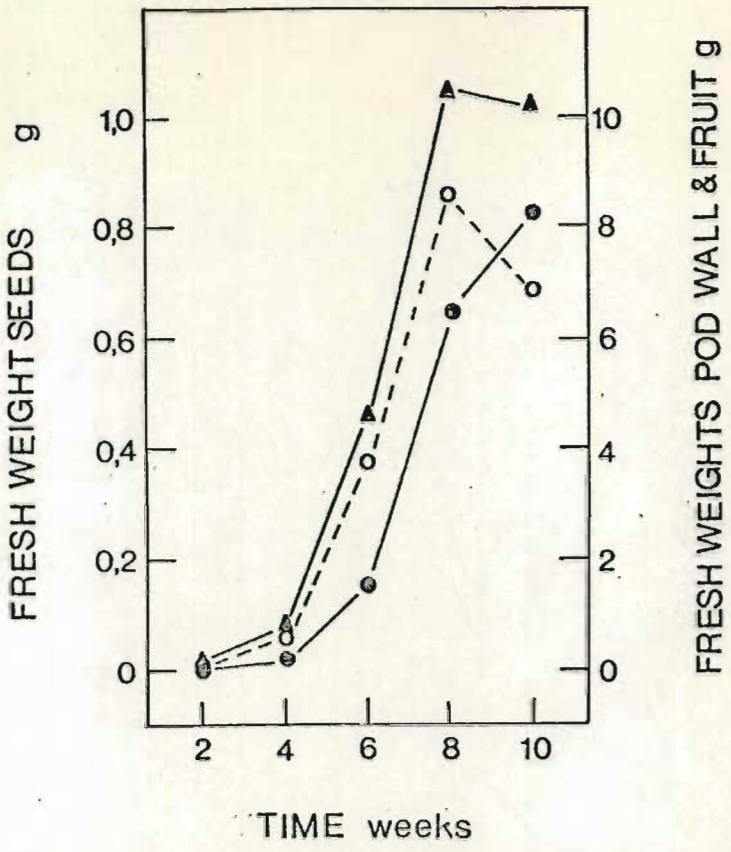



Figure 2:5. Changes in the average weights of the fruits (▲——▲), seeds (●——●), and pod walls (o——o) during development (see also Table 2:5).

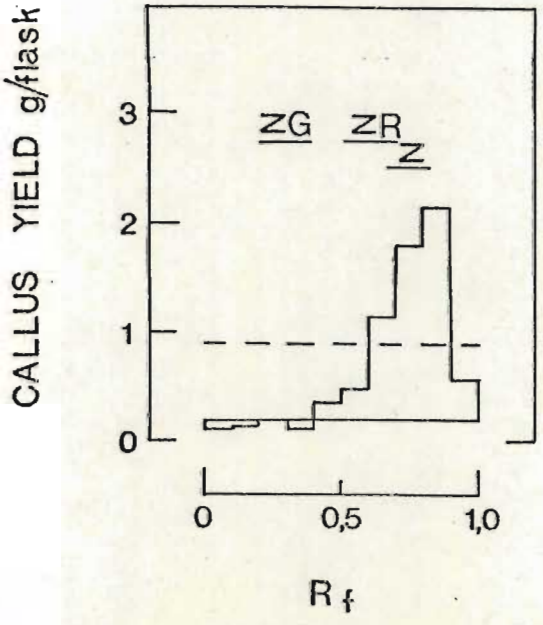



Figure 2:6. Cytokinin activity in 16 grammes of fruit material collected from the primary inflorescences of 20 plants at 2 weeks after anthesis of the lowest flower on the terminal inflorescence. The extract was purified on Dowex 50 cation exchange resin and the ammonia eluate separated on paper with *iso*-propanol:25 percent ammonium hydroxide:water (10:1:1 v/v). Callus grown on 10 microgrammes per litre kinetin yielded 0,70 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. Broken line indicates confidence limit at the level P = 0,01.

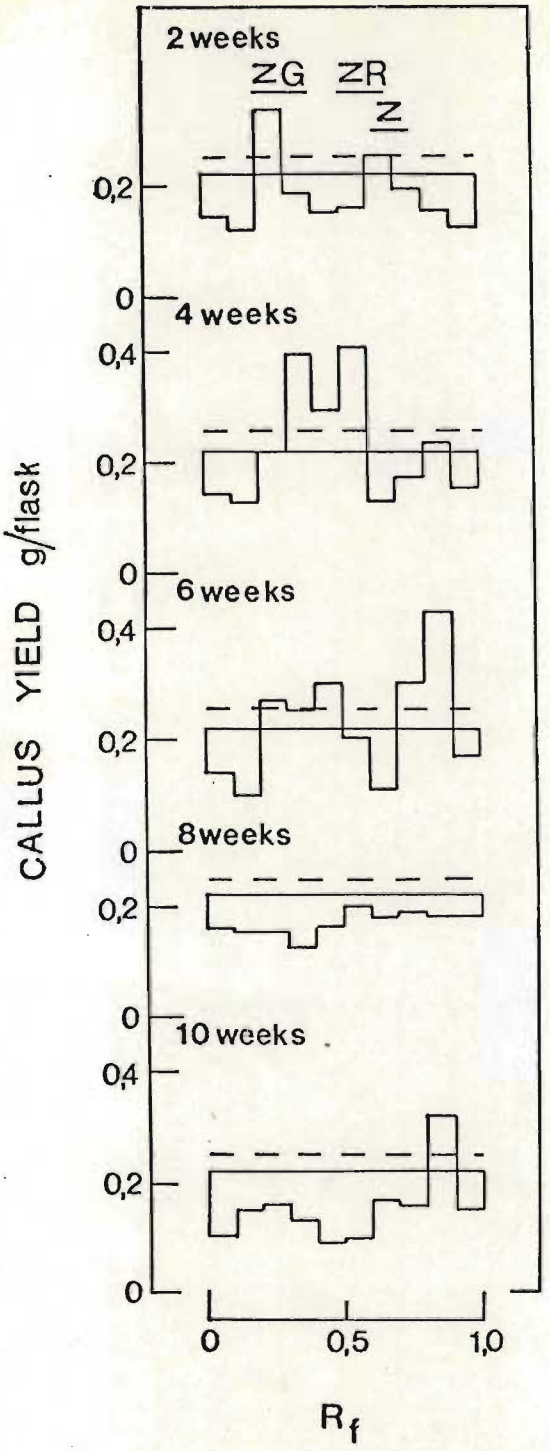



Figure 2:7. Cytokinin activity in the sap exuding from the fruit stalks at 2, 4, 6, 8 and 10 weeks after anthesis of the first fruit on the primary inflorescence. The crude sap was concentrated under vacuum, taken up in 80 percent ethanol, streaked onto Whatman No. 1 chromatography paper and separated with *iso*-propanol:25 percent ammonium hydroxide:water (10:1:1 v/v). Callus grown on 10 microgrammes per litre kinetin yielded 1,37 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. Broken line indicates confidence limit at the level P = 0,01.

were present in this fraction of the pod wall extracts at 4 and 6 weeks after anthesis (Figure 2:9). Two small peaks of activity which co-eluted with the glucoside cytokinins could be detected, while a third peak of activity co-eluted with zeatin riboside (Figure 2:9). At 8 and 10 weeks after anthesis there was a high level of activity at the elution volume corresponding to zeatin glucoside (Figure 2:4). Treatment of these extracts with β-glucosidase resulted in the disappearance of activity in this region and the appearance of activity co-eluting with zeatin and zeatin riboside (Figure 2:9) thus indicating the presence of zeatin glucoside and zeatin riboside glucoside-like compounds in these extracts. Cytokinins co-eluting with zeatin and zeatin riboside were present in Fraction B ($R_f$ 0,5-0,9) of 4-, 6-, 8- and 10-week-old pods (Figure 2:9).

(iii) Seeds

The results of the assay of paper chromatograms of seed extracts showed that the cytokinin activity present in the seeds decreased between 4 and 10 weeks after anthesis (Figure 2:10). At 4 weeks the seeds contained 61,71 KE per gramme seed material while at 10 weeks they contained 3,56 KE per gramme seed material (Table 2:5). The sample of mature seed exhibited 2,03 KE per gramme of seed material. At 4 and at 6 weeks activity was present in both A ($R_f$ 0,15-0,5) and B ($R_f$ 0,5-0,9) fractions of the paper chromatograms. However, by 10 weeks after anthesis activity was present predominantly in the B fraction and co-chromatographed

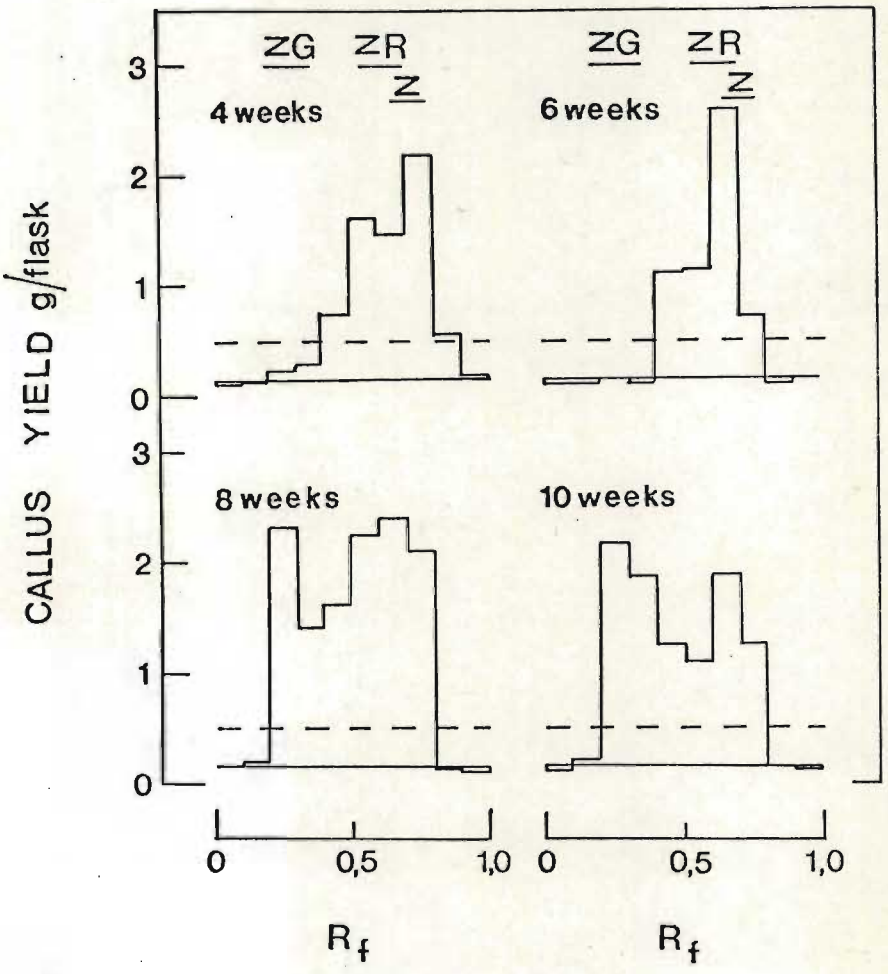



Figure 2:8. Cytokinin activity in 25 grammes of pod wall material collected at 4, 6, 8 and 10 weeks after anthesis of the first flower on the primary inflorescences. The extracts were purified on Dowex 50 cation exchange resin and the ammonia eluates separated on paper with *iso*-propanol:25 percent ammonium hydroxide:water (10:1:1 v/v). Callus grown on 10 microgrammes per litre kinetin yielded 0,86 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. Broken lines indicate the confidence limit at the level P = 0,01.

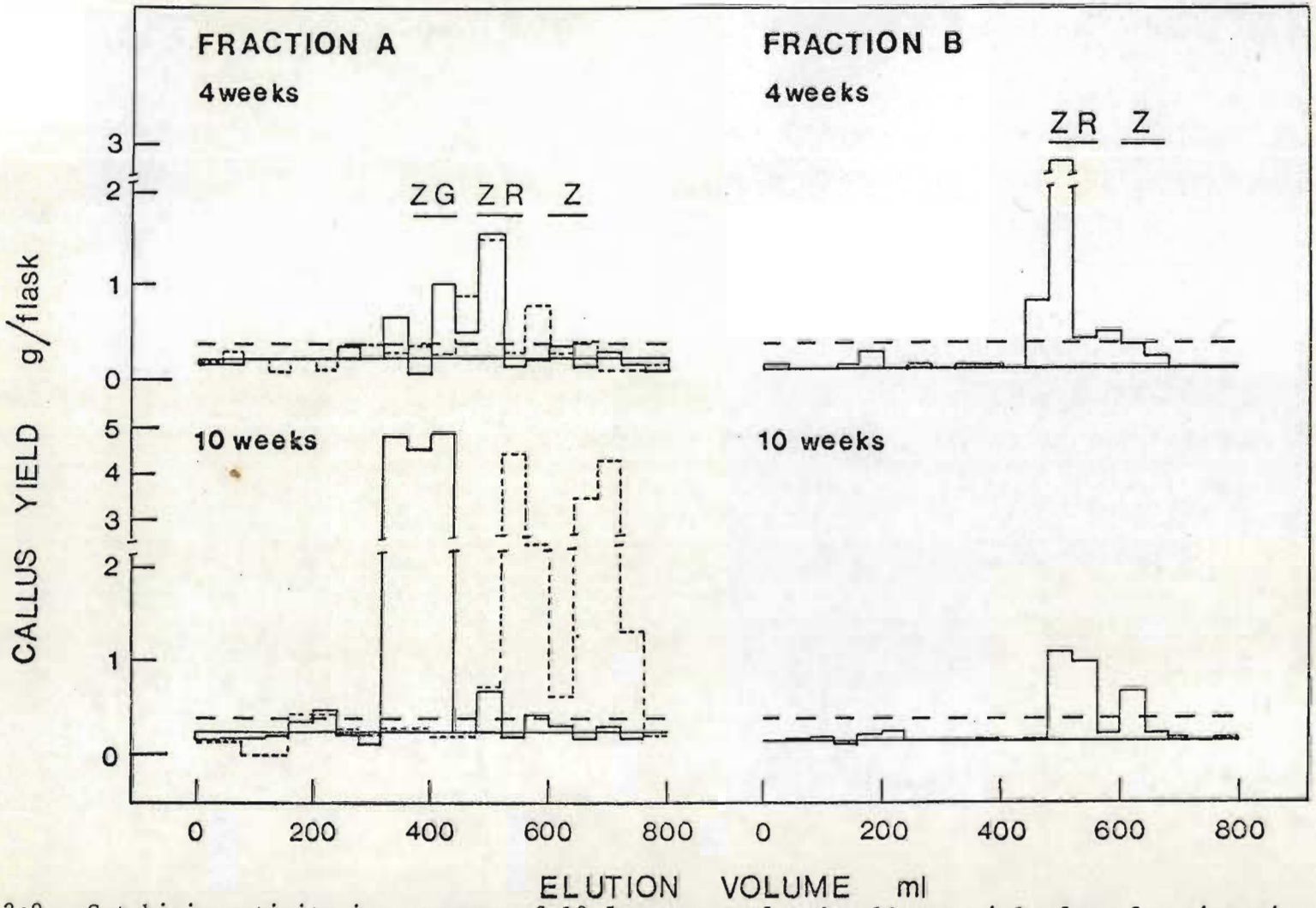



Figure 2:9. Cytokinin activity in extracts of 12,5 grammes of pod wall material after fractionation on a Sephadex LH-20 column The pod wall material collected at 4 and 10 weeks after anthesis was purified using Dowex 50 cation exchange resin and the ammonia eluates were separated on paper with *iso*-propanol:25 percent ammonium hydroxide:water (10:1:1 v/v). The activity was eluted from $R_f$ 0,15-0,5 (Fraction A) and from $R_f$ 0,5-0,9 (Fraction B) of paper chromatograms and then fractionated on Sephadex. Callus grown on 10 micrograms per litre kinetin yielded 2,30 grammes fresh weight. Broken-lined histogram indicates activity after treatment with β-glucosidase. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. Broken line indicates confidence limit at the level P = 0,01.

with zeatin and zeatin riboside (Figure 2:10).

These results were confirmed by column chromatography of A and B fractions of seed extracts (Figure 2:11). Two peaks of activity were present in the A fraction of 6-week-old seeds. The peak at elution volume 160-280 co-eluted with what have previously been described as nucleotide cytokinins (see Experiment 1). The second peak at elution volume 320-440 millilitres co-eluted with the glucoside cytokinins. Treatment of the activity in the A fraction with β-glucosidase caused this activity to co-elute with zeatin and zeatin riboside. The peak at 160-280 millilitres was not affected by glucosidase treatment. Although activity co-eluting with the glucoside cytokinins was not detected in the A fraction at 10 weeks. Cytokinins co-eluting with zeatin and zeatin riboside were present in the B fraction at 6 and at 10 weeks. KOSHIMIZU *et al.* (1967) have isolated and identified dihydro-zeatin in *Lupinus luteus* L. fruits and seeds. Extracts of white lupin seeds were therefore also examined for the possible presence of dihydro-zeatin using potassium permanganate to oxidise unsaturated compounds. The results (Figure 2:12) indicate that after treatment of 2 grammes of the B fraction of 8-week-old seed extracts the callus yield at elution volumes corresponding to zeatin and zeatin riboside on Sephadex LH-20 was reduced by 63,1 percent and 41,9 percent respectively, thereby introducing the possibility of the occurrence of dihydro-zeatin and

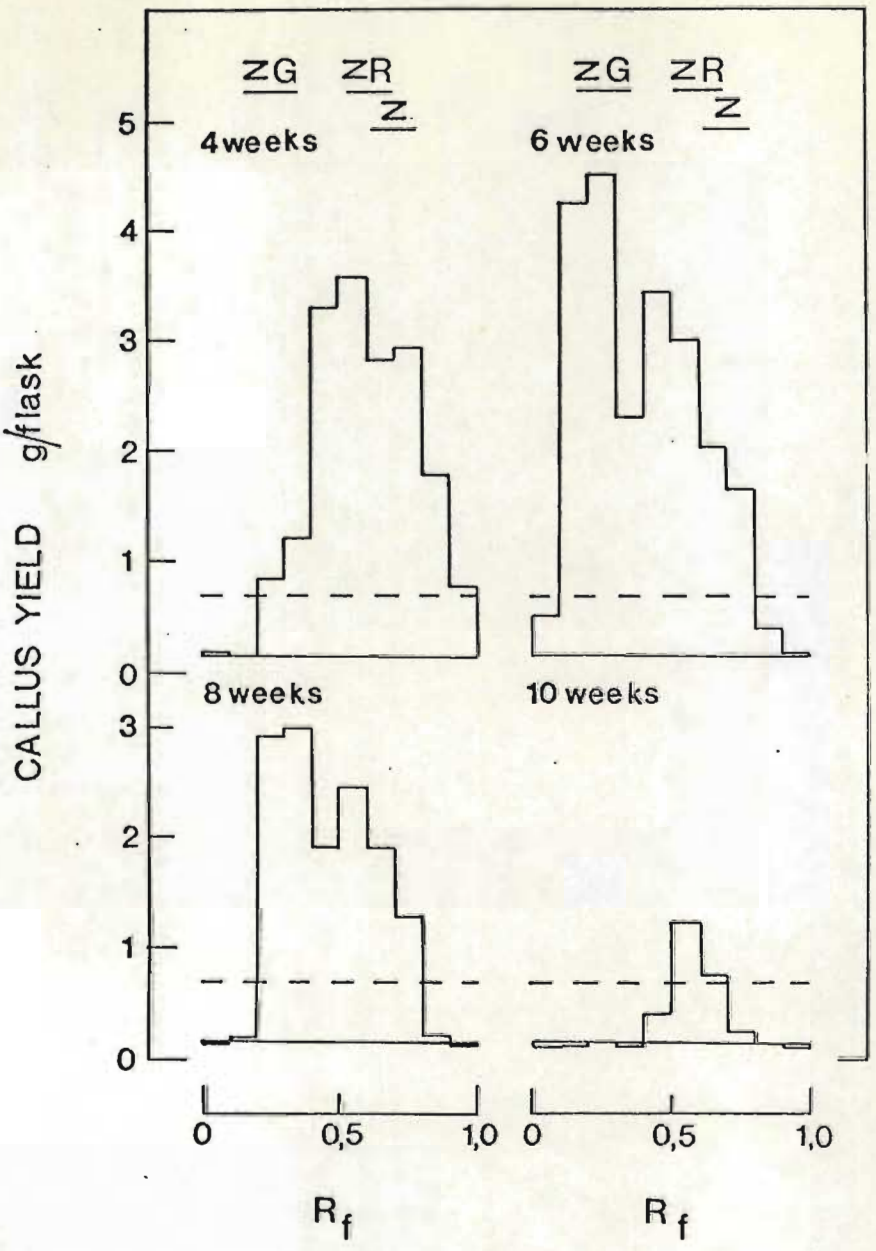



Figure 2:10. Cytokinin activity in extracts of seed material collected at 4, 6, 8 and 10 week intervals after anthesis. At 4 weeks 8 grammes of material was analysed and thereafter 12,5 grammes. The extracts were purified on Dowex 50 cation exchange resin and the ammonium eluates separated on paper with *iso*-propanol:25 percent ammonium hydroxide:water (10:1:1 v/v). Callus grown on 10 microgrammes per litre kinetin yielded 0,70 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. Broken line indicates confidence limit at the level P = 0,01.

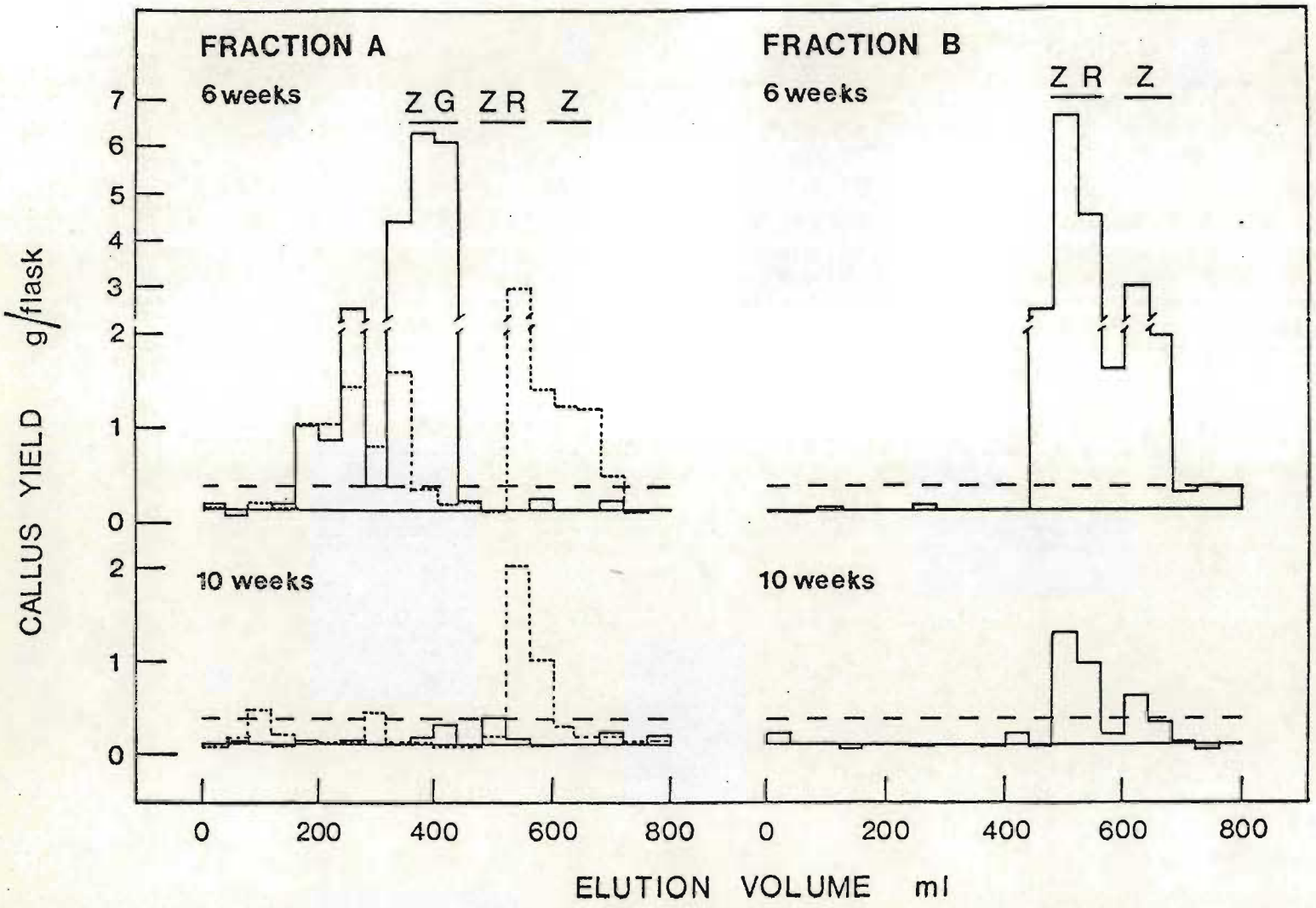



Figure 2:11. Cytokinin activity in extracts of 6,25 grammes of seed material after fractionation on a Sephadex LH-20 column. The seed material, collected at 6 and 10 weeks after anthesis, was purified using Dowex 50 cation exchange resin and the ammonia eluates separated on paper with *iso*-propanol:25 percent ammonium hydroxide:water (10:1:1 v/v). The cytokinins were eluted from $R_f$ 0,15-0,5 (Fraction A) and from $R_f$ 0,5-0,9 (Fraction B) of paper chromatograms and then fractionated on Sephadex. Callus grown on 10 microgrammes per litre kinetin yielded 0,92 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. Broken-lined graph represents result after β-glucosidase treatment. Broken line indicates the confidence limit at the level P = 0,01.

dihydro-zeatin riboside in white lupin seeds.

## Discussion

In the white lupin, as in other annuals, development of the fruits leads ultimately to a reduction in growth, and subsequently to whole plant senescence. The decreased levels of cytokinin activity in the root exudate which accompany floral development in the sunflower have been suggested to lead to shoot senescence (SITTON *et al.*, 1967). Although the results of Experiment 1 indicated that there was an overall decrease in the cytokinin levels of the root exudate during the vegetative and reproductive phases of growth, a closer examination of cytokinin levels in fruiting plants has indicated that the level of cytokinin activity may fluctuate during the fruiting period. It was low at the early stages of fruit development (2 weeks after anthesis), and reached higher levels between 4 and 8 weeks after anthesis (Table 2:2, Figures 2:1 and 2:2). Root exudate production was low at 10 weeks after anthesis and the 2 millilitres of root exudate collected from 20 plants, was not examined for cytokinin activity. Although the level of cytokinin activity in the root exudate increased during fruit development, the diversity of cytokinins present in the root exudate was reduced, especially if this result is compared with that recorded from young vegetative plants (Experiment 1).

