TRANSPORT AND METABOLISM OF 8(^{14}C)*t*-ZEATIN

By

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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.

M. J. Hutton.

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ABSTRACT

A review of the literature established that there were areas of cytokinin transport which needed further investigation, in order to determine the function and/or mode of action of cytokinins during certain stages of plant development. Radioactive zeatin was applied to plant systems suitable for determining more about specific problems of cytokinin transport. The metabolism of the radioactive zeatin was monitored in relation to transport.

The metabolism in, and possible export of, radioactive zeatin out of immature, mature and senescing *Ginkgo biloba* leaves was monitored using explants. The results showed that approximately the same percentage radioactivity was exported from the leaves at all three stages of their development. This indicates that these deciduous leaves could potentially export cytokinins, but the results were not regarded as being significant. Cytokinins would not have been expected to be transported out of expanding leaves, which rapidly utilize cytokinins, and in comparison greater cytokinin export would have been expected to occur from senescing leaves, but this did not occur. The results could indicate that cytokinin glucosides in deciduous leaves are primarily inactivation products rather than storage compounds.

The metabolism and transport of radioactive zeatin, applied to the leaves of *Citrus sinesis* trees, was monitored

during the flush of new growth following a dormant period. Some of the radioactive zeatin applied to these leaves appeared to be utilized in the new shoot growth. This could imply that accumulated cytokinin glucosides in these evergreen leaves are exported out of the leaves and re-utilized, and thus fulfil a storage function; although the extent to which export occurred as opposed to inter-conversion and/or catabolic metabolism in the leaves could not be determined.

The distribution of radioactive zeatin applied to the xylem and phloem of *Phaseolus vulgaris* plants at three stages of development was also monitored. There appeared to be preferential transport of cytokinins in the transpiration stream, that is, applied to the xylem. The vegetative apices, buds and flowers appeared to be the major sinks for radioactive compounds in the vegetative and flowering plants, and the leaves were the most important sink in the fruiting plants. Radioactive zeatin in the phloem appeared to move passively with the assimilate stream. The transport of radioactive zeatin applied to the primary leaves appeared to confirm that cytokinins in the phloem were transported along with the assimilate stream. The results also indicated that the accumulated cytokinin glucosides in these annual leaves were primarily inactivation products, involved in regulating leaf metabolism, although some re-utilization of these cytokinins could potentially take place.

The extent to which cytokinins are transported between the cotyledons and the embryonic axis of *Phaseolus vulgaris* seeds during the early stages of germination was also monitored using radioactive zeatin. The results indicated that cytokinins are transported from the radicle to the cotyledons and could thus be involved in the initiation of hydrolytic enzyme activity. Cotyledonary cytokinins did not appear to be transported to the radicle to any extent and are probably mainly involved in cotyledon enlargement.

The metabolism of the radioactive zeatin was essentially similar in all the plant tissues to which it was applied. The same three radioactive peaks were detected on paper chromatograms of all extracts. Column chromatography indicated that ribosylation, side chain reduction, oxidation (involving breaking of the double bond in the side chain) and possibly glucosylation were all involved in the metabolism of the radioactive zeatin. Oxidation appeared to be the most important metabolic step, with N-(purin-6-yl) glycine and possibly trihydroxyzeatin apparently the main oxidation products formed. The significance of this oxidative metabolic pathway was assessed.

There are, however, a number of potentially limiting factors to be considered when assessing the significance of the transport and metabolism of an exogenously applied cytokinin. Taking these factors, such as the unphysiological cytokinin concentration applied, into account, it appeared that the transport of the radioactive zeatin

could have reflected normal cytokinin transport occurring in the tissues. The main metabolic pathway, oxidation, did not, however, appear to be the main metabolic pathway in the plant tissues and may have been the result of the exogenous application of zeatin. The fact that metabolism did not appear to be the normal metabolism of the tissues, could potentially have affected transport.

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INTRODUCTION

Plants undergo an orderly sequence of events during their life cycle which must involve a complex control system to ensure integration and co-ordination (WAREING, 1977). Cytokinins, a group of plant growth regulators, form part of this control system which regulates plant growth and development (WAREING, 1977; LETHAM, 1978). It is, therefore, necessary to understand the fundamentals of cytokinins and their mode of action, in order to assess their role in the plant. The availability of radioactive zeatin made it expedient to examine various aspects of cytokinin transport and metabolism. An extensive literature survey was undertaken in order to determine specific problems related to cytokinin transport, to summarize the literature on cytokinin metabolism and also to put the information on cytokinins into perspective, so that it could be related to the experimental results obtained. Although much of the information in this literature survey is not specifically related to cytokinin transport and metabolism, it has been included as it was felt that issues, such as the mechanisms and sites of cytokinin biosynthesis and the distribution of cytokinins throughout the plant, would also be relevant to transport and metabolism studies.

The existence of cytokinins, which were originally regarded as specific cell division compounds, was recognized as early as 1913 by HABERLANDT. Later plant tissue culture experiments (CAPLIN and STEWARD, 1948; STEWARD and CAPLIN,

1952; JABLONSKI and SKOOG, 1954) strengthened the idea of cell division inducing compounds and eventually led to the discovery of kinetin (6-furfurylaminopurine) (MILLER, SKOOG, OKUMURA, VON SALTZA and STRONG, 1955, 1956). Kinetin was originally extracted from autoclaved herring sperm DNA and because of its ability to bring about cytokinesis, it was termed kinetin (MILLER, 1961). Kinetin is not regarded as a natural compound, but rather as an experimental artifact, and it was not until 1963 that the first naturally occurring cytokinin, 6-(4-hydroxy-3-methyl-*trans*-2-butenyl-amino) purine (zeatin) was extracted from immature *Zea mays* L. kernels (LETHAM, 1963). This group of cell division stimulating compounds was originally termed kinins (MILLER, SKOOG, OKUMURA, VON SALTZA and STRONG, 1956) and cytokinins was the generic name later given to these compounds (SKOOG, STRONG and MILLER, 1965). Since the isolation of zeatin, many other naturally occurring cytokinins, including ribosylzeatin, dihydrozeatin, dihydroribosylzeatin and their glucosylated derivatives, have been extracted from diverse higher plant genera, bacteria, fungi and algae, as well as some insects (LETHAM, 1978). Cytokinins can thus be regarded as being ubiquitous (KENDE, 1971). Apart from promoting cell division, cytokinins have also been shown to be involved in other physiological processes, including cell enlargement, senescence, nutrient mobilization and germination. Although the exact mechanism of cytokinin action has not yet been conclusively determined, it is possible that they are active at the level of the gene (BURROWS, 1975).

Many cytokinin definitions tend to favour comparison with kinetin (SKOOG and ARMSTRONG, 1970; LETHAM, 1967a, 1978; BURROWS, 1975). HALL (1973) defined cytokinins as compounds which promote growth and differentiation in cultured cells. This is a flexible definition which could include synthetic and natural cytokinins as well as those compounds which do not meet the structural requirements for a cytokinin. SKOOG and ARMSTRONG (1970) defined the structural requirements for high cytokinin activity as an intact adenine moiety with an N^6-substituent of moderate molecular length. There are compounds such as dihydroconiferyl alcohol (LEE, PURSE, PRYCE, HORGAN and WAREING, 1981), which do not fulfil these structural requirements but are active in cytokinin bioassays. If these non-adenine compounds are to be regarded as cytokinins, then a flexible cytokinin definition is preferable. For the purpose of this thesis, however, cytokinins are to be regarded as N^6-substituted adenines which promote growth in the soyabean callus bioassay.

Identification and assessment of cytokinin levels by means of bioassays is often regarded as being inaccurate and an approximation (LETHAM, 1967b, 1978; DEKHUIJZEN and GEVERS, 1975; HORGAN, PALNI, SCOTT and MCGAW, 1981). The physical techniques of gas liquid chromatography, mass spectrometry and high-pressure liquid chromatography (CARNES, BRENNER and ANDERSON, 1975; HAHN, 1975; HORGAN, HEWETT, HORGAN, PURSE and WAREING, 1975; WANG and HORGAN, 1978; SUMMONS, ENTSCH, LETHAM, GOLLNOW and MACLEOD, 1980) are often regarded

as more accurate methods of cytokinin identification. Although these sensitive techniques are invaluable for the identification of active cytokinins as well as the detection of weakly active compounds or inactive substituted adenines, bioassays will always be an important and fundamental part of cytokinin research. Cytokinin bioassays are necessary to assess the biological activity of separated compounds. Physical techniques and bioassays should complement each other (SUMMONS, ENTSCH, LETHAM, GOLLNOW and MACLEOD, 1980) when assessing cytokinins. This is not always possible as much of the expensive equipment necessary for these physical techniques is not always readily available for routine analyses. Several cytokinin bioassays exist, which include the soyabean cotyledon (*Glycine max* L. cv. Acme) (MILLER, 1963, 1965), tobacco pith (*Nicotiana tobaccum* L.) (MURASHIGE and SKOOG, 1962) and the carrot phloem (*Daucus carota* L.) (LETHAM, 1967b) tissue culture bioassays as well as the oat leaf senescence (*Avena sativa* L.) (VARGA and BRUINSMA, 1973), *Amaranthus caudatus* L. betacyanin (BIDDINGTON and THOMAS, 1973), lettuce (*Lactuca sativa* L.) seed germination (MILLER, 1958) and the soyabean hypocotyl (MANOS and GOLDTHWAITE, 1975) assays. Although the tissue culture bioassays are time consuming and risk high rates of contamination, they are considered to be the most sensitive and reliable of the cytokinin bioassays because of their specificity, wide range of sensitivity and because they are performed under sterile conditions (KENDE, 1971).

Meristematic regions are often regarded as potential sites

of cytokinin production because of the high cytokinin activity usually recorded in these tissues. When determining sites of biosynthesis it must, however, be remembered that high cytokinin levels need not necessarily reflect a site of synthesis, but may represent a sink or storage site (VAN STADEN and DAVEY, 1979) and that all tissues may have a latent capacity for cytokinin production (MUIRA and MILLER, 1969). GOLDACRE (1959) proposed that cytokinin production may be a normal accompaniment of cell division and he suggested the root as a site of cytokinin synthesis. VAN STADEN, CHOVEAUX and DIMALLA (1980) have, however, shown that cytokinin production is not necessarily linked to the actual occurrence or completion of cell division. Seeds (LETHAM and WILLIAMS, 1969; BLUMENFELD and GAZIT, 1971; HAHN, DE ZACKS and KENDE, 1974; NAGAR and RAJA RAO, 1981),buds (KANNANGARA and BOOTH, 1974) and the cambium (SKENE, 1972a) have also been suggested as sites of cytokinin production but, as yet, only the root has been conclusively shown to synthesize these compounds.

CHINBALL (1939, 1954) proposed that a hormone-like factor from the roots controlled protein degradation in leaves and hence leaf senescence. RICHMOND and LANG (1957) observed that kinetin delayed detached leaf senescence and they proposed that kinetin-like compounds were CHINBALL'S postulated root-factor. Endogenous cytokinins were subsequently extracted from roots and root exudate and circumstantial evidence accumulated, suggesting that roots produced cytokinins. Applying stress to root systems re-

sulted in decreased cytokinin activity in the shoot which suggested that shoots are dependent on root produced cytokinins (ITAI and VAADIA, 1965; ITAI, RICHMOND and VAADIA, 1968; BURROWS and CARR, 1969; ITAI and VAADIA, 1971). KENDE (1971) found that the cytokinin activity in the roots of decapitated *Helianthus annuus* L. did not decrease for four days after shoot decapitation, which also suggests that roots are capable of synthesizing cytokinins. KENDE and SITTON (1967) observed that root removal resulted in physiological symptoms in the shoot which were typical of a cytokinin deficiency and which could be overcome by cytokinin treatment. This type of evidence favoured the roots as a site of cytokinin production, but no direct evidence existed for this proposal and some researchers regarded the shoot as the site of production of root cytokinins (TORREY and LOOMIS, 1967 ; RADIN and LOOMIS, 1971). Cytokinins have been shown to accumulate in rootless explants (SKENE, 1972a; CHEN and PETSCHOW, 1978a; VAN STADEN and CHOVEAUX, 1980; VAN STADEN and DIMALLA, 1981) and it has been suggested that cytokinin biosynthetic sites are located in the shoot in addition to the root. It has, however, also been proposed that cytokinins, which accumulate in rootless explants, may have been released from inactive storage cytokinins rather than actually synthesized in the shoot, or alternatively, they may have been synthesized by potential root primordia present in the shoot (CHEN and PETSCHOW, 1978a; VAN STADEN and CHOVEAUX, 1980). FEATONBY-SMITH and VAN STADEN (1981) and FORSYTH and VAN STADEN (1981) pointed out that when considering shoot tissue as a possible

site of cytokinin biosynthesis, the presence of root primordia must be verified anatomically as they have been shown to produce cytokinins very early in their development, that is, even before rupturing the epidermis.

ENGELBRECHT (1972) provided further evidence for root cytokinin production by demonstrating that adventitious root formation on the petioles of detached *Phaseolus vulgaris* L. leaves resulted in an increase in the cytokinin content of the leaf. HENSON and WAREING (1977a) also demonstrated that removal of the roots of *Xanthium strumarium* L. resulted in decreased cytokinin activity in the detached leaves and buds. What can be regarded as conclusive evidence for cytokinin synthesis in roots, was provided by VAN STADEN and SMITH (1978), who eliminated microbes and the shoot as potential cytokinin sources, and were able to demonstrate that excised aseptically cultured *Zea mays* and *Lycopersicon esculentum* Mill. radicles accumulated cytokinins and released them into the culture medium.

It is now generally accepted that the roots produce cytokinins, but it appears as if it is the root tip which is the actual site of synthesis. WEISS and VAADIA (1965) and SHORT and TORREY (1972) detected high cytokinin activity in root-apex extracts of *Helianthus annuus* and *Pisum sativum* L. plants, but detected little, if any, activity in the physiologically older root tissue. FELDMAN (1979) has shown that interaction between the proximal meristem and the quiescent centre of a root is necessary for cytokinin

production, which provides further evidence for the root tip as the actual site of cytokinin synthesis in the root. The root tip may be capable of producing cytokinins, but it has been proposed that the developing radicle only acquires this capacity once it reaches a certain maturity (SMITH, 1977). Low cytokinin levels have been recorded in the developing radicles of a number of species. Three-day old *Zea mays* radicles were not able to synthesize cytokinins (SMITH, 1977), and two-day old *Pisum sativum* seedlings showed low cytokinin levels (THIMANN, SHIBAOKA and MARTIN, 1970), whereas older *Pisum sativum* roots have been shown to synthesize cytokinins (SHORT and TORREY, 1972). BROWN and VAN STADEN (1973) could not detect cytokinin activity in germinating radicles of *Protea compacta* R. Br. and *Leucadendron daphnoides* Meisn. This absence of activity in the initial stages of germination may, however, reflect rapid utilization rather than an inability to synthesize cytokinins. Developing lateral and adventitious roots have been shown to synthesize cytokinins during the initial stages of their development, that is, before even rupturing the epidermis (FEATONBY-SMITH and VAN STADEN, 1981; FORSYTH and VAN STADEN, 1981). This could, therefore, suggest that the developing radicle is capable of cytokinin synthesis during the initial stages of its growth and the low cytokinin levels often detected could be the result of rapid utilization of these compounds. Although the root primordia produce cytokinins, it has been suggested that the shoot moderates this production (BEEVER and WOOLHOUSE, 1974; DAVEY and VAN STADEN, 1976; PALMER,

HORGAN and WAREING, 1981b). HENSON and WAREING (1977b) have proposed the existence of a shoot-to-root signal capable of moderating cytokinin production in the root system of *Xanthium strumarium*.

Having established that roots produce cytokinins, it is necessary to determine the fate of these compounds. It has been shown that the cytokinins produced in the roots are transported in the xylem sap to the shoot which is said to be dependent on these cytokinins for normal functioning (PETERSON and FLETCHER, 1973; WAREING, HORGAN, HENSON and DAVIS, 1976; WAREING, 1977 and VONK, 1979). Ribosylzeatin is considered to be the main translocational cytokinin form in the xylem (GORDON, LETHAM and PARKER, 1974; LETHAM, 1974; DAVEY and VAN STADEN, 1976; HENSON and WAREING, 1976; LETHAM, PARKER, DUKE, SUMMONS and MACLEOD, 1976), although zeatin (VAN STADEN and DAVEY, 1976) and dihydrozeatin (PURSE, HORGAN, HORGAN and WAREING, 1976) have also been extracted from xylem sap. The cytokinins in the transpiration stream appear to be utilized differently at various stages of shoot development. Cytokinins from the roots of vegetative and flowering *Lupinus albus* L. plants accumulate in fully expanded mature leaves, but are utilized in rapidly growing apical regions and in young expanding leaves, whereas the cytokinins in the transpiration stream of fruiting plants accumulate in the leaves and fruit (DAVEY and VAN STADEN, 1978a,b). HEWETT and WAREING (1973b) also observed considerable quantitative and qualitative differences in leaves of different ages of

Populus robusta Schneid.

The cytokinin activity of immature expanding leaves is usually low (VAN STADEN, 1976a; DAVEY and VAN STADEN, 1978a; HENSON, 1978a; HENDRY, VAN STADEN and ALLEN, 1982) probably due to the rapid utilization of cytokinins in these organs (HENDRY, VAN STADEN and ALLEN, 1982). HEWETT and WAREING (1973b) observed that cytokinin activity was at a maximum in expanding *Populus robusta* leaves which is contrary to the above suggestion. They do, however, suggest that cytokinin diversity is at a maximum in expanding *Populus robusta* leaves which is supported by other workers. Zeatin and ribosylzeatin are the predominant cytokinins in expanding leaves accompanied by very low or undetectable levels of cytokinin glucosides (ENGELBRECHT, 1971; LORENZI, HORGAN and WAREING, 1975; VAN STADEN, 1976a,b; HENSON, 1978a). As leaves mature, there is usually an increase in cytokinin activity (VAN STADEN, 1976a,b; HENSON, 1978a; VAN STADEN and DAVEY, 1981b), as well as a change in the predominant cytokinin form. Cytokinin glucosides appear to be the major cytokinins in mature and senescing leaves (ENGELBRECHT, 1971; HEWETT and WAREING, 1973b; VAN STADEN, 1976a,b; DAVEY and VAN STADEN, 1978a; HENSON, 1978a,b; DUKE, LETHAM, PARKER, MACLEOD and SUMMONS, 1979; VONK and DAVELAAR, 1981). It has been suggested that once the apical sink activity ceases, cytokinins in the xylem sap are diverted to the leaves where they are converted to cytokinin glucosides. DAVEY and VAN STADEN (1978a,b) have demonstrated this in the annual, *Lupinus albus* which

rapidly utilizes cytokinins in young expanding leaves and accumulates cytokinin glucosides in fully expanded leaves. HENSON (1978a,b) observed a similar trend in the deciduous species, *Alnus glutinosa* (L.) Gaertn. During spring, the immature leaves exhibited low cytokinin levels with very little cytokinin glucosides detectable. These levels increased in the autumn before the mature leaves were shed. In evergreen species, cytokinin activity increases during the growing season with zeatin and ribosylzeatin predominating in the leaves in spring and summer. During autumn and winter, cytokinin glucosides accumulate in the leaves but their levels decrease again the following spring, accompanied by a corresponding increase in zeatin and ribosylzeatin (LORENZI, HORGAN and WAREING, 1975; HENDRY, VAN STADEN and ALLAN, 1982). Leaves, therefore, appear to metabolize root-produced cytokinins differently at different stages of maturity and seem to acquire the capacity for glucosylation as they mature (HENSON, 1978a). It has been suggested that all functional tissue has the capacity for glucosylation (VAN STADEN and DAVEY, 1979), but in intact plant systems, mature and senescing leaves appear to be the primary site of cytokinin glucoside accumulation. Cytokinin glucosides are thought to be formed *in situ* in leaves (VAN STADEN, 1976b; HENSON,1978a,b; 1978a; HOAD, LOVEYS and SKENE, 1977). Cytokinin glucosides have been extracted from leaves, phloem (HALL and BAKER, 1972; HENSON and WAREING, 1976; VAN STADEN and BROWN, 1977, 1978; DAVEY and VAN STADEN, 1978c), roots (YOSHIDA and ORITANI, 1972; HENSON and WHEELER, 1977a; VAN STADEN and DIMALLA,

1977), seeds and fruits (DAVEY and VAN STADEN, 1978c; SUMMONS, ENTSCH, LETHAM, GOLLNOW and MACLEOD, 1980), and honeydew (VAN STADEN, 1976b), but as yet there has only been one report of the occurrence of these compounds in xylem sap. VAN STADEN and DIMALLA (1980) reported the occurrence of cytokinin glucosides in the root sap of *Bougainvilla* 'San Diego Red'. These authors did not rule out the possibility that these cytokinin glucosides in the xylem sap could have been transported laterally from the phloem. Therefore, until conclusive evidence is provided for the occurrence of glucosides in root exudate, it is perhaps better not to regard cytokinin glucosides as a normal constituent of xylem sap. The fact that cytokinin glucosides accumulate in leaves and are not normally detected in xylem exudate, implies that these compounds are synthesized in the leaves from cytokinins arriving in the transpiration stream (ENGELBRECHT, 1972; HEWETT and WAREING, 1973b; ALVIM, HEWETT and SAUNDERS, 1976; HENSON and WAREING, 1976; VAN STADEN, 1976b; HENSON and WHEELER, 1977b; HOAD, LOVEYS and SKENE, 1977).

There have been various suggestions for the physiological significance of cytokinin glucosides, but their exact function remains to be elucidated. These suggestions include storage compounds (PARKER and LETHAM, 1973; WAREING, HORGAN, HENSON and DAVIS, 1976; HENSON and WHEELER, 1977a; VAN STADEN and DAVEY, 1979; VONK and DAVELAAR, 1981), inactivation or detoxification products (HOAD, LOVEYS and SKENE, 1977; PARKER, LETHAM, GOLLNOW, SUMMONS, DUKE and

MACLEOD, 1978), translocatable forms (VAN STADEN, 1976b; VAN STADEN and DIMALLA, 1980), active cytokinin forms (FOX, CORNETTE, DELEUZE, DYSON, GIERSAK, NIU, ZAPATA and MCCHESNEY, 1973), and they have also been suggested to regulate cytokinin levels and to confer metabolic stability on cytokinin activity (HENSON and WHEELER, 1977a; PARKER, LETHAM, GOLLNOW, SUMMONS, DUKE and MACLEOD, 1978; VAN STADEN and DIMALLA, 1980; PALMER, SCOTT and HORGAN, 1981). The suggestion of storage compounds, especially if used in its widest sense to accommodate inactivation and transport compounds (VAN STADEN and DAVEY, 1979), is probably the most commonly accepted function of cytokinin glucosides.

Glucosides are stable cytokinin metabolites. This stability is apparently due to the O-glucosyl moiety and saturated side chain which are structural features which confer resistance to cytokinin oxidase type enzyme (WHITTY and HALL, 1974) degradation (PARKER and LETHAM, 1973; VAN STADEN and PAPAPHILIPPOU, 1977; PARKER, LETHAM, GOLLNOW, SUMMONS, DUKE and MACLEOD, 1978; SUMMONS, LETHAM, GOLLNOW, PARKER, ENTSCH, JOHNSON, MACLEOD and ROLFE, 1981). Glucosides of zeatin, ribosylzeatin and dihydrozeatin have been detected in a number of plant species and are said to be characteristic of mature and ageing tissue (DAVEY and VAN STADEN, 1978b). The glucosylating capacity of zeatin and dihydrozeatin appears to be different (HENSON,1978a; PALMER, SCOTT and HORGAN, 1981). Dihydrozeatin has a greater glucosylation capacity than zeatin, and its glucosides are also more stable than zeatin glucosides. The significance of these

differences is not, however, clear.

The glucoside metabolites of zeatin and its derivatives are the N^7-glucoside (raphanatin), N^9-glucoside and the O-glucoside, as well as three derivatives of the O-glucoside. These glucosides appear to have different properties. The 7-glucosides are extremely stable compounds, persisting for long periods in plant tissues (FOX, CORNETTE, DELEUZE, DYSON, GIERSAK, NIU, ZAPATA and MCCHESNEY, 1973; PARKER and LETHAM, 1973; GORDON, LETHAM and PARKER, 1974), and have been suggested to be the active cytokinin form as well as storage compounds. HOAD, LOVEYS and SKENE (1977) and PALMER, SCOTT and HORGAN (1981) have suggested that O-glucosides are storage compounds as they can be hydrolyzed by β-glucosidase enzymes, whereas the 7- and 9-glucosides may be inactivation or detoxification products, as they are resistant to β-glucosidase treatment and as such, represent irreversible metabolites. It may, therefore, be possible that cytokinin glucosides do not have a common function, but may, in fact, have a number of functions depending on where they are formed, the type of glucoside formed and whether they are glucosides of zeatin or dihydrozeatin.

Cytokinin glucosides accumulate in leaves as the result of continuous cytokinin transport to the shoot (DAVEY and VAN STADEN, 1978a; PALMER, HORGAN and WAREING, 1981b), suggesting that these compounds are inactivation or storage forms. The O-glucosides of dihydrozeatin and dihydroribosylzeatin have been suggested to be the major glucosides in leaves

(PALMER, HORGAN and WAREING, 1981a,b). Non-glucosylated cytokinins delay leaf senescence (VARGA and BRUINSMA, 1973; BIDDINGTON and THOMAS, 1978; DUMBROFF and WALKER, 1979), whereas glucosylated cytokinins do not appear to have this capacity. ENGELBRECHT (1971) proposed that senescing leaves compartmentalized these accumulated cytokinin glucosides which were then unable to prevent the leaf from fading. Cytokinin glucosides can thus be considered to be inactive forms, which are apparently not able to bring about the physiological responses normally associated with zeatin and ribosylzeatin (VAN STADEN and DAVEY, 1979). Cytokinin glucosides are, however, biologically active, but are less active than zeatin and ribosylzeatin in the soyabean callus bioassay (PETERSON and MILLER, 1977; VAN STADEN and PAPAPHILIPPOU, 1977; LETHAM, 1978). The actual biological activity of these glucosides has, however, been questioned. HENSON (1975a) has proposed that glucosides may only be active in bioassays by virtue of their hydrolysis in the test tissue, as they can be readily hydrolyzed to active cytokinins. If the cytokinin glucosides, which accumulate in leaves are to be regarded as storage compounds, then, as such, they represent a potential reservoir of active cytokinins which can be re-utilized. In deciduous species, if these cytokinins are not to be lost to the system, they must be exported out of the leaves before they abscise (VAN STADEN and BROWN, 1977; NETTING, 1979). These glucosides would, therefore, be exported via the living phloem to storage organs or to meristematic areas, where they could be utilized. Gluco-

sides have been extracted from phloem and their levels have been shown to increase in autumn before the leaves are shed (VAN STADEN, 1976b; VAN STADEN and BROWN, 1978). Glucosides may, in fact, also represent stable translocatable forms facilitating transport through the living phloem (VAN STADEN and BROWN, 1978; VAN STADEN and DIMALLA, 1980). If glucosides and/or their hydrolyzed products are not exported out of leaves, then their accumulation in leaves could represent an inactivation or detoxification process. The term storage could encompass transport and detoxification, as suggested by VAN STADEN and DAVEY (1979). There is evidence available which suggests that glucosides or their hydrolyzed forms may be exported out of leaves, but it is not conclusive. KANNANGARA and BOOTH (1974) suggested that some of the cytokinins detected in *Dahlia variabilis* (Willd.) Desf. buds may have been exported from the leaves via the phloem as these cytokinins were not detected in the xylem. VAN STADEN and DAVEY (1981a) have reported that zeatin glucoside was exported from mature *Lupinus albus* leaves to which radioactive zeatin was applied, although the amount exported was very small. VAN STADEN (1982a) obtained similar results when radioactive zeatin was applied to mature rose leaves. In this case, however, the small amount of radioactive cytokinin exported from the leaves was apparently sufficient to stimulate the growth of the axillary bud associated with the treated leaves. VONK and DAVELAAR (1981) are also of the opinion that cytokinins in the phloem sap of *Yucca flaccida* Haw. inflorescences originate in the leaves. ^{14}C-O-

glucosylzeatin applied to gall tissue was rapidly degraded (HORGAN, PALNI, SCOTT and MCGAW, 1981). This implies that cytokinin glucosides could be re-utilized but it does not reflect the endogenous situation in which O-glucosylzeatin accumulates.

Contrary to the above suggestions, SKENE (1972b) has proposed that cytokinins are not exported in the assimilate stream, as he observed that cytokinin activity in grape vine leaves (*Vitis vinifera* L.) decreased in both the presence and absence of the phloem. He was not, however, able to explain the decrease in activity in the absence of the phloem. A similar response was also observed by PALMER, HORGAN and WAREING (1981a), who reported that cytokinin glucoside levels decreased in attached and detached *Phaseolus vulgaris* leaves. These authors suggested that in attached leaves, glucoside metabolism may result in breakdown or transport compounds being formed, and that if glucosides are exported, they are not exported *per se*. The possibility does, therefore, exist that cytokinin glucoside may be exported from mature deciduous and annual leaves, but this does not appear to be the case in the leaves of evergreen plants. The high cytokinin glucoside levels in evergreen leaves in autumn are followed by a decrease in spring with a corresponding increase in the levels of zeatin and ribosylzeatin. It appears that the cytokinin glucosides which accumulate during the periods of slow growth, are hydrolyzed to active cytokinins within the leaf during periods of active growth (HENDRY, VAN STADEN

and ALLAN, 1982). The accumulated glucosides could also undergo destructive matabolism during the periods of active growth, rather than being converted to active forms. There is, as yet, no conclusive evidence for the export or utilization of cytokinin glucosides and their proposed function as a storage compound which can be re-utilized, therefore remains in question. PALMER, HORGAN and WAREING (1981a) have implied that glucosides in senescing leaves may represent a basal level of leaf cytokinin which will never be exported or further metabolized.

Inactivation or detoxification products are also possible functions of cytokinin glucosides. Cytokinin glucosides appear to accumulate in ageing tissue, possibly suggesting that this is a detoxification mechanism as this tissue will eventually be lost to the system. Cytokinin glucosides can also potentially be metabolized from excess cytokinin in all functional tissue (VAN STADEN and DAVEY, 1979). Glucosylation could, therefore, also be a mechanism for regulating cytokinin levels by inactivating excess free cytokinins in tissues,as cytokinin glucosides have been shown to occur in fruits and seeds (VAN STADEN, 1976d; DAVEY and VAN STADEN, 1978c), in roots (YOSHIDA and ORITANI, 1972; VAN STADEN and DIMALLA, 1977), in gall tissue (PETERSON and MILLER, 1977) and callus (HORGAN, 1975; VAN STADEN and DAVEY, 1977). Glucosylation could, therefore, be a means of regulating cytokinin levels as supra-optimal cytokinin concentrations have been shown to be inhibitory (CHEN, 1981). FOX, CORNETTE, DELEUZE, DYSON, GIERSAK,

NIU, ZAPATA and MCCHESNEY (1973) and GAWER, LALOUE, TERRINE and GUERN (1977) do not regard glucosylation as a detoxification process. These authors regard the facts that glucosides are biologically active and that they do not increase proportionally to the amount of supplied cytokinin as evidence that cytokinin glucosides do not represent detoxification products. They regard storage as a more likely function for glucosylated cytokinins.

Despite the many suggestions for the functions of cytokinin glucosides, this problem essentially remains unsolved. Fundamental questions, such as whether cytokinin glucosides are exported and/or re-utilized, and if they are exported, whether they are exported *per se* or metabolized prior to export, remain to be answered. These facts will help to establish the physiological significance of this common group of cytokinins.

Developing seeds are rich in cytokinins, having a large diversity of cytokinins (DAVEY and VAN STADEN, 1978c), as well as a high cytokinin concentration (MILLER, 1965; BLUMENFELD and GAZIT, 1970; BURROWS and CARR, 1970; LETHAM, 1973; DAVEY and VAN STADEN, 1978c; DAVEY and VAN STADEN, 1981). Seeds were originally used as a source of cytokinin isolation (LETHAM, 1963) which led to seeds being regarded as a potential site of cytokinin biosynthesis. There is, however, no conclusive evidence that seeds are capable of synthesizing their own cytokinins.

Seed cytokinins can be accounted for by *de novo* synthesis in the seeds themselves, by cytokinins arriving in the transpiration stream from the roots, either directly or indirectly via other plant parts, or by a combination of both processes. The evidence for *de novo* cytokinin synthesis does not appear to be very convincing. BLUMENFELD and GAZIT (1971) proposed that the embryo of *Persea americana* L. (avocado) was capable of cytokinin synthesis, because they observed that callus derived from the embryo was capable of producing cytokinins. Whether this can be regarded as evidence that seeds synthesize cytokinins, is debatable. PETERSON and FLETCHER (1973) reported that fruits developed on rootless tobacco, tomato, pear and bean plants, and they concluded that seeds did not depend exclusively on root-produced cytokinins. They did not, however, rule out the possibility that other plant parts may have supplied cytokinins to the developing fruit. HAHN, DE ZACKS and KENDE (1974) provided what they considered to be conclusive evidence that seeds synthesize cytokinins. They observed that cytokinin levels in seeds of pea pods cultured independently of a root system, increased. They did not, however, eliminate the seed pod which has subsequently been shown to contain high cytokinin levels (DAVEY and VAN STADEN, 1978c; SUMMONS, LETHAM, GOLLNOW, PARKER, ENTSCH, JOHNSON, MACLEOD and ROLFE, 1981) and as such, represents a potential cytokinin reservoir for the developing seeds. KRECHTING, VARGA and BRUINSMA (1978) and VAN STADEN and BUTTON (1978) both repeated HAHN'S original experiment of aseptic seed pod culture, but

younger pods, that is, five days, as opposed to ten days, were used in these experiments. The cytokinin levels decreased in these aseptically cultured pea fruits suggesting that seeds are not capable of cytokinin synthesis, and that seeds, therefore, depend on the rest of the plant for these compounds. Further evidence for seeds synthesizing cytokinins, was demonstrated by SUMMONS, LETHAM, GOLLNOW, PARKER, ENTSCH, JOHNSON, MACLEOD and ROLFE (1981). Immature seeds of *Lupinus luteus* L. were excised from pods and were incubated on a medium containing (^{3}H)adenosine for 75 hours. The bulk of the adenosine was not metabolized but O-glucosyldihydrozeatin (0,3 per cent of the extracted radioactivity) was the cytokinin formed. This was taken as evidence that seeds are capable of cytokinin synthesis. These research workers do, however, point out that this may only be the case in an *in vitro* system and may not necessarily occur in the intact plant. VAN STADEN and CHOVEAUX (1981) injected 8(^{14}C)adenine into young developing fruits of *Lupinus albus in situ*, and after five days, none of the recovered radioactivity was associated with the cytokinin activity which was detected in the developing pods. These authors concluded that adenine was not incorporated into the endogenous cytokinins and thus a large proportion of the cytokinins associated with the fruit are not formed *in situ*. These different results could perhaps be explained in terms of maturity of seeds to which the labelled adenosine and adenine were applied. It has, however, also not been unequivocally proved that adenine and adenosine are the precursors of

cytokinin biosynthesis.

There is also some evidence which implies that the roots supply the seeds with some, if not all, of their cytokinins (BURROWS and CARR, 1970; VARGA and BRUINSMA, 1974; HOAD, LOVEYS and SKENE, 1977; COLBERT and BEEVER, 1981). It has frequently been observed that disbudding and fruit removal results in an increase in the cytokinin concentration of the xylem sap and leaves (SETH and WAREING, 1967; WAREING, HORGAN, HENSON and DAVIS, 1976; HOAD, LOVEYS and SKENE, 1977 and COLBERT and BEEVER, 1981). These observations could imply that the developing fruit competes with the leaves for root-produced cytokinins, and that these cytokinins are preferentially transported to the stronger sink, that is, the seeds. VARGA and BRUINSMA (1974) ascribe cytokinin activity to differences in the relative sink activities of fruit and foliage in attracting cytokinins from the roots. The fact that low cytokinin levels accumulate in parthenocarpic fruit as opposed to the high cytokinin levels of seeded fruit, is usually ascribed to the low sink capacity and competition of seedless fruits, rather than being due to the seeds being able to synthesize cytokinins (VARGA and BRUINSMA, 1974). The above can, however, only be regarded as circumstantial evidence for seed cytokinins originating in the roots.

It has also been reported that floral initiation appears to result in a decrease in cytokinin activity in the root exudate (VAN STADEN and WAREING, 1972; DAVEY and VAN

STADEN, 1978a; COLBERT and BEEVER, 1981). This could imply that the shoot is perhaps a more important source of seed cytokinins than the root. It could, therefore, be suggested that some seed cytokinins originate in the leaves. Cytokinins have been extracted from the phloem sap passing into developing *Lupinus albus* pods, indicating that at least some of the seed cytokinins are translocated from other parts of the plant (DAVEY and VAN STADEN, 1978c). VONK and DAVELAAR (1981) support this suggestion and have proposed that cytokinin glucosides, which accumulate in mature *Yucca flaccida* leaves, are converted by β-glucosidase in the leaves and stem and are transported through the inflorescence stalk as zeatin nucleotides. As a result of radioactive experiments, VAN STADEN and DAVEY (1981a) also suggested that leaves of fruiting *Lupinus albus* plants represent a potential source for seed cytokinins. These authors, however, suggested that zeatin glucosides were exported *per se* and were not metabolized. DAVEY and VAN STADEN (1981) also observed that radioactive zeatin applied to the xylem of fruiting lupin plants was preferentially transported to the leaves, with very little radioactivity recorded in the seeds. These results indicate that not all seed cytokinins are synthesized *in situ* and that root-produced cytokinins are apparently transported indirectly to the seeds, via the leaves. SUMMONS, LETHAM, GOLLNOW, PARKER, ENTSCH, JOHNSON, MACLEOD and ROLFE (1981) have also demonstrated that (^{3}H)-ribosylzeatin, supplied to de-rooted *Lupinus angustifolius* L. shoots, was not preferentially transported to the developing seeds, but rather to

the lateral shoots.

Developing seeds may well have the capacity for cytokinin production, but it is apparent that cytokinins from the rest of the plant also contribute to the seed cytokinin. Root-produced cytokinins appear to be initially transported to the leaves where they are metabolized and are subsequently exported to the developing fruit.

The possibility that developing seeds may synthesize cytokinins makes it possible that germinating seeds may also have this capacity. NOEL and VAN STADEN (1976) and SMITH and VAN STADEN (1979) suggested that substances other than food reserves are required for radicle growth. PINFIELD and STOBART (1972) reported that cytokinins adopt a role in radicle growth. Cytokinins are now considered to be involved in axes growth and in the initiation of hydrolysis of food reserves in the cotyledons.

Cytokinins have been extracted from the cotyledons of *Phaseolus vulgaris* (HUTTON, VAN STADEN and DAVEY, 1982), *Cucurbita pepo* L. (pumpkin) (UHEDA and KURAISHI, 1977; RYBICKA, ENGELBRECHT, MIKULOVICH and KULAEVA, 1977 and *Lupinus albus* (DAVEY, 1978) seeds. It is generally agreed that cotyledons utilize stored cytokinins rather than synthesizing these compounds. Cytokinin glucosides, which are considered to be storage cytokinins, are not always detected in mature seeds. DAVEY and VAN STADEN (1978c) were unable to detect cytokinin glucosides in mature *Lupinus* albus seeds,

but SMITH (1977) and SUMMONS, ENTSCH, LETHAM, GOLLNOW and MACLEOD (1980) detected glucosides in *Zea mays* kernels. SMITH (1977) proposed that these intact glucosides are transported to the embryonic axis where they are converted to active forms. RYBICKA, ENGELBRECHT, MIKULOVICH and KULAEVA (1977) also supported the concept of cytokinin utilization rather than synthesis, as they observed that the cytokinin content of detached cotyledons decreased, while that of intact cotyledons initially decrease but was followed by an increase, presumably once the radicle started to synthesize cytokinins.

While it is generally accepted that the radicle is a site of cytokinin biosynthesis, a number of studies have indicated that during the initial stages of development, these organs contain very low or undetectable levels of endogenous cytokinins (THIMANN, SHIBOAKA and MARTIN, 1970; WEBB, VAN STADEN and WAREING, 1973; BROWN and VAN STADEN, 1973; BOROWSKA and RUDNICKI, 1975; SMITH, 1977). SMITH and VAN STADEN (1978) suggested that the cytokinins in the embryo of germinating *Zea mays* kernels, initially maintain radicle growth but cytokinins from the endosperm are necessary for further radicle growth, up until the time the radicle starts to produce cytokinins. This could imply that the developing radicle is dependent on the endosperm/cotyledons for cytokinins during its early development. Contrary to this suggestion, TZOU, GALSON and SONDHEIMER (1973) and GORDON, LETHAM and PARKER (1974) have reported a barrier to cytokinin transport out of cotyledons. This suggests that the radicle has sufficient reserves for its

utilization, before it is able to produce cytokinins. Alternatively, the radicle may, in fact, synthesize cytokinins earlier than detected, with the low cytokinin levels detected being due to rapid utilization in this rapidly growing organ.

Apart from being implicated in radicle development, cytokinins are also thought to be involved in the mobilization of food reserves in the cotyledons. The embryonic axis is dependent on the food reserves of the cotyledons, which are hydrolyzed and transported to the axis (DAVIES and CHAPMAN, 1979, 1980; HOCKING, 1980). As early as 1963, VARNER, BALCE and HAUNG indicated that a factor from the embryonic axis controlled food mobilization in the cotyledons. It is now generally accepted that the embryonic axis control of reserve mobilization is via the production of hormones, which are transported to the cotyledons where they initiate hydrolytic enzyme activity (DAVIES and CHAPMAN, 1979, 1980; GEPSTEIN and ILAN, 1979; JACOBSEN, HIGGINS and ZWAR, 1979; MOROHASHI, 1980). In monocotyledons, gibberellins exported from the axis control reserve breakdown. In the dicotyledons reserve mobilization appears to be affected by cytokinins rather than by gibberellins (PENNER and ASHTON, 1967; GEPSTEIN and ILAN, 1980; METIVIER and PAULILO, 1980a). It has been shown that applied cytokinins, such as benzyladenine, increase hydrolytic enzyme activity in detached cotyledons (GEPSTEIN and ILAN, 1979; METIVIER and PAULILO, 1980a,b) and that exogenous cytokinins can partially or completely replace

the axial requirement for normal enzyme action (PENNER and ASHTON, 1967; VAN ONCKELEN, CAUBERGS and DE GREEF, 1977; DAVIES and CHAPMAN, 1979; GEPSTEIN and ILAN, 1980; METIVIER and PAULILO, 1980b; MURRAY and ADAMS, 1980). This type of evidence does make it seem likely that the radicle supplies cytokinins to the cotyledons, but there is no direct evidence for this cytokinin transport. The extent of cytokinin interaction between the cotyledons and the radicle has not yet been determined.

Cytokinins are essential for the outgrowth of lateral buds (PROCHÁZKA, 1981). High cytokinin levels have been detected in buds of woody and herbaceous species prior to bud burst (ENGELBRECHT, 1971; HEWETT and WAREING, 1973a; VAN STADEN and BROWN, 1977; VAN STADEN and DIMALLA, 1978). These high cytokinin levels, together with the fact that buds are meristematically active regions led to the suggestion that buds are sites of cytokinin synthesis (KANNANGARA and BOOTH, 1974). LEE, KESSLER and THIMANN (1974) also proposed that buds synthesize cytokinins as a result of experiments obtained with the antibiotic hadacidin, which is reported to interfere with cytokinin biosynthesis. There is, however, relatively little evidence to support the proposal that buds are sites of cytokinin synthesis. The roots and/or the cambium (VAN STADEN, 1979c) are usually regarded as the main source of bud cytokinins.

Cytokinin levels in the xylem sap have been shown to increase prior to bud burst with a maximum usually occurring

at or just before bud burst. This has been demonstrated in a number of species, including *Malus sylvestris* (L.) Mill. (LUCKWILL and WHYTE, 1968), *Populus robusta* (HEWETT and WAREING, 1973a), *Acer saccharum* Marsh. (DUMBROFF and BROWN, 1976) and *Salix viminalis* L. (ALVIM, HEWETT and SAUNDERS, 1976). This increase in xylem sap cytokinins suggests that root-produced cytokinins, which are transported directly to the bud, are probably involved in the induction of bud burst in the intact plant. Further support for the concept that bud cytokinins originate in roots, was provided by experiments which demonstrated that leaf removal resulted in increased cytokinin in buds (HEWETT and WAREING, 1977b) and that bud removal resulted in increased cytokinin activity in leaves (BEEVER and WOOLHOUSE, 1974) and in the xylem sap (COLBERT and BEEVER, 1981). These increases suggest that there is competition between the leaves and buds for root-produced cytokinins. The cytokinin levels of excised buds of *Xanthium strumarium* decreased by 80 per cent within 24 hours after being detached from the plant, which further suggests that these buds are not capable of synthesizing cytokinins, but are dependent on the rest of the plant for cytokinins (HENSON and WAREING, 1977a). VAN STADEN and BROWN (1978) observed an accumulation of cytokinins in the bark and buds below the girdle, which they had made in *Salix babylonica* L. trees. They concluded that cytokinins from the root are transported via the phloem to the buds and suggested that in the presence of a functional root system, buds do not synthesize cytokinins, but utilize cytokinins transported

to them.

Although it is possible that roots supply buds with cytokinins, there are results which are not in accord with this view. HEWETT and WAREING (1973a) demonstrated that the dormant lateral buds of excised *Populus robusta* twigs could be induced to burst as a result of chilling. This bud burst occurred in the absence of a root system, but the possibility that stored cytokinins were used in bud burst cannot be ignored. PROCHÁZKA (1981) is also of the opinion that roots may not play a primary role in the release of lateral buds. Using ^{14}C-kinetin he observed that the flow of cytokinins from the roots of decapitated *Pisum sativum* seedlings to the lateral buds was only significantly higher 96 hours after decapitation, that is, after visible bud growth was observed. GOODWIN, GOLLNOW and LETHAM (1978) have suggested that the increase in xylem sap cytokinins prior to bud burst may serve to support or induce the rapid growth that follows bud burst rather than initiating bud burst, or may even be unrelated to bud growth and be concerned with cambium activation, or some other aspect of shoot growth. However, as VAN STADEN (1979c) pointed out, the occurrence of cytokinins in an organ can only be related to synthesis if the possibility of transport from other organs or tissues is completely eliminated. This is extremely difficult to do in the case of buds, making it difficult to assess whether they are capable of synthesizing cytokinins. From the available evidence, it appears that even if buds do synthesize cytokinins, they also appear to

receive cytokinins from other parts of the plant.

It has been suggested that the cambium is capable of cytokinin biosynthesis (SKENE, 1972a; VAN STADEN, 1979c; VAN STADEN and CHOVEAUX, 1980; VAN STADEN and DIMALLA, 1981), but it is possible that stored cytokinins or lateral transport from the xylem may account for cytokinins in the cambium. VAN STADEN (1979c) is of the opinion that callus derived from the exposed cambial region of excised *Salix babylonica* buds is responsible for the accumulation of cytokinins in the buds. Having eliminated the xylem and phloem as potential cytokinin sources,he thus proposed that the dividing cambium synthesizes cytokinins. It does not, however, appear to be generally accepted that the cambium synthesizes cytokinins.

Generally, it can be said that there is considerable evidence that the roots synthesize cytokinins; the same cannot, however, be said for the other meristematic tissues that have been proposed as sites of cytokinin synthesis. Therefore, until further evidence can be provided, the roots can only be regarded as a site of cytokinin biosynthesis. In addition to establishing the sites of synthesis, the actual mechanism of cytokinin biosynthesis has also received much attention. Despite the research carried out in this field, the biosynthetic pathway for cytokinins remains in dispute, with two main schools of thought on this subject.

There are basically two types of cytokinins in cells. Namely, free cytokinins and those which are associated with RNA, that is, they occur as bases of tRNA. Cytokinins appear to be ubiquitous components of tRNA (SKOOG and ARMSTRONG, 1970) occurring in the tRNA of micro-organisms, plants and animals, whereas free cytokinins appear to be mainly restricted to the higher plants (CHEN and MELITZ, 1979). The significance of these two kinds of cytokinins has not been determined. Fundamental differences appear to exist between tRNA and free cytokinins. tRNA cytokinins are usually *cis* isomers, whereas free cytokinins are predominantly *trans* isomers (BURROWS, 1978b). Free cytokinins are also regarded as the physiologically active forms (MUIRA and HALL, 1973; BURROWS, 1975) as it has been established that isopentenyladenosine is only active once it has been released from tRNA. Cytokinins such as *cis*-ribosylzeatin and 6-(3-Methylbut-2-enylamino)-2-methylthio-9-β-D-ribofuranosylpurine, which are present in tRNA have never been identified as free,naturally occurring cytokinins in plants (LETHAM, 1978). These differences between tRNA and free cytokinins tend to suggest that they arise by different biosynthetic pathways and that they possibly have different functions.

Cytokinins have always been associated with nucleic acids partly because of the circumstances which led to their discovery and partly because they affect cell division and protein synthesis (VAN STADEN and DAVEY, 1979). The cytokinin, isopentenyladenosine, was identified as a con-

stituent of tRNA in brewers yeast in 1966 (ZACHAU, DÜTTING and FELDMAN, 1966) and also from crude yeast and calf liver tRNA by HALL, CSONKA, DAVID and MCLENNAN (1967), which resulted in DNA and RNA being regarded as potential sources of cytokinins. Other cytokinins were subsequently extracted from tRNA and include isopentenyladenine, 2-methylthio-isopentenyladenosine, 2-methylthio-isopentenyladenine and *cis*-ribosylzeatin (MUIRA, ARMSTRONG and SKOOG, 1975; BURROWS, 1978a). The biosynthesis of tRNA cytokinins has been established, with the terminal step in the biosynthetic pathway involving the transfer of isopentenyl pyrophosphate to a suitable tRNA, which lacks this modification, to yield isopentenyladenosine. Isopentenyladenosine then serves as a substrate for further oxidation and substitution (BURROWS, 1978a). Mevalonic acid serves as the precursor for isopentenyl pyrophosphate in tRNA. The establishment of the biosynthesis of tRNA cytokinins led to the suggestion that free cytokinins may also be synthesized via a similar pathway, that is, free cytokinins arise from tRNA. This proposal is not, however, universally accepted and there are two schools of thought on the biosynthesis of free cytokinins.

One school, which is supported by researchers such as BURROWS (1978a,b), STUCHBURY, PALNI, HORGAN and WAREING (1979); NISHINARI and SYONO (1980a,b) and CHEN (1981), advocates the *de novo* biosynthesis of free cytokinins from adenine monomers. This biosynthetic pathway would, therefore, be independent of tRNA. The opposing school of

thought favours the release of free cytokinins as intact by-products of tRNA degradation. tRNA would thus form an integral part of the synthesis of free cytokinins. This idea is supported by workers such as KLEMEN and KLÄMBT (1974); MAASS and KLÄMBT (1981a,b) and HELBACH and KLÄMBT (1981). There is evidence favouring both of these biosynthetic pathways and it has been proposed that both pathways may, in fact, be operative (BURROWS, 1978b; BARNES, TIEN and GRAY, 1980; WANG, BEUTLEMANN and COVE, 1981). BARNES, TIEN and GRAY (1980) have suggested that tRNA degradation supplies a basal level of cytokinins, while *de novo* synthesis occurs in response to environmental and physiological changes. Biosynthesis of this nature would allow manipulation of hormone metabolism without interfering with the fundamental RNA metabolism. It has also been suggested that tRNA degradation is responsible for free cytokinin production in bacteria and that *de novo* free cytokinin synthesis may be more important in higher plants (HALL, 1973; LETHAM, 1978). HAHN, HEITMAN and BLUMBACH (1976) calculated that sufficient cytokinin was released from tRNA in the bacterium, *Agrobacterium tumefaciens*, to account for all the cytokinins found, but similar calculations for higher plants (MUIRA and HALL, 1973; BURROWS and FUELL, 1981; KLEMEN and KLÄMBT, 1974) are not always consistent and are, therefore, not conclusive.

Direct and indirect evidence exists for the *de novo* synthesis of free cytokinins. Most research workers who favour this mode of biosynthesis, argue that the turnover

of tRNA is not likely to be such that it would supply the cytokinin levels detected in tissues (MUIRA and HALL, 1973; LETHAM, 1978 ; STUCHBURY, PALNI, HORGAN and WAREING, 1979). BURROWS and FUELL (1981) suggested that the tRNA contribution to free cytokinins is not likely to exceed 2 per cent in *Solanum tuberosum* L. cells. BARNES, TIEN and GRAY (1980) put this figure slightly higher, but still said that tRNA degradation is not likely to account for more than 40 per cent of free cytokinin levels. These authors suggest that although tRNA does contribute to the free cytokinin pool, the amount actually released by tRNA is only a small fraction of the total. MAASS and KLÄMBT (1981a,b), who favour free cytokinin synthesis via tRNA degradation, contradict the above suggestions on the rate of turnover and contributions made by tRNA to the free cytokinin pool. They estimate the half-life of tRNA in *Phaseolus vulgaris* roots to be 65 hours and have reported that the appearance of free cytokinins, such as zeatin and dihydrozeatin, shows a direct relationship to the rate of tRNA degradation. These authors, therefore, suggest that free cytokinin synthesis is dependent on tRNA degradation. Other attempts have been made to determine the half-life of tRNA (KLEMEN and KLÄMBT, 1974), but the results are not always consistent. The half-life of tRNA often appears to reflect the metabolism of the cell, which suggests that tRNA degradation could potentially supply the cell with free cytokinins. There are, however, arguments against this suggestion. SHORT and TORREY (1972) found that the root tips of *Pisum sativum* seedlings contained 27 per cent more

free cytokinins than cytokinins bound in tRNA, which would necessitate a high rate of tRNA turnover. WAREING, HORGAN, HENSON and DAVIS (1976) also question whether the rate of turnover of tRNA is sufficient to supply free cytokinins. These authors remarked that if cytokinins are released during tRNA degradation, then why should leaves require cytokinins from other parts of the plant, unless the amount of cytokinin released is insufficient. Until consistent data concerning the half-life of tRNA can be provided, the possibility that tRNA degradation is capable of supplying the levels of free cytokinins occurring in plant cells, will remain in question.

Conclusive evidence has, however, been provided for *de novo* cytokinin synthesis. ^{14}C-Adenine and ^{14}C-adenosine have been used in experiments to demonstrate their direct incorporation into cytokinins of the *trans* form, that is, free cytokinins (CHEN and ECKERT, 1976; BURROWS, 1978b; NISHINARI and SYONO, 1980a,b; HORGAN, PALNI, SCOTT and MCGAW, 1981). In many of these radioactive studies, no radioactivity was recovered from isopentenyladenosine, the main cytokinin occurring in tRNA. In 1978, TAYA, TANAKA and NISHIMURA isolated an enzyme from the slime mould, *Dictyostelium discoideum*, which was capable of catalyzing the synthesis of cytokinin nucleotides. This enzyme was subsequently isolated from a higher plant (CHEN and MELITZ, 1979), and this was regarded as conclusive evidence for the synthesis of free cytokinins independent of the degradation of tRNA. What can perhaps be regarded as the final proof

for *de novo* cytokinin biosynthesis, was provided by NISHINARI and SYONO (1980a) and CHEN (1981), who demonstrated the synthesis of cytokinin nucleotides in cell free systems. Despite this evidence, MAASS and KLÄMBT (1981b) still doubt that a *de novo* synthesis is involved in cytokinin production. They propose that cytokinin biogenesis may depend on tRNA breakdown at 40 to 50 per cent and for the other 50 to 60 per cent on the breakdown of isopentenylated mRNA and eventually oligonucleotides as their intermediate degradation products, which can be isopentenylated.

Despite the evidence for *de novo* cytokinins, the complete mechanism for the synthesis of free cytokinins has not yet been determined. Adenine and adenosine have both been suggested as possible precursors of free cytokinins (CHEN and PETSCHOW, 1978b; NISHINARI and SYONO, 1980a,b). The origin of the isopentenyl side chain has not been determined but NISHINARI and SYONO (1980a) have proposed that the isopentenyl group is transferred directly to adenosine to form isopentenyladenosine. Isopentenyladenosine is then converted to zeatin via isopentenyladenine. A scheme of the synthesis of free cytokinins in an *in vitro* system, as suggested by NISHINARI and SYONO (1980a) is shown in Diagram 1.

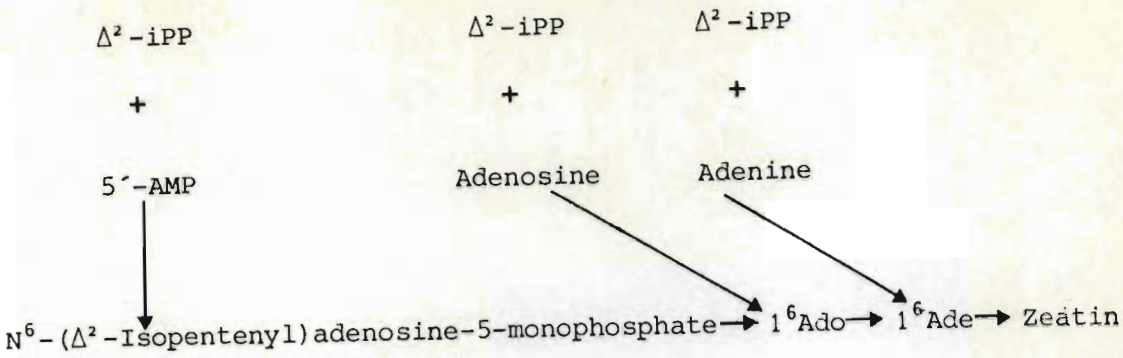


Diagram 1 Scheme for the synthesis and interconversion of cytokinins (NISHINARI and SYONO, 1980a). i^6Ado = isopentenyladenosine; i^6Ade = isopentenyladenine; Δ^2-iPP = isopentenyl pyrophosphate; AMP = adenosine monophosphate.

In an *in vivo* system NISHINARI and SYONO (1980b) demonstrated that isopentenyladenosine was converted to zeatin via ribosylzeatin. They have, however, not resolved the differences in cytokinin synthesis observed between *in vitro* and *in vivo* systems. It is apparent that *de novo* biosynthesis does occur in cells, but it is probable that tRNA degradation is also responsible for maintaining an adequate supply of free cytokinins in plant tissues.

The number of endogenous cytokinins extracted from plants is approximately 25 (ENTSCH, LETHAM, PARKER, SUMMONS and GOLLNOW, 1979) with as many as 16 metabolites being formed from exogenously supplied zeatin in one plant system (PARKER, LETHAM, GOLLNOW, SUMMONS, DUKE and MACLEOD, 1978). In order to assess the role of cytokinins in root-shoot interrelations, it is essential to establish the function of the various cytokinins, as well as their rates of turnover in the plant and in various organs (WAREING, HORGAN, HENSON and DAVIS, 1976). Establishing the functions of cytokinins is essential if these compounds are to be regarded as sig-

nificant in plants. Determining the rate of cytokinin turnover may also be important as it has been suggested (WHITTY and HALL, 1974) that it may be a means of conveying information necessary for cellular growth.

CHEN (1981) has proposed that when a cytokinin enters a plant from an exogenous source, or is synthesized in the cell, many factors, such as the relative activities of enzymes and relative concentration and distribution of cytokinins, determine whether it is to be converted to an 'active form' (a cytokinin free base?), a 'storage form' (cytokinin glucosides?), a 'transport form' (cytokinin nucleotides and/or glucosides?) or a 'degraded form' (adenine, adenosine, inosine). These proposed functions for the various cytokinins are not conclusive and as yet, it has not even been determined which is the actual active component responsible for cytokinin activity. Zeatin and its derivatives are often regarded as being active *per se* because they are active in cytokinin bioassays. There is, however, no evidence that zeatin, which is often regarded as the most active cytokinin (LETHAM, 1967b), itself or a metabolite of zeatin is the active component. This is a fundamental question of cytokinin action which needs to be resolved. HECHT, FRYE, WERNER, HAWRELAK, SKOOG and SCHMITZ (1975) have suggested that the free base cytokinins (zeatin and ribosylzeatin) may best fit the requirements of the "active" compound. CHEN and KRISTOPEIT (1981) hypothesized that an adequate level of active cytokinin in plant cells may be provided through the deribosylation of cytokinin ribosides in concert with other cytokinin meta-

bolic enzymes. HALL (1973), however, is of the opinion that there is no one "active form" of cytokinin. He suggested that all the components of the metabolic system through their dynamic interconversions with other metabolic events, express the ultimate response. More recently, VAN STADEN, DREWES and HUTTON (1982) suggested that trihydroxyzeatin, a biologically active intermediate of zeatin oxidation, may in fact be the active cytokinin component.

Closely linked to the active cytokinin component are the structural requirements for cytokinin activity. SKOOG and ARMSTRONG (1970) defined the structural requirements for high cytokinin activity as an N^6-substituent of moderate molecular length. The expression of cytokinin activity is, however, also dependent on the spatial arrangement, as well as the type of atoms in the N^6-substituent (HECHT, 1979). MATSUBARA (1980) has emphasized the importance of factors such as the absence of a terminal carboxyl group, the presence of a double bond at the 2,3-position, introduction of a second methyl group at the 3-position, hydroxylation at the 4-position and correct stereochemistry of the substituents attached to the double bond as an important feature of the side chain. VAN STADEN, DREWES and HUTTON (1982) following the detection that there was very little difference in the biological activities of *trans*-zeatin and dihydrozeatin, the one of which can be oxidized and the other not, concluded that biological activity (if dependent on a purine molecule with intact C_5 side chain) is largely associated with the chirality present at C_3. These authors also

concluded that a hydroxyl group at the 4-position of the side chain enhances activity.

Cytokinin metabolism studies can potentially yield information concerning the functions and rates of turnover of cytokinins, the active cytokinin form, as well as information about cytokinin biosynthesis. That is, metabolic studies can attempt to elucidate the molecular basis for the diverse growth regulatory activities of cytokinins (COWLEY, DUKE, LIEPA, MACLEOD and LETHAM, 1978). There have been numerous studies on cytokinin metabolism, but no common metabolic pattern has emerged. Cytokinin metabolism apparently changes quantitatively and qualitatively during different stages of development, in various organs and under different physiological conditions (CHEN and KRISTOPEIT, 1981). Cytokinin metabolism has also been said to vary in different species (PALMER, SCOTT and HORGAN, 1981).

Radioactivity labelled cytokinins have been invaluable in yielding information concerning cytokinin metabolism and the distribution of these metabolites. These studies, which involve the application of exogenous cytokinins, do have certain limitations which must be realized if they are to be regarded as being meaningful. It is usually questioned whether exogenously applied cytokinins actually undergo the same metabolism as endogenous cytokinins. STUCHBURY, PALNI, HORGAN and WAREING (1979) proposed that metabolism observed should reflect the true capabilities of the tissues, since it is the distribution of enzymes which

determines which cytokinins are formed. This would mean that exogenous and endogenous cytokinins could potentially undergo similar metabolism. The relative activities of cytokinin metabolic enzymes, such as glucosidases, cytokinin oxidase (WHITTY and HALL, 1974), adenosine phosphorylase and adenosine nucleotidase (CHEN and PETSCHOW, 1978b; NISHINARI and SYONO, 1980b; CHEN and KRISTOPEIT, 1981) are, however, affected by the relative concentrations and distributions of cytokinins (CHEN, 1981). The physiological concentration of the supplied cytokinin could thus have an effect on the metabolism detected. Endogenous cytokinin levels are usually regarded as being very low. Any exogenously applied cytokinin can usually be regarded as excess and the concentrations of these exogenous compounds can also be regarded as unphysiological. It can, therefore, be asked whether the metabolism of unphysiological cytokinin concentrations can be regarded as being indicative of the metabolism of the low concentrations of cytokinins naturally occurring in plant tissues. The normal complement of endogenous cytokinins must also be considered when applying exogenous cytokinins to a plant system. Although radioactive metabolites are usually converted to compounds which have been shown to occur endogenously, it may not necessarily mean that this metabolism reflects the metabolism of the naturally occurring compounds (HORGAN, PALNI, SCOTT and MCGAW, 1981).