As in Experiment 1, the cytokinin activity in the leaf extracts increased during the course of fruit development. The levels of cytokinin activity co-eluting with the

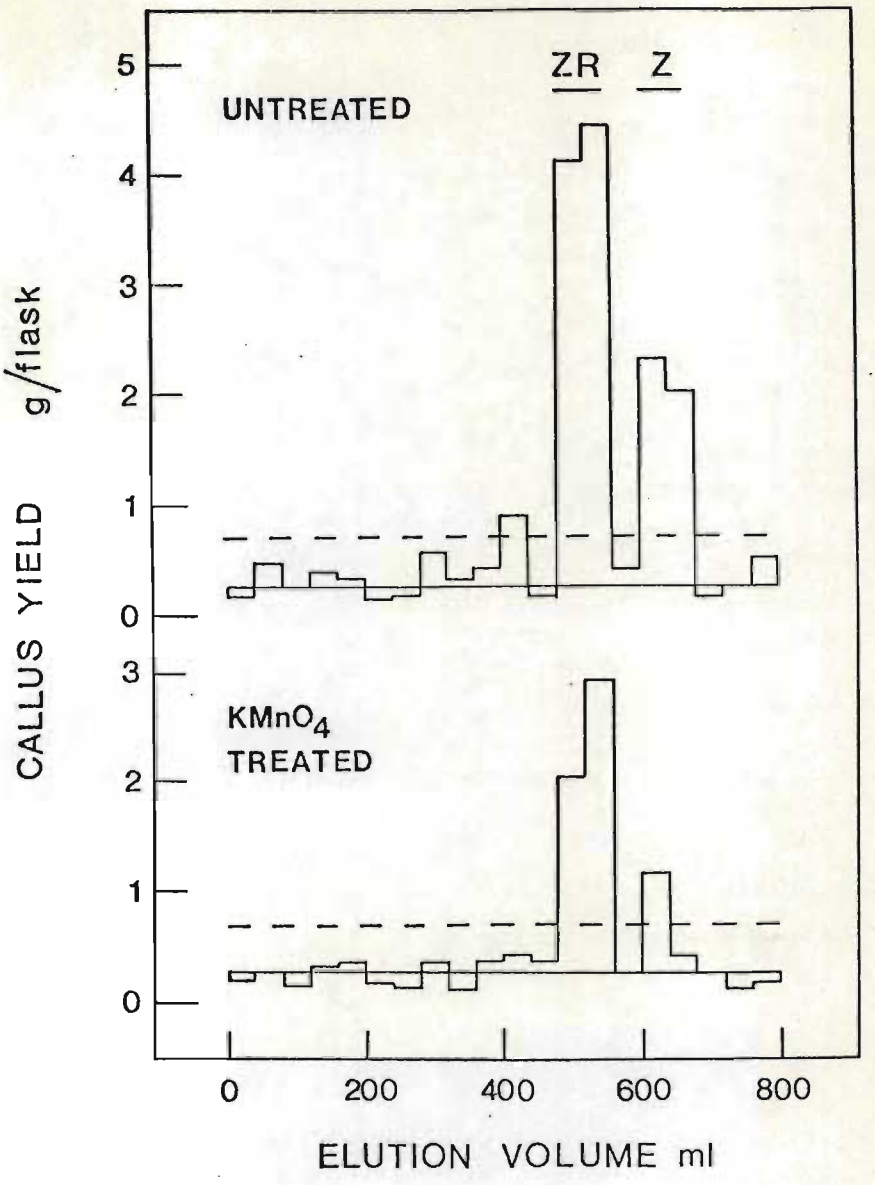



Figure 2: 2. Cytokinin activity in extracts of 2 grammes of seed material collected at 8 weeks after anthesis before and after treatment with potassium permanganate. The seed material was purified using Dowex 50 cation exchange resin and the ammonia eluates separated on paper with *iso*-propanol:25 percent ammonium hydroxide:water (10:1:1 v/v). The cytokinins were then eluted from $R_f$ 0,5-0,9 of paper chromatograms treated, and fractionated on Sephadex LH-20. Callus grown on 10 microgrammes per litre kinetin yielded 1,50 grammes fresh weight. Z = zeatin; ZR = zeatin riboside. Broken line indicates confidence limit at the level P = 0,01.

glucoside cytokinins increased from low and undetectable levels at 2, 4 and 6 weeks to higher levels at 8 and 10 weeks. The appearance of glucoside cytokinins at high levels in mature senescing leaves has been noted by a number of workers (HEWETT and WAREING, 1973b; VAN STADEN, 1976b; 1976d; WAREING *et al.*, 1976). WAREING *et al.* (1976) indicated that the glucoside cytokinins in the leaf tissues may be derived from zeatin and zeatin riboside imported via the transpiration stream. Leaf tissue has the ability to convert zeatin and its riboside to their glucoside forms (LETHAM *et al.*, 1976). Such a conversion may be a means of inactivating excess cytokinin entering the leaves in the transpiration stream (HENSON and WAREING, 1976). As the free base cytokinins are present in the root exudate of white lupins, and as high levels of glucoside cytokinins are present in mature and senescing leaves (8 and 10 weeks after anthesis), it seems likely that these processes take place in the white lupin leaves. As cytokinins applied to leaf tissues have been shown to promote the mobilisation of nutrients to regions of their high concentration (MOTHES and ENGELBRECHT, 1961), the inactivation of the cytokinins in the leaves, by conversion to the glucoside form, would be of advantage to the plant. It would prevent the leaf from acting as a sink for photosynthates, and allow for the translocation of these compounds to the non-photosynthetic parts of the plant and to the seeds. Cytokinins in the senescent leaves appear to be lost to the plant upon leaf abscission.

Although the cytokinins present in the transpiration

TABLE 2:5. The cytokinin activity in the seeds, fruits, pod walls and apices of white lupin plants during development. The callus yield calculated from paper chromatograms is expressed as microgramme kinetin equivalents (KE).

| Time, weeks after anthesis | 2 | 4 | 6 | 8 | 10 |
|---|---|---|---|---|---|
| Cytokinin activity in seeds, KE $g^{-1}$ | | 61,71 | 55,60 | 29,30 | 3,56 |
| Average weight of seeds, g | | 0,023±0,01 | 0,159±0,09 | 0,635±0,14 | 0,851±0,09 |
| Cytokinin activity in seeds KE $seed^{-1}$ | | 1,42 | 8,84 | 18,60 | 3,02 |
| Average number of seeds $fruit^{-1}$ | | 4,60±0,93 | 4,36±1,19 | 4,28±1,25 | 4,20±1,15 |
| Cytokinin activity in seeds KE $seed^{-1} fruit^{-1}$ | | 6,52 | 38,54 | 79,60 | 12,68 |
| Cytokinin activity in pod walls, KE $g^{-1}$ | | 5,67 | 4,32 | 10,67 | 9,08 |
| Average weight of pod walls, g | | 0,81±0,45 | 4,05±1,68 | 8,97±2,57 | 7,08±2,13 |
| Cytokinin activity in pod wall, KE $pod\ wall^{-1}$ | | 4,57 | 17,50 | 95,70 | 64,28 |
| Cytokinin activity in fruit KE $fruit^{-1}$ | 1,38 | 11,09 | 56,04 | 175,30 | 76,96 |
| Average number of fruits at terminal apex | 4,80±1,20 | 3,70±0,90 | 3,30±0,07 | 3,80±0,89 | 3,80±1,00 |
| Cytokinin activity in fruits at terminal apex, KE $plant^{-1}$ | 6,62 | 41,03 | 184,93 | 665,00 | 292,45 |

stream appeared to accumulate in the leaves, as in the previous experiment, cytokinins were also present in the young fruits on the primary inflorescence (Table 2:5). However, the accumulation of glucoside cytokinins in older leaves may also be indicative of the fact that these compounds are utilized more readily in younger leaves than in older leaves.

The high level of activity recorded in the young fruits (Figure 2:6) when compared with the level in the primary inflorescence of flowering plants (Experiment 1) indicates that cytokinins accumulate in the growing fruit tissues. Furthermore the level of cytokinin activity in the fruits on the primary inflorescence continued to increase up to 8 weeks after anthesis. As was pointed out in the introduction to this experiment, the origin of the cytokinins in the fruit tissues is unclear. The root tissues are considered to be the major if not the only region of cytokinin production in the plant (VAN STADEN and SMITH, 1978). Assuming that this situation exists in the plant then, if the cytokinin activity in the root exudate is indicative of the amount of cytokinin transported from the root tissues to the shoot tissues, it would appear as if insufficient cytokinin activity was detected in the root exudate between 6 and 8 weeks after anthesis to allow for the high levels of activity in the fruits to have originated in the roots and for it to have been translocated to the shoot only in the xylem sap. If the roots are the only source of cytokinins to the above ground parts of the plant, the possibility that cytokinins are not only translocated to the shoot in the root exudate but may also

be translocated to the shoot via the phloem must be considered. In the white lupin, cytokinins were present in the sap exuding from the severed fruit stalk or pedicel (Figure 2:7). This exudate is considered to be composed largely of phloem sap (PATE, SHARKEY and LEWIS, 1974), and cytokinins may therefore be translocated within the phloem of white lupins.

Although the roots have been considered to be sites of cytokinin biosynthesis in the plant, alternative sites have also been proposed. The high levels of cytokinin activity recorded in the seed tissues of many species have been considered to be indicative of a region of cytokinin biosynthesis (LETHAM and WILLIAMS, 1969). However, as in senescent leaf tissues, this high level may merely represent a site of cytokinin inactivation. The presence of high levels of glucoside cytokinins in the young white lupin seeds (Figures 2:10 and 2:11) would appear to endorse the latter idea.

The presence of cytokinins in the fruit sap indicates that some, if not all, the cytokinins present in fruit tissues may originate in parts of the plant body other than the fruit itself. The relocation of cytokinins within the shoot, to regions of intense metabolic activity, such as occur in developing pods and seeds, may therefore also contribute to the high levels of cytokinin activity detected in the developing fruits. Indeed, there appears to be an interrelationship between the cytokinin levels in various regions of the shoots of different plants for VARGA and

BRUINSMA (1974) have shown that reduction of foliage causes a large increase in cytokinins in the fruits of the tomato, while HOAD *et al.* (1977) working with the grape, indicated that fruit removal from rooted cuttings resulted in an increased level of cytokinin glucoside in the leaf tissues.

The cytokinins present in fruits and seeds have been investigated by numerous workers (MILLER, 1965; KOSHIMIZU *et al.*, 1970; BLUMENFELD and GAZIT, 1970; LETHAM, 1974; HAHN *et al.*, 1974; VAN STADEN and DREWES, 1975; VAN STADEN and STEWART, 1975; SCHULMAN and LAVEE, 1976). These compounds and other plant growth regulators, seem to be involved in a source-sink type mechanism for the attraction of metabolites and mineral elements to developing fruits (SETH and WAREING, 1967; LUCKWILL, 1977). In the white lupin, the pod wall and endosperm act as temporary reservoirs for carbohydrate and nitrogenous compounds which are subsequently incorporated into the embryo of the developing seed (PATE, SHARKEY and ATKINS, 1977). Fruit development also depends largely on a supply of these compounds for building new tissues (LUCKWILL, 1977). In the pod wall tissues a high level of cytokinin activity was recorded at 8 weeks after anthesis. The fresh weight of the pod declines after this time, and it has been shown that between 8 and 12 weeks after anthesis, the pod transfers 16 percent of its nitrogen to the seed (PATE *et al.*, 1977). The potential of the pod wall to act as a storage organ appears to be maximal after 8 weeks. At this time high levels of both free base and bound forms of cytokinin were recorded in the pod tissues. As in pea seeds (BURROWS and CARR, 1970) there

appeared to be a high level of cytokinin activity (on the basis of the activity per gramme seeds fresh weight) when the endosperm was present. However, as the degree of seed development varies between fruits, it remains for further experiments using seeds harvested at more regimented time intervals to assess the significance of this finding.

During the early stages of fruit development in the white lupin (4 weeks after anthesis), the extracts of pod wall material exhibited low levels of cytokinin activity (Figure 2:8). At this time the cytokinins present co-eluted mainly with zeatin and zeatin riboside. However, with maturity, there was an increase in the level of cytokinin activity in the pods, which was characterized by an increase in the levels of activity co-eluting with zeatin and zeatin riboside, as well as by the appearance of glucoside cytokinins in the pod wall tissues. Thus, the pattern of cytokinin accumulation in the leaves and in the pod walls of the white lupin is similar.

In the seed tissues high levels of cytokinin activity (on activity per gramme basis) were present during the early stages of growth and these decrease to low levels in the maturing seeds at the terminal shoot apex (Table 2:5). The onset of maturity in the seeds is characterized by a decrease in the cytokinin glucosides and nucleotides, in addition to the overall decreased levels of cytokinins co-eluting with zeatin and zeatin riboside. These cytokinins may either be utilised during the course of seed development or translocated away from the maturing seeds. The presence of glucoside

cytokinins in the seeds, however, would appear to indicate that these cytokinins are stored in the young seed as an inactive form, for later use during maturation. HEWETT and WAREING (1973b) suggested such a function for similar compounds in poplar leaves. LORENZI *et al.* (1975) were able to demonstrate that glucoside cytokinins accumulated in *Picea sitchensis* leaves during the growing season may be converted to the free base and nucleoside forms when active growth is resumed in spring. Furthermore, soybean callus also has the ability to hydrolyse zeatin glucoside when it is incorporated into the growing medium (VAN STADEN and PAPAPHILIPPOU, 1977). ENGELBRECHT (1972) suggested that compounds co-eluting with the nucleoside and glucoside cytokinins, which were later shown to occur in leaves (HEWETT and WAREING, 1973b), may undergo reversible sequestration to the free base forms. It is possible that such sequestration may make the cytokinins in the seeds readily available for utilisation during seed development and maturation.

Compounds in white lupin seed extracts exhibiting cytokinin activity, and co-eluting with zeatin and zeatin riboside after potassium permanganate treatment, were probably dihydro-derivatives of zeatin and its riboside (Figure 2:12). Although the significance to the plant of compounds with a saturated side chain is not understood, WAREING *et al.* (1976) have suggested that these compounds are more resistant to attack by enzymes of the cytokinin oxidase type. Purified "cytokinin oxidase" type enzymes from maize have been shown to readily cleave cytokinin side chains of the *iso*-pentenyl type, while saturated side chains are not attacked (WHITTY

and HALL, 1974). The present results indicate the possible presence of dihydro-zeatin and its riboside in lupin seeds, and also stress the need for a greater understanding of the precise nature of the cytokinins present in a tissue in order to assess their importance to plant growth and development.

# PART II

## THE EMBRYONIC PLANT

### EXPERIMENT 3

### Cytokinin Levels in Maturing White Lupin Fruits in Relation to the Growth and Development of the Embryo

#### Introduction

During the reproductive growth phase of the white lupin, fruits are formed on the primary inflorescence and subsequently on the secondary inflorescences. Previous experiments have shown that the cytokinin activity in the fruit increases and then decreases during the course of development. In these studies fluctuating cytokinin levels in the fruits were related to the growth and cytokinin status of the whole plant. Enclosed within the pod wall and testa of the fruit is a young embryonic plant which, during this first phase of its growth, is dependent on the parent plant for a supply of nutrients until its maturation. Cytokinins appear to be involved in some systems in the process of nutrient mobilization (MOTHES *et al.*, 1959; 1961; MOTHES and ENGELBRECHT, 1961) and it is significant that plant growth substances including the cytokinins have been closely linked with the nutrient status of developing fruits (LUCKWILL, 1977). These considerations, in addition to the fact that embryology is a further aspect of whole plant growth, prompted a closer examination of cytokinin levels in the fruits and seeds in relation to the growth and development of the embryo.

Plate 3:1. The white lupin flower with ovary exposed at anthesis (top left). The senescent flower at 3 days after anthesis with developing fruit exposed (top right). From left to right in lower row are developing fruits at 6, 9 and 12 days after anthesis.

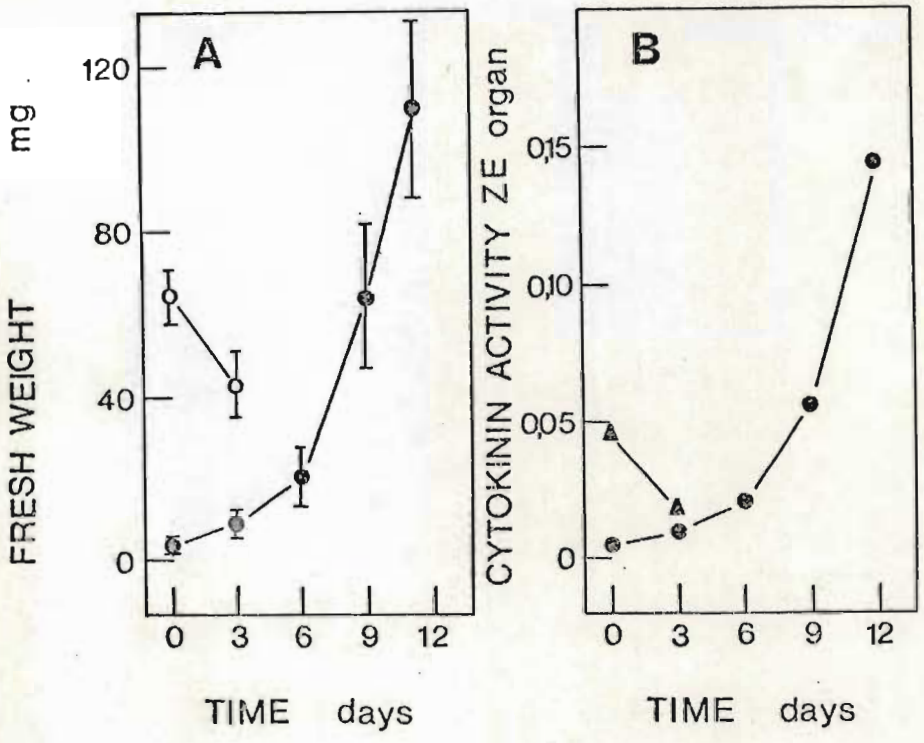



Figure 3:1. A. The changing fresh weights of flowers, ovaries and developing fruits of white lupins from anthesis to 12 days after anthesis. B. Changing cytokinin levels in the flower parts, ovaries and developing white lupin fruits from anthesis to 12 days after anthesis. The callus yield after column chromatography on Sephadex LH-20 was expressed as microgram zeatin equivalents per organ.

It has been established that the growth of the white lupin fruit is not rapid during the first two weeks after anthesis (PATE *et al.*, 1977). However, at the time of flowering, a slight increase in the level of cytokinin activity was observed in the inflorescence (Experiment 1). This was considered to be due to the presence of young developing fruits on the inflorescence. In order to establish the cytokinin status of the flowers and young fruits the flower at anthesis was selected as the starting point for this study. The growth of the developing embryo was monitored at the gross morphological, light microscope and electron microscope level.

Experimental Procedure

For convenience, this study was divided into two parts. In the first section developmental changes taking place during fruit development are discussed, with special attention being given to anatomical changes in the embryo. Embryonic material was studied at the light and electron microscope level according to procedures outlined in the materials and methods. Material was examined at two-weekly intervals after anthesis of the flower. In order to supplement this, some additional material was collected and examined at intermediate stages.

In the second section the cytokinin levels in the flower parts and developing fruits were examined first at anthesis and at 3 days after anthesis. Thereafter the developing fruits were examined at 6, 9 and 12 days after anthesis, and then at two weekly intervals after anthesis. Floral parts, excluding ovaries (i.e. petals, sepals, stamens), and ovaries

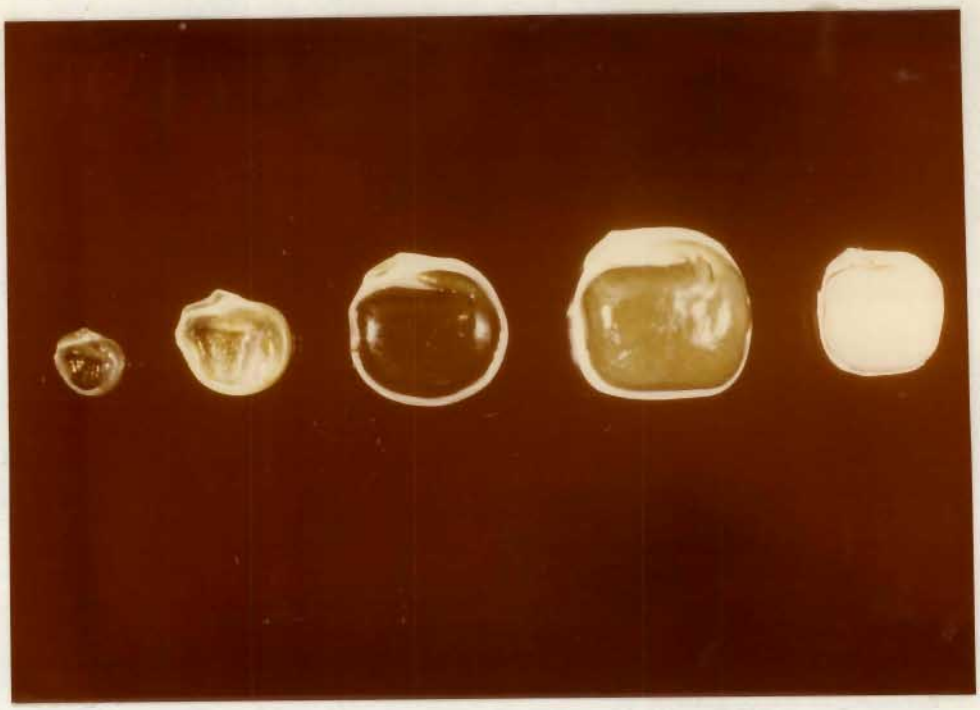


Plate 3:2. The white lupin seed at 4, 6, 8 and 10 weeks after anthesis and at maturity with part of the testa removed to expose the embryo.

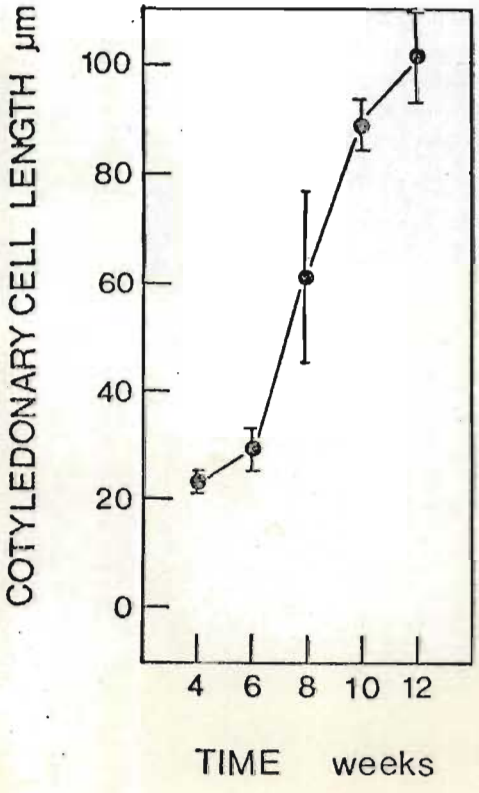



Figure 3:2. Increase in length of cotyledonary cells between 4 and 12 weeks after anthesis.

were analysed for cytokinin activity at anthesis and at 3 days after anthesis. Thereafter, the level of cytokinin activity in the developing fruits was monitored. Floral parts were extracted and assayed in 8 gramme batches while 2,5 grammes of ovary and developing fruit material was used at all developmental stages up to 12 days after anthesis. At two weeks after anthesis, although the fruits were small, they were divided into pod wall and seed fractions. At later developmental stages the seed was broken up into its component parts and each analysed separately for cytokinin activity. Four week old seeds were divided into testa and endosperm fractions, while the embryos and suspensors were removed from a further sample of thirty seeds, for separate analysis. Six and eight week old seeds were divided into testa, endosperm and embryo fractions, and at 10 weeks after anthesis the endosperm was no longer present and the seeds therefore were divided only into embryo and testa fractions.

Although attempts were made to extract material of the same organ or part thereof in batches of uniform fresh weights, this proved to be exceedingly difficult as only limited amounts of material were available, and the fresh weight changes in the fruits and different parts of the fruits were considerable at different developmental stages. Pod material was extracted and analysed in 5 gramme batches. The 2-week-old seed sample weighed 0,88 grammes. Testa material was extracted at 4, 6, 8 and 10 weeks after anthesis in 2,5 gramme lots, while endosperm material harvested at 4, 6 and 8 weeks after anthesis was analysed in 0,25 gramme quantities. At 4 weeks after anthesis the embryos and suspensors removed

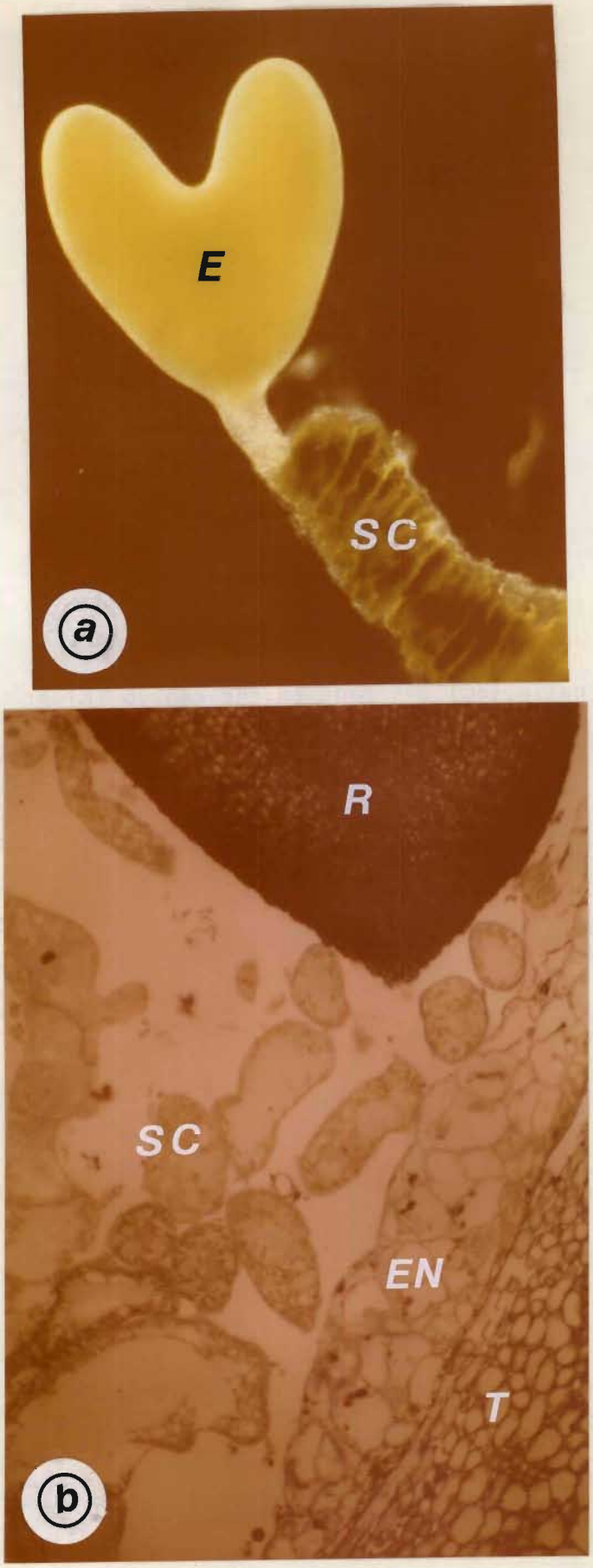
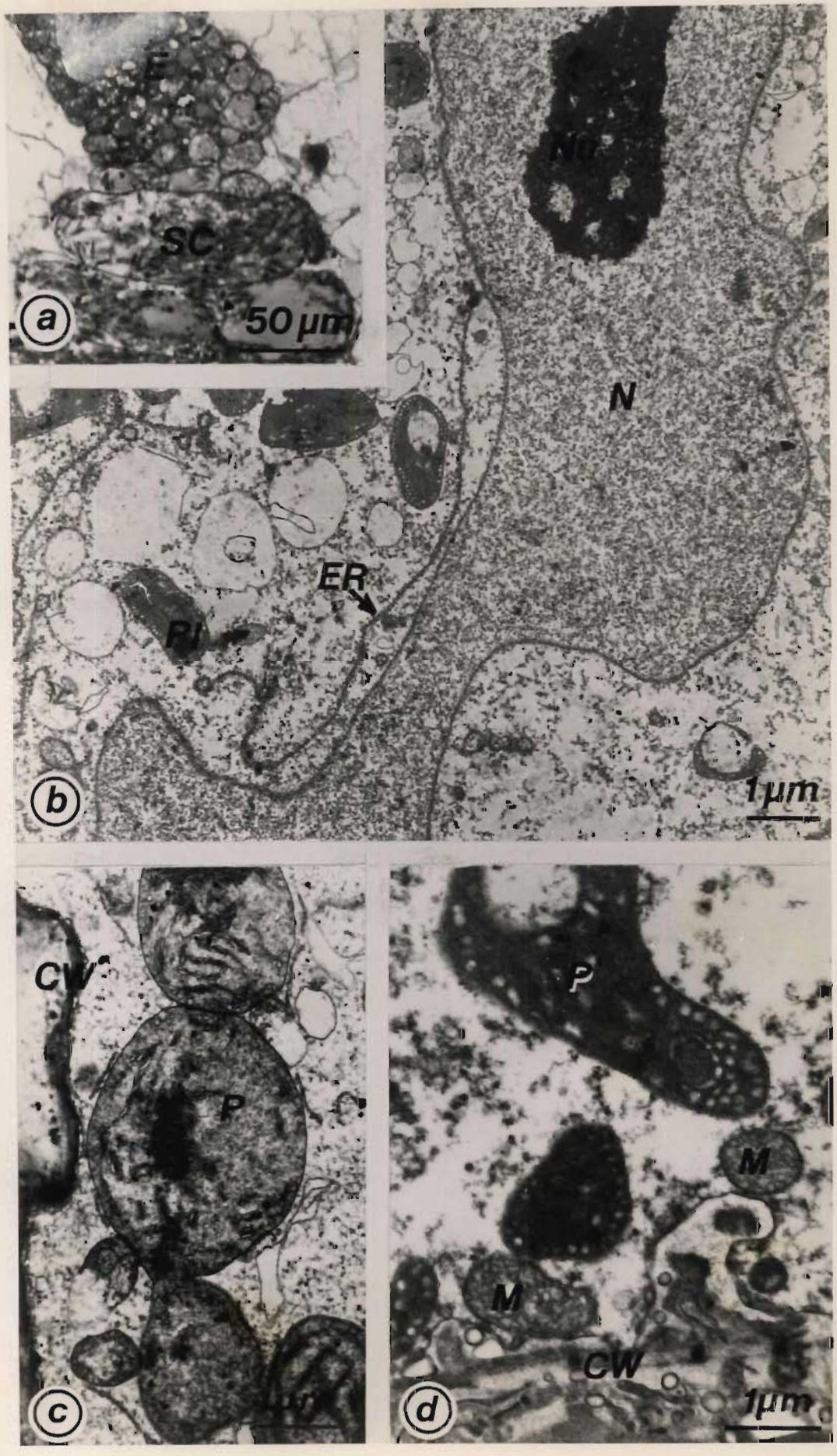



Plate 3:3. a. The embryo (E) and suspensor (SC) at 4 weeks after anthesis. Notice the difference in size between the embryo cells and the suspensor cells. b. The developing radicle (R) displaces the suspensor cells. SC = suspensor cells; T = testa; EN = endosperm.


E
SC
a
50 µm
Nu
N
ER
Pl
b
1 µm
CW
P
c
P
M
M
CW
d
1µm

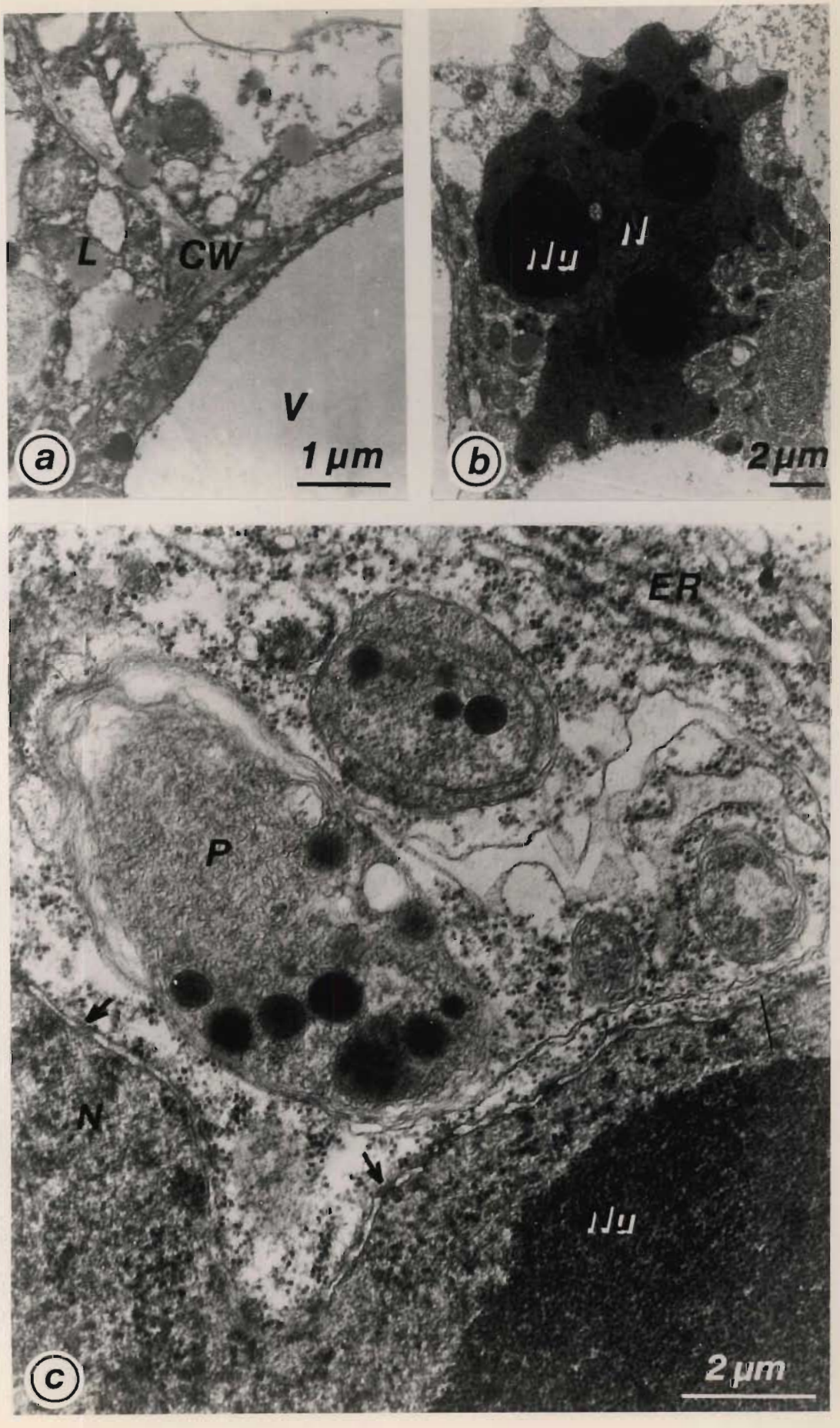
from thirty seeds were analysed separately. Six-week-old embryos weighed 0,88 grammes prior to analysis while at 8 and 10 weeks material was analysed for cytokinin activity in 3 gramme batches. Procedures outlined in the Materials and Methods were followed.

## Results

### *Morphological, anatomical and ultrastructural changes during embryo development*

In Plate 3:1 the structure of the flower at anthesis, and the developing fruit at 3 day intervals after anthesis are illustrated. Although cross pollination may be effected in this species (bees were often seen visiting the flowers), the anthers ripen prior to anthesis, and self pollination also appeared to take place. The flowers begin to senesce soon after anthesis, and at 3 days after anthesis the petals were browning, by 6 days most of the petals had abscised and the developing fruit had increased in size and fresh weight. This increase in fresh weight continued between 6 and 12 days after anthesis (Figure 3:1A). During this period and up to approximately four weeks after anthesis, the increased fresh weight of the fruit was due mainly to the growth of the pod wall. By 8 weeks after anthesis however, the pod wall was declining and the growth of the seed contributed considerably to the increase in fresh weight of the fruit. The development of the white lupin seed between 4 weeks after anthesis and maturity is illustrated in Plate 3:2.

The period between 4 and 8 weeks after anthesis was characterized by the rapid development of the embryo (Plate


L
CW
V
1 µm
a
N
Nu
2 µm
b
ER
P
N
Nu
2 µm
c

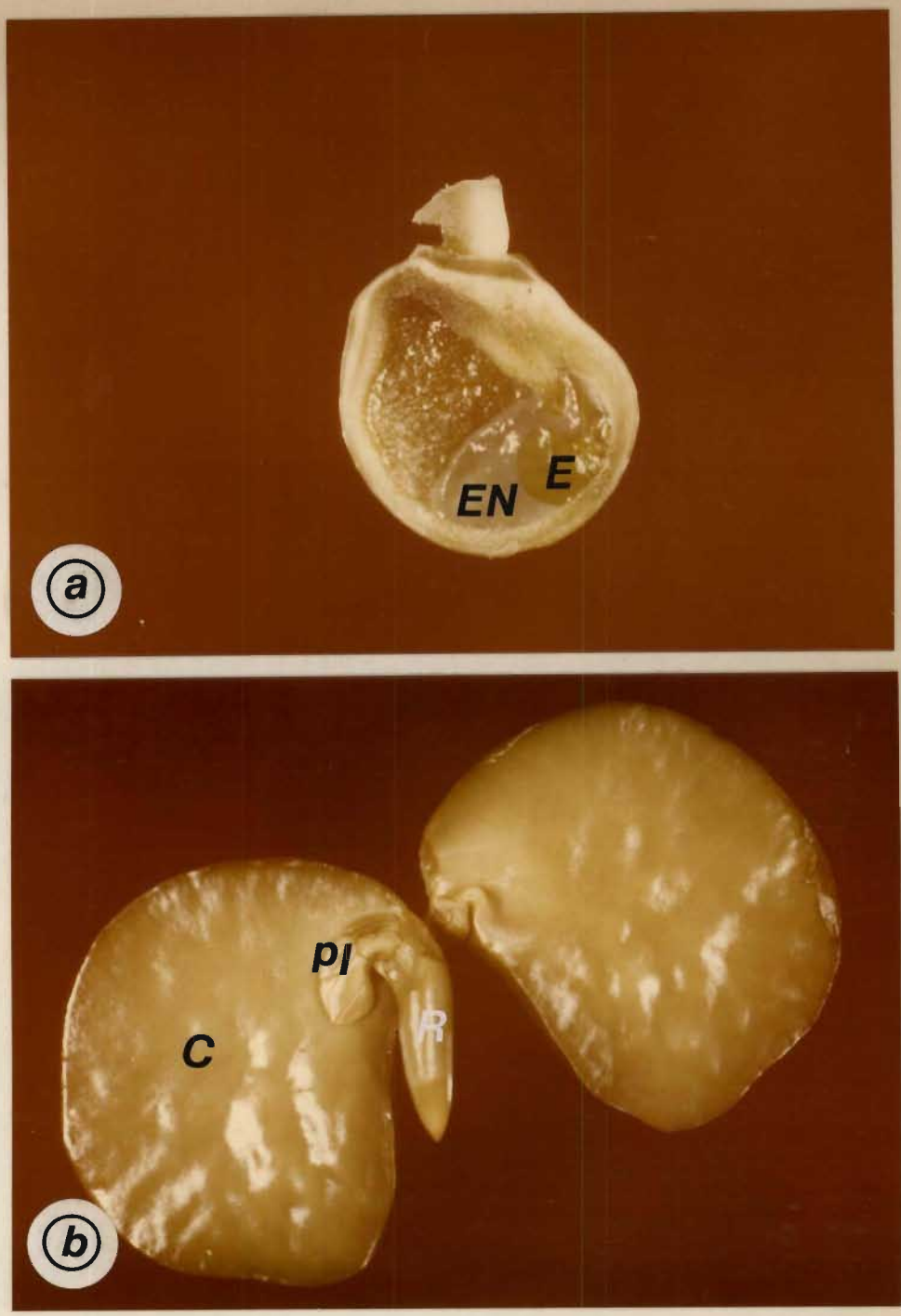
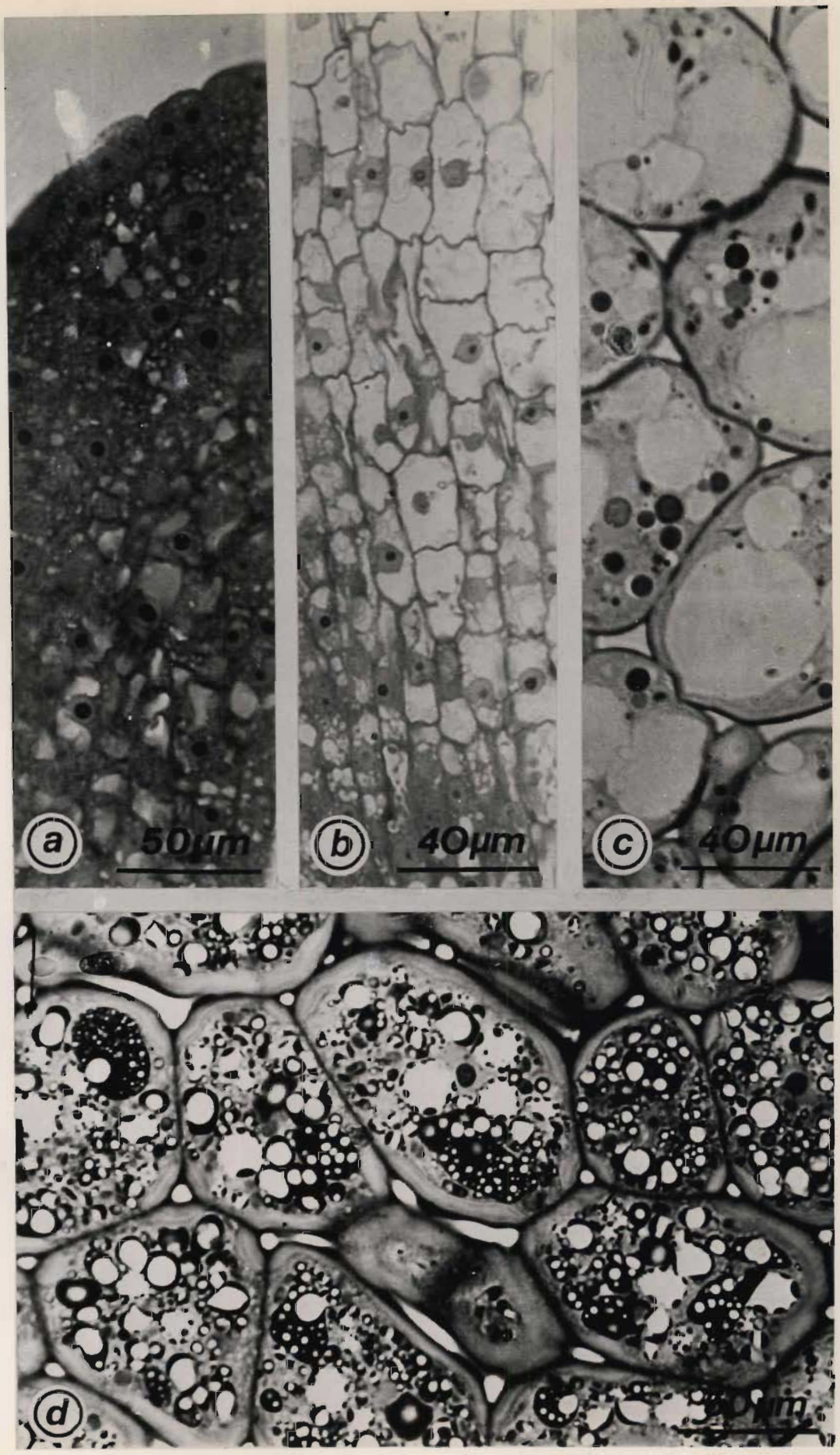
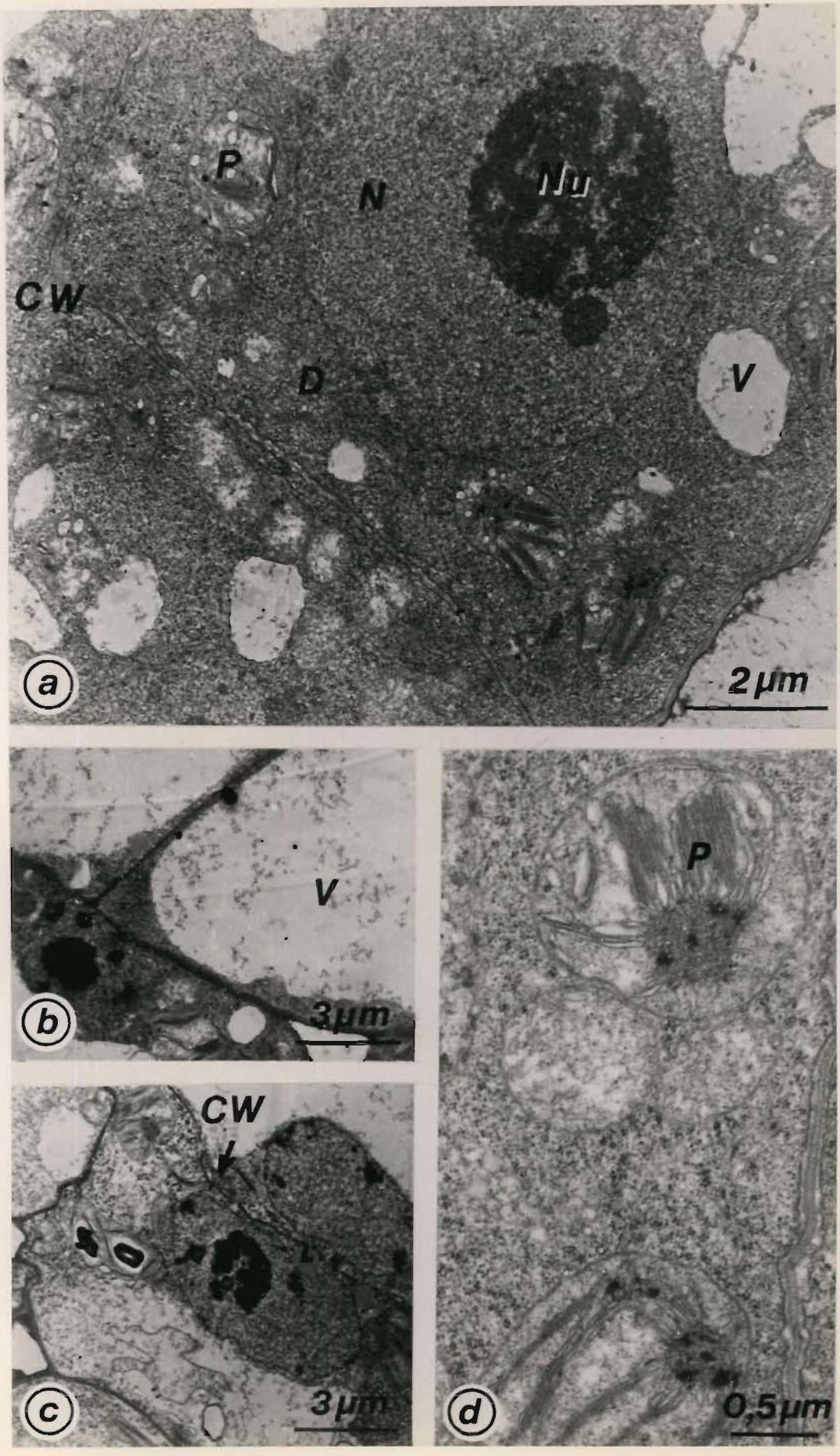



Plate 3:6. a. The 6-week-old embryo (E) encased in cellular endosperm (EN). b. The embryo at 12 weeks after anthesis. The radicle (R) and plumule (PL) are well developed, and the cotyledons (C) have expanded to form the most conspicuous embryonic organ.


a
50µm
b
40µm
c
40µm
d

3:2). At 4 weeks after anthesis, the embryo is at the globular or heart shaped stage of development and is suspended by means of a multicellular suspensor in the semi-fluid semi-cellular endosperm (Plate 3:3). The suspensor at this stage attaches the embryo to the embryo sac wall. The suspensor is made up of two different cell types, the cells adjacent to the embryo are smaller and brick shaped, while the basal part of the suspensor is made up of larger more irregularly shaped cells. The suspensor cells are much larger than the embryo cells at this stage of development (Plate 3:3; Plate 3:4). Some unique ultrastructural features characterize the suspensor cells and include large nuclei with projections which ramify throughout the cytoplasm, transfer cell-like walls, large active mitochondria and dense chloroplasts (Plate 3:4). Transfer cell walls are present along the interface between the endosperm and the suspensor cells and the suspensor cells and the embryo. The endosperm cells in this region are thin walled and highly vacuolate. The nuclei of these cells are large and have numerous nucleoli. Endosperm cell cytoplasm appears to be highly active. Rough endoplasmic reticulum and ribosomes occur in abundance (Plate 3:5). The embryonic cells at this stage are small and meristematic. They are thin walled with dense cytoplasm, the rounded nuclei have prominent nucleoli.

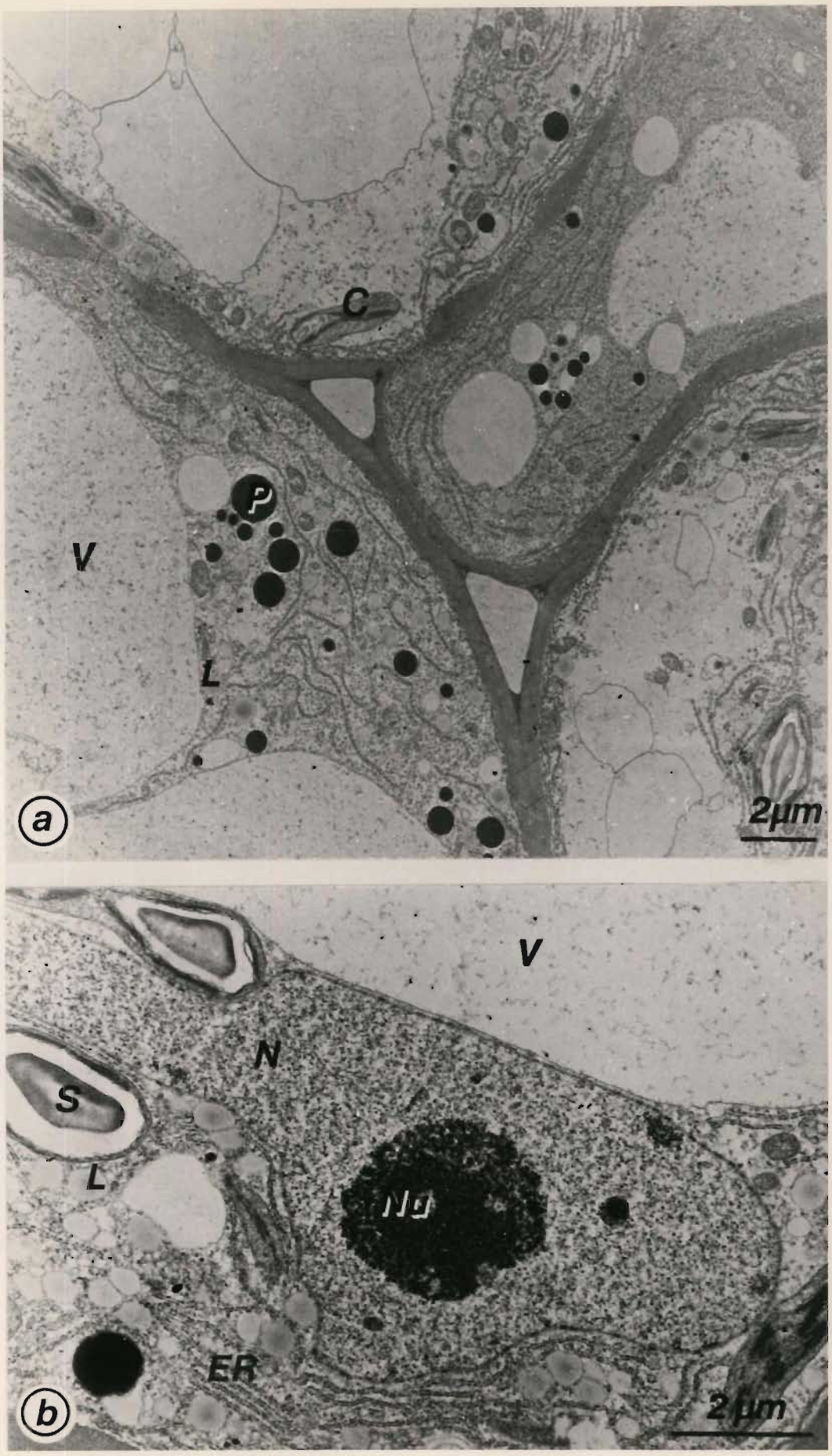
At 4 weeks after anthesis the embryonic cotyledons are visible. Between four and six weeks after anthesis, further development of the embryo results in increased cotyledon size


P
N
Nu
CW
D
V
a
2 µm
V
b
3µm
CW
c
3µm
P
d
0,5µm

and the development of the young radicle. As the radicle end of the embryo develops it pushes aside the suspensor cells, though some of these cells may remain attached to the tip of the radicle (Plate 3:3b and Plate 3:6a). Although cell division is still evident at 6 weeks after anthesis, cell expansion is beginning to take place and the cells of the embryo are highly vacuolate (Plate 3:7 and Plate 3:8). Lipid and some starch are present in the 6-week-old embryo cells.

The embryo increases rapidly in fresh weight between 6 and 12 weeks after anthesis, and a mature radicle and plumule are formed (Plate 3:6b). However, by far the most prominent organs developed during this phase of growth are the cotyledons which expand to fill the embryo sac. The growth of the cotyledons is mostly due to cell expansion (Plate 3:7), and the length of the cotyledon cells increases considerably between 6 and 12 weeks after anthesis (Figure 3:2). Dividing chloroplasts may be observed in the cotyledon cells between 6 and 8 weeks after anthesis (Plate 3:10). Over this period carbohydrate, lipid and protein reserves are accumulated in the cotyledonary cells. The contents of the protein bodies stained positively for protein with iodine and mecuric bromphenol blue.. Lipid was located in the cytoplasm using Sudan III stain. The periodic acid Schiff's reaction for carbohydrate confirmed the presence of carbohydrate in the chloroplasts and in the thickening cell walls.