Despite these potential limitations, the fate of radioactive cytokinins is usually regarded as being indicative of the normal situation. There are, however, examples which

contradict this assumption. HORGAN, PALNI, SCOTT and MCGAW (1981) applied ^{14}C-O-glucosylzeatin to gall tissue and it was rapidly degraded, resulting in a low concentration in this tissue. This does not appear to be the fate of endogenous zeatin glucosides, which accumulate in gall tissue. This suggests different metabolic pathways for exogenous and endogenous cytokinins. Results of this nature could result in ambiguity in the interpretation of metabolic studies. There does not, however, seem to be an alternative to exogenous cytokinin studies for determining cytokinin metabolism. Therefore, when undertaking studies involving the application of exogenous radioactive cytokinins, particular attention should perhaps be paid to the normal endogenous cytokinin content of the tissue, the amount of hormone supplied and to any modifications of the plant system which could result in a wounding response. Wounded or pruned tissue can be a source of cytokinins through associated autolyzing tissue (CARLSON and LARSON, 1977), which could have an effect on the results detected. These precautions may increase the validity of radioactive metabolism studies which are necessary to understand cytokinin action.

Cytokinin metabolism is an elaborate and complex process resulting in the formation of a variety of biologically active derivatives of the original cytokinin (VAN STADEN, DREWES and HUTTON, 1982). Cytokinins can basically be metabolized in three possible ways. That is;

(1) by modification of the purine ring (PARKER and LETHAM, 1973; GORDON, LETHAM and PARKER, 1974; VAN STADEN, 1981b) to form metabolites such as lupinic acid (DUKE, MACLEOD, SUMMONS, LETHAM and PARKER, 1978) and ribosylated and glucosylated (N^7 and N^9-glucosides) derivatives;

(2) by side chain modification (PARKER, LETHAM, WILSON, JENKINS, MACLEOD and SUMMONS, 1975; LETHAM, PARKER, DUKE, SUMMONS and MACLEOD, 1976) to form metabolites resulting from glucosylation (O-glucosides), reduction (dihydrozeatin) and oxidation (6-(2,3,4-trihydroxy-3-methylbutylamino)purine) and,

(3) by side chain cleavage (SUMMONS, ENTSCH, LETHAM, GOLLNOW and MACLEOD, 1980; VAN STADEN, 1981a,b) resulting in the formation of adenine, adenosine, urea and N-(purin-6-yl) glycine (VAN STADEN, DREWES and HUTTON, 1982).

VAN STADEN, DREWES and HUTTON (1982) regard 6-(2,3,4-trihydroxy-3-methylbutylamino)purine (trihydroxyzeatin), an oxidation product of zeatin, as a metabolite of zeatin resulting from side chain cleavage. It may, however, be more accurate to regard trihydroxyzeatin as a metabolite resulting from side chain modification as there are still five carbon atoms in the isopentenyl side chain. N-(Purin-6-yl) glycine (purinyl glycine), the end-product of zeatin oxidation (MILLER, 1965; LETHAM, SHANNON and MCDONALD, 1967; VAN STADEN, DREWES and HUTTON, 1982), can be said to be formed as the result of side chain cleavage

as only two carbon atoms remain in the side chain. Cytokinins can be metabolized by a combination of the above three metabolic pathways in one system.

Cytokinin metabolism has been investigated using synthetic and natural cytokinins, namely benzyladenine, kinetin, zeatin and zeatin derivatives. It has been suggested that the metabolism of natural and synthetic cytokinins is similar (SONDHEIMER and TZOU, 1971), but zeatin metabolism appears to be a more complex process. Benzylaminoriboside was recorded as the major metabolite formed from 6-benzylaminopurine in a number of plant species (MCCALLA, MOORE and OSBORNE, 1962; DOREE and SADORGE, 1968; WOOLLEY and WAREING, 1972; RAMINA, 1979), whereas other research workers established the 3, 7 and 9-glucosides of benzylaminopurine as major metabolites (FOX, CORNETTE, DELEUZE, DYSON, GIERSAK, NIU, ZAPATA and MCCHESNEY, 1973; WILSON, GORDON, LETHAM and PARKER, 1974; LETHAM, WILSON, PARKER, JENKINS, MACLEOD and SUMMONS, 1975; PALNI, TAO and LETHAM, 1982). FOX and his co-workers suggested that the riboside of benzylaminopurine was an intermediatory precursor for the glucosides, which are the end-products of metabolism. They also proposed that due to their stability, the 7-glucosides, which are also metabolites of zeatin, are the active cytokinin compounds. These authors do, however, point out that the bulk of the benzylaminopurine entering the plant tissue undergoes side chain cleavage to form several purine metabolites and it is the remainder of the hormone which is glucosylated. Benzyladenine thus appears to undergo ribosylation,

glucosylation and side chain cleavage. The latter metabolic pathway could be a mechanism for disposing of excess applied cytokinin, as purine derivatives do not appear to be active in cytokinin bioassays, whereas the ribosylated and glucosylated derivatives are biologically active.

The 3-glucoside (LETHAM, WILSON, PARKER, JENKINS, MACLEOD and SUMMONS, 1975) and the 7-glucoside of benzylaminopurine (FOX, CORNETTE, DELEUZE, DYSON, GIERSAK, NIU, ZAPATA and MCCHESNEY, 1973; WILSON, GORDON, LETHAM and PARKER, 1974; GAWER, LALOUE, TERRINE and GUERN, 1977; COWLEY, DUKE, LIEPA, MACLEOD and LETHAM, 1978; ENTSCH and LETHAM, 1979) are considered to be unusual structures by virtue of the nature of the sugar moiety involved and the position of the glycosidic linkages. The 3-glucosides also differ from the 7- and 9-glucosides in that they are biologically more active and they are also more sensitive to β-glucosidase enzyme activity (LETHAM, WILSON, PARKER, JENKINS, MACLEOD and SUMMONS, 1975). The physiological significance of these different glucosides has not yet been resolved. GAWER, LALOUE, TERRINE and GUERN (1977) argue against these metabolites being active forms of cytokinin as they have shown that the 7-glucoside of benzyladenine was less active than benzyladenine in the soyabean bioassay. They do not, however, rule out the possibility that they may be active via re-utilization, the rate of which could be a limiting factor of biological activity. These authors,as well as FOX and co-workers (1973) also dismiss the possibility that these metabolites are detoxification products as their concentration does not increase proportionally to the increase

in the supplied cytokinin, and because they are biologically active. The concept of glucosides being storage metabolites (PARKER, WILSON, LETHAM, COWLEY and MACLEOD, 1973), which would mean that they could be re-utilized, is supported by GAWER, LALOUE, TERRINE and GUERN (1977). They have demonstrated that the 7-glucoside of benzyladenine, applied to tobacco cells, was metabolized to benzyladenosine-5-phosphate, which suggests possible re-utilization. There is, however, no unequivocal evidence for the function of the glucosides of benzyladenine, although as with the glucosides of zeatin, storage is probably the most favoured concept.

Zeatin metabolism is more complex than benzyladenine metabolism, with as many as 16 metabolites being identified from *Lupinus* seedlings (PARKER, LETHAM, GOLLNOW, SUMMONS, DUKE and MACLEOD, 1978). Zeatin and ribosylzeatin appear to undergo similar metabolism when applied to plant tissue (VAN STADEN and DAVEY, 1977; SUMMONS, ENTSCH, LETHAM, GOLLNOW and MACLEOD, 1980; LETHAM and PALNI, 1981), and it is implied that these two cytokinins are interchangeable. The metabolites of exogenously applied zeatin can be divided into four groups (ENTSCH, LETHAM, PARKER, SUMMONS and GOLLNOW, 1980), namely;

(1) simple derivatives of zeatin, including dihydrozeatin, ribosylzeatin, dihydro-ribosylzeatin, zeatin nucleotides and dihydrozeatin nucleotides;

(2) glucoside metabolites, which include the 7-, 9-

and O-glucosides of zeatin, ribosylzeatin and dihydrozeatin;

(3) amino acid metabolites which include lupinic acid and dihydrolupinic acid and,

(4) products of N^6-side chain cleavage which include adenine, adenosine and adenine nucleotides.

LETHAM and PALNI (1981) have proposed a comprehensive model for zeatin metabolism, which is indicated in Diagram 2. Zeatin biosynthesis is also included in this model.

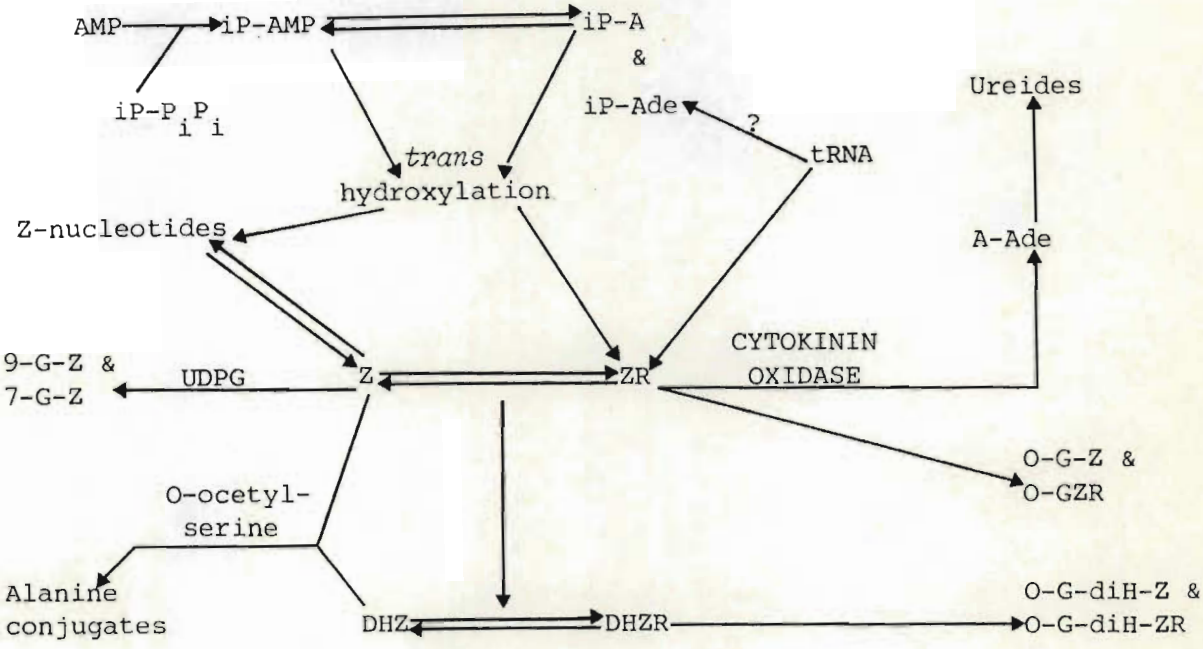


Diagram 2 Representation of the basic metabolism of natural cytokinins (LETHAM and PALNI, 1981). Z = zeatin; ZR = ribosylzeatin; DHZ = dihydrozeatin; DHZR = dihydro-ribosylzeatin; A = adenosine; Ade = adenine; iP = isopentenyl; G = glucosyl; diH = dihydro.

One of the earliest studies on the metabolism of radioactive zeatin was carried out by SONDHEIMER and TZOU (1971), who detected zeatin, ribosylzeatin, zeatin-5-ribotide and their

dihydro derivatives as the major metabolites in the excised axes of *Phaseolus vulgaris*. Ribosylzeatin was, however, detected as the major metabolite of zeatin in *Fraxinus americana* L. embryos, with the 5′-mono, di and triphosphates of zeatin being recorded as minor metabolites. No dihydro-derivatives were detected in these embryos (TZOU, GALSON and SONDHEIMER, 1973). Dihydro derivatives have subsequently not been identified as major metabolites in most zeatin metabolism studies. Nucleotides of zeatin and ribosylzeatin are usually recorded as metabolites of exogenous zeatin and often represent the major metabolites in some tissues (TZOU, GALSON and SONDHEIMER, 1973; VONK and DAVELAAR, 1981). Since the identification of the 7-glucoside (raphanatin) (PARKER and LETHAM, 1973) as a major metabolite of zeatin, 7-, 9- and O-glucosides of zeatin, ribosylzeatin and their dihydro derivatives, have subsequently been identified as major metabolites in a number of plant tissues (GORDON, LETHAM and PARKER, 1974; LETHAM, PARKER, DUKE, SUMMONS and MACLEOD, 1976; VAN STADEN and DAVEY, 1977; COWLEY, DUKE, LIEPA, MACLEOD and LETHAM, 1978; DUKE, LETHAM, PARKER, MACLEOD and SUMMONS, 1978, 1979; HENSON, 1978a,b; ENTSCH, LETHAM, PARKER, SUMMONS and GOLLNOW, 1980; SUMMONS, ENTSCH, LETHAM, GOLLNOW and MACLEOD, 1980; HORGAN, PALNI, SCOTT and MCGAW, 1981; PALMER, HORGAN and WAREING, 1981a,b) including leaves, seeds, roots and callus. Adenine and adenosine have been detected as minor zeatin metabolites (PARKER and LETHAM, 1973; GORDON, LETHAM and PARKER, 1974), although they are often regarded as major metabolites (PARKER and LETHAM, 1974; DUKE, LETHAM, PARKER, MACLEOD and SUMMONS, 1979; HENSON, 1978a; SUMMONS, ENTSCH,

LETHAM, GOLLNOW and MACLEOD, 1980; PALMER, SCOTT and HORGAN, 1981). Adenine and adenosine result from cleavage of the isopentenyl side chain.

The formation of adenine and adenosine as metabolites of zeatin is an interesting concept. These compounds are formed by complete removal of the isopentenyl side chain, which is cleaved by cytokinin oxidase (WHITTY and HALL, 1974) type enzymes (SUMMONS, ENTSCH, LETHAM, GOLLNOW and MACLEOD, 1980). The reaction is apparently complete as no intermediates are recorded. Adenine and adenosine are not active in the soyabean callus bioassay (VAN STADEN and DAVEY, 1977; VAN STADEN, 1979a). This could imply that if side chain cleavage is to be regarded as a major metabolic pathway, then zeatin and ribosylzeatin are active *per se* and that adenine and adenosine represent breakdown products resulting from utilization, that adenine and adenosine are active by means of incorporation back into the system, or alternatively, the formation of these compounds could be a mechanism for disposing of excess cytokinins. HECHT (1979) has proposed that plant tissues are not responsive to exogenous cytokinins as they utilize their own endogenous cytokinin supply. This could imply that the bulk of exogenously supplied cytokinins may be broken down to adenine and adenosine, with only a small fraction undergoing cytokinin metabolism, as was suggested by FOX, CORNETTE, DELEUZE, DYSON, GIERSAK, NIU, ZAPATA and MCCHESNEY in 1973. This concept would, however, question the earlier assumption that the metabolism of exogenous cytokinins is indicative of the

normal metabolism of the cell. Side chain cleavage by cytokinin oxidase type enzymes implies that this is an oxidation reaction. VAN STADEN and DREWES (1982) have reported that potassium permanganate oxidation of zeatin resulted in the formation of a number of compounds. These compounds had similar elution volumes to the radioactive peaks formed when radioactive zeatin was applied to germinating *Zea mays* kernels (VAN STADEN, 1981a,b). VAN STADEN and DREWES (1982) have identified two of these compounds as 6-(2,3,4-trihydroxy-3-methylbutylamino)purine (trihydroxyzeatin) and N-(purin-6-yl)glycine (purinyl glycine). Trihydroxyzeatin and purinyl glycine have both been previously recorded in plant tissue (MILLER, 1965; LETHAM, SHANNON and MCDONALD,1967; LETHAM, 1973). This implies that potassium permanganate oxidation reflects a naturally occurring oxidation pathway for zeatin which is operative in plant tissues. Purinyl glycine has been reported as the inactive end-product of zeatin oxidation which suggests that trihydroxyzeatin is an intermediate in this reaction. Potassium permanganate oxidation of zeatin as suggested by VAN STADEN, DREWES and HUTTON (1982) is shown in Diagram 3.

Trihydroxyzeatin, a biologically active intermediate oxidation product, co-chromatographs with adenosine both on paper (R_f 0,36) and on a Sephadex LH-20 column eluted with 10 per cent methanol. WHITTY and HALL (1974) detected unstable intermediates in oxidation reactions with cytokinin oxidase enzymes. They reported that isopentenylaldehyde could have been one of the intermediates, but this was not

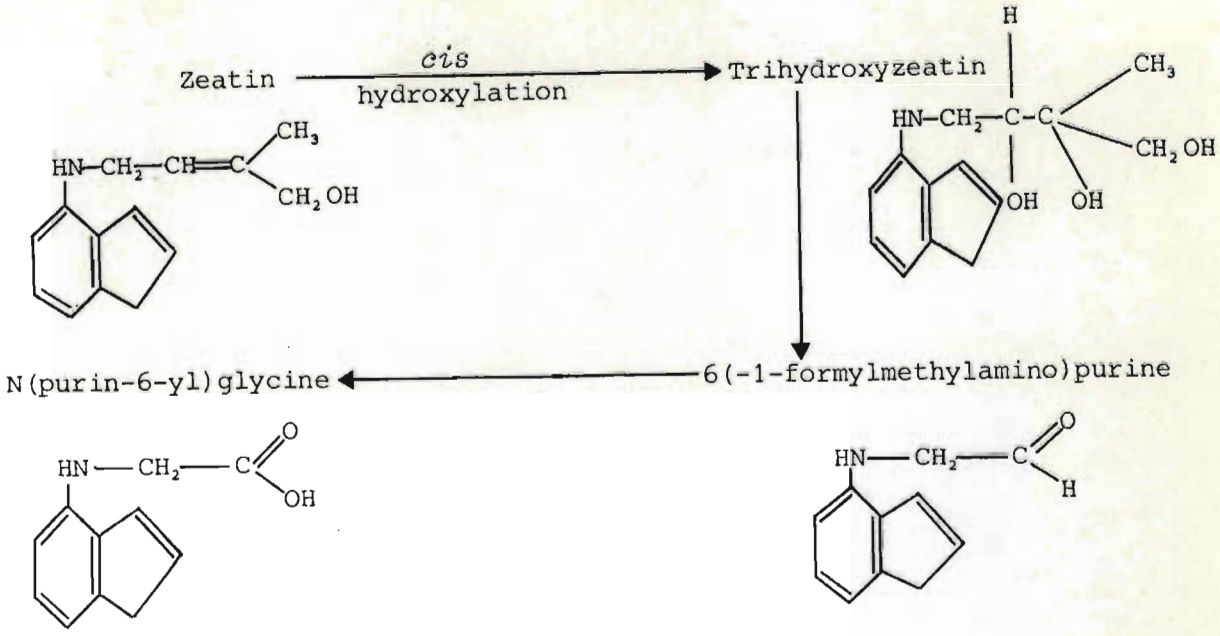


Diagram 3 Potassium permanganate oxidation of zeatin to N(purin-6-yl) glycine (VAN STADEN, DREWES and HUTTON, 1982).

confirmed. They also reported that cytokinin oxidase treatment of isopentenyladenosine and ribosylzeatin resulted in the formation of adenosine. VAN STADEN, DREWES and HUTTON (1982) also detected a compound co-chromatographing with adenosine following the oxidation of isopentenyl-adenine but mass spectrometry revealed that this compound was actually 6-(2,3-dihydroxy-3,3-dimethylbutylamino)purine. It would thus appear as if the oxidation products of zeatin, ribosylzeatin, isopentenyladenine and isopentenyladenosine could have been incorrectly identified on numerous occasions in the past.

Trihydroxyzeatin is biologically very active. VAN STADEN, DREWES and HUTTON (1982) have shown that trihydroxyzeatin is as active as zeatin in the soyabean callus bioassay, except at the concentration of 10^{-4} Molar at which trihydroxy-

zeatin is more active than zeatin. This could imply that zeatin is converted to trihydroxyzeatin, which could thus account for the similar responses. Trihydroxyzeatin, which co-elutes with zeatin glucoside and adenosine, could perhaps be the active form of zeatin (VAN STADEN, DREWES and HUTTON, 1982). This concept could be supported by the fact that zeatin and its glucosylated derivatives are not stable in plant tissues (HENSON, 1978b). The co-elution of zeatin glucoside, adenosine and trihydroxyzeatin further implies that some of the previously identified zeatin metabolites possibly need verification. Purinyl glycine is reported to be weakly, biologically active (MILLER, 1965) and is unaffected by alkaline phosphatase (VAN STADEN, DREWES and HUTTON, 1982). While not actually identifying purinyl glycine, all the evidence presented by HENSON and WHEELER (1977a) indicated that this compound was formed after the application of zeatin to root nodules. Their product was very polar and co-chromatographed with adenosine monophosphate, it did not respond to alkaline phosphatase treatment and gave a weak response with the soyabean callus bioassay. YOKOTA, UEDA and TAKAHASHI (1981) have also reported the occurrence of a compound in immature seeds of *Dolichos lablab* L. which was more polar than glucoside cytokinins, but was not a ribotide cytokinin. Purinyl glycine has similar chromatographic properties to tho e associated with the previously recorded cytokinin nucleotides (R_f 0,03 on paper chromatograms and an elution volume of 180 to 280 millilitres on a Sephadex LH-20 column eluted with 10 per cent methanol (VAN STADEN, DREWES and HUTTON, 1982). This

could also imply that the previous reports of cytokinin nucleotides need further investigation. Zeatin oxidation resulting in side chain reduction/cleavage may thus be an important metabolic pathway in plant tissues.

Relatively little attention has been paid to the metabolism of dihydrozeatin. This cytokinin and its glucosylated derivatives are reported to be more stable than zeatin (HENSON, 1978b). The glucosylation capacity of dihydrozeatin is also reported to be greater than that of zeatin (HENSON, 1978b). The stability of dihydrozeatin is said to be due to it being resistant to enzymes of the cytokinin oxidase type (HENSON, 1978b; VAN STADEN, 1981a), and this stability is said to facilitate O-glucosylation. Dihydro derivatives are reported to be the metabolites which accumulate in leaves and more recently, PALMER, HORGAN and WAREING (1981b) have suggested a zeatin-dihydrozeatin interconversion mechanism in *Phaseolus vulgaris* plants. These authors have suggested that this interconversion and the relative activities of cytokinin oxidase enzymes maintain a decreasing concentration gradient of zeatin derivatives and an increasing gradient of dihydrozeatin derivatives from the roots to the lamina. The significance of this selective degradation or interconversion of cytokinins has not been determined, but it has been suggested that this process could be important in controlling the relative levels of active cytokinins in the plant (PALMER, HORGAN and WAREING, 1981b). The amount of biological activity associated with dihydrozeatin, relative to that of zeatin does not appear to

be clear. SUMMONS, ENTSCH, LETHAM, GOLLNOW and MACLEOD (1980) reported low cytokinin activity associated with dihydrozeatin, whereas HENSON (1978b) and VAN STADEN, DREWES and HUTTON (1982) reported that dihydrozeatin was as active, or even more active, than zeatin in the soyabean callus bioassay. The biological activity of dihydrozeatin and zeatin may well be dependent on the bioassay system used. Dihydrozeatin does, however, exhibit biological activity and its importance as a metabolite of zeatin due to its stability could suggest that it is involved in regulating active cytokinin levels. The stability of these cytokinins could imply that they are not active *per se*.

It can thus be seen that zeatin metabolism is indeed a complex process. Zeatin usually undergoes more than one type of metabolism in a plant tissue, with the physiological activity of the tissue apparently affecting the number of metabolites formed. VAN STADEN (1981a,b) detected more zeatin metabolites in the biologically more active embryonic axis of *Zea mays* kernels than in the endosperm. Despite the numerous metabolic studies undertaken, the significance of the metabolites of zeatin, as well as the active cytokinin component have not yet been unequivocally determined. When assessing zeatin metabolism, it must, however, be remembered that the formation of a large number of metabolites from zeatin may not necessarily be related to the formation of functional metabolites, but may be related to storage and/or inactivation of the applied cytokinin.

Kinetin is used extensively in cytokinin studies to bring about, and to monitor physiological effects normally associated with endogenous cytokinins. Callus, for tissue culture bioassays, is also maintained on kinetin yet, relatively little is known about its metabolism. EL SADI (1972) and PROCHÁZKA, SCHRAUDOLF and ŠONKA (1977), working with *Zea mays* and *Pisum sativum* seedlings suggested that kinetin is metabolized to adenine, adenosine and other purine derivatives with some free kinetin remaining. MIERNYK and BLAYDES (1977) carried out a more comprehensive study on ^{14}C-kinetin metabolism in germinating *Lactuca sativa* seeds. In this system, the kinetin was initially metabolized to a bound storage form, 9-riboside-5-monophosphate. Free kinetin was later released from this storage form. Minor metabolites formed were adenosine-5-monophosphate and inosine-5-monophosphate. Diagram 4 illustrates MIERNYK and BLAYDES' proposed model for kinetin metabolism.

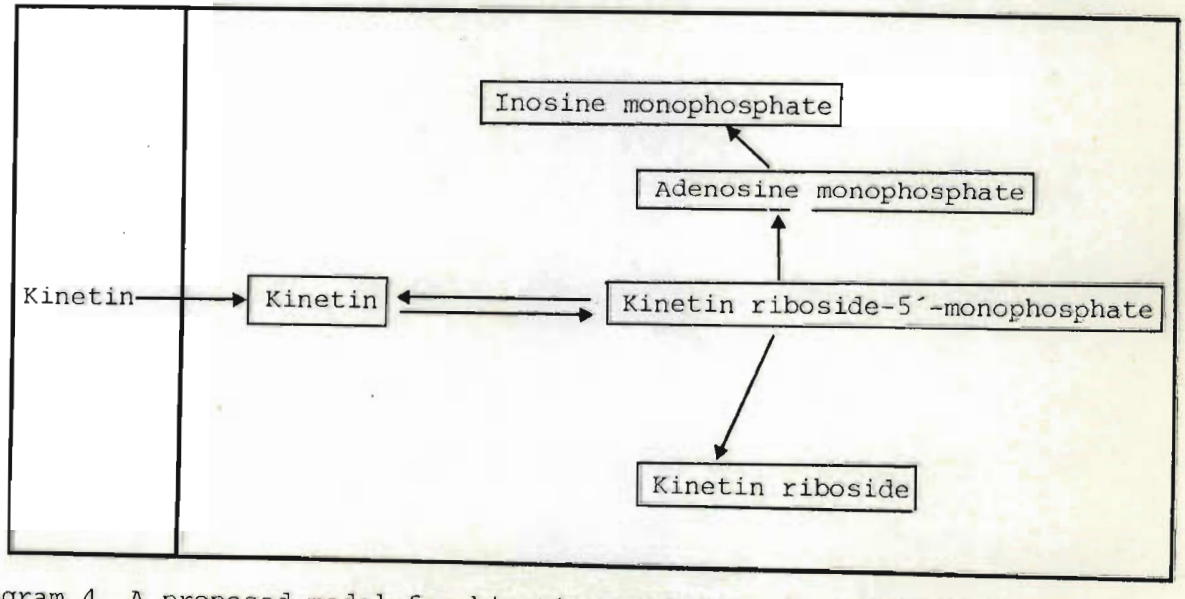


Diagram 4 A proposed model for kinetin metabolism in germinating lettuce seeds (MIERNYK and BLAYDES, 1977). [ ] - represent intercellular pool of each metabolite.

During short term metabolism, kinetin appears to undergo side chain modification and side chain cleavage. Due to the frequent use of kinetin in cytokinin experiments, the metabolism of kinetin needs to be verified as well as the long term metabolism investigated.

This literature survey has perhaps served to highlight some of the areas of cytokinin transport and metabolism which warrant further investigation. GOODWIN, GOLLNOW and LETHAM (1978) have pointed out that, although our knowledge of metabolites of exogenous cytokinins has advanced, there is little information available concerning the regulation of cytokinin metabolism, the tissues where it occurs and the significance of the diversity of metabolites. They also said that the information concerning the distribution of xylem sap cytokinins in the organs of the shoot and the regulation of their translocation is poor. Basic questions such as whether xylem sap cytokinins contribute to the high levels of cytokinins detected in immature fruit and developing seed, or whether xylem sap cytokinins are largely destined for the actively growing apex, and if so, are they transported first to the leaves and then to the apex or directly to the apex, also need to be answered. This thesis by no means attempts to answer all of these questions, but it was hoped that the results would contribute to solving some of these problems. 8(^{14}C)*t*-Zeatin was, therefore, used as a tool to determine more about the transport and metabolism of zeatin in specific plant systems. Radioactive zeatin was applied to deciduous, evergreen and annual leaves in order to

determine whether leaf cytokinins contribute to the cytokinins in the rest of the plant and thereby also to obtain some information on the role of cytokinin glucosides. Zeatin was also applied to germinating bean seeds and to bean plants during their development, to determine the distribution of root-produced cytokinins at various stages of plant growth.

MATERIALS AND METHODS

The transport and metabolism of 8(^{14}C)*t*-zeatin was examined using four different plant systems. *Ginkgo biloba* L. explants with leaves at three stages of maturity; dormant *Citrus sinesis* (L.) Osbeck trees; *Phaseolus vulgaris* plants at three stages of development and germinating *Phaseolus vulgaris* seeds were all used as experimental material. The actual experiments undertaken using the above plant systems, will be explained in detail in Chapters 1, 2, 3 and 4 respectively. In general terms it can be said that 8(^{14}C)*t*-zeatin was applied to plant material which was then extracted for cytokinins in order to evaluate the metabolism and transport of the radioactive zeatin. After paper and/or column chromatography, the extracts were subjected to bioassays and radioassays in an attempt to determine cytokinin-like activity and the radioactivity, if any, associated with it. It must be noted that cytokinins were not actually identified by mass spectrometry but were only tentatively identified on a basis of co-elution with authentic cytokinin standards and with the use of various chemical and enzyme treatments.

1.0 Application of 8(^{14}C)*t*-Zeatin

8(^{14}C)*t*-Zeatin (specific activity 441,1 MBq mmol^{-1}) was synthesized and then purified by Dr. J. Corse in 1979, using high performance liquid chromatography. This zeatin was redissolved in 20 per cent ethanol and its purity was checked

before it was used. Figure 1A shows the single radioactive peak, which co-elutes with authentic zeatin, obtained after separating 5 microlitres of 8(^{14}C)*t*-zeatin on Whatman No. 1 chromatography paper with *iso*-propanol:25 per cent ammonium hydroxide:water (10:1:1 v/v) (PAW). A single radioactive peak was also obtained after passing 5 microlitres of 8(^{14}C)*t*-zeatin through Dowex 50 cation exchange resin (Figure 1B). This peak co-eluted with authentic zeatin indicating that the radioactively labelled zeatin was not hydrolyzed by the cation exchange resin. From Figures 1B and 1C it can also be seen that 98,7 per cent of the radioactivity applied to the Dowex 50 resin was recovered in the ammonia fraction with only 1,3 per cent remaining in the aqueous fraction. The biological activity associated with 5 microlitres of radioactive zeatin is shown in Figure 1D. Fractionation of 8(^{14}C)*t*-zeatin on a Sephadex LH-20 column, eluted with 10 per cent methanol, also resulted in a single radioactive peak which co-eluted with zeatin (Figure 2). It can, therefore, be said that the radioactive zeatin used in the experiments was relatively pure.

Except where otherwise stated, 5 microlitres of 8(^{14}C)*t*-zeatin was applied to each sample via a microsyringe. Five microlitres of labelled zeatin gave approximately 8 x 10^5 disintegrations per minute (dpm).

2.0 Extraction and Purification of Plant Material for Cytokinins

The cytokinins were extracted from the plant material

Figure 1A 8(^{14}C)*t*-Zeatin applied to Whatman No. 1 chromatography paper and separated with PAW.

B 8(^{14}C)*t*-Zeatin applied to Dowex 50 cation exchange resin and eluted with 5M NH_4OH. This ammonia fraction constituted 98,7 per cent of the total radioactivity recovered.

C The aqueous fraction of 8(^{14}C)*t*-zeatin applied to a Dowex 50 column. This fraction constituted 1,3 per cent of the total radioactivity recovered.

D The biological activity, as detected by the soyabean callus bioassay, associated with five microlitres 8(^{14}C)*t*-zeatin. Z = zeatin; ZR = ribosylzeatin

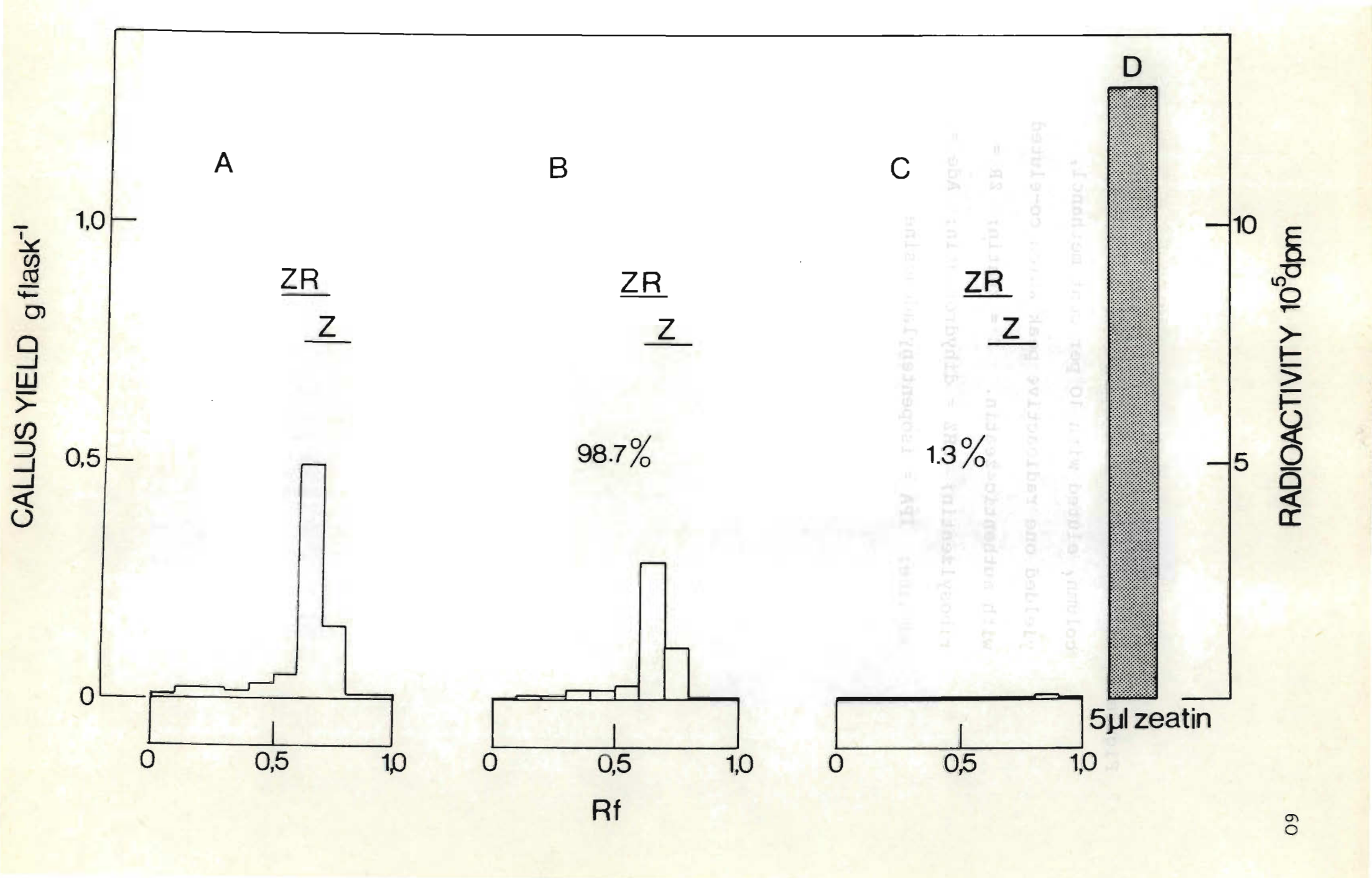
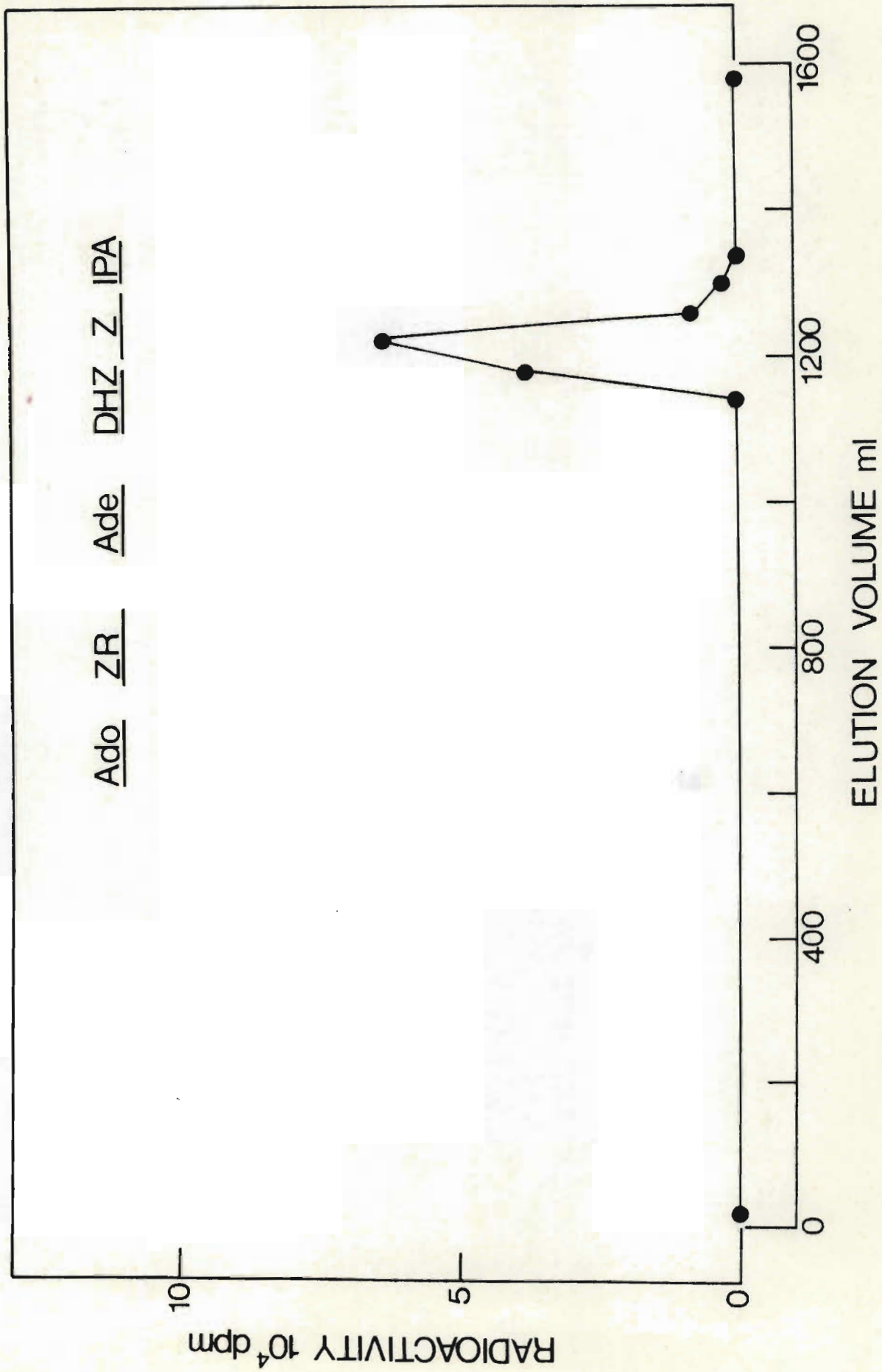

A
B
C
D
ZR
Z
98.7%
1.3%
CALLUS YIELD g flask⁻¹
RADIOACTIVITY 10⁵dpm
1,0
0,5
0
10
5
0
0,5
1,0
Rf
5µl zeatin

Figure 2 8(^{14}C)*t*-Zeatin passed through a Sephadex LH-20 column, eluted with 10 per cent methanol, yielded one radioactive peak which co-eluted with authentic zeatin. Z = zeatin; ZR = ribosylzeatin; DHZ = dihydrozeatin; Ade = adenine; IPA = isopentenyladenosine.


Ado ZR Ade DHZ Z IPA
RADIOACTIVITY 10^4 dpm
0
5
10
0
400
800
1200
1600
ELUTION VOLUME ml

analyzed by homogenizing the samples in 80 per cent ethanol and allowing them to stand overnight at 5°C. The extracts were then filtered through Whatman No. 1 filter paper and the residues washed with 80 per cent ethanol. The filtrates were then concentrated to dryness under vacuum at 40°C. The residues were redissolved in 50 millilitres of 80 per cent ethanol and the pH of the ethanolic extracts was adjusted to 2,5 with dilute hydrochloric acid. Appropriate quantities of Dowex 50W-X8 cation exchange resin (J.T. Baker Chemical Co. Phillipsburg, N.J.; H^+ form, 20-50 mesh, 2,5 x 25 centimetres), based on a fresh weight basis, were shaken in the extracts for one hour. The extracts were then passed through the exchange resin at a flow rate of 40 millilitres per hour. The columns were washed with 100 millilitres of distilled water, followed by 100 millilitres of 80 per cent ethanol. The combined aqueous and ethanolic eluates constituted the aqueous fraction. In those samples where the aqueous fraction was analyzed, the pH was adjusted to 7,0 with dilute sodium hydroxide, and the fraction was then concentrated to dryness under vacuum. The residues were resuspended in 3 millilitres of 80 per cent ethanol and strip-loaded onto Whatman No. 1 chromatography paper. The cytokinins were eluted from the cation exchange resin with 100 millilitres of 5N ammonium hydroxide. This constituted the ammonia fraction. The ammonia eluates were concentrated to dryness under vacuum and the residues taken up in 3 millilitres of 80 per cent ethanol. These ethanolic fractions were then strip-loaded onto Whatman No. 1 chromatography paper.

Dowex 50 cation exchange resin has been widely used to purify cytokinin extracts (ENGELBRECHT, 1971; HEWETT and WAREING, 1973c; VAN STADEN, 1976b; WANG, THOMPSON and HORGAN, 1977). The use of strongly acidic cation exchange resins is often criticized and it has been suggested that there is a danger of hydrolyzing the active compounds using these resins (TEGELY, WITHAM and KRASNUK, 1971; DEKHUIJZEN and GEVERS, 1975; LETHAM, 1978). LETHAM (1978) suggested that strongly acidic resins were not necessary for cytokinin purifications and that the use of cellulose phosphate columns may, in fact, be preferable. MILLER (1975), however, confirmed the reliability of Dowex 50 as he obtained similar results when he used two techniques which omitted Dowex 50 and a technique in which Dowex 50 was used. VAN STADEN (1976c) also demonstrated that zeatin, ribosylzeatin and their glucosylated derivatives could be efficiently purified from plant material by means of Dowex 50 cation exchange resin.

3.0 Chromatographic Techniques

3.1 Paper chromatography

Extracts were strip-loaded, in a one centimetre strip, onto sheets of Whatman No. 1 chromatography paper. The chromatograms were separated in descending chromatography tanks, using *iso*-propanol:25 per cent ammonium hydroxide: water (10:1:1 v/v) (PAW), until the solvent front was approximately 30 centimetres from the origin. The

chromatograms were then oven dried (25°C) for 24 hours. The dry chromatograms were divided into 10 equal R_f strips and were either assayed for radioactivity and/or cytokinin activity or stored at -20°C until analyzed.

3.2 Column chromatography

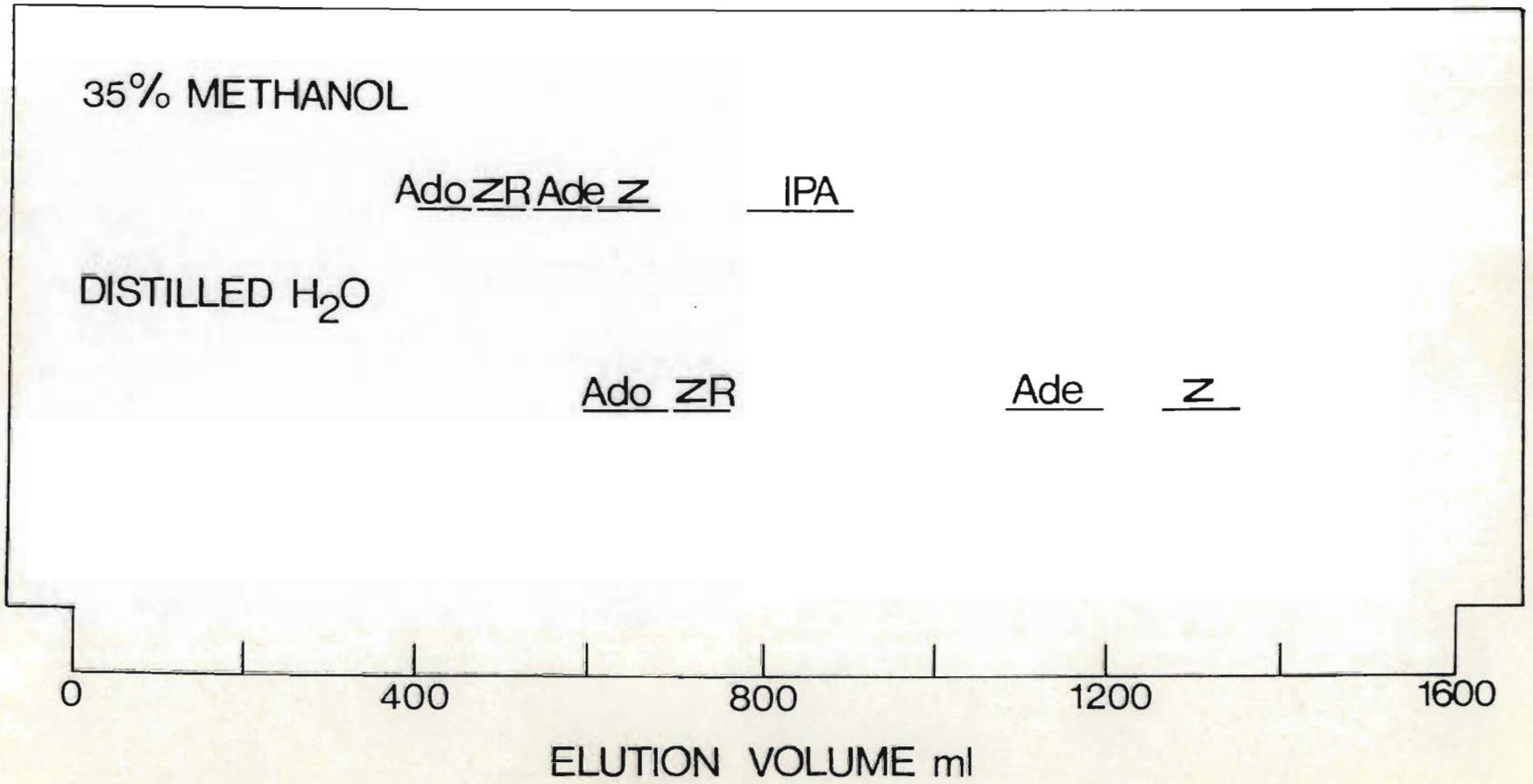
Column chromatography was used to fractionate extracts so that the cytokinins could be identified on a basis of co-elution with authentic cytokinins. The physical techniques of high-pressure liquid chromatography, gas-liquid chromatography and mass spectrometry are sensitive and reliable techniques for cytokinin identification. Although bioassays are often regarded as approximations, they constitute an integral part of cytokinin research, as they are essential for determining the biological activity of separated compounds. It may also be advantageous to assess the biological activity of a compound before it is identified by means of physical techniques, rather than initially determining its physical properties. Column chromatographic techniques are most frequently used to purify extracts prior to bioassay.

The column technique commonly used today is one in which cytokinins are fractioned on a Sephadex LH-20 column with 35 per cent ethanol as the solvent (ARMSTRONG, BURROWS, EVANS and SKOOG, 1969). This system can separate zeatin from ribosylzeatin but it does not efficiently separate adenine and adenosine, which may serve as precursors for

the synthesis of zeatin and ribosylzeatin (VAN STADEN, 1979a; STUCHBURY, PALNI, HORGAN and WAREING, 1979) from these cytokinins. A further disadvantage of this system is that none of the dihydro derivatives of the different cytokinins can be separated. Attempts have been made to improve the separation of cytokinins. Zeatin and dihydrozeatin have been separated on a Sephadex G-10 column eluted with distilled water (BURROWS, 1978a). Other systems which have been used include 5 per cent ethanol with Sephadex G-10 (VAN STADEN and DIMALLA, 1980) and 20 per cent ethanol with Sephadex LH-20 (SMITH and VAN STADEN, 1978; DEKHUIJZEN, 1980). Better separation of a wide range of cytokinins, including the cytokinin glucosides, was achieved by using 35 per cent methanol (HENSON and WHEELER, 1977a) or 20 per cent methanol (HENSON, 1978a) in conjunction with Sephadex LH-20. Adenine, dihydrozeatin and zeatin could not be separated completely from one another using these systems. As column chromatography was the only technique readily available in our laboratories for separating cytokinins, it was attempted to develope an efficient column chromatographic technique to separate cytokinins prior to assay.

A range of ethanol and methanol concentrations were used in an attempt to obtain the best separation of adenine derivatives and authentic cytokinins on Sephadex G-10 and Sephadex LH-20 columns. Distilled water and 35 per cent methanol gave the best separation on Sephadex G-10 columns (93 x 2,5 centimetres) (Figure 3). However, the compounds eluted too close together for use in routine analyses of plant ex-

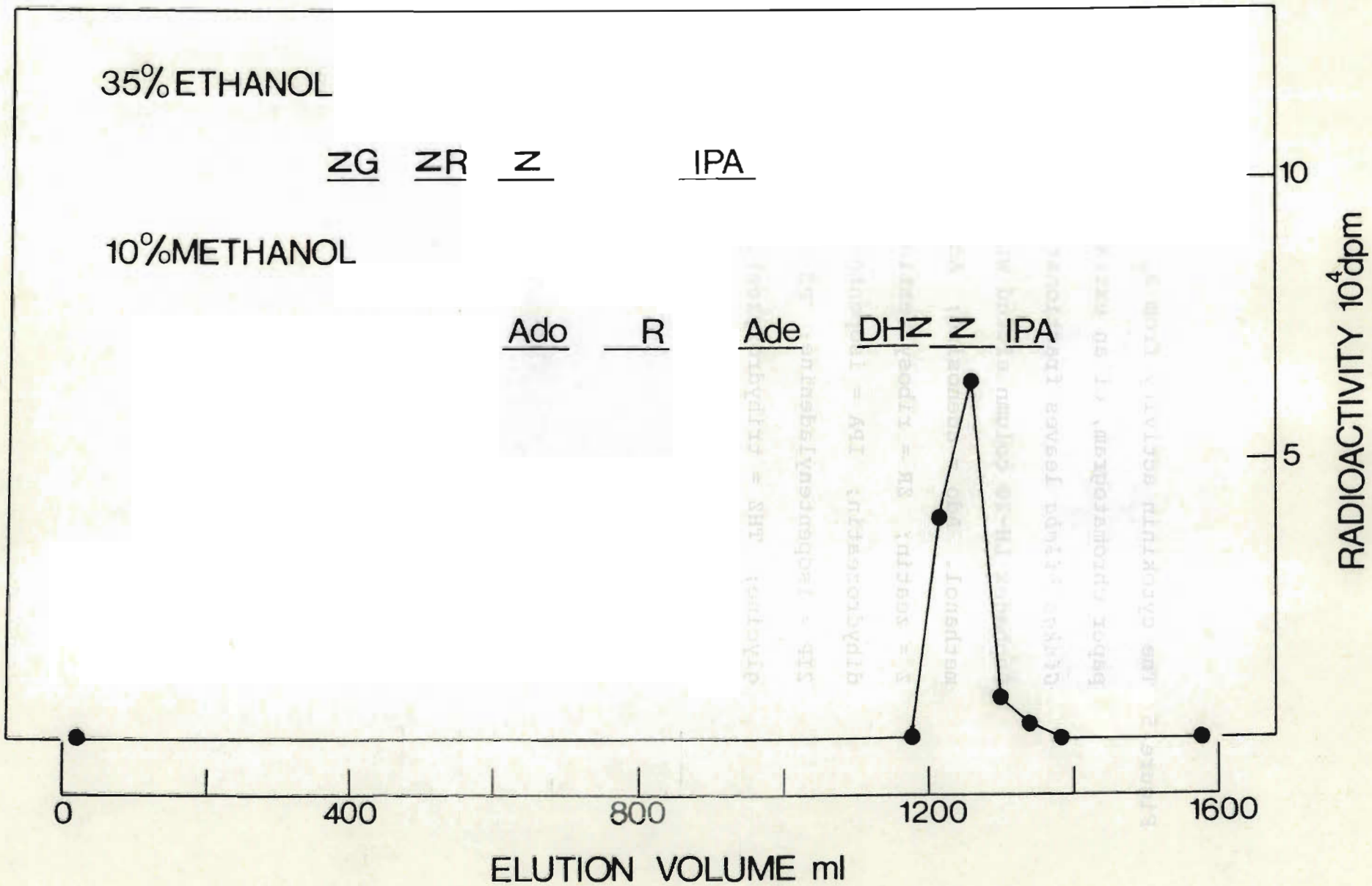
Figure 3 The separation of cytokinins achieved using a Sephadex G-10 column eluted with distilled water and 35 per cent methanol, as detected by ultra-violet absorbance (254 nanometres). Ado = adenosine; Ade = adenine; Z = zeatin; ZR = ribosylzeatin; IPA = isopentenyladenosine.


35% METHANOL
Ado ZR
Ade Z
IPA
DISTILLED H_2O
Ado ZR
Ade Z
0
400
800
1200
1600
ELUTION VOLUME ml

tracts which often contain impurities. Very efficient separation was achieved eluting a Sephadex LH-20 column (90 x 2,5 centimetres) with 10 per cent methanol (HUTTON and VAN STADEN, 1981) at a flow rate of 15 millilitres an hour at 20°C. As can be seen from Figure 4, adenosine, ribosylzeatin, adenine, dihydrozeatin and zeatin can all be separated, with distinct troughs between them, using this system. The elution volume of zeatin was confirmed using 8(^{14}C)*t*-zeatin as a marker (Figure 4). The elution volumes of the different compounds, as detected by ultra-violet absorbance (254 nanometres), remained unchanged when they were all applied simultaneously to the column. The efficiency of separation was also maintained when separating a plant extract. The histogram in Figure 5 represents the cytokinin activity in R_f 0,5-1,0, detected by means of the soyabean callus bioassay, in a leaf extract of *Ginkgo biloba* which had been purified by means of Dowex 50 and paper chromatographs before separation on a Sephadex LH-20 column eluted with 10 per cent methanol.

Isopentenyladenosine could not be completely separated from zeatin using 10 per cent methanol. The presence of isopentenyladenosine can be detected by fractionation on a Sephadex LH-20 column eluted with 35 per cent ethanol as it is separated from zeatin using this solvent system (Figure 4). Ten per cent methanol also does not separate adenosine, zeatin glucoside and trihydroxyzeatin which co-elute using this solvent system (Figure 5). Sephadex LH-20 columns eluted with 10 per cent methanol were, however, used for the separation of cytokinin extracts, as this system achieved

Figure 4 The separation of cytokinins on a Sephadex LH-20 column, eluted with 10 per cent methanol compared with using 35 per cent ethanol as the solvent, as detected by ultraviolet absorbance (254 nanometres). The elution volume of zeatin was confirmed using 8(^{14}C)*t*-zeatin as a marker. Ado = adenosine; Ade = adenine; Z = zeatin; ZR = ribosylzeatin; IPA = isopentenyladenosine; DHZ = dihydrozeatin.


35% ETHANOL
ZG
ZR
Z
IPA
10% METHANOL
Ado
R
Ade
DHZ
Z
IPA
10
5
RADIOACTIVITY 10^4dpm
0
400
800
1200
1600
ELUTION VOLUME ml

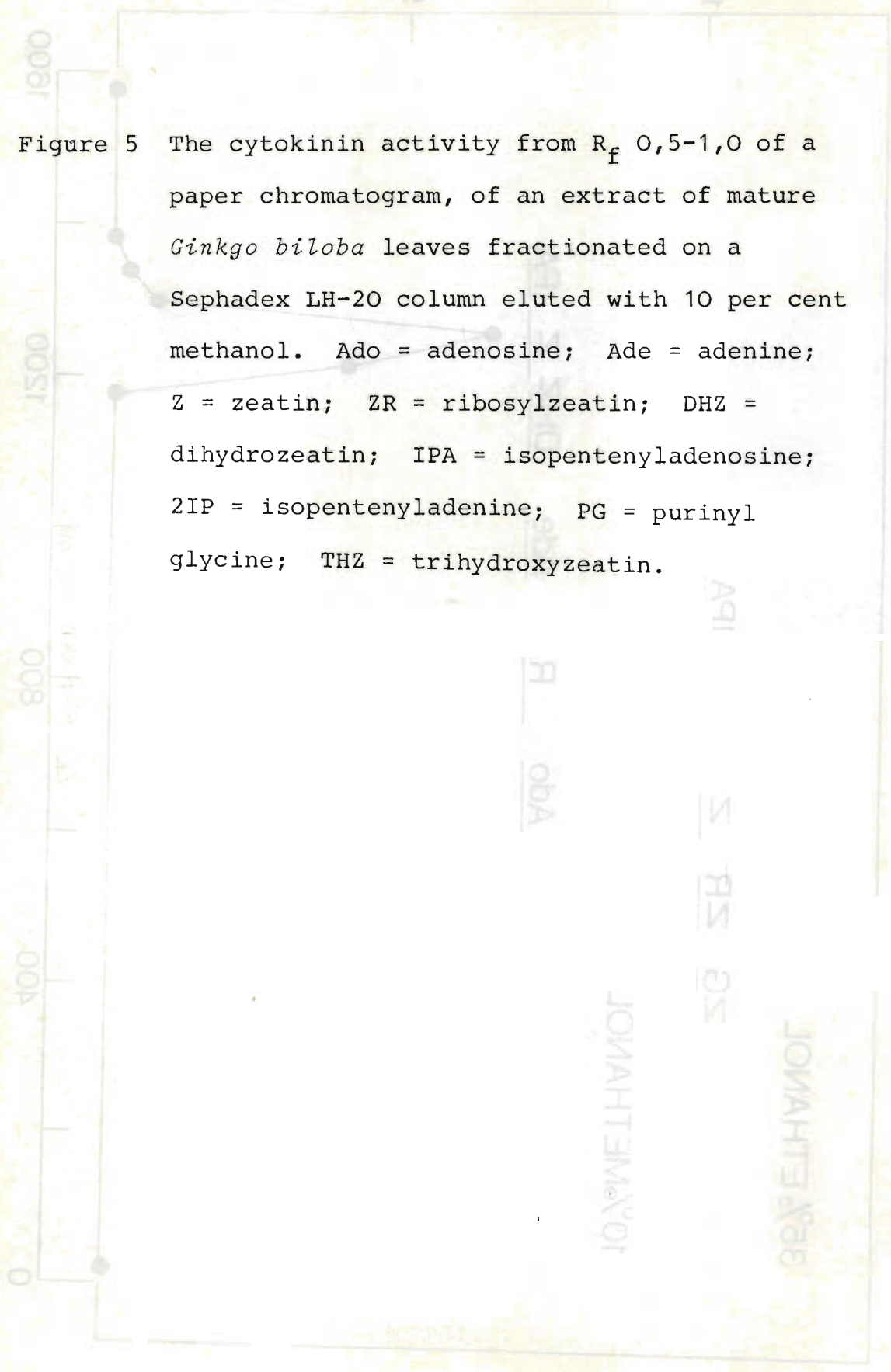
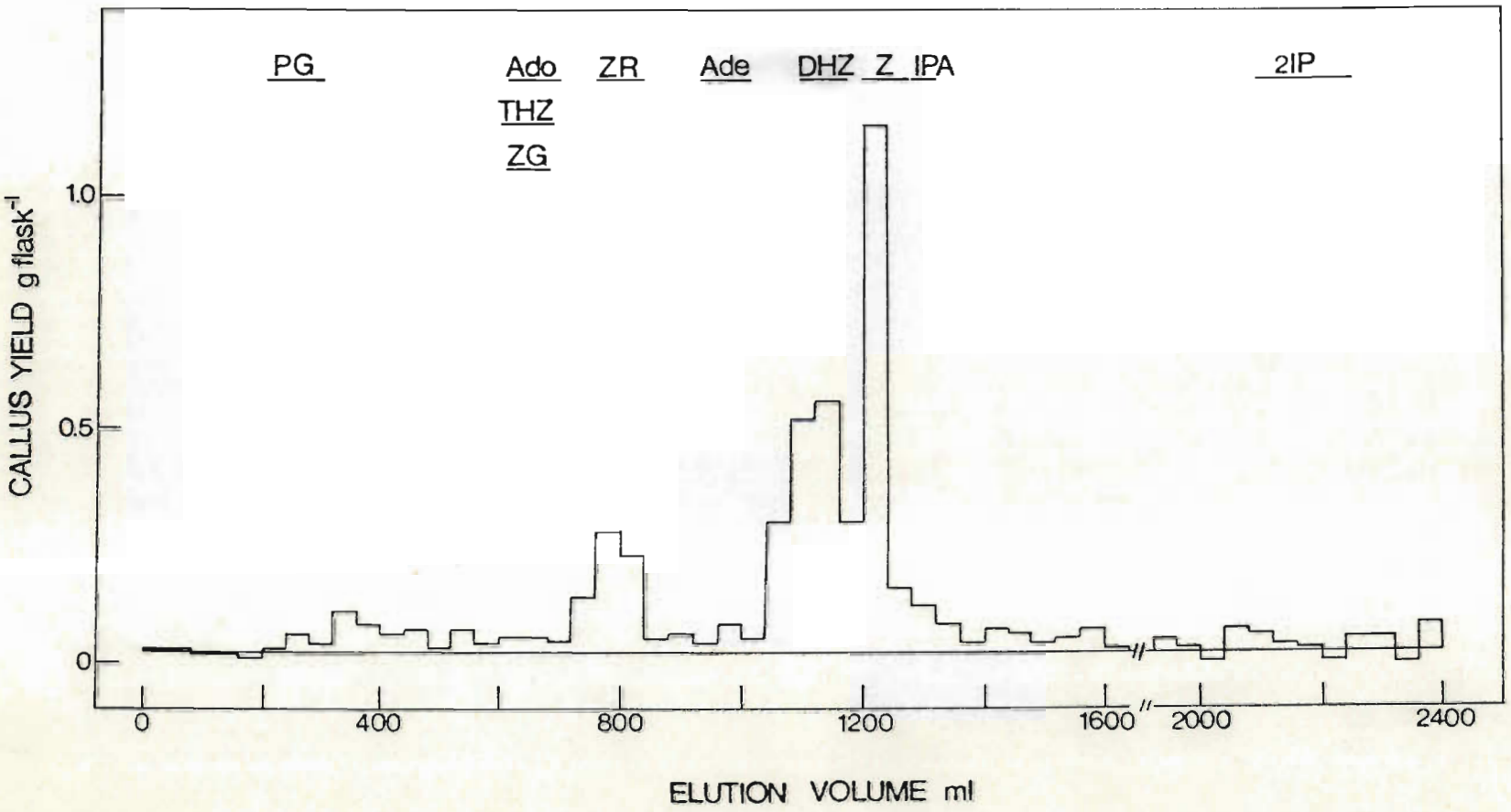


Figure 5 The cytokinin activity from R_f 0,5-1,0 of a paper chromatogram, of an extract of mature *Ginkgo biloba* leaves fractionated on a Sephadex LH-20 column eluted with 10 per cent methanol. Ado = adenosine; Ade = adenine; Z = zeatin; ZR = ribosylzeatin; DHZ = dihydrozeatin; IPA = isopentenyladenosine; 2IP = isopentenyladenine; PG = purinyl glycine; THZ = trihydroxyzeatin.