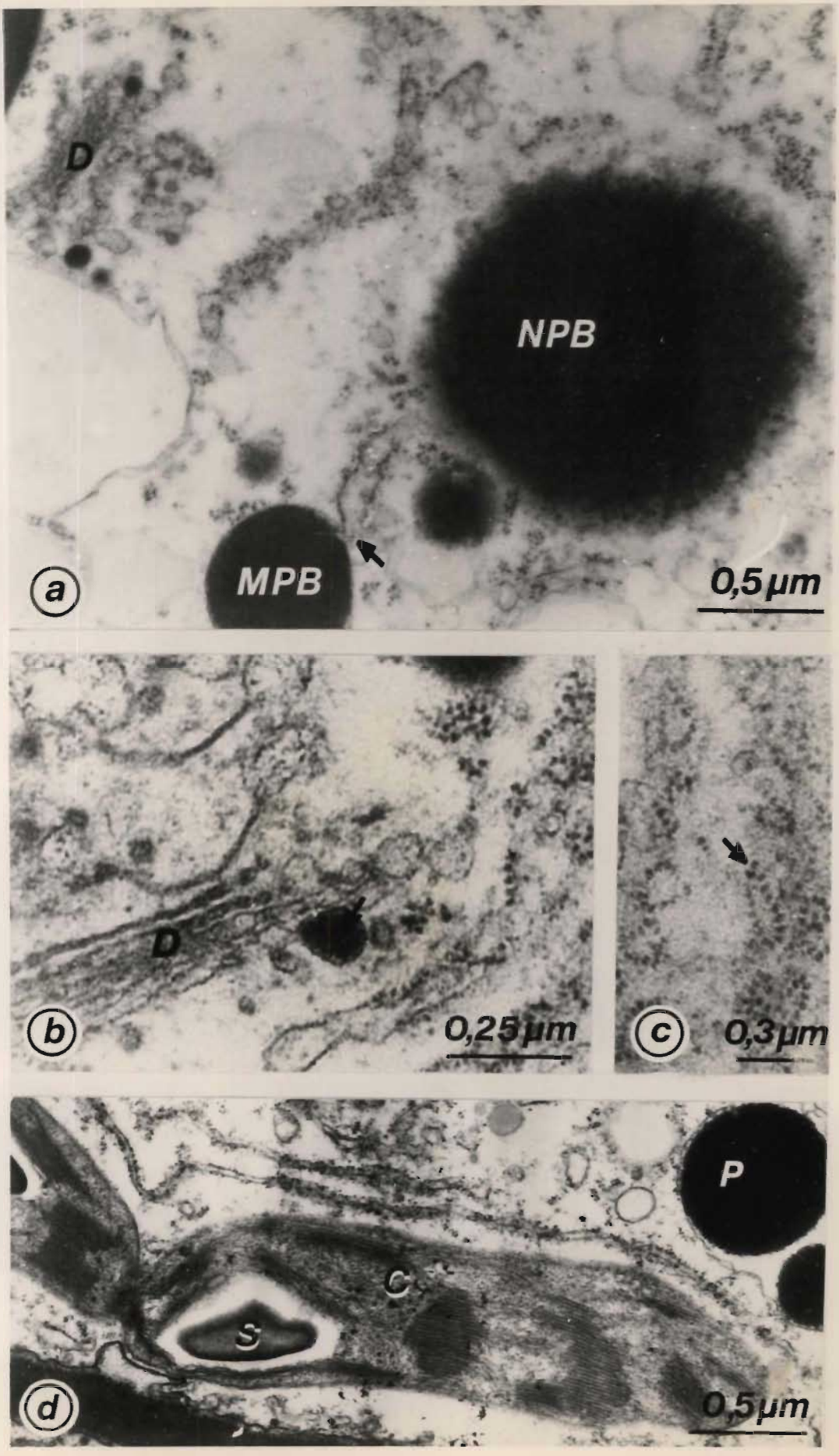
Although lipid and starch reserves were observed in 6-week-old cotyledons, evidence of protein synthesis was apparent only at 7 to 8 weeks after anthesis, and coincides


C
P
V
L
a
2μm
V
N
S
L
Ng
ER
b
2μm

with an increase in nuclear size and a proliferation of rough endoplasmic reticulum in the cotyledonary cells (Plate 3:9). At early stages of protein accumulation, two kinds of proteinaceous aggregation were observed, a membrane bound aggregation and a non-membrane bound one. The membrane bound proteinaceous aggregation was closely associated with the endoplasmic reticulum (Plate 3:10). Further features of the cells which were actively synthesizing protein were the presence of cleavages in the ribosome particles making up the polyribosome, and the fact that dark, protein-like membrane bound bodies were budded off the dictyosomes (Plate 3:10). Protein was deposited along the periphery of protein vacuoles and subsequently appeared to disperse throughout the protein vacuole (Plate 3:11). At 12 weeks after anthesis the protein bodies occupied a large volume of the cell. The nucleus at this stage was lobed with a contracted nucleolus. Lipid and starch reserves were present in the cytoplasm surrounding the protein bodies (Plate 3:11). The thick cell walls at this stage of development did not stain positively for lignin.

*Changes in the cytokinin levels in white lupin fruits and seeds during embryo development*

Cytokinin activity was detected in the flower parts and in the ovaries at anthesis. The level of cytokinin activity in the flower parts declined as the flower began to senesce. However, the activity in the ovaries (developing fruits) increased after anthesis, thereby closely paralleling the increase in fresh weight up to 12 days after anthesis. The level of the cytokinins in the flower parts, ovaries and


D
NPB
MPB
a
0,5µm
D
b
0,25µm
c
0,3µm
P
C
S
d
0,5µm

developing fruits was very low, and although some peaks of activity co-eluted with authentic markers the possible identity of other peaks of activity was difficult to assess.

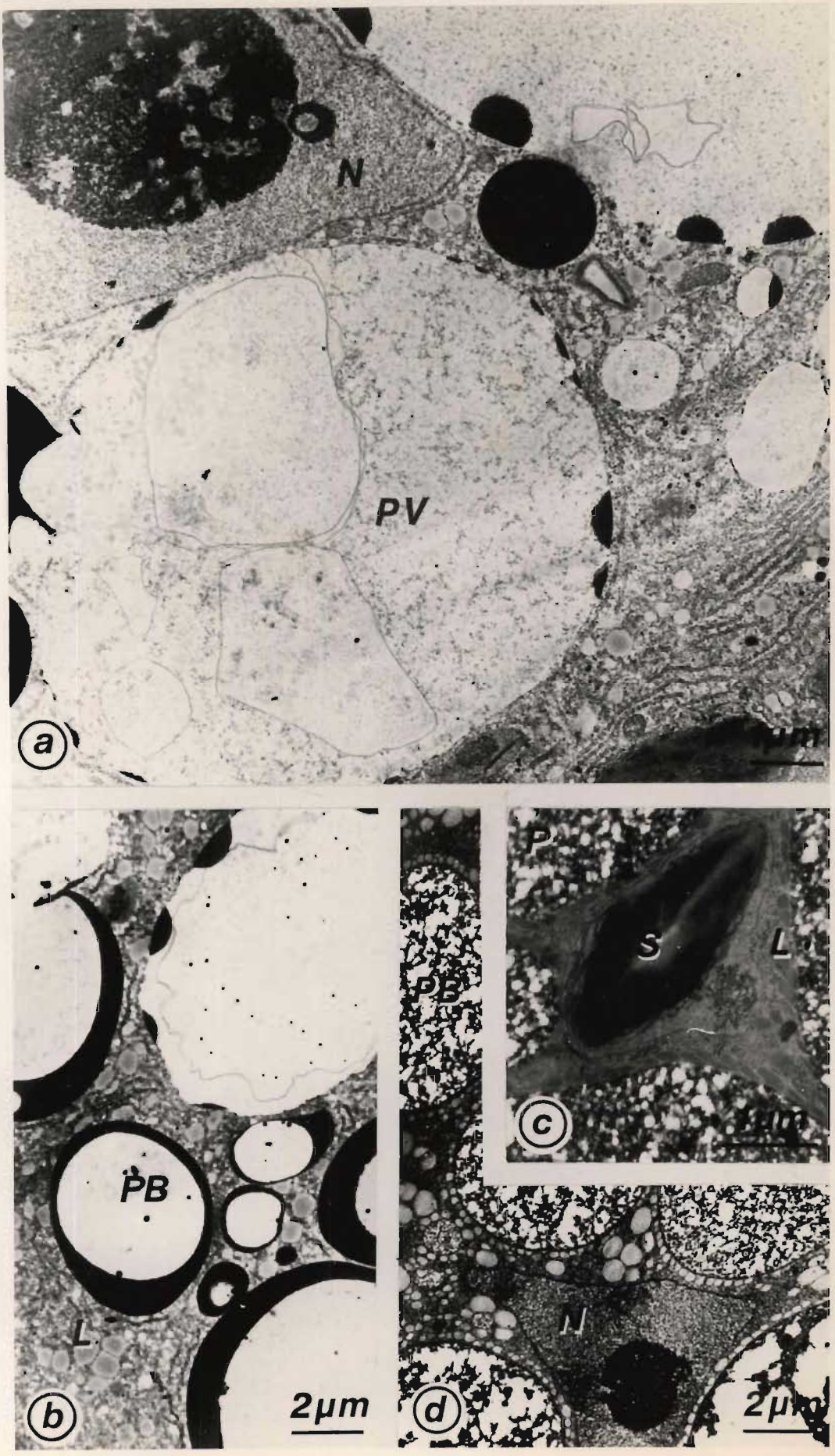
Two-week-old seeds and pods were analysed separately for cytokinin activity, and at later developmental stages the embryos, endosperms and testas were divided into separate fractions.

(i) Pod wall

The cytokinin changes taking place in the pod wall were essentially similar to those recorded in Experiment 2. The overall level of cytokinin activity in the pod wall increased with increasing fresh weight of the pod wall between 2 and 10 weeks after anthesis (Figure 3:3). The increase in cytokinin activity was due mainly to an increase in the level of slow moving cytokinins. There was however, also an increase in the level of activity co-eluting with zeatin and zeatin riboside (Figure 3:4). As in Experiment 2, this result was confirmed using column chromatography.

(ii) Seeds

In Figures 3:5a-f the averaged results of bioassays of both paper and column chromatography show that the total amount of cytokinin activity in the seed changes during growth and development. At 6 weeks the highest level of cytokinin activity was recorded irrespective of whether the results were expressed on the basis of activity per gramme seed material or on the basis of activity per seed.


N
PV
a
PB
L
b
2μm
PB
P
S
L
c
N
d
2μm

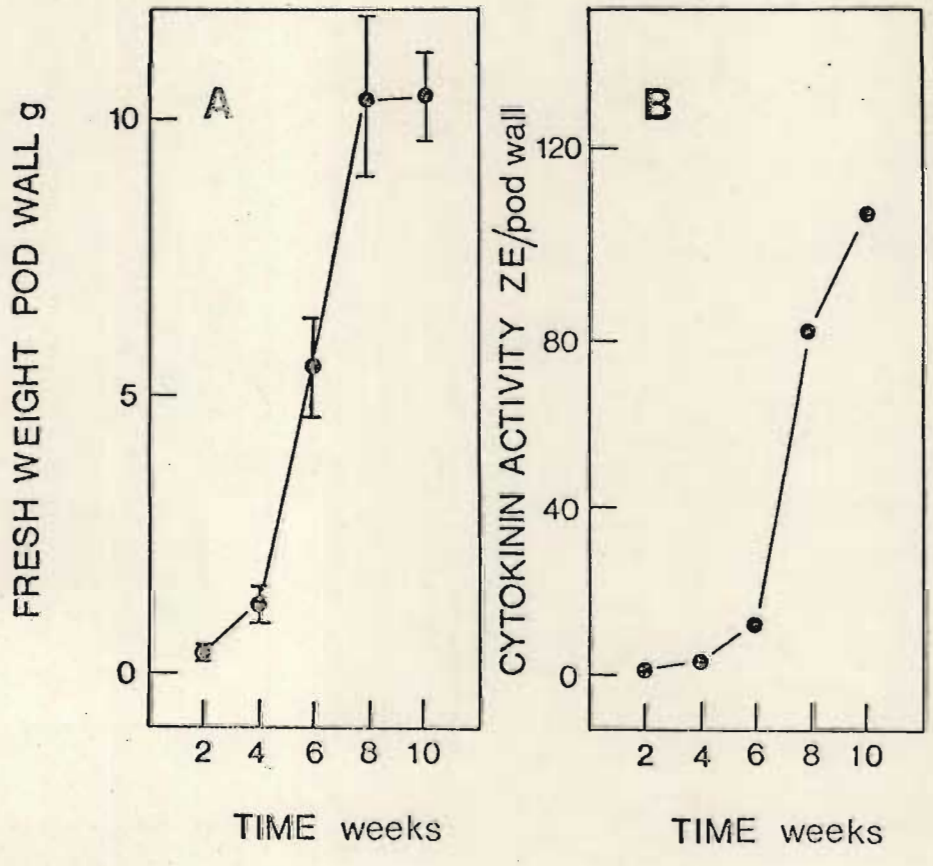



Figure 3:3. A. Increase in fresh weight of the pod wall between 2 and 10 weeks after anthesis. B. Cytokinin activity in the pod walls between 2 and 10 weeks after anthesis. The results of paper chromatography were expressed as zeatin equivalents (ZE) per pod wall.

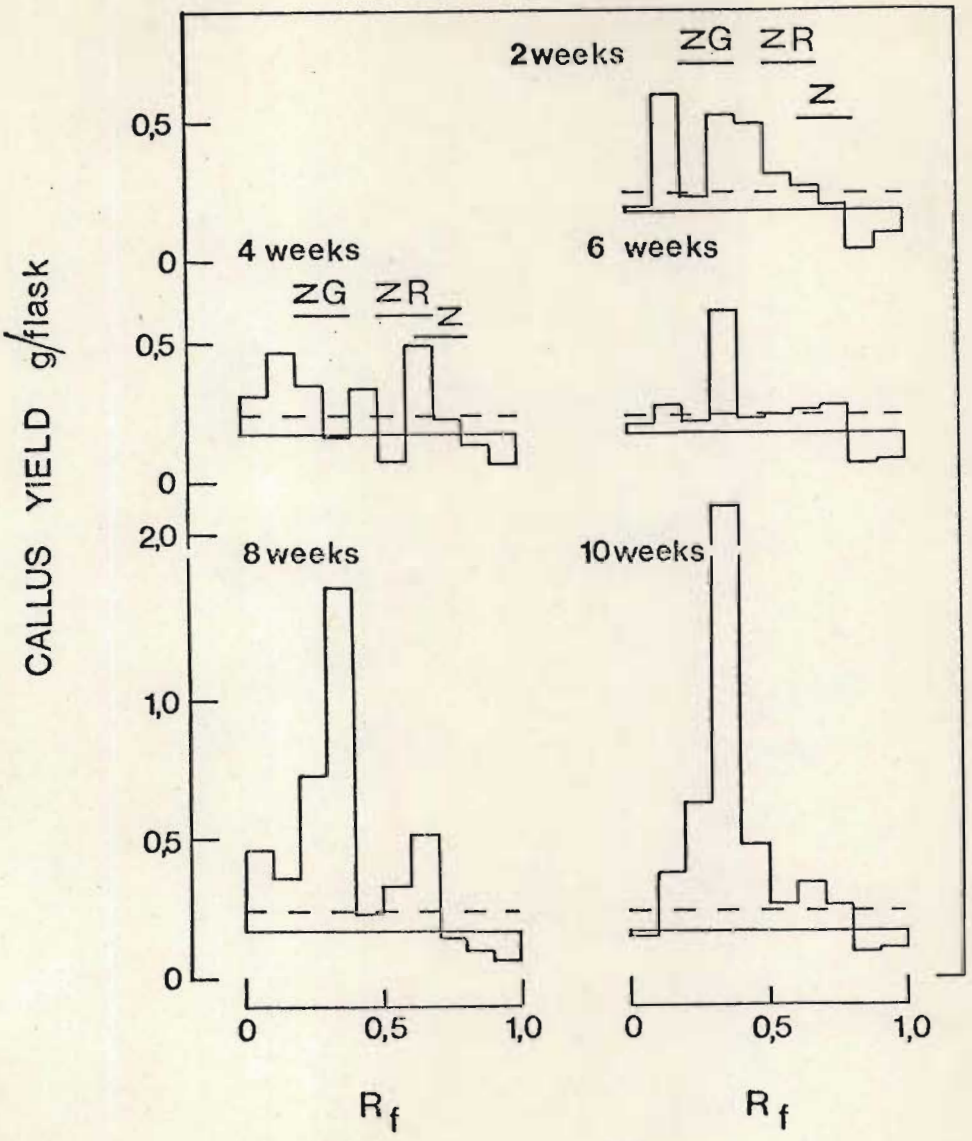



Figure 3:4. The results of paper chromatography of extracts of 5 grammes of pod wall material harvested at 2, 4, 6, 8 and 10 weeks after anthesis. Callus grown on 5 microgrammes per litre zeatin yielded 0,66 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. The broken line indicates the confidence limit at the level P = 0,01.

Analysis of entire 2-week-old seeds for cytokinin activity showed that low levels of activity were present (Figure 3:5d and e). However by 4 weeks after anthesis, the total level of activity was increasing (Figure 3:5 d and e). The higher level of activity was apparently associated with the endosperm rather than with the testa (including both embryo and suspensor) (Figure 3:5f). Separate analysis of paper chromatograms of embryo and suspensor extracts showed that some of the activity ($\pm$ 16 percent) of the total activity of the seed at this time was present in the embryos and suspensors (Figure 3:7). The level of activity in each suspensor was slightly higher than in each embryo (0,075 microgramme zeatin equivalents (ZE) per suspensor compared with 0,069 ZE per embryo). The highest level of cytokinin activity was detected in 6-week-old seeds (Figures 3:5d and e). At this time compounds exhibiting cytokinin activity could be detected in the embryos, endosperms and testas (Figure 3:5f). However, most of the activity was still located in the endosperm (Figure 3:5f).

By 8 weeks after anthesis, the cytokinin activity in the seeds had started decreasing (Figure 3:5d and e). This decrease appeared to be due to a decrease in the level of activity in endosperm extracts. The level of cytokinin activity in the seeds continued to decrease and was low in 10-week-old seeds. At 10 weeks most of the activity in the seed was in the testa (Figure 3:5f).

Following column chromatography on Sephadex LH-20, five

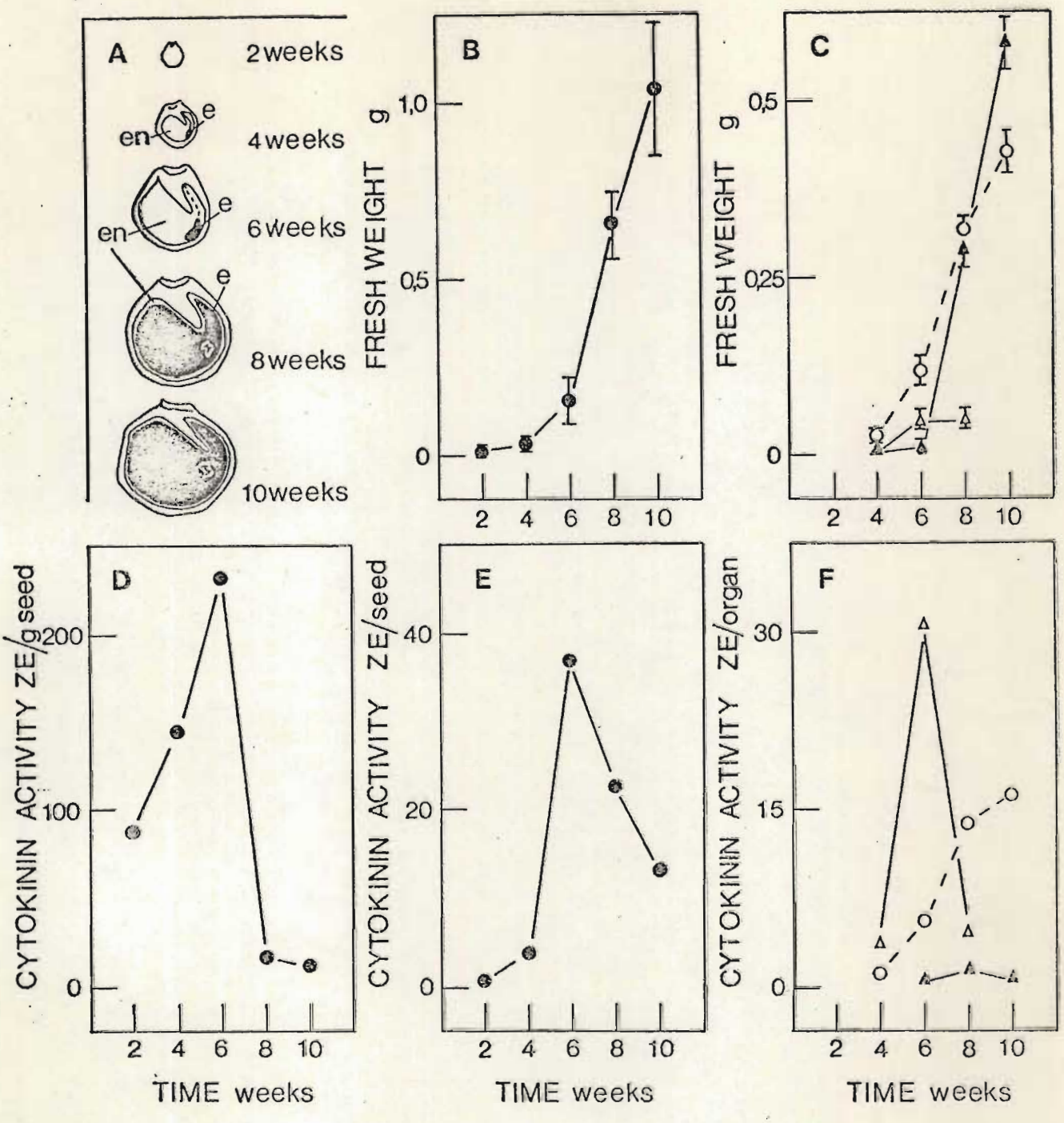



Figure 3:5. The pattern of growth and the changing cytokinin activity in white lupin seeds and parts thereof during development.
●——● whole seed; o----o testa; Δ——Δ endosperm; ▲——▲ embryo.
The values indicating cytokinin activity on these graphs were derived by expressing the callus yield from bioassays as microgramme zeatin equivalents (ZE). Standards were included at the time of assay of each separate bioassay. The values above represent the average of the results of two bioassays, one after paper chromatography and one after fractionation on Sephadex LH-20. e = embryo; en = endosperm.

different peaks of cytokinin activity were detected in extracts of 2-week-old seed (Figure 3:6). Two peaks co-eluted with authentic zeatin and zeatin riboside, while the remaining peaks had elution volumes of 200-280 millilitres, 360-480 millilitres and 800-840 millilitres, respectively (Figure 3:6).

In the testas activity was present in both fast- and slow-moving fractions of paper chromatograms of material harvested at 4, 6, 8 and 10 weeks after anthesis (Figure 3:8). Column chromatography of these extracts showed that four peaks of activity were present in the testas. At 6 weeks after anthesis two of these peaks co-eluted with zeatin and zeatin riboside. The remaining peaks eluted off Sephadex at elution volumes of 320 to 360 millilitres and 360 to 440 millilitres (Figure 3:9).

Cytokinin activity in the embryos co-eluted primarily with zeatin riboside at 6, 8 and 10 weeks. At 6 weeks activity was also detected at elution volumes of 200-240 millilitres, 280-320 millilitres and 800-840 millilitres (Figure 3:10).

High levels of cytokinin activity were present in both fast- and slow-moving fractions of paper chromatograms of endosperm extracts (Figure 3:11). The chromatograms were therefore divided into two fractions, Fraction A ($R_f$ 0,05-0,5) and Fraction B ($R_f$ 0,5-1,0). Three major peaks of activity were detected after the fractionation of activity present in Fraction A through a Sephadex LH-20 column eluted with 20 percent ethanol. One peak

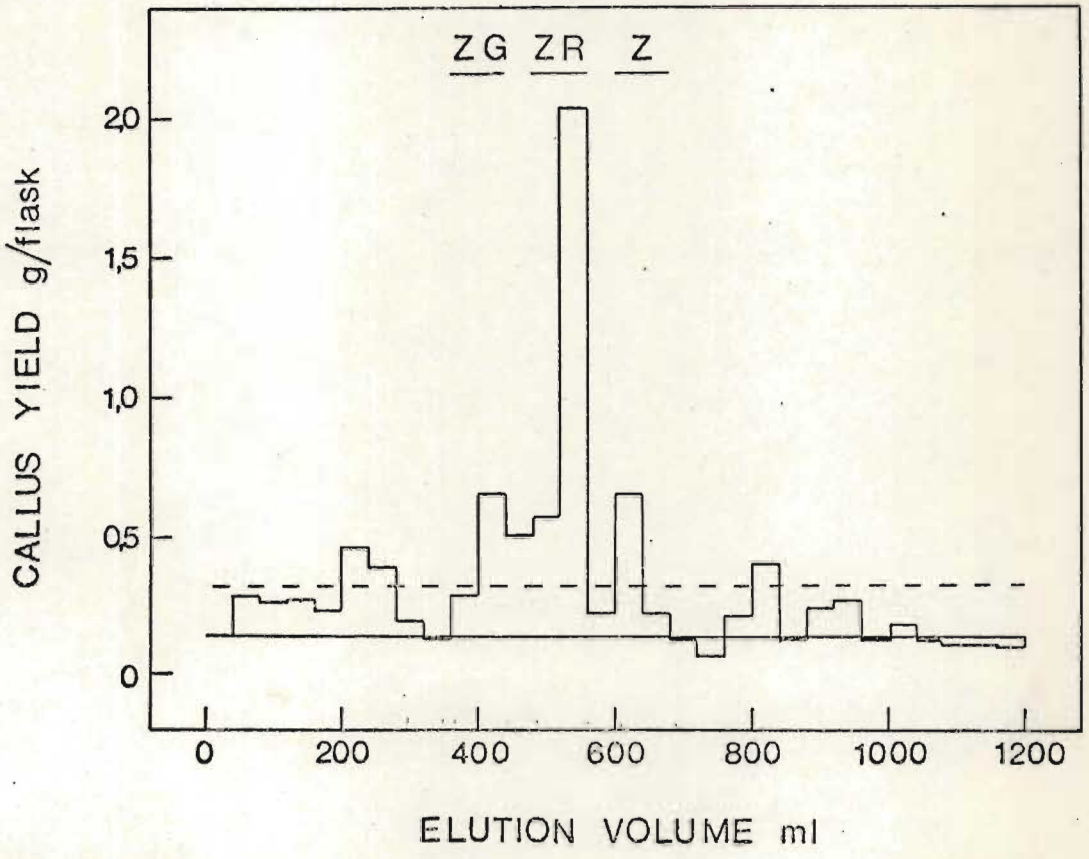



Figure 3:6. The cytokinin activity in 0,88 grammes fresh weight of seed harvested at 2 weeks after anthesis. The seed material was fractionated on a column packed with Sephadex LH-20 and eluted with 35 percent ethanol. Callus grown on 5 microgrammes per litre zeatin yielded 0,53 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. The broken line indicates the confidence limit at the level P = 0,01.

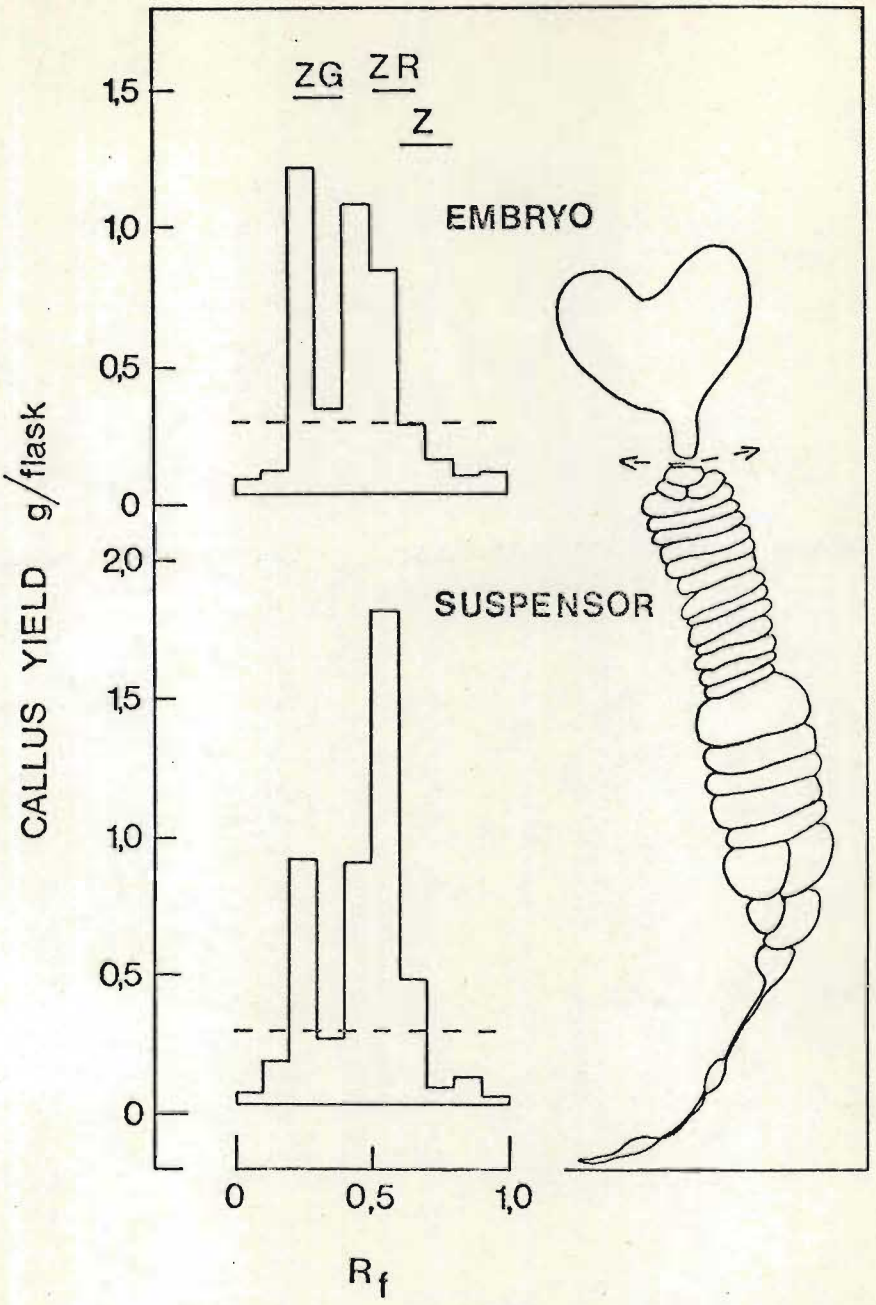



Figure 3:7. The cytokinin activity in 4-week-old embryos and suspensors of white lupin seeds. The embryos and suspensors removed from 30 seeds, were ground in a glass homogeniser in 80 percent ethanol. The ethanolic extract was reduced to dryness under vacuum and taken up in 1 millilitre of 80 percent ethanol and strip loaded onto Whatman No. 1 chromatography paper. The chromatograms were run in *iso*-propanol: 25 percent ammonium hydroxide:water (10:1:1 v/v), divided into 10 $R_f$ strips and assayed. Callus grown on 5 microgrammes per litre zeatin yielded 2,56 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. The broken lines indicate the confidence limit at the level P = 0,01.

co-eluted with zeatin riboside, while the remaining peaks had elution volumes of 240 to 320 millilitres and 440 to 520 millilitres, respectively. Cytokinins in the B fraction co-eluted with zeatin riboside and zeatin glucoside on Sephadex LH-20 columns eluted with 35 percent ethanol. In 4- and 8-week-old embryos, low levels of activity co-eluting with zeatin were also recorded, while in 4-week-old embryos some activity was detected in the A fraction at the elution volume 360 to 440 millilitres (Figure 3:12).

Discussion

Examination of the cytokinin levels in the flowers at anthesis indicated that, at the time of flowering, the level of cytokinin activity in the flower parts (excluding the ovaries) was higher than the level in the ovary (Figure 3:1B). As the flower senesced, so the level of activity in the flower parts decreased while that in the developing fruits increased. Decreasing levels of cytokinin activity have been reported to occur in *Rosa* sp. as the flower ages (MAYAK, HALEVY and KATZ, 1972) while the application of kinetin has been shown to delay senescence in the flowers of *Dianthus caryophyllus* (EISINGER, 1977). In the white lupin, decreasing levels of cytokinin activity in the flower parts may contribute to the senescence of these organs. The isolation of compounds which exhibit cytokinin activity from the flower parts and young developing fruits of the white lupin supports an earlier suggestion (Experiment 1) that the detection of some cytokinin activity at the flowering terminal apices may be attributed to the presence of flowers and immature fruits.

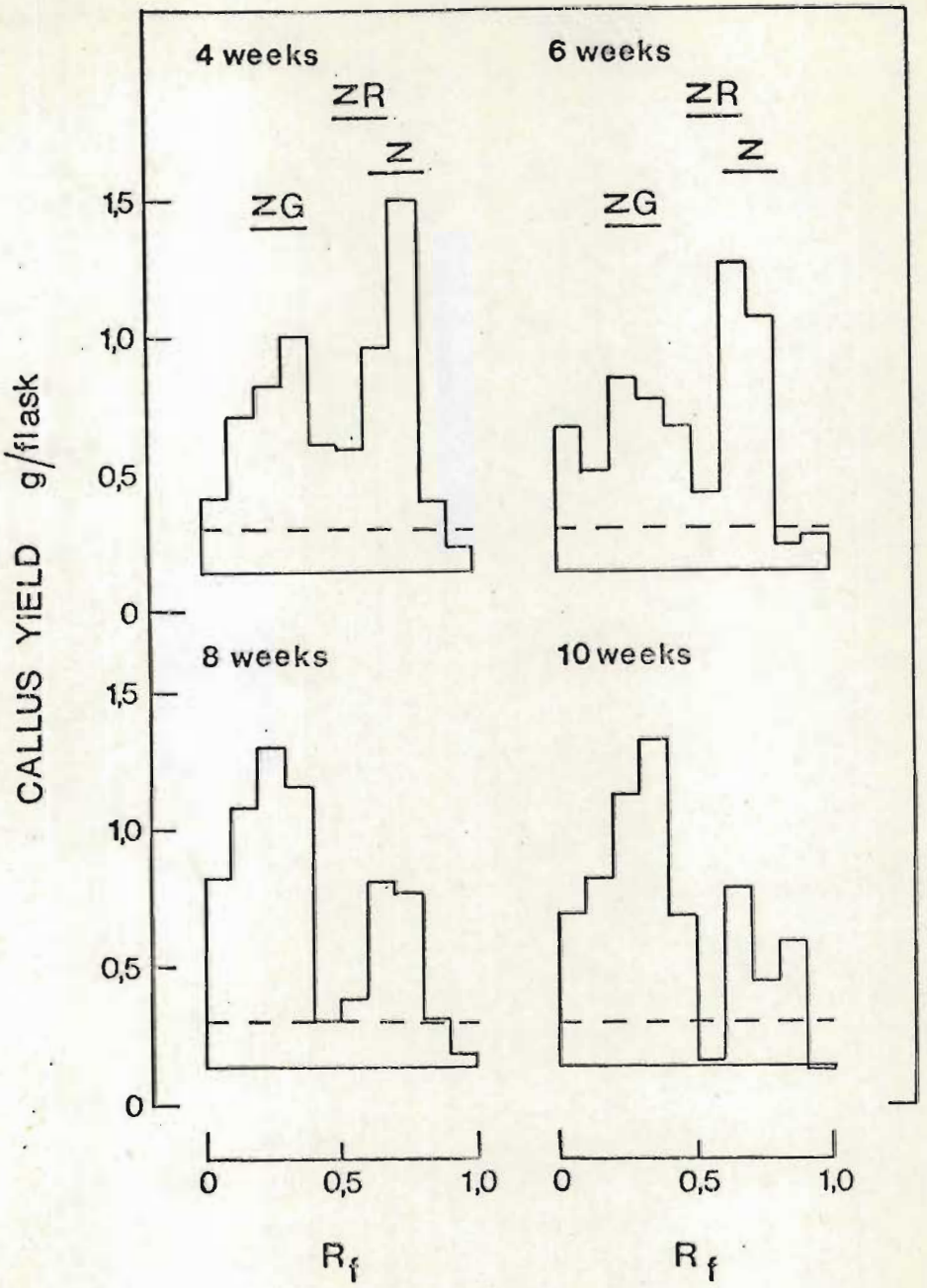



Figure 3:8. Cytokinin activity in extracts of 2,5 grammes of testa material following paper chromatography using an *iso*-propanol:25 percent ammonium hydroxide:water (10:1:1 v/v) solvent system. Material was harvested for extraction at 4, 6, 8 and 10 week intervals after anthesis. Callus grown on 5 microgrammes per litre zeatin yielded 0,62 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. The broken line represents the confidence limit at the level P = 0,01.

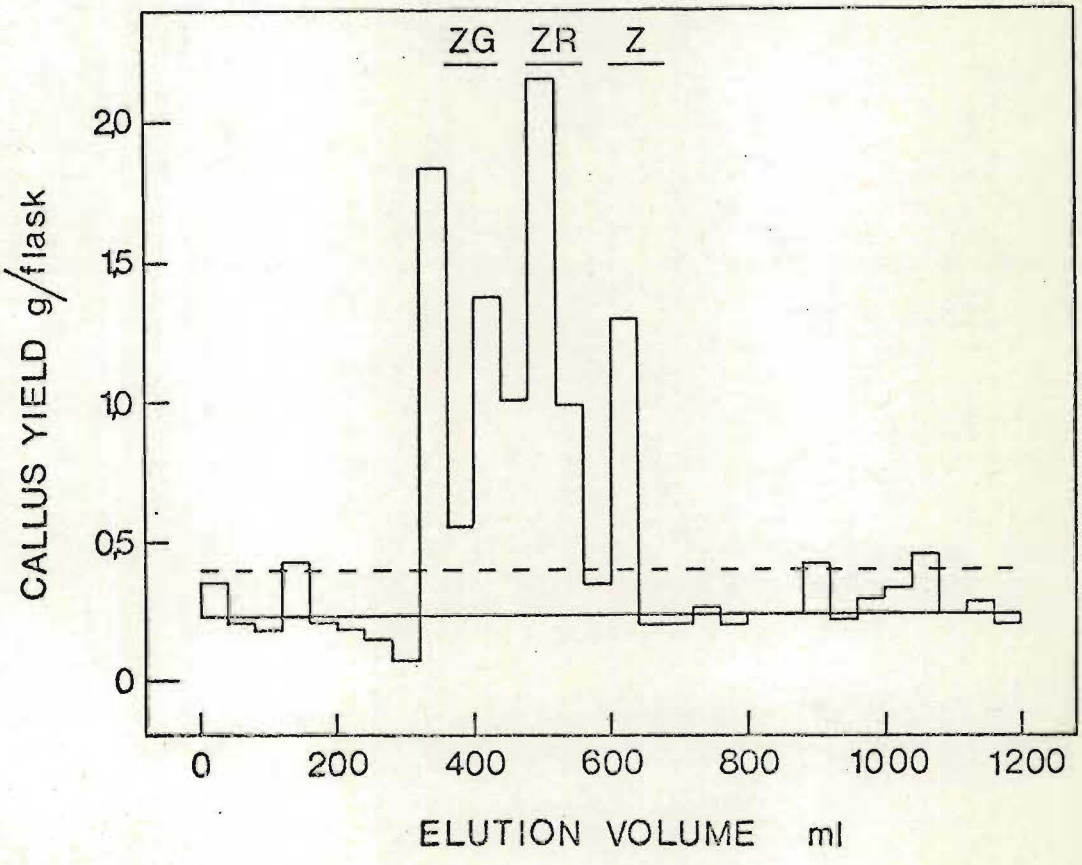



Figure 3:9. The cytokinin activity in an extract of 2,5 grammes of testa material following fractionation on a column packed with Sephadex LH-20 and eluted with 35 percent ethanol. The testas were collected from 6-week-old seeds. Callus grown on 5 microgrammes per litre zeatin yielded 0,53 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. The broken line represents the confidence limit at the level P = 0,01.

Between anthesis and 12 days after anthesis, cytokinin activity in the young developing fruits continued to increase in the whole fruit up to 6 weeks after anthesis, after which it again decreased. This pattern is essentially similar to that recorded in Experiment 2, though due to differences in the method of sampling, the peak in cytokinin activity in Experiment 2 occurred at 8 weeks after anthesis. It has been shown that in the avocado (GAZIT and BLUMENFELD, 1970), grape (LILOV and ANDONOVA, 1976) and tomato (DAVEY and VAN STADEN, 1978; DESAI and CHISM, 1978) there are decreasing levels of cytokinin activity in the fruit tissues as the tissues mature. However, in these species fruit ripening was often examined only after maturity had been reached. This consideration and the differences in maturation patterns between these fruits and legume fruits do not facilitate a comparison of the present results to those of these workers. In legumes the development of the embryo appears to be dependent on a transference of nutrients initially from the endosperm and later from the testa and pod wall tissues (PATE *et al.*, 1977). Thus, it is not unexpected that the results of KRECHTING *et al.* (1978) and the present investigation indicated that cytokinin levels initially increase and only later decrease.

KRECHTING *et al.* (1978) showed that the relative level of cytokinin activity in pea seeds and pods may change with time. Likewise, in the white lupin, separate analysis of pod wall and seed material indicated similar fluctuations. In the white lupin fruit at 2 weeks after anthesis, total

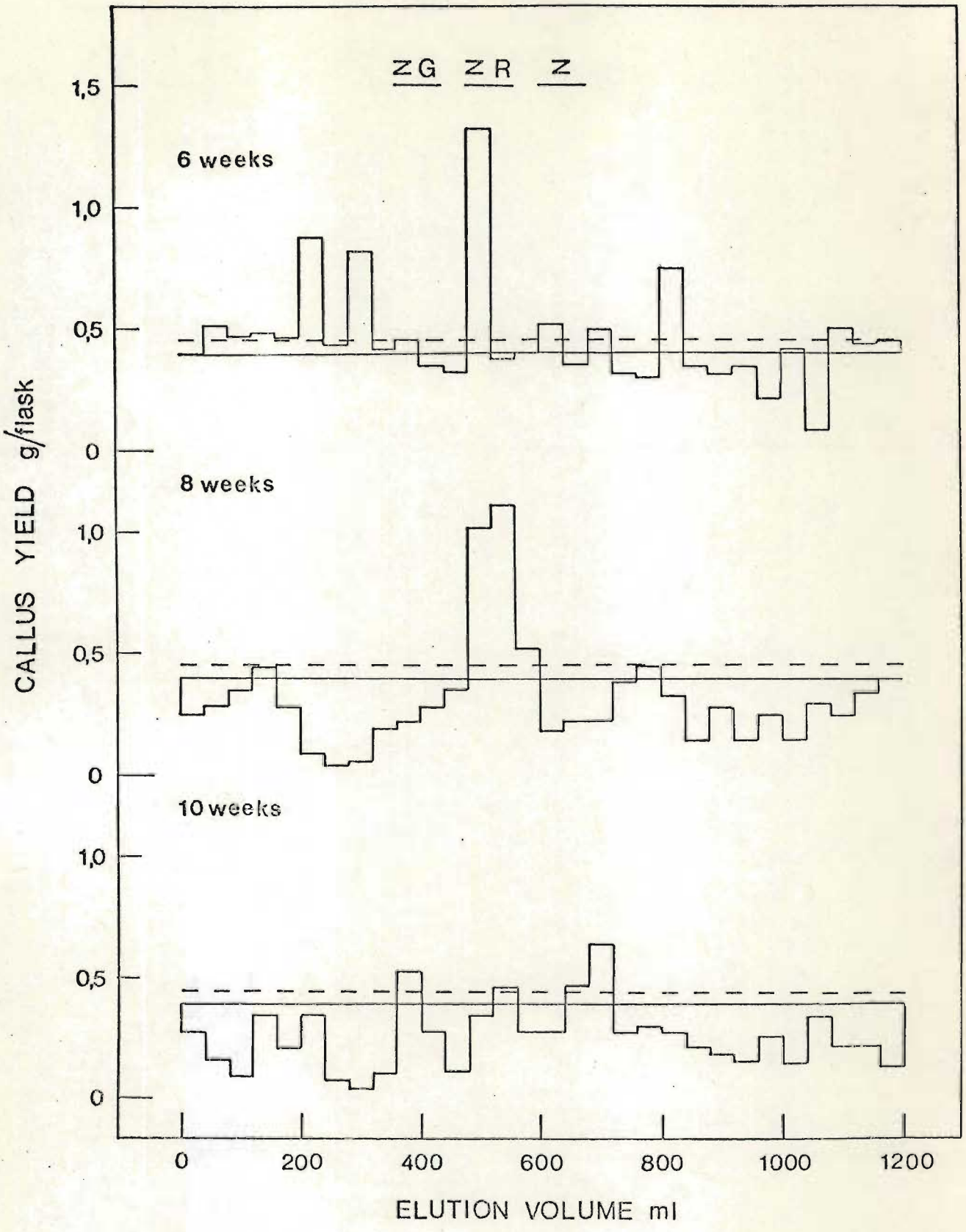



Figure 3:10. Cytokinin activity in an extract of embryos harvested at 6, 8 and 10 week intervals after anthesis. The embryo extracts were fractionated on a Sephadex LH-20 column eluted with 35 percent ethanol. At 6 weeks 0,08 grammes of embryo material was extracted while at 8 and 10 weeks 3 grammes of material was used. Callus grown on 5 microgrammes per litre zeatin yielded 1,68 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. The broken line represents the confidence limit at the level P = 0,01.

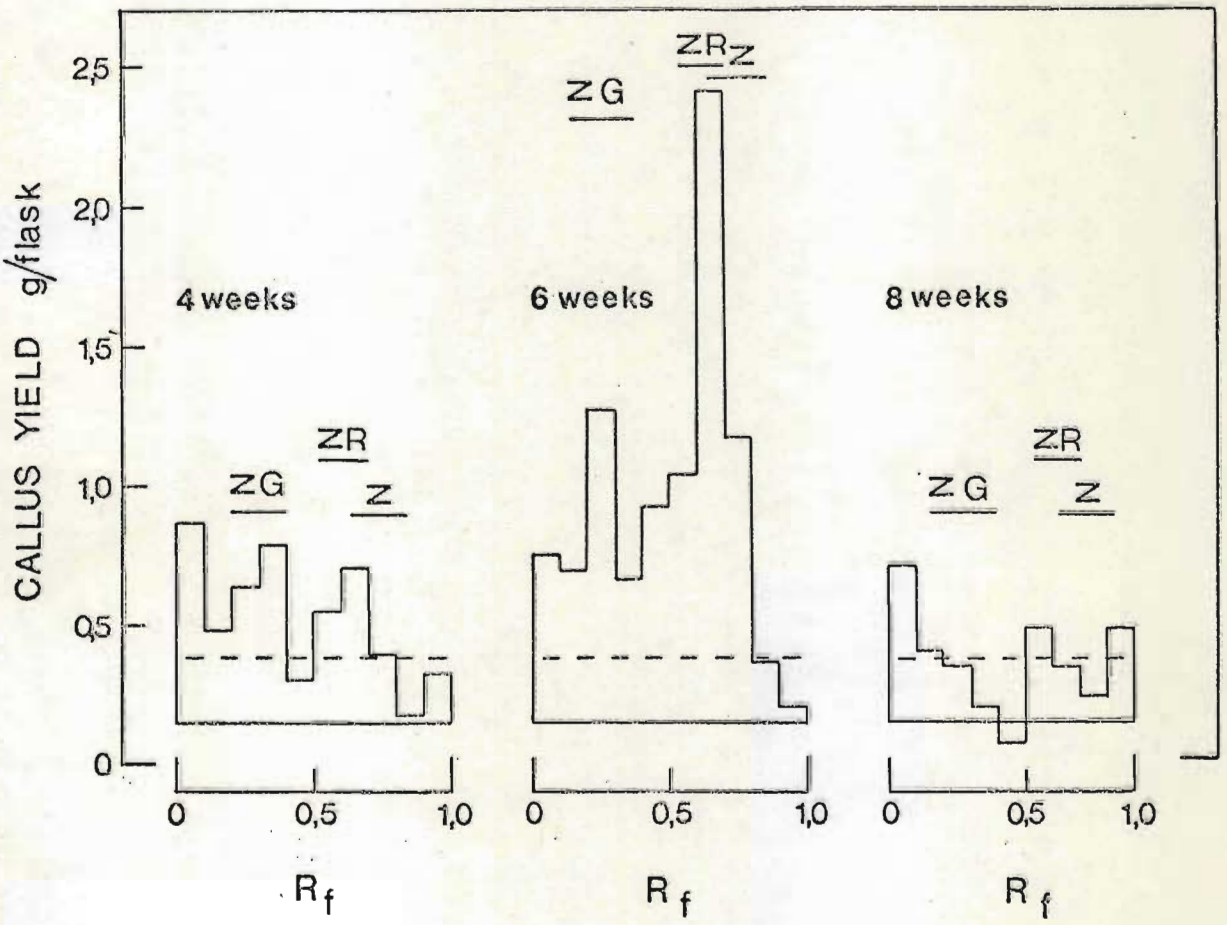



Figure 3:11. The cytokinin activity in 0,25 grammes of endosperm material collected from seed harvested at 4, 6 and 8 weeks after anthesis. The extracts were strip loaded onto paper chromatograms and separated with *iso*-propanol:25 percent ammonium hydroxide:water (10:1:1 v/v). Callus grown on 5 microgrammes per litre zeatin yielded 0,68 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. The broken line represents the confidence limit at the level P = 0,01.

cytokinin levels in the pod wall were higher (0,99 ZE per pod wall) than the level in the seed (0,62 ZE per seed). By 4 weeks after anthesis, however, the activity associated with the pod wall increased to 3,40 ZE per pod wall, while that in the seed had increased to a higher level (4,79 ZE per seed). This trend continued and at 6 weeks after anthesis, activity associated with the pod wall measured 13,00 ZE per pod wall, while that in the seed increased to 37,16 ZE per seed. At later developmental stages the level in the seed dropped, although the level of activity in the pod wall continued to increase. This pattern of changing levels of cytokinin activity in the pod wall and seed could be explained in terms of the transference of cytokinins from a site of synthesis (or accumulation) in the seed to the pod wall. However, such a suggestion is not in accord with current evidence provided by KRECHTING *et al.* (1978) and VAN STADEN and BUTTON (1978) who demonstrated that cytokinins were not accumulated within detached fruits cultured *in vitro*. Furthermore, cytokinins appeared to pass into the fruit in the fruit sap (Experiment 2). The build-up of these substances in the pod wall tissues may, therefore, be interpreted as a reduction in the withdrawal of cytokinins from the pod wall by the maturing seed.

The changing levels of cytokinin activity in the pod wall and in the seed tissues may be more meaningfully explained if one examines the distribution of activity within different parts of the seed, and attempts to assess the function of the tissues at the time at which the levels were high. Between anthesis, and 6 weeks after anthesis,

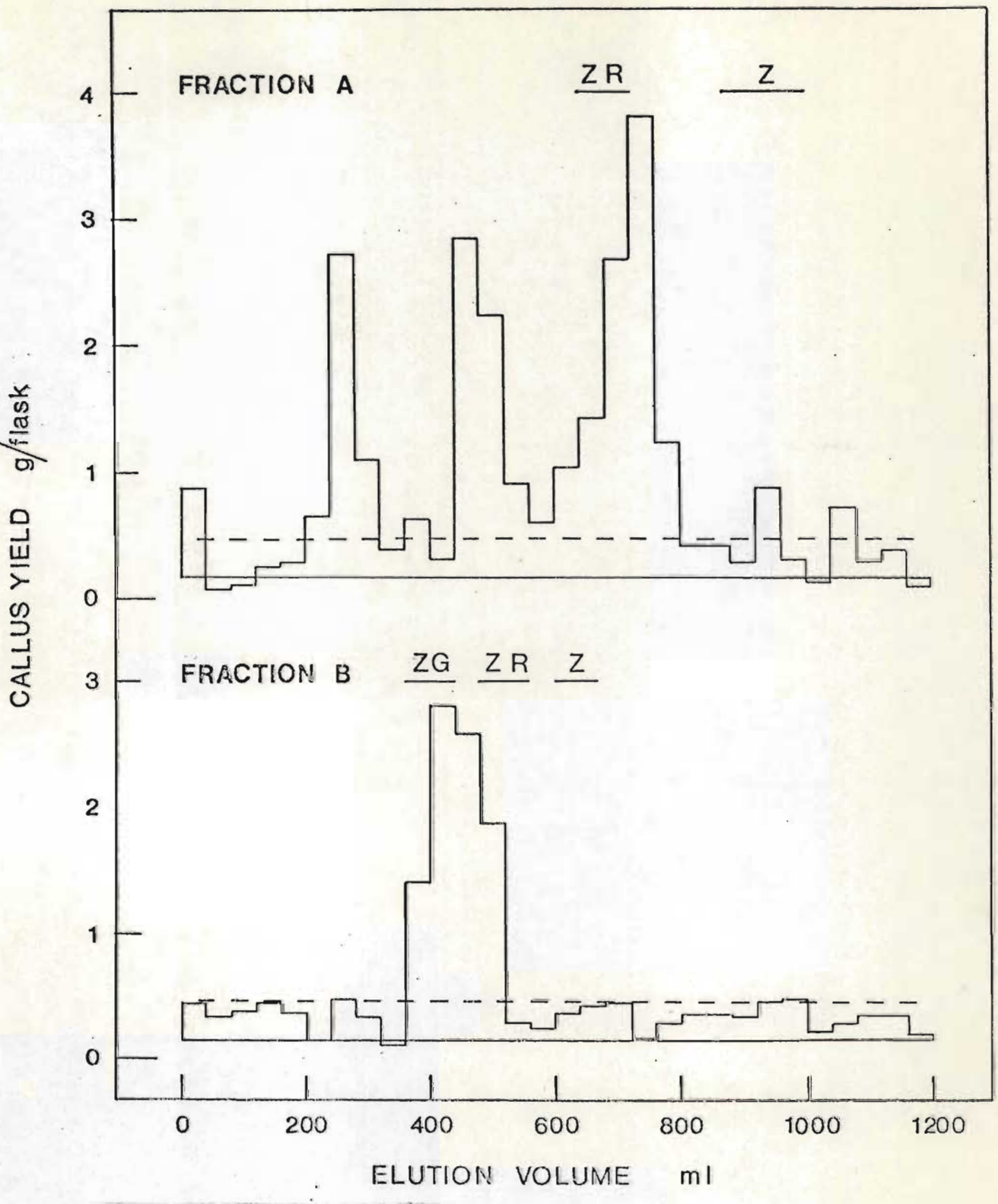



Figure 3:12. Cytokinin activity in 0,25 grammes of endosperm material following fractionation on Sephadex LH-20 eluted with 20 percent (Fraction A) and 35 percent (Fraction B) ethanol. The extracts of endosperm material collected from 6-week-old seeds were initially chromatographed on paper. The paper chromatograms were divided into two fractions: Fraction A, $R_f$ 0,05-0,5 and Fraction B, $R_f$ 0,5-1,0, and the activity eluted and run on Sephadex LH-20. Callus grown on 5 microgrammes per litre zeatin yielded 0,73 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. The broken line represents the confidence limit at the level P = 0,01.

the immature white lupin seed was a highly meristematic structure. Indeed, the present results indicate that divisions occurred in the embryo up to 6 weeks after anthesis. Cytokinins have been shown to promote cell division in plant cells (MILLER, 1965) and have been considered to be produced in the meristematic areas of the plant (GOLDACRE, 1959). It is possible, therefore, that the cytokinins present within the seed were produced as a result of this active division. However, current evidence does not favour the seed as a site of synthesis (KRECHTING *et al.*, 1978; VAN STADEN and BUTTON, 1978). Furthermore, although most of the seed tissues were meristematic over this period of time the activity was not present at similar levels in all seed tissues, and low levels were associated with the embryo.

In the white lupin, as in the pea (BURROWS and CARR, 1970), high levels of cytokinin activity in the seeds coincided with a period when there was a large proportion of endosperm present in the seed. High levels of cytokinin activity were also shown to occur in the endosperm of the avocado for the period over which the endosperm persisted (GAZIT and BLUMENFELD, 1970). In view of these facts, it is not unexpected that the endosperm in the white lupin exhibited far higher levels of cytokinin activity than other parts of the seed. At 6 weeks after anthesis 84,12 percent of the activity in the seed was associated with the endosperm. Micrographs of the endosperm tissue show that it is metabolically very active (Plate 3:5) and biochemical studies suggest that it is involved in the storage and

conversion of nutrients entering the seed (ATKINS, PATE and SHARKEY, 1975). It is of interest that between 4 and 6 weeks after anthesis, the cytokinin content of the endosperm increased, a period during which it has been established that pools of sugars and amino acids are formed in the white lupin seed (ATKINS *et al.*, 1975). In the 'competing sinks' hypothesis it has been suggested that the attraction of nutrients needed for the building of new tissues limits fruit growth, and that high concentrations of hormones found in the seed are necessary in order to create a strong physiological sink capable of competing with the remainder of the plant for nutrients (LUCKWILL, 1977). Cytokinins have been implicated in such an hypothesis (LUCKWILL, 1977) and it has been demonstrated that sugars and amino acids can be transported preferentially to regions of high cytokinin levels (MOTHES and ENGELBRECHT, 1961). Cytokinins in the endosperm may be involved in such a phenomenon during the early stages of seed growth, but this function may later be transferred to the pod wall, as was suggested in Experiment 2. The glucoside cytokinins present in the endosperm and pod wall may form a pool from which active free base cytokinins are sequestered.