PG
Ado
THZ
ZG
ZR
Ade
DHZ
Z
IPA
2IP
CALLUS YIELD g flask⁻¹
1.0
0.5
0
0
400
800
1200
1600
2000
2400
ELUTION VOLUME ml

the most efficient separation.

Bioassays or radioassays of paper chromatograms indicated the presence of active cell division components or radioactive peaks in different zones of the chromatograms. These regions of the chromatograms were eluted with distilled water and a graded ethanol concentration series, that is 30, 50, 80 and 100 per cent ethanol, filtered and concentrated to dryness under vacuum. The residue was taken up in one millilitre of 10 per cent methanol and put onto a Sephadex LH-20 column. If the amount of material to be analyzed was very small, the extracts were not initially purified by means of paper chromatography, but were applied directly to the column. That is, after concentrating the ammonia fraction, the extract was immediately redissolved in one millilitre of 10 per cent methanol and applied to a column. The paper chromatograms were usually eluted either as a whole fraction or divided into three fractions, that is R_f 0-0,2; R_f 0,2-0,5 and R_f 0,5-1,0.

After elution through the columns, the fractions were combined in 40 millilitre fractions in erlenmeyer flasks. In radioactive extracts an aliquot of each fraction was used for a radioassay and the remainder was dried on a hot plate (30°C) in a stream of air and either assayed for cell division or stored for further analysis. In all other extracts, the 40 millilitre fractions were dried and assayed for cytokinin activity using the soyabean callus bioassay (MILLER, 1963; 1965).

4.0 Chemical and Enzyme Treatments

4.1 β-glucosidase treatment

The polar cytokinins on paper chromatograms frequently contain cytokinin glucosides which can be hydrolyzed by β-glucosidase (YOSHIDA and ORITANI, 1972; PARKER, LETHAM, WILSON, JENKINS, MACLEOD and SUMMONS, 1975; VAN STADEN, 1976a). To test for the presence of cytokinin glucosides, the R_f zones 0,0 to 0,5 were eluted from paper chromatograms, using a graded ethanol concentration series, filtered and concentrated to dryness under vacuum. The extract was then taken up in five millilitres of 0,02 Molar *tris* buffer at pH 5,2 and incubated with 1,5 milligrammes of β-glucosidase (Sigma - from almonds) for 24 hours at 25°C. Biologically active or radioactive peaks from column chromatography, were resuspended in 80 per cent ethanol and then concentrated to dryness. The residue was taken up in buffer and incubated with β-glucosidase. The reaction was terminated by adding four millilitres of 80 per cent ethanol to the reaction mixture. The fraction was then filtered, concentrated to dryness and taken up in one millilitre of 10 per cent methanol and applied to a Sephadex LH-20 column or taken up in three millilitres of 80 per cent ethanol and strip-loaded onto Whatman No. 1 chromatography paper and separated using PAW. The treated and control fractions were then assayed for biological and/or radioactivity.

When using β-glucosidase to determine the presence of

cytokinin glucosides, it must be remembered that only the O-glucosides of zeatin and its derivatives are hydrolyzed by β-glucosidase. The 7- and 9-glucosides appear to be resistant to this enzyme (HOAD, LOVEYS and SKENE, 1977; PALMER, SCOTT and HORGAN, 1981). Compounds remaining after β-glucosidase treatment may, therefore, represent the 7- and 9-cytokinin glucosides as well as other possible compounds.

4.2 Potassium permanganate oxidation

Potassium permanganate oxidation was undertaken in order to determine the presence of cytokinins with an unsaturated side chain. Fractions of paper or column chromatograms were eluted, concentrated to dryness and taken up in a few millilitres of distilled water. A few drops of 0,01 per cent aqueous solution of potassium permanganate was added to the extract until the permanganate colour persisted for more than a few seconds (MILLER, 1965). Excess 80 per cent ethanol was then added to complete the decomposition of the permanganate. After concentrating to dryness, the residue was taken up in one millilitre of 10 per cent methanol and applied to a Sephadex LH-20 column or the residue was redissolved in three millilitres of 80 per cent ethanol and applied to paper chromatograms. Treated fractions and untreated controls were assayed for biological and radioactivity.

If a fraction contains cytokinins with an unsaturated side

chain it will be oxidized and inactivated immediately by the neutral solution of potassium permanganate resulting in the complete loss of activity in the bioassay system, and a loss or shift of the radioactive peak in the radioassay. The double bond in the allyl group (isopentenyl side chain) of zeatin is broken and oxidized by such treatments and the inactive N-(purin-6-yl) glycine is formed (MILLER, 1965). Any activity which persists following potassium permanganate oxidation, is due to other compounds which co-chromatograph with zeatin or unsaturated zeatin derivatives.

4.3 Alkaline phosphate treatment

Alkaline phosphatase treatment was carried out according to the procedure outlined by MILLER (1965). Extracts were concentrated to dryness and taken up in two millilitres of buffer (0,01 Molar magnesium chloride; 0,1 Molar *tris*-hydroxymethylaminoethane at pH 8,2) to which two milligrammes of alkaline phosphatase (Sigma - from calf intestine mucosa) was added. The extract was incubated at 32°C for 24 hours. The reaction was terminated by the addition of 80 per cent ethanol. Treated fractions and untreated controls were assayed for biological and radioactivity.

Alkaline phosphatase treatment causes the hydrolysis of certain fractions active as cytokinins to yield compounds which co-chromatograph with zeatin and ribosylzeatin (VAN STADEN and DAVEY, 1977; VAN STADEN and MENARY, 1976). This is taken to indicate the presence of a nucleotide of

zeatin (VAN STADEN and MENARY, 1976) or phosphorylated zeatin (VAN STADEN and DAVEY, 1977).

5.0 Soyabean Callus Bioassay

The soyabean cotyledonary callus bioassay was used to determine cytokinin activity of fractions from paper and column chromatography. Tissue culture bioassays are regarded as being the most sensitive of the cytokinin assays. The soyabean (*Glycine max* L. cv. Acme) callus bioassay exhibits a linear relationship between response and concentration over a wide range of cytokinin concentrations, and it is probably the best tissue culture assay to use (VAN STADEN and DAVEY, 1979). Advantages of this bioassay are that microbial growth is eliminated and also that no natural cytokinins have been detected in soyabean callus maintained on kinetin (VAN STADEN and DAVEY, 1977). The main disadvantage of this system is that it is time consuming.

Callus was obtained from the cotyledons of soyabeans according to the procedure described by MILLER (1963; 1965) and was maintained by three-weekly sub-culture. Four stock solutions were prepared and the nutrient medium was made up as outlined in Table 1. Either 15 or 20 millilitres of medium was added to 25 or 50 millilitre erlenmeyer flasks which contained 0,15 and 0,20 grammes (one per cent) of agar respectively. The flasks were stoppered with non-absorbent cotton wool bungs which were then covered with aluminium foil. The flasks were then autoclaved at a pressure of

Table 1 Basal medium for soyabean callus bioassay (adapted from MILLER, 1963; 1965).

Stock Solution	Chemical	$g\ell^{-1}$ Stock Solution	mℓ Stock Solution per Litre Medium
Stock 1	KH_2PO_4	3,0	100
	KNO_3	10,0	
	NH_4NO_3	10,0	
	$Ca(NO_3)_2.4H_2O$	5,0	
	$MgSO_4.7H_2O$	0,175	
	KCl	0,65	
	$MnSO_4.4H_2O$	0,14	
Stock 2	NaFeEDTA	1,32	10
	$ZnSO_4.7H_2O$	0,38	
	H_3BO_3	0,16	
	KI	0,08	
	$Cu(NO_3)_2.3H_2O$	0,035	
	$(NH_4)_6MO_7O_{24}.4H_2O$	0,01	
Stock 3	Myo-inositol	10,0	10
	Nicotinic acid	0,2	
	Pyridoxine HCl	0,08	
	Thiamine	0,08	
Stock 4	NAA	0,02	10
Additional	Sucrose		30 $g\ell^{-1}$ medium
	Agar		10 $g\ell^{-1}$ medium
pH adjusted to 5,8 with NaOH			

1,05 bars for 20 minutes before being transferred to a sterile transfer chamber. Once the agar had set, three pieces of soyabean stock callus, each weighing approximately 20 milligrammes, were placed on the medium in each flask. The flasks were then incubated in a growth room where a constant temperature (26°C $\pm$ 2°C) and continuous low light intensity (cool white fluorescent tubes) were maintained. After 21 days, the three pieces of callus were weighed simultaneously. The amount of callus growth was plotted on a histogram relative to the control value. Kinetin standards were included with each bioassay. To estimate gross levels of cytokinin activity, results were expressed as kinetin equivalents.

6.0 Radioassay Techniques

Whenever the radioactivity, which was associated with the different R_f zones from paper chromatograms was determined, the paper was incorporated directly into the glass scintillation vials. Only a proportion of each paper chromatogram was counted and the total radioactivity was calculated from this. One millilitre of absolute methanol followed by 10 millilitres of scintillation cocktail (4,0 grammes PPO and 0,2 grammes POPOP ℓ^{-1} toluene) was added to each vial. When column chromatography was employed, aliquots of each 40 millilitre fraction were incorporated into the counting vials and then air dried at room temperature. The residues were redissolved in one millilitre of absolute methanol and 10 millilitres of scintillation cocktail was

added to each vial. After standing for 12 hours, the samples were counted in a liquid scintillation spectrometer (Packard Tri-Carb Model 3380). Correction for quenching was carried out using the channels ratio method and results were expressed as disintegration per minute (dpm). The remainder of the paper chromatograms and column fractions were assayed for cytokinin activity which could then be correlated with radioactivity.

7.0 Electron Microscope Techniques

Sections of immature, mature and senescing *Ginkgo biloba* leaves were prepared for electron microscopy. Leaf material was fixed at 4°C in 6 per cent glutaraldehyde, buffered at pH 7,2 with 0,05 molar sodium-cacodylate, for 24 hours. The fixed material was then washed three times for periods of 30 minutes each, in 0,05 Molar sodium-cacodylate buffer, before being dehydrated with a graded alcohol series. Prior to embedding in epon-araldite resin, the material underwent two washes of 15 minutes each in propylene oxide. The material was then infiltrated with epon-araldite resin, poured into moulds and polymerized for 48 hours at 70°C. The material was then sectioned with a diamond knife on a LKB microtome. The sections were stained with uranyl acetate and lead citrate (REYNOLDS, 1963). The sections were examined using an Hitachi HU 11E electron microscope at an accelerating voltage of 50 kV and photographed.

CHAPTER ONE

THE TRANSPORT AND METABOLISM OF 8(^{14}C) *t*-ZEATIN APPLIED TO *GINKGO BILOBA* LEAVES

1.1 Introduction

Root-produced cytokinins transported to leaves are metabolized/utilized differently at various stages of leaf development. The cytokinin activity of immature expanding leaves is reported to be low (VAN STADEN, 1976b; HENSON, 1978b), probably due to the rapid utilization of cytokinins in these leaves (HENDRY, VAN STADEN and ALLAN, 1982). High cytokinin activity has been recorded in mature and senescing leaves (HEWETT and WAREING, 1973b; VAN STADEN, 1976a,b; HENSON, 1978a,b). Cytokinin glucoside levels, which are extremely low or undetectable in immature leaves, are responsible for most of the activity detected once leaves begin to mature and senesce (VAN STADEN, 1976b; HENSON, 1978b).

One of the suggested functions of these accumulated cytokinin glucosides is that of storage compounds (PARKER and LETHAM, 1973; WAREING, HORGAN, HENSON and DAVIS, 1976; HENSON and WHEELER, 1977a; DAVEY and VAN STADEN, 1981; VONK and DAVELAAR, 1981). Storage implies re-utilization and in deciduous species cytokinin glucosides, such as zeatin glucoside or ribosylzeatin glucoside,should be re-utilized if they are to fulfil their proposed role as storage compounds. That is, glucosides or their hydro-

lyzed products, namely zeatin or ribosylzeatin, must be exported to meristematic or storage regions before the leaves abscise (VAN STADEN and BROWN, 1978). There is as yet, no conclusive evidence that cytokinin glucosides are re-utilized and that they do fulfil a storage function in leaves.

In these experiments 8(^{14}C)*t*-zeatin was applied to immature, mature and senescing leaves of *Ginkgo biloba* L. explants in an attempt to assess the metabolism of zeatin arriving in these leaves, as well as the possible transport of zeatin and/or its metabolites, out of the leaves at different stages of development. The formation of O-glucosides from exogenous zeatin has been reported previously in *Alnus glutinosa* (HENSON and WHEELER, 1977a; HENSON, 1978a), *Populus alba* (LETHAM, PARKER, DUKE, SUMMONS and MACLEOD, 1976) and in *Lupinus* species (PARKER, LETHAM, GOLLNOW, SUMMONS, DUKE and MACLEOD, 1978; VAN STADEN and DAVEY, 1981a) leaves. As no radioactive labelled cytokinin glucosides were available, it was hoped that some of the 8(^{14}C)*t*-zeatin applied to the leaves would be glucosylated and the fate of these glucosides could then be determined.

1.2 Experimental Procedure

1.2.1 Endogenous cytokinin levels and 8(^{14}C)*t*-zeatin experiments

Ginkgo biloba explants consisting of three leaves, a shoot

of approximately 5,5 centimetres and a short side shoot, were used in all experiments (Figure 1.1). Explants were used which had immature green leaves (that is, before the leaves were fully expanded - spring), mature green leaves (that is, fully expanded leaves - middle of summer) and yellow senescing leaves (that is, just prior to abscission - autumn). The months in which these explants were harvested are shown in Diagram 1.1. Explants with mature and senescing leaves are shown in Plate 1.1.

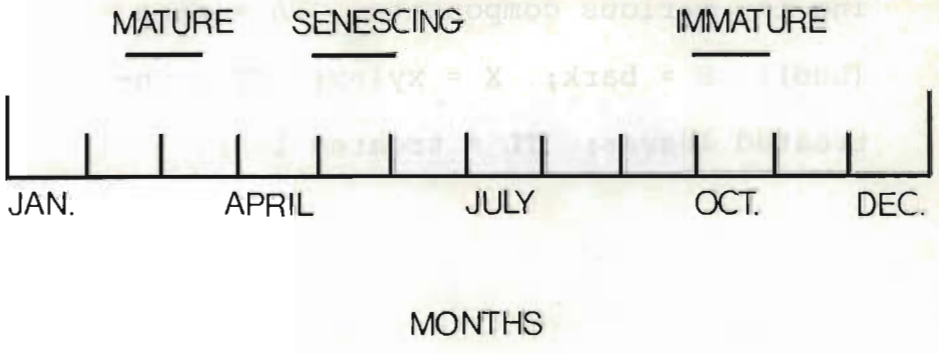
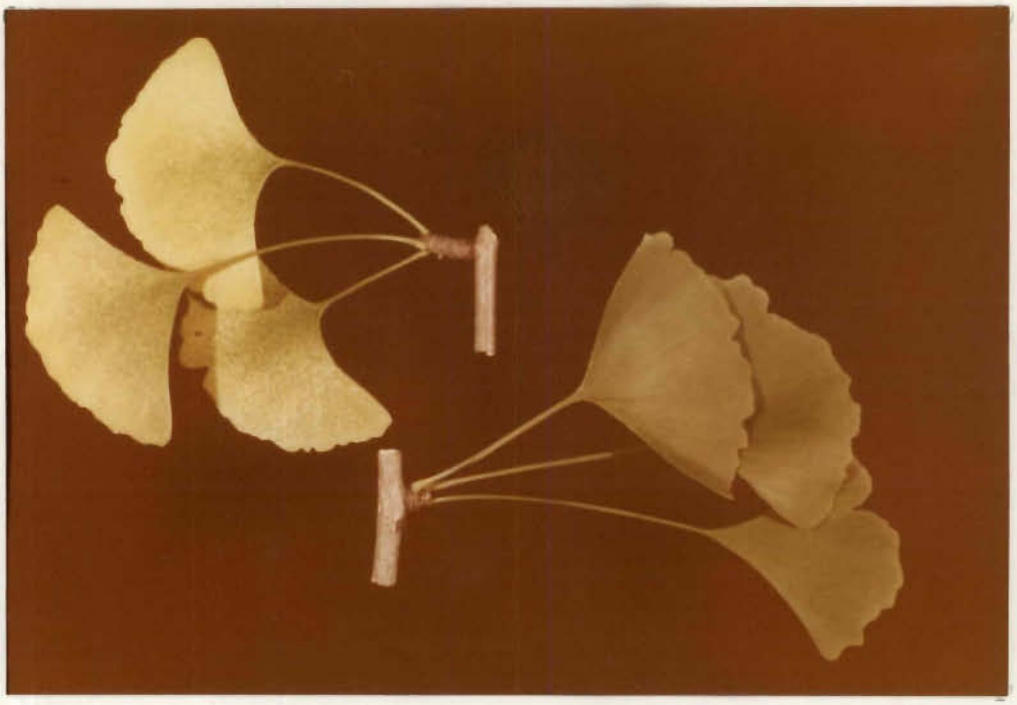


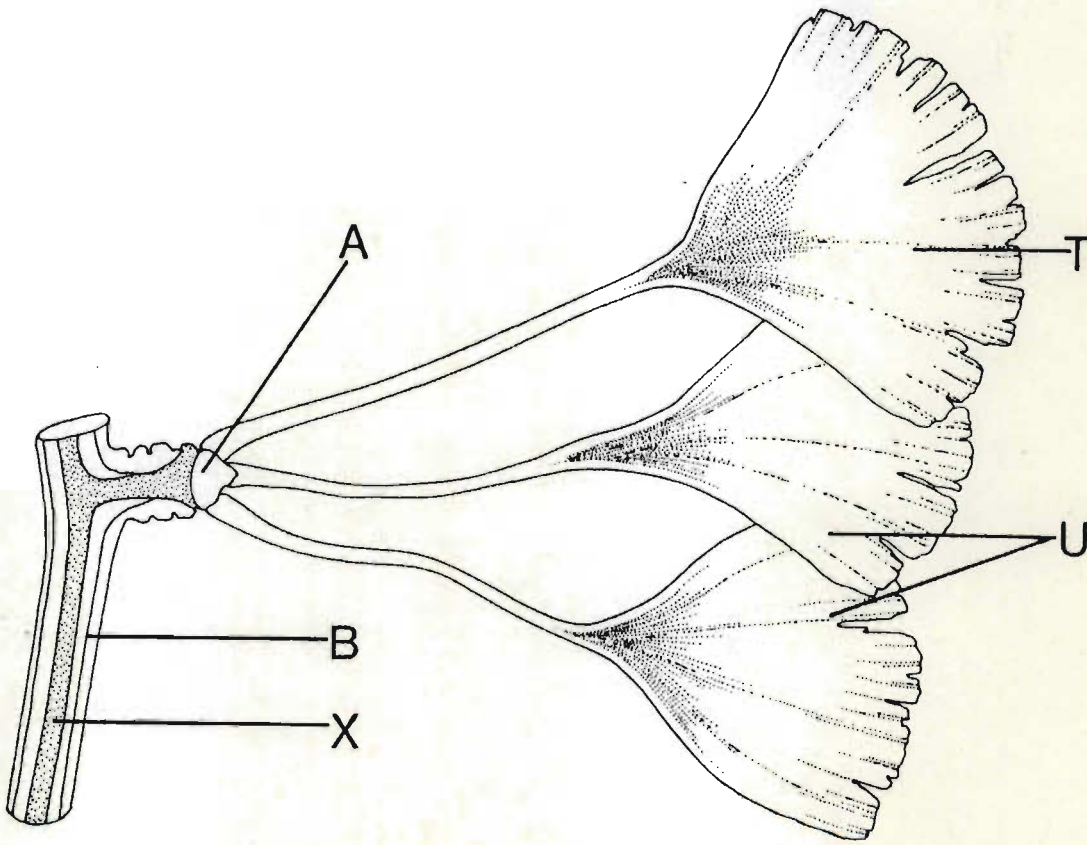
Diagram 1.1 The months at which the explants were harvested. The stage of development of the leaves at these times is indicated.

Explants were placed in 50 millilitre beakers containing 10 millilitres of distilled water. Thirty explants were used for each experiment and five microlitres of 8(^{14}C) *t*-zeatin (approximately 8 x 10^5 dpm) were applied, via a microsyringe, to one of the three leaves of each explant. The treated leaf was identified by marking it with a felt pen. Following the application of the labelled zeatin, the explants were placed in a growth room which was maintained at a constant temperature (25°C) and at an 18 hour photoperiod. After 48 hours, and in the case

Plate 1.1 *Ginkgo biloba* explants with mature and senescing leaves.

Figure 1.1 Diagram of a *Ginkgo biloba* explant showing the various components. A = apex (bud); B = bark; X = xylem; UT = untreated leaves; TL = treated leaf.




A
T
U
B
X

of explants with senescing leaves 48 and 96 hours, the experiments were terminated and the explant components harvested. These components consisted of treated leaves, untreated leaves, bark, xylem and the apices (Figure 1.1). These components were weighed and frozen at -20°C until analyzed for biological and radioactivity. The water in which the explants were standing was also frozen and later assayed for cytokinin activity. The endogenous cytokinin content of the components of these explants was also determined by means of paper chromatography. The cytokinins in the leaves were further characterized by column chromatography.

The techniques for the cytokinin extraction, soyabean callus bioassay, radioassay, paper and column chromatography are all described in detail in the Materials and Methods section. The explant components were extracted for cytokinin activity and purified by means of paper chromatography. Portions of the paper chromatograms were used for bioassays and radioassays with the remainder of the chromatograms being used for column chromatography. Aliquots of fractions from column chromatography were used for radioassays with the rest of the fraction being used to assess biological activity or for further identification of the separated compounds by means of chemical and enzyme treatments. These treatments are also described in the Materials and Methods section.

1.2.2 Re-application of radioactive peak 1 and radioactive peak 2

Radioactive peaks 1 and 2, which ran at R_f 0,0-0,2 and R_f 0,2-0,5 respectively on paper chromatograms, were the peaks formed from the radioactive zeatin applied to the *Ginkgo biloba* leaves. These peaks were re-applied to explants with mature leaves in order to determine whether the radioactive compounds associated with these peaks would be exported out of these leaves to a greater extent than the radioactive zeatin, and also to determine whether they would undergo any further metabolism. Radioactive peak 2 appeared to contain glucosylated compounds, and by applying this peak to leaves it was also hoped to investigate the fate of cytokinin glucosides. Radioactive peak 1 and 2 were, therefore, eluted from paper chromatograms using a graded ethanol concentration series. The eluant was filtered, concentrated to dryness and taken up in 0,5 millilitres of 20 per cent ethanol. These radioactive peaks were then applied to mature leaves of *Ginkgo biloba* explants. The explant components were harvested after 48 hours and deep frozen at -20°C until analyzed for biological and radioactivity. The same methods were used as for analyzing the explant components following the application of 8(^{14}C)*t*-zeatin.

1.3 Results

Before commencing with the results and discussion, it is necessary to point out that none of the cytokinins, endo-

genous and metabolites of the 8(^{14}C)*t*-zeatin, were actually identified by chemical or physical techniques. VAN STADEN and DAVEY (1979) pointed out that unless cytokinins are actually identified by chemical techniques they should not be referred to as cytokinins, but should be qualified by reference to the bioassay system employed. In this study, cytokinins were only tentatively identified by paper and column chromatography as well as by various chemical and enzyme treatments. For convenience, however, cytokinins are often referred to specifically, even though they were not definitely characterized.

1.3.1 Ultra-structure of *Ginkgo biloba* leaves

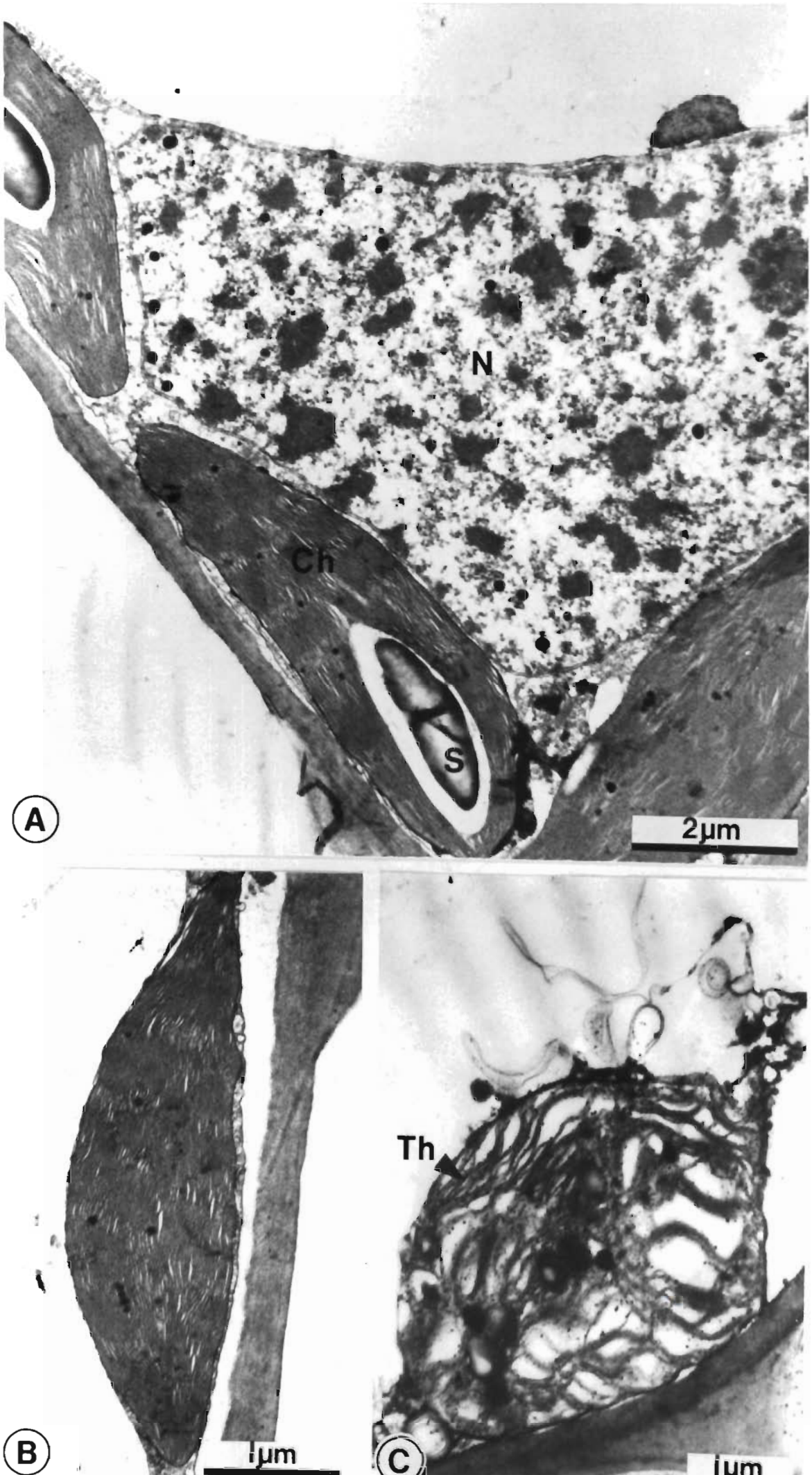
The ultra-structure of immature, mature and senescing *Ginkgo biloba* leaves was examined using the transmission electron microscope. This was carried out in order to determine whether any possible changes in cytokinin metabolism could be related to ultra-structural changes in the leaves. From Plate 1.2 it can be seen that the cells of immature green leaves were organized and contained large nuclei and chloroplasts in which starch was beginning to accumulate. The cells of mature leaves were also well organized with large, well defined chloroplasts. In the senescing leaves the cells were disorganized. The chloroplasts of these cells were beginning to degenerate, and lipid globules had begun to appear in the chloroplasts. The results of the metabolism studies showed that metabolic rates were fastest in young leaves and decreased as the leaves senesced. These metabolic rates could be anticipated

Plate 1.2 Chloroplasts of *Ginkgo biloba* leaves at different stages of leaf development. Anatomical effects of senescence are reported to be seen most clearly in chloroplasts (THIMANN, 1977).

A Well defined chloroplasts, in which starch was beginning to accumulate, of expanding immature leaves. Large active nucleus was also present.

B Well defined chloroplast in mature leaves.

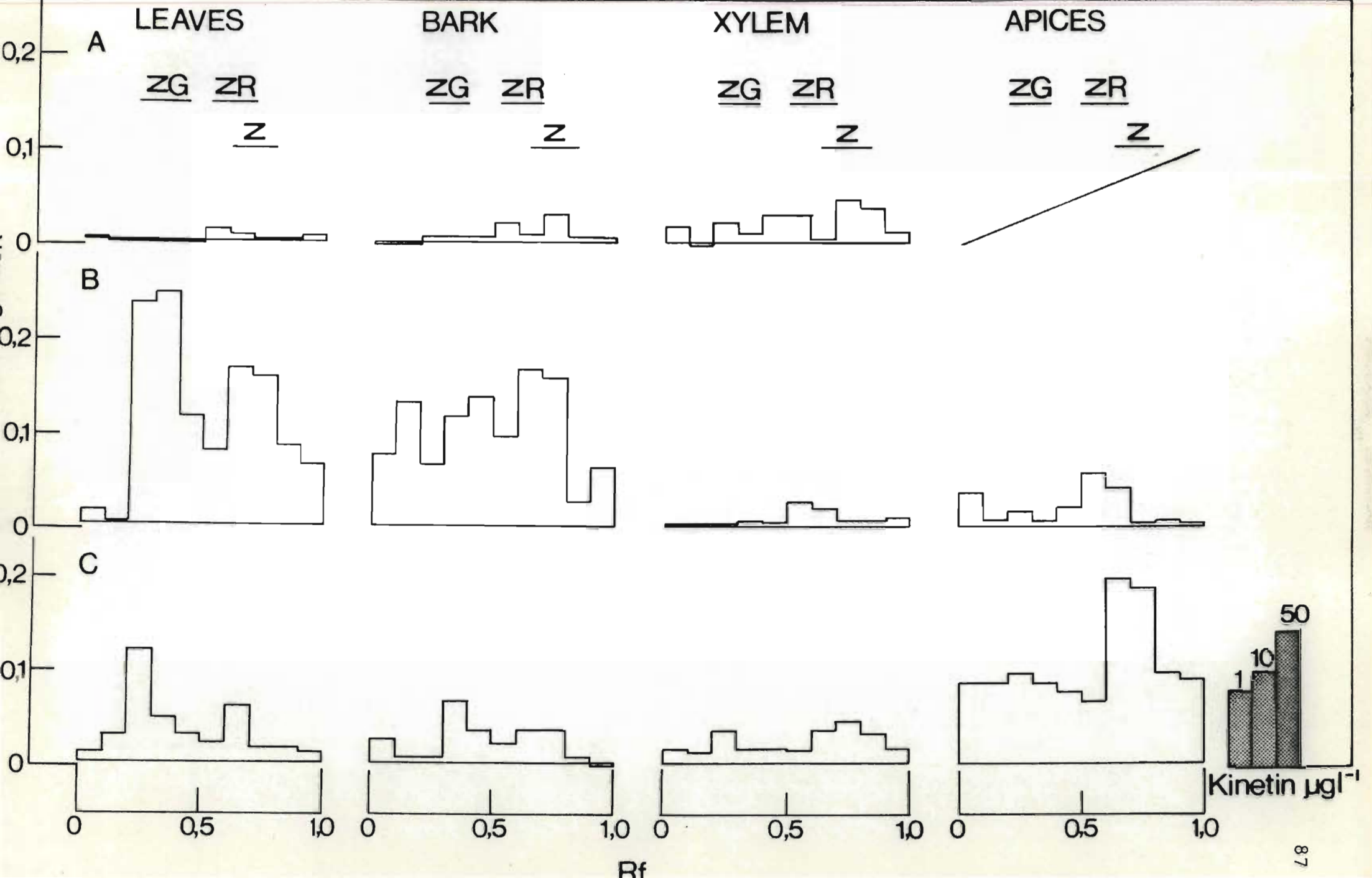
C Chloroplast in a senescing leaf. Thylakoids becoming distorted and breaking down. Ch = chloroplast; N = nucleus; S = starch; Th = thylakoids.


N
Ch
S
A
2µm
B
1µm
Th
C
1µm

if one takes the ultra-structural changes in these leaves into account. Although the proportions were different, the same three radioactive peaks were detected in these leaves, irrespective of their age and associated ultra-structural differences.

1.3.2 Endogenous cytokinins in *Ginkgo biloba* explants

The endogenous cytokinin content of the *Ginkgo biloba* explants was determined by means of paper chromatography (Figure 1.2). Explants harvested in spring, that is, with immature green leaves, contained relatively little cytokinin activity (Figure 1.2A). In these explants the highest cytokinin levels were recorded in the xylem. This was probably the result of root-produced cytokinins being transported to the leaves via the transpiration stream. The very low cytokinin activity detected in the leaves was probably due to the rapid utilization of cytokinins in these rapidly dividing leaves. No cytokinin glucosides were detected in the leaves, with only low levels of compounds co-chromatographing with authentic zeatin and ribosylzeatin being detected. Compounds co-chromatographing with zeatin glucoside were detected in the xylem tissue. This does not seem to be a common occurrence, but fractionation of a xylem extract on a Sephadex LH-20 column, eluted with 10 per cent methanol, showed that the glucoside levels were actually insignificant while the zeatin and ribosylzeatin levels were significant at the level $p = 0,01$. The cytokinin activity of explants with mature green leaves increased compared to those explants with immature leaves


LEAVES
BARK
XYLEM
APICES
A
B
C
ZG
ZR
Z
0,2
0,1
0
0,5
1,0
Rf
1
10
50
Kinetin µgl⁻¹

(Figure 1.2B). The accumulation of compounds co-chromatographing with cytokinin glucosides, as well as an increase in the zeatin and ribosylzeatin levels, were responsible for the high activity recorded in mature leaves. The bark of these explants also exhibited high cytokinin activity with three peaks being recorded. Two of these peaks co-chromatographed with authentic zeatin, ribosylzeatin and zeatin glucoside. The low cytokinin activity recorded in the apices was due to compounds co-chromatographing with zeatin, ribosylzeatin and zeatin glucoside. Explants harvested in autumn, which had yellow senescing leaves, showed a decrease in activity compared to explants with mature leaves (Figure 1.2C). Compounds co-chromatographing with cytokinin glucosides predominated in the leaves and bark of these explants. The highest cytokinin levels in these explants were recorded in the apices with compounds co-chromatographing with zeatin and ribosylzeatin forming the largest peak of activity. The high cytokinin activity detected in the apices appeared to correspond to the decrease in cytokinin activity in the senescing leaves which suggested that cytokinins may be exported from these leaves to the apices. It must also be remembered that root-produced cytokinins may have been responsible for the cytokinin activity recorded in the apices. That is, root-produced cytokinins may be responsible for supplying cytokinins to the buds and cytokinins from the leaves may, in fact, not make a significant contribution to the cytokinin content of the buds.

The cytokinin content of the leaves of the three explant systems was further investigated by means of column chromatography (Figure 1.3). The low, almost undetectable cytokinin activity co-chromatographing with zeatin and ribosylzeatin in immature green leaves detected on paper chromatograms (Figure 1.2A), was confirmed by column chromatography. Three major and two minor peaks of activity were detected in mature leaves. The results of chemical and enzyme treatments, which will be dealt with later, suggested that the major peaks co-eluted with ribosylzeatin glucoside, zeatin glucoside and ribosylzeatin. The other active peaks appeared to co-elute with zeatin and dihydrozeatin. The major peaks of activity detected in senescing leaves co-eluted with the suspected ribosylzeatin glucoside and zeatin glucoside. Smaller biologically active peaks in these leaves had similar elution volumes to authentic ribosylzeatin, zeatin and dihydrozeatin.

Chemical and enzyme treatments were used to more conclusively determine the nature of the cytokinins occurring in these leaves. Figure 1.4 shows that on paper chromatograms, β-glucosidase treatment of R_f 0,0 to 0,5 of a mature leaf extract resulted in the complete shift of the predominant polar peak, which co-eluted with zeatin glucoside. This confirmed the presence of O-glucoside cytokinins and also suggested that adenosine and trihydroxyzeatin, which co-elute with zeatin glucoside, were not present.

Potassium permanganate oxidation of R_f 0,5 to 1,0 of mature leaves resulted in a reduction but not the complete loss of activity associated with the non-polar peak. This

Figure 1.3 The cytokinin activity in 20 grammes of (A) immature; (B) mature and (C) senescing leaves following fractionation on a Sephadex LH-20 column eluted with 10 per cent methanol. The leaf extracts were initially purified by paper chromatography. PG = purinyl glycine; THZ = trihydroxyzeatin; Ado = adenosine; ZR = ribosylzeatin; ZG = zeatin glucoside; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.

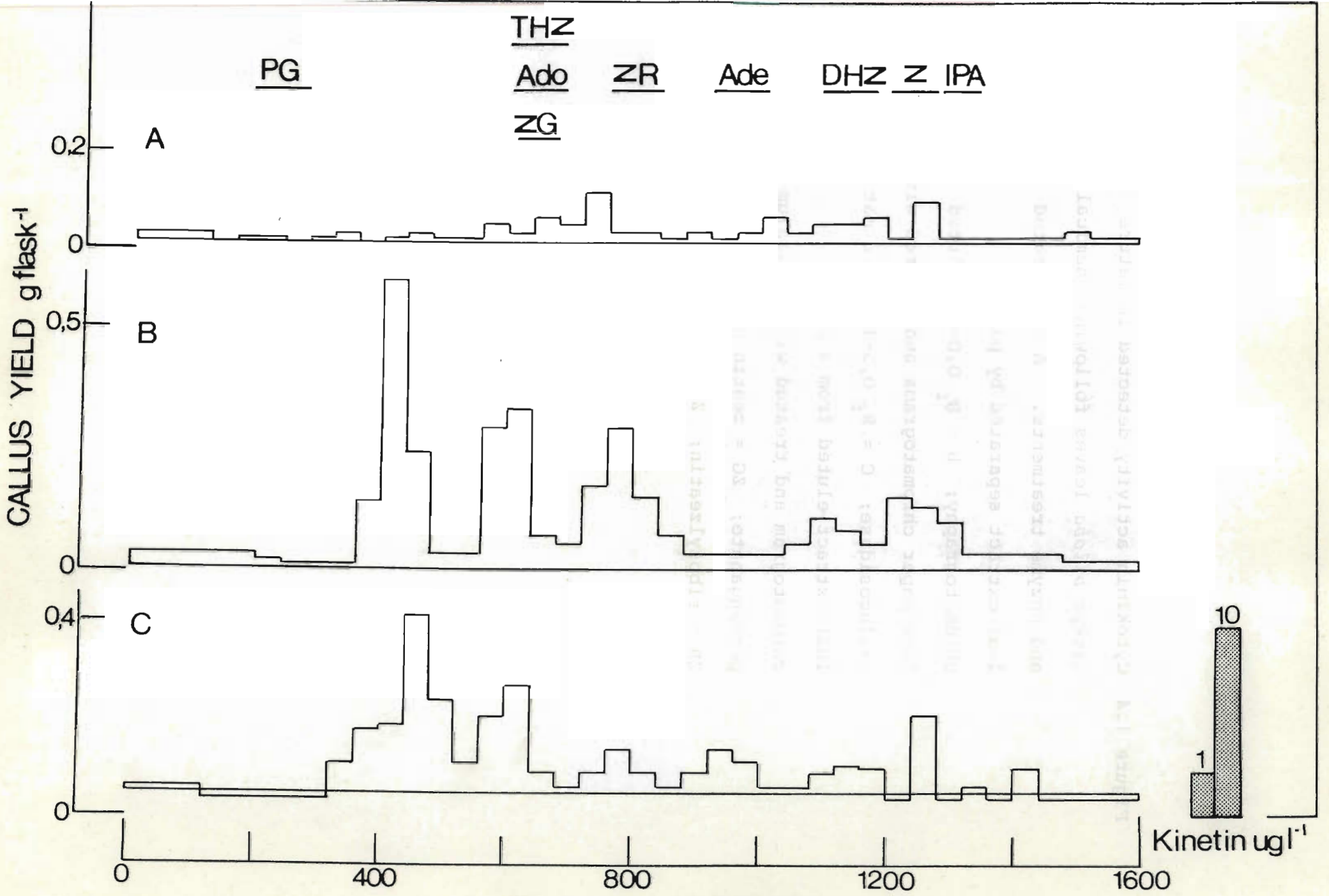
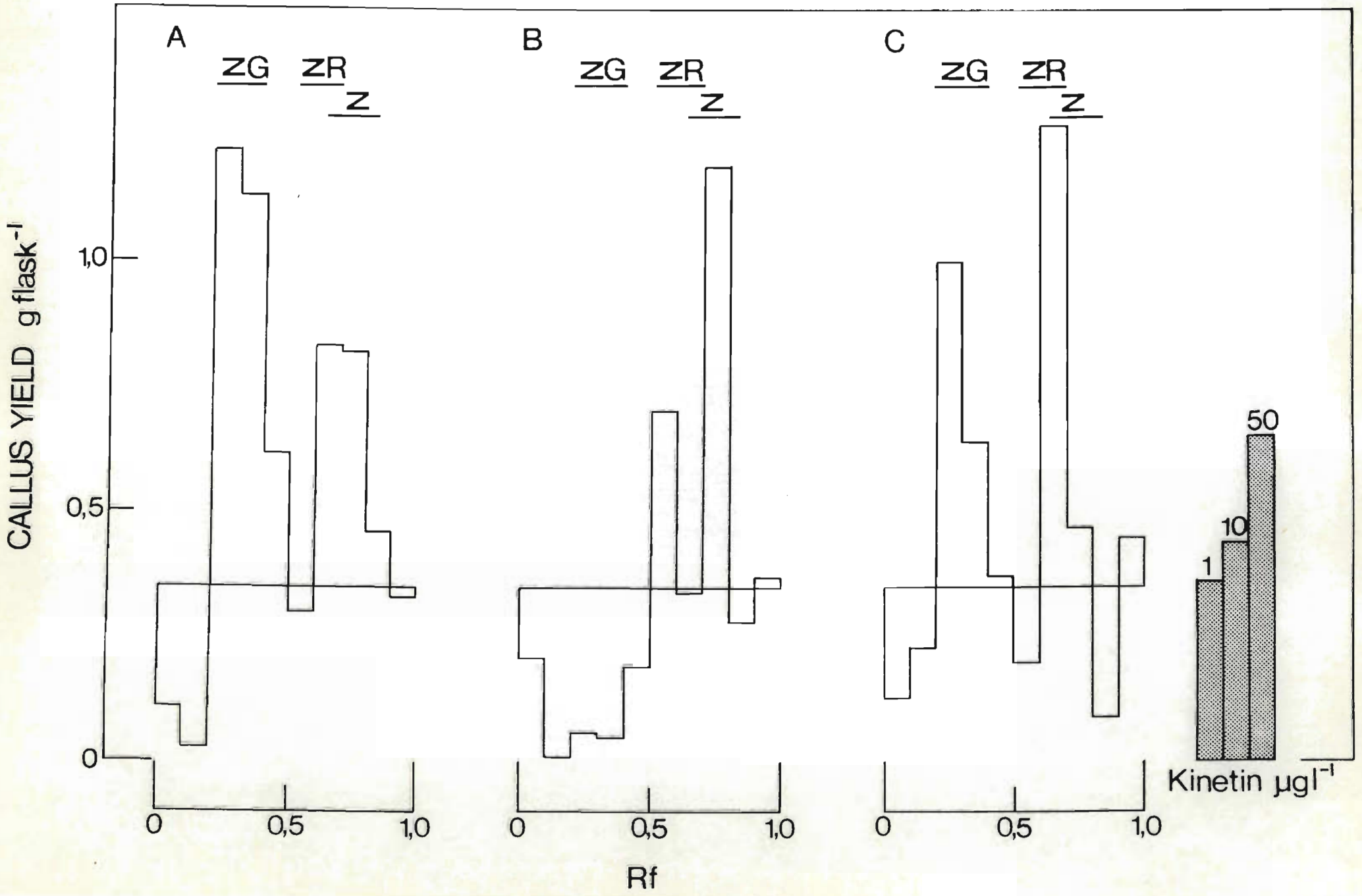

THZ
PG
Ado
ZR
Ade
DHZ
Z
IPA
ZG
A
B
C
0,2
0
0,5
0
0,4
0
CALLUS YIELD g flask-1
0
400
800
1200
1600
Kinetin ugl-1
1
10

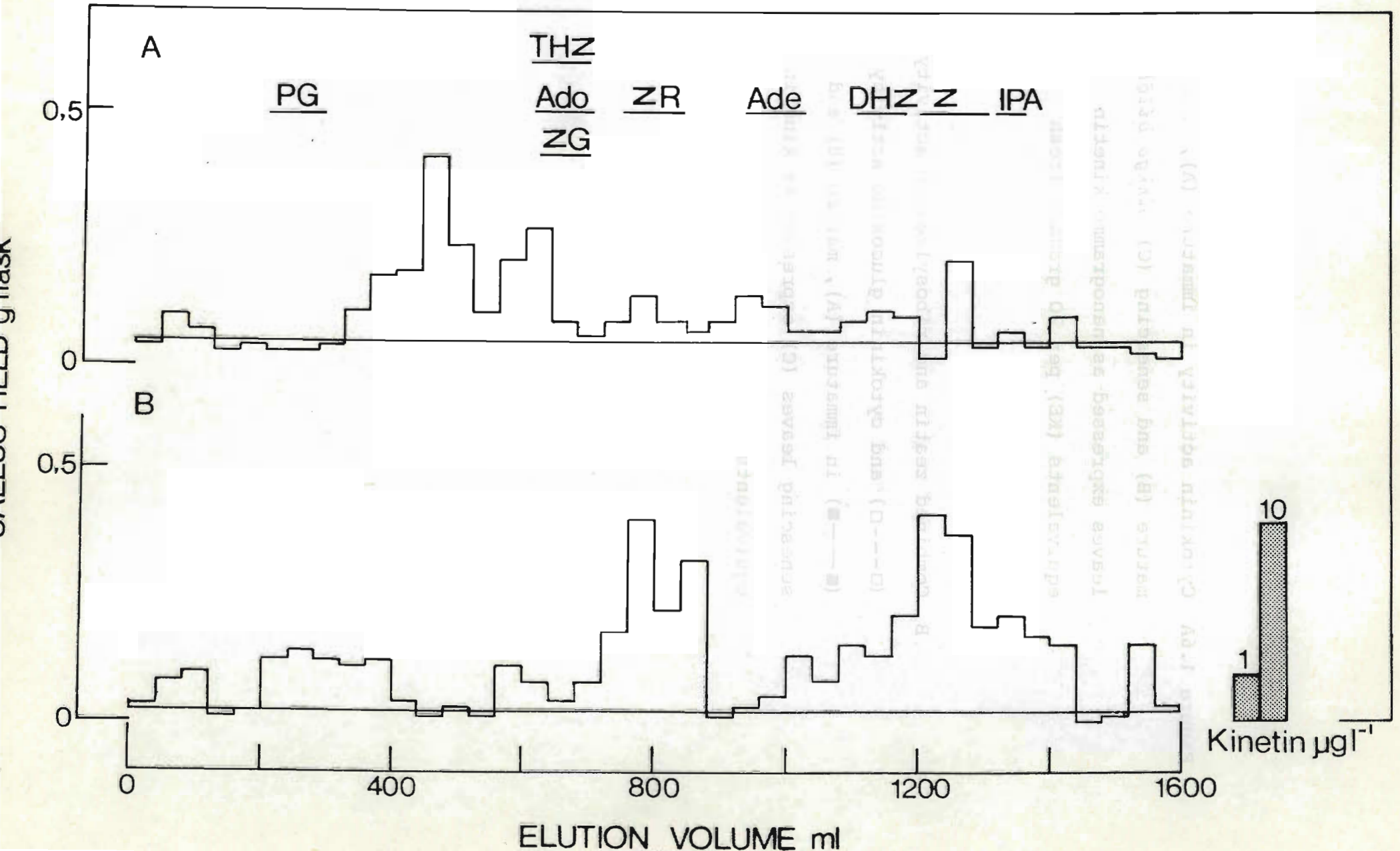
Figure 1.4 Cytokinin activity detected in mature *Ginkgo biloba* leaves following chemical and enzyme treatments. A = untreated leaf extract separated by paper chromatography; B = R_f 0,0-0,5 eluted from paper chromatograms and treated with β-glucosidase; C = R_f 0,5-1,0 of a mature leaf extract eluted from a paper chromatogram and treated with potassium permanganate; ZG = zeatin glucoside; ZR = ribosylzeatin; Z = zeatin.

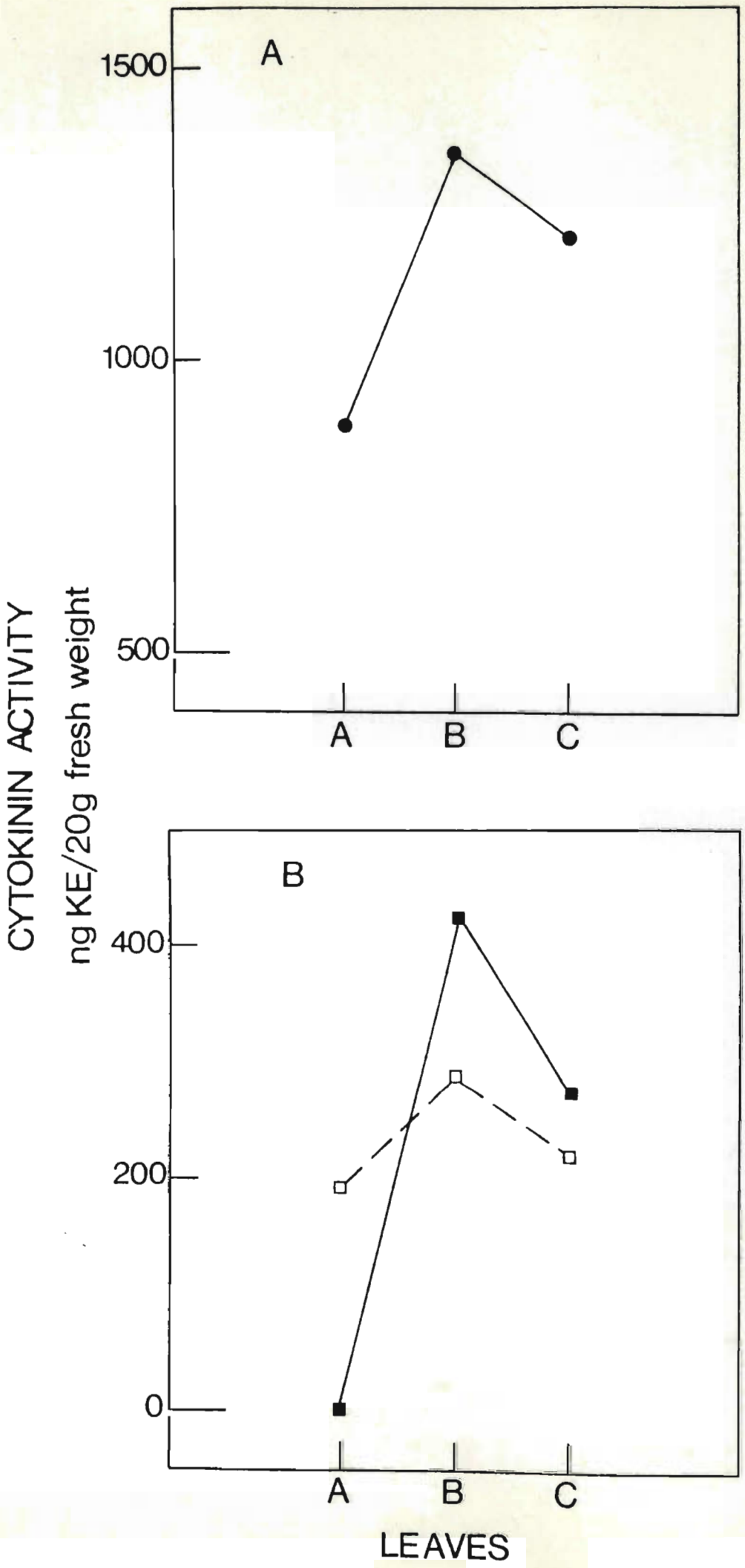

A
ZG
ZR
Z
B
ZG
ZR
Z
C
ZG
ZR
Z
CALLUS YIELD g flask⁻¹
1,0
0,5
0
0
0,5
1,0
Rf
50
10
1
Kinetin µgl⁻¹

suggested that compounds other than zeatin and ribosylzeatin were also components of this peak. Cytokinins such as dihydrozeatin and dihydroribosylzeatin, which are not oxidized by potassium permanganate, may also have been present. Column chromatography of R_f 0,0 to 0,5 of a senescing leaf extract treated with β-glucosidase resulted in the loss of the two major peaks of activity and an increase in activity of the peaks which co-eluted with ribosylzeatin, dihydrozeatin and zeatin (Figure 1.5). These results confirmed that the glucosylated compounds present in the leaves were the O-glucosides of zeatin, ribosylzeatin, dihydrozeatin and possibly dihydroribosylzeatin.

Expressing the cytokinin activity recorded in the leaves quantitatively, that is, as nanogramme kinetin equivalents per 20 grammes fresh weight (Figure 1.6A), confirmed that the cytokinin levels were low in immature leaves, reached a maximum in mature leaves and began to decrease once the leaves had begun to senesce. If the suspected cytokinin glucoside and combined zeatin and ribosylzeatin content of the leaves is also expressed as nanogramme kinetin equivalents per 20 grammes fresh weight (Figure 1.6B), then it can be seen that cytokinin glucosides were absent in immature leaves, reached a maximum in mature leaves and decreased in senescing leaves. Zeatin and ribosylzeatin levels also reached a maximum in mature leaves and decreased in senescing leaves but their levels were lower than the glucoside levels.

Figure 1.5 The cytokinin activity in untreated senescing leaves fractionated on a Sephadex LH-20 column eluted with 10 per cent methanol. The extract was initially purified by paper chromatography (A). R_f 0,0-0,5 of a senescing leaf extract treated with β-glucosidase and separated by column chromatography (B). PG = purinyl glycine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; Ado = adenosine; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.


A
THZ
PG
Ado
ZR
Ade
DHZ
Z
IPA
ZG
0,5
0
B
0,5
0
10
1
Kinetin µgl⁻¹
0
400
800
1200
1600
ELUTION VOLUME ml

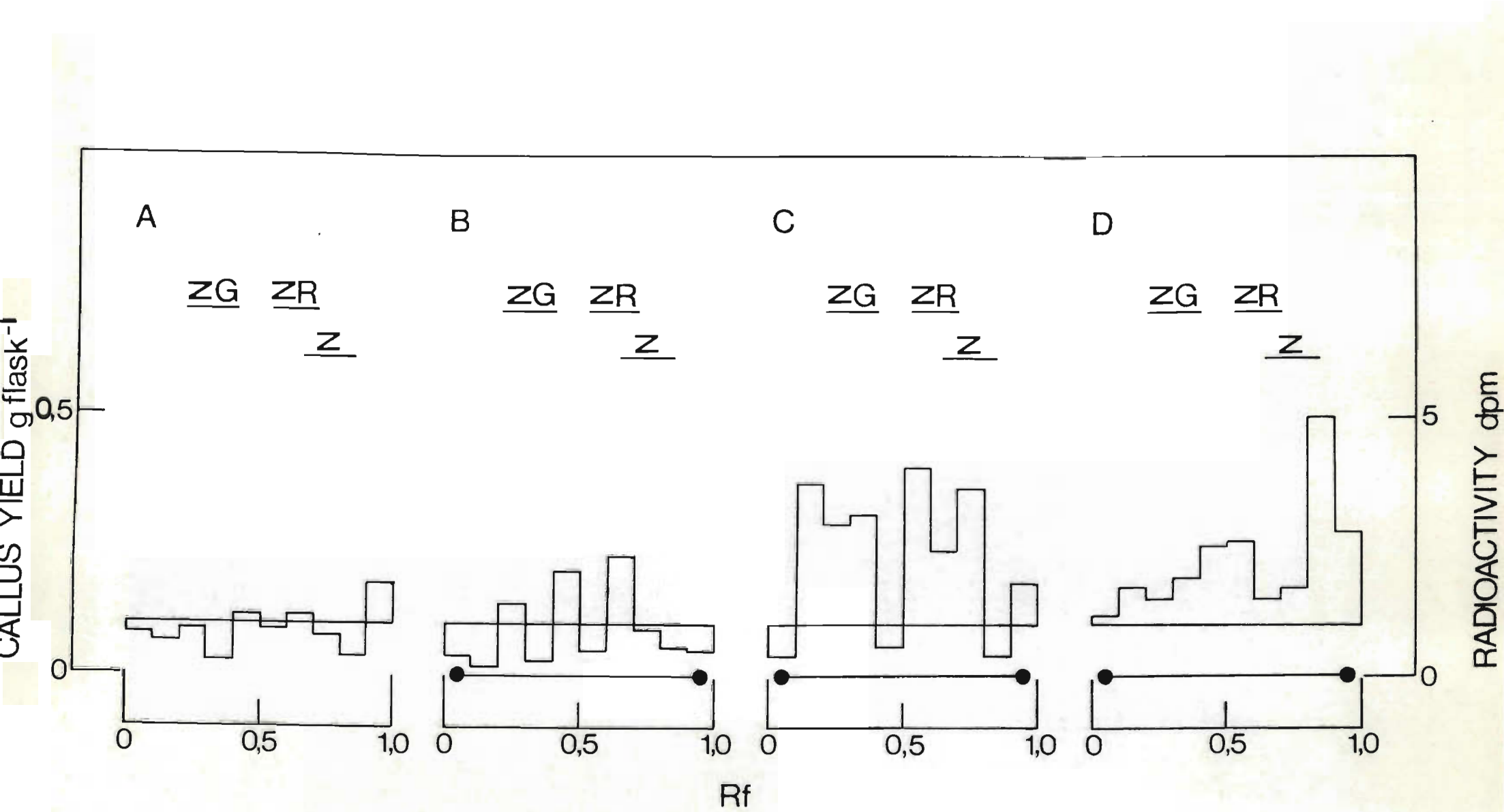

A
1500
1000
500
A
B
C
B
400
200
0
A
B
C
CYTOKININ ACTIVITY
ng KE/20g fresh weight
LEAVES

1.3.3 8(^{14}C)*t*-Zeatin applied to the leaves of *Ginkgo biloba* explants

8(^{14}C)*t*-Zeatin was applied to immature, mature and senescing leaves of *Ginkgo biloba* explants and its subsequent metabolism and transport was monitored. Zeatin appeared to be an endogenous cytokinin in *Ginkgo biloba* leaves and was also present in the xylem, which suggested that it was probably naturally transported to the leaves from the roots. Using radioactive zeatin, it was hoped to determine the fate of zeatin at three stages of leaf development, as well as the possible transport of zeatin and/or its metabolites out of the leaves.

Treated explants were maintained in distilled water for 48 hours, except for explants with senescing leaves which were incubated for 48 and 96 hours. The experimental period was extended in the case of senescing leaves as it was thought that metabolism would be slower in these leaves and a longer period was thus allowed. The water in which the explants were incubated was analyzed to determine whether any radio or biological activity was lost from the system. From Figure 1.7 it can be seen that no radioactivity was detected in these water extracts. Distilled water does not cause soyabean callus growth, but the distilled water in which the explants were maintained, did result in callus growth. This biological activity was not further investigated as no radioactivity was associated with it. The biological activity was probably due to cytokinins being leached from the cut stem surface or was possibly the re-

Figure 1.7 Radioactivity (●——●) and biological activity (histograms) of the distilled water in which the explants were maintained. Radioactive zeatin was applied to leaves of these explants. A = biological activity of distilled water; B = distilled water from explants with immature leaves; C = distilled water from explants with mature leaves; D = distilled water from explants with senescing leaves. Z = zeatin; ZR = ribosylzeatin; ZG = zeatin glucoside. Callus grown on 50 microgrammes per litre kinetin yielded 0,58 microgrammes fresh weight.


A
B
C
D
ZG
ZR
Z
0,5
0
5
0
0,5
1,0
Rf
CALLUS YIELD g flask-1
RADIOACTIVITY dpm

sult of cytokinin synthesis in a response to wounding (CARLSON and LARSON, 1977).

Transport of 8(^{14}C)*t*-zeatin applied to *Ginkgo biloba* leaves

From Table 1.1 it can be seen that less than 2 per cent of the radioactivity recovered was exported from the leaves to which it was applied during the 48 hour experimental period.

Table 1.1 The distribution of radioactivity (% dpm/explant component) 48 hours after applying 8(^{14}C)*t*-zeatin to (1) immature; (2) mature and (3) senescing leaves of *Ginkgo biloba* explants. 3a represents treated senescing leaves incubated for 96 hours.

Explant Component	Radioactivity (% dpm Recovered/Component)			
	(1) Immature	(2) Mature	(3) Senescing 48 Hour	(3a) Senescing 96 Hour
Treated leaves	98,70	98,07	99,83	99,05
Untreated leaves	0,10	0,41	0,04	0,44
Xylem	0,60	0,04	0	0
Bark	0,60	0,80	0,12	0,50
Apices	-	0,68	0,01	0,01

The radioactivity, which was exported out of the leaves, was recovered in all of the other explant components analyzed. The apex material was relatively small in relation to the other explant components, which could cause a distortion when results are expressed per explant com-

ponent. The results were, therefore, also expressed per one gramme fresh weight (Table 1.2). This did not change the overall trend but may have placed the results in perspective in relation to the different sizes of the explant components. Less than 3 per cent of the total radioactivity recovered was still only exported from the treated leaves when the results were expressed per one gramme fresh weight. In all cases results have been expressed per explant component and per one gramme fresh weight. Although these results do not always correspond, it was decided that those results expressed per one gramme fresh weight were more meaningful and were the results which were thus discussed.

Table 1.2 Distribution of radioactivity (% dpm/one gramme fresh weight) 48 hours after applying 8(^{14}C)*t*-zeatin to (1) immature; (2) mature and (3) senescing leaves of *Ginkgo biloba* explants. 3a represents treated senescing leaves incubated for 96 hours.

Explant Component	Radioactivity (% dpm Recovered/one Gramme Fresh Weight)			
	(1) Immature	(2) Mature	(3) Senescing 48 Hour	(3a) Senescing 96 Hour
Treated leaves	98,85	97,78	99,86	99,36
Untreated leaves	0,05	0,23	0,02	0,29
Xylem	0,50	0,02	0	0
Bark	0,60	0,80	0,12	0,35
Apices	-	1,18	0,001	0,001

If the radioactivity recovered is expressed in terms of those organs to which it was exported (Table 1.3), it can be seen that when expressed per explant component or per one gramme fresh weight, the bark appeared to be the major component to which radioactivity was exported. It was only in the mature explant system, that by expressing the results per one gramme fresh weight, the apices became the most important recipient. It appears that once the leaves began to senesce the untreated leaves represented a stronger sink than the apices for the cytokinins exported out of these leaves. The decreased export of cytokinins to the apices may also have been the result of these organs becoming dormant. If cytokinins are exported to the apices from the leaves then it appears as if the mature leaves are most important in this respect.

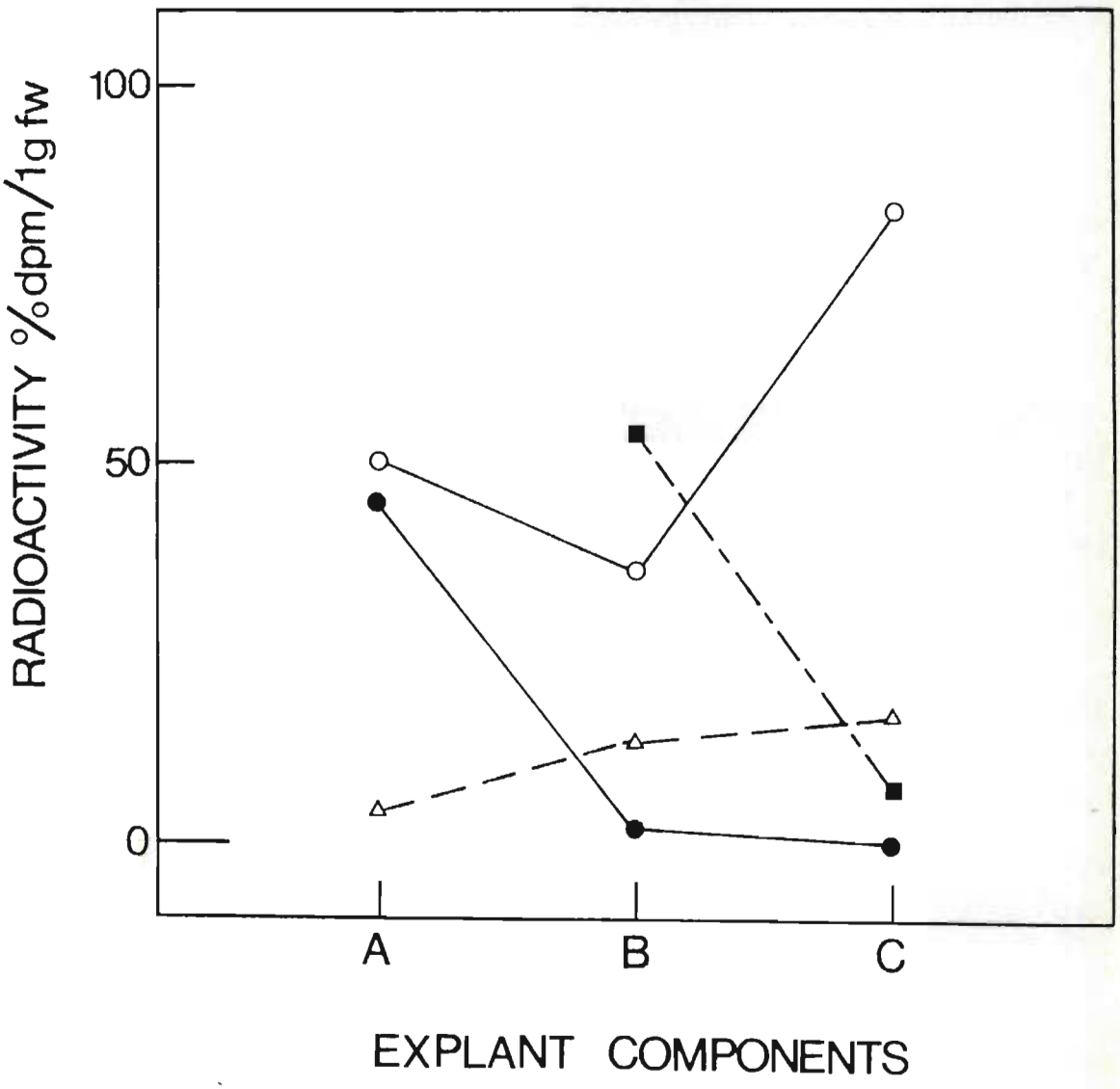
Table 1.3 Distribution of radioactivity (A expressed as % dpm/explant component and B expressed as % dpm/ one gramme fresh weight) in those explant components to which radioactive compounds were translocated. The radioactivity was recovered from paper chromatograms and (1) represents explants with immature leaves; (2) mature and (3) senescing leaves.

Explant Component	Percentage radioactivity (dpm) recovered/untreated component							
	(1) Immature		(2) Mature		(3) Senescing 48 Hour		(3a) Senescing 96 Hour	
	A	B	A	B	A	B	A	B
Untreated leaves	7,05	4,5	21,38	10,29	23,37	16,40	47,88	45,01
Xylem	46,01	45,09	1,81	0,94	0	0	0	0
Bark	46,94	50,66	41,35	35,88	76,51	82,90	52,02	54,39
Apices	-	-	35,46	53,89	0,12	0,70	0,10	0,60

If the radioactivity exported to the explant components is represented graphically over time or leaf maturation, that is, the immature through to the senescing system (Figure 1.8) then it can be seen that the amount of radioactivity exported to the untreated leaves increased slightly as the leaves matured. The radioactivity in the xylem and the apices decreased with leaf maturation. The radioactivity in the bark fluctuated, decreasing in the mature system probably due to the higher levels of radioactivity being transported to the apices. The high levels of radioactivity in the bark of senescing explants could suggest that some of the radioactivity exported from the leaves was stored in the bark. It must, however, be remembered that the exported radioactivity only represented a very small percentage of the total radioactivity recovered. This then poses the question as to whether this exported radioactivity can be regarded as being significant in terms of the cytokinin levels present in these tissues and the unphysiological cytokinin concentration applied to the explants.

Metabolism of 8(^{14}C)*t*-zeatin applied to *Ginkgo biloba* leaves

The radioactive metabolites formed from the 8(^{14}C)*t*-zeatin applied to the *Ginkgo biloba* leaves eluted as three radioactive peaks on paper chromatograms. The first two peaks, which eluted at R_f 0,0-0,2 and R_f 0,2-0,5, were referred to as radioactive peak 1 and 2 respectively. The last peak, which eluted at R_f 0,5-1,0, probably contained the radioactivity associated with the labelled zeatin. Ribosyl-


RADIOACTIVITY %dpm/1g fw
100
50
0
A
B
C
EXPLANT COMPONENTS

zeatin and dihydrozeatin, which could potentially be metabolites of zeatin would, however, also elute at R_f 0,5-1,0. This last peak was, therefore, referred to as radioactive peak 3, although it may not have contained any zeatin metabolites.

In all the explant components, radioactive peak 1 appeared to be the smallest peak, with peaks 2 and 3 representing the major radioactive components. The distribution of these radioactive peaks in the explant components is shown in Table 1.4. Assuming that radioactive peak 3 represented a large proportion of the original zeatin applied to the system, then metabolism appeared to be most rapid in the immature explants. Fifty-two per cent of the applied zeatin was metabolized in the immature leaves with 25 and 14 per cent being metabolized in the mature and senescing leaves respectively. Rapid zeatin metabolism could have been anticipated in the expanding immature leaves. In the immature explant system, radioactive peak 2 was the major peak in the untreated leaves and xylem, while peak 3 predominated in the bark and treated leaves. In the mature and senescing systems, radioactive peak 3 was the major radioactive peak in all of the explant components. If the radioactive peaks formed in the senescing *Ginkgo biloba* explants are compared 48 and 96 hours after applying the labelled zeatin, it can be seen that the amount of zeatin metabolized in the treated leaves did not appear to change. The proportions of the radioactive peaks detected in the explant components other than the treated leaves was,

Table 1.4 The distribution of radioactive peaks 1 (R_f 0,0-0,2); 2 (R_f 0,2-0,5) and 3 R_f 0,5-1,0) after applying 8(^{14}C)*t*-zeatin to (A) immature; (^{14}C) (b) mature; senescing - 48 hours and (D) senescing - 96 hours, leaves of *Ginkgo biloba* explants. Radioactivity is expressed as % dpm recovered/radioactive peak.

	Explant Component	Radioactivity (% dpm/Radioactive Peak)		
		Radioactive Peak 1 (R_f 0,0-0,2)	Radioactive Peak 2 (R_f 0,2-0,5)	Radioactive Peak 3 (R_f 0,5-1,0)
A	Immature System			
	Treated leaves	12,50	39,20	48,30
	Untreated leaves	0	91,00	9,00
	Xylem	25,00	47,00	28,00
	Bark	14,00	23,00	63,00
B	Mature System			
	Treated leaves	5,20	19,40	75,40
	Untreated leaves	0	17,20	82,80
	Xylem	0	30,40	69,60
	Bark	8,60	15,40	76,00
	Apices	5,40	14,30	80,30
C	Senescing System-48 Hour			
	Treated leaves	3,40	10,30	86,30
	Untreated leaves	0	0	100
	Xylem	0	0	0
	Bark	0	9,30	90,70
	Apices	0	0	100
D	Senescing System-96 Hour			
	Treated leaves	2,40	10,00	87,60
	Untreated leaves	0	11,00	89,00
	Xylem	0	0	0
	Bark	19,00	16,00	65,00
	Apices	0	0	0

however, affected. Radioactive peak 2, which was absent after 48 hours appeared after 96 hours in the untreated leaves. In the bark, the percentage radioactivity associated with radioactive peak 2 increased after 96 hours and was accompanied by the appearance of radioactive compounds chromatographing at R_f 0,0-0,2. Radioactive peak 1 (R_f 0,0-0,2) was absent in many explant components. Where present, it usually represented the smallest percentage of radioactivity. Radioactive peak 1 was present in the highest amounts in the rapidly metabolizing immature system. This, together with the fact that in the senescing system radioactive peak 1 was absent after 48 hours and was only detected after 96 hours incubation, suggested that this peak could be the final product of the metabolic pathway associated with the applied zeatin. That is, zeatin → radioactive peak 2 → radioactive peak 1, could represent the metabolic pathway of the applied zeatin.

If the percentage radioactivity associated with each radioactive peak is expressed per explant (Table 1.5), it can be seen that radioactive peak 2 was the major peak in the immature explants. Radioactive peak 3, which could represent metabolites of zeatin but probably represented the original radioactive zeatin, was the major peak in the mature and senescing explants. The highest percentage of radioactivity associated with peak 1 occurred in the immature explants. This peak only increased in the slowly metabolizing senescing system after 96 hours in-

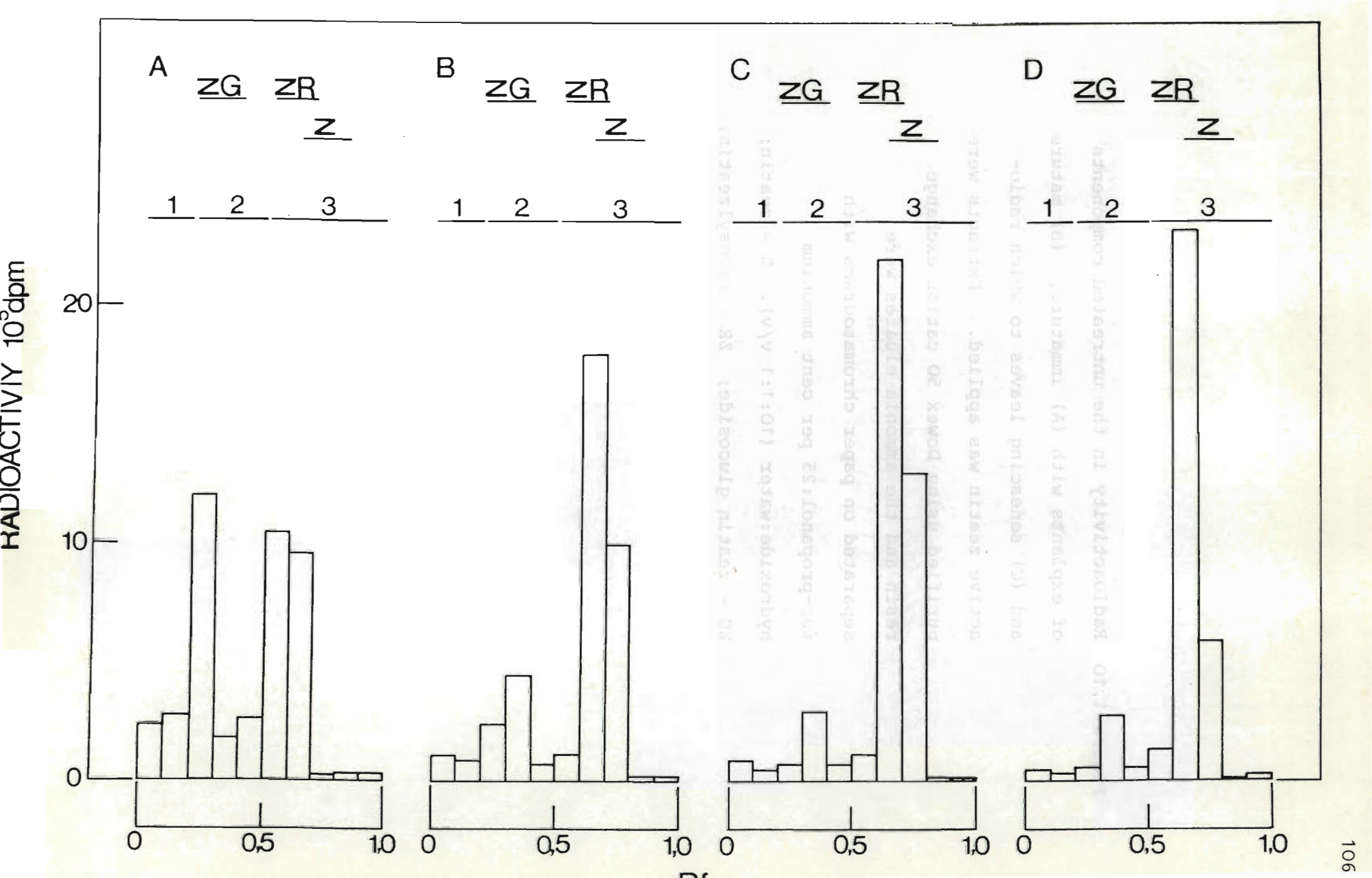
Table 1.5 The percentage of radioactive peaks 1(R_f 0,0-0,2); 2 (R_f 0,2-0,5) and 3 (R_f 0,5-1,0) recovered in (A) immature; (B) mature; (C) senescing (48 hours) and (D) senescing (96 hours) *Ginkgo biloba* explants after applying 8(^{14}C)*t*-zeatin to the leaves of these explants.

Explant	Radioactivity (% dpm/Radioactive Peak/Explant		
	Radioactive Peak 1 (R_f 0,0-0,2)	Radioactive Peak 2 (R_f 0,2-0,5)	Radioactive Peak 3 (R_f 0,5-1,0)
(A) Immature	12,88	50,05	37,07
(B) Mature	3,84	19,34	76,82
(C) Senescing 48 Hour	0,85	4,90	94,25
(D) Senescing 96 Hour	5,35	9,25	85,40

cubation.

If the radioactive peaks formed in the various components of the explants are compared to the endogenous cytokinin content of these components (Figures 1.2, 1.9 and 1.10), it can be seen that the radioactive peaks formed did not always co-chromatograph with the endogenous cytokinin activity. This may have been the result of endogenous cytokinins and the radioactive compounds both causing soyabean callus growth. This implies that the metabolism recorded may not refelct the normal metabolic pathway operating in these tissues. Some of the radioactive peaks did, however, correspond to the endogenous cytokinins, which could suggest that the metabolic pathway of the

Figure 1.9 The radioactivity detected from paper chromatograms of (A) immature, (b) mature, (C) senescing leaves incubated for 48 hours and (D) senescing leaves incubated for 96 hours, leaves to which 8(^{14}C)*t*-zeatin was applied. Radioactive peak 1 (R_f 0,0-0,2) (1); radioactive peak 2 (R_f 0,2-0,5) (2) and radioactive peak 3 (R_f 0,5-1,0) (3) formed from the radioactive zeatin are indicated on the figures. ZG = zeatin glucoside; ZR = ribosylzeatin; Z = zeatin.


A
ZG
ZR
Z
1
2
3
B
ZG
ZR
Z
1
2
3
C
ZG
ZR
Z
1
2
3
D
ZG
ZR
Z
1
2
3
RADIOACTIVIY 10³dpm
20
10
0
0
0,5
1,0
0
0,5
1,0
0
0,5
1,0
0
0,5
1,0
Rf

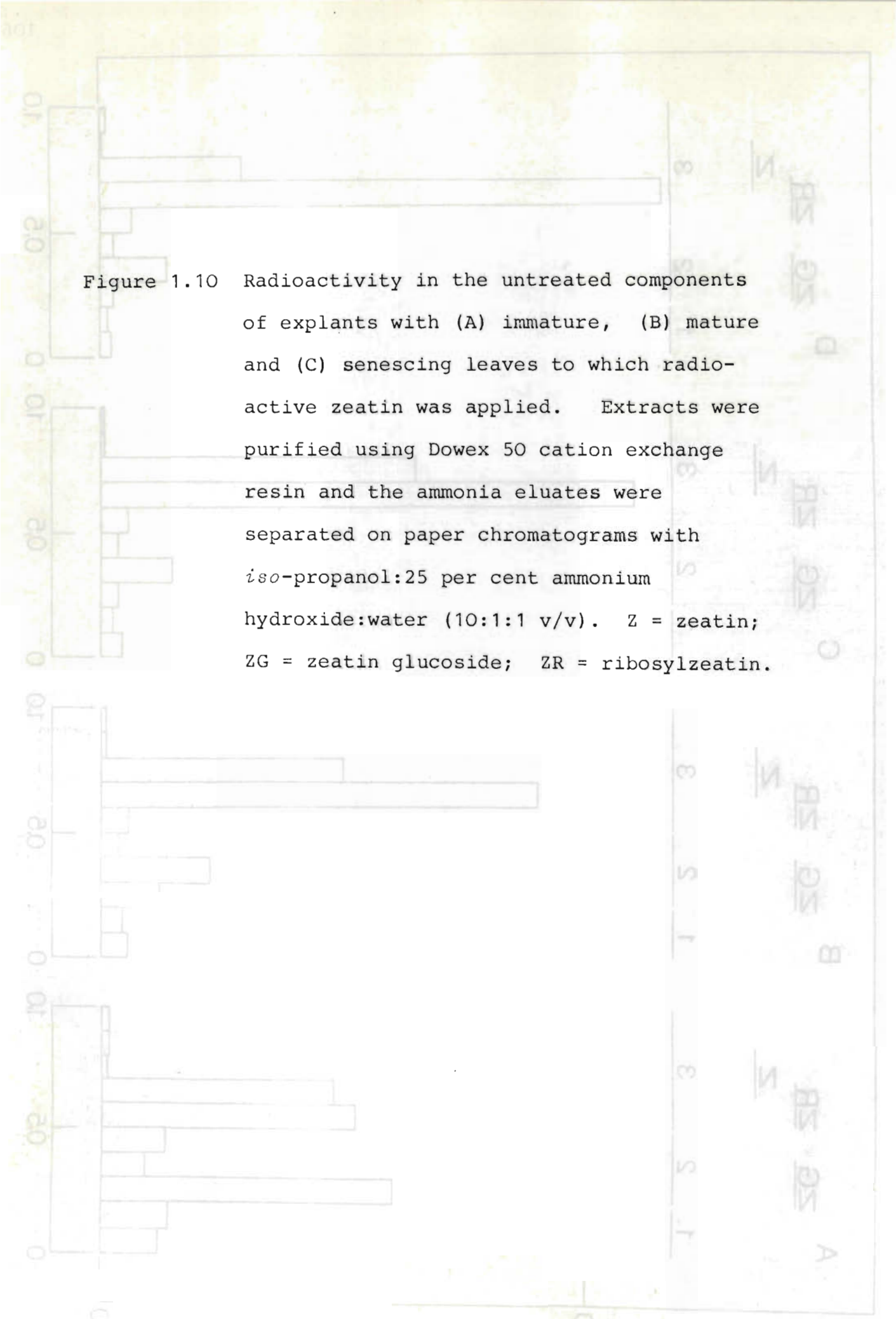
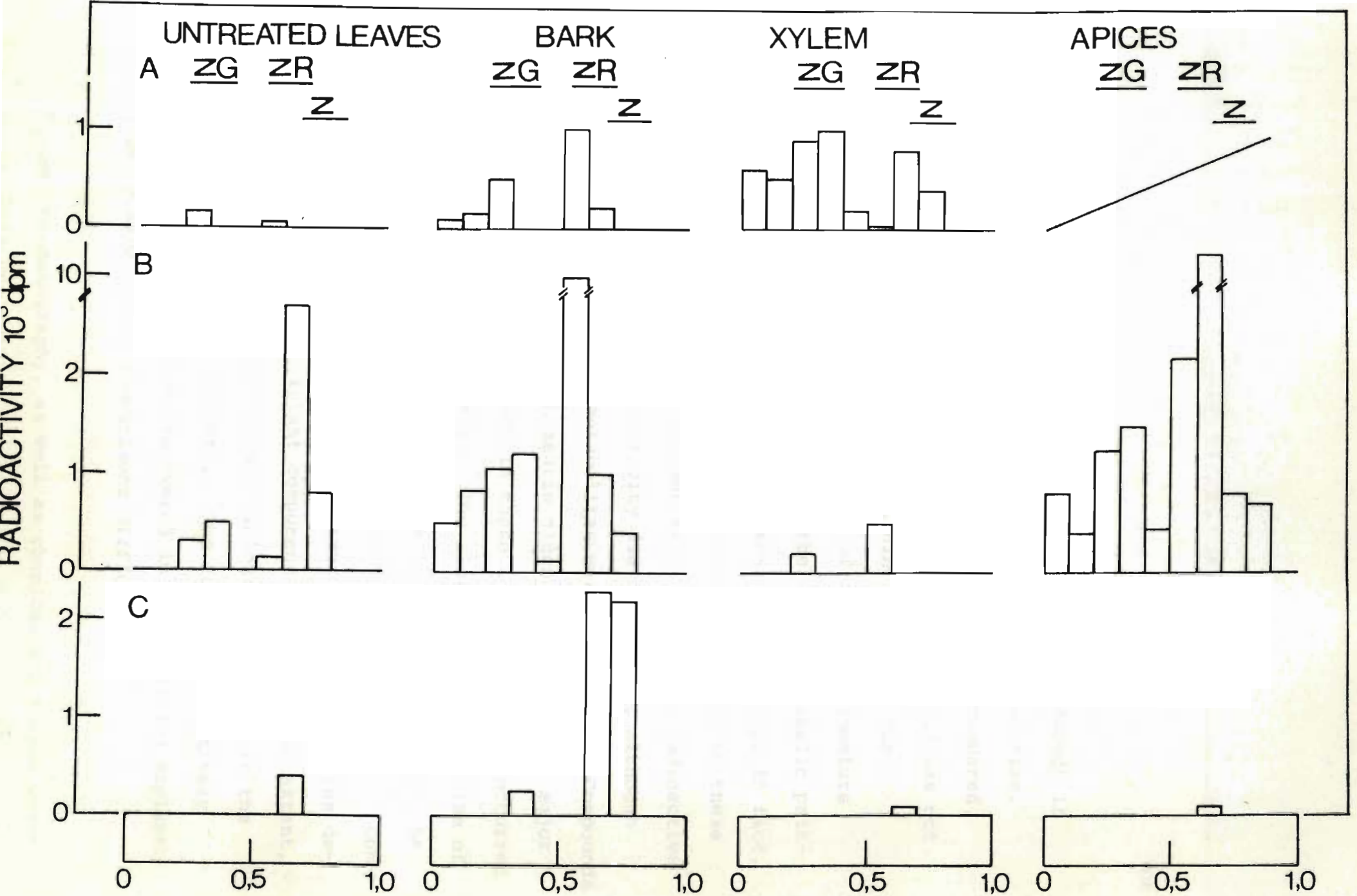


Figure 1.10 Radioactivity in the untreated components of explants with (A) immature, (B) mature and (C) senescing leaves to which radioactive zeatin was applied. Extracts were purified using Dowex 50 cation exchange resin and the ammonia eluates were separated on paper chromatograms with *iso*-propanol:25 per cent ammonium hydroxide:water (10:1:1 v/v). Z = zeatin; ZG = zeatin glucoside; ZR = ribosylzeatin.


UNTREATED LEAVES
BARK
XYLEM
APICES
A
B
C
ZG
ZR
Z
RADIOACTIVITY 10³ dpm
0
1
10
2
0,5
1,0

(R_f 0,5-1,0), for column chromatography. The small percentage of radioactivity usually recovered at radioactive peak 1 (R_f 0,0-0,2) did not make it feasible to deal with this peak separately and it was thus combined with radioactive peak 2 (R_f 0,2-0,5). Figures 1.11, 1.12, 1.13 and 1.14 illustrate the A and B fractions of the treated immature, mature, senescing (48 and 96 hours) leaves fractionated on Sephadex LH-20 columns eluted with 10 per cent methanol. The biological activity of the A fraction of the immature leaves was unfortunately lost due to the callus becoming autonomous. Approximately nine peaks of activity could be recognized in these leaf extracts after fractionation by column chromatography. These nine radioactive peaks were referred to as A, B, C, D, E, F, G, H1 and H2. Under standard conditions of flow rate and temperature, the elution volumes of these radioactive peaks is shown in Diagram 1.2. These peaks are also marked on the appropriate Figures, as well as the percentage radioactivity associated with each peak.

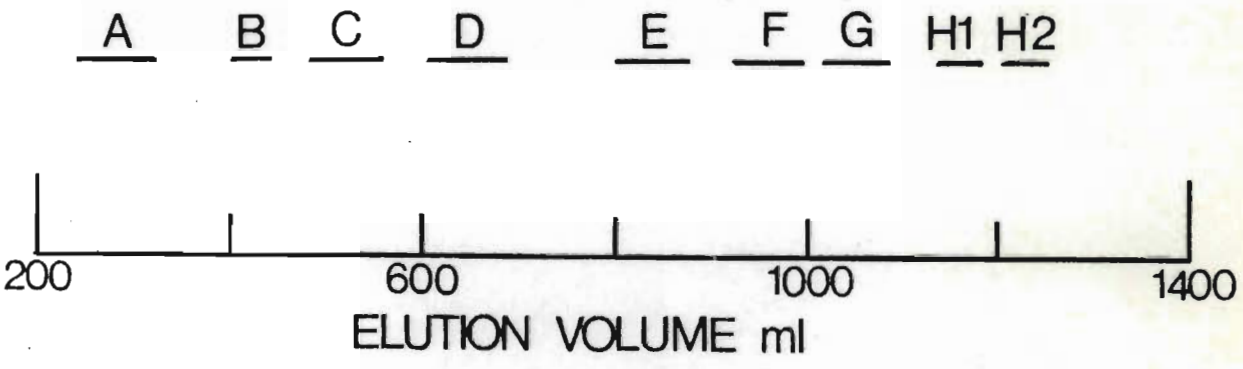


Diagram 1.2 The approximate elution volumes of the nine peaks of radioactivity detected in the leaf extracts following the application of 8(^{14}C)*t*-zeatin to these leaves.

Figure 1.11 Radioactivity (●——●) and biological activity (histograms) in the A and B fractions of immature *Ginkgo biloba* leaves to which 8(^{14}C)*t*-zeatin was applied. Fraction A represents R_f 0,0-0,5 of paper chromatograms and fraction B represents R_f 0,5-1,0. The extracts were fractionated on a Sephadex LH-20 column eluted with 10 per cent methanol. Radioactive peaks A, B, C, D, E, G and H are indicated as well as the percentage radioactivity associated with each peak. PG = purinyl glycine; Ado = adenosine; ZG = zeatin glucoside; THZ = trihydroxy-zeatin; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.

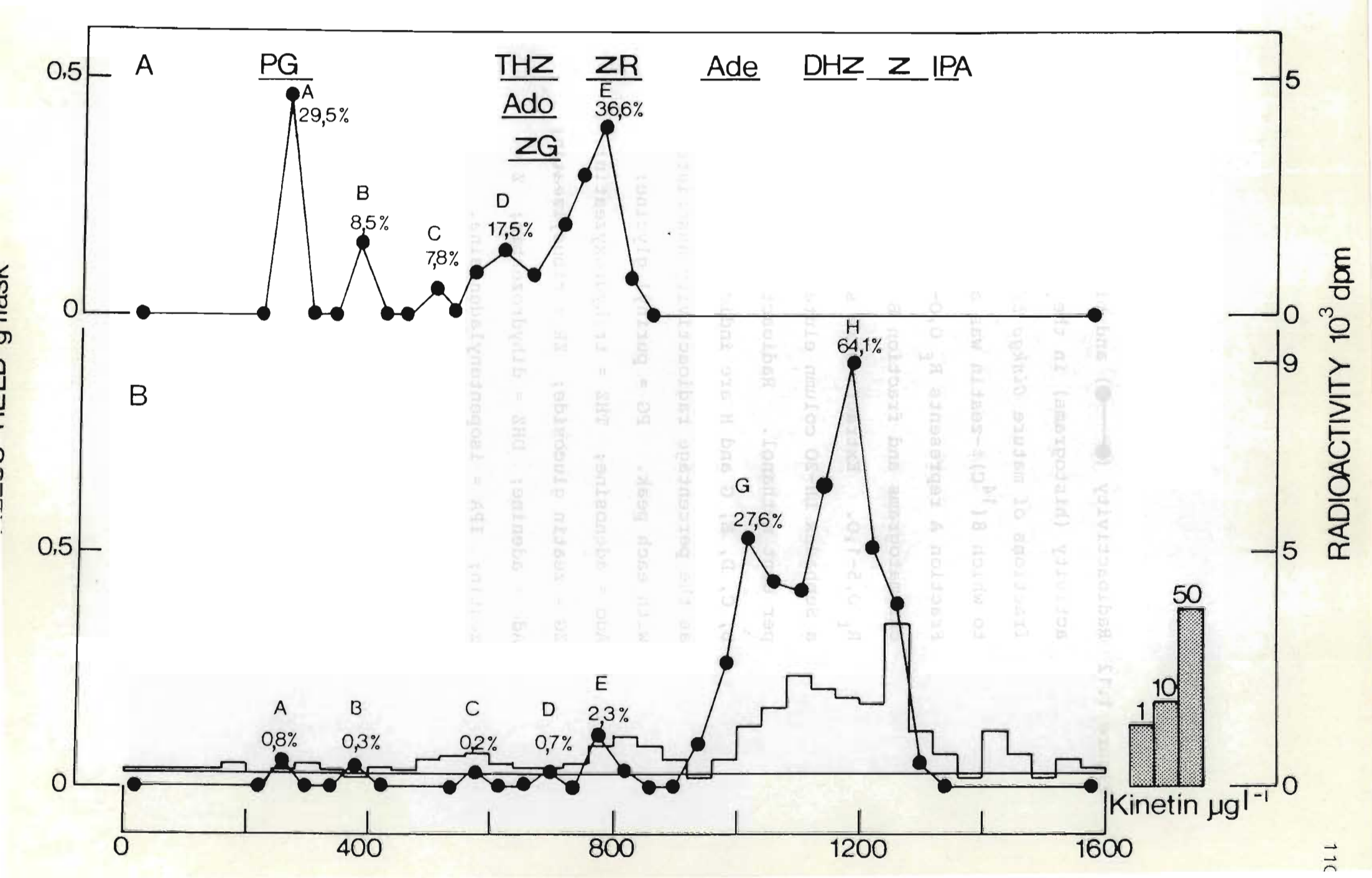
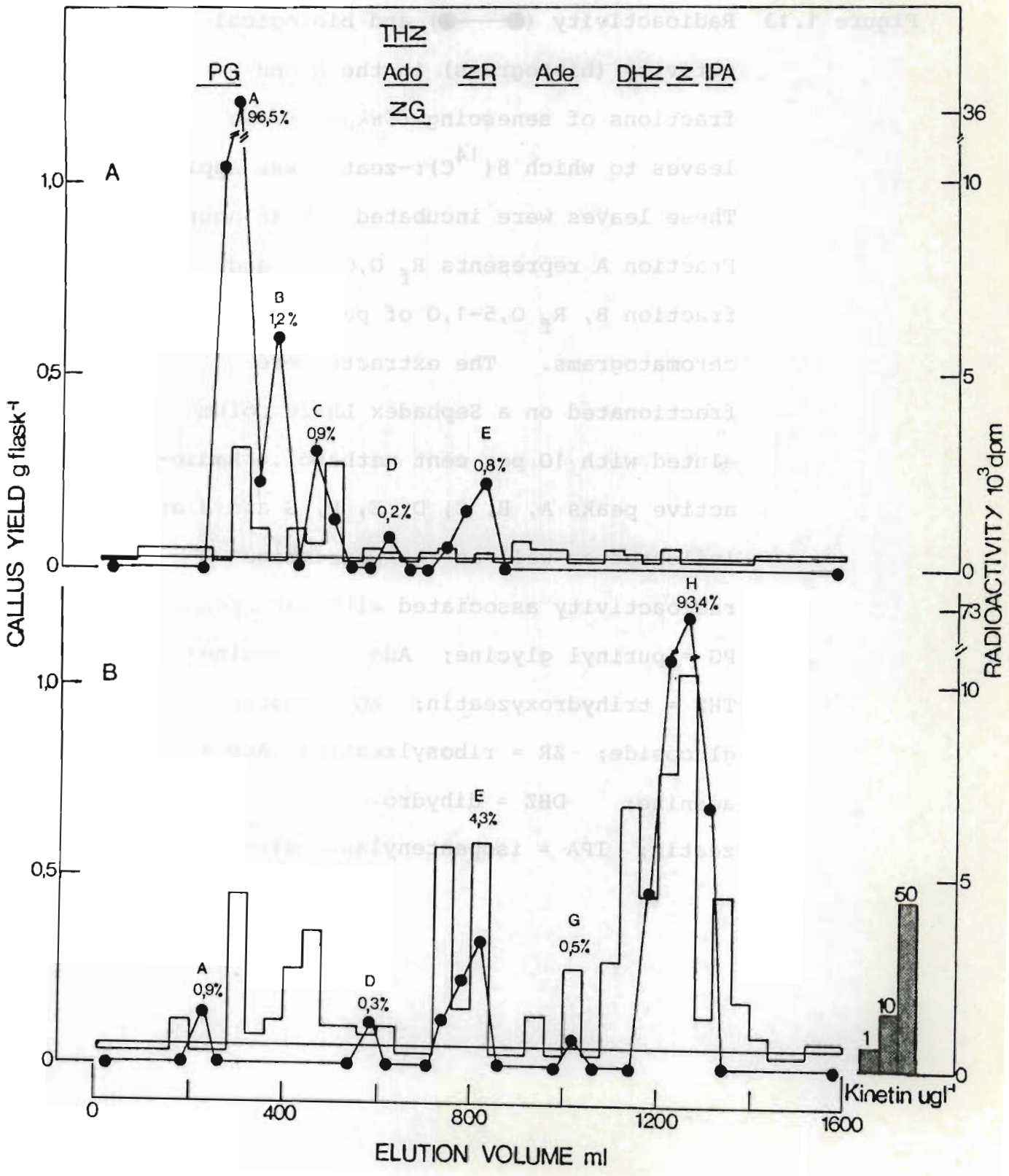

A
PG
THZ
Ado
ZG
ZR
Ade
DHZ
Z
IPA
A
29,5%
B
8,5%
C
7,8%
D
17,5%
E
36,6%
B
H
64,1%
G
27,6%
A
0,8%
B
0,3%
C
0,2%
D
0,7%
E
2,3%
0,5
0
5
9
0
400
800
1200
1600
1
10
50
Kinetin µgl^{-1}
RADIOACTIVITY 10^3 dpm

Figure 1.12 Radioactivity (●——●) and biological activity (histograms) in the A and B fractions of mature *Ginkgo biloba* leaves to which 8(^{14}C)*t*-zeatin was applied. Fraction A represents R_f 0,0-0,5 of paper chromatograms and fraction B represents R_f 0,5-1,0. Extracts were separated on a Sephadex LH-20 column eluted with 10 per cent methanol. Radioactive peaks A, B, C, D, E, G and H are indicated as well as the percentage radioactivity associated with each peak. PG = purinyl glycine; Ado = adenosine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.


THZ
PG
Ado
ZR
Ade
DHZ
Z
IPA
ZG
A
96,5%
B
1,2%
C
0,9%
D
0,2%
E
0,8%
A
1,0
0,5
0
36
10
5
0
B
H
93,4%
E
4,3%
G
0,5%
A
0,9%
D
0,3%
1,0
0,5
0
73
10
5
0
50
10
1
Kinetin ugl-1
0
400
800
1200
1600
ELUTION VOLUME ml
CALLUS YIELD g flask-1
RADIOACTIVITY 10³dpm

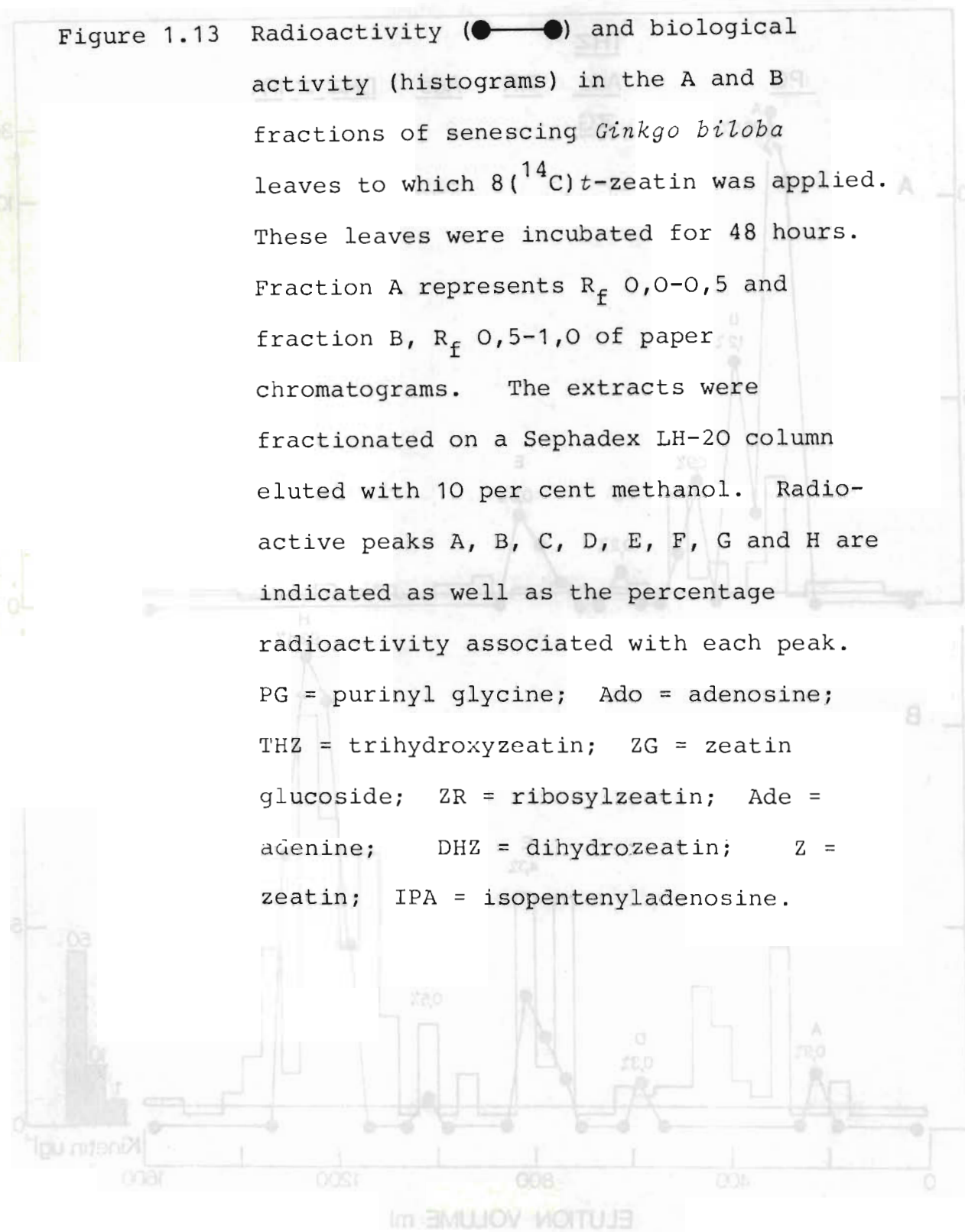


Figure 1.13 Radioactivity (●——●) and biological activity (histograms) in the A and B fractions of senescing *Ginkgo biloba* leaves to which 8(^{14}C)*t*-zeatin was applied. These leaves were incubated for 48 hours. Fraction A represents R_f 0,0-0,5 and fraction B, R_f 0,5-1,0 of paper chromatograms. The extracts were fractionated on a Sephadex LH-20 column eluted with 10 per cent methanol. Radioactive peaks A, B, C, D, E, F, G and H are indicated as well as the percentage radioactivity associated with each peak. PG = purinyl glycine; Ado = adenosine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.

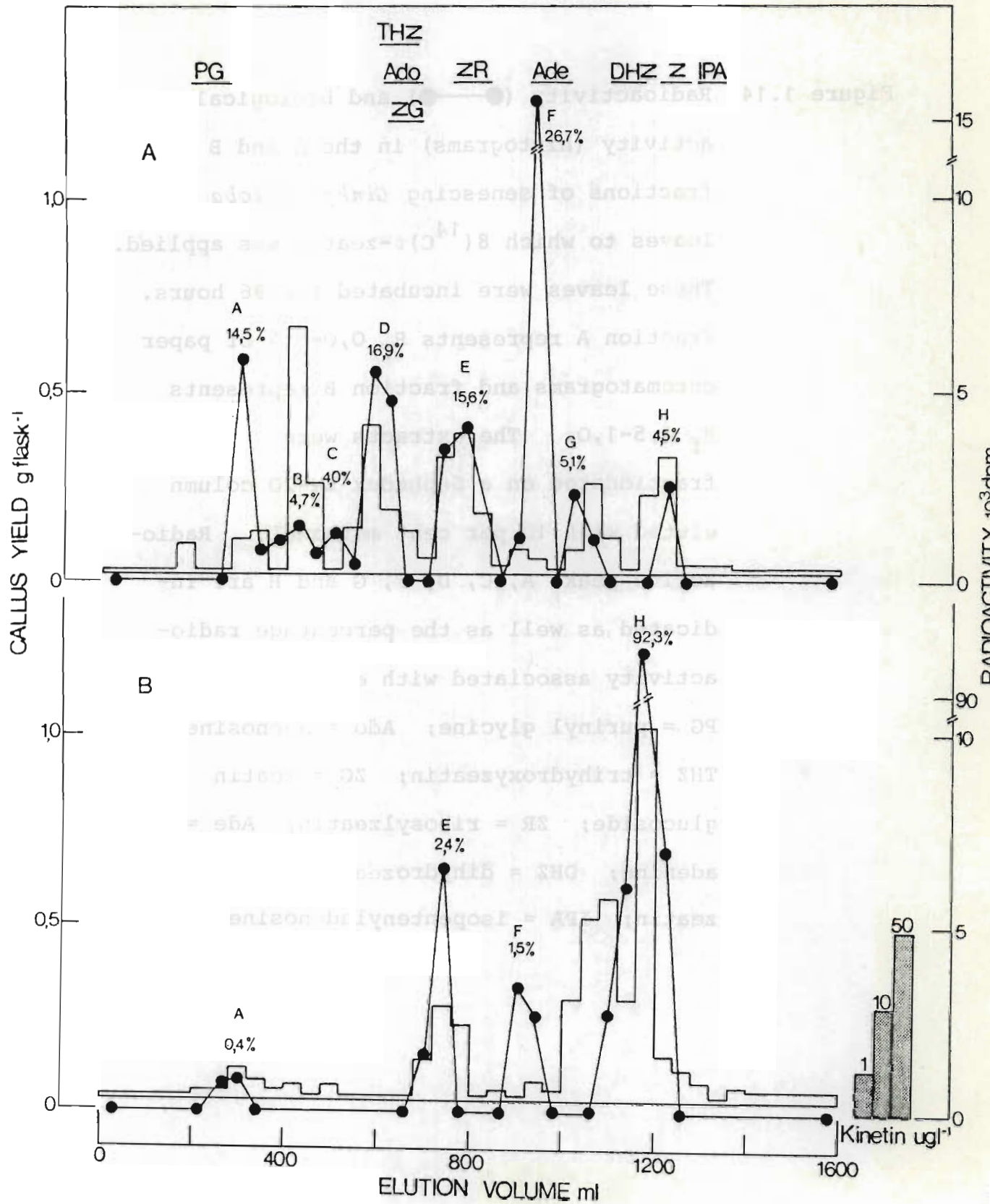
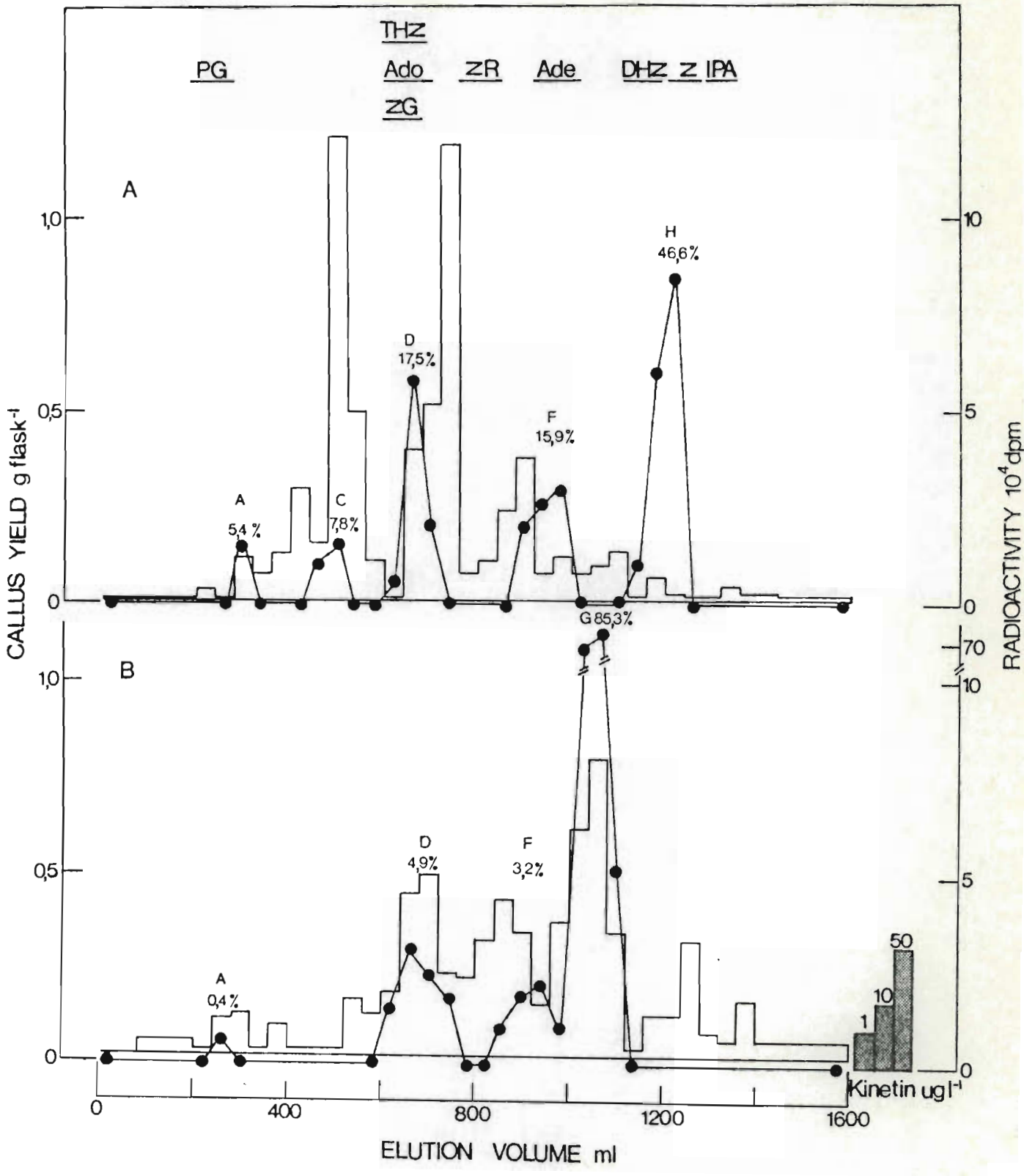

THZ
PG
Ado
ZR
Ade
DHZ
Z
IPA
ZG
A
B
C
D
E
F
G
H
14,5 %
4,7%
4,0%
16,9%
15,6%
26,7%
5,1%
4,5%
0,4%
2,4%
1,5%
92,3%
CALLUS YIELD g flask⁻¹
RADIOACTIVITY 10³dpm
Kinetin ugl⁻¹
ELUTION VOLUME ml
0
400
800
1200
1600
1,0
0,5
15
10
5
90
1
50

Figure 1.14 Radioactivity (●——●) and biological activity (histograms) in the A and B fractions of senescing *Ginkgo biloba* leaves to which 8(^{14}C)*t*-zeatin was applied. These leaves were incubated for 96 hours. Fraction A represents R_f 0,0-0,5 of paper chromatograms and fraction B represents R_f 0,5-1,0. The extracts were fractionated on a Sephadex LH-20 column eluted with 10 per cent methanol. Radioactive peaks A, C, D, F, G and H are indicated as well as the percentage radioactivity associated with each peak. PG = purinyl glycine; Ado = adenosine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.


THZ
PG
Ado
ZR
Ade
DHZ
Z
IPA
ZG
A
B
H
46,6%
D
17,5%
F
15,9%
A
5,4 %
C
7,8%
G 85,3%
D
4,9%
F
3,2%
A
0,4%
1,0
0,5
0
10
5
70
50
1
CALLUS YIELD g flask-1
RADIOACTIVITY 10⁴dpm
Kinetin ug l-1
0
400
800
1200
1600
ELUTION VOLUME ml

It was often difficult to clearly distinguish between the last two radioactive peaks, which were thus referred to as H1 and H2 rather than as 2 distinct peaks. In many instances, there was also an overlap of peaks occurring in the A and B fractions, probably due to the incomplete separation of compounds by paper chromatography. It could be said that peaks A to D eluted in the A fractions and peaks E to H2 in the B fractions. Seven of these peaks co-eluted with authentic cytokinin standards. Peak A (280-320 millilitres) co-eluted with purinyl glycine, peak C (480 millilitres) eluted with the suspected ribosylzeatin glucoside and/or dihydroribosylzeatin glucoside, peak D (600-640 millilitres) co-eluted with adenosine, trihydroxy-zeatin and zeatin glucoside, peak E (800-840 millilitres) co-eluted with ribosylzeatin, peak F (920-960 millilitres) co-eluted with adenine and peaks H1 and H2 (1120-1240 millilitres) co-eluted with dihydrozeatin and zeatin, respectively. It was assumed that these radioactive peaks were the same in all extracts. They were further identified by chemical and enzyme treatments.

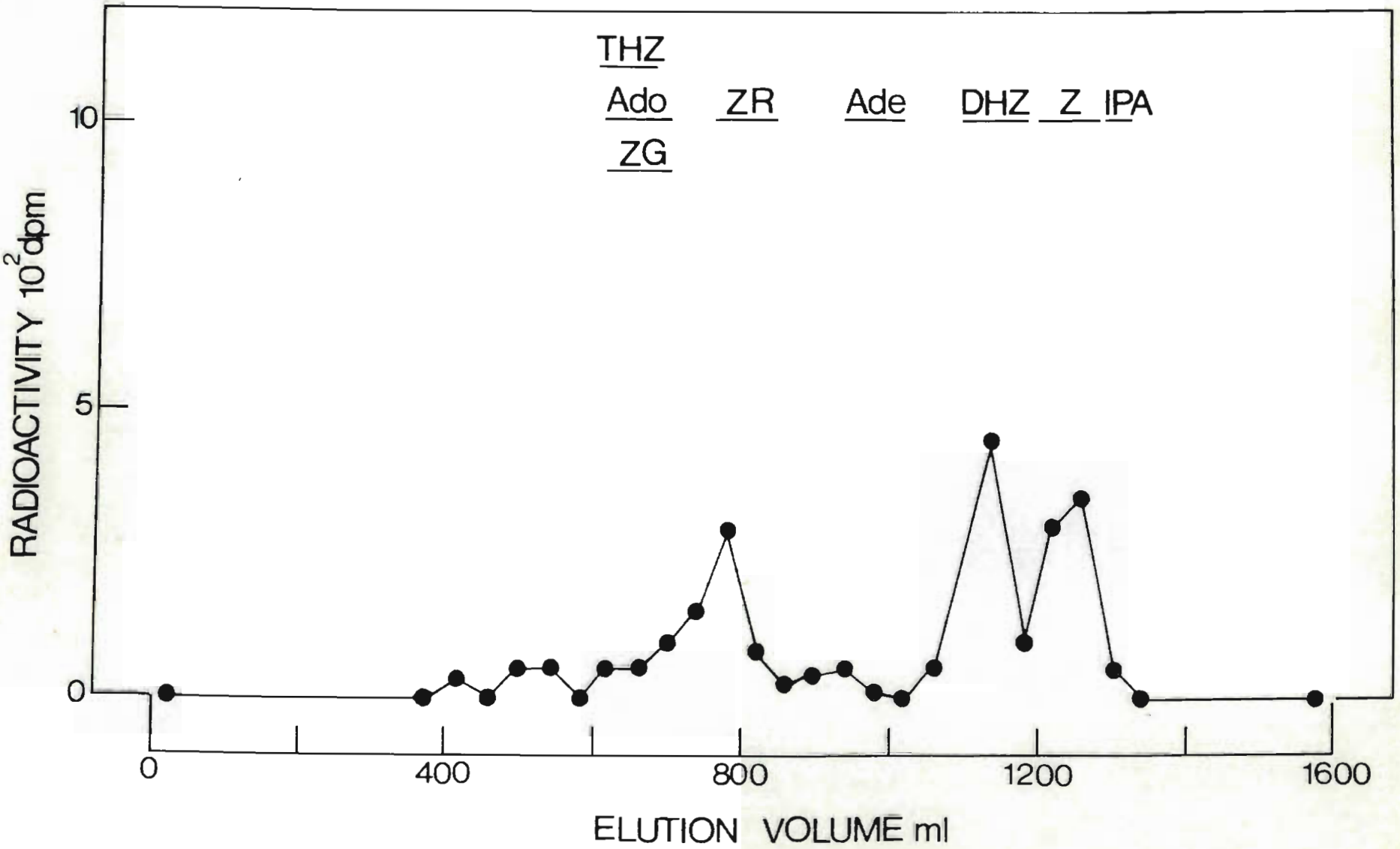
Relatively little biological activity was associated with peak A which was detected in both the A and B fractions of the extracts. The comparatively low levels of this peak in the B fractions of the extracts was probably the result of poor separation by paper chromatography. Peak A appeared to be common to all leaf extracts, but was the major radioactive metabolite in the A fraction of the immature and mature leaves. Alkaline phosphatase, β-

glucosidase and potassium permanganate treatment did not change the chromatographic properties of peak A. This peak co-eluted with purinyl glycine, which is reported to be the biologically inactive end-product of zeatin oxidation (MILLER, 1965; LETHAM, SHANNON and MCDONALD, 1967). The similar chromatographic behaviour of peak A and purinyl glycine (VAN STADEN, DREWES and HUTTON, 1982), together with the fact that they are both biologically inactive, implied that peak A may have been purinyl glycine.

Radioactive peak B was not present in all leaf extracts and in terms of the percentage radioactivity associated with it, it was considered to be relatively insignificant. Although this peak was biologically active, it did not co-elute with any authentic cytokinin standards. Radioactive peak B was also unaffected by alkaline phosphatase or β-glucosidase treatment. The nature of this peak was not further investigated.

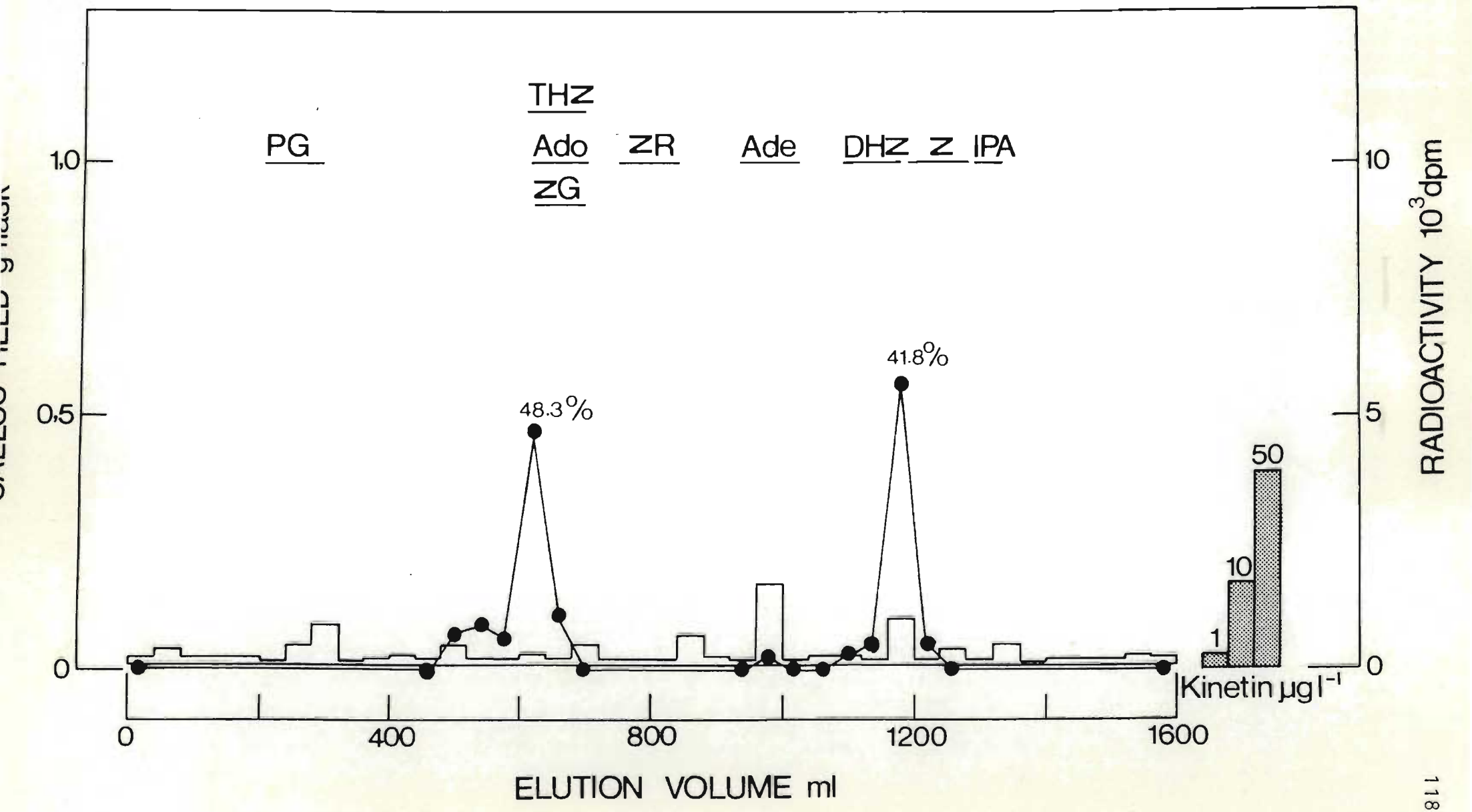
Radioactive peak C was only present in the A fractions of the leaf extracts. This peak appeared to be biologically very active. Alkaline phosphatase treatment did not affect the chromatograph behaviour of this peak, but β-glucosidase treatment resulted in a loss of radioactivity associated with this peak and the formation of new radioactive peaks (Figure 1.15). These peaks co-eluted with ribosylzeatin, dihydrozeatin and zeatin. This suggested that zeatin glucoside, ribosylzeatin glucoside and dihydrozeatin glucoside were all components of this peak.

Figure 1.15 Radioactivity detected following β-glucosidase treatment of radioactive peak C. Radioactive peak C, from the A fraction of treated mature *Ginkgo biloba* leaves (Figure 1.12) was redissolved, treated with β-glucosidase and then fractionated on a Sephadex LH-20 column eluted with 10 per cent methanol. PG = purinyl glycine; Ado = adenosine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.


THZ
Ado
ZR
Ade
DHZ
Z
IPA
ZG
10
5
0
RADIOACTIVITY 10^2dpm
0
400
800
1200
1600
ELUTION VOLUME ml

Radioactive peak D, which had an elution volume of 600 to 640 millilitres, co-eluted with authentic adenosine, trihydroxyzeatin and zeatin glucoside, all of which have approximately the same elution volume using this solvent system. This peak was insignificant in terms of radio- and biological activity in immature and mature leaves. It was, however, a major peak in senescing leaves. Figure 1.16 shows that β-glucosidase treatment did have some effect on the chromatographic behaviour of this peak. Forty-two per cent of the radioactivity originally associated with this peak shifted and co-eluted with zeatin, suggesting that zeatin-O-glucoside was a component of this peak. Forty-eight per cent of the radioactivity associated with peak D was, however, not affected by β-glucosidase which implied that compounds other than the cytokinin O-glucosides were also present in this peak. Relatively little biological activity was associated with the bioassay following β-glucosidase treatment, but no biological activity was associated with the original peak D (Figure 1.16). The absence of biological activity could suggest that adenosine, which is biologically inactive in the soyabean callus bioassay (VAN STADEN, 1979a), may also have been a component of peak D. The absence of biological activity also implies that the 7- and 9-cytokinin glucosides, which are not affected by β-glucosidase (LETHAM, WILSON, PARKER, JENKINS, MACLEOD and SUMMONS, 1975), and trihydroxyzeatin, which is biologically very active (VAN STADEN, DREWES and HUTTON, 1982) were not components of this peak. The low levels of cytokinin activity detected in this bioassay should,

Figure 1.16 Radioactivity (●——●) and biological activity (histogram) detected following β-glucosidase treatment of radioactive peak D. Radioactive peak D (elution volume 600 to 640 millilitres) from the A fraction of treated senescing *Ginkgo biloba* leaves (Figure 1.13) was re-dissolved, treated with β-glucosidase and then fractionated on a Sephadex LH-20 column eluted with 10 per cent methanol. The percentage radioactivity associated with the radioactive peaks is indicated. PG = purinyl glycine; Ado = adenosine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.