A tissue such as the endosperm, which appears to contain high levels of cytokinins, must be able to regulate the supply of these compounds to the young embryo. It has been suggested that the suspensor is able to regulate the supply of growth substances entering the embryo during the early stages of development (PRZYBYLLOK and NAGL, 1977). Cytokinins were present in the white lupin suspensors

(Figure 3:6). This observation, and the transfer cell-type structure of the suspensor cells suggest that these cells may transfer substances including cytokinins to the embryo.

The presence of high levels of cytokinin activity co-eluting with the ribosides and glucosides of zeatin are suggestive of a further means of regulating the supply of cytokinins within the seed tissues. The glucosylation of cytokinins is considered to be a means of inactivation (HENSON and WAREING, 1976; VAN STADEN and PAPAPHILIPPOU, 1977) and thus storage (HEWETT and WAREING, 1973b) of these compounds. The cytokinins co-eluting with the glucosides of zeatin may therefore be stored forms.

Cytokinin levels in testa of the white lupin increased during the course of seed development. Difficulty was experienced in removing the endosperm completely from the testa and it is possible that much of the activity associated with the testa was due to the presence of the residual endosperm material. As the embryo expanded to fill the embryo sac the endosperm became closely appressed to the testa.

The pattern of increasing levels of activity in the seeds up to 6 weeks, after which they decrease, is similar to that which has been demonstrated to occur in pumpkin seeds (GUPTA and MAHESHWARI, 1970). It was reported that cytokinin levels were low in both very young and very old seeds of this species (GUPTA and MAHESHWARI, 1970). The increase in cytokinin activity in the white lupin seed

occurred during a period of increasing fresh weight. However, after 6 weeks, the fresh weight of the seed continued to increase, while the cytokinin level declined. This observation contrasts with that of PRAKASH and MAHESHWARI (1970), who demonstrated that in the watermelon the cytokinin level in the whole seed was maximal at 11 days after pollination, whereas seed size increase was most rapid between 4 and 9 days after anthesis. These workers suggested that, rather than contributing to the growth of the seed, changes in cytokinin levels were a mere accompaniment of growth. Such an approach towards the presence of these growth substances ignores the growth regulatory functions attributed to these compounds in plants.

The application of excess cytokinin to plant tissues, has shown that these growth regulators may be utilized by the plant in order to bring about numerous responses in the tissues to which they are applied. Some of the activities observed to be taking place in the embryonic cells during the course of seed development resemble these responses of plant tissues to the application of cytokinins. It is possible that cytokinins in the seed, rather than being transferred to the pod wall tissues (as was suggested earlier) are utilized in order to promote the subcellular activities of the growing embryo. Ultrastructural studies indicate that cell division and cell expansion take place in the embryo during periods of high cytokinin levels in the seed (Plate 3:7; Plate 3:8). As cytokinins have been shown to induce both cell division (MILLER, 1965) and

cell expansion (GORDON and LETHAM, 1975), it is possible that cytokinins are utilized within the seed to promote these activities. Applied cytokinins have been demonstrated to be involved in the promotion of chloroplast division (BOASSON and LAETSCH, 1969; BOASSON, BONNER and LAETSCH, 1972) and differentiation (HARVEY, LU and FLETCHER, 1974), phenomena observed in the expanding cotyledonary cells of the white lupin (Plate 3:10). There also appears to be a close association between cytokinins and the protein synthesizing mechanism. It has been shown that applied cytokinins assist in the maintenance of protein synthesis in leaves (OSBORNE, 1962) and can, in conjunction with auxin, induce increased nuclear size, endopolyploidy and increased RNA synthesis in legume roots (LIBBENGA and TORREY, 1974; SHININGER and TORREY, 1974; GORDON, LETHAM and BEEVER, 1975; SHININGER and POLLY, 1977). As the period during which there is a decrease in cytokinins in lupin seeds is also a period during which protein is synthesized and the nucleus increases in size (Plates 3:9 and 3:10), it is possible that cytokinins are utilized in order to promote these activities in the embryo. However, it remains for further research to reveal whether cytokinins are indeed utilized in these processes.

EXPERIMENT 4

Cytokinins in the Growing Seedling

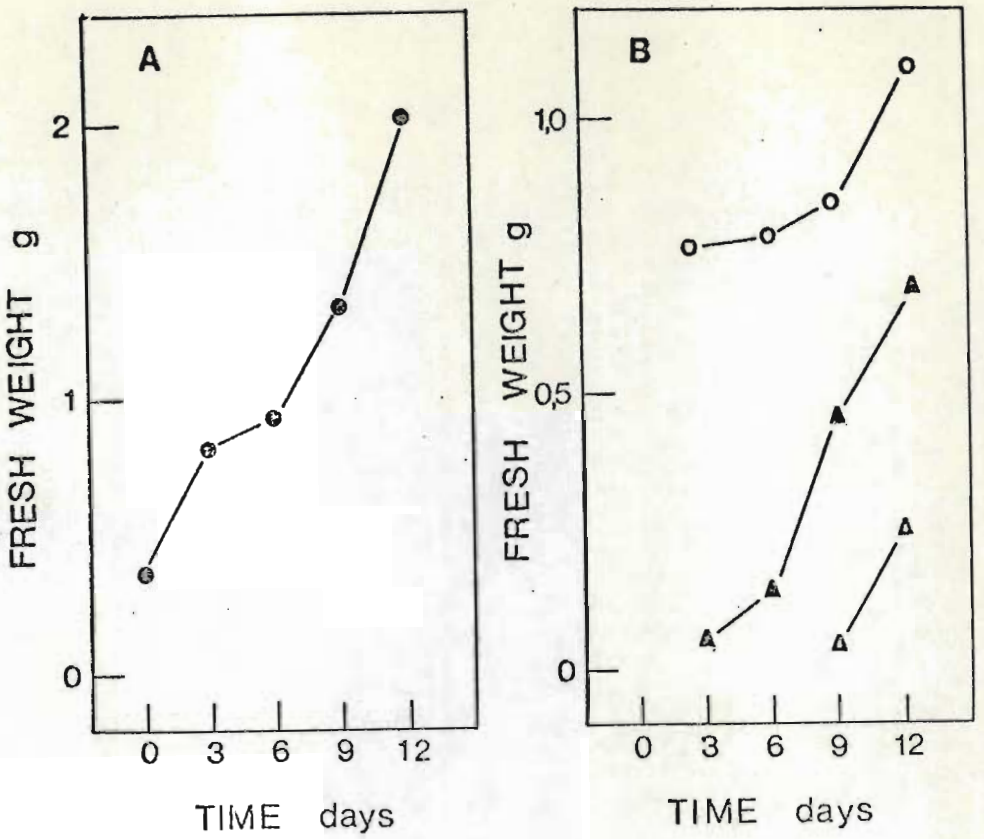
## Introduction

In the previous experiment cytokinin levels in the plant were examined during the course of vegetative growth, flowering and fruit development. Attention was also given to the involvement of cytokinins in the development of the embryonic plant within the seed (Experiment 3). The involvement of cytokinins in the development of the mature embryo into a young seedling is a phase of white lupin growth which has been neglected. It was therefore the intention of the present experiment to investigate the cytokinin levels in the embryo from the time of planting until 12 days after planting.

## Experimental Procedure

White lupin seeds were planted in trays of moistened vermiculite and harvested at 3, 6, 9 and 12 day intervals after planting. A sample of dry seed was retained and represented day 0. The seeds had germinated by 3 days after planting. At day 3 and, at later sampling times, only seeds germinated by day 3 were harvested.

At day 0 whole embryos were analysed for cytokinins while at later times the seedlings were divided into cotyledons and radicles (3 and 6 days after planting) and cotyledons, radicle and young shoots (plumule) (9 and 12 days after planting). All plant material was extracted and analysed for cytokinin activity according to procedures




C

WHITE LUPIN SEEDLING DEVELOPMENT

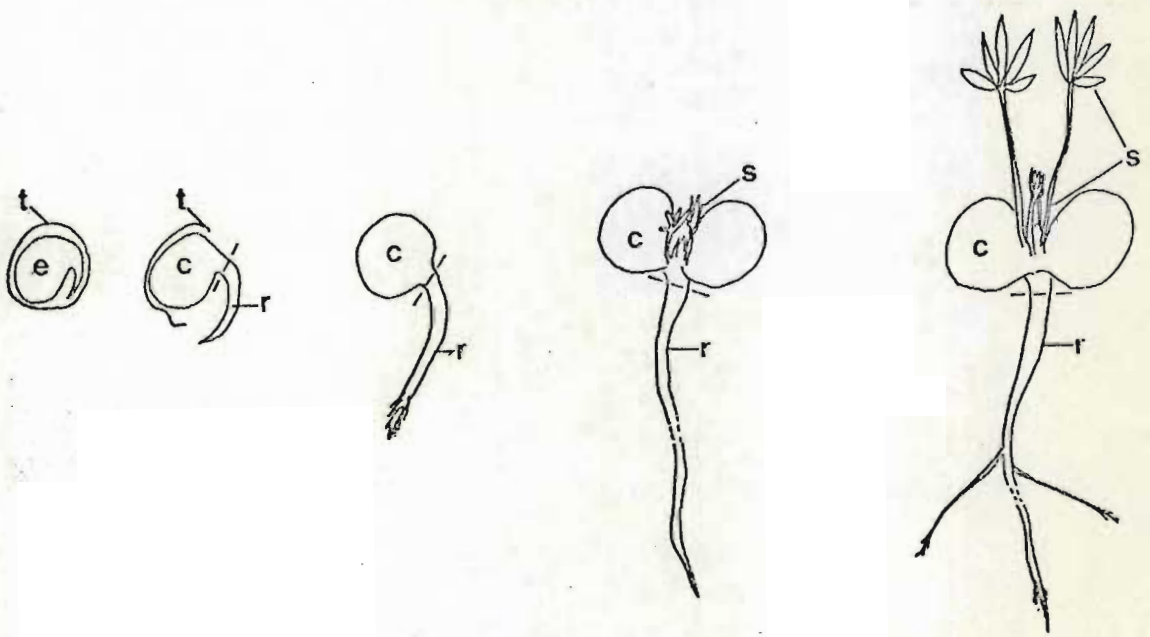



Figure 4:1. A. Change in fresh weight of the white lupin during seedling development, from maturity to 12 days after planting. B. Change in fresh weight of the cotyledons (o——o), radicles (▲——▲) and young shoots (Δ——Δ) over a 12 day period after planting. C. Diagrammatic representation of seedling development in the white lupin. c = cotyledon, e = embryo, r = radicle, s = young shoot.

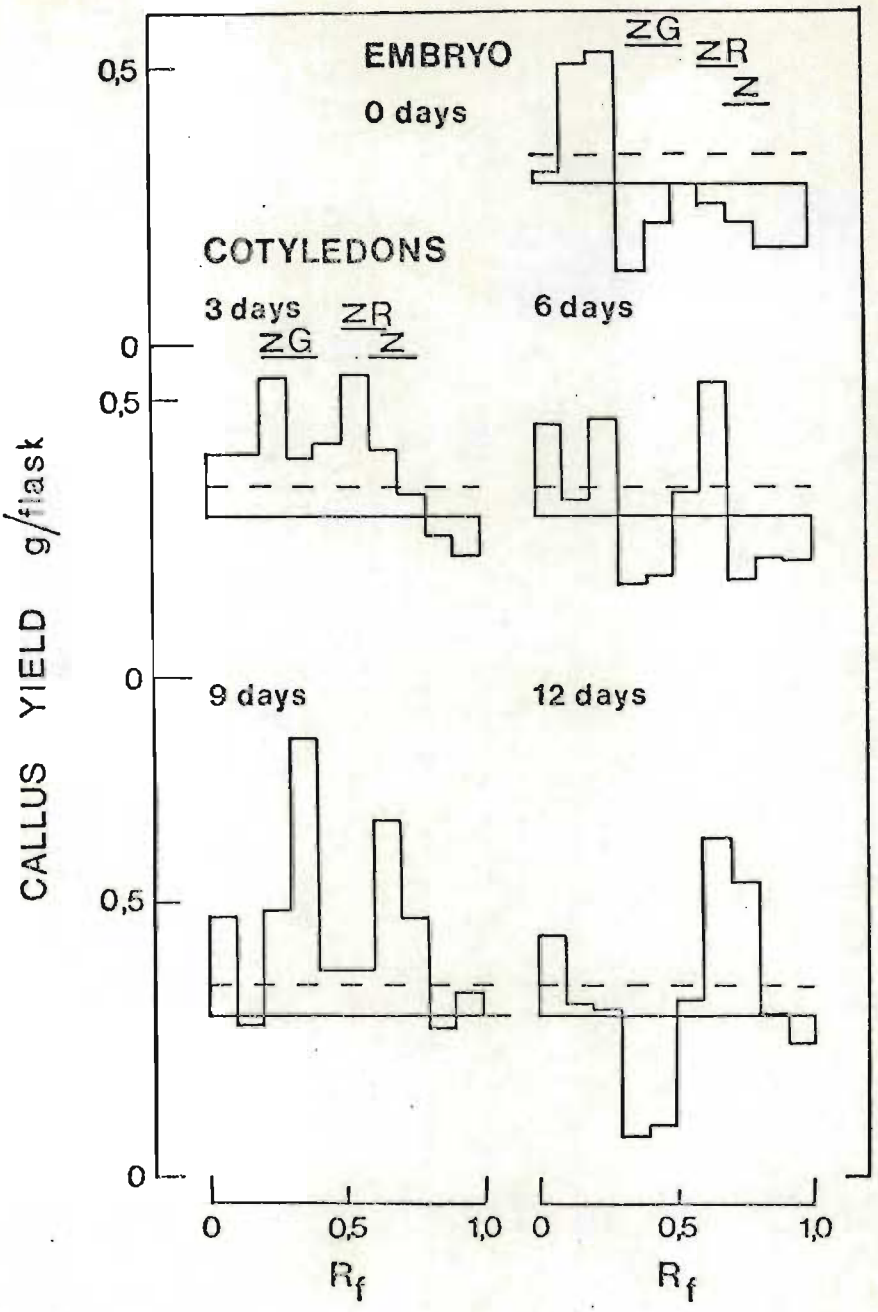



Figure 4:2. Cytokinin activity in extracts of 7,5 grammes of mature embryos and cotyledons. The ethanolic extracts were purified using Dowex 50 cation exchange resin. The ammonia eluates were reduced, and strip loaded onto chromatograms which were then separated with *iso*-propanol:25 percent ammonium hydroxide:water (10:1:1 v/v). Callus grown on 5 microgrammes per litre zeatin yielded 0,63 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. The broken line indicates the confidence limit at the level P = 0,01.

outlined in the Materials and Methods. As in the previous experiments, attempts were made to extract uniform batches of material. The whole embryos and cotyledons were extracted in 7,5 gramme lots, the radicles in 5 gramme lots and the young shoots in 6 gramme lots.

Results and Discussion

Figure 4:1 shows that the seedling increased in fresh weight over the 12 day experimental period. These changes in fresh weight were accompanied by morphological changes which are illustrated in Figure 4:2. Elongation of the radicle preceded the growth of the young shoot. The cotyledons initially orange, became bright green in colour. Cytokinin activity was detected in extracts of the whole embryo and cotyledons after assay of paper chromatograms (Figure 4:3). The level of activity in the whole embryo was low. However, the activity in the cotyledons fluctuated over the experimental period but showed an overall increase up to 9 days after planting, after which it decreased. A slow-moving fraction predominated in the mature embryo, while both fast- and slow-moving cytokinin-like compounds were detected in the cotyledons. Column chromatography of the whole embryo and cotyledons at 12 days after planting, indicated the presence of compounds co-eluting with zeatin, zeatin riboside and the glucoside cytokinins (Figure 4:4). The radicles at 3 days after planting contained very low levels of cytokinin activity (Figure 4:5). The level increased gradually, however, and at 12 days after planting was higher. This activity

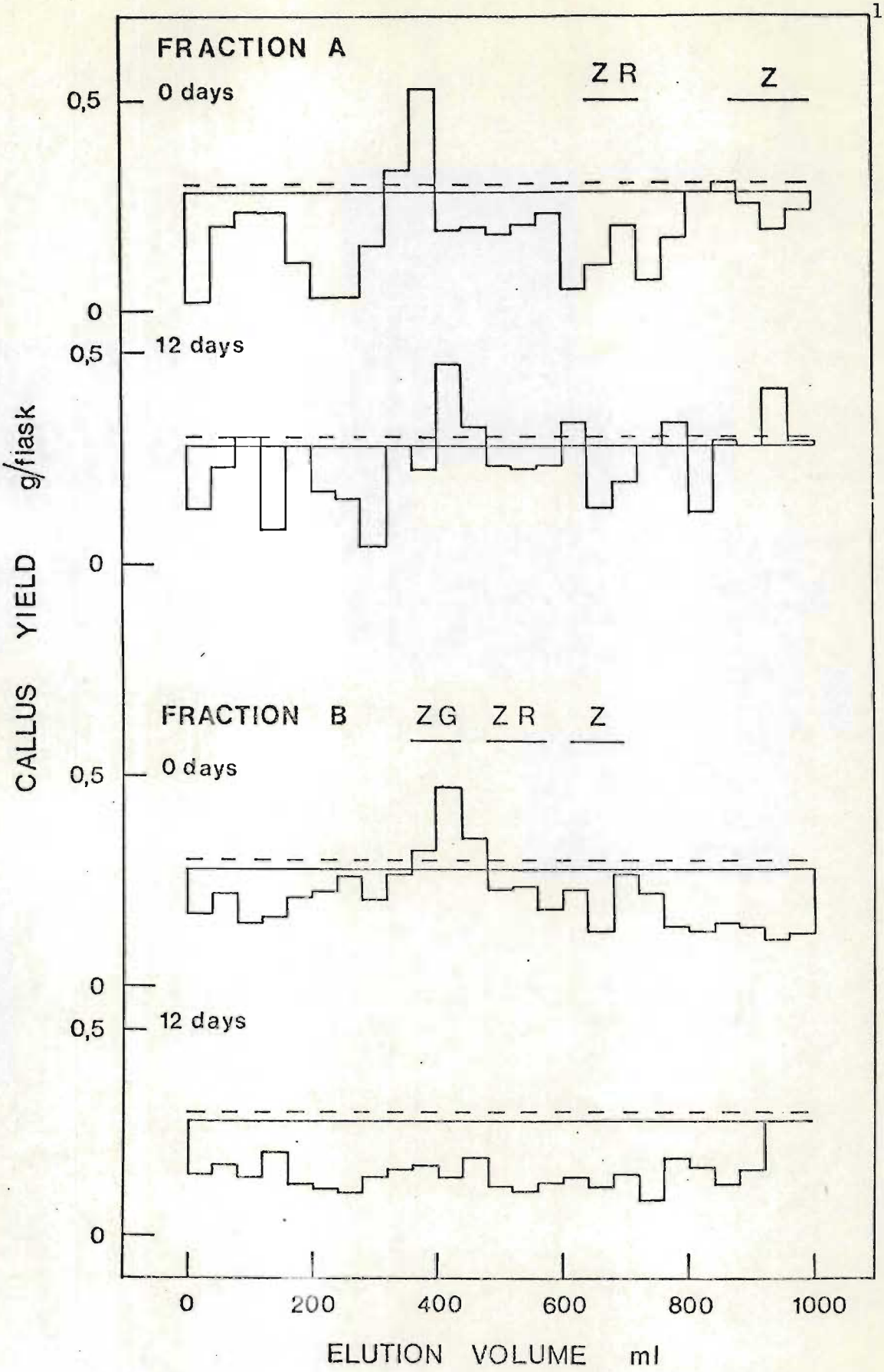



Figure 4:3. Cytokinin activity in extracts of 7,5 grammes of mature embryos (0 days) and cotyledons (12 days) following fractionation on Sephadex LH-20. Fraction A represents the activity at $R_f$ 0,05-0,5 of paper chromatograms and was fractionated on a column eluted with 20 percent ethanol. Fraction B represents the activity at $R_f$ 0,5-1,0 of paper chromatograms and was fractionated on a column eluted with 35 percent ethanol. Callus grown on 5 micrograms per litre zeatin yielded 1,09 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. The

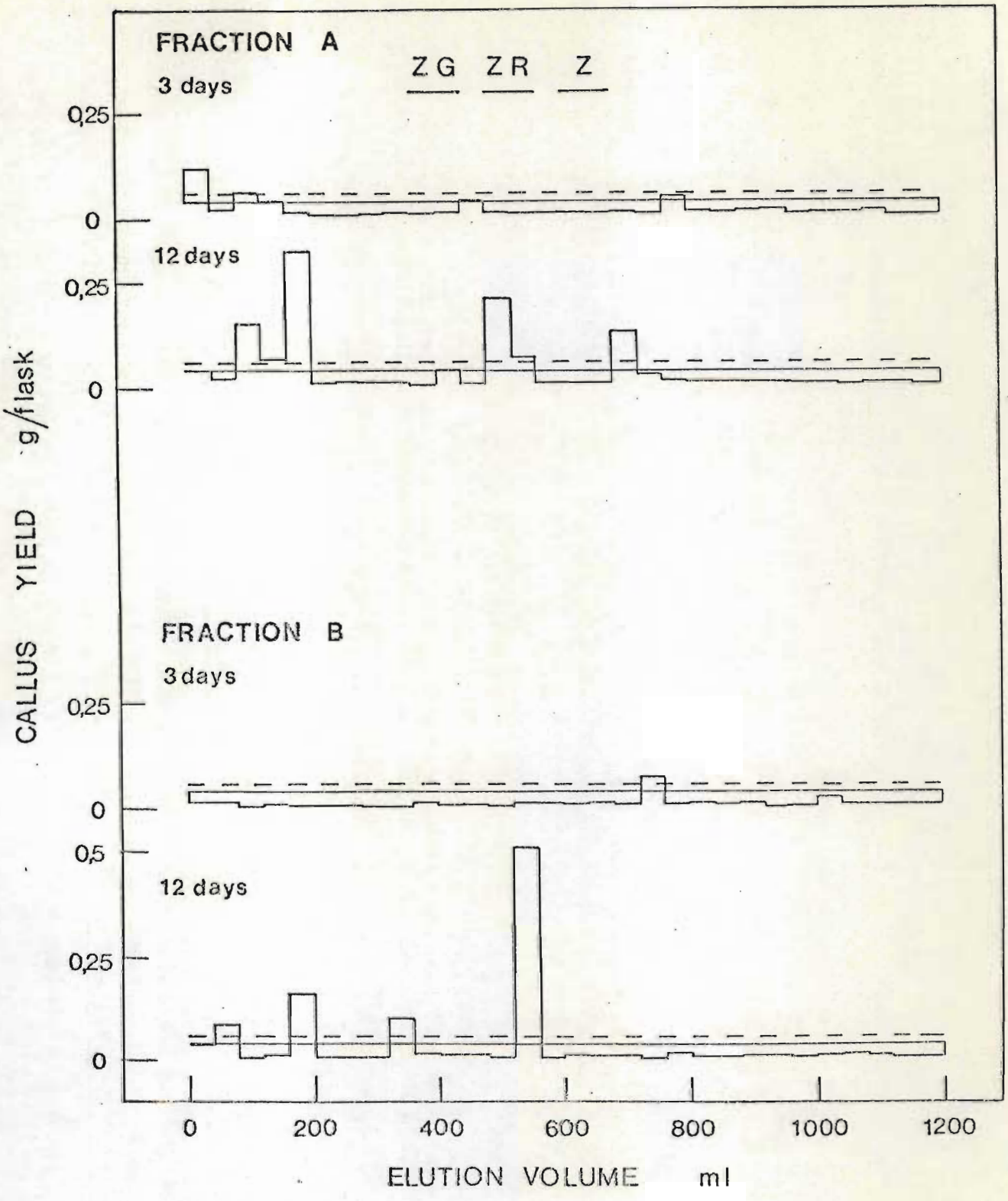



Figure 4:4. Cytokinin activity in extracts of 5 grammes of white lupin radicles harvested at 3 and 12 days after planting. Fraction A represents the activity at $R_f$ 0,05-0,5 of paper chromatograms and Fraction B, $R_f$ 0,5-1,0. The extracts were fractionated on a Sephadex LH-20 column eluted with 35 percent ethanol. Callus grown on 5 micrograms per litre zeatin yielded 0,83 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. The broken line indicates the confidence limit at the level P = 0,01.