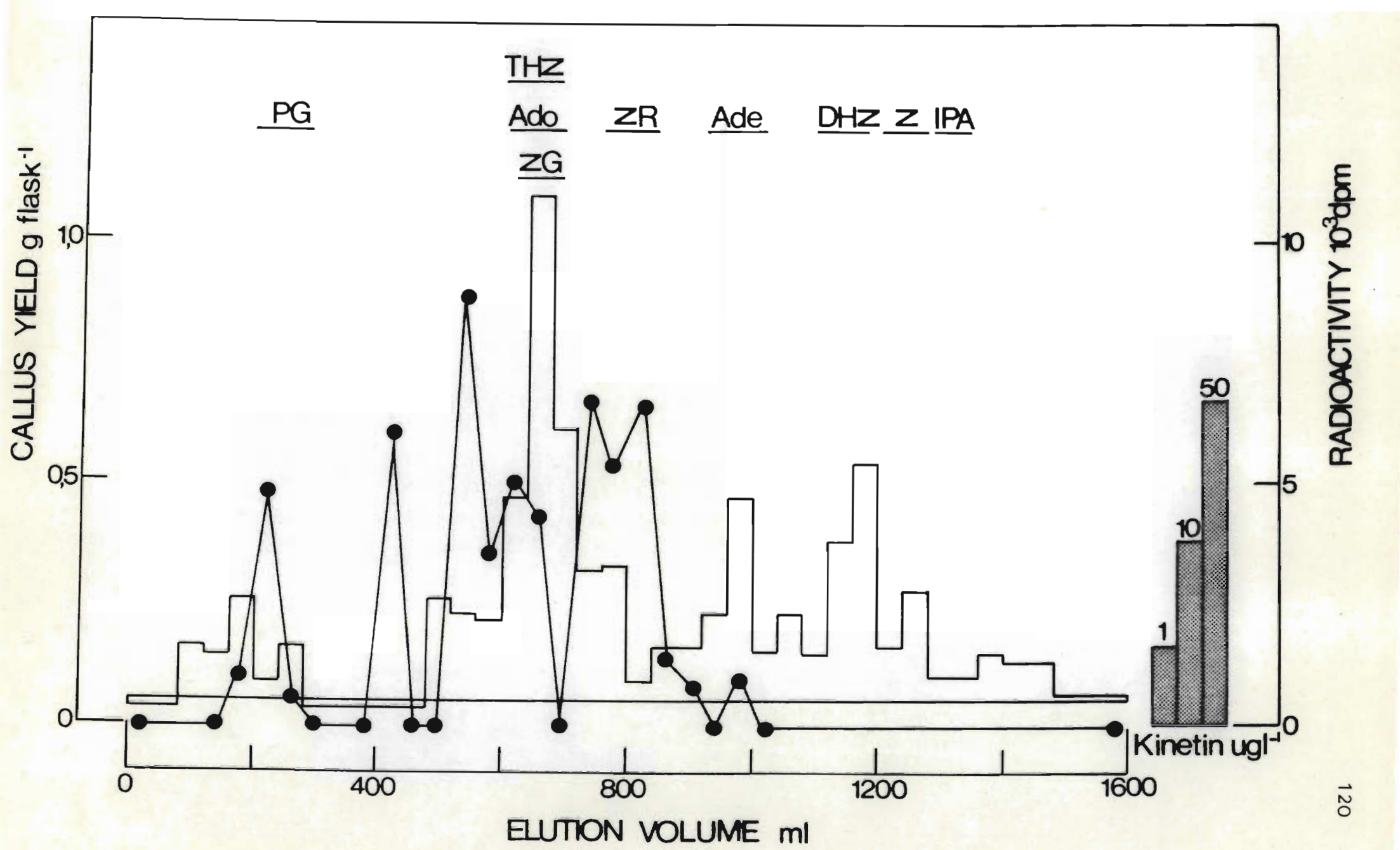

THZ
PG
Ado
ZR
Ade
DHZ
Z
IPA
ZG
1,0
0,5
0
10
5
0
48.3%
41.8%
50
10
1
Kinetin µg l⁻¹
0
400
800
1200
1600
ELUTION VOLUME ml
RADIOACTIVITY 10³dpm

however, be taken into consideration. The presence of compounds co-eluting with purinyl glycine could, therefore, imply that trihydroxyzeatin, an intermediate in the oxidation reaction resulting in purinyl glycine, was, in fact, a component of this peak.

Radioactive peak E was an important peak in all leaf extracts and was biologically active. This peak co-eluted with authentic ribosylzeatin. Potassium permanganate oxidation (Figure 1.17) resulted in 76 per cent of the radioactivity originally associated with this peak shifting. The radioactive peaks resulting from this oxidation treatment were similar to those peaks formed when zeatin was oxidized with potassium permanganate (Figure 1.19). The fact that not all the radioactivity shifted following potassium permanganate oxidation suggested that dihydroribosylzeatin may also have been a component of peak E.

Peak F was only detected in the A and B fractions of the senescing leaf extracts and was the main radioactive peak in the A fraction of senescing leaves incubated for 48 hours. Relatively little biological activity was associated with this peak which co-eluted with adenine. Adenine, which is inactive in the soyabean callus bioassay (VAN STADEN, 1979a) has been reported to be a major metabolite of zeatin, resulting from side chain cleavage (SUMMONS, ENTSCH, LETHAM, GOLLNOW and MACLEOD, 1980). However, as relatively little biological activity was associated with this radioactive peak, it was not regarded as being im-

Figure 1.17 Radioactivity (●——●) and biological activity (histogram) following potassium permanganate oxidation of radioactive peak E (elution volume 800 to 840 millilitres). Radioactive peak E, from the B fraction of treated senescing *Ginkgo biloba* leaves (Figure 1.13) was redissolved, treated with potassium permanganate and then fractionated on a Sephadex LH-20 column eluted with 10 per cent methanol. PG = purinyl glycine; Ado = adenosine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.

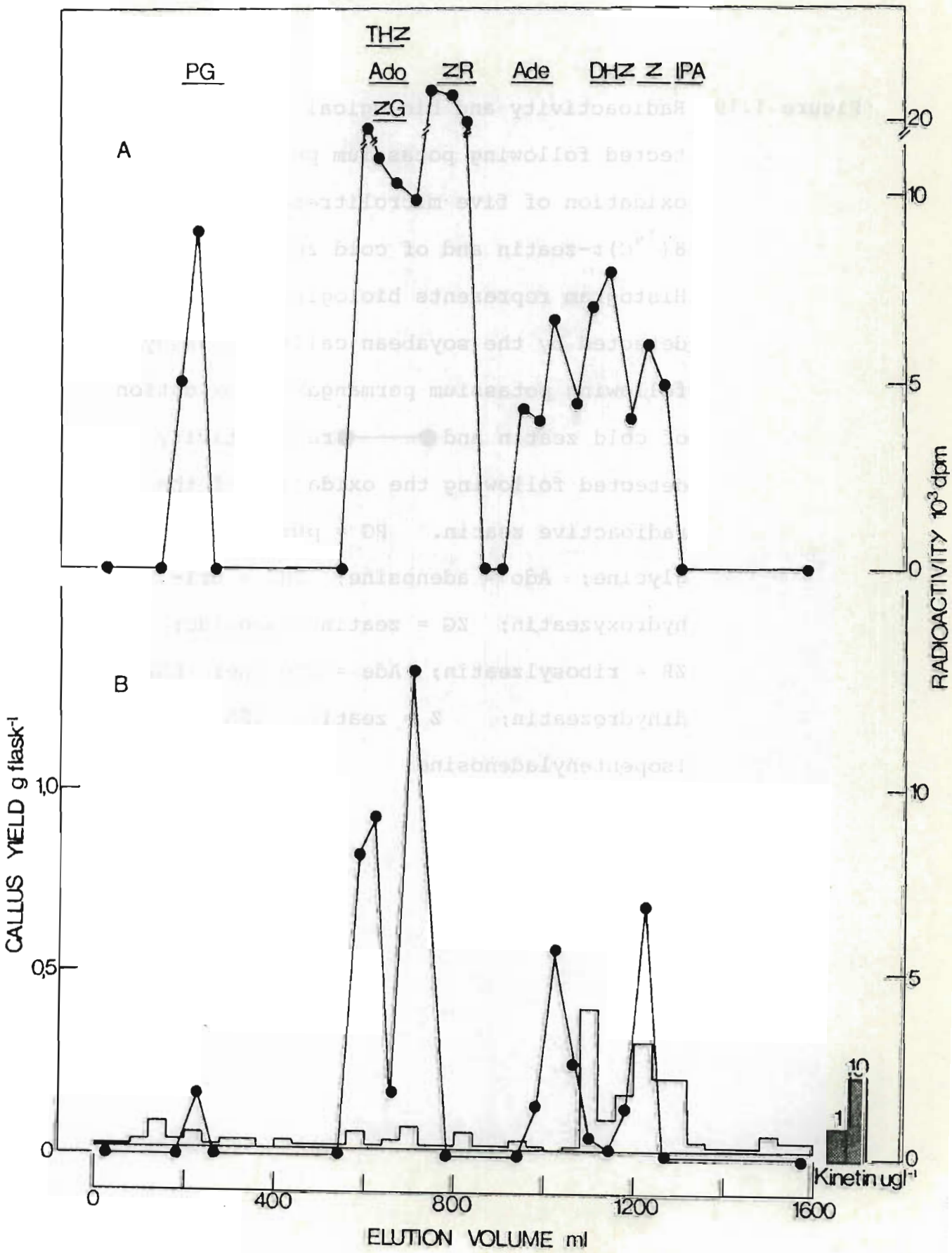

THZ
PG
Ado
ZR
Ade
DHZ
Z
IPA
ZG
CALLUS YIELD g flask-1
1,0
0,5
0
RADIOACTIVITY 10-3dpm
10
5
0
50
10
1
Kinetin ugl-1
0
400
800
1200
1600
ELUTION VOLUME ml

portant and was, therefore, not investigated further.

Radioactive peak G occurred predominantly in the B fractions of the leaf extracts. This peak was biologically active but did not co-elute with any of the authentic cytokinin standards. It did, however, co-elute with one of the peaks detected following potassium permanganate oxidation of cold and radioactive zeatin (Figure 1.19). This peak was not identified and was not further investigated.

Peak H1 and peak H2 were often difficult to separate because there was no distinct trough between their elution volumes and because of the high biological activity associated with these peaks. These peaks, which co-eluted with dihydrozeatin and zeatin, were the major radioactive peaks in the B fractions of immature, mature and senescing leaves (48 hours). These radioactive peaks were not detected in senescing leaves incubated for 96 hours. The radioactivity associated with zeatin probably represented the original radioactive zeatin applied to leaves which was not metabolized. Figure 1.18A and 1.18B illustrate the peaks formed following potassium permanganate oxidation of these peaks. In both cases some of the original peak remained, suggesting that dihydrozeatin, as well as zeatin, was, in fact, a component of these peaks. Five microlitres of 8(^{14}C)*t*-zeatin, as well as cold zeatin were also oxidized by potassium permanganate. The peaks formed following this oxidation process are shown in Figure 1.19. The radioactive and biological peaks formed, co-eluted and

Figure 1.18 Radioactivity (●——●) and biological activity (histograms) detected following potassium permanganate oxidation of radioactive peak H, which co-elutes with zeatin and dihydrozeatin. Radioactive peak H from (A) the B fraction of senescing *Ginkgo biloba* leaves (Figure 1.13) and (B) the B fraction of mature leaves (Figure 1.12) was redissolved, treated with potassium permanganate and then fractionated on a Sephadex LH-20 column eluted with 10 per cent methanol. PG = purinyl glycine; Ado = adenosine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.


THZ
PG
Ado
ZR
Ade
DHZ
Z
IPA
ZG
A
B
20
10
5
0
RADIOACTIVITY 10^3 dpm
CALLUS YIELD g flask^{-1}
1,0
0,5
0
400
800
1200
1600
ELUTION VOLUME ml
Kinetin ugl^{-1}

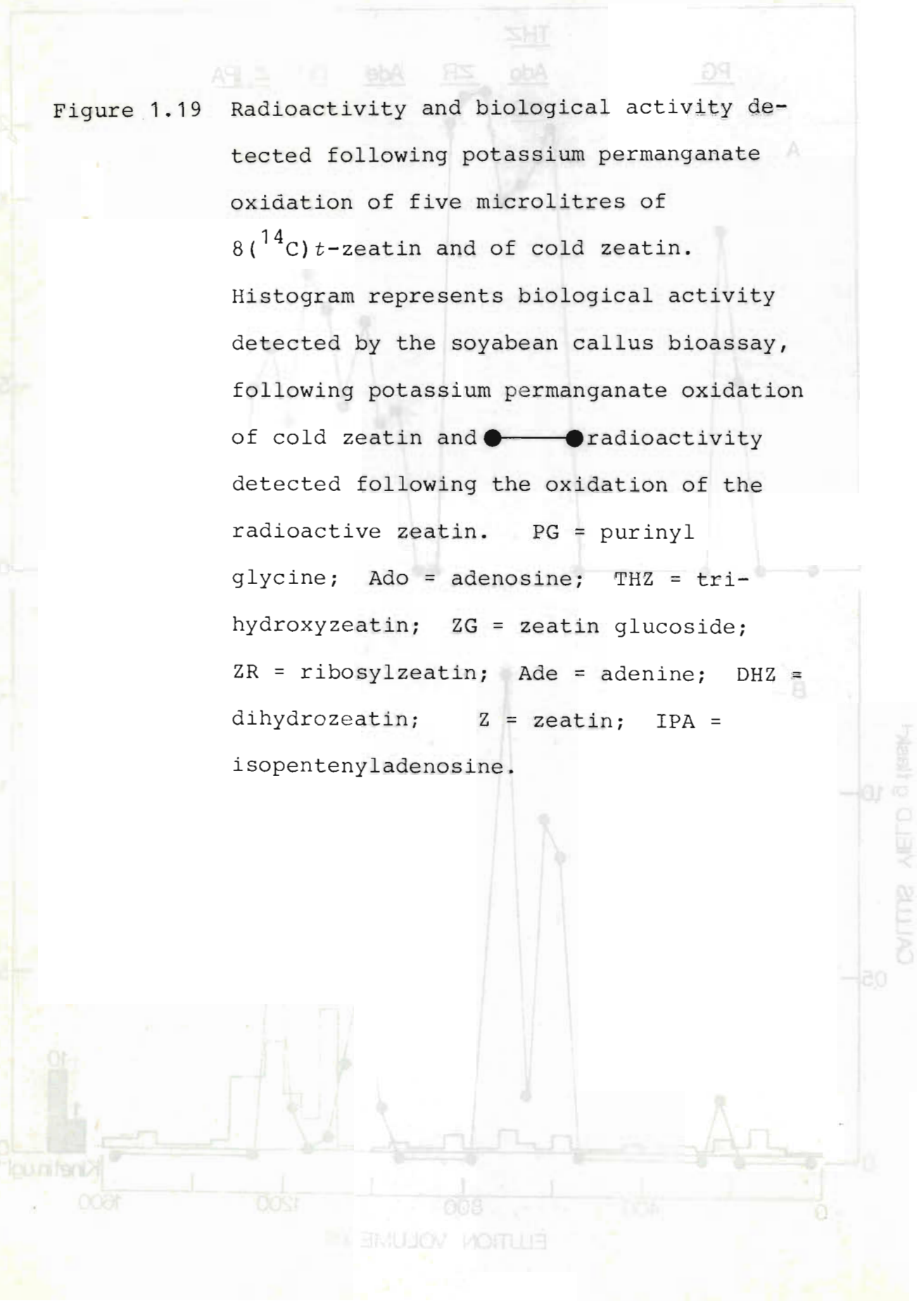
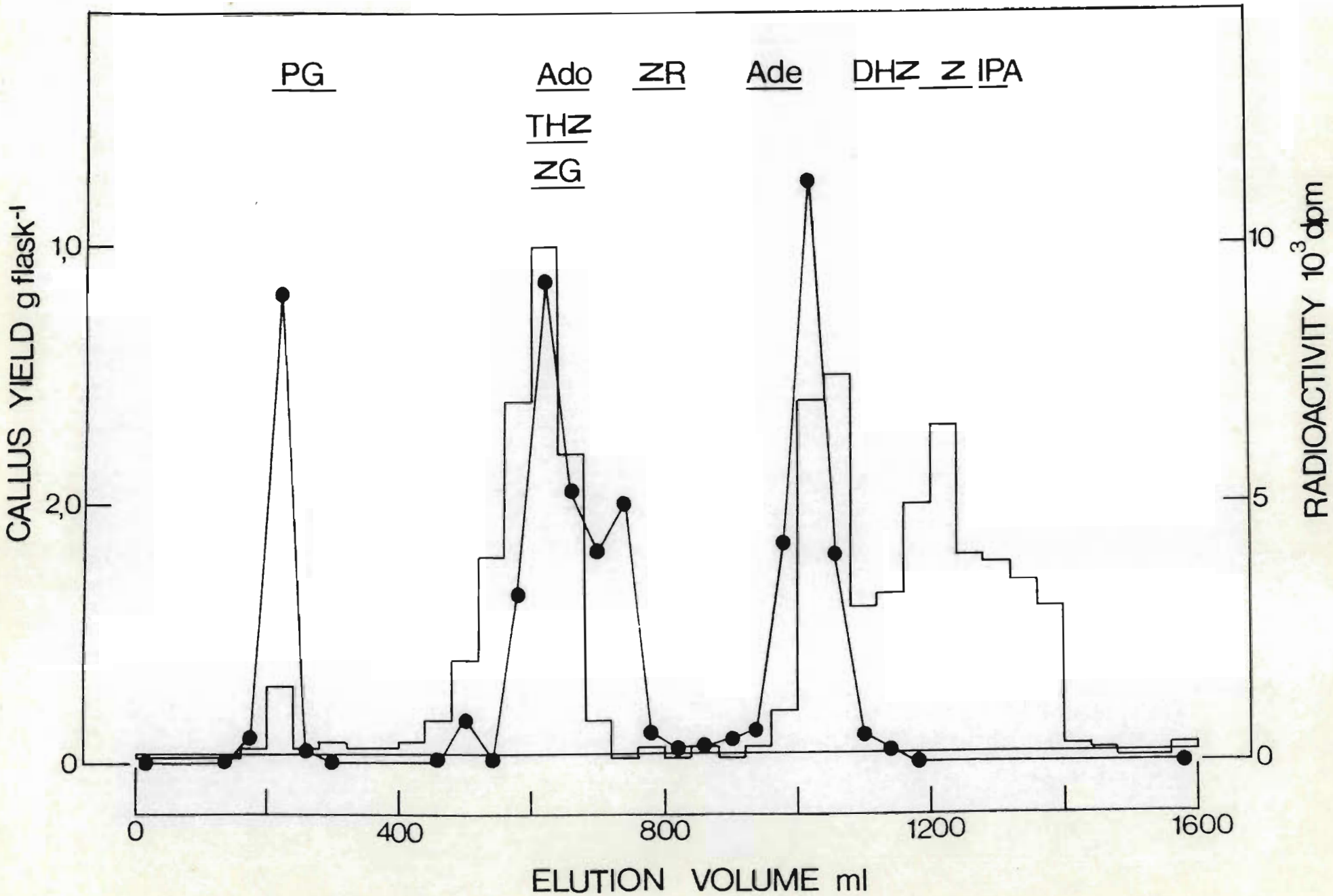


Figure 1.19 Radioactivity and biological activity detected following potassium permanganate oxidation of five microlitres of 8(^{14}C)*t*-zeatin and of cold zeatin. Histogram represents biological activity detected by the soyabean callus bioassay, following potassium permanganate oxidation of cold zeatin and ●——● radioactivity detected following the oxidation of the radioactive zeatin. PG = purinyl glycine; Ado = adenosine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.


PG
Ado
THZ
ZG
ZR
Ade
DHZ
Z
IPA
CALLUS YIELD g flask-1
1,0
2,0
0
RADIOACTIVITY 10^3 dpm
10
5
0
0
400
800
1200
1600
ELUTION VOLUME ml

they also co-eluted with the peaks formed as a result of the oxidation of radioactive peak H. The radioactive peaks formed also corresponded to some of the other peaks formed from the radioactive zeatin applied to the *Ginkgo biloba* leaves, namely radioactive peaks A, C, D, E and G. This suggested that oxidation may be one of the metabolic pathways of the applied zeatin.

Generally it can be said that peak A which was thought to be purinyl glycine, was important in the A fraction of immature and mature leaves. Cytokinin glucosides (peaks C and D) became more important in senescing leaves. Ribosylzeatin, dihydrozeatin and zeatin appeared to be important in the B fraction of all the leaf extracts, although the zeatin probably represented residual 8(^{14}C)*t*-zeatin applied to the leaves. Adenine and other unidentified oxidation products appeared to assume importance in the B fractions of senescing leaves. The possible metabolic pathways of the exogenous zeatin are indicated in Diagram 1.3. Ribosylation and side chain reduction appeared to be operative in all leaf extracts. Oxidation, resulting in the formation of purinyl glycine, appeared to be more important in immature and mature leaves than senescing leaves and glucosylation appeared to be a feature of senescing leaves.

The radioactive peaks detected in the treated leaf extracts were also recorded in the untreated explant components. The radioactive peaks recovered in some of these components

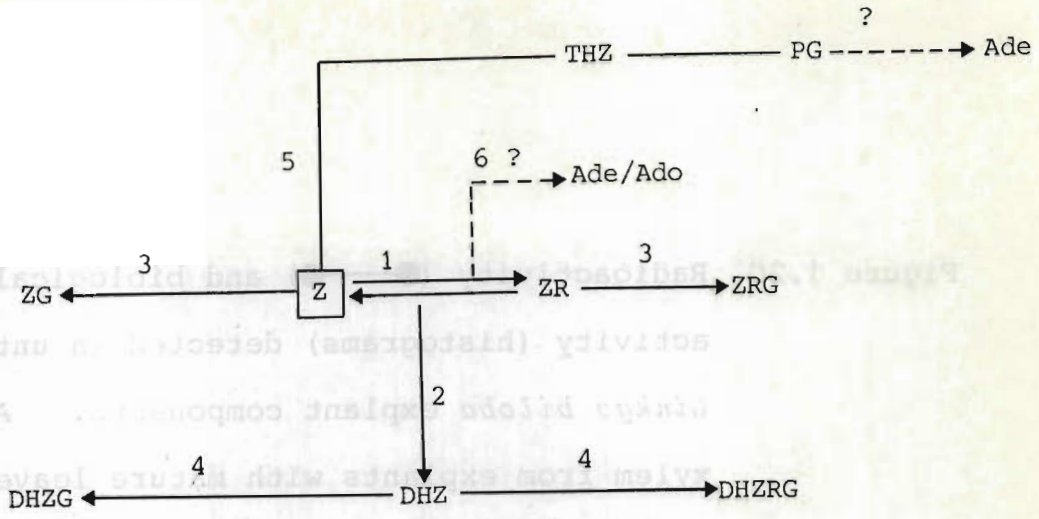


Diagram 1.3 The possible metabolic pathway for 8(^{14}C)*t*-zeatin applied to *Ginkgo biloba* leaves. 1 = ribosylation; 2 = side chain reduction; 3 and 4 = glucosylation; 5 = oxidation and 6 = oxidation involving side chain cleavage. Z = zeatin; ZR = ribosylzeatin; ZRG = ribosylzeatin glucoside; ZG = zeatin glucoside; DHZ = dihydrozeatin; DHZR = dihydroribosylzeatin; DHZG = dihydrozeatin glucoside; DHZRG = dihydroribosylzeatin glucoside; Ade = adenine; Ado = adenosine; THZ = trihydroxyzeatin; PG = purinyl glycine.

are shown in Figures 1.20 and 1.21. In some components, all nine radioactive peaks were detected, whereas in other components fewer peaks were detected, possibly reflecting the metabolic capabilities of the tissues.

1.3.4 Re-application of the metabolites of 8(^{14}C)*t*-zeatin to mature leaves of *Ginkgo biloba* explants

Radioactive peak 2 formed from the 8(^{14}C)*t*-zeatin, applied to *Ginkgo biloba* leaves, appeared to contain some glucosylated compounds. In an attempt to determine whether these glucosylated compounds were exported out of the leaves before they were shed, this radioactive peak was eluted from paper chromatograms of mature and senescing

Figure 1.20 Radioactivity (●——●) and biological activity (histograms) detected in untreated *Ginkgo biloba* explant components. A = xylem from explants with mature leaves; B = untreated leaves of explants with senescing leaves; C = apex (bud) material of explants with senescing leaves. Extracts were fractionated on a Sephadex LH-20 column eluted with 10 per cent methanol. Radioactive peaks A, B, C, D, E, F, G and H are indicated on the figures. PG = purinyl glycine; Ado = adenosine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.

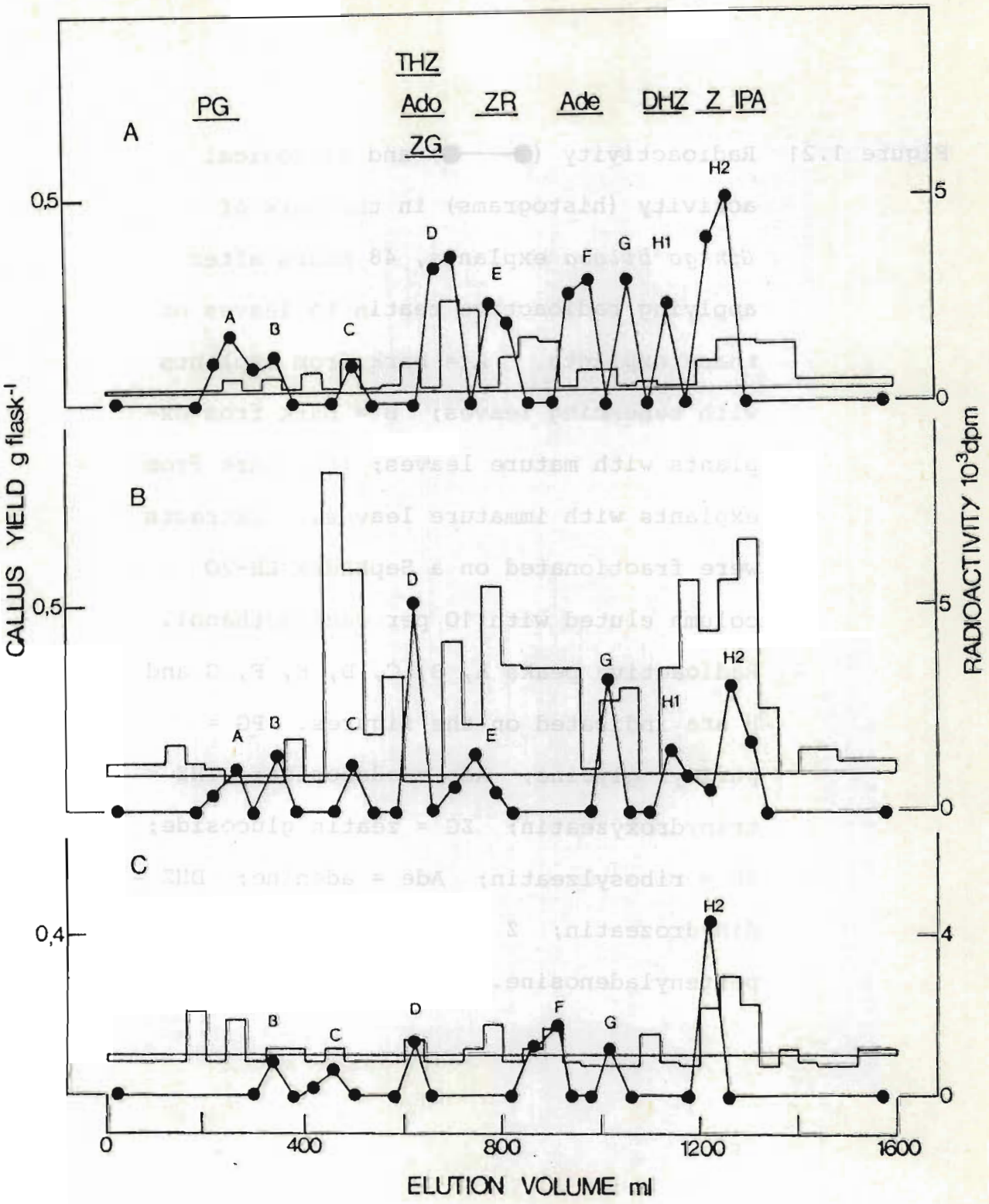
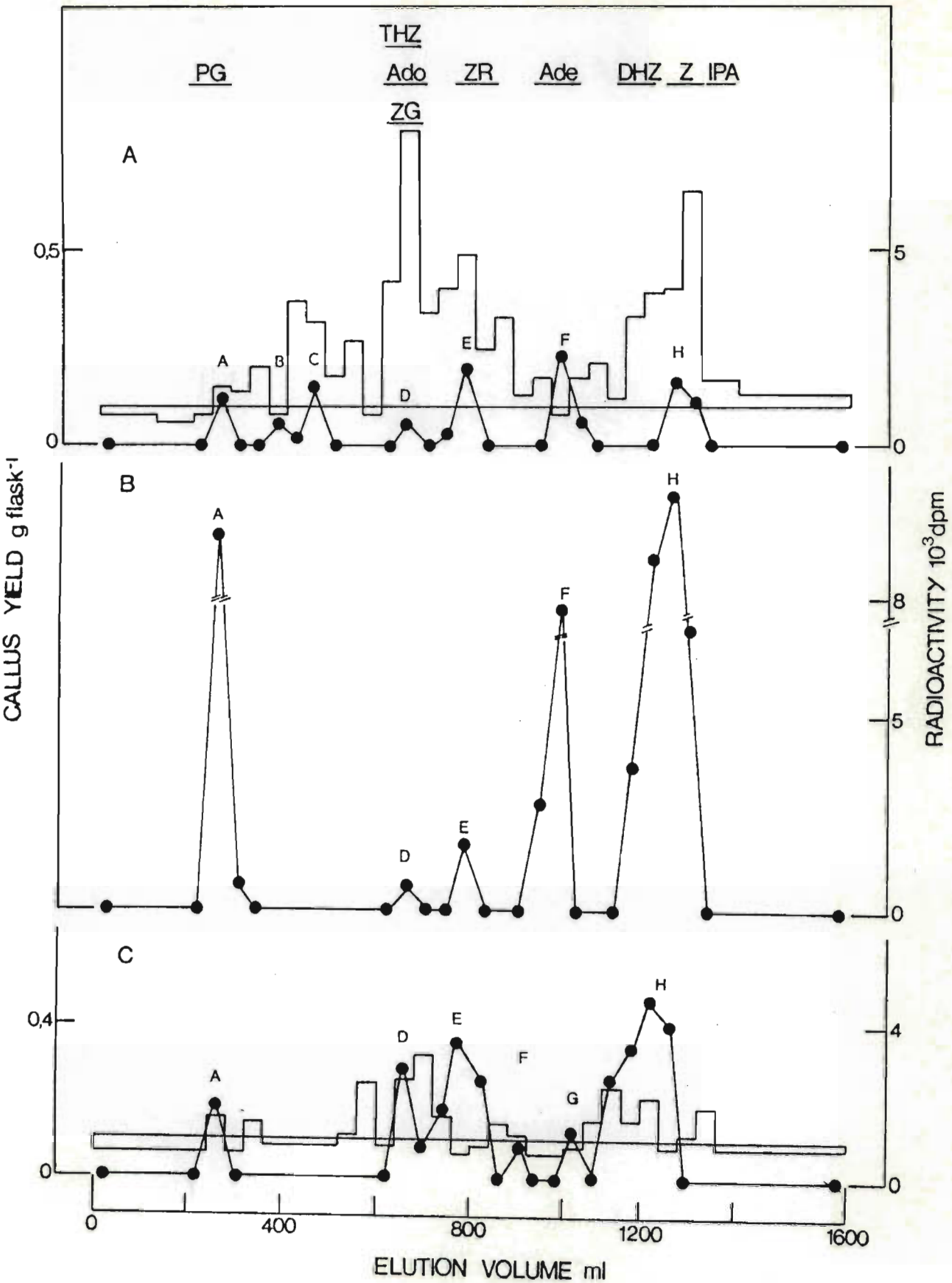

THZ
PG
Ado
ZR
Ade
DHZ
Z
IPA
ZG
A
B
C
D
E
F
G
H1
H2
0,5
0,5
0,4
5
5
4
0
0
0
0
400
800
1200
1600
CALLUS YIELD g flask-1
RADIOACTIVITY 10³dpm
ELUTION VOLUME ml

Figure 1.21 Radioactivity (●——●) and biological activity (histograms) in the bark of *Ginkgo biloba* explants, 48 hours after applying radioactive zeatin to leaves of these explants. A = bark from explants with senescing leaves; B = bark from explants with mature leaves; C = bark from explants with immature leaves. Extracts were fractionated on a Sephadex LH-20 column eluted with 10 per cent methanol. Radioactive peaks A, B, C, D, E, F, G and H are indicated on the figures. PG = purinyl glycine; Ado = adenosine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.


THZ
PG
Ado
ZR
Ade
DHZ
Z
IPA
ZG
A
B
C
D
E
F
G
H
0,5
0
5
0
8
5
0
0,4
0
4
0
0
400
800
1200
1600
ELUTION VOLUME ml
CALLUS YIELD g flask-1
RADIOACTIVITY 10³dpm

leaves and re-applied to mature leaves of explants. It must, however, be remembered that radioactive peak 2 (R_f 0,2-0,5) did not only contain glucosylated compounds. This makes any speculation about the transport and metabolism of glucosylated compounds difficult. Radioactive peak 1 (R_f 0,0-0,2) and radioactive peak 2 (R_f 0,2-0,5) were also both re-applied to mature leaves in order to determine whether these radioactive peaks would undergo further transport and/or metabolism.

Radioactive peak 2 (R_f 0,2-0,5) did not appear to be exported from *Ginkgo biloba* leaves to a much greater extent than was the radioactive zeatin over the 48 hour experimental time period (Table 1.6). Although in terms of the limited experimental period and the low cytokinin levels naturally present in plant tissue, the increase of 6 per cent exported radioactivity could be regarded as significant. Expressing the results per one gramme fresh weight as opposed to per explant component, did not affect the amount of radioactivity exported from the leaves, but did affect the distribution of the exported radioactivity. If the results were expressed per explant component, the highest percentage of radioactivity was recovered in the untreated leaves, with the lowest percentage being detected in the apices. If, however, the results were expressed per one gramme fresh weight, the highest percentages of radioactivity were recorded in the xylem and apices (Table 1.7).

Table 1.6 The distribution of radioactivity (% dpm/component and % dpm/one gramme fresh weight) 48 hours after applying radioactive peak 2 (R_f 0,2-0,5) to mature leaves of *Ginkgo biloba* explants.

Explant Component	Radioactivity	
	% dpm/explant component	% dpm/one gramme fresh weight
Treated leaves	92,60	92,40
Untreated leaves	3,50	1,96
Xylem	2,90	2,53
Bark	0,80	0,81
Apices	0,20	2,30

Table 1.7 The distribution of radioactivity (% dpm/explant component and % dpm/one gramme fresh weight) in those explant components to which radioactive compounds were translocated, 48 hours after re-applying radioactive peak 2 to the leaves of *Ginkgo biloba* explants.

Explant Component	Radioactivity	
	% dpm/explant component	% dpm/one gramme fresh weight
Untreated leaves	46,83	25,80
Xylem	40,30	33,31
Bark	10,26	10,60
Apices	2,61	30,29

Radioactive peak 2 did not appear to be metabolized to any extent in the leaves to which it was applied, but it was metabolized in the untreated components to which it was

transported (Table 1.8). Three radioactive peaks were detected on paper chromatograms following the re-application of radioactive peak 2. These radioactive peaks, which had the same R_f values as the radioactive peaks detected following the application of 8(^{14}C)*t*-zeatin to the explants, were referred to as radioactive metabolites 1, 2 and 3 (Table 1.8). Metabolite 3, which co-chromatographed with zeatin, ribosylzeatin and dihydrozeatin, was the major metabolite recovered in the xylem, bark and apices. Radioactive peak 3, which had the same R_f value as metabolite 3, was the major peak recovered in these components following the application of 8(^{14}C)*t*-zeatin to mature leaves. The presence of radioactive compounds in the xylem suggested that lateral transport took place. Metabolite 2 was the major peak in the untreated leaves. This metabolite,

Table 1.8 The distribution of radioactive metabolites 1 (R_f 0,0-0,2); 2 (R_f 0,2-0,5) and 3 (R_f 0,5-1,0) 48 hours after applying radioactive peak 2 (R_f 0,2-0,5) to mature leaves of *Ginkgo biloba* explants. Radioactivity is expressed as % dpm/metabolite.

Explant Component	Radioactivity (% dpm/Metabolite)		
	Metabolite 1 (R_f 0,0-0,2)	Metabolite 2 (R_f 0,2-0,5)	Metabolite 3 (R_f 0,5-1,0)
Treated leaves	4,40	90,30	5,30
Untreated leaves	17,00	83,00	0
Xylem	0	7,20	92,80
Bark	6,40	11,80	81,80
Apices	0	0	100

which had the same R_f value as the radioactive peak originally applied to the leaves, did not, however, correspond to the major radioactive peak, that is, peak 3, which was detected in the leaves following the application of 8(^{14}C)*t*-zeatin. From Figure 1.22A it can be seen that the radioactivity recovered in the untreated explants co-chromatographed with the biological activity associated with the components. That is, zeatin and ribosylzeatin appeared to represent the main cytokinins in the xylem and bark with cytokinin glucosides predominating in the leaves. The occurrence of different cytokinin metabolites in the various tissues illustrates the different functions, and thus metabolic capacities of these tissues.

In order to determine whether radioactive metabolites 1, 2 and 3 were similar to the original radioactive peaks formed from the radioactive zeatin, these metabolites were fractionated by column chromatography (Figure 1.23). These three metabolites were separated individually on a Sephadex LH-20 column eluted with 10 per cent methanol. If Figures 1.23 and 1.12 are compared with metabolites 1 and 2 constituting the A fraction and metabolite 3 the B fraction, it can be seen that some of the peaks detected appeared to be similar to the peaks detected following the application of radioactive zeatin to mature leaves. Peaks A, B, D, E, F and H appeared to be common to the radioactive peaks and metabolites. Radioactive metabolite 3 appeared to consist of zeatin and ribosylzeatin, possibly suggesting that some of the radioactivity associated with radioactive

Figure 1.22 Radioactivity (●——●) and biological activity (histograms) in the untreated *Ginkgo biloba* components following the re-application of radioactive peaks 1 and 2 to leaves of these explants. A = radioactive peak 2 (R_f 0,2-0,5) eluted from paper chromatograms and re-applied to mature leaves of explants; B = radioactive peak 1 (R_f 0,0-0,2) eluted from paper chromatograms and re-applied to mature leaves of explants. Extracts were purified using Dowex 50 cation exchange resin and the ammonia eluates were separated on paper chromatograms with *iso*-propanol:25 per cent ammonium hydroxide:water (10:1:1 v/v). ZG = zeatin glucoside; ZR = ribosylzeatin; Z = zeatin.

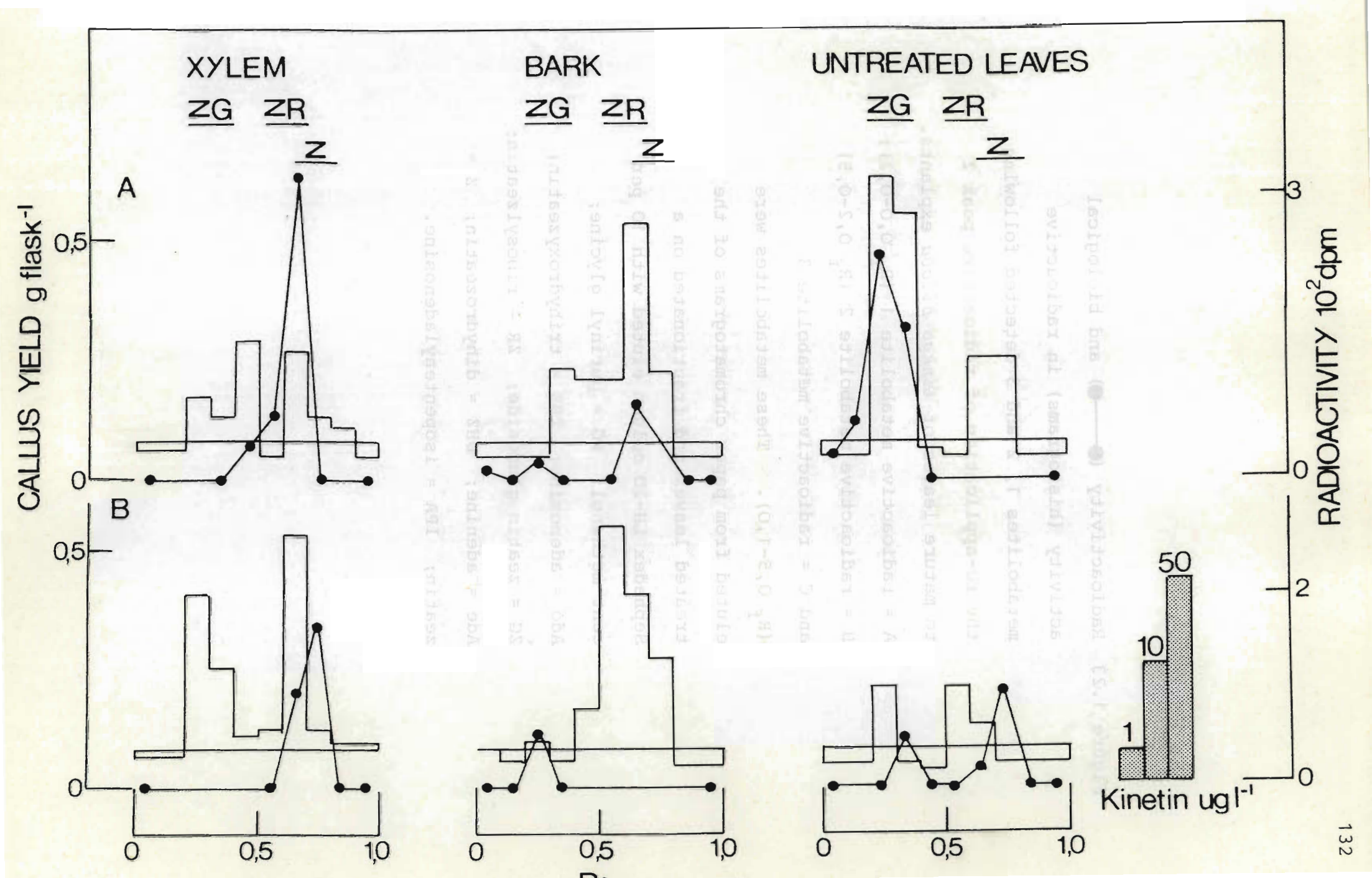
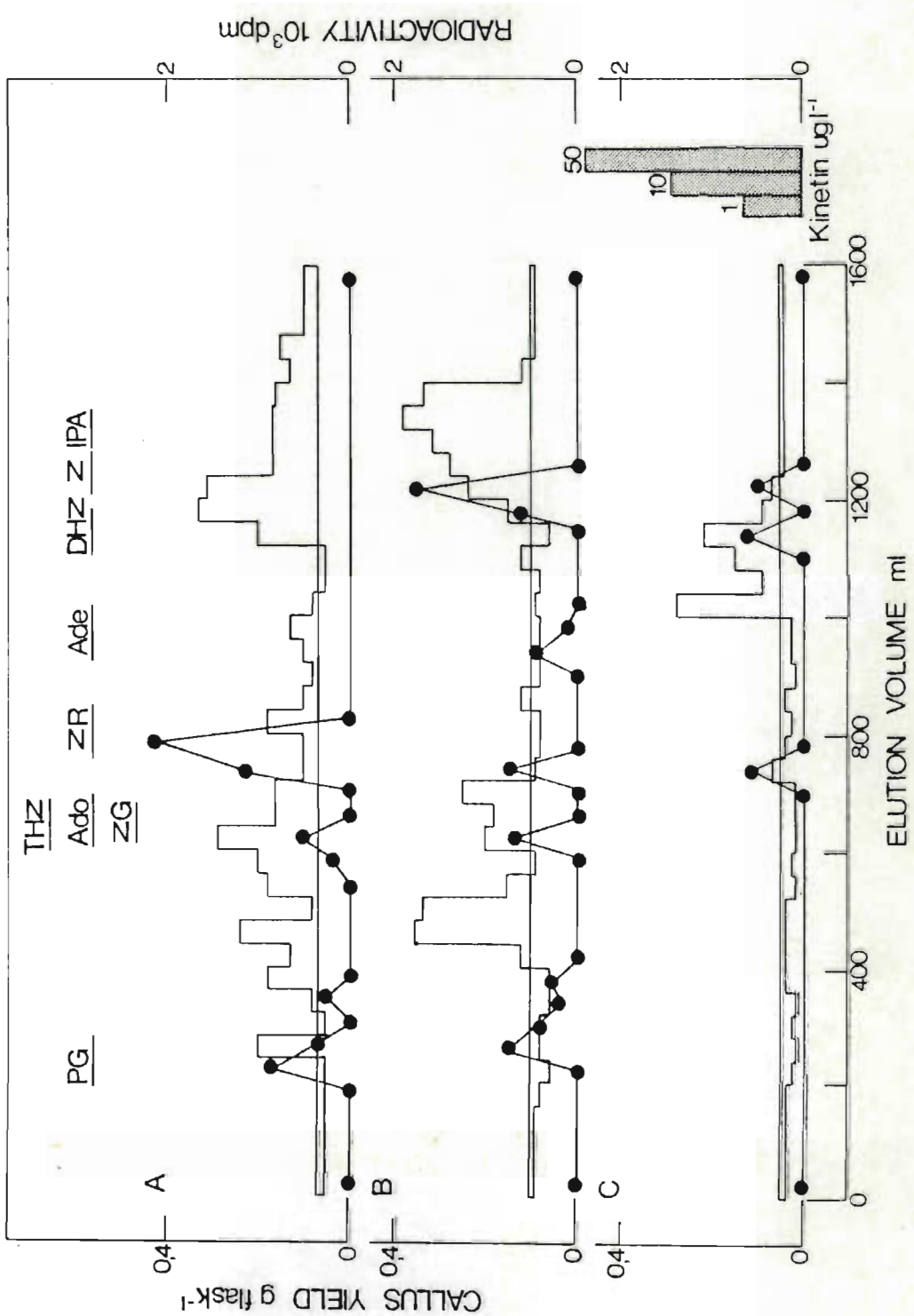

XYLEM
ZG ZR
Z
BARK
ZG ZR
Z
UNTREATED LEAVES
ZG ZR
Z
A
B
CALLUS YIELD g flask-1
0,5
0
0,5
0
0
0,5
1,0
0
0,5
1,0
0
0,5
1,0
3
2
0
RADIOACTIVITY 10²dpm
50
10
1
Kinetin ug l-1

Figure 1.23 Radioactivity (●——●) and biological activity (histograms) in radioactive metabolites 1, 2 and 3 detected following the re-application of radioactive peak 2 to mature leaves of *Ginkgo biloba* explants. A = radioactive metabolite 1 (R_f 0,0-0,2); B = radioactive metabolite 2 (R_f 0,2-0,5) and C = radioactive metabolite 3 (R_f 0,5-1,0). These metabolites were eluted from paper chromatograms of the treated leaves and fractionated on a Sephadex LH-20 column eluted with 10 per cent methanol. PG = purinyl glycine; Ado = adenosine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.


RADIOACTIVITY 10^3dpm
CALLUS YIELD g flask^{-1}
Kinetin ugl^{-1}
ELUTION VOLUME ml
A
B
C
PG
THZ
Ado
ZG
ZR
Ade
DHZ
Z
IPA

peak 2 was converted back to these cytokinins, although incomplete separation by paper chromatography could have accounted for the detection of these compounds.

From Table 1.9 it can be seen that the radioactivity associated with radioactive peak 1 (R_f 0,0-0,2) also did not appear to be exported out of mature *Ginkgo biloba* leaves to any extent. Less than 5 per cent of the recovered radioactivity was exported out of the leaves in 48 hours. This figure is higher than that exported out of the leaves treated with radioactive zeatin, but is lower than the 8 per cent exported following the reapplication of radioactive peak 2 to mature leaves. The exported radioactivity was mainly detected in the xylem. Expressing the results per one gramme fresh weight or per explant component did not affect the transport pattern observed (Table 1.10). The high radioactivity recorded in the xylem was probably the result of lateral transport.

Table 1.9 The distribution of radioactivity (% dpm/explant component and % dpm/one gramme fresh weight) 48 hours after applying radioactive peak 1 (R_f 0,0-0,2) to mature leaves of *Ginkgo biloba* explants.

Explant Component	Radioactivity	
	% dpm/explant component	% dpm/one gramme fresh weight
Treated leaves	95,04	95,84
Untreated leaves	0,28	0,16
Xylem	4,60	3,32
Bark	0,05	0,06
Apices	0,03	0,62

Table 1.10 The distribution of radioactivity (% dpm/explant component and % dpm/one gramme fresh weight) in those explants to which radioactive compounds were translocated 48 hours after re-applying radioactive peak 1 to the leaves of *Ginkgo biloba* explants.

Explant Component	Radioactivity	
	% dpm/explant component	% dpm/one gramme fresh weight
Untreated leaves	5,90	3,75
Xylem	92,36	79,82
Bark	1,04	1,42
Apices	0,70	15,00

Radioactive peak 1 resulted in the same three radioactive metabolites on paper chromatograms as did radioactive peak 2. These metabolites were also referred to as radioactive metabolites 1, 2 and 3, although it was realized that the radioactivity associated with metabolite 1 probably represented the original radioactivity of peak 1. Following re-application, radioactive peak 1 was not exported to the same extent as radioactive peak 2 but the results of paper chromatography suggested that it was more extensively metabolized (Table 1.11). In the treated leaves, 48 per cent of the radioactivity originally thought to be associated with radioactive peak 1, was detected at R_f 0,2-0,5. That is, at the R_f values of metabolite 2 or radioactive peak 2. Some of the radioactivity recovered as metabolite 2 may, however, represent residual radioactive peak 2 which did not separate from radioactive peak 1 on the paper chromatograms. No metabolite 1 was recorded in

Table 1.11 The distribution of radioactive metabolites 1 (R_f 0,0-0,2); 2 (R_f 0,2-0,5) and 3 (R_f 0,5-1,0) 48 hours after applying radioactive peak 1 (R_f 0,0-0,2) to mature leaves of *Ginkgo biloba* explants. Radioactivity is expressed as % dpm/metabolite.

Explant Component	Radioactivity (% dpm/Metabolite)		
	Metabolite 1 (R_f 0,0-0,2)	Metabolite 2 (R_f 0,2-0,5)	Metabolite 3 (R_f 0,5-1,0)
Treated leaves	50,60	48,20	1,20
Untreated leaves	0	27,30	72,70
Xylem	0	0	100
Bark	0	100	0
Apices	0	100	0

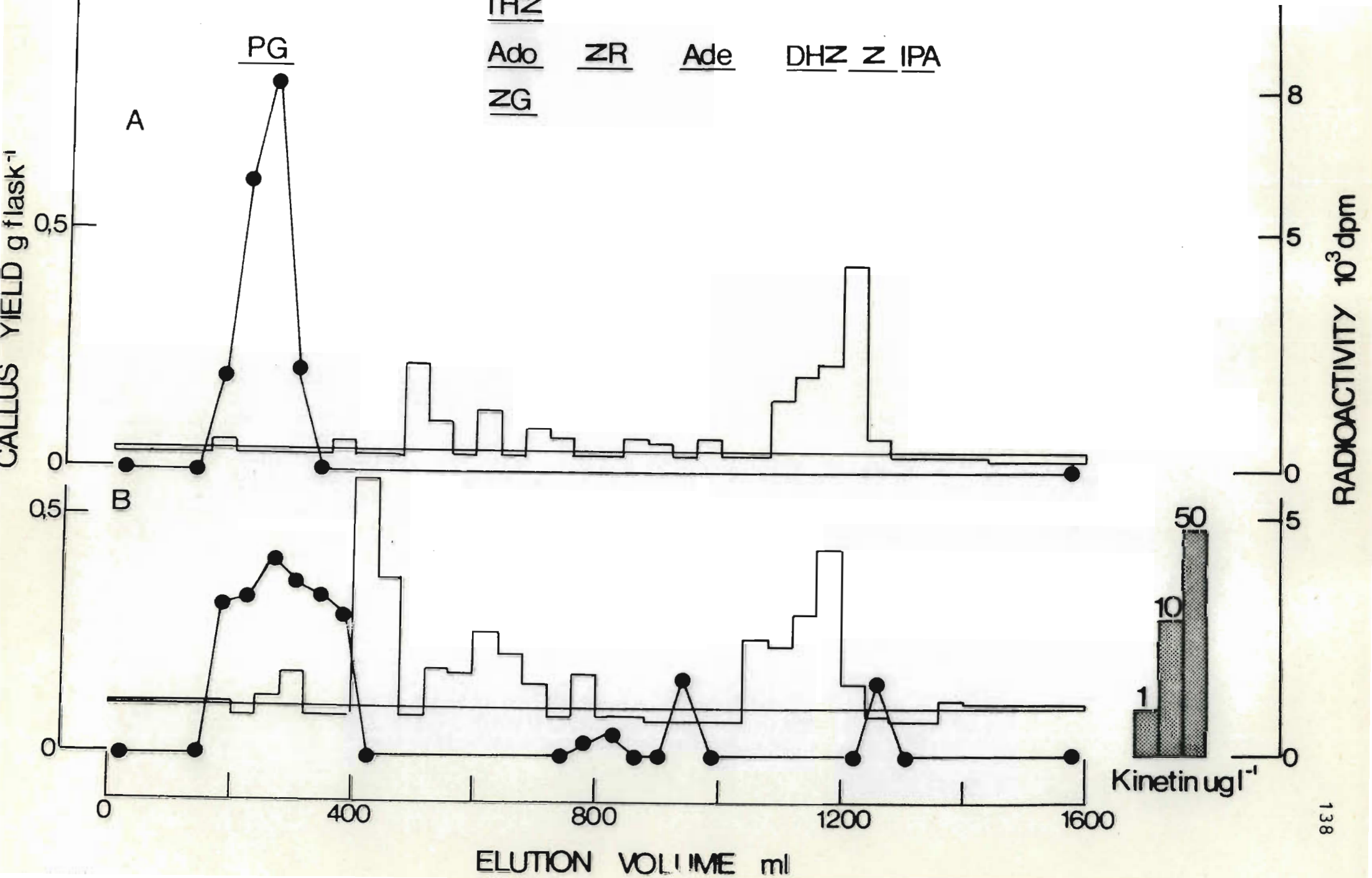
the radioactivity which was exported from the treated leaves. Metabolite 2 (R_f 0,2-0,5), which co-eluted with cytokinin glucosides, was the major metabolite in the bark and apices and metabolite 3 (R_f 0,5-1,0) was the major metabolite in the xylem and untreated leaves (Table 1.11).

The biological activity associated with the radioactivity recovered in the untreated explant components is shown in Figure 1.22B. The radio- and biological activity did not always correspond, although this might not have been expected as the endogenous cytokinins and the applied compounds could both cause growth of the soyabean callus bioassay. The same radioactive metabolite was detected in the xylem following the re-application of radioactive peaks

1 and 2. In the untreated leaves, two metabolites were detected following the re-application of peak 1 compared with the single metabolite detected when peak 2 was applied to the leaves. A single radioactive peak was detected in bark which corresponded to a minor metabolite detected in the bark of those explants to which radioactive peak 1 was re-applied (Figures 1.22A and B).

Column chromatography of radioactive metabolites 1 and 2 detected following the re-application of radioactive peak 1 (Figure 1.24), revealed a common compound in both of these metabolites. This compound had an elution volume of 200 to 320 millilitres, which would, therefore, co-elute with purinyl glycine. This compound appeared to have a more definite elution volume when metabolite 1 was fractionated on a Sephadex LH-20 column. When metabolite 2 was separated by column chromatography, it appeared as if another compound could have been incorporated in the major peak. Metabolite 2 also contained other smaller radioactive peaks which appeared to co-chromatograph with zeatin and ribosylzeatin. The occurrence of a single major radioactive peak in both metabolite 1 and 2 following column chromatography, appeared to contradict the paper chromatography results. Paper chromatography suggested that two distinct metabolites existed, namely metabolite 1 and 2. Column chromatography, however, showed that there was only one major peak which suggests that the separation of the extract by paper chromatography was not very efficient.

Figure 1.24 Radioactivity (●——●) and biological activity (histograms) in radioactive metabolites 1 and 2 detected following the re-application of radioactive peak 1 (R_f 0,0-0,2) to mature leaves of *Ginkgo biloba* explants. A = radioactive metabolite 1 (R_f 0,0-0,2); B = radioactive metabolite 2 (R_f 0,2-0,5). These radioactive metabolites were eluted from paper chromatograms of the treated leaves and fractionated on a Sephadex LH-20 column eluted with 10 per cent methanol. PG = purinyl glycine; Ado = adenosine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.


THZ
PG
Ado
ZR
Ade
DHZ
Z
IPA
ZG
A
B
CALLUS YIELD g flask⁻¹
0,5
0
RADIOACTIVITY 10³dpm
8
5
0
50
10
1
Kinetin ugl⁻¹
0
400
800
1200
1600
ELUTION VOLUME ml

Generally it can be said that the re-applied radioactive peaks were exported from the treated leaves to a greater extent than the radioactive zeatin itself. The significance of the exported radioactivity is difficult to assess, but in terms of the experimental time period of 48 hours and the excessive cytokinin concentration applied to the leaves, it is probably important. Lateral transport from the bark to the xylem appears to occur to a greater extent following the re-application of the radioactive peaks than following the application of radioactive zeatin.

1.4 Discussion

Ginkgo biloba leaves exhibit similar seasonal trends in cytokinin activity to those recorded for other deciduous species. That is, low cytokinin activity, mainly due to compounds co-eluting with zeatin and ribosylzeatin, was recorded in immature leaves in spring. Cytokinin activity increased in mature leaves, mainly as a result of the accumulation of cytokinin glucosides. Cytokinin levels decreased slightly in senescing leaves in autumn and glucosides were the major cytokinins in these leaves. Similar increases in cytokinin activity and glucosylation capacity with leaf maturation have been previously recorded in the leaves of *Populus* species (ENGELBRECHT, 1971; HEWETT and WAREING, 1973b), *Acer platanoides* L. (ENGELBRECHT, 1971), *Ginkgo biloba* (VAN STADEN, 1976b), *Salix babylonica* (VAN STADEN, 1977; VAN STADEN and DAVEY, 1981b) and *Alnus glutinosa* (HENSON, 1978a,b). From this study, the endo-

genous cytokinins which were tentatively identified in the *Ginkgo biloba* leaves included zeatin, ribosylzeatin, dihydrozeatin and their glucosylated derivatives, as well as possibly dihydroribosylzeatin. Purinyl glycine and other oxidation products of zeatin, which appeared to be metabolites of the radioactive zeatin, did not occur as endogenous cytokinins in these leaves.

Although it has often been agreed that naturally occurring cytokinins are not very effective in delaying senescence (VARGA and BRUINSMA, 1973; BIDDINGTON and THOMAS, 1978; DUMBROFF and WALKER, 1979), ribosylzeatin has been reported to be more effective than zeatin in delaying leaf senescence (DUMBROFF and WALKER, 1979). VAN STADEN (1976b) has reported that the ratio of zeatin to ribosylzeatin in *Ginkgo biloba* leaves changed with time, with yellow senescing leaves containing low ribosylzeatin and high zeatin levels. Higher levels of ribosylzeatin than zeatin were also recorded in mature leaves in this study (Figure 1.3). These observations were based on the fact that zeatin and ribosylzeatin are equally active in the soyabean callus bioassay (VAN STADEN and DAVEY, 1977). The high cytokinin glucoside levels recorded in mature and senescing leaves do not appear to be effective in delaying leaf senescence, which could thus suggest that a continuous supply of ribosylzeatin and zeatin is necessary to delay leaf senescence. PALMER, HORGAN and WAREING (1981a) have also suggested that a continuous supply of cytokinin, rather than a high level of cytokinin which may be inactivated by

metabolism and/or compartmentation in the leaf, may be required to prevent leaf senescence. The actual activity of the accumulated cytokinin glucosides in leaves has also been questioned. It has been suggested that cytokinin glucosides are not involved in the physiological responses normally associated with cytokinins (VAN STADEN and DAVEY, 1979) and it has also been proposed that glucosides may only be active by virtue of their hydrolysis in test tissue, but may, in fact, be inactive in the endogenous situation (HENSON, 1978a). This could support the suggestion that a continuous cytokinin supply is necessary to delay leaf senescence.

Cytokinin glucosides are often regarded as storage compounds (PARKER and LETHAM, 1973; WAREING, HORGAN, HENSON and DAVIS, 1976; HENSON and WHEELER, 1977a; VAN STADEN and DAVEY, 1979; VONK and DAVELAAR, 1981). The accumulation of cytokinin glucosides in mature deciduous leaves, and the fact that these compounds can potentially be hydrolyzed to free bases and their ribosides, led to the suggestion that cytokinin glucosides represent a potential cytokinin reservoir in these leaves. This reservoir should be exported out of the leaves before they abscise if these cytokinins are not to be lost to the system (VAN STADEN and BROWN, 1978). In the *Ginkgo biloba* explants, the endogenous activity of the bark followed a similar pattern to that of the leaves. That is, cytokinin activity and glucoside levels increased in the bark as the leaves matured. No cytokinin glucosides were detected in

the xylem and the presence of these cytokinins in the bark implied that they may have originated in the leaves. The maximum export of cytokinins appeared to occur from mature leaves. This can, however, only be regarded as circumstantial evidence for the export of cytokinins from these leaves. The cytokinin activity in the apices of explants with senescing leaves was high, while that in the bark and leaves of these explants decreased. This decrease in cytokinin activity in the bark could suggest a decrease in cytokinin export to the buds, which were becoming dormant. The cytokinins in the leaves may, therefore, have been transported to the buds at an earlier stage, that is, predominantly from mature leaves. The cytokinins in the buds could, however, have been transported directly from the roots (VAN STADEN and DAVEY, 1979) or may have been synthesized in the buds themselves (KANNANGARA and BOOTH, 1974). The presence of cytokinins in the bark of senescing explants suggests that cytokinins from the leaves are stored in the bark.

Earlier work regarding the export of cytokinins from leaves is contradictory. SKENE (1972b) proposed that cytokinins were not exported out of leaves as cytokinin activity in *Vitis vinifera* leaves decreased in both the presence and absence of the phloem, whereas KANNANGARA and BOOTH (1974) suggested that cytokinins were transported out of *Dahlia variabilis* (Willd) Desf. leaves. The increase of cytokinin glucoside activity in the phloem of *Salix babylonica* in autumn (VAN STADEN and BROWN, 1978) has also been re-

garded as evidence that cytokinins are exported out of leaves. This can, however, also only be regarded as circumstantial evidence for the export of cytokinins. The radioactive zeatin applied to *Ginkgo biloba* leaves was exported out of these leaves, although the significance of the percentage radioactivity exported was difficult to determine. The radioactive zeatin applied to the leaves did undergo some glucosylation but less than 2 per cent of the radioactivity, associated with zeatin and/or its metabolites, was exported out of expanding, mature and senescing leaves. When assessing the significance of this low percentage of radioactivity exported out of the treated leaves, there are a number of factors which must be considered. These include the unphysiological cytokinin concentration applied to the leaves, the limited experimental period of 48 hours and interruption of phloem transport in the explants. It must also be realized that all the radioactivity would probably never all be exported out of the leaves to which it was applied.

It is only logical to assume that any concentration of exogenously applied cytokinin will be excess in terms of the endogenous cytokinin levels in the tissue. This in itself, poses the question whether the transport and metabolism of exogenous cytokinins can be regarded as being normal. The application of a single cytokinin will also create an imbalance with regard to the diversity of cytokinins naturally present in any tissue. The above factors could have a profound effect on the metabolism and/

or transport of the radioactive zeatin applied to the leaves. Another factor to be considered is that the export of radioactive compounds was only monitored over a 48 hour period. It cannot be expected that most of the radioactivity be exported out of the leaves during this period as export is probably a continuous process. The application of radioactive zeatin at an isolated time may also not be representative of the whole process. It could be suggested that monitoring export over a longer period would perhaps result in an increase in exported radioactivity. The export of radioactivity from senescing leaves was recorded over 48 and 96 hours. In this case, the extended experimental period did not increase the amount of radioactivity exported but did affect the proportions of zeatin metabolites formed. That is, there was an increase in radioactive peaks 1 and 2, which are the radioactive peaks formed from the applied zeatin. It was also found that following the re-application of radioactive peaks 1 and 2 to mature leaves, a greater percentage of radioactivity was exported to the untreated components than following the application of zeatin to these leaves. This could imply that the radioactive zeatin had to be metabolized before it was transported out of the leaves and that a longer experimental period, especially in the case of immature and mature leaves which appeared to have a faster rate of zeatin metabolism than senescing leaves, could potentially result in increased export from the leaves. The fact that explants were used in the experiments must also be considered in relation to transport. The use of explants would result in

interruption of phloem transport. RAMINA, PIMPINI, BONIOLO and BERGAMASCO (1979) have pointed out that translocation and adsorption of exogenous and endogenous growth substances can be affected in explants because different organs can, by correlative phenomena affect these processes. Interruption of phloem transport, could thus affect the transport of radioactive compounds out of the leaves. In terms of endogenous cytokinin activity, it must also be remembered that high cytokinin levels have been detected in leaves after they have abscised (ENGELBRECHT, 1971; VAN STADEN, 1976b). This could, therefore, suggest that if cytokinin export does take place under natural conditions, only a small proportion of the total cytokinin content of the leaf is involved. Taking the above points into consideration, the small percentage radioactivity exported out of leaves may, in fact, be relevant.

Other research workers have regarded the small percentage of radioactivity exported out of leaves as being relevant. VAN STADEN (1982a) suggested that the small percentage of applied zeatin and/or its metabolites exported from mature *Rosa hybrida* leaves was sufficient to stimulate growth of axillary buds associated with the treated leaves. In this experiment, only 1, 4 and 0,4 per cent of the total radioactivity was exported out of the treated leaves after four and eight days, respectively. VAN STADEN and DAVEY (1981a) reported that less than 1 per cent of the radioactivity, associated with the radioactive zeatin was exported out of treated *Lupinus albus* leaves. VONK and DAVELAAR (1981),

using radioactive cytokinins, also reported that cytokinins were transported out of *Yucca flaccida* leaves. In the case of *Ginkgo* leaves, however, it might have been anticipated that the amount of cytokinin exported from mature and senescing leaves would have exceeded that exported from young expanding leaves. It has been suggested that cytokinins are rapidly utilized in expanding leaves HENSON, 1978b; HENDRY, VAN STADEN and ALLAN, 1982). Rapid utilization would not favour the export of cytokinins out of these leaves. The accumulation of cytokinins in mature and senescing leaves makes the possibility of export from these leaves more feasible. Despite the earlier considerations, the fact that approximately equal amounts of radioactivity were exported from the leaves of all three ages could suggest that this export may not have been important. This exported radioactivity may have represented passive transport along with the assimilate stream, resulting from the excess cytokinin applied to the leaves, rather than active transport out of the leaves.

Although the approximately equal amounts of radioactivity exported from immature, mature and senescing leaves may not favour active transport out of these leaves, the fact that a definite distribution pattern appeared to exist, is interesting and could imply that the exported radioactivity was significant. The bark appeared to be the primary recipient in the explants with immature and senescing leaves. The highest percentage of radioactivity was recorded in the apices of explants with mature leaves. Cytokinins

would be exported from the leaves via the phloem. The fact that radioactivity was detected in the xylem, implies that lateral transport must have occurred. VAN STADEN and HUTTON (1981) have previously reported that lateral transport from bark to xylem did take place in dormant *Salix babylonica* cuttings. Lateral transport occurred most extensively in the explants with immature leaves and did not appear to take place in explants with senescing leaves. Lateral transport took place fairly extensively following the re-application of radioactive peaks 1 and 2 to the mature leaves. This is interesting with regard to the rapid metabolism of the applied zeatin in immature leaves which resulted in a greater percentage of radioactive peaks 1 and 2 being formed. This could imply that the formation of these radioactive peaks facilitated lateral transport. The export pattern detected suggests that leaf cytokinins do contribute to the cytokinin content of the buds, as suggested by VAN STADEN, SPIEGELSTEIN, ZIESLIN and HALEVY (1981) and VAN STADEN (1982a). Mature leaves appeared to be most important in this respect. Once the leaves had begun to senesce and the buds had begun to become dormant, the cytokinins exported out of the leaves appeared to be stored in the bark rather than be exported to the buds. A greater percentage of radioactivity appeared to be exported to the untreated senescing leaves than to the dormant buds. VAN STADEN and DAVEY (1981b) have proposed that the bark could play an important function in regulating cytokinin levels which could account for the accumulation of radioactive compounds

in the bark of the senescing explants. To a certain extent, these results correspond to the endogenous cytokinin activity recorded. That is, cytokinins appear to be primarily exported from mature leaves to the apices, rather than from the senescing leaves. If, however, the export of the radioactive compounds is merely a passive transport and a function of the assimilate stream, then the distribution pattern detected may, in fact, not be relevant.

Although it is difficult to assess the significance of the exported radioactivity, it is possible that some of the cytokinins, which accumulate in leaves, are exported out of the leaves before they are shed. Not all cytokinins are, however, exported out of senescing leaves. ENGELBRECHT (1971), VAN STADEN (1976b) and VAN STADEN and DAVEY (1981b) have all reported high cytokinin activity in leaves of *Populus tremula* L., *Acer platanoides* L., *Ginkgo biloba* and *Salix babylonica* once they had senesced and fallen from the trees. PALMER, HORGAN and WAREING (1981a) have proposed that the cytokinins remaining in senescing leaves may represent a basal level of leaf cytokinin which will never be exported or further metabolized. So, although cytokinin glucosides could represent a potential reservoir, which could be re-utilized, they could also fulfil another function in leaves.

Leaf senescence must be regarded as an integral part of leaf development as well as the ultimate survival of the tree. During development, deciduous leaves can be said

to undergo changes in status. According to THOMAS and STODDART (1980), during the early stages of leaf development, the leaf is predominantly heterotrophic, becoming a net exporter of photosynthates, when mature. Thereafter, the leaf is a declining exporter of photosynthetic carbon but becomes a major source of nitrogen, phosphorus, potassium and other minerals as a result of nutrient mobilization during senescence. If the various cytokinins present during leaf development are considered, a possible role for cytokinin glucosides emerges. Zeatin and ribosylzeatin are the predominant cytokinins in young expanding leaves. These cytokinins are reported to be effective in delaying leaf senescence by being able to delay DNA, RNA, chlorophyll and protein degradation (THOMAS, 1975; THIMANN, 1977; NAITO, TSUJI and HATAKEYAMA, 1978) and are also thought to be involved in nutrient mobilization. These cytokinins could, therefore, have an important role in rapidly dividing immature leaves with regard to cell division and nutrient mobilization. Cytokinin glucosides are apparently not able to bring about nutrient mobilization and are not able to delay leaf senescence (VAN STADEN and DAVEY, 1979). That is, cytokinin glucosides are not able to bring about the physiological processes normally associated with zeatin and ribosylzeatin. The glucosylation capacity of mature leaves could, in fact, be a function of senescence and could be involved in the regulation of leaf metabolism. The accumulation of these cytokinins could also facilitate the transport of nutrients out of the leaves before they senesce. That is, glucosylation 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 plants and to the buds. It appears that the sink capacity of leaves is such that a large proportion of root-produced cytokinins are continually transported to the leaves. It may, therefore, be possible that the functional cytokinins (zeatin and ribosylzeatin) arriving in the leaves becomes excessive once the leaves begin to mature and senesce. Glucosylation could, therefore, be a mechanism for inactivating this excess, which would then eventually be lost to the system. Dihydro glucosides, which are reported to be more stable than zeatin glucosides (HENSON, 1978a), have often been reported as the major glucosides in leaves (WANG, THOMPSON and HORGAN, 1977; PALMER, HORGAN and WAREING, 1981a,b) which could support the proposed inactivation function of cytokinin glucosides. Although storage and re-utilization is a possible function of cytokinin glucosides, it is difficult to assess the extent to which this actually occurs in the intact tree. The stress situation created by the use of an explant may have some effect on leaf cytokinin re-utilization. Inactivation would perhaps appear to be the major function of cytokinin glucosides which accumulate in mature and senescing leaves.

The same three radioactive peaks were detected on paper chromatograms following the application of 8(^{14}C)*t*-zeatin to expanding, mature and senescing *Ginkgo biloba* leaves. Although the proportions of these radioactive peaks

differed, the same radioactive compounds were formed irrespective of whether the leaves were actively dividing or degenerating. This could imply that the enzymes necessary for zeatin metabolism were present in general cytoplasm. VAN STADEN (1979b) has previously reported that cell-free extracts from senescing *Ginkgo biloba* leaves had the capacity to metabolize applied zeatin, although it appeared that cell-free extracts were less efficient in the conversion of zeatin to zeatin glucoside. As with transport, it is difficult to determine whether the metabolism observed reflects the metabolic fate of the cytokinins arriving in the leaves via the transpiration stream. Some of the nine radioactive peaks formed from the radioactive zeatin co-chromatographed with the endogenous cytokinins found in the leaves. This does not, however, necessarily mean that the observed metabolic pathway may be the normal pathway operating at that time in the leaves. The radioactive zeatin appeared to be metabolized most rapidly in expanding immature leaves. The major radioactive peaks in these leaves co-eluted with ribosylzeatin, dihydrozeatin and zeatin. The latter peak probably represented the original radioactive zeatin applied to the leaves. Other radioactive peaks in these leaves co-eluted with purinyl glycine, zeatin glucoside and ribosylzeatin glucoside, which do not appear to be endogenous cytokinins occurring in these leaves. The latter three compounds chromatographed at R_f 0,0-0,5 on paper chromatograms and accounted for 51 per cent of the recovered radioactivity. Zeatin, ribosylzeatin and their dihydro derivatives are normally the only

detectable cytokinins in immature *Ginkgo biloba* leaves. Glucosylation and oxidation are apparently not normal features of these leaves which tends to suggest that the metabolism of the exogenous zeatin was not indicative of the metabolism of the endogenous zeatin arriving in these leaves. The application of zeatin to these leaves may have resulted in exceeding the level of functional cytokinins capable of being utilized by immature leaves. The excess cytokinin could, therefore, have been inactivated by means of oxidation and to a lesser extent, by glucosylation. The formation of glucosylation and oxidation products would thus have theoretically advanced the leaf to the next stage of metabolic development, which may, in some way, account for the unexpected export of cytokinins from the immature leaves. Metabolism of the labelled zeatin appeared to be slower in the mature and senescing leaves. Except for two unidentified peaks, as well as a radioactive peak which co-eluted with purinyl glycine, the other radioactive peaks appeared to correspond to the endogenous cytokinin complement of these leaves. The ratio of glucosylated and non-polar radioactive compounds did not, however, correspond to the ratio of endogenous cytokinins, which was probably the result of the slow rate of metabolism of the exogenously applied zeatin in these leaves. Compounds co-eluting with ribosylzeatin, zeatin glucoside, ribosylzeatin glucoside and their dihydro derivatives as well as purinyl glycine and adenine, appeared to be the main metabolites of the radioactive zeatin in the mature and senescing leaves. Inactivation of cytokinins, arriving

in mature and senescing leaves, by means of glucosylation appeared to be a normal feature of these leaves, but oxidation did not appear to be a major metabolic pathway normally operative in these leaves. To a large extent, the radioactivity recovered in the untreated components of the explants corresponded to the endogenous cytokinin content of these components. This suggested that the exported radioactivity followed the normal metabolic pathway operating in these untreated components. The low percentage of exported radioactivity may have been more representative of the cytokinin concentrations of these components than the exogenous zeatin applied to the leaves, and may thus result in the normal metabolic pathway being followed.

The 8(^{14}C)*t*-zeatin applied to the *Ginkgo biloba* leaves, therefore,appeared to undergo a number of metabolic steps (Diagram 1.3). Ribosylation (VAN STADEN and DAVEY, 1977; VONK and DAVELAAR, 1981; HUTTON and VAN STADEN, 1982), side chain reduction resulting in the formation of dihydro derivatives (SONDHEIMER and TZOU, 1971), glucosylation and oxidation (VAN STADEN and HUTTON, 1982), all appeared to be processes involved in the metabolism of the radioactive zeatin. Oxidation, possibly resulting in the formation of purinyl glycine, appeared to be important in actively growing leaves and glucosylation was more important in senescing leaves. This could perhaps be related to the status of a leaf during its development and the possible functions of cytokinin glucosides in leaves. That is,

photosynthates would be expected to be predominantly exported from senescing leaves and the accumulation of cytokinin glucosides would enhance this process. Glucosylation would, therefore, not be feasible in immature leaves which are regarded as being predominantly heterotrophic (THOMAS and STODDART, 1980). Metabolic studies carried out on leaves thus far do not seem to reflect a common metabolic pattern, although leaf age and leaf type must be taken into account when considering this. Glucosylation, ribosylation, side chain reduction and oxidation resulting from side chain cleavage have all previously been recorded in the leaves of deciduous species. LETHAM, PARKER, DUKE, SUMMONS and MACLEOD (1976) reported that dihydrozeatin, zeatin and dihydroribosylzeatin glucoside were the major metabolites of H^3-zeatin applied to detached mature *Populus alba* leaves. HENSON (1978a) reported adenine, adenosine, zeatin glucoside and dihydrozeatin as the major metabolites of radioactive zeatin fed to detached *Alnus glutinosa* leaves. DUKE, LETHAM, PARKER, MACLEOD and SUMMONS (1979) also applied H^3-zeatin to excised *Populus albus* and they recorded adenosine, zeatin-O-glucoside, dihydrozeatin-O-glucoside and dihydroribosylzeatin-O-glucoside as the major metabolites. Adenine, adenosine and glucosylated cytokinins, therefore appear to be the compounds most commonly recorded in these deciduous leaves. These compounds, especially adenine and adenosine, did not appear to be major metabolites in the leaves of the *Ginkgo biloba* explants. A number of the radioactive peaks were detected in the *Ginkgo biloba* which co-chromatographed with peaks which

were formed following the oxidation of zeatin with potassium permanganate (Figure 1.19) (VAN STADEN, DREWES and HUTTON, 1982). Two of these peaks, namely radioactive peaks D and F co-eluted with adenosine and adenine, respectively but the biological activity associated with these peaks implied that they may not have been adenosine and adenine, as those compounds are inactive in the soyabean callus bioassay (VAN STADEN and DAVEY, 1977; VAN STADEN, 1979a). The radioactive peak co-eluting with adenine was not identified. Zeatin glucoside and trihydroxyzeatin have similar elution volumes to adenosine in the chromatographic system used and could thus have been constituents of this peak. This could suggest that further consideration should be given to previous reports that adenine and adenosine are metabolites of zeatin. The radioactive peak co-eluting with purinyl glycine also corresponded with one of the compounds formed following the oxidation of zeatin. Purinyl glycine is the end-product of zeatin oxidation (MILLER, 1965; LETHAM, SHANNON and MCDONALD, 1967) and trihydroxyzeatin is reported to be an intermediate in this oxidation reaction (VAN STADEN, DREWES and HUTTON, 1982) and has previously been recorded in plant tissue (LETHAM, 1973). The presence of purinyl glycine could, therefore, imply that trihydroxyzeatin was a component of the radioactive peak co-eluting with adenosine. The detection of a compound co-eluting with purinyl glycine, especially in the actively growing leaf tissue is interesting. This compound, which has similar chromatographic properties (VAN STADEN, DREWES and HUTTON, 1982) to the cytokinin nucleotides (VONK and DAVELAAR, 1981) has not been previously recorded as a metabolite of zeatin

in leaves. Although the applied zeatin was readily metabolized, the re-application of radioactive peaks 1 and 2 revealed that this metabolism may also have been reversible. The significance of this is not clear but it could be suggested that some metabolites were storage and/or transport forms which could be re-utilized when necessary.

The fact that radioactivity was exported to a greater extent following the re-application of radioactive peaks 1 and 2 than following the application of zeatin, implies that zeatin is not exported from the leaves *per se* but is metabolized prior to transport. The detection of radioactive peak 3, which co-eluted with zeatin and probably represented unmetabolized radioactive zeatin as the major peak in the bark of all extracts, following the application of zeatin to the leaves, contradicts the above statement. Column chromatography of the bark extracts (Figure 1.21) confirmed that compounds co-eluting with zeatin were, in fact, the major cytokinins in the bark, although smaller peaks co-eluting with purinyl glycine, ribosylzeatin, adenine and trihydroxyzeatin, zeatin glucoside and adenosine, were also detected. The fact that radioactive peak 3 was the major peak in the apices and untreated leaves also implies that zeatin was exported *per se*. It is difficult to reconcile these conflicting observations, especially as radioactive metabolite 3 (which has the same R_f values as radioactive peak 3) was the major metabolite in the bark and xylem following the re-application of radioactive peak 2, whereas metabolite 2 (which has the same R_f values as

radioactive peak 2) was the major metabolite in the bark following the re-application of radioactive peak 1. VONK and DAVELAAR (1981) proposed that glucosides were hydrolyzed prior to being transported out of *Yucca flaccida* leaves, whereas VAN STADEN (1982a) has reported that, although it was difficult to determine whether zeatin or its metabolites were exported from *Rosa hybrida* leaves, there were indications that a certain amount of unmetabolized zeatin was exported from the mature leaves. Although the results of these experiments are not conclusive, it would appear that zeatin was the cytokinin primarily involved in export, although further metabolism probably occurred in the bark as suggested by the presence of endogenous cytokinins co-eluting with zeatin glucoside in the bark. This would imply that cytokinin glucosides are hydrolyzed prior to being exported out of the leaves. The excess zeatin applied to the leaves should, however, also be considered when determining which cytokinins are actually involved in transport.

In conclusion, it can be said that *Ginkgo biloba* leaves do have the potential to export cytokinin from the leaves and that cytokinin glucosides could, therefore, fulfil a storage function. The extent to which this occurs under natural conditions could perhaps be more accurately assessed using intact trees. Not all the cytokinins which accumulate in leaves are, however, exported, suggesting that inactivation by means of glucosylation, which may be a mechanism for regulating leaf metabolism, is also a function of the accumulated cytokinin glucosides. Although ribosylation,

side chain reduction and glucosylation, which appear to be normal metabolic pathways of the leaves, did take place, oxidation also appeared to be an important metabolic pathway for the exogenously applied zeatin. In terms of endogenous cytokinin activity, oxidation does not appear to be a normal metabolic pathway of the leaves, suggesting that this could be a mechanism for dealing with the unphysiological concentration of zeatin applied to the leaves.

CHAPTER TWO

THE TRANSPORT AND METABOLISM OF 8(^{14}C)*t*-ZEATIN APPLIED TO *CITRUS SINESIS* LEAVES

2.1 Introduction

Comparatively few studies have been carried out on the cytokinin activity of leaves of evergreen species. The work done by LORENZI, HORGAN and WAREING (1975) on *Picea sitchensis* Carriere, by VAN STADEN (1978) on *Encephalartos natalensis* Dyer and Verdoorn, *Podocarpus henkelii* Stapf. and *Welwitschia mirabilis* Hook., by ILAN and GOREN (1979), who investigated cytokinins in senescing lemon leaves and by HENDRY, VAN STADEN and ALLAN (1982) on *Citrus sinesis* (L.) Osbeck, probably constitutes all the research published on endogenous cytokinins in evergreen leaves. The leaves of evergreen species exhibit seasonal fluctuations in cytokinin activity with qualitative and quantitative changes taking place. High zeatin and ribosylzeatin levels, and almost undetectable cytokinin glucoside levels are reported to be present in the leaves in spring and summer. The situation appears to be reversed in autumn and winter. Cytokinin glucoside levels are high and zeatin and ribosylzeatin levels are low during these periods of slow growth (LORENZI, HORGAN and WAREING, 1975; HENDRY, VAN STADEN and ALLAN, 1982). Cytokinin glucosides also accumulate in mature and senescing deciduous leaves in autumn (HEWETT and WAREING, 1973b; VAN STADEN, 1976b, 1977; HENSON, 1978a,b). Most of the accumulated cytokinin glucosides in deciduous leaves

appear to be lost from the plants when the leaves abscise (ENGELBRECHT, 1971). The leaves of citrus trees are reported to abscise after 17 to 24 months on the tree (HENDRY, 1980). Cytokinin glucosides are, therefore, not lost to trees by leaf abscission. The exact fate of these compounds has not been conclusively determined. LORENZI, HORGAN and WAREING (1975) have proposed that cytokinin glucosides are inactivation or storage compounds. These authors suggested a possible interconversion of cytokinin glucosides and zeatin and ribosylzeatin, as there appears to be a reciprocal relationship between these cytokinins. This proposed interconversion of cytokinins is based on the observation that cytokinin glucoside levels decrease simultaneously with the increase in zeatin and ribosylzeatin levels. HENDRY, VAN STADEN and ALLAN (1982) have also suggested that unless cytokinin glucosides are catabolically metabolized or exported out of the leaves to the developing shoot during the flush of new growth, then it is probable that they are hydrolyzed to zeatin and ribosylzeatin, as suggested by LORENZI, HORGAN and WAREING (1975).

The aim of this experiment was to determine whether cytokinins are exported from evergreen leaves following a quiescent period. HENDRY, VAN STADEN and ALLAN (1982) have suggested that cytokinin remobilization and utilization or export to the new shoot may occur from mature *Citrus sinesis* leaves following quiescent periods. Radioactive zeatin was, therefore, applied to dormant *Citrus sinesis* trees and its transport and metabolism was monitored during

the new flush of growth. That is, are leaf cytokinins utilized in the buds and new shoots associated with the flush of growth. Although zeatin glucoside was not applied to these leaves, it was also hoped to obtain some information on the fate of cytokinin glucosides during the next growing season.

2.2 Experimental Procedure

Evergreen plants, which were just emerging from a period of winter dormancy, were needed in order to monitor possible cytokinin movement from leaves during the new flush of growth. Dormancy was induced in 12 Valencia orange trees (*Citrus sinesis*) in order to synchronize cycles for simultaneous sampling. Dormancy can be induced by drought or cold temperatures (REUTHER, 1977). A temperature below 13°C is reported to be sufficient to induce the cessation of shoot extension (HENDRY, 1980). The citrus trees were therefore maintained in a growth chamber at 10°C for five weeks, which is a sufficiently long period to induce dormancy. An eight hour photoperiod and 95 per cent relative humidity were also maintained in the growth chamber. The trees were watered with Hoagland's mineral nutrient solution before being placed in the growth chamber. This was repeated at three weekly intervals. After five weeks, the conditions in the growth chamber were gradually changed to conditions which would initiate bud burst. That is, the temperature was gradually increased to a 30/20°C diurnal temperature regime, the humidity was reduced to a 60/40

per cent diurnal regime and the photoperiod was extended to 16 hours. Prior to initiating these changes, the trees were treated with radioactive zeatin, that is, while the trees were still under dormant conditions. Five microlitres of 8(^{14}C)*t*-zeatin (approximately 8 x 10^5 dpm) was applied via a microsyringe, to four leaves near the apical regions of each plant (Diagram 2.1). The treated leaves were marked for later recognition. The trees were harvested at three intervals after treatment. Four trees were harvested after 6 days at low temperatures, that is, before temperature was increased. A further four trees were harvested 6 days after the temperatures had begun to increase (that is, 12 days after treatment) and the buds had begun to swell. The last four trees were harvested after 12 days, during which the 30/20°C temperature regime was reached (that is, 18 days after treatment). By 18 days after applying the radioactive zeatin to the leaves, a small shoot had emerged. These three sampling times were referred to as 6, 12 and 18 days after treatment. The time intervals at which the trees were harvested were chosen as HENDRY (1980) had previously reported that the buds of dormant citrus trees swell a few days after emergence from the cold and that bud burst occurred after 10 to 15 days, with no new bud burst occurring after the first three weeks following cold emergence. The harvested trees were divided into treated leaves, untreated leaves above and below the treated leaves, roots, xylem, bark and buds/new shoots (Diagram 2.1). The components were weighed and deep frozen at -20°C until analyzed for cytokinin activity.

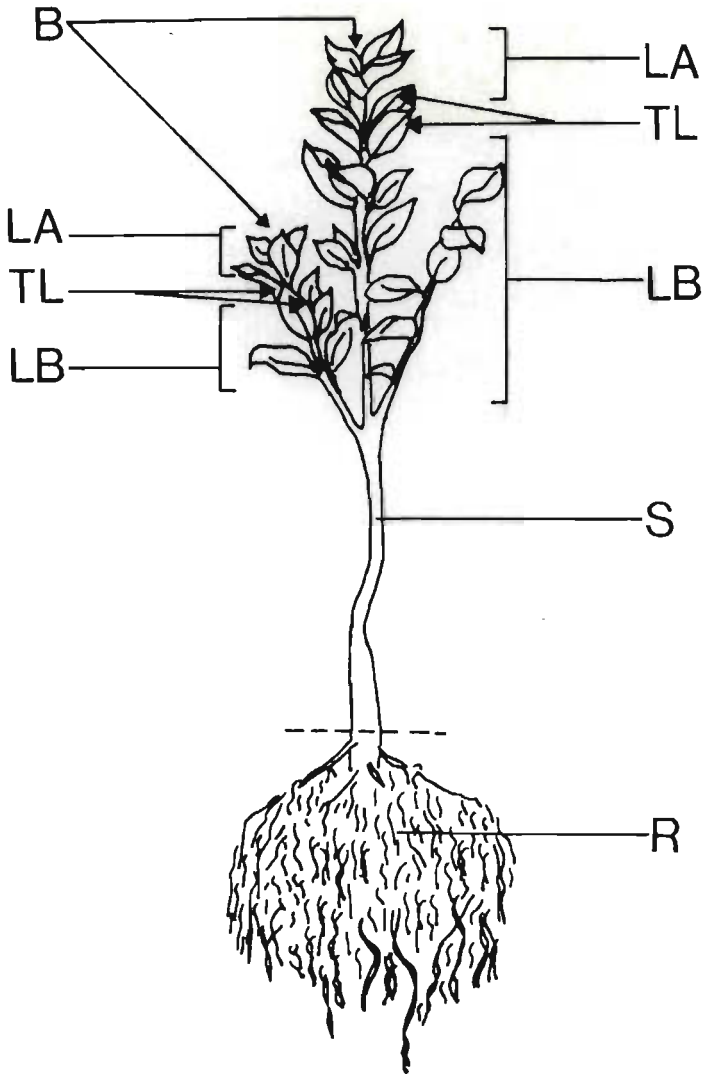


Diagram 2.1 Line diagram of the *Citrus sinesis* trees used in the experiments showing the various components analyzed. Not all trees were branched. Branched and unbranched trees were combined at each sampling time. A small portion of the stem tissue associated with the buds was included with the bud samples. R = roots; S = stem, which were separated into xylem and bark; TL = treated leaves; LA = untreated leaves above the treated leaves; LB = untreated leaves below the treated leaves; B = buds and subsequent new shoots (Redrawn from HENDRY, 1980).

The cytokinin extraction, paper and column chromatography, bioassay and radioassay techniques are explained in detail in the Materials and Methods section. The different components were extracted for cytokinins and strip-loaded onto paper chromatograms. Portions of these chromatograms were used for radioassays and the rest of the chromatograms were used to detect biological activity using the soyabean callus bioassay (MILLER, 1963, 1965). The treated leaves were fractionated on a Sephadex LH-20 column eluted with 10 per cent methanol. The buds and new shoots were not initially separated by paper chromatography but were immediately fractionated by column chromatography because of the small amounts of material available.

2.3 Results

2.3.1 Transport of 8(^{14}C)*t*-zeatin applied to the leaves of *Citrus sinesis* trees

From Table 2.1 it can be seen that less than 5 per cent of the radioactivity recovered from the components of the *Citrus sinesis* trees, was exported out of the leaves to which it was applied. If the results are expressed per one gramme fresh weight (Table 2.1) which could place the results in perspective by allowing for any possible distortion due to the bud and new shoot material being relatively small in comparison to the other components, it can be seen that the amount of radioactivity exported out of the leaves was not affected. The percentage radioactivity recovered in the components to which radioactivity was exported did change when the results were expressed per one gramme fresh weight.

Table 2.1 The distribution of radioactivity (A expressed as % dpm/component and B expressed as % dpm/one gramme fresh weight) recovered in the components of *Citrus sinesis* trees 6, 12 and 18 days after applying radioactive zeatin to leaves of these trees.

Component	Radioactivity (% dpm)					
	6 Days		12 Days		18 Days	
	A	B	A	B	A	B
Treated leaves	95,70	97,05	95,40	94,47	97,40	98,78
Untreated leaves above	0,01	0,07	0,86	0,57	0,18	0,37
Untreated leaves below	0,82	0,21	1,93	0,40	0,97	0,18
Buds/new shoots	0	0	0,86	4,21	0,08	0,39
Bark	3,00	2,27	0,32	0,21	0,15	0,12
Xylem	0,40	0,27	0,13	0,10	0,21	0,12
Roots	0,60	0,13	0,26	0,04	0,29	0,04

Table 2.2 shows the percentage radioactivity recovered in the untreated components to which radioactivity was exported, expressed per component and per one gramme fresh weight. Expressing the results in these two ways, affects the transport pattern. As mentioned earlier, results expressed per one gramme fresh weight were considered to be more significant, and were the results which were discussed. While the trees were still in dormant conditions, no radioactivity was detected in the dormant buds. Most of the radioactivity was recovered in the bark and a greater percentage radioactivity was detected in the leaves below

Table 2.2 The distribution of radioactivity (A expressed as % dpm/component and B expressed as % dpm/one gramme fresh weight) in the untreated components, to which radioactive compounds were translocated 6, 12 and 18 days after applying radioactive zeatin to leaves of *Citrus sinesis* trees. Radioactivity was from paper chromatograms.

Component	Radioactivity (% dpm)					
	6 Days		12 Days		18 Days	
	A	B	A	B	A	B
Untreated leaves above	2,00	2,44	18,85	10,24	6,99	30,38
Untreated leaves below	17,10	7,08	42,08	7,17	37,13	14,67
Buds/new shoots	0	0	23,38	76,22	30,56	31,60
Bark	62,02	76,90	7,09	3,84	6,02	10,04
Xylem	7,45	9,23	2,84	1,75	8,14	9,89
Roots	11,43	4,35	5,75	0,78	11,16	3,42

the treated leaves and in the roots than in leaves above the treated leaves. After 6 days at the higher temperature (that is, 12 days after treatment) most of the exported radioactivity was detected in the buds, with leaves above the treated leaves having the second highest percentage of radioactivity. Comparatively little radioactivity was recorded in the xylem, bark and roots. Eighteen days after applying zeatin to the leaves, the highest percentage radioactivity was recorded in the new shoots and leaves above the treated leaves. There was, however, a decrease in radioactivity in the new shoots and an increase in the leaves above the treated leaves, compared to 12 days after

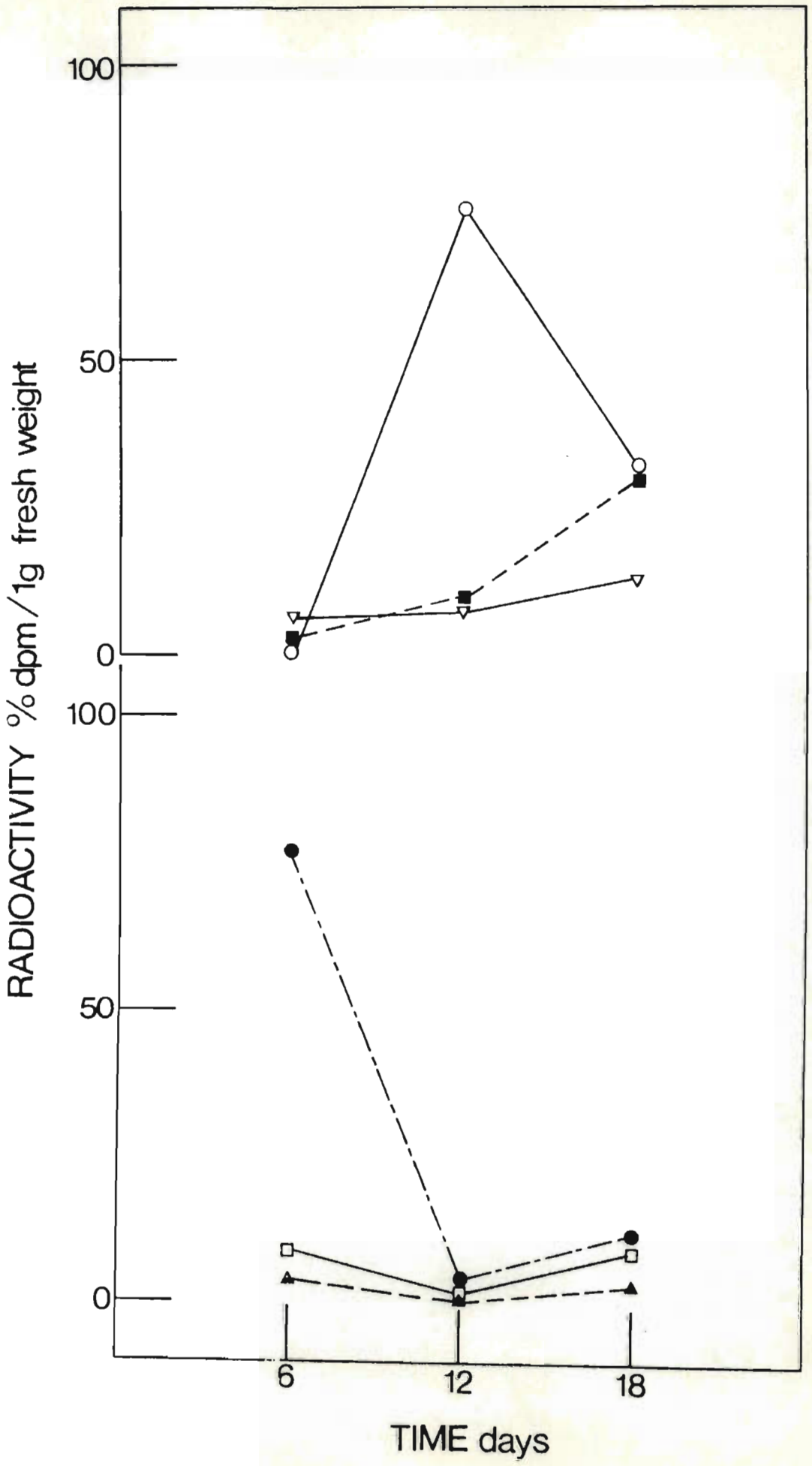
treatment. This could suggest that radioactive compounds were rapidly utilized in these actively growing shoots or that the mature leaves had a greater sink capacity than the new shoots for cytokinins exported from the leaves. The newly developing shoot could, however, receive cytokinins from the roots, rather than from the leaves, which could perhaps also account for the decrease in radioactivity in the new shoots.

The percentage radioactivity exported to the various tree components over time is shown in Figure 2.1. This Figure shows that the radioactivity detected in the untreated leaves increased over time, with the increase being more pronounced in leaves above the treated leaves. The radioactivity in the new shoots reached a maximum 12 days after applying the labelled zeatin to leaves, and then decreased. The radioactivity in the bark was highest at the first sampling time and thereafter decreased. Low levels of radioactivity were recorded in the xylem and roots throughout the experimental period.

2.3.2 Metabolism of 8(^{14}C)*t*-zeatin applied to leaves of *Citrus sinesis* trees

Three radioactive peaks were detected on paper chromatograms of the components of the *Citrus sinesis* trees following the application of radioactive zeatin to the leaves. These radioactive peaks had the same R_f values as the radioactive peaks detected in the *Ginkgo biloba* explants and were, therefore, also referred to as radioactive peaks 1, 2

Figure 2.1 The percentage radioactivity exported to the untreated components of the *Citrus sinesis* trees 6, 12 and 18 days after applying radioactive zeatin to some of the leaves. Results are expressed as the percentage radioactivity recovered per one gramme fresh weight. □———□= xylem; ●—·—●= bark; ▲----▲ = roots;○———○ = buds/new shoots; ■----■ = leaves above the treated leaves; ▽———▽ = leaves below the treated leaves.


100
50
0
100
50
0
RADIOACTIVITY % dpm/1g fresh weight
6
12
18
TIME days

and 3. The distribution of radioactive peaks 1 (R_f 0,0-0,2), 2 (R_f0,2-0,5) and 3 (R_f 0,5-1,0) in the treated leaves is shown in Figure 2.2 and the distribution of these peaks in all the components analyzed is shown in Table 2.3. The radioactive zeatin was fairly extensively metabolized in the leaves to which it was applied. While still maintained under dormant conditions, radioactive peak 2, which co-eluted with trihydroxyzeatin, adenosine and zeatin glucoside, was the major peak detected in the treated leaves. With the increase in temperature, radioactive peak 3, which co-eluted with zeatin and ribosylzeatin, appeared to be the major peak in these leaves. Approximately equal quantities of radioactive peaks 2 and 3 were present in the treated leaves after 18 days. Radioactive peak 1 increased slightly in the treated leaves and the untreated components during the experimental period. The radioactivity detected in the untreated leaves followed a similar pattern to that of the treated leaves. Radioactive peak 2 was the major peak in the xylem, bark and roots at all sampling times. The high levels of radioactive peak 2 in the bark could suggest that if cytokinins are exported from these evergreen leaves, then zeatin and ribosylzeatin, which would be components of peak 3 (R_f 0,5-1,0),are not involved in this export and could, therefore, imply that cytokinin glucosides are exported *per se*. Radioactivity in the xylem also implied that lateral transport occurred.

If the total percentage of each radioactive peak recovered at each sampling time is expressed per whole tree (Table 2.4),

Figure 2.2 The distribution of radioactive peaks 1 (R_f 0,0-0,2), 2 (R_f 0,2-0,5) and 3 (R_f 0,5-1,0) in the leaves to which radioactive zeatin was applied (treated leaves). Treated leaves were harvested after 6 (A), 12 (B) and 18 (C) days. Extracts were separated on paper chromatograms with *iso*-propanol:25 per cent ammonium hydroxide: water (10:1:1 v/v). Z = zeatin; ZR = ribosylzeatin; ZG = zeatin glucoside.

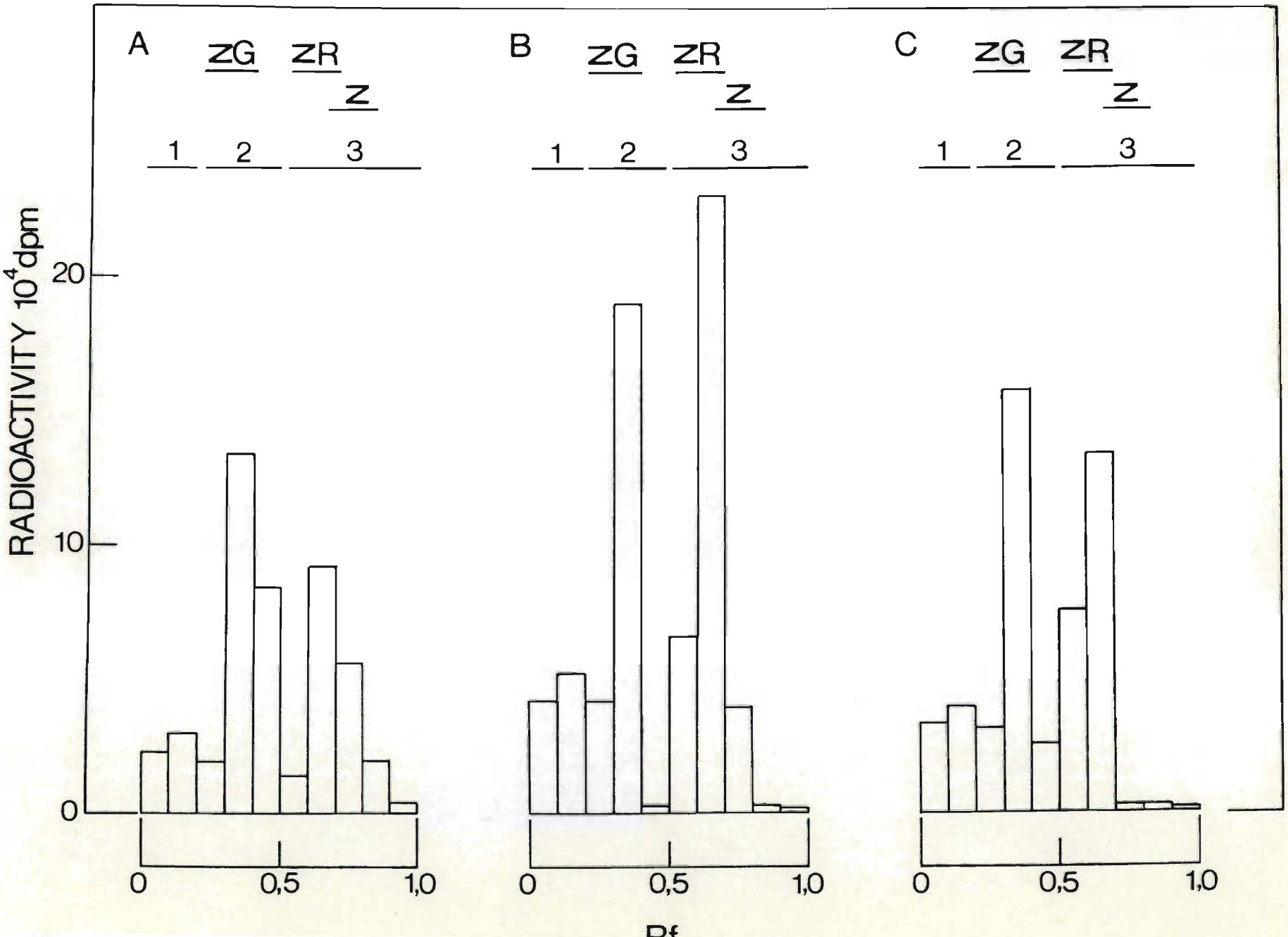

A
ZG
ZR
Z
1
2
3
B
ZG
ZR
Z
1
2
3
C
ZG
ZR
Z
1
2
3
RADIOACTIVITY 10^4dpm
20
10
0
0
0,5
1,0
0
0,5
1,0
0
0,5
1,0
Rf

Table 2.3 The distribution of radioactive peaks 1 (R_f 0,0-0,2), 2 (R_f 0,2-0,5) and 3 (R_f 0,5-1,0) in the components of *Citrus sinesis* trees 6, 12 and 18 days after applying radioactive zeatin to leaves of these trees. Radioactivity is expressed as % dpm recovered per radioactive peak.

Component	Radioactivity (% dpm/Radioactive Peak)		
	Radioactive Peak 1 (R_f 0,0-0,2)	Radioactive Peak 2 (R_f 0,2-0,5)	Radioactive Peak 3 (R_f 0,5-1,0)
6 days after treatment			
Treated leaves	11,7	51,9	36,4
Untreated leaves above	0	97,4	2,6
Untreated leaves below	0,6	63,3	36,1
Bark	7,3	58,6	34,1
Xylem	5,6	69,8	24,6
Roots	13,4	77,1	9,5
12 days after treatment			
Treated leaves	13,1	34,9	52,0
Untreated leaves above	3,0	19,6	77,4
Untreated leaves below	8,2	41,7	50,1
Bark	15,5	70,7	13,8
Xylem	17,0	75,3	7,7
Roots	13,3	66,3	20,4
18 days after treatment			
Treated leaves	14,8	43,2	42,0
Untreated leaves above	15,2	63,5	21,3
Untreated leaves below	1,0	44,2	54,8
Bark	15,6	84,4	0
Xylem	15,3	52,9	31,8
Roots	20,5	76,8	2,7

Table 2.4 The total percentage of radioactive peaks 1 (R_f 0,0-0,2), 2 (R_f 0,2-0,5) and 3 (R_f 0,5-1,0) recovered in all components of the *Citrus sinesis* trees 6, 12 and 18 days after applying radioactive zeatin to leaves of these trees.

Days after applying 8(^{14}C)*t*-zeatin to leaves	Radioactivity (% dpm/Radioactive Peak/tree		
	Radioactive Peak 1 (R_f 0,0-0,2)	Radioactive Peak 2 (R_f 0,2-0,5)	Radioactive Peak 3 (R_f 0,5-1,0)
6 days	6,43	69,68	23,89
12 days	11,68	51,42	36,90
18 days	13,73	60,83	25,44

then it can be seen that radioactive peak 2 was the major peak formed at all three sampling times, but was most prominent in the trees which were maintained under dormant conditions. The level of radioactive peak 3 fluctuated while that of radioactive peak 1 increased with time.

Fractionation of the treated *Citrus sinesis* leaves on a Sephadex LH-20 column resulted in eight radioactive peaks. These peaks were referred to as radioactive peaks A, B, C, D, E, F, G and H and they are shown in Figure 2.3. If the paper chromatograms of these leaf extracts are divided into A (R_f 0,0-0,5) and B (R_f 0,5-1,0) fractions before column chromatography, it could be seen that radioactive peaks A, B, C, D and F were associated with the A fraction and that peaks E, G and H were primarily associated with the B fraction (Figure 2.4). The eight radioactive peaks

Figure 2.3 Radioactivity (●——●) and biological activity (histograms) in the treated *Citrus sinesis* leaves. Radioactive zeatin was applied to leaves which were harvested after 6 (A), 12 (B) and 18 (C) days. The eight radioactive peaks detected (A, B, C, D, E, F, G and H), as well as the percentage radioactivity associated with each peak, are indicated. PG = purinyl glycine; Ado = adenosine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; IPA = isopentenyladenosine; Z = zeatin.

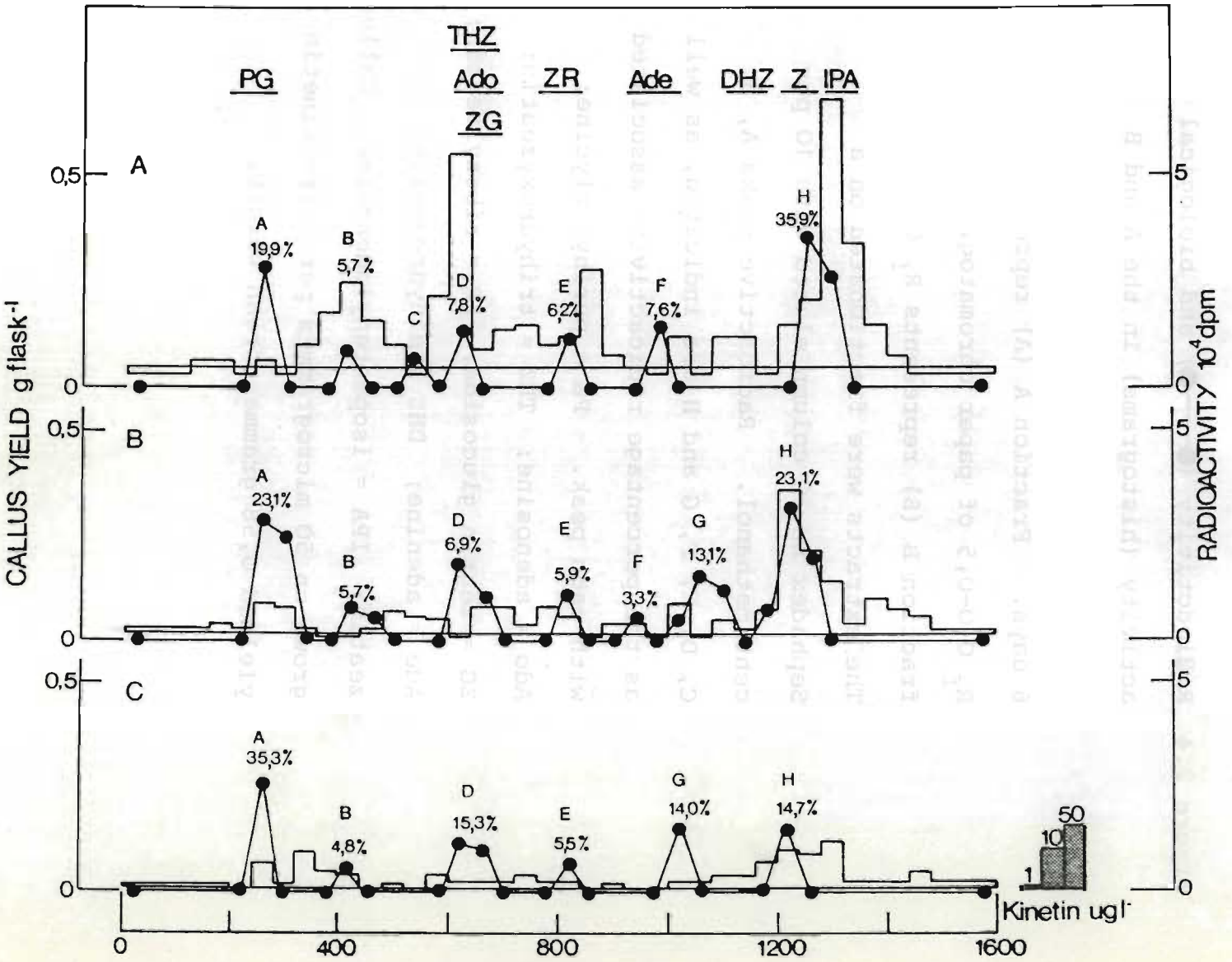
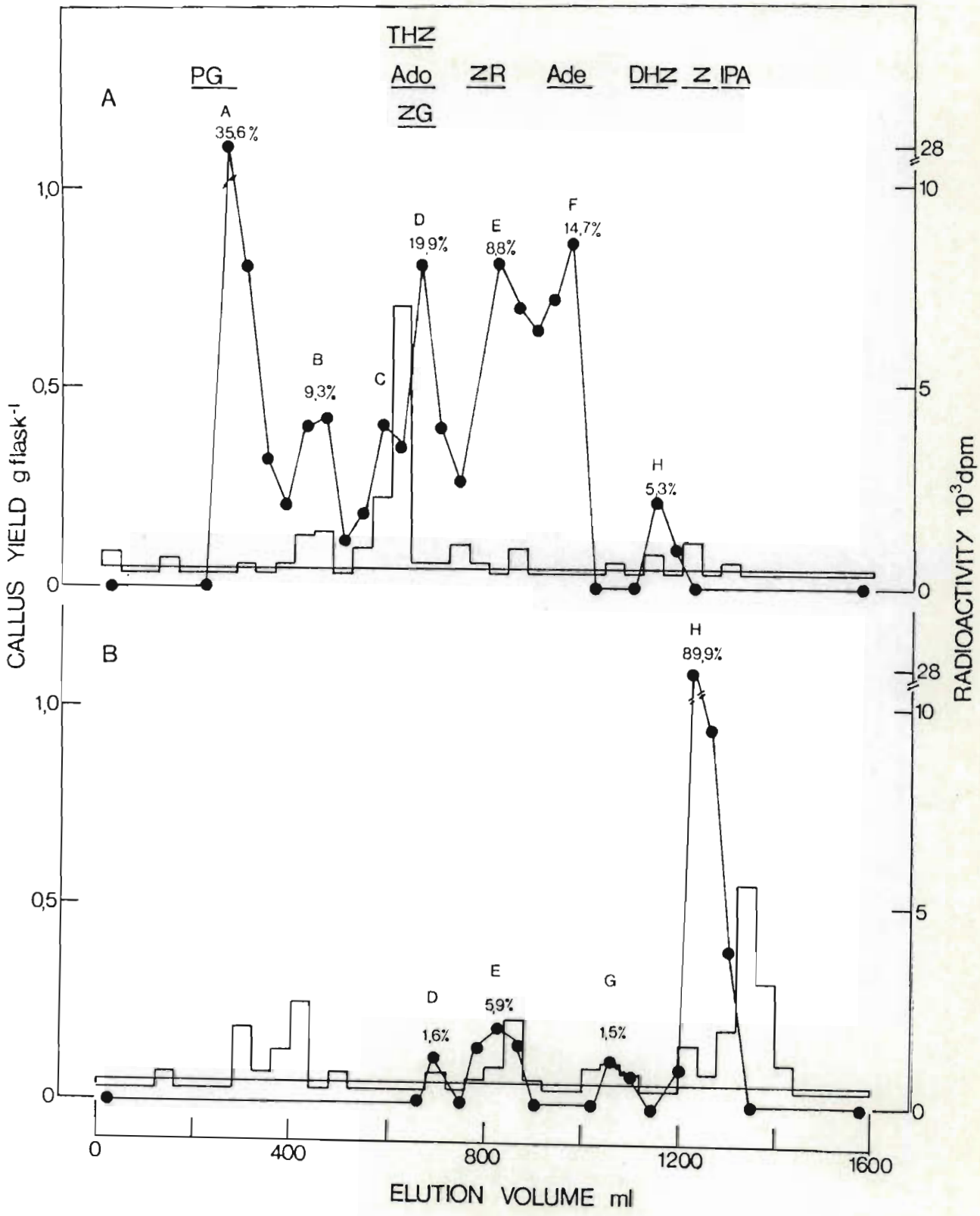

THZ
PG
Ado
ZR
Ade
DHZ
Z
IPA
ZG
A
B
C
0,5
0
5
A 19,9%
B 5,7%
C
D 7,8%
E 6,2%
F 7,6%
H 35,9%
A 23,1%
B 5,7%
D 6,9%
E 5,9%
F 3,3%
G 13,1%
H 23,1%
A 35,3%
B 4,8%
D 15,3%
E 5,5%
G 14,0%
H 14,7%
CALLUS YIELD g flask-1
RADIOACTIVITY 10-4dpm
1
10
50
Kinetin ug l-1
0
400
800
1200
1600
ELUTION VOLUME ml

Figure 2.4 Radioactivity (●——●) and biological activity (histograms) in the A and B fractions of treated leaves harvested after 6 days. Fraction A (A) represents R_f 0,0-0,5 of paper chromatograms and fraction B (B) represents R_f 0,5-1,0. The extracts were fractionated on a Sephadex LH-20 column eluted with 10 per cent methanol. Radioactive peaks A, B, C, D, E, F, G and H are indicated, as well as the percentage radioactivity associated with each peak. PG = purinyl glycine; Ado = adenosine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine. Callus grown on 50 microgrammes per litre kinetin yielded 0,58 grammes fresh weight.

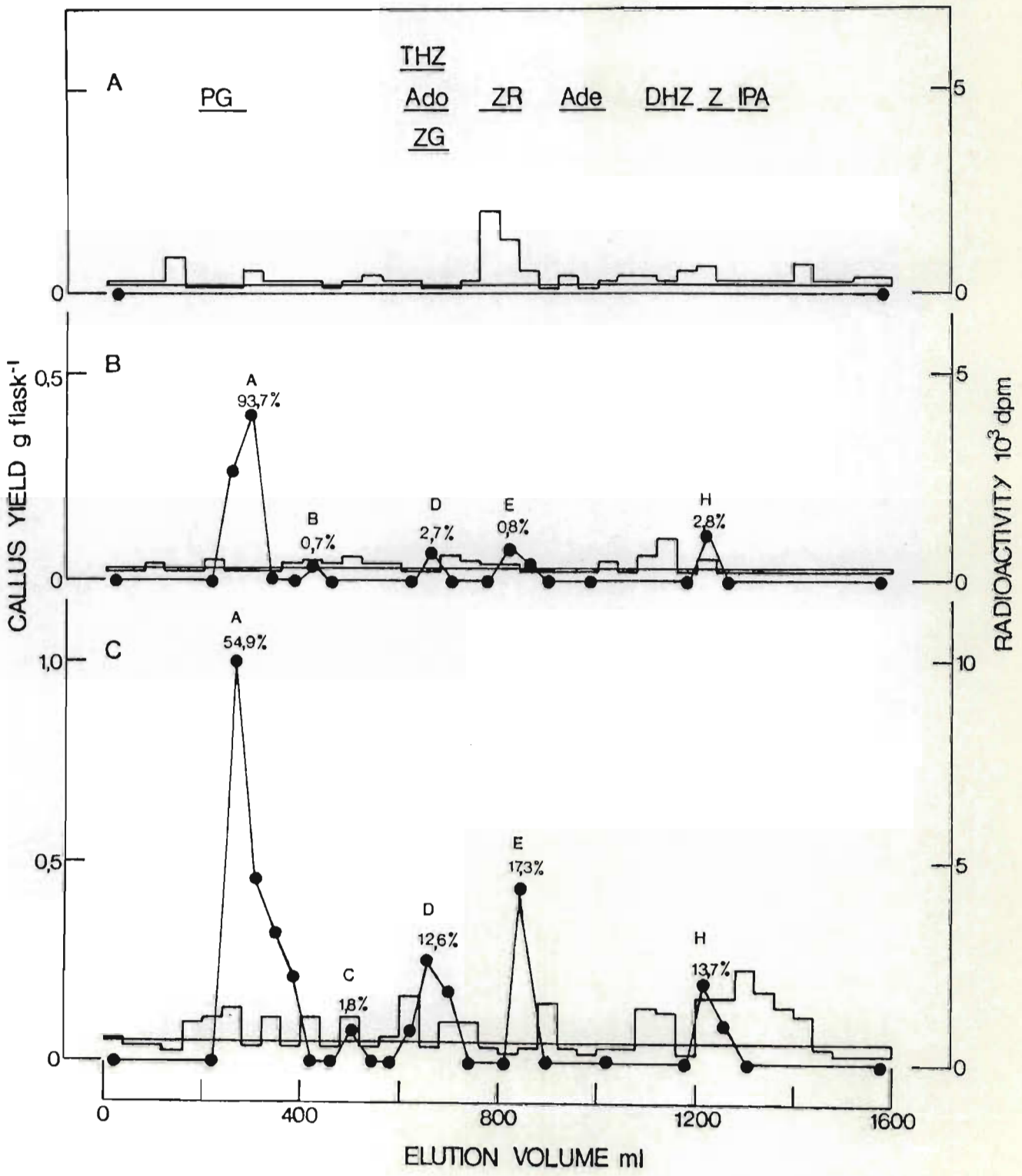

THZ
PG
Ado
ZR
Ade
DHZ
Z
IPA
ZG
A
B
A
35,6%
B
9,3%
C
D
19,9%
E
8,8%
F
14,7%
H
5,3%
H
89,9%
D
1,6%
E
5,9%
G
1,5%
CALLUS YIELD g flask⁻¹
RADIOACTIVITY 10³dpm
1,0
0,5
0
28
10
5
0
400
800
1200
1600
ELUTION VOLUME ml

were only tentatively identified on the basis of co-elution with authentic cytokinins. Radioactive peak A, which had an elution volume of 240 to 320 millilitres, was relatively inactive and co-eluted with purinyl glycine. Purinyl glycine is also biologically inactive (MILLER, 1965). Radioactive peak A was a major peak at all three sampling times and the radioactivity associated with this peak increased with time. Radioactive peak B was biologically active but did not co-elute with any cytokinin standards. Relatively little radioactivity was associated with this peak. Radioactive peak C was also a minor peak in the A fractions of the treated leaves and was not investigated. Radioactive peak D was biologically active and increased with time. This peak co-eluted with zeatin glucoside, adenosine and trihydroxyzeatin. The presence of the peak co-eluting with purinyl glycine suggests that trihydroxyzeatin was a component of this peak, as it is an intermediate in the oxidation reaction resulting in the formation of purinyl glycine (VAN STADEN, DREWES and HUTTON, 1982). Radioactive peak E co-eluted with ribosylzeatin, but only appeared to be a minor peak. Radioactive peak F was also a minor peak. This peak co-eluted with adenine, which is also inactive in the soyabean callus bioassay (VAN STADEN, 1979a). The radioactivity associated with peak G increased with time. This peak did not co-elute with any of the cytokinin standards and could, therefore, not be identified. This radioactive peak did, however, have a similar elution volume to an unidentified compound detected following the oxidation of radioactive zeatin

(Figure 1.19). Radioactive peak H co-eluted with zeatin and probably represented the original radioactive zeatin applied to the leaves. The activity associated with this biologically very active peak decreased with time.

Relatively little bud and new shoot material was associated with the flush of growth. This material was, therefore, not separated by paper chromatography, but following Dowex 50 purification, was immediately separated on Sephadex LH-20 columns eluted with 10 per cent methanol. From Figure 2.5 it can be seen that five radioactive peaks were associated with this material. These peaks were referred to as A, B, D, E and H, in accordance with the nomenclature given to the radioactive peaks detected in the treated leaves. Relatively little biological activity was associated with this radioactivity, which was probably the result of rapid cytokinin utilization in these actively dividing tissues. No radioactivity was detected in buds of trees maintained in dormant conditions. The biological activity detected in these buds co-eluted with ribosylzeatin and to a lesser extent with dihydrozeatin and zeatin. As the temperatures were increased, radioactivity was detected in the regions of new growth. The diversity of radioactive compounds in the flush of growth appeared to increase with time. Twelve days after applying labelled zeatin to these trees, a peak which co-eluted with purinyl glycine was the major compound detected in the buds, with minor peaks co-eluting with zeatin, ribosylzeatin and trihydroxyzeatin, adenosine, zeatin glucoside, also present (Figure 2.5B).

Figure 2.5 Radioactivity (●——●) and biological activity (histograms) in the bud and new shoot material associated with the new flush of growth of *Citrus sinesis* trees. Extracts were separated on Sephadex LH-20 columns eluted with 10 per cent methanol. A = buds from trees harvested 6 days after applying radioactive zeatin to leaves; B = shoot material harvested after 12 days; C = new shoot material harvested after 18 days. Radioactive peaks A, B, D, E and H are indicated as well as the percentage radioactivity associated with each peak. PG = purinyl glycine; Ado = adenosine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine. Callus grown on 50 microgrammes per litre kinetin yielded 0,36 grammes fresh weight.


A
PG
THZ
Ado
ZG
ZR
Ade
DHZ
Z
IPA
B
A
93,7%
B
0,7%
D
2,7%
E
0,8%
H
2,8%
C
A
54,9%
C
1,8%
D
12,6%
E
17,3%
H
13,7%
CALLUS YIELD g flask-1
RADIOACTIVITY 10-3 dpm
ELUTION VOLUME ml
0
400
800
1200
1600
0,5
1,0
5
10

Eighteen days after treatment, the radioactive peak which co-eluted with purinyl glycine was still the major peak, but the percentage radioactivity associated with this peak had decreased and the radioactive compounds co-eluting with zeatin (radioactive peak H), ribosylzeatin (peak E) and trihydroxyzeatin, zeatin glucoside, adenosine (peak D), all of which have the same elution volume, appeared to become more important (Figure 2.5C). Radioactive peak B, which was also recorded in this material, decreased slightly with time. This peak did not co-elute with any authentic cytokinin standards. The biological activity associated with radioactive peak D implied that adenosine, which is biologically inactive in the soyabean callus bioassay (VAN STADEN, 1979a), was not a component of this peak. Zeatin glucoside levels are reported to decrease during bud swell (HENDRY, 1980) which could suggest that glucosides were also not a major component of radioactive peak D. This could imply that trihydroxyzeatin was the major component of peak D. Radioactive peaks F and G, both of which appeared to be oxidation products, were absent in the new flush of growth. It is also interesting to note that the percentage of radioactivity co-eluting with purinyl glycine increased with time in the treated leaves and decreased in the buds and new shoots.