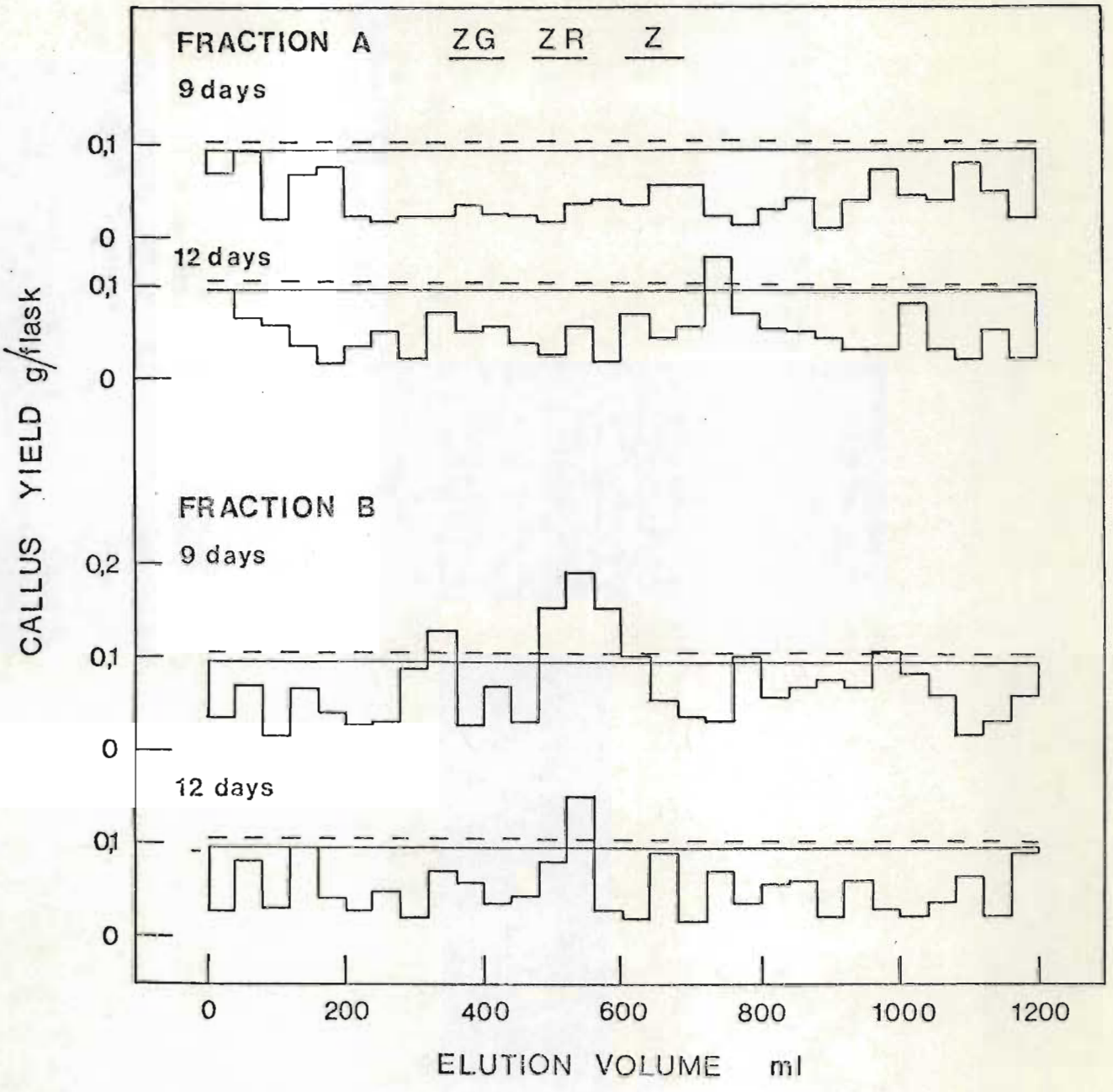



Figure 4:5. Cytokinin activity in 6 gramme extracts of young shoots harvested at 9 and 12 days after planting. Fraction A represents $R_f$ 0,05-0,5 of paper chromatograms and Fraction B, $R_f$ 0,5-1,0. The extracts were fractionated on a Sephadex LH-20 column eluted with 35 percent ethanol. Callus grown on 5 microgrammes per litre zeatin yielded 0,60 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. The broken line indicates the confidence limit at the level P = 0,01.

co-eluted on Sephadex LH-20 with zeatin riboside (Figure 4:5). Cytokinin activity was detected in the young shoots at 9 and 12 days after planting, but the levels of activity were very low (Figure 4:6). These low levels of cytokinin activity detected in developing white lupin seedling tissues are consistent with previous reports that seedlings contain low levels of cytokinin. BROWN and VAN STADEN (1973) reported that the level of cytokinin activity in germinated *Protea compacta* seeds was low, while THIMANN *et al.* (1970) reported that pea seedlings exhibited low levels of cytokinin activity. In contrast however, SMITH (1977) reported that the maize seedlings contain higher levels of cytokinin activity. As maize is an endospermous seed while the other seeds are exendospermous at maturity, it is possible that the source of cytokinins in these different seed types may differ. In the maize seed it would appear as if the endosperm is a rich source of cytokinin to the growing embryo. Furthermore, it has been suggested that the radicle in maize is dependent on a supply of cytokinin for its initial growth and only acquires the capacity for cytokinin synthesis after reaching a certain stage of maturity (SMITH, 1977). In the white lupin seed no endosperm is present in the seed at maturity. As the seed matures the testa may contain increased levels of cytokinin activity (Experiment 3). Thus the testa could possibly act as a source of cytokinins to the young embryo, but it is discarded at an early stage. The cytokinins in the seedling are low, and this may indicate that the white lupin seedling does not require high levels of cytokinin for

its growth. However, it should be stressed that low levels of cytokinins are not necessarily indicative of a paucity of the substance and can merely indicate that they are rapidly utilized. It is therefore possible that cytokinin synthesis is initiated (possibly in the young radicle) relatively early in the course of seedling growth (before or just after the testa is discarded) but that these cytokinins are utilized by the rapidly growing seedling. Increasing levels of cytokinin activity in the cotyledons and radicles would appear to support this suggestion.

# PART III

## CYTOKININ IDENTIFICATION

### EXPERIMENT 5

### Identification of the Cytokinins in White Lupin Fruits

#### Introduction

In the literature review it was mentioned that, although an understanding of the role of cytokinins in plant tissues may be enhanced by quantitative studies, the overall concept of their involvement in plant growth processes is improved by the identification of the active compounds present in plants. Throughout this project use has been made of column chromatography to ascertain by means of co-elution with authentic markers, the possible nature of the cytokinins present in white lupin extracts. However, the limitations of such a technique were emphasised when the presence of a possible dihydro-derivative of zeatin was indicated in white lupin extracts at the elution volume corresponding to authentic zeatin. A programme designed not only to isolate cell division inducing factors commonly present in white lupin extracts, but also to ascertain the chemical identity of these compounds was therefore initiated. Extracts of white lupin fruits had been shown to contain a high level of cytokinin activity, as well as a number of different cytokinins. In view of these facts the cytokinin complement of fruit extracts was studied.

Experimental Procedure and Results

The basic methods of Dowex purification, chromatography and bioassay used in this study are outlined in the Materials and Methods section and shall not be elaborated on in this procedural outline. Quantitative differences in materials, necessitated by a mass extraction and purification procedure, will be quoted wherever necessary.

Two and a half kilograms of white lupin fruits (including seeds and pod walls) were homogenised in a Wareing blender in 5 litres of 80 percent ethanol. The homogenate was allowed to stand overnight at 5°C. It was then filtered through Whatman No. 1[1] filter paper and the filtrate concentrated to dryness on a flash evaporator. The residue was taken up in 500 millilitres of 80 percent ethanol, the pH adjusted to 2,5 with hydrochloric acid and this ethanolic extract passed through a column packed with 500 grammes of Dowex 50 (20-40 US mesh) cation exchange resin. Five hundred millilitres of 80 percent ethanol were then used to wash the column. The eluate and the wash were discarded. Compounds adhering to the resin were eluted with 2 litres of 5N ammonium hydroxide. When the ammonia eluate had been concentrated to dryness under vacuum, the residue was taken up in 40 millilitres of 80 percent ethanol and strip loaded onto 20 sheets of Whatman No. 3 mm chromatography paper. The chromatograms were then separated in *iso*-propanol: 25 percent ammonium hydroxide:water (10:1:1 v/v) (PAW) and air dried. The compounds present on these chromatograms

[1]Whatman No. 1 filter paper was used throughout this experiment.

between $R_f$ 0,05-1,0 were eluted with 2,5 litres of 80 percent ethanol. The eluate was filtered and concentrated to dryness. The residue was taken up in 20 millilitres of 80 percent ethanol and strip loaded onto 10 sheets of Whatman No. 1 chromatography paper. Once these chromatograms had been separated in PAW, the elution procedure was repeated. The resulting residue was taken up in 15 millilitres of 80 percent ethanol, applied to 9 sheets of Whatman No. 1 chromatography paper (equivalent of 277 grammes of fruit material per sheet) and again separated in PAW and air dried. A five centimetre strip (equivalent of 34 grammes of fruit material) of one chromatogram was then divided into 10 $R_f$ zones and these were assayed for cytokinin activity.

High levels of cytokinin activity were detected between $R_f$ 0,3-0,8. As the glucoside cytokinins run between $R_f$ 0,2-0,4 on paper and zeatin and zeatin riboside between $R_f$ 0,5-0,85 the chromatograms were divided into two fractions, an A fraction ($R_f$ 0,05-0,5) and a B fraction ($R_f$ 0,5-1,0). The activity in both fractions was eluted separately with 2,5 litres of 80 percent ethanol, and the eluates were filtered and reduced to dryness. The residues were taken up in 20 millilitres (A fraction), 5 millilitres (B fraction) of 80 percent ethanol, streaked onto 4 (A fraction) and 1 (B fraction) sheets of Whatman No. 1 chromatography paper and run in PAW. The chromatograms were again assayed for cytokinin activity.

Activity was detected in both fast- and slow-moving regions of these chromatograms. They were therefore divided into two sections: $R_f$ 0,05-0,5 and $R_f$ 0,5-1,0. The activity

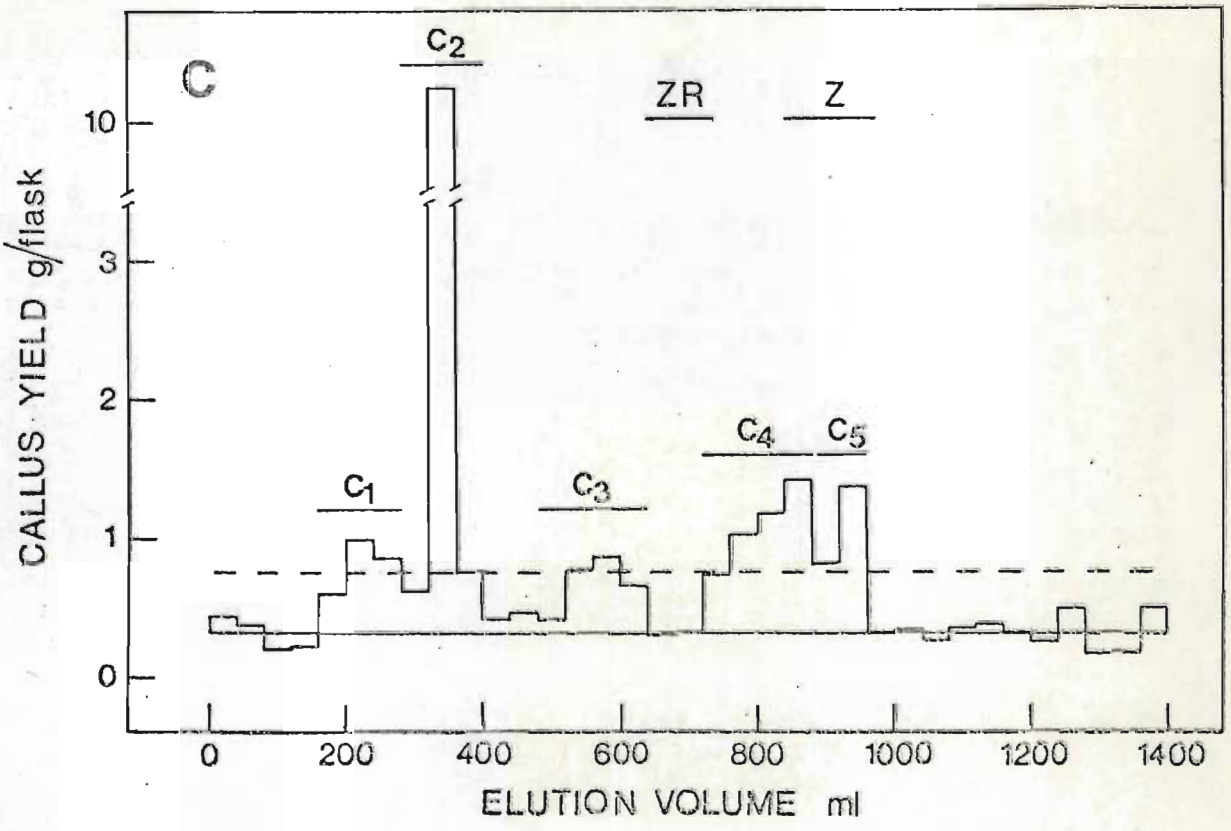



Figure 5:1. The distribution of cytokinin activity in Fraction A after fractionation on a Sephadex LH-20 column eluted with 20 percent ethanol. The equivalent of 27,5 grammes fresh weight fruit material was used. Callus grown on 5 microgrammes per litre zeatin yielded 0,45 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. The broken line indicates the confidence limit at the level P = 0,01.

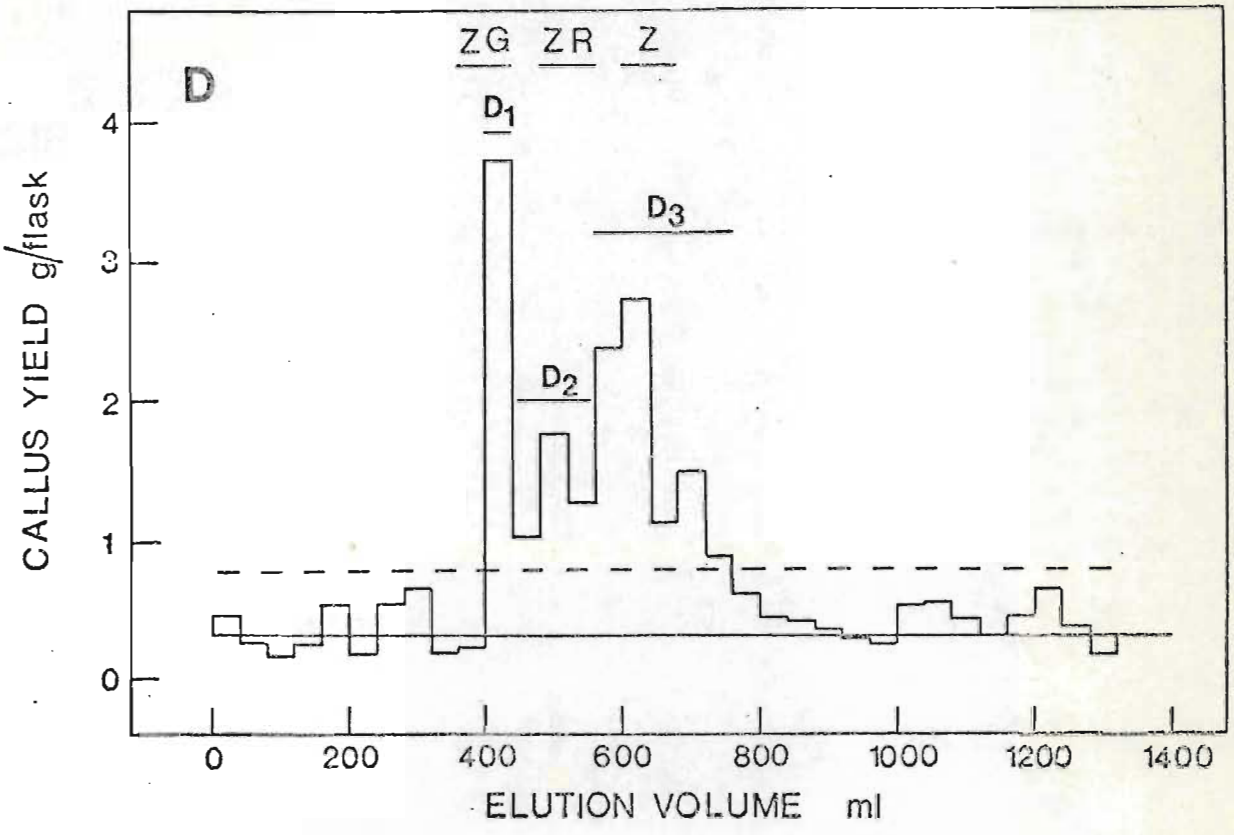



Figure 5:2. The distribution of the cytokinin activity in Fraction B after separation on a Sephadex LH-20 column eluted with 35 percent ethanol. The equivalent of 27,5 grammes fresh weight fruit material was assayed. Callus grown on 5 microgrammes per litre zeatin yielded 0,45 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. The broken line indicates the confidence limit at the level P = 0,01.

at $R_f$ 0,05-0,5 from chromatograms of both A and B fractions was combined to give fraction C, while the activity at $R_f$ 0,5-1,0 from chromatograms of both A and B fractions was combined to give fraction D. In both instances the activity was eluted, filtered and concentrated to dryness.

*Fraction C.* The residue of fraction C was taken up in 4 millilitres of 80 percent ethanol, applied to a Sephadex LH-20 column and eluted with 20 percent ethanol. Forty millilitre fractions were collected, and the equivalent of 27,5 grammes fruit material was removed from each fraction for assay before the remainder was air dried and stored. Five major peaks of activity were detected in Fraction C and denoted C 1-5 (Figure 5:1).

*Fraction D.* The residue of Fraction D was taken up in 4 millilitres of 80 percent ethanol, applied to a Sephadex LH-20 column and eluted with 35 percent ethanol. Forty millilitre fractions were collected and the equivalent of 27,4 grammes of fruit material removed from each fraction and assayed. The remainder of the fraction was air dried and stored. Three major peaks of activity were detected in Fraction D and denoted D 1-3 (Figure 5:2). The peaks of activity derived in the above manner were combined as follows:

| Designation | elution volumes (ml) 20% column | | 35% column |
|---|---|---|---|
| $C_1$ | 160 - 280 | | |
| $C_2$ | 280 - 400 | | |
| $C_3 + D_1 = C_3D_1$ | 480 - 640 | + | 400 - 440 |
| $C_4 + D_2 = C_4D_2$ | 520 - 880 | + | 440 - 560 |
| $C_5 + D_3 = C_5D_3$ | 880 - 960 | + | 560 - 760 |

*Fraction $C_1$.* This fraction was the most impure of the five

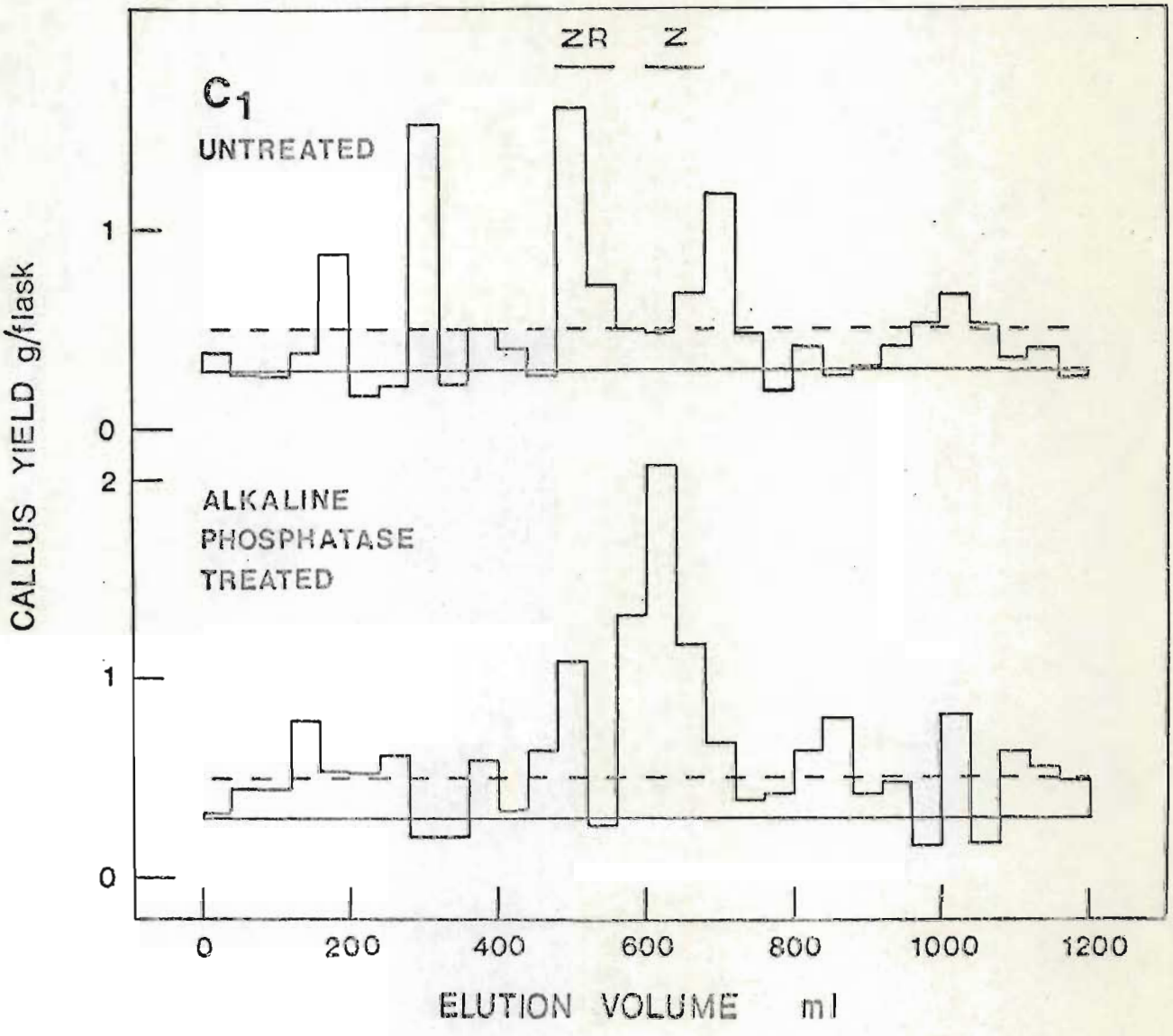



Figure 5:3. The cytokinin activity in Fraction $C_1$ following fractionation of untreated and alkaline phosphatase treated samples on a Sephadex LH-20 column eluted with 35 percent ethanol. The equivalent of 55 grammes of fruit material was used for each sample. Callus grown on 5 microgrammes per litre zeatin yielded 2,49 grammes fresh weight. Z = zeatin; ZR = zeatin riboside. The broken line indicates the confidence limit at the level P = 0,01.

fractions. Two samples (equivalent of 2 x 56 grammes fruit material) were removed. The first sample was fractionated on a Sephadex LH-20 column eluted with 5 percent ethanol. Forty millilitre fractions were collected. The remaining sample was treated with alkaline phosphatase (see Materials and Methods) and passed through Sephadex LH-20 in a similar manner. The fractions from both treatments were air dried and assayed simultaneously. Figure 5:3 shows that this activity eluted at 280-320 millilitres and that treatment with alkaline phosphatase caused a reduction in activity at this elution volume and an increase in activity at an elution volume co-eluting with authentic zeatin.

*Fraction $C_2$.* Fraction $C_2$ co-eluted on a Sephadex LH-20 eluted with 20 percent ethanol, with cytokinins which have been previously described as zeatin riboside glucoside and dihydro-zeatin riboside glucoside (SMITH, 1977). In order to test for the possible presence of these compounds three samples (equivalent of 27,5 grammes fruit material) were removed. One sample served as a control while the remaining samples were treated with β-glucosidase, and β-glucosidase and potassium permanganate, respectively. These samples were then applied to a Sephadex LH-20 column and eluted with 35 percent ethanol. Forty millilitre fractions were collected, dried and assayed. The activity in the untreated sample eluted at 320-440 millilitres (Figure 5:4). Treatment with β-glucosidase resulted in a movement of this activity to an elution volume corresponding to that of authentic zeatin riboside. Treatment with β-glucosidase and potassium permanganate resulted in an increase in activity at elution volume

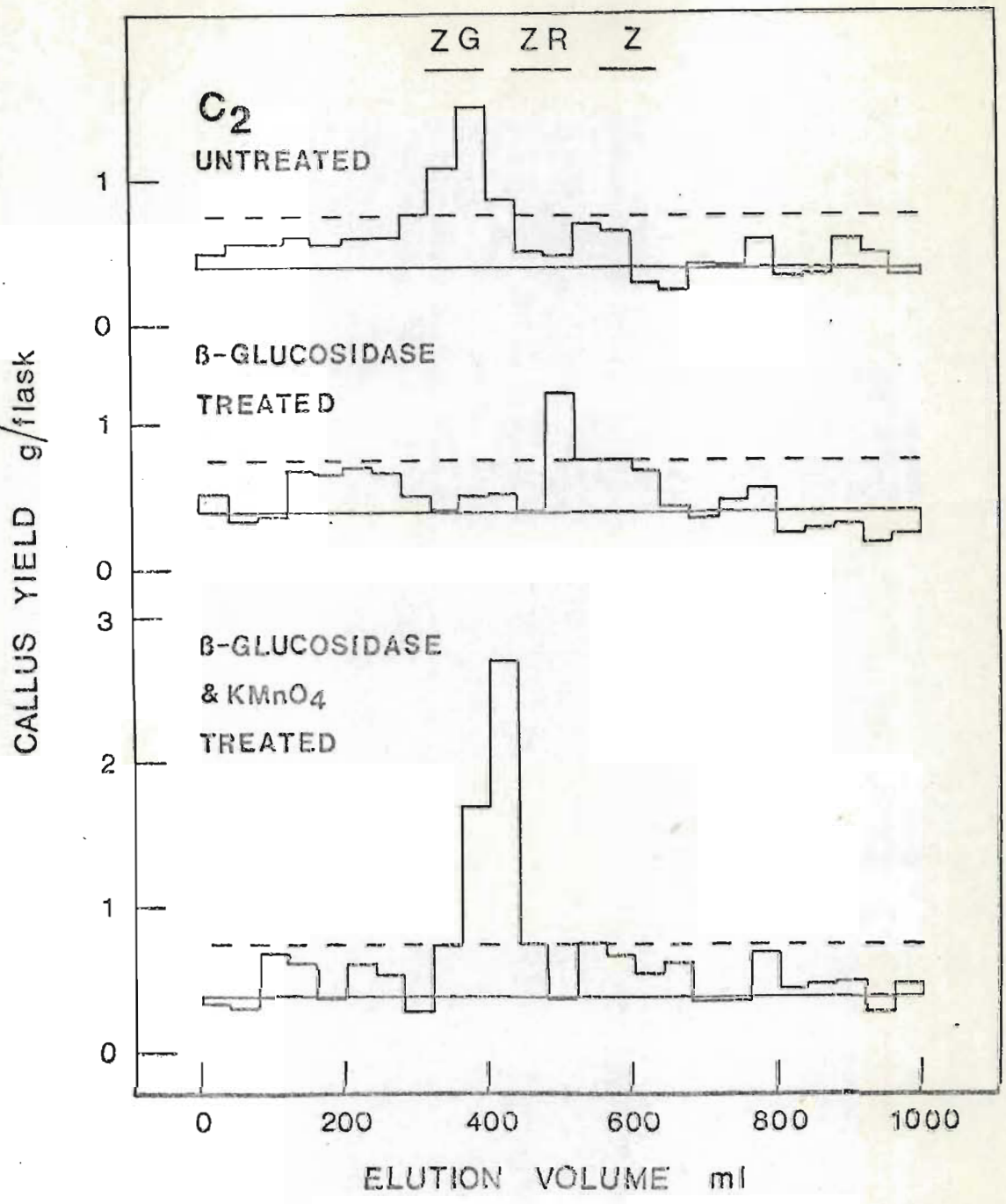



Figure 5:4. The distribution of cytokinin activity in three samples of 27,5 grammes of lupin fruit material following fractionation on a Sephadex LH-20 column eluted with 35 percent ethanol. One sample served as a control while the remaining samples were β-glucosidase, and β-glucosidase and potassium permanganate treated, respectively. Callus grown on 5 microgrammes per litre zeatin yielded 0,97 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. The broken line indicates the confidence limit at the level P = 0,01.

320-440. There was no activity at the elution volume corresponding to zeatin riboside. This rather unexpected result indicated the possible presence of other compounds co-eluting with what appeared to be initially glucosylated zeatin riboside.