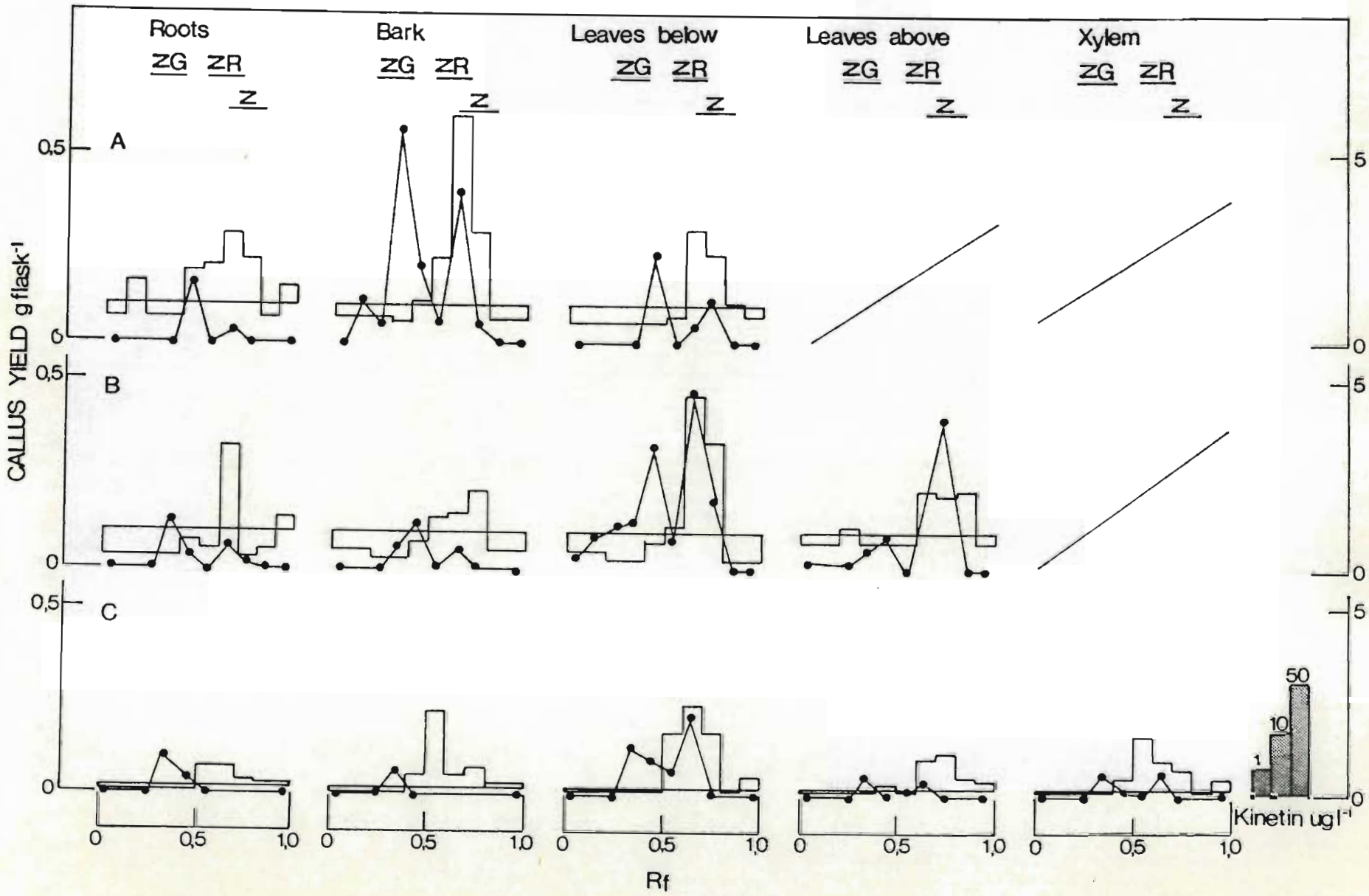
Most of the untreated components of the *Citrus sinesis* trees were not fractionated by column chromatography because of the small amounts of radioactivity associated with them. Where sufficient material was available, the paper chromato-

grams of these extracts were assayed for cytokinin activity using the soyabean callus bioassay (Figure 2.6). Radioactivity and biological activity did not always co-chromatograph in these components, which could imply that the metabolism of the radioactive compounds was not indicative of the endogenous situation. Compounds co-eluting with zeatin and ribosylzeatin appeared to be the only biologically active cytokinins, with no cytokinin glucosides being detected. Contrary to this, a non-polar radioactive peak, which co-eluted with zeatin glucoside, but was inactive, was detected in all components. A radioactive peak co-eluting with zeatin and ribosylzeatin was, however, recorded in some components.

2.4 Discussion

The flush of growth produced by evergreen plants during the warmer winter months is governed by a number of factors, of which cycles of cytokinins and other plant growth regulators are said to be important (HENDRY, 1980). Although there are many factors to be considered, the results of these experiments suggest that cytokinins from the leaves are involved in the new flush of growth. Cytokinins have been implicated in bud burst (VAN STADEN, 1979c), but the extent to which cytokinins from leaves, from the roots or cytokinins synthesized in the buds themselves, are involved in this process, is difficult to assess and would necessitate further investigation. Radioactivity, associated with the radioactive zeatin, was exported out of the *Citrus sinesis* leaves while the trees were still dormant, but was only de-

Figure 2.6 Radioactivity (●——●) and biological activity (histograms) in the untreated components of the *Citrus sinesis* trees. Components are from trees harvested 6 (A), 12 (B) and 18 (C) days after applying radioactive zeatin to leaves of the trees. Extracts were separated on paper chromatograms with *iso*-propanol:25 per cent ammonium hydroxide:water (10:1:1 v/v). Z = zeatin; ZR = ribosylzeatin; ZG = zeatin glucoside.


Roots
Bark
Leaves below
Leaves above
Xylem
ZG ZR Z
A
B
C
0,5
0
CALLUS YIELD g flask⁻¹
RADIOACTIVITY 10³dpm
5
0
0,5
1,0
Rf
1
10
50
Kinetin ug l⁻¹

tected in the buds and new shoots once the temperatures had increased. This suggests that leaf cytokinins could be involved in the initiation of bud burst and in subsequent new shoot growth. This assumption was made despite the fact that less than 5 per cent of the recovered radioactivity was exported out of the leaves to which the radioactive zeatin was applied. However, as mentioned in the *Ginkgo biloba* experiments, certain factors must be considered when assessing the significance of the exported radioactivity. It must be remembered that the radioactive zeatin was only applied to a few leaves of each *Citrus sinesis* tree. It therefore cannot be expected that these few leaves would be responsible for supplying the buds and new shoots with their total cytokinin complement. Another factor to be considered is the localized application of radioactive zeatin to the leaf, which would mean that there is probably transport of this cytokinin within the leaf, as well as out of the leaf. In this respect, a greater export of radioactivity might be obtained if radioactive zeatin was applied evenly to the whole leaf. Other factors to be considered are the time of application, the unphysiological concentration of zeatin applied to the leaves and the endogenous cytokinin complement of the leaves in relation to the applied zeatin. Considering the above facts it is only logical to assume that all the radioactivity would not be exported out of the leaves, and this suggests that the exported radioactivity is significant. The fact that a definite export pattern appeared to exist, strengthened the assumption that cytokinins are exported out of evergreen leaves following a

quiescent period and are involved in new shoot growth. It could, however, also be argued that the exported radioactivity was merely transported passively in the assimilate stream, as a result of the large amount of zeatin applied to the leaves. HENDRY (1980) has also proposed that root-produced cytokinins could be supplied to the new shoot rather than to the leaves, which could imply that cytokinins in the transpiration stream are primarily involved in bud burst and subsequent growth.

It is interesting to note that the exported radioactivity was only detected in the buds once the temperatures had been increased and active growth had been resumed. HENDRY (1980) reported that there were two critical periods of cytokinin utilization in flush development. The first period being during bud swell, immediately after warmer temperatures were experienced. The results of this experiment appeared to fit in with this first period of cytokinin utilization. Before warmer temperatures were experienced, radioactivity was mainly associated with the bark. VAN STADEN and DAVEY (1981b) have proposed that the bark could have an important role in the regulation of cytokinin levels in different tissues at different times of the year, which could perhaps account for the radioactivity detected in the bark. Radioactivity was only detected in the buds when environmental conditions favoured bud burst. Once new shoot growth had been initiated, the percentage radioactivity recorded in the new shoot material decreased. HENDRY (1980) has suggested that mature *Citrus sinesis*

leaves are less competitive sinks during flush emergence than the flush itself. This could imply that the decrease in radioactivity in the new flush of growth reflected rapid utilization, rather than preferential transport to the untreated mature leaves. The metabolic study also appeared to indicate that the cytokinins arriving in the buds and new shoot material were rapidly utilized. The major radioactive peak in these tissues, which co-eluted with purinyl glycine, appeared to decrease during new shoot growth, suggesting utilization of cytokinins arriving in the new shoots.

Seasonal cytokinin studies on evergreen leaves have led to the proposal that unless cytokinin glucosides, which accumulate during autumn and winter, are catabolically metabolized or exported from the leaves (HENDRY, 1980), they are probably hydrolyzed to zeatin and ribosylzeatin (LORENZI, HORGAN and WAREING, 1975; HENDRY, VAN STADEN and ALLAN, 1982) and would, therefore, represent storage compounds. The results of this experiment suggest that at least some cytokinins are exported from the leaves. The metabolic study indicated that zeatin was not involved in transport which could imply that cytokinin glucosides are not hydrolyzed before being exported from the leaves. Radioactive peak 2 (R_f 0,2-0,5), which was formed from the radioactive zeatin and co-eluted with zeatin glucoside, trihydroxyzeatin and adenosine on paper chromatograms, was the major radioactive peak in the bark. The small amount of radioactive peak 3 (R_f 0,5-1,0; which co-eluted with

zeatin) initially detected in the bark, decreased and was absent after 18 days. The radioactive zeatin was, therefore, metabolized in the leaves and in the bark, perhaps emphasizing the role of the bark in controlling cytokinin levels (VAN STADEN and DAVEY, 1981b). Increasing levels of radioactive peak 1 (R_f 0,0-0,2; which co-eluted with purinyl glycine) in the bark and the fact that the major radioactive peak in the buds and new shoots co-eluted with purinyl glycine, suggested that radioactive peak 2 was also metabolized in the bark. This could imply that cytokinin glucosides are primarily exported *per se* and perhaps undergo metabolism in the bark. VAN STADEN (1976a) has suggested that cytokinin glucosides are transported as intact molecules as they were detected in the honeydew collected from *Salix babylonica*. These results, therefore, support the idea of export of cytokinin glucosides, but do not rule out the possibility of an interconversion of zeatin and ribosylzeatin glucosides; and zeatin and ribosylzeatin. The increase in zeatin and ribosylzeatin levels in these leaves in spring (LORENZI, HORGAN and WAREING, 1975) could, perhaps, also be accounted for by the transport of root-produced cytokinins to the leaves.

Cytokinin glucosides, therefore, appear to represent storage compounds in evergreen leaves. The accumulation of these compounds during autumn and winter could thus be a mechanism for regulating leaf metabolism. Cytokinin glucosides could prevent the leaves from acting as a sink for photosynthates during the winter. Cytokinin glucosides could,

Purinyl glycine, and radioactive peaks D and G, both of which could have been oxidation products, appeared to increase in these leaves. This then raises the possibility that the metabolism observed is not indicative of normal metabolism in the leaves, which could perhaps be related to the exogenous application of zeatin. If the metabolism observed does not reflect the endogenous situation, then the transport of cytokinins from leaves can possibly be questioned. The accumulation of zeatin and ribosylzeatin in the new shoot does, however, suggest that this is the normal metabolic pathway in this tissue and could imply that the transport of radioactive compounds does reflect the endogenous situation.

The export of radioactivity, associated with the radioactive zeatin, from the *Citrus sinesis* leaves implies that cytokinin glucosides are exported from these leaves. The final proof for the re-utilization of cytokinin glucosides in the new flush of growth would, however, be obtained using radioactive zeatin glucosides, although the problems of applying unphysiological cytokinin concentrations would still be present.

CHAPTER THREE

THE TRANSPORT AND METABOLISM OF 8(^{14}C)*t*-ZEATIN APPLIED TO *PHASEOLUS VULGARIS* PLANTS

3.1 Introduction

Leaf senescence is an integral part of leaf development (THOMAS and STODDART, 1980) and of the life cycle of the plant. Senescing leaves are reported to be a source of minerals as a result of nutrient mobilization during senescence (THOMAS and STODDART, 1980). In annual plants, leaf senescence appears to be synchronized with fruit development (WAREING and SETH, 1967). It has frequently been reported that prevention of pollination or the removal of developing seeds and fruit will delay leaf senescence (MOLISCH, 1938; WAREING and SETH, 1967; NOODEN and LEOPOLD, 1978). Seeds therefore appear to have the ability to mobilize and accumulate minerals from other plant parts (WAREING and SETH, 1967) and the high cytokinin content of developing seeds has been implicated in nutrient mobilization (MOTHES and ENGELBRECHT, 1961; WAREING and SETH, 1967; NOODEN and LEOPOLD, 1978; TURVEY and PATRICK, 1979). As in deciduous and evergreen leaves, cytokinin glucosides accumulate in mature annual leaves (WANG, THOMPSON and HORGAN, 1977; DAVEY and VAN STADEN, 1978a). Cytokinin glucosides are apparently unable to mobilize nutrients (VAN STADEN and DAVEY, 1979) and mature leaves are, therefore, not a competitive nutrient sink for seed cytokinins. The accumulation of cytokinin glucosides in mature annual leaves

could thus be regarded as a function of leaf senescence and nutrient mobilization out of the leaves (DAVEY, 1978).

The origin of the high levels and diversity of cytokinins in developing seeds and fruit (LETHAM, 1963, 1973; DAVEY and VAN STADEN, 1977; SUMMONS, ENTSCH, GOLLNOW and MACLEOD, 1980) has not been conclusively determined. It has been suggested that seeds synthesize cytokinins (BLUMENFELD and GAZIT, 1971; HAHN, DE ZACKS and KENDE, 1974; NAGAR and RAJA RAO, 1981), but this has been disputed (VAN STADEN and BUTTON, 1978; KRECHTING, VARGA and BRUINSMA, 1978). In addition to synthesis within the seed, cytokinins could also be transported directly from the roots to the fruit or indirectly via the leaves. It has been proposed that the developing fruit may be a stronger sink than the leaves for root-produced cytokinins (VARGA and BRUINSMA, 1974), but DAVEY and VAN STADEN (1981) have suggested that no preferential site of cytokinin mobilization exists and that cytokinin transport is a function of the transpiration stream. It has also been proposed that cytokinins from the leaves are transported to the seeds. DAVEY and VAN STADEN (1978c) detected cytokinins in the phloem sap of *Lupinus albus*, which suggested that some seed cytokinins could originate in the leaves. Using radioactive zeatin, VAN STADEN and DAVEY (1981a) subsequently reported that a small percentage of the radioactive zeatin applied to the primary leaves of fruiting *Lupinus* plants was exported to the developing fruit. VONK and DAVELAAR (1981) have also suggested that cytokinin glucosides, which accumulate in *Yucca flaccida* leaves, are

exported as nucleotides through the inflorescence stalk.

Seed cytokinins can thus be said to be involved in seed development and leaf senescence and these experiments were carried out in an attempt to determine the origin of seed cytokinins more conclusively. Radioactive zeatin was applied to the stem of vegetative, flowering and fruiting *Phaseolus vulgaris* plants to determine the distribution of root-produced cytokinins during development. That is, to determine whether preferential sites of cytokinin mobilization exist during development. Radioactive zeatin was also applied to primary leaves to determine whether leaf cytokinins contribute to the cytokinin content of seeds. By applying cytokinins to leaves, it was also hoped to obtain some information on the role of glucosides in mature annual leaves.

3.2 Experiment Procedure

3.2.1 8(^{14}C)*t*-zeatin applied to the xylem and phloem of *Phaseolus vulgaris* plants

Phaseolus vulgaris L. (cv. Contender) plants were grown in a greenhouse and were treated with radioactive zeatin at three stages of development. These developmental stages, vegetative, flowering and fruiting, were defined as follows : Vegetative plants had two trifoliate leaves and a vegetative apical bud, flowering plants had floral buds with the maximum of two open flowers and fruiting plants had developing fruit of approximately one centimetre in length. Sixteen

plants were treated at each stage of development. Control plants were also harvested to determine endogenous cytokinin activity at the three stages of development.

At each of these developmental stages, five microlitres 8(^{14}C)*t*-zeatin (8 x 10^5 dpm) was applied, via a micro-syringe, to the xylem and to the phloem of the stem of each plant. When the zeatin was applied to the xylem, it was injected into the stem just above the soil. The zeatin was applied to the phloem, in the middle of the stem, by gently scraping the surface of the stem and allowing the zeatin to be absorbed. It cannot, however, conclusively be stated that the zeatin was applied only to the xylem or to the phloem because of the proximity of these tissues. The treated stem area was marked for later identification. The plants were harvested 48 hours after treatment and were divided into treated leaves, stem, roots, primary and trifoliate leaves, vegetative apices; buds and flowers; fruit (depending on the stage of development) and senesced petals in fruiting plants only. When zeatin was applied to the phloem, the untreated stem was divided into two regions, namely the stem above and below the treated stem area. The plant components were weighed and frozen at -20°C until extracted for cytokinins.

3.2.2 8(^{14}C)*t*-zeatin applied to primary leaves of *Phaseolus vulgaris* plants

Phaseolus vulgaris (bean) plants were grown in the greenhouse. At anthesis, five microlitres of 8(^{14}C)*t*-zeatin was applied,

via a microsyringe, to the primary leaves of 20 plants. The plants were harvested 5 and 10 days after treatment. The developing fruit were approximately one and four centimetres in length respectively at these harvesting times and weighed approximately 0,017 and 0,18 grammes respectively. Ten plants were used at each sampling time and they were divided into treated primary leaves, trifoliate leaves and stem, roots and fruit. The components were weighed and frozen at -20°C until extracted for cytokinins.

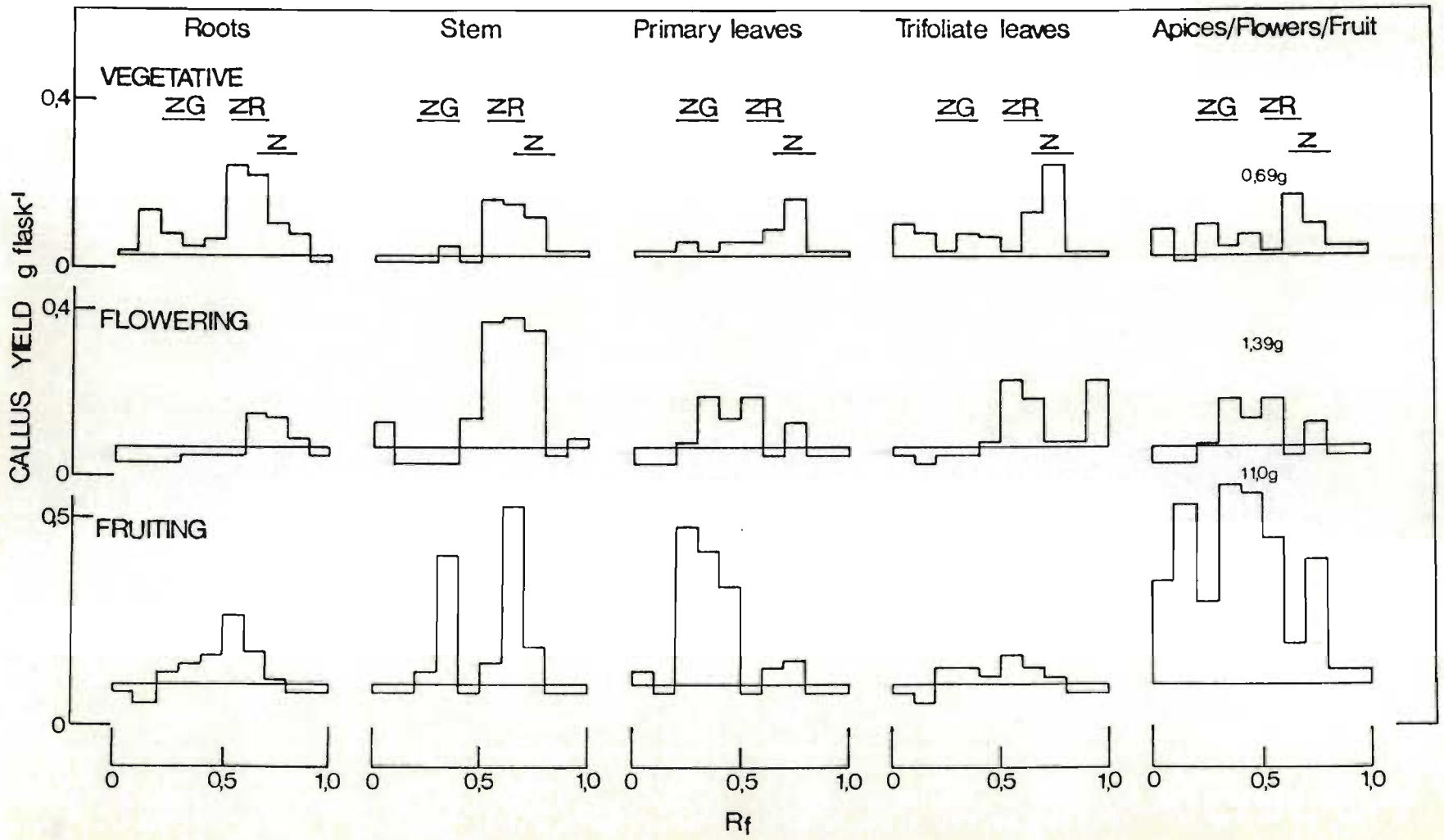
The techniques for cytokinin extraction, the soyabean callus bioassays, radioassays and paper and column chromatography are described in detail in the Materials and Methods section. All plant components were extracted for cytokinins and separated by paper chromatography. A portion of each chromatogram was used for a radioassay and the rest of the chromatogram was either used for a bioassay or column chromatography.

3.3 Results

3.3.1 Endogenous cytokinin activity in *Phaseolus vulgaris* plants

Phaseolus vulgaris plants reached anthesis approximately six weeks after planting. Cytokinin activity fluctuated in the components of these plants during their growth and development (Figure 3.1). Cytokinin activity increased in the primary leaves and was accompanied by an accumulation

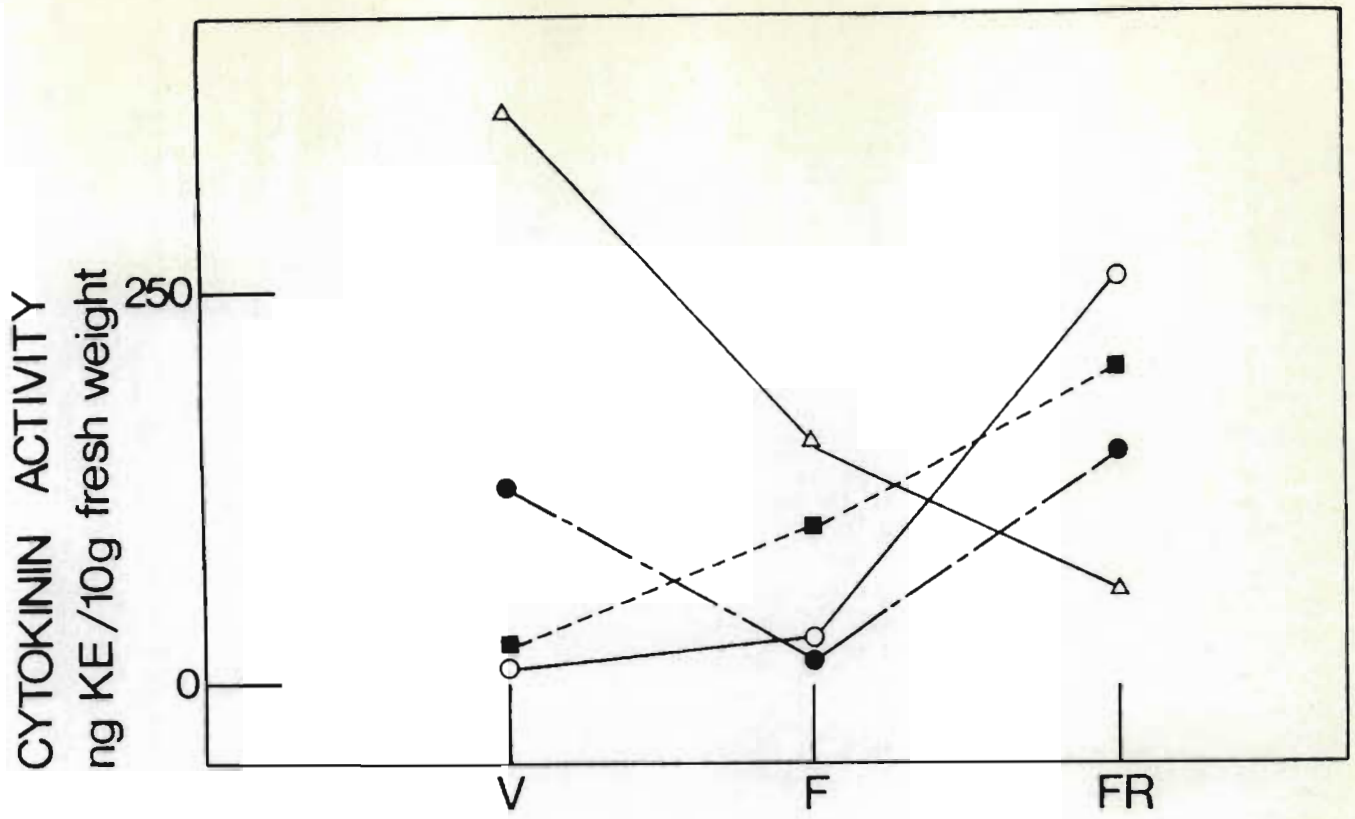
Figure 3.1 Cytokinin activity in the components of vegetative, flowering and fruiting *Phaseolus vulgaris* plants. Activity is expressed per 10 grammes fresh weight except for the apices, buds, flowers and fruit which are expressed per component. Extracts were separated on paper chromatograms with *iso*-propanol:25 per cent ammonium hydroxide:water (10:1:1 v/v). Callus grown on 50 microgrammes per litre kinetin yielded 0,78 grammes fresh weight. Z = zeatin; ZR = ribosylzeatin; ZG = zeatin glucoside.

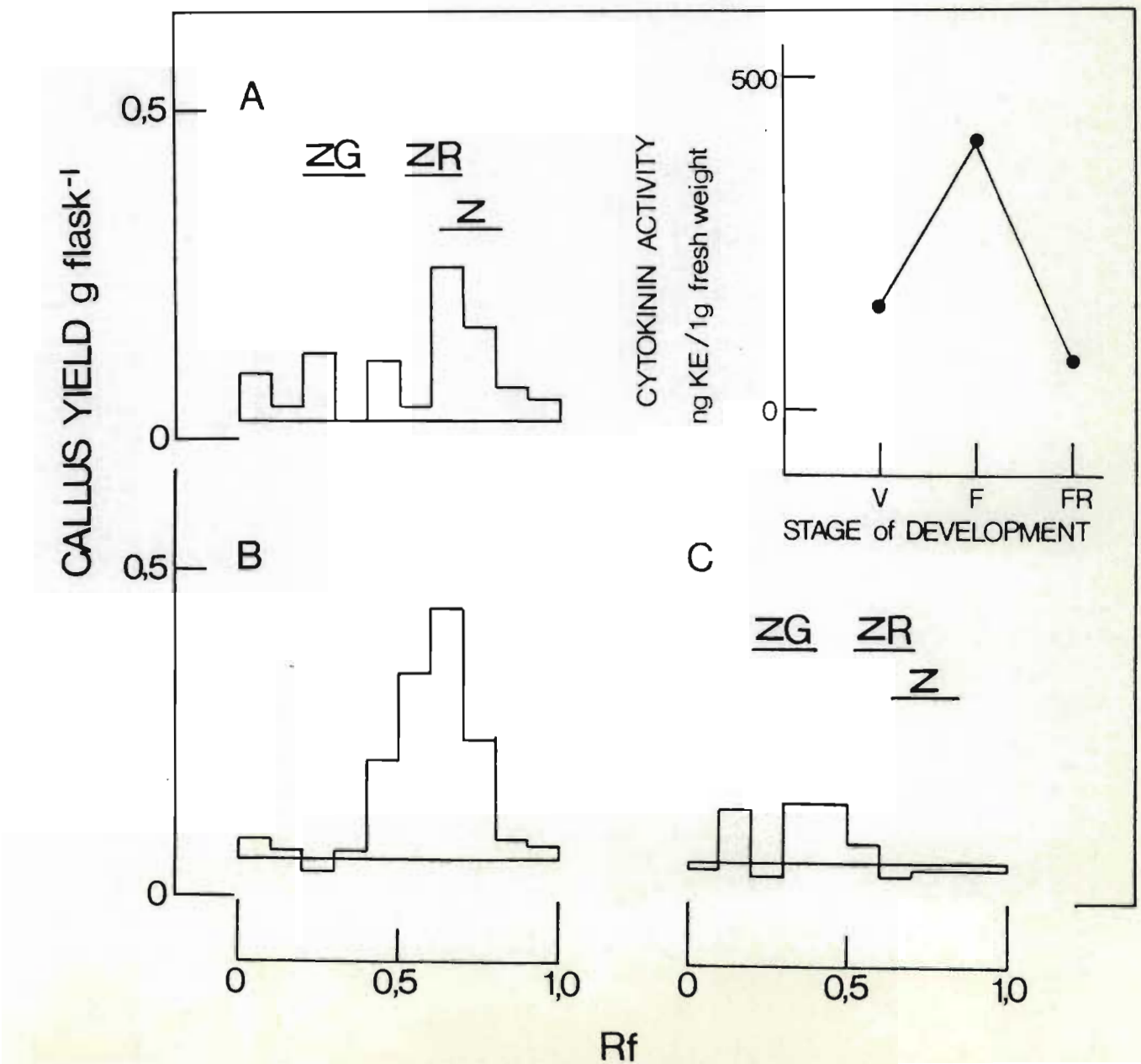

Roots
Stem
Primary leaves
Trifoliate leaves
Apices/Flowers/Fruit
VEGETATIVE
FLOWERING
FRUITING
ZG
ZR
Z
0,69g
1,39g
11,0g
0,4
0,4
0,5
0
0,5
1,0
Rf
CALLUS YIELD g flask-1

of compounds co-eluting with cytokinin glucosides in fruiting plants. Contrary to this, cytokinin activity decreased in trifoliate leaves and glucoside levels were also low. Cytokinin levels fluctuated in the roots, decreasing during flowering. Cytokinin activity increased in the stem, with cytokinin glucosides being detected in the stems of fruiting plants. These trends in cytokinin activity in the various components are seen more clearly when activity is expressed quantitatively in terms of kinetin equivalents (Figure 3.2). If cytokinin activity in the vegetative apices, buds and flowers and fruit was expressed per component (Figure 3.1) and not as a constant weight, it appeared that cytokinin activity in these components increased during plant development. If, however, cytokinin activity was expressed per one gramme fresh weight, the cytokinin levels appeared to reach a maximum in the buds and flowers and decreased as the fruits developed (Figure 3.3). As mentioned previously, it was felt that large fresh weight differences between components could cause distortions when results were expressed per component and expressing cytokinin activity of the apices, flowers and fruit per one gramme fresh weight may have resulted in a better indication of cytokinin activity. Components co-eluting with zeatin and ribosylzeatin appeared to be the major cytokinins in the vegetative apices and buds and flowers, with cytokinin glucosides accumulating in the developing fruit. Glucosylation, therefore, only appeared to occur in the fruiting plants.

Figure 3.2 Cytokinin activity in the components of vegetative, flowering and fruiting *Phaseolus vulgaris* plants expressed as nanogramme kinetin equivalents per 10 grammes fresh weight. V = vegetative; F = flowering; FR = fruiting; ●—·—● = roots; O——O = primary leaves; Δ——Δ = trifoliate leaves; ■------■ = stem.

Figure 3.3 Cytokinin activity in the vegetative apices (A), buds and flowers(B) and fruit (C) of *Phaseolus vulgaris* plants expressed per one gramme fresh weight. Insert represent the activity expressed as nanogramme kinetin equivalents. Z = zeatin; ZR = ribosylzeatin; ZG = zeatin glucoside; V = vegetative; F = flowering; FR = fruiting.


CYTOKININ ACTIVITY
ng KE/10g fresh weight
250
0
V
F
FR


CALLUS YIELD g flask⁻¹
A
0,5
0
ZG
ZR
Z
B
0,5
0
0
0,5
1,0
C
ZG
ZR
Z
0
0,5
1,0
Rf
CYTOKININ ACTIVITY
ng KE/1g fresh weight
500
0
V
F
FR
STAGE of DEVELOPMENT

3.3.2 The transport and metabolism of 8(^{14}C)*t*-zeatin applied to the stem of *Phaseolus vulgaris* plants

Transport of radioactive zeatin applied to the xylem of bean plants

A relatively large proportion of radioactive zeatin was transported from the treated stem area to other plant components. The radioactivity recovered in the treated stem area could represent excess zeatin, which the plant was not capable of utilizing during the 48 hour experimental period. Table 3.1 shows the percentage radioactivity recovered in the components of vegetative, flowering and fruiting plants expressed per component and per one gramme fresh weight.

Table 3.1 The percentage radioactivity (A expressed as % dpm/component and B expressed as % dpm/one gramme fresh weight) in the components of vegetative, flowering and fruiting *Phaseolus vulgaris* plants 48 hours after applying radioactive zeatin to the xylem.

Plant Component	Radioactivity (% dpm)					
	Stage of Plant Development					
	Vegetative		Flowering		Fruiting	
	A	B	A	B	A	B
Primary leaves	13,50	2,79	17,75	4,97	12,20	14,98
Trifoliate leaves	9,40	3,04	11,35	7,70	12,57	19,35
Stem	14,80	3,56	17,86	10,20	8,91	13,59
Roots	4,10	2,01	2,93	0,54	2,91	2,50
Apices/flowers/fruit	3,30	9,36	2,67	11,57	1,60	9,00
Treated stem	54,90	79,24	47,44	65,32	61,42	32,74
Senesced petals	-	-	-	-	0,32	7,84

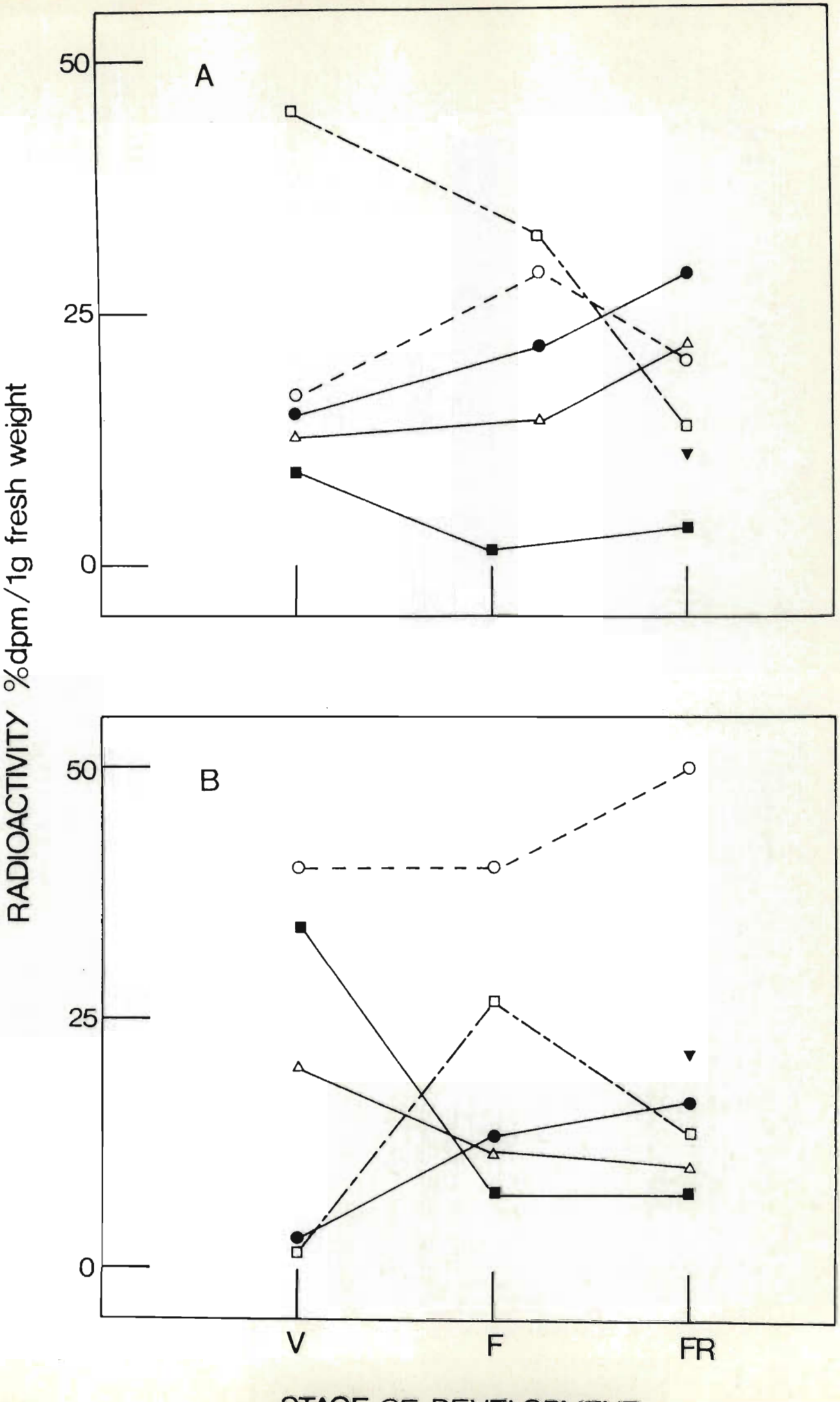
Expressing the results in these two ways did affect the overall trends. The results expressed per one gramme fresh weight were the results which were discussed. The highest percentage radioactivity was recovered in the apices, buds and flowers of vegetative and flowering plants. During fruit development relatively little radioactivity was recovered in the fruit, with the highest percentages of radioactivity recorded in the leaves and stem tissue. The percentage radioactivity in the untreated components only, is shown in Figure 3.4A. From this Figure it can be seen that radioactivity transported to the apical regions (apices, buds and flowers, fruit) decreased with plant development. The radioactivity recovered in the primary and trifoliate leaves, increased with plant growth, with slightly more radioactivity being recorded in the trifoliate leaves. Radioactivity in the roots decreased, while that in the stem fluctuated, reaching a maximum in flowering plants. It is interesting to note that the percentage radioactivity recovered in the senesced petals, which were found on the soil below the fruiting plants, or loosely attached to the ends of the developing fruit, was only slightly lower than that recovered in the fruit.

Transport of radioactive zeatin applied to the phloem of bean plants

It can be assumed that if cytokinins are exported out of leaves, they would be transported in the phloem. Radioactive zeatin was, therefore, applied to the phloem to de-

Figure 3.4A The percentage radioactivity recovered in the untreated components to which radioactivity was transported 48 hours after applying radioactive zeatin to the xylem of vegetative, flowering and fruiting *Phaseolus vulgaris* plants. Only that radioactivity which was exported from the treated stem area was considered and was expressed as % dpm per one gramme fresh weight.

B The percentage radioactivity in the untreated components to which radioactivity was transported 48 hours after applying radioactive zeatin to the phloem of vegetative, flowering and fruiting plants. △——△ = primary leaves; ●——● = trifoliate leaves; O----O = stem; ■——■ = roots; ▼ = senesced petals; □—·—□ = vegetative apices, buds, flowers and fruit; V = vegetative; F = flowering; FR = fruiting.


A
50
25
0
B
50
25
0
V
F
FR
RADIOACTIVITY %dpm/1g fresh weight
STAGE OF DEVELOPMENT

termine the fate of cytokinins in the assimilate stream. From Table 3.2, it can be seen that radioactivity, associated with the radioactive zeatin applied to the phloem, was transported away from the treated stem area and that the distribution pattern was different when zeatin was applied to the xylem. The percentage radioactivity in the untreated components only, is shown in Figure 3.4B. From this Figure, it can be seen that the radioactivity exported to primary leaves decreased with time, while that in the trifoliate leaves increased slightly. Radioactivity decreased in the roots and increased in the stem during plant growth. The radioactivity transported to the vegetative apices was low, appeared to reach a maximum in the buds and flowers and decreased in the developing fruit. A relatively high percentage of the exported radioactivity was also detected in the senesced petals, which suggests that at least some of the radioactivity detected in the buds and flowers is not utilized in fruit development.

Metabolism of 8(^{14}C)*t*-zeatin applied to the xylem and phloem of bean plants

Paper chromatographic separation of all the plant components resulted in three radioactive peaks. The first two peaks, which eluted at R_f 0,0-0,2 and R_f 0,2-0,5 respectively, were referred to as radioactive peaks 1 and 2. The last peak (R_f 0,5-1,0), which probably contained radioactivity associated with the labelled zeatin, as well as possibly

Table 3.2 The percentage radioactivity (A expressed as % dpm/component and B expressed as % dpm/one gramme fresh weight) in the components of vegetative, flowering and fruiting *Phaseolus vulgaris* plants 48 hours after applying radioactive zeatin to the phloem.

Plant Component	Radioactivity (% dpm)					
	Stage of Plant Development					
	Vegetative		Flowering		Fruiting	
	A	B	A	B	A	B
Primary leaves	2,65	6,44	7,97	1,75	6,24	1,29
Trifoliate leaves	1,70	0,91	2,94	1,92	9,88	2,63
Roots	20,50	11,22	7,34	1,13	5,37	0,75
Apices/flowers/fruit	0,30	0,78	0,91	4,01	0,10	1,75
Treated stem	29,50	67,77	64,23	85,02	55,68	77,70
Stem above treated stem	3,90	1,90	3,38	1,20	5,26	1,83
Stem below treated stem	17,60	10,98	13,32	4,79	15,05	10,35
Senesced petals	-	-	-	-	1,52	3,70

ribosylzeatin and dihydrozeatin, was referred to as radioactive peak 3. The distribution of these peaks in the plant components, following the application of radioactive zeatin to the xylem and phloem, is shown in Tables 3.3 and 3.4 respectively. Following the application of zeatin to the xylem, radioactive peak 2 was the major peak in the vegetative and flowering plants, with radioactive peak 1 becoming more important in the fruiting plants. The metabolic pattern appeared to be slightly different when zeatin was applied to the phloem. There was no distinct radioactive peak in the components of the vegetative plants. Radioactive peak 2 appeared to be the major peak in the flowering and fruiting plants. The different amounts of

Table 3.3 The distribution of radioactive peaks 1 (R_f 0,0-0,2), 2 (R_f 0,2-0,5) and 3 (R_f 0,5-1,0) in the components of (A) vegetative; (B) flowering and (C) fruiting bean plants 48 hours after applying radioactive zeatin to the xylem. Radioactivity is expressed as % dpm recovered/radioactive peak.

Plant Components	Radioactivity (% dpm)		
	Radioactive Peak 1 (R_f 0,0-0,2)	Radioactive Peak 2 (R_f 0,2-0,5)	Radioactive Peak 3 (R_f 0,5-1,0)
A Vegetative			
Primary leaves	30,40	55,80	13,80
Trifoliate leaves	36,50	49,80	13,70
Stem	25,00	47,20	27,80
Roots	28,90	44,30	26,80
Apices/Flowers/Fruit	25,90	47,50	26,60
Treated stem	25,00	41,80	33,20
B Flowering			
Primary leaves	35,05	50,13	14,82
Trifoliate leaves	31,55	47,99	20,46
Stem	33,15	50,00	16,85
Roots	22,16	70,65	7,19
Apices/Flowers/Fruit	23,99	48,08	27,93
Treated stem	27,73	46,99	25,28
C Fruiting			
Primary leaves	43,49	43,72	12,79
Trifoliate leaves	43,34	44,70	11,96
Stem	49,04	42,68	8,28
Roots	31,43	37,14	31,43
Apices/Flowers/Fruit	49,56	30,97	19,47
Treated stem	30,82	44,42	24,76
Senesced petals	46,67	51,11	2,22

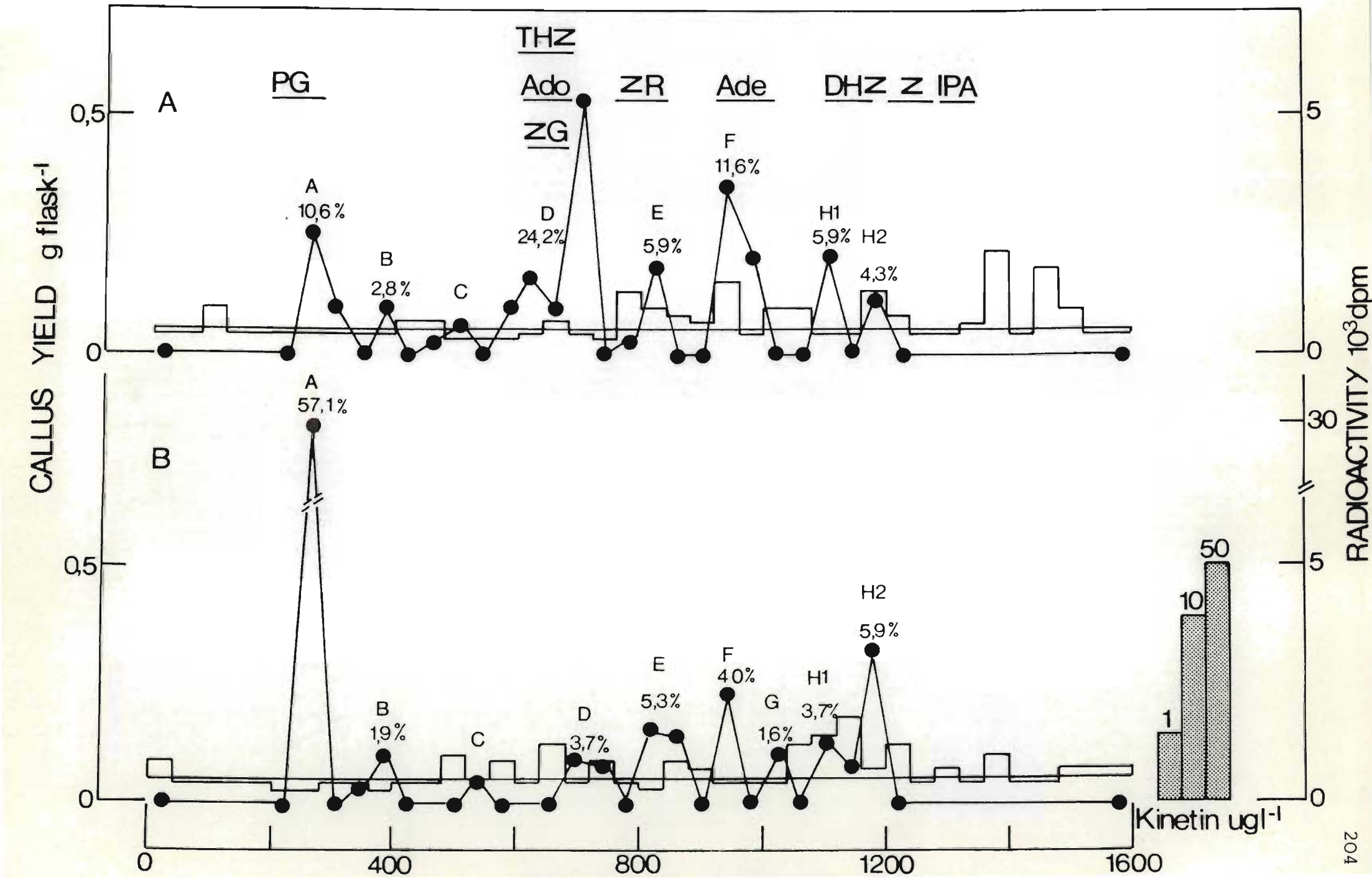
Table 3.4 The distribution of radioactive peaks 1 (R_f 0,0-0,2), 2 (R_f 0,2-0,5) and 3 (R_f 0,5-1,0) in the components of (A) vegetative; (B) flowering and (C) fruiting bean plants, 48 hours after applying radioactive zeatin to the phloem. Radioactivity is expressed as % dpm recovered/radioactive peak.

Plant Components	Radioactivity (% dpm)		
	Radioactive Peak 1 (R_f 0,0-0,2)	Radioactive Peak 2 (R_f 0,2-0,5)	Radioactive Peak 3 (R_f 0,5-1,0)
A Vegetative			
Primary leaves	35,80	24,50	39,70
Trifoliate leaves	1,50	56,30	42,20
Roots	21,10	33,40	45,50
Apices/flowers/fruit	30,30	54,80	14,90
Treated stem area	18,50	37,10	44,40
Stem above treated area	52,70	37,40	9,90
Stem below treated area	25,20	12,90	61,90
B Flowering			
Primary leaves	19,64	37,45	42,91
Trifoliate leaves	29,63	46,42	23,95
Roots	36,76	41,50	21,74
Apices/flowers/fruit	8,73	58,73	32,54
Treated stem area	24,12	39,82	36,06
Stem above treated area	39,34	43,98	16,68
Stem below treated area	20,94	41,10	37,96
C Fruiting			
Primary leaves	31,69	51,95	16,36
Trifoliate leaves	25,96	52,05	21,99
Roots	26,13	37,59	36,28
Apices/flowers/fruit	18,51	41,85	39,64
Treated stem area	25,95	45,98	28,07
Stem above treated area	27,31	54,91	17,78
Stem below treated area	21,52	47,96	30,53
Senesced petals	24,40	38,49	37,11

radioactivity associated with the three radioactive peaks following the application of zeatin to the xylem and phloem probably reflects the different transport patterns and thus the metabolic capacities of the different tissues.

Column chromatography of the components of plants to which zeatin had been applied to the xylem, showed that a number of radioactive compounds were actually present in the three radioactive peaks detected on the paper chromatograms. The paper chromatograms were not divided into the three radioactive peaks, but were separated on Sephadex LH-20 columns as one extract. It was felt that the occurrence of the same three radioactive peaks in all systems examined thus far, did not necessitate separating these peaks individually. Nine radioactive peaks were detected in the treated stem of flowering and fruiting plants following fractionation by column chromatography (Figure 3.5). The elution volumes of these peaks were similar to the radioactive peaks recorded in the *Ginkgo biloba* and *Citrus sinesis* leaves, and were, therefore, also referred to as radioactive peaks A, B, C, D, E, F, G, H1 and H2. These peaks were only tentatively identified on the basis of co-elution with cytokinin standards. Radioactive peak A co-eluted with purinyl glycine and peak C co-eluted with the suspected ribosylzeatin glucoside. Radioactive peak D co-eluted with three compounds which have similar elution volumes in this chromatographic system. These three compounds are adenosine, trihydroxyzeatin and zeatin glucoside. Peaks E and F co-eluted with ribosylzeatin and

Figure 3.5 Radioactivity (●——●) and biological activity (histograms) in the treated stem of (A) flowering and (B) fruiting *Phaseolus vulgaris* plants 48 hours after applying radioactive zeatin to the xylem of the treated stem area. Extracts were separated on Sephadex LH-20 columns eluted with 10 per cent methanol. Radioactive peaks A, B, C, D, E, F, G, H1 and H2 are indicated as well as the percentage radioactivity associated with each peak. PG = purinyl glycine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; Ado = adenosine; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.

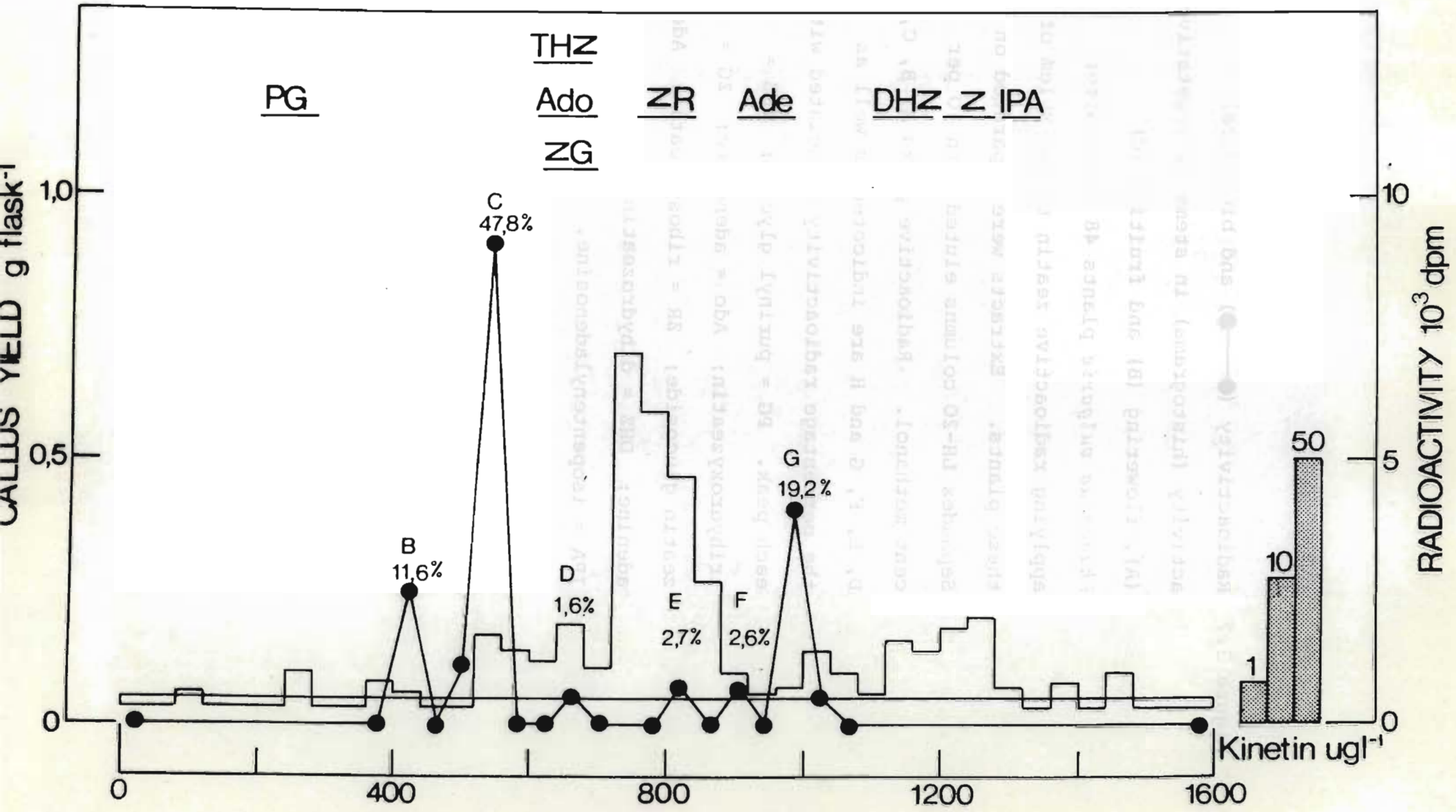

THZ
PG
Ado
ZR
Ade
DHZ Z IPA
ZG
A
B
A
10,6%
B
2,8%
C
D
24,2%
E
5,9%
F
11,6%
H1
5,9%
H2
4,3%
A
57,1%
B
1,9%
C
D
3,7%
E
5,3%
F
4,0%
G
1,6%
H1
3,7%
H2
5,9%
0,5
0
5
30
1
10
50
0
400
800
1200
1600
CALLUS YIELD g flask-1
RADIOACTIVITY 10³dpm
Kinetin ugl-1

adenine respectively. Peaks H1 and H2 co-eluted with dihydrozeatin and zeatin respectively. Radioactive peaks B and G did not co-elute with any of the cytokinin standards and were not identified. From Figure 3.5 it can be seen that radioactive peaks A, D and F were the major peaks in the treated stem area of flowering plants and peak A was the major peak in the fruiting plants.

The other plant compounds were also separated by column chromatography (Figures 3.6, 3.7, 3.8, 3.9 and 3.10). Some of the nine radioactive peaks detected in the treated stem were also detected in the untreated components. There were insufficient vegetative apices, buds and flowers for column chromatography. Three major and three minor peaks were, however, detected in the developing fruit (Figure 3.6). These peaks did not co-chromatograph with the biologically active peaks, which co-eluted with ribosylzeatin and zeatin. The radioactive peaks detected in the other components did not appear to follow any specific pattern. Radioactive peak A, which co-eluted with purinyl glycine, appeared to be an important compound, especially in flowering and fruiting plants. Radioactive peak G, which had a similar elution volume to an oxidation product of zeatin, (Figure 1.19), was also an important compound.

The untreated components of plants to which zeatin had been applied to the phloem, were not fractionated by column chromatography. The paper chromatograms of these extracts were assayed for cytokinin activity using the soyabean

Figure 3.6 Radioactivity (●——●) and biological activity (histogram) in the developing fruit of *Phaseolus vulgaris* plants 48 hours after applying radioactive zeatin to the xylem of these plants. The extract was fractionated on a Sephadex LH-20 column eluted with 10 per cent methanol. Radioactive peaks B, C, D, E, F and G are indicated as well as the percentage radioactivity associated with each peak. PG = purinyl glycine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; Ado = adenosine; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.


THZ
PG
Ado
ZR
Ade
DHZ
Z
IPA
ZG
CALLUS YIELD g flask-1
1,0
0,5
0
C
47,8%
B
11,6%
D
1,6%
E
2,7%
F
2,6%
G
19,2%
10
5
0
RADIOACTIVITY 10-3 dpm
50
10
1
Kinetin ugl-1
0
400
800
1200
1600

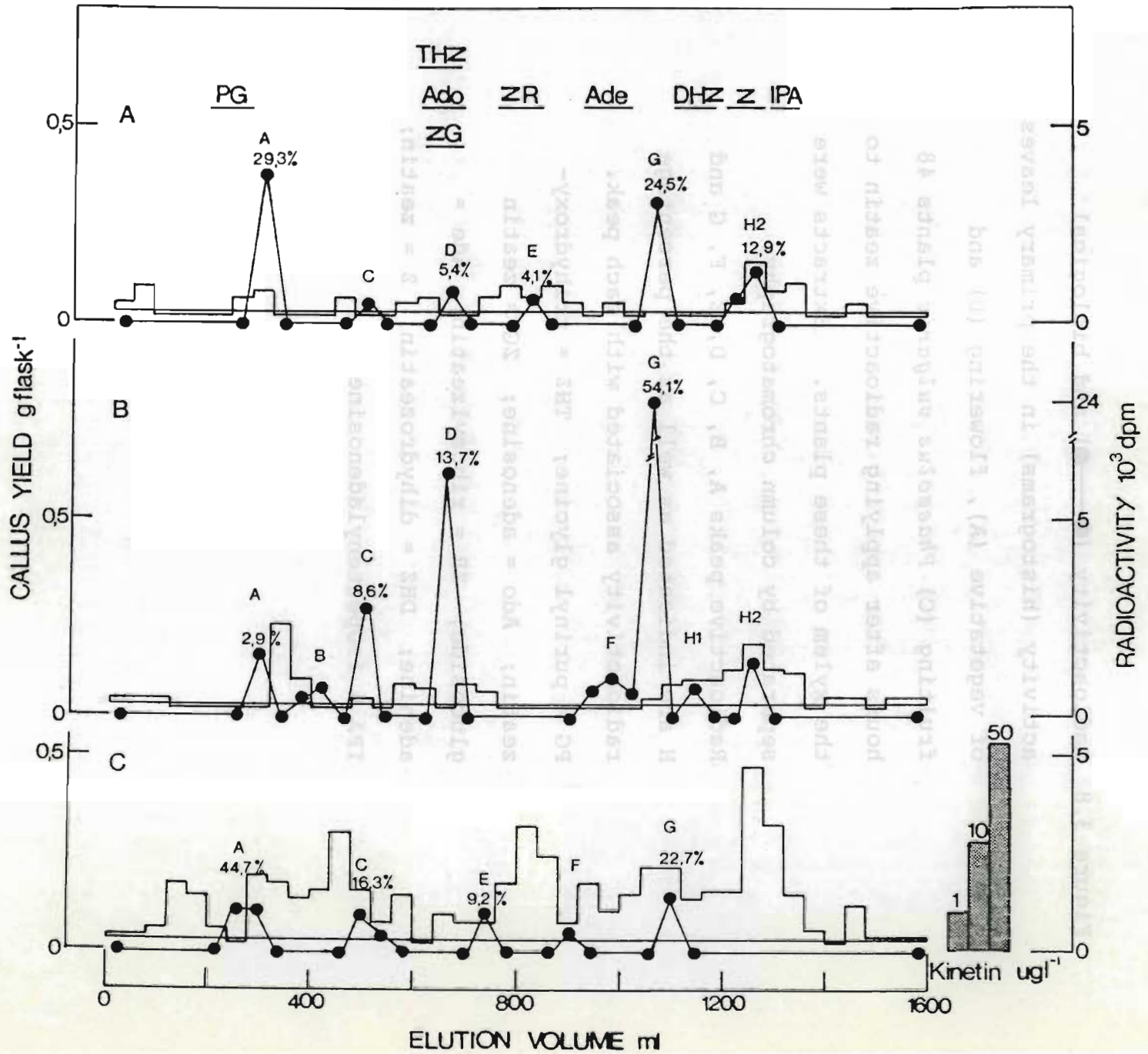
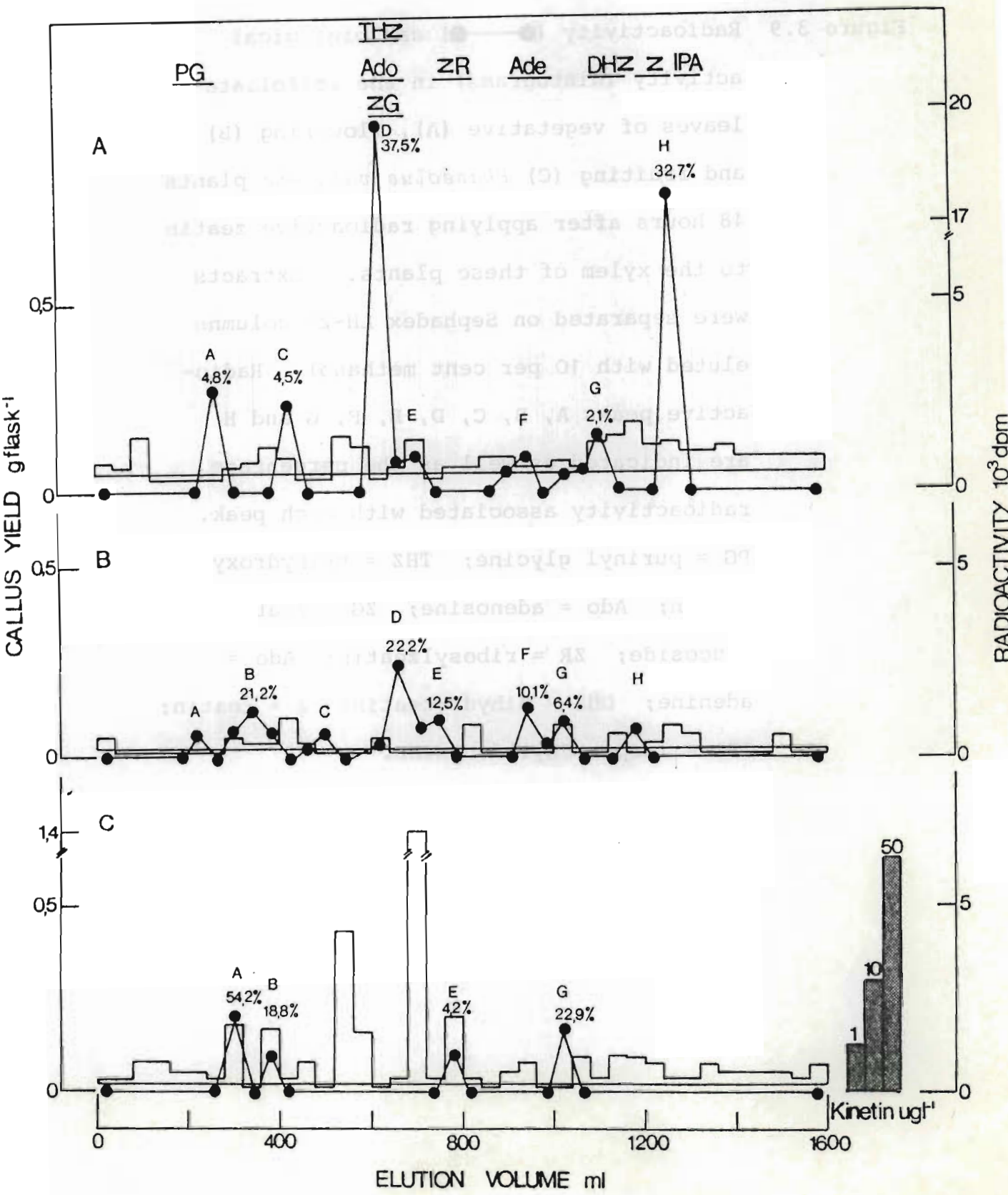

THZ
PG
Ado
ZR
Ade
DHZ
Z
IPA
ZG
A
B
C
A 29,3%
C
D 5,4%
E 4,1%
G 24,5%
H2 12,9%
A 2,9%
B
C 8,6%
D 13,7%
F
G 54,1%
H1
H2
A 44,7%
C 16,3%
E 9,2%
F
G 22,7%
0,5
0
5
24
50
10
1
CALLUS YIELD g flask-1
RADIOACTIVITY 10³ dpm
Kinetin ugl-1
0
400
800
1200
1600
ELUTION VOLUME ml

Figure 3.8 Radioactivity (●——●) and biological activity (histograms) in the primary leaves of vegetative (A), flowering (B) and fruiting (C) *Phaseolus vulgaris* plants 48 hours after applying radioactive zeatin to the xylem of these plants. Extracts were separated by column chromatography. Radioactive peaks A, B, C, D, E, F, G and H are indicated as well as the percentage radioactivity associated with each peak. PG = purinyl glycine; THZ = trihydroxy-zeatin; Ado = adenosine; ZG = zeatin glucoside; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.