*Fraction* $C_3D_1$. The combined $C_3$ and $D_1$ fractions were re-dissolved in 2 millilitres of 20 percent ethanol, and applied to a Sephadex LH-20 column and eluted with 20 percent ethanol. Forty millilitre fractions were collected, and the equivalent of 27,5 grammes of fruit material removed for assay. The remaining liquid in each flask was air dried and the residue stored. From the results of the assay (Figure 5:5) it was evident that there were two peaks of activity present, one eluting at elution volume 460-500 millilitres ($C_3D_{1a}$) and the other with authentic zeatin riboside ($C_3D_{1b}$). $C_3D_{1b}$ was combined with $C_4D_2$ (see Fraction $C_4D_2$). Three samples of $C_3D_{1a}$ were removed (equivalent of 27,5 grammes of fruit material). One sample acted as a control while the remaining samples were treated with β-glucosidase, and β-glucosidase and potassium permanganate, respectively. These samples were then applied to a Sephadex LH-20 column and eluted with 35 percent ethanol. Forty millilitre samples were collected, air dried and assayed. The activity in the untreated sample was present at an elution volume of 340-440 millilitres (Figure 5:6). Treatment of this fraction with β-glucosidase resulted in a reduction in activity associated with this elution volume and an increase in activity co-eluting with authentic zeatin and zeatin riboside. Treatment with β-glucosidase and potassium permanganate resulted in a reduction in activity

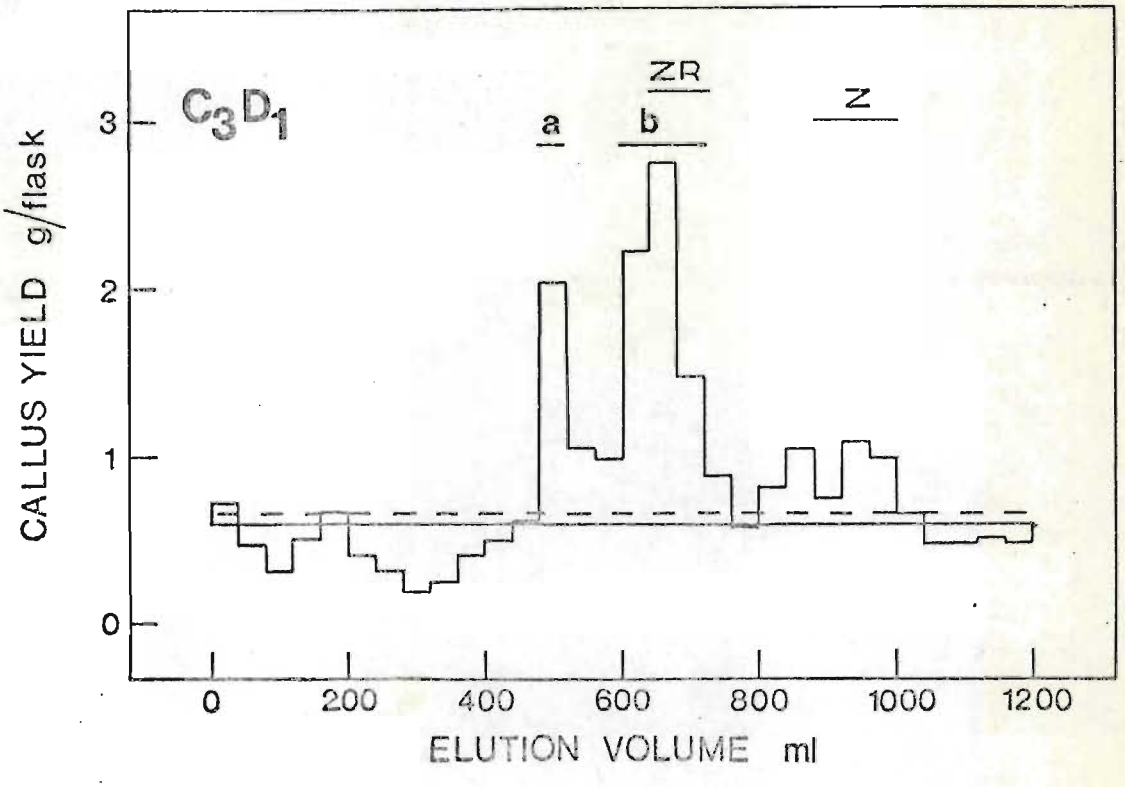



Figure 5:5. The distribution of cytokinin activity in Fraction $C_3D_1$ following fractionation on a Sephadex LH-20 column eluted with 20 percent ethanol. Forty millilitre fractions were collected and the equivalent of 27,5 grammes removed for assay. Two peaks of cytokinin activity were detected and designated a ($C_3D_1a$) and b ($C_3D_1b$). Callus grown on 5 microgrammes per litre zeatin yielded 4,00 grammes fresh weight. Z = zeatin; ZR = zeatin riboside. The broken line indicates the confidence limit at the level P = 0,01.

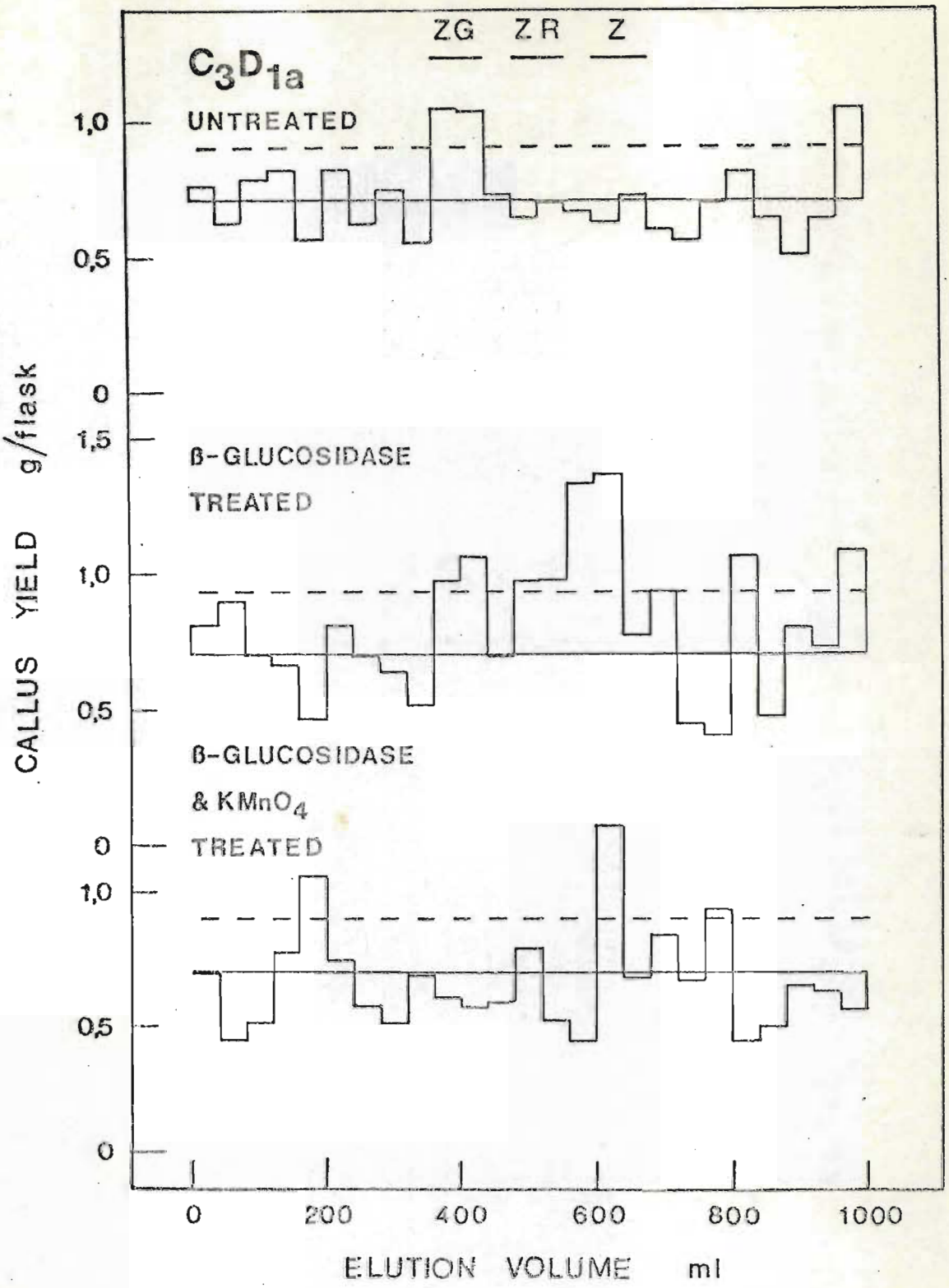



Figure 5:6. The cytokinin activity in $C_3D_{1a}$ following fractionation of untreated, β-glucosidase, and β-glucosidase and potassium permanganate treated, samples on a Sephadex LH-20 column eluted with 35 percent ethanol. The equivalent of 27,5 grammes of fruit material was used for each treatment. Callus grown on 5 microgrammes per litre zeatin yielded 2,49 grammes fresh weight. Z = zeatin; ZR = zeatin riboside; ZG = zeatin glucoside. The broken lines indicate the confidence limit at the level P = 0,01.

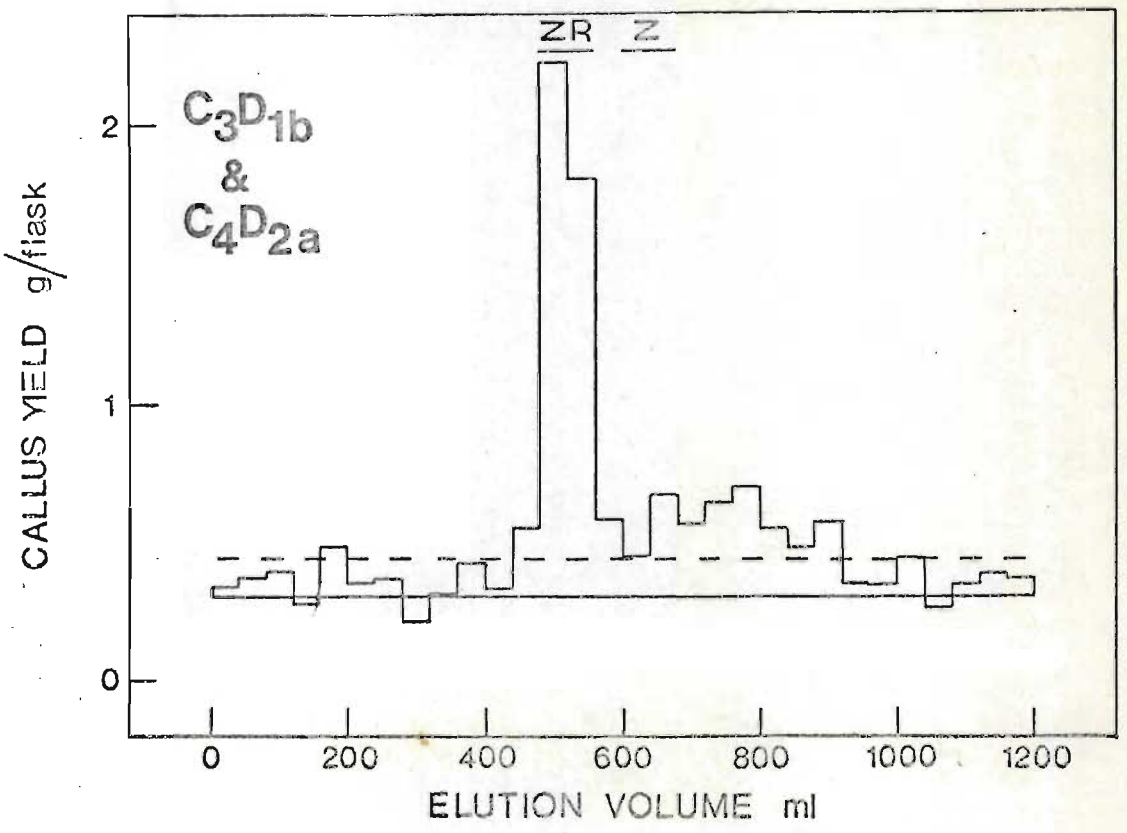



Figure 5:7. The distribution of cytokinin activity in the combined fractions $C_3D_{1b}$ and $C_4D_{2a}$ following separation on a Sephadex LH-20 column eluted with 35 percent ethanol. Forty millilitre fractions were collected, and the equivalent of 27,5 grammes removed for assay. Callus grown on 5 microgrammes per litre zeatin yielded 4,24 grammes fresh weight. Z = zeatin; ZR = zeatin riboside. The broken line indicates the confidence limit at the level P = 0,01.

co-eluting with authentic zeatin riboside and zeatin though some activity co-eluting with zeatin remained. This result suggested that a glucosylated form of zeatin was present in this fraction and, furthermore, that a dihydro-derivative could possibly be present.

*Fraction* $C_4D_2$. This combined sample was dissolved in 1 millilitre of 35 percent ethanol and applied to a Sephadex LH-20 column and eluted with 35 percent ethanol. Forty millilitre fractions were collected, and half a millilitre (equivalent of 27,5 grammes fruit material) of each fraction was removed and assayed for cytokinin activity. Activity was detected at elution volumes 480-560 millilitres ($C_4D_{2a}$) and 600-680 ($C_4D_{2b}$) millilitres. Fraction $C_4D_{2a}$ was combined with Fraction $C_5D_3$ (see Fraction $C_5D_3$). Fraction $C_4D_{2a}$ was however combined with Fraction $C_3D_{1b}$. This combined fraction was then applied to a Sephadex LH-20 column and eluted with 35 percent ethanol. Forty millilitre fractions were collected and half a millilitre removed from each and assayed. The activity in this assay was present at the elution volume corresponding to authentic zeatin riboside (Figure 5:7).

*Fraction* $C_5D_3$. The residue of this combined fraction was taken up in 2 millilitres of 35 percent ethanol and applied to a Sephadex LH-20 column and eluted with 35 percent ethanol. Forty millilitre fractions were collected and the equivalent of 27,5 grammes of fruit material removed for assay before the remainder of the fraction was air dried. The results of the assay indicated that the majority of the activity in

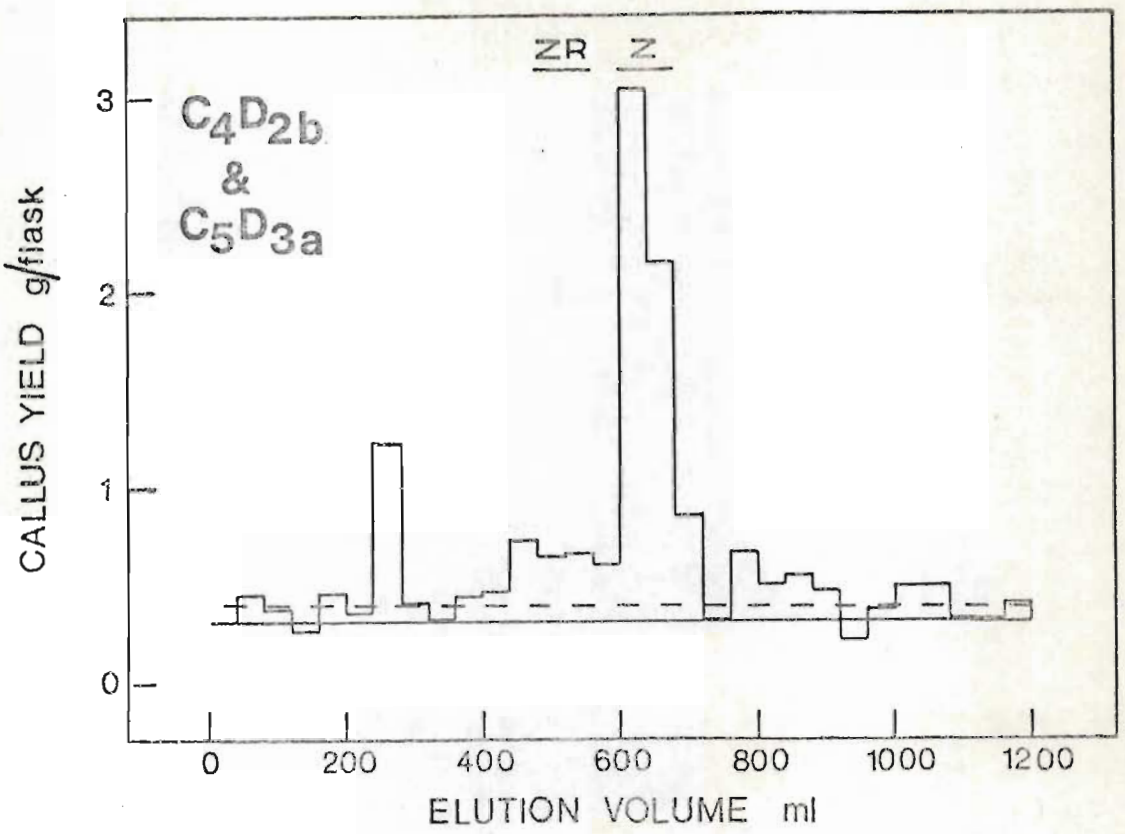



Figure 5:8. The distribution of cytokinin activity in the combined $C_5D_{3a}$ (elution volume 560-600 millilitres) and $C_4D_{2b}$ fractions following fractionation on a Sephadex LH-20 column eluted with 35 percent ethanol. Forty millilitre fractions were collected and the equivalent of 27,5 grammes fruit material removed for assay. Callus grown on 5 microgrammes per litre zeatin yielded 4,24 grammes fresh weight. Z = zeatin; ZR = zeatin riboside. The broken line indicates the confidence limit at the level P = 0,01.

this sample was present at an elution volume of 520-600 millilitres ($C_5D_{3a}$). The activity at this elution volume was combined with fraction $C_4D_{2b}$, taken up in 1 millilitre of 35 percent ethanol and again passed through a Sephadex LH-20 column eluted with 35 percent ethanol. Forty millilitre fractions were collected and the equivalent of 27,5 grammes of fruit material removed for assay before the remainder of each sample was air dried for storage. The assay showed clearly that the activity in this fraction co-eluted distinctly with authentic zeatin (Figure 5:8).

The active fractions derived by passing $C_4D_{2a}$ + $C_3D_{1b}$ and $C_5D_{3a}$ + $C_4D_{2b}$ (Figures 5:7 and 5:8) through Sephadex LH-20 columns were analyzed using low resolution mass spectrometric techniques by Professor S.E. Drewes of the Department of Chemistry at the University of Natal, Pietermaritzburg. The activity at the elution volume 600-680 millilitres (Figure 5:8) was positively identified as zeatin (Figure 5:9). The results also indicated that the active peak which co-eluted with zeatin riboside at an elution volume of 480-560 millilitres was an adenine derivative. In view of the above findings it would appear that zeatin is present in extracts of white lupin fruits, and that the other cell division inducing compounds are either derivatives of zeatin, or similar to the other naturally occurring cytokinins which are $N^6$-substituted derivatives of adenine.

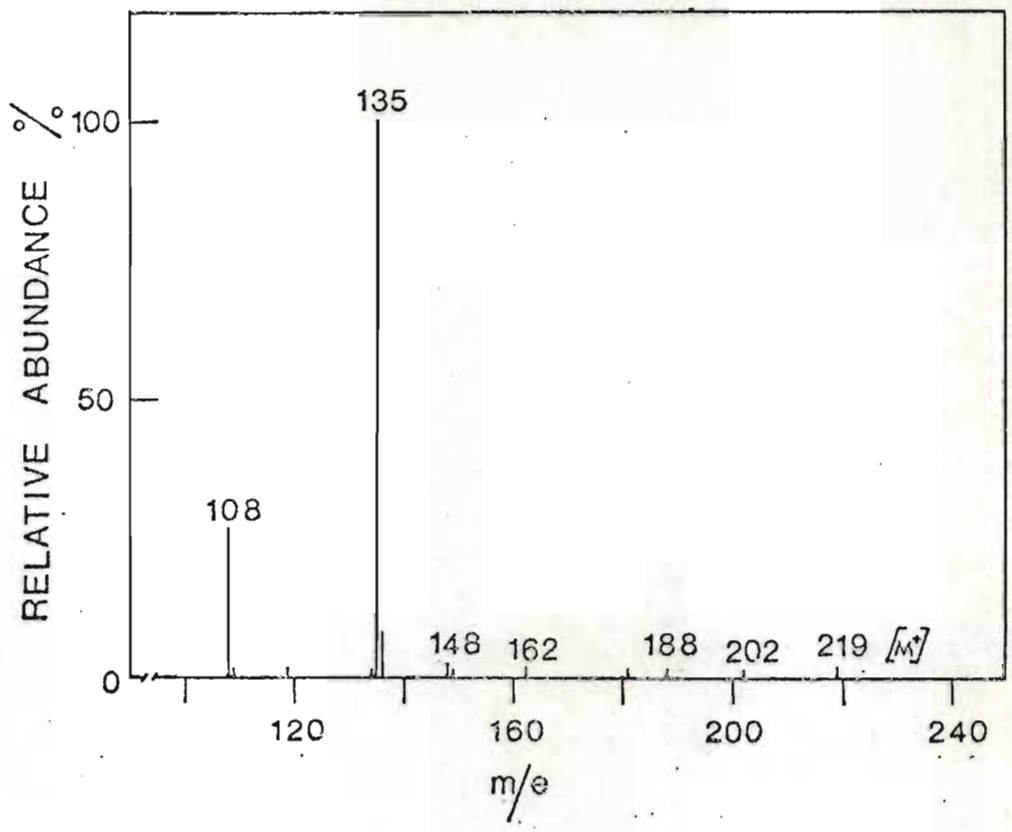



Figure 5:9. Mass spectrum of the active compound co-eluting with zeatin in $C_4D_2$ + $C_5D_3$ (Figure 5:8).

# CONCLUSION

The occurrence of cytokinins in a wide variety of plants and plant organs was noted in the literature review, and it was recognised that these growth substances change both quantitatively and qualitatively during plant growth. However, although it was indicated that the roots alone have been proven to be sites of cytokinin synthesis, it was stressed that the simple root-to-shoot type transference of cytokinins that was originally proposed, has been complicated by the isolation of cytokinin-like compounds from phloem sap. The present study attempted to synthesize our knowledge of changing levels of cytokinins in a plant by investigating cytokinin changes throughout the growth cycle. It is unfortunate that changing levels of other growth regulators have not been investigated in a similar system, as one regulator often appears to interact with another in order to induce growth responses.

Cytokinin-like compounds were detected at varying levels in all plant organs during whole plant growth. These compounds had chromatographic properties on paper and on Sephadex LH-20 similar to the cytokinins which have been isolated from other plants (HENSON and WAREING, 1974; 1976; 1977a; 1977b; VAN STADEN, 1976b; 1976c; 1976d; 1977). Analysis of the cytokinins in white lupin fruit tissues showed that two of these substances co-eluted on Sephadex LH-20 with authentic zeatin and zeatin riboside, respectively. Mass spectrometric analysis of these cytokinin-like compounds

showed that the substance co-eluting with zeatin was indeed zeatin, while analysis of the activity co-eluting with zeatin riboside indicated the presence of an adenine derivative at this elution volume. Another peak of activity co-eluted on Sephadex with zeatin glucoside and could be hydrolyzed by β-glucosidase to yield a peak of activity co-eluting with zeatin in the same system. The two remaining major peaks of activity recorded from white lupin fruit extracts could be hydrolyzed by β-glucosidase to yield a peak of activity co-eluting with zeatin riboside on Sephadex LH-20, and by alkaline phosphatase to yield a peak of activity co-eluting with zeatin on Sephadex LH-20, respectively. The results also indicated that the dihydro-derivatives of these cytokinins could occur in the white lupin, but did not exclude the possibility that other cytokinin-like compounds might be present in white lupin extracts.

Cytokinins were shown to occur in root exudates, leaves and fruiting apices of white lupin plants. Significant levels of activity were, however, not detected in the vegetative terminal apices or in the terminal apex when it was in flower. The total level of activity in the translocation stream of the plants was much greater than that which accumulated in the leaves and apices up to the time of flowering. In view of the suggestion by HENSON (1978) that cytokinins are more rapidly metabolized in young leaves, this observation was considered indicative of the rapid utilization of cytokinins in the actively growing

young plant. Such an hypothesis is supported by the fact that in other parts of the plant, such as embryos and young seedlings, active growth was not accompanied by appreciable increases in the cytokinin levels. The possibility that cytokinins in the young shoot could have been translocated back to the roots in the phloem cannot be excluded. However, studies outlined in the literature review, which have involved the removal of apical tissues, have indicated that cytokinins are not necessarily translocated to the roots but may accumulate in shoot tissues. An investigation of the cytokinin levels in the mature root tissues was not made as the facilities for the liquid culture of large numbers of mature plants were not available. However, were such a study to be made in the future, it would add greatly to our knowledge of the cytokinin status of the plant, but would have to take into consideration the effect of nodulation on cytokinin production by the roots.

After flowering, the level of activity in the terminal apices increased, although the amount of cytokinin in the root exudate decreased. This was contrary to earlier findings (DAVEY and VAN STADEN, 1976) and the fruiting stage of growth was therefore examined more closely. These studies showed that although insufficiently high levels of cytokinin were present in the root exudate to account for the high level in the fruits, cytokinins were present in the sap passing into the fruits. As this sap is composed largely of phloem exudate (PATE *et al.*, 1974) it was

concluded that cytokinins may be present in the phloem of the white lupin and at least some of the cytokinins present in the fruits can originate in other parts of the plant. This finding is also in accordance with the suggestion of HOAD *et al.* (1977) that the zeatin riboside present in the grape can be derived from the roots. Cytokinin levels in the leaves were also shown to increase during the course of fruit development. This increase was due to the accumulation of cytokinin glucosides in the leaves as they matured and approached senescence. As was pointed out in the literature review, little evidence has been accumulated to refute the idea that the cytokinins in the leaves originate in the root tissues. However, the detection of cytokinins in phloem sap points to the possibility that there may be reciprocal transport of cytokinins between organs of the shoot. In the white lupin, the levels of cytokinin activity in the leaf tissues do not reach the high levels of activity recorded in the fruit tissues. Thus while it seems unlikely that the leaves supply cytokinins to the developing fruits, it is equally unlikely that cytokinins are donated to the leaves by the fruits. The fruits in this system and in other systems (VARGA and BRUINSMA, 1974; HOAD *et al.*, 1977) appear to be sites of cytokinin accumulation. Studies utilizing labelled cytokinins will show whether or not reciprocal transport of cytokinins takes place between organs of the shoot.

The significance of the accumulation of cytokinins in the pod wall and seed tissues was also explored. It

became apparent that cytokinin levels in the embryos were relatively low, while those in the adjacent endosperm, testa and pod wall were higher. The latter tissues act as transitory stores for nutrients which are accumulated in the cotyledons of the developing embryo (PATE *et al.*, 1977) and it was concluded that cytokinins were probably involved in the promotion of nutrient mobilization to these tissues. In endospermous seeds cytokinin-like compounds have been shown to be present in the endosperm of the mature seed, and appear to be utilised by the growing embryo upon germination (STEWARD and CAPLIN, 1952; SMITH, 1977). As the cytokinins in the exendospermous seed of the white lupin decreased as maturity approached, it was suggested that these compounds were utilized by the maturing embryo. Cytokinins promote cell division, cell expansion and protein synthesis (OSBORNE, 1962: NAITO, TSUJI and HATAKEYAMA, 1978): all of which were shown to take place in the developing white lupin embryo.

Cytokinins were also isolated from the suspensor of the white lupin. This is the first report of the occurrence of these compounds in the suspensor. As the suspensor cells were transfer cell-like in structure, it was suggested that the organ might transport not only nutrients but also growth substances to the young embryo.

Where high levels of cytokinin activity were recorded in white lupin extracts (mature to senescing leaves and pod walls, testas and endosperms), the glucoside cytokinins predominated. This observation is particularly significant

if one considers that the glucoside cytokinins are more active in the soybean callus bioassay in higher concentrations than zeatin and zeatin riboside (VAN STADEN and PAPAPHILIPPOU, 1977).

In the literature review, it was pointed out that while compounds such as zeatin and zeatin riboside have come to be recognised as active forms of cytokinin, the glucosylated and phosphorylated derivatives of these cytokinins are regarded as bound forms, which may either be stored in a particular tissue, or merely represent inactivation products of the free forms. Other workers have shown that cytokinins applied to plant tissues are often rapidly metabolized to the glucosylated forms (LETHAM *et al.*, 1976; HENSON and WHEELER, 1977c). In view of these considerations, the significance of the presence of cytokinins in plant tissues would appear to be moderated by the ability of this tissue to utilise the available cytokinin. In leaves it has been demonstrated that sensitivity to applied cytokinins changes with increasing age of the leaf (NAITO *et al.*, 1978), and it is possible that as the leaves and pod walls approach senescence their ability to utilise the endogenous cytokinins declines. In the developing seed tissues, however, this ability does not appear to be lost and is probably transferred to the young seedling.

In conclusion, it can be stated that this study of cytokinin levels in the annual plant during its growth cycle, has pointed out that high levels of cytokinins do

not accumulate in actively growing tissues, unless these tissues act as temporary reservoirs for nutrients. But it is stressed that caution should be exercised in considering nutrient mobilization the only function of these compounds. Their proposed utilization in actively growing tissues probably influences numerous growth responses. This aspect of cytokinin involvement in plant growth will only be understood when more is known of the sites of action and modes of utilization of these substances.

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