THZ
PG
Ado
ZR
Ade
DHZ
Z
IPA
ZG
A
B
C
D 37,5%
H 32,7%
A 4,8%
C 4,5%
G 2,1%
E
F
D 22,2%
B 21,2%
E 12,5%
F 10,1%
G 6,4%
H
A 54,2%
B 18,8%
E 4,2%
G 22,9%
0,5
1,4
0
20
17
5
50
10
1
Kinetin ugl-1
0
400
800
1200
1600
ELUTION VOLUME ml
CALLUS YIELD gflask-1
RADIOACTIVITY 10-3 dpm

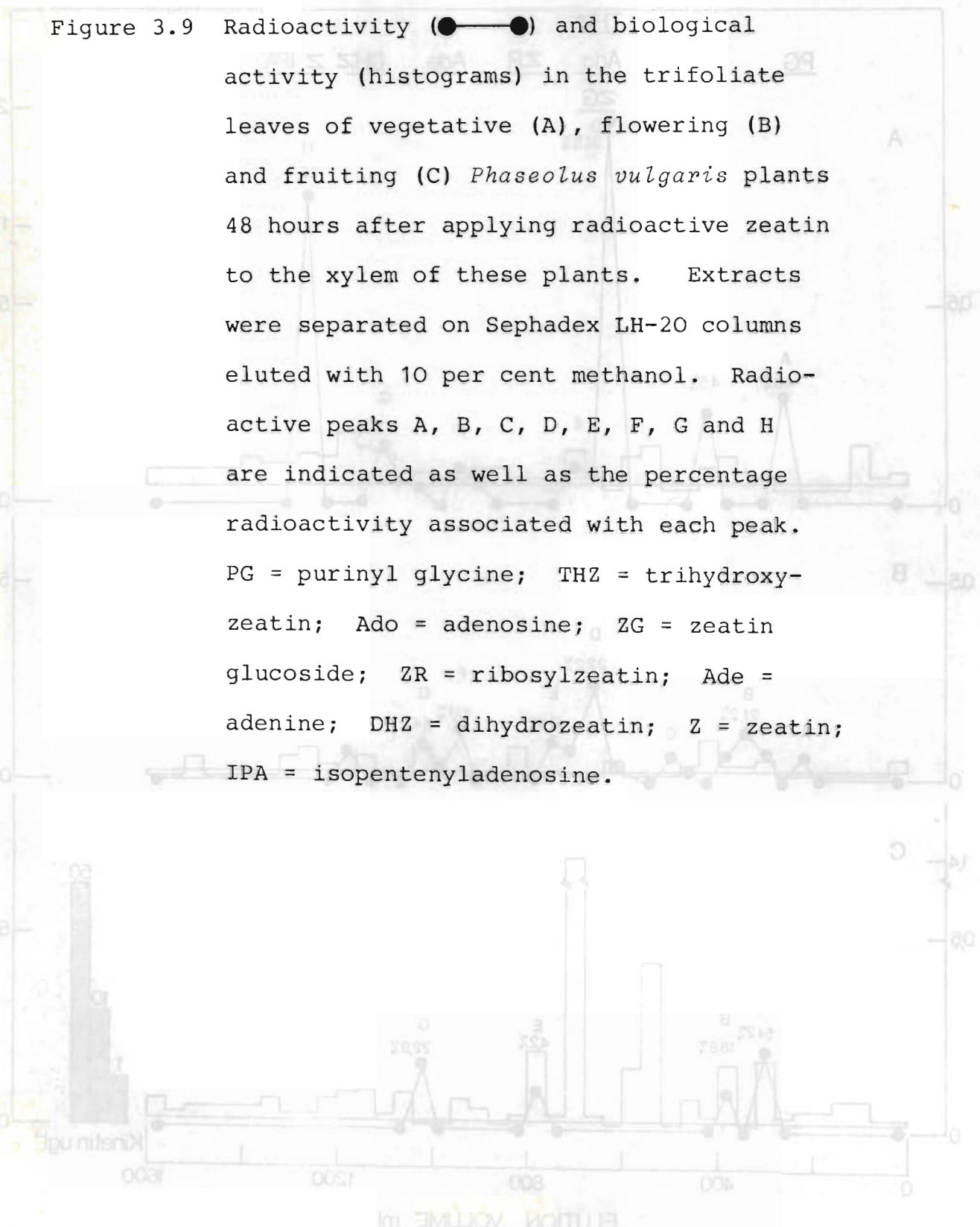
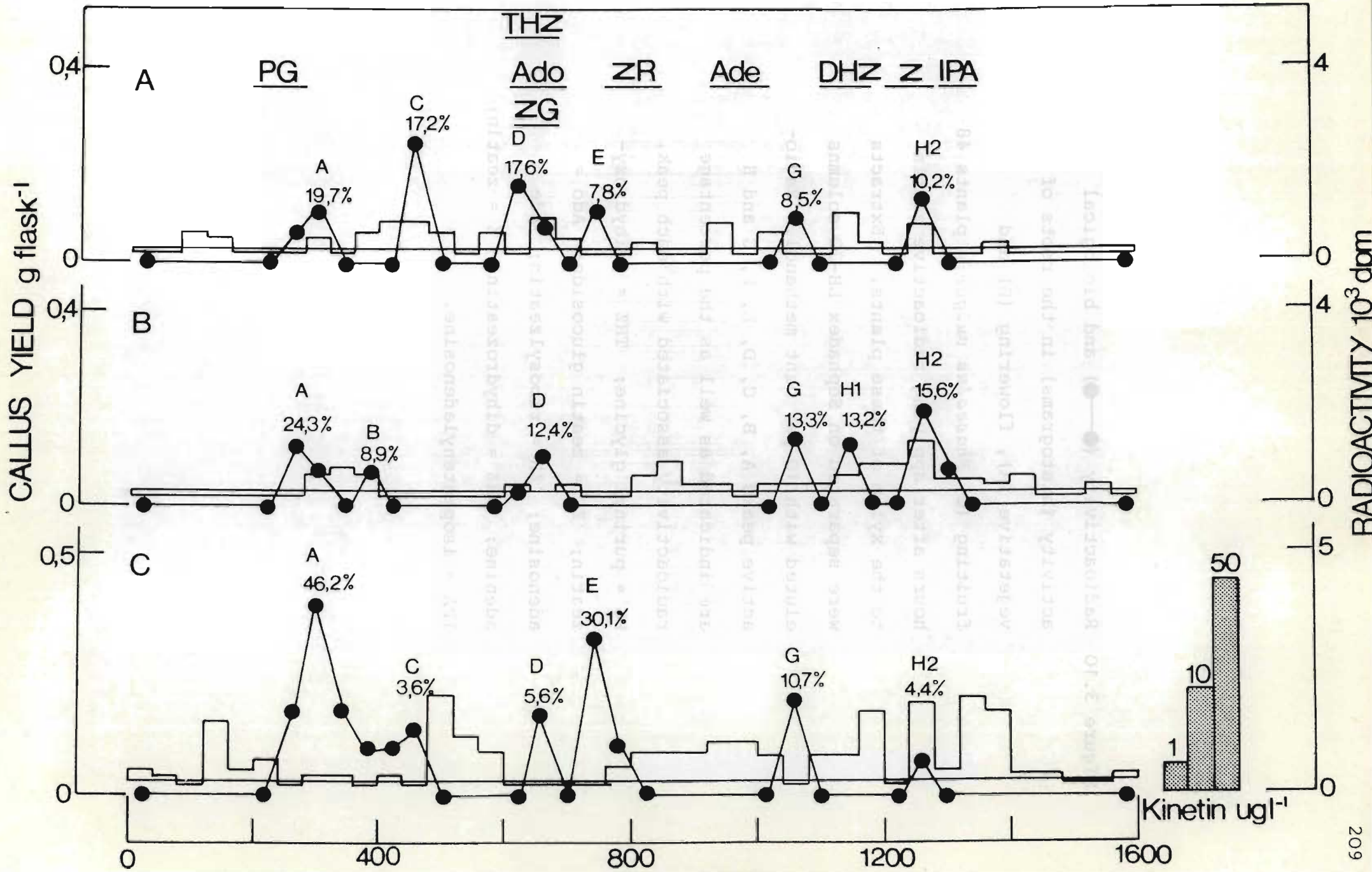


Figure 3.9 Radioactivity (●——●) and biological activity (histograms) in the trifoliate leaves of vegetative (A), flowering (B) and fruiting (C) *Phaseolus vulgaris* plants 48 hours after applying radioactive zeatin to the xylem of these plants. Extracts were separated on Sephadex LH-20 columns eluted with 10 per cent methanol. Radioactive peaks A, B, C, D, E, F, G and H are indicated as well as the percentage radioactivity associated with each peak. PG = purinyl glycine; THZ = trihydroxyzeatin; Ado = adenosine; ZG = zeatin glucoside; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.


THZ
PG
Ado
ZR
Ade
DHZ
Z
IPA
ZG
A
B
C
0,4
0
0,5
4
5
A
19,7%
C
17,2%
D
17,6%
E
7,8%
G
8,5%
H2
10,2%
A
24,3%
B
8,9%
D
12,4%
G
13,3%
H1
13,2%
H2
15,6%
A
46,2%
C
3,6%
D
5,6%
E
30,1%
G
10,7%
H2
4,4%
1
10
50
Kinetin ugl⁻¹
0
400
800
1200
1600
CALLUS YIELD g flask⁻¹
RADIOACTIVITY 10³ dpm

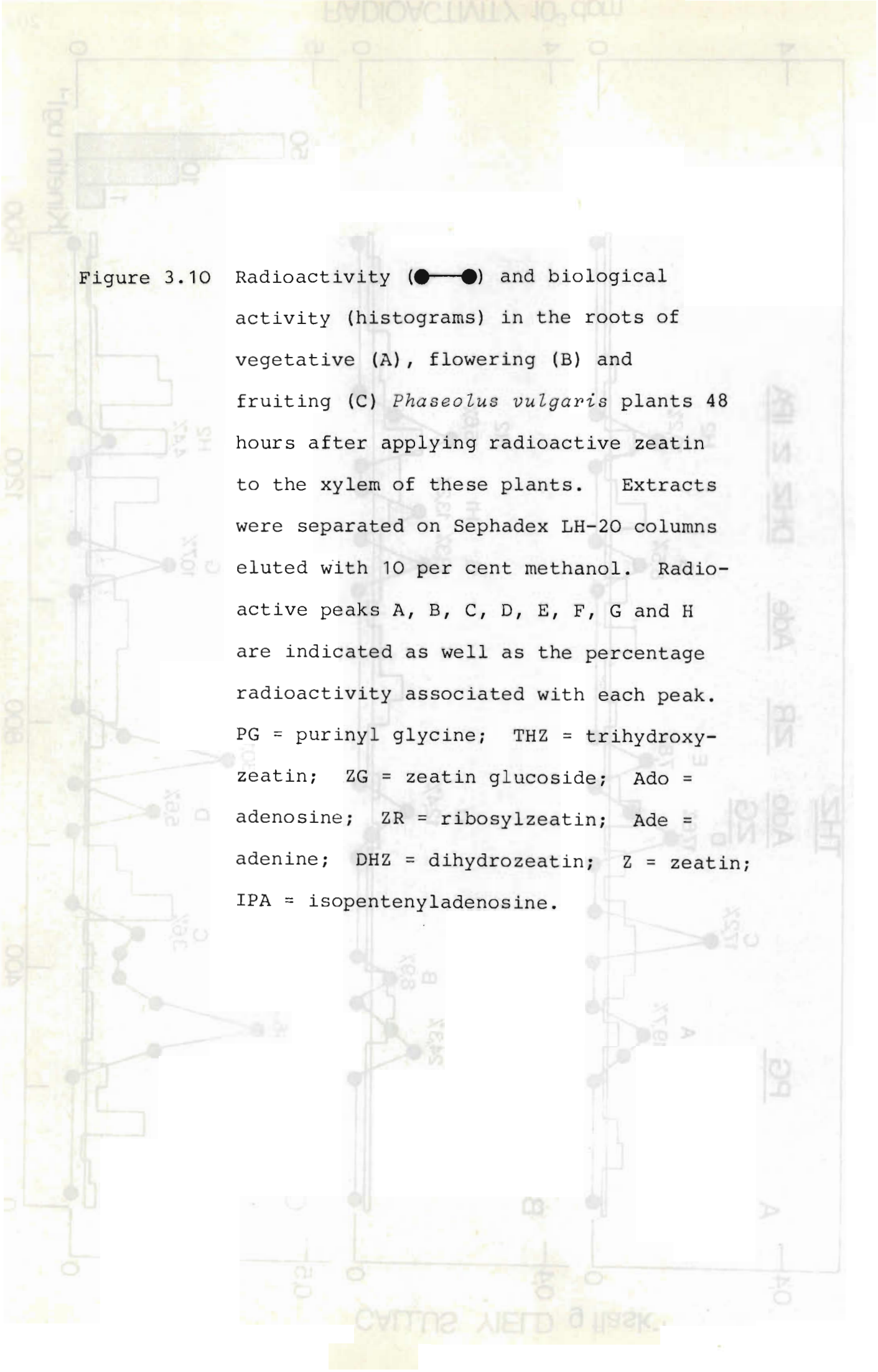
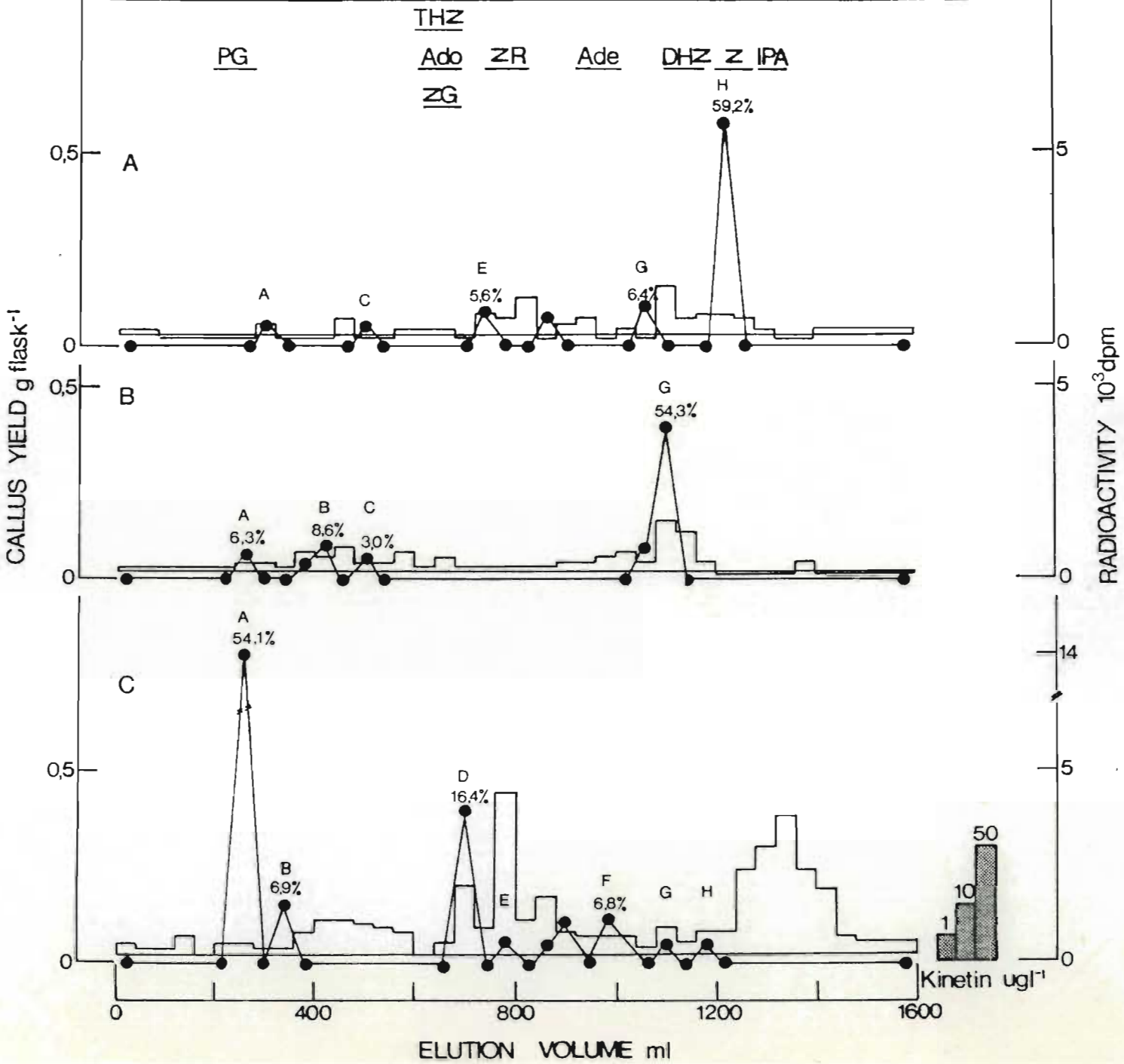


Figure 3.10 Radioactivity (●—●) and biological activity (histograms) in the roots of vegetative (A), flowering (B) and fruiting (C) *Phaseolus vulgaris* plants 48 hours after applying radioactive zeatin to the xylem of these plants. Extracts were separated on Sephadex LH-20 columns eluted with 10 per cent methanol. Radioactive peaks A, B, C, D, E, F, G and H are indicated as well as the percentage radioactivity associated with each peak. PG = purinyl glycine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; Ado = adenosine; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.


THZ
PG
Ado
ZR
Ade
DHZ
Z
IPA
ZG
A
B
C
H
59,2%
E
5,6%
G
6,4%
54,3%
6,3%
8,6%
3,0%
54,1%
D
16,4%
6,9%
F
6,8%
0,5
0
5
14
50
10
1
CALLUS YIELD g flask⁻¹
RADIOACTIVITY 10³dpm
Kinetin ugl⁻¹
0
400
800
1200
1600
ELUTION VOLUME ml

callus bioassay. From Figure 3.11 it can be seen that there were essentially two radioactive peaks, a polar and a non-polar peak. These radioactive peaks did not co-chromatograph very well with the biological activity.

3.3.3 Transport and metabolism of 8(^{14}C)*t*-zeatin applied to primary leaves of fruiting *Phaseolus vulgaris* plants

Transport of radioactive zeatin applied to primary bean leaves

If cytokinins are exported out of leaves, it might be expected that cytokinin glucosides, which have been suggested to be storage compounds, or their hydrolyzed products would be transported out of the leaves. Radioactive zeatin was, therefore, applied to primary leaves of fruiting *Phaseolus vulgaris* plants as it was thought that these leaves would have high cytokinin glucoside levels.

From Table 3.5 it can be seen that less than 3 per cent of the radioactivity was exported out of the primary leaves. In terms of that radioactivity which was exported, it can be seen that after five days, the developing fruit received most of the radioactivity. After ten days, however, the highest percentage radioactivity was recorded in the trifoliate leaves and stem. Radioactivity detected in the roots and fruits thus decreased with time and increased in the trifoliate leaves and stem. The decrease in radio-

Figure 3.11 Radioactivity (●——●) and biological activity (histograms) in the components of vegetative (A), flowering (B) and fruiting (C) *Phaseolus vulgaris* plants 48 hours after radioactive zeatin to the phloem of these plants. Extracts were separated on paper chromatograms with *iso*-propanol:25 per cent ammonium hydroxide:water (10:1:1 v/v). Callus grown on 50 microgrammes per litre kinetin yielded 0,56 grammes fresh weight. Z = zeatin; ZR = ribosylzeatin; ZG = zeatin glucoside.

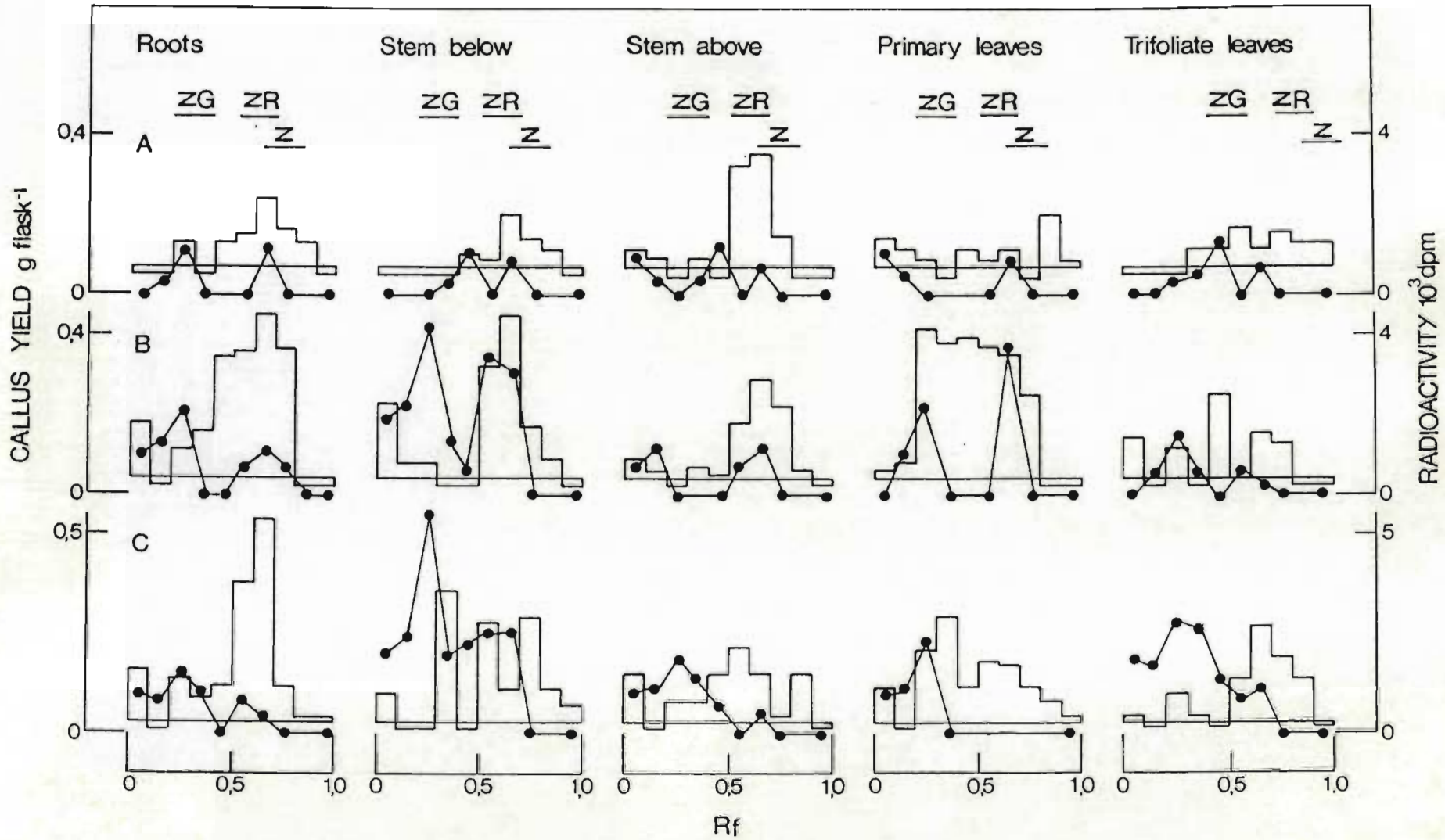

Roots
Stem below
Stem above
Primary leaves
Trifoliate leaves
ZG ZR Z
A
B
C
0,4
0,5
0
4
5
CALLUS YIELD g flask-1
RADIOACTIVITY 10³dpm
0 0,5 1,0
Rf

Table 3.5 The distribution of radioactivity (A expressed as % dpm/component and B expressed as % dpm/one gramme fresh weight) 5 and 10 days after applying 8(^{14}C)*t*-zeatin to the primary leaves of fruiting *Phaseolus vulgaris* plants.

Plant Component	Radioactivity (% dpm)			
	Days After Treatment			
	A	B	A	B
Treated primary leaves	98,75	97,98	95,20	97,12
Trifoliate leaves and stem	0,97	0,56	4,60	2,33
Roots	0,22	0,56	0,12	0,38
Fruit	0,06	0,89	0,08	0,17

activity in the fruit could be the result of rapid cytokinin utilization in the fruit or preferential transport to the leaves following the initial stages of fruit development.

Metabolism of radioactive zeatin applied to the primary leaves

Paper chromatographic separation of the extracts resulted in three radioactive peaks, which had the same R_f values as the three peaks detected following the application of zeatin to the stem of the *Phaseolus vulgaris* plants. These peaks were also called radioactive peaks 1, 2 and 3. The distribution of these peaks in the treated leaves is shown in Table 3.6. Radioactive peaks 1 and 2 increased and radioactive peak 3, which probably contained residual

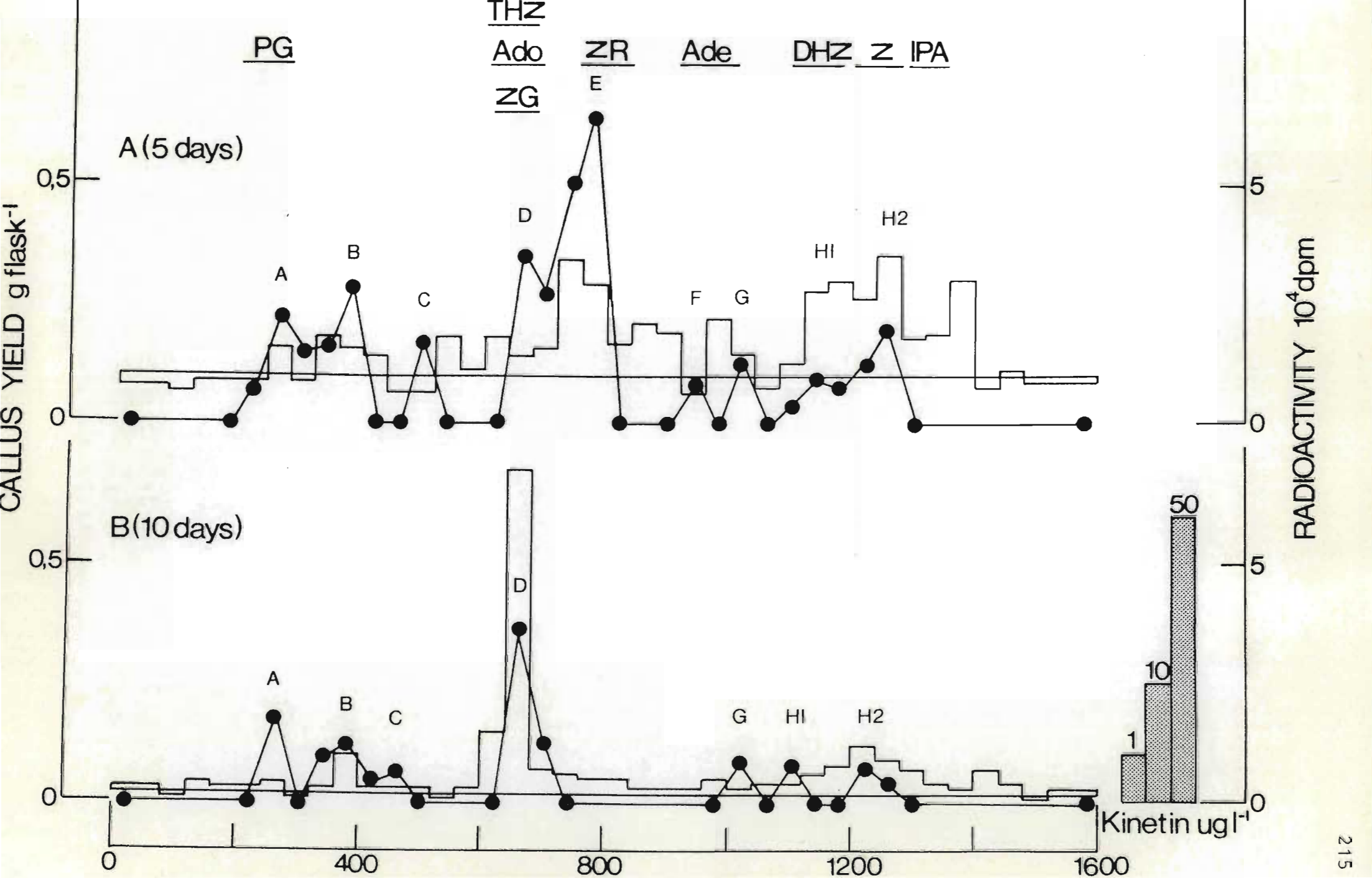
Table 3.6 The distribution of radioactive peaks 1 (R_f 0,0-0,2), 2 (R_f 0,2-0,5) and 3 (R_f 0,5-1,0) in the primary leaves 5 and 10 days after applying 8(^{14}C)*t*-zeatin to these leaves.

Plant Component	Sampling Time (days)	Radioactivity (% dpm/Radioactive Peak)		
		Radioactive Peak 1 (R_f 0,0-0,2)	Radioactive Peak 2 (R_f 0,2-0,5)	Radioactive Peak 3 (R_f 0,5-1,0)
Treated primary leaves	5	20,80	46,00	33,20
	10	28,70	58,90	12,40

radioactive zeatin, decreased with time.

After fractionation by column chromatography, nine radioactive peaks were detected in the treated primary leaves. These peaks were also referred to as radioactive peaks A, B, C, D, E, F, G, H1 and H2 (Figure 3.12). These peaks were only tentatively identified. Peak A, which co-elutes with purinyl glycine was not affected by alkaline phosphatase treatment. The chromatographic properties of peak B were not affected by β-glucosidase or potassium permanganate treatment and it did not co-elute with any cytokinin standards. Ribosylzeatin glucoside appears to have a similar elution volume to radioactive peak C. This peak was, however, not affected by β-glucosidase treatment. This does not positively eliminate glucosides as components of this peak, as 7- and 9-glucosides are not affected by β-glucosidase. The tentative identification of peaks D to H2 has been mentioned earlier. The other

Figure 3.12 Radioactivity (●——●) and biological activity (histograms) in the treated primary leaves of fruiting *Phaseolus vulgaris* plants 5 (A) and 10 (B) days after applying radioactive zeatin to these leaves. Extracts were separated on Sephadex LH-20 columns eluted with 10 per cent methanol. Radioactive peaks A, B, C, D, E, F, G and H are indicated in the figures. PG = purinyl glycine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; Ado = adenosine; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.

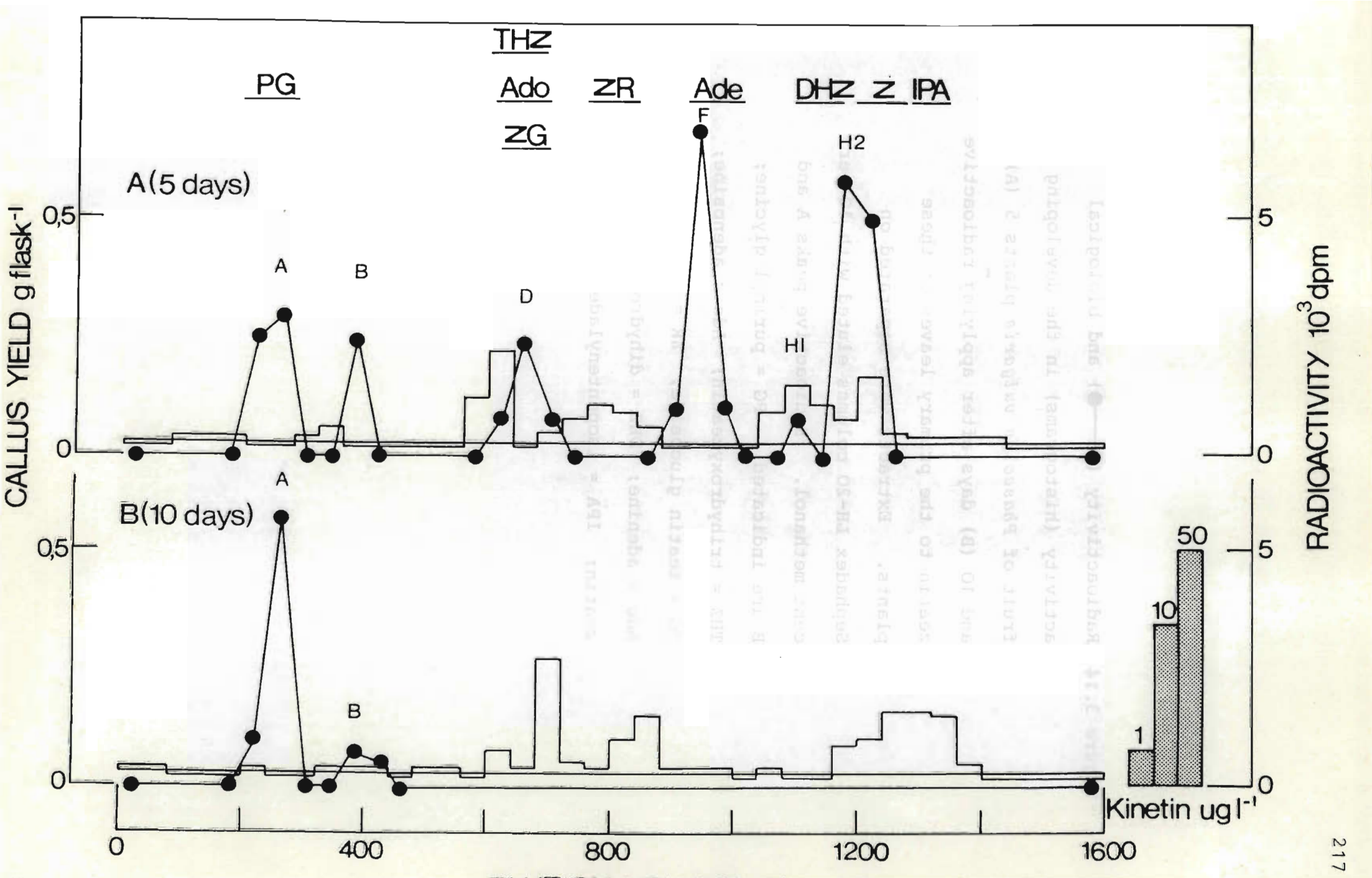

THZ
PG
Ado
ZR
Ade
DHZ
Z
IPA
ZG
E
A (5 days)
0,5
0
D
H2
B
HI
A
C
F
G
CALLUS YIELD g flask-1
5
0
RADIOACTIVITY 10^4dpm
B (10 days)
0,5
D
50
A
10
B
C
G
HI
H2
1
0
Kinetin ug l-1
0
400
800
1200
1600

plant components were also separated by column chromatography. Figure 3.13 illustrates the radioactivity associated with the stem and trifoliate leaves. Five days after treatment, radioactive peaks co-eluting with zeatin and adenosine were the major peaks, but after ten days the major peak co-eluted with purinyl glycine. Only one radioactive peak was detected in the fruit (Figure 3.14). This peak, which co-eluted with purinyl glycine, did not co-chromatograph with the biological activity in the fruit.

3.4 Discussion

Cytokinins produced in the roots are transported to the shoot via the transpiration stream. Endogenous cytokinin experiments indicated that cytokinin levels increased in the primary leaves and stem tissue, but decreased in the trifoliate leaves during plant development. The cytokinin levels in the roots fluctuated, decreasing during bud development and increasing during flowering and the early stages of fruit development. DAVEY and VAN STADEN (1976) reported fluctuations in the root exudate of *Lycopersicon esculentum* Mill. plants during their development. That is, cytokinin levels decreased during flower bud development and increased at anthesis. These authors have proposed that a decrease in cytokinins may be a prerequisite for flowering. Cytokinin levels in the vegetative apices, buds and flowers, and fruit, depended on how the results were expressed. If expressed per constant fresh weight, cytokinin activity reached a maximum in the

Figure 3.13 Radioactivity (●——●) and biological activity (histograms) in the stem and trifoliate leaves of fruiting *Phaseolus vulgaris* plants 5 (A) and 10 (B) days after applying radioactive zeatin to the primary leaves of these plants. Extracts were separated on Sephadex LH-20 columns eluted with 10 per cent methanol. Radioactive peaks A, B, D, F and H are indicated. PG = purinyl glycine; THZ = trihydroxyzeatin; Ado = adenosine; ZG = zeatin glucoside; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.


THZ
PG
Ado
ZR
Ade
DHZ
Z
IPA
ZG
F
H2
A(5 days)
A
B
D
HI
B(10 days)
A
B
CALLUS YIELD g flask-1
RADIOACTIVITY 10^3 dpm
0,5
0
5
50
10
1
Kinetin ug l-1
0
400
800
1200
1600

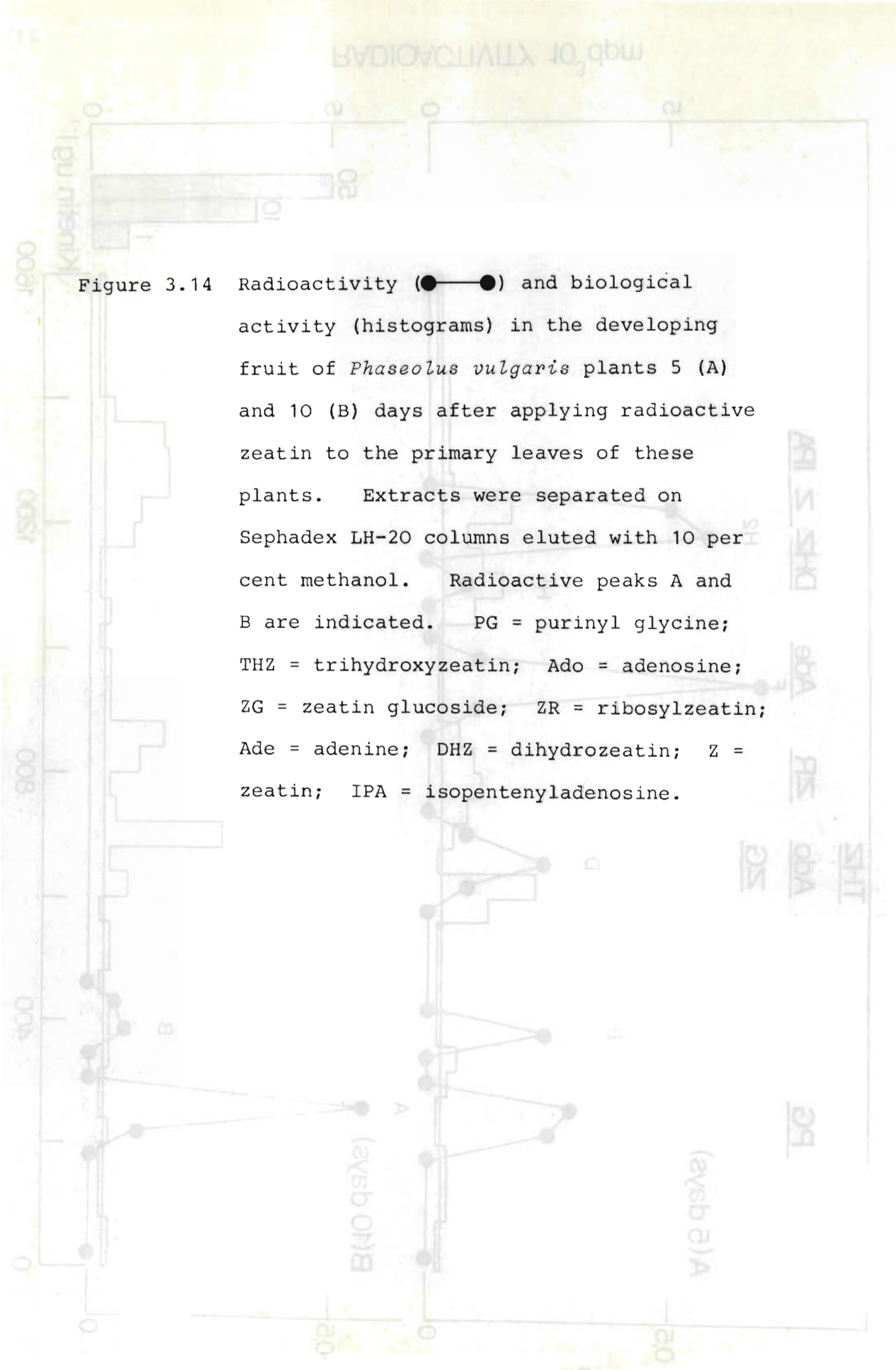
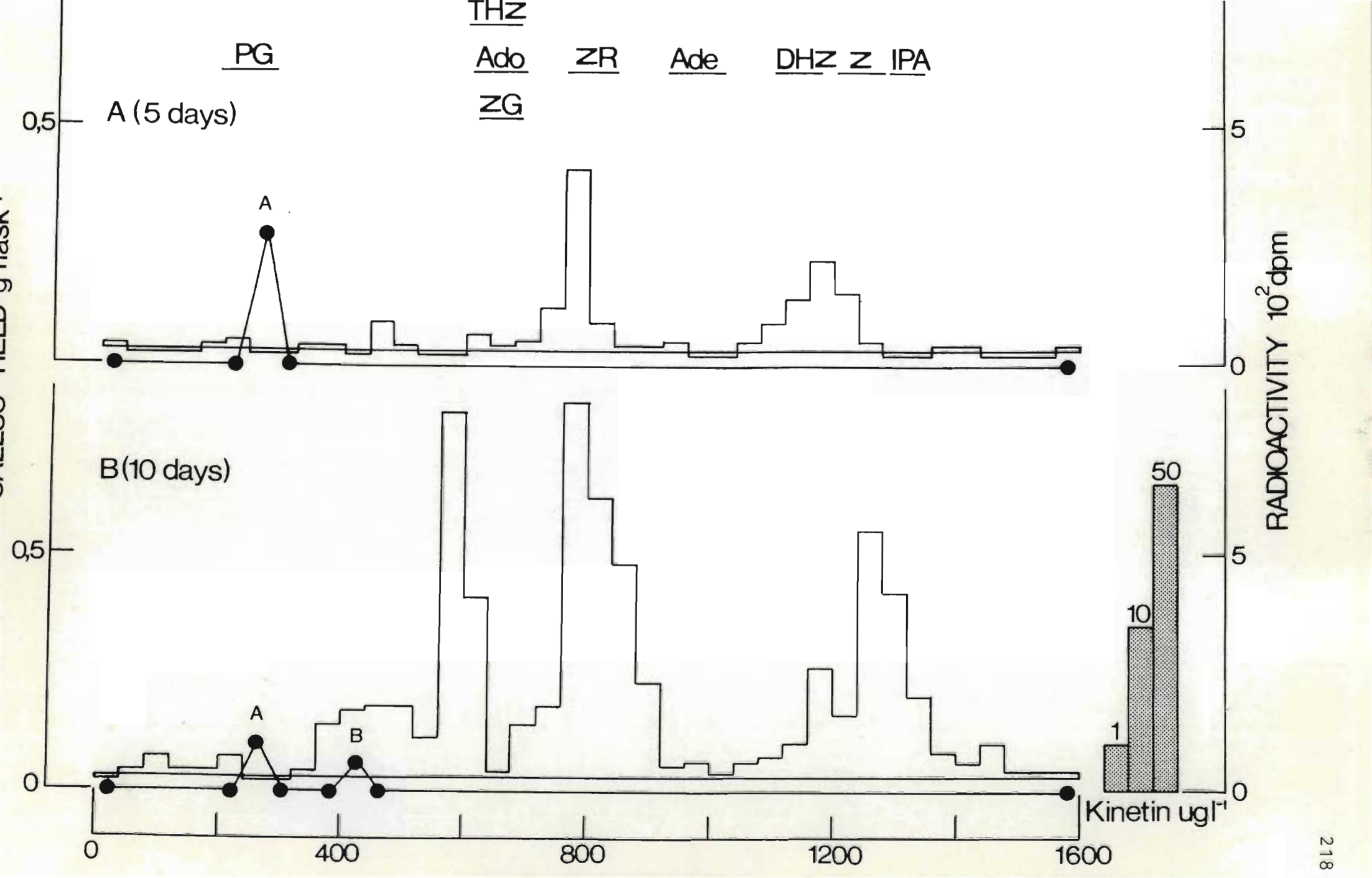


Figure 3.14 Radioactivity (●——●) and biological activity (histograms) in the developing fruit of *Phaseolus vulgaris* plants 5 (A) and 10 (B) days after applying radioactive zeatin to the primary leaves of these plants. Extracts were separated on Sephadex LH-20 columns eluted with 10 per cent methanol. Radioactive peaks A and B are indicated. PG = purinyl glycine; THZ = trihydroxyzeatin; Ado = adenosine; ZG = zeatin glucoside; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.


THz
PG
Ado
ZR
Ade
DHZ
Z
IPA
ZG
A (5 days)
0,5
A
5
0
RADIOACTIVITY 10^2 dpm
B(10 days)
0,5
50
10
1
A
B
5
0
Kinetin ugl^{-1}
0
400
800
1200
1600

buds and flowers and then decreased. If, however, the results were expressed per component, cytokinin activity appeared to increase and was highest in the developing fruit. It is difficult to determine whether it is more accurate to express results per one gramme fresh weight or per actual component. It can be said that cytokinin activity should ideally be expressed per cell and that expressing results per one gramme fresh weight may give a better indication of cytokinin activity per cell, than if results are expressed per component. It could, however, also be argued that components with large weight differences, such as flowers and fruit, are not comparable in terms of cell numbers. Expressing cytokinin activity per component may, therefore, also give a valid indication of cytokinin activity. Throughout these experiments, results expressed per one gramme fresh weight were the results discussed, but results expressed per component should also be borne in mind.

It has been suggested that cytokinins in the transpiration stream are not transported to preferential sites, but that cytokinin transport is rather a function of the transpiration stream (RAMINA, PIMPINI, BONIOLO and BERGAMASCO, 1979; DAVEY and VAN STADEN, 1981). DAVEY and VAN STADEN (1981) based their suggestion on the fact that in fruiting *Lupinus albus* plants, cytokinins were predominantly transported to the leaves. They did not, however, take cytokinin transport in earlier stages of development into account. The results of these experiments suggested that

cytokinins are, in fact, transported to definite sites. If cytokinins were passively transported in the transpiration stream, the highest percentages of radioactivity would have been expected to be recovered in the leaves, which did not occur. The highest percentage radioactivity was recorded in the vegetative apices and buds and flowers of vegetative and flowering plants and in the leaves of fruiting plants. Endogenous cytokinin activity was low in the vegetative apices and the high percentage radioactivity transported to these organs therefore supports the suggestion that cytokinins are rapidly utilized in these actively dividing tissues (DAVEY and VAN STADEN, 1978c). In fruiting plants, the leaves appeared to be a stronger sink than the fruit for cytokinins in the transpiration stream. DAVEY and VAN STADEN (1981) and SUMMONS, LETHAM, GOLLNOW, PARKER, ENTSCH, JOHNSON, MACLEOD and ROLFE (1981) have also reported that radioactive cytokinins are preferentially transported to leaves rather than to the developing seeds. It would thus seem that during fruit maturation, cytokinins are preferentially transported to the leaves, which would thus disagree with the proposal of VARGA and BRUINSMA (1974) that the developing fruit may be a stronger sink than the leaves for root-produced cytokinins. The decrease in radioactivity in the fruit could suggest that the developing seeds do not rely exclusively on cytokinins in the transpiration stream. Seeds may thus be capable of synthesizing cytokinins or may also receive cytokinins from the leaves. The fact that endogenous cytokinin activity in the trifoliate leaves decreased while radioactivity in-

creased in these leaves during development, suggests that cytokinins could have been exported from the leaves. Rapid cytokinin utilization in the developing seeds could, however, have accounted for the decrease in radioactivity in the fruit, rather than preferential transport to the leaves.

Some of the cytokinins in the fruit do appear to originate in the roots, but the vegetative apices, buds and flowers appear to be more important sinks for root-produced cytokinins than the developing seeds. It is, however, difficult to establish whether this cytokinin supply is sufficient to account for all the cytokinins in the fruits, especially in terms of the 48 hour experimental period. Conclusive evidence as to whether seeds are capable of cytokinin synthesis is necessary to determine the significance of root-produced cytokinins arriving in the developing fruit. It is interesting to note that following the application of radioactive zeatin to the xylem and phloem, a relatively high percentage of radioactivity was detected in the senesced petals. This implies that not all of the cytokinin transported to the flowers was incorporated into the developing seeds. The high cytokinin content of the flowers could perhaps attract nutrients during the initial stages of seed development, that is, until seeds can synthesize or accumulate sufficient cytokinins for this purpose. PATE and FARRINGTON (1981) observed that *Lupinus angustifolius* flowers attract assimilates most strongly at the bud stage, but lose sink strength after opening. This

could also account for the radioactivity in the faded petals. These authors also observed a resurgence of sink strength once the petals had senesced and the young fruit had begun to elongate.

The distribution pattern recorded following the application of radioactive zeatin to the phloem suggested a more passive movement of cytokinins in the assimilate stream. During fruit development, the mobilization centre for photosynthates shifts away from the roots, stem, apex and young leaves to the developing fruit (NOODEN and LEOPOLD, 1978). The transport of radioactive compounds in the phloem appeared to follow this pattern. That is, in vegetative plants, radioactivity was primarily transported to the roots, whereas in flowering and fruiting plants, the buds, flowers and developing fruit were more important in terms of radioactivity. Lateral transport from the phloem to the xylem may also have had an effect on the transport patterns detected. Although radioactive zeatin was not applied to the phloem of flower shoots, it can be suggested that if cytokinins are exported out of leaves, then they are transported passively in the assimilate stream rather than being actively transported to definite sites.

The export of radioactivity out of primary leaves of fruiting plants could confirm the suggestion that cytokinins exported out of leaves move passively in the assimilate stream. As in evergreen and deciduous leaves, relatively

little radioactivity was actually exported out of the treated leaves. The significance of this exported radioactivity in terms of the whole plant and of the large amount of zeatin applied to the leaves, is difficult to assess. VAN STADEN and DAVEY (1981a) also only recorded a very low percentage radioactivity exported out of leaves of fruiting *Lupinus albus* plants. In this experiment, the amount of radioactivity exported out of the leaves did not increase with time, which could perhaps imply that the exported radioactivity was not significant. Of the radioactivity which was exported, the highest percentage was recorded in the fruit after five days, but after ten days it was recorded in the trifoliate leaves and stem. VAN STADEN and DAVEY (1981a) reported that radioactive zeatin was not exported out of fruit. This, together with the fact that there was no increase in radioactivity being exported out of leaves after ten days, suggests that if cytokinins are exported out of leaves, then they are only transported to the fruit during the initial stages of fruit development and they are rapidly utilized in the fruit. NAGAR, INDIRA IYER and SICAR (1982) reported that cytokinin levels were high in the early stages of fruit growth of *Moringa pterigosperma* Gaertn. and them remained constant. This could account for the decrease in radioactive compounds in the fruit. It is, therefore, possible that cytokinins from the leaves do contribute towards the high cytokinin content of seeds, but do not appear to be as important as the roots in this respect. VAN STADEN and DAVEY (1981a) and VONK and DAVELAAR (1981) have also pro-

posed that cytokinins are exported out of leaves and that these cytokinins contribute to seed cytokinins.

In retrospect, however, a better indication of cytokinin export from leaves may have been obtained if radioactive zeatin had been applied to the trifoliate leaves, instead of the primary leaves. Cytokinin activity in the trifoliate leaves decreased during development and increased in the primary leaves. Radioactivity, however, increased in both the primary and trifoliate leaves, suggesting cytokinin export out of trifoliate rather than primary leaves. PATE and FARRINGTON (1981) also reported that assimilate flow from the leaves to the inflorescence of *Lupinus angustifolius* was usually confined to the organs on the same or adjacent phyllotaxis. That is, fruit are nourished mainly by foliar organs in closest proximity. If cytokinins are exported in the assimilate stream, it is possible that more cytokinin would be transported out of trifoliate leaves, which are in closer proximity to the fruit than the primary leaves. Trifoliate leaves could, therefore, potentially have been a more likely source of seed cytokinins than the primary leaves.

The low percentage radioactivity exported from the primary leaves implies that most of the cytokinin glucosides accumulated in these leaves are not re-utilized. Glucosylation could thus be a mechanism for preventing the leaves from acting as a sink for photosynthates and thereby allow the translocation of these compounds to the develop-

ing seeds. Cytokinin glucosides in annual leaves could, therefore, essentially fulfil the same role as in deciduous and evergreen leaves. That is, to regulate leaf metabolism and facilitate nutrient mobilization to reproductive organs.

The decrease in radioactive compounds co-eluting with zeatin, and the increase in compounds co-eluting with purinyl glycine in the stem and trifoliate leaves of plants following the application of zeatin to the primary leaves, as well as detection of purinyl glycine in the fruits of these plants, implies that if cytokinins are exported out of the leaves, then zeatin is not primarily involved in this transport. This could imply that if cytokinin glucosides are re-utilized, then they may be hydrolyzed in the leaves and stem but undergo further metabolism in the stem before entering the fruit. Previous reports on the export of cytokinin glucosides and/or their hydrolyzed products from leaves, are contradictory. VAN STADEN and DAVEY (1981a) suggested that zeatin glucoside was exported out of *Lupinus albus* leaves, whereas VONK and DAVELAAR (1981) have proposed that cytokinin glucosides are metabolized in the leaves and shoot tissue and are transported in the inflorescence stalk of *Yucca flaccida* plants as cytokinin nucleotides. It is interesting to note that purinyl glycine and cytokinin nucleotides have similar chromatographic properties (VAN STADEN, DREWES and HUTTON, 1982) which could imply that purinyl glycine is also a translocatable form. PALMER, HORGAN and WAREING (1981a)

reported that dihydrozeatin glucoside levels decreased in detached *Phaseolus vulgaris* leaves as well as in attached leaves following bud burst. They suggested that if glucosides are exported, then they are first metabolized. The availability of radioactive cytokinin glucosides, therefore, appears necessary to determine whether cytokinin glucosides are exported *per se* or hydrolyzed prior to export.

Radioactive zeatin applied to the stem and leaves of *Phaseolus vulgaris* plants was rapidly metabolized, with the same nine radioactive peaks detected as in *Ginkgo biloba* and *Citrus sinesis* leaves following fractionation of radioactive peaks 1 (R_f 0,0-0,2), 2 (R_f 0,2-0,5) and 3 (R_f 0,5-1,0) by column chromatography. Oxidation appeared to be the most important metabolic pathway for the applied zeatin, with ribosylation, side chain reduction and possibly glucosylation also occurring. Metabolites resulting from oxidation included compounds co-eluting with purinyl glycine, trihydroxyzeatin, an unidentified compound co-eluting with a compound detected following potassium permanganate oxidation of zeatin and possibly adenine. The significance of compounds co-eluting with purinyl glycine, which is the biologically inactive end-product of zeatin oxidation (MILLER, 1965; LETHAM, SHANNON and MCDONALD, 1967) is not clear. This compound, which did not co-chromatograph with the biologically active peaks, appeared to be important in the untreated components to which radioactivity was transported, especially in the fruiting plants.

This could suggest that purinyl glycine is an inactive translocatable form, which can possibly be converted back to active compounds when they are required. However, the fact that these oxidation products are not detected as normal cytokinin components of *Phaseolus vulgaris* plants, raises the possibility that zeatin oxidation is not a normal metabolic pathway, but rather the result of the excess zeatin supplied to the system. PALMER, SCOTT and HORGAN (1981) have reported urea and ureides as major metabolites and adenine, adenosine and glucosylated conjugates as minor metabolites following the application of radioactive zeatin to detached *Phaseolus vulgaris* leaves. Adenosine co-eluted with the trihydroxyzeatin detected in this system and further investigation could show that these are, in fact, the same compound. PALMER, SCOTT and HORGAN (1981) suggested that a similar system to the cytokinin oxidase enzyme isolated from *Zea mays* (WHITTY and HALL, 1974) may be significant in the regulation of cytokinin levels in *Phaseolus vulgaris* leaves. Metabolism results from these experiments also suggest that oxidation (side chain cleavage) rather than side chain modification, is the major form of metabolism. However, oxidation in these experiments would involve breaking of the double bound in the side chain to form trihydroxyzeatin and cleavage of the side chain to form purinyl glycine, rather than complete side chain cleavage to form adenine,as suggested by PALMER, SCOTT and HORGAN (1981).

CHAPTER FOUR

THE TRANSPORT AND METABOLISM OF 8(^{14}C)*t*-ZEATIN APPLIED TO GERMINATING *PHASEOLUS VULGARIS* SEEDS

4.1 Introduction

Cytokinins are considered to be involved in the initiation of germination and embryonic axis growth (PINFIELD and STOBART, 1972) but the exact involvement of cytokinins in these processes has not been clearly defined. Cytokinins have been extracted from the cotyledons of diverse genera (UHEDA and KURAISHI, 1977; DAVEY, 1978; HUTTON, VAN STADEN and DAVEY, 1982) and these cytokinins have been implicated in cotyledon enlargement (HUFF and ROSS, 1975), and in the initiation of radicle growth (PINFIELD and STOBART, 1972; NOEL and VAN STADEN, 1976; SMITH and VAN STADEN, 1979). It is generally agreed that radicles synthesize cytokinins (VAN STADEN and DAVEY, 1979) but low or undetectable cytokinin levels have been recorded during early radicle development (THIMANN, SHIBAOKA and MARTIN, 1970; WEBB, WAREING and VAN STADEN, 1973). SMITH and VAN STADEN (1978) have proposed that in germinating *Zea mays* kernels, cytokinins in the embryo maintain radicle growth but cytokinins from the endosperm are necessary for further radicle growth up to the time when these organs start synthesizing cytokinins. VAN STADEN (1981a) has, however, subsequently shown that radioactive zeatin, applied to the endosperm of *Zea mays* kernels was not involved in radicle protrusion or the early stages of germination. Low cytokinin levels in

radicles could thus be the result of rapid utilization or export of cytokinins to the cotyledons, rather than the inability to synthesize these compounds. Other authors have also proposed that there is a barrier to cytokinin transport out of cotyledons (TZOU, GALSON and SONDHEIMER, 1973; GORDON, LETHAM and PARKER, 1974). This could imply that cotyledonary cytokinins are involved in cotyledon enlargement rather than in the initiation of radicle growth.

Cytokinins have also been implicated in the regulation of hydrolytic enzyme activity in cotyledons (GEPSTEIN and ILAN, 1979, 1980). Circumstantial evidence suggests that cytokinins released by the embryonic axis may regulate hydrolytic enzyme activity in cotyledons of dicotyledonous seeds. Exogenously applied cytokinins have been shown to increase hydrolytic enzyme activity (GEPSTEIN and ILAN, 1979; METIVIER and PAULILO, 1980a) and exogenous cytokinins can apparently partially or completely replace the axial requirement for normal enzyme action (PENNER and ASHTON, 1967; VAN ONCKELEN, CAUBERGS and DE GREEF, 1977; DAVIES and CHAPMAN, 1979; GEPSTEIN and ILAN, 1980; METIVIER and PAULILO, 1980b; MURRAY and ADAMS, 1980). Endogenous cytokinin activity also indicates cytokinin transport from the embryonic axes to the cotyledons, as the presence of the embryonic axis has been shown to increase cytokinin levels in the cotyledons (RYBICKA, ENGELBRECHT, MIKULOVICH and KULAEVA, 1977; VAN ONCKELEN, CAUBERGS and DE GREEF, 1977; HUTTON, VAN STADEN and DAVEY, 1982). Cytokinins from the embryonic axis, therefore, appear to be involved

in the mobilization of cotyledonary reserves on which the axis is dependent, but there is no direct evidence for the transport of cytokinins from the embryonic axis.

The extent to which cytokinins are actually transported between the cotyledons and the embryonic axis during the early stages of germination was, therefore, investigated in these experiments. Radioactive zeatin was applied to the radicle tip and to the cotyledons of germinating *Phaseolus vulgaris* seeds, and its subsequent transport and metabolism was monitored. By applying radioactive zeatin to the radicle it was hoped to determine whether cytokinins from the embryonic axis are transported to the cotyledons and thus initiate hydrolytic enzyme activity. By investigating possible cytokinin transport out of cotyledons, it was hoped to determine whether these cytokinins are involved in radicle growth in dicotyledonous seeds.

4.2 Experimental Procedure

4.2.1 Endogenous cytokinin experiments

Endogenous cytokinin activity was determined in the cotyledons, radicles and plumules of germinating *Phaseolus vulgaris* (cv. Contender) seeds. Seeds were imbibed for 12 hours to facilitate the removal of the testae. These seeds were then incubated on moist filter paper in petri dishes at 20°C in the dark. Twenty seeds were sampled at 0 (that is, after 12 hours imbibition), 24, 48 and 96 hours. At each sampling time, the seeds were divided into

cotyledons, radicles and plumules. These components were deep frozen at -20°C until analyzed for cytokinin activity. In the second experiment, the embryonic axes were removed after the 12 hour incubation period and the excised cotyledons were then incubated for a further 0, 24, 48, 72 and 96 hours respectively. The third experiment was conducted over a four day incubation period and involved the removal of the embryonic axis at different times after commencing with the experiment and then incubating the cotyledons on their own for the remainder of the experiment (four days). At the end of the four day experimental period, all cotyledonary material was collected and deep frozen until analyzed. Cotyledons were therefore collected from controls (embryonic axis present all the time), and where the axes had been removed for four and two days respectively.

4.2.2 Application of 8(^{14}C)*t*-zeatin to the radicle tip of germinating seeds

Phaseolus vulgaris seeds were imbibed for 12 hours to facilitate the removal of their testae. One microlitre of 8(^{14}C)*t*-zeatin (approximately 2 x 10^5 dpm) was then applied via a microsyringe to the radicle tip of 20 seeds. Prior to applying the radioactive cytokinin, a small strip of Parafilm was placed between the radicle and the cotyledons to prevent any zeatin spreading on to the cotyledons. The seeds were germinated in repli dishes so as to prevent them from falling over and zeatin diffusing on to the filter paper on which they were incubated. The seeds were then incubated at 20°C in the dark for 24 and 48 hours. At the

end of the incubation times, the seeds were divided into cotyledons, radicles (including the hypocotyl) and plumules. The collected material was deep frozen until extracted for cytokinins. All experiments were repeated at least once.

4.2.3 Application of 8(^{14}C) *t*-zeatin to the cotyledons of germinating seeds

The testae were removed from seeds following a 12 hour imbibition period. One microlitre of 8(^{14}C) *t*-zeatin was then applied via a microsyringe to the outer surface of one cotyledon of each of 20 seeds. Prior to applying the radioactive cytokinin, the surface of the cotyledon was gently damaged to facilitate the uptake of the hormone. The seeds were then incubated in repli dishes at 20°C in the dark for 24 and 48 hours. At these sampling times, the seeds were divided into treated cotyledons, untreated cotyledons, radicles and plumules. The collected material was then deep frozen until extracted for cytokinins. All experiments were repeated at least once.

4.2.4 Re-application of radioactive peaks 1 and 2 to the cotyledons of germinating seeds

Radioactive peak 1 (R_f 0,0-0,2) and radioactive peak 2 (R_f 0,2-0,5), formed from the radioactive zeatin, were re-applied to the cotyledons of germinating seeds in an attempt to learn more about the nature of these metabolites. Cotyledons were fed labelled zeatin and then incubated for 48 hours. The cotyledonary material was extracted with ethanol and purified with Dowex 50. After paper chromato-

graphy, the R_f zones which yielded the radioactive peaks (R_f 0,0-0,2 and R_f 0,2-0,5) were eluted with ethanol, concentrated and the radioactive metabolites re-applied to the cotyledons of 12 hour imbibed seeds. After 24 and 48 hours the seeds were harvested and their cotyledons, radicles and plumules analyzed for radioactivity.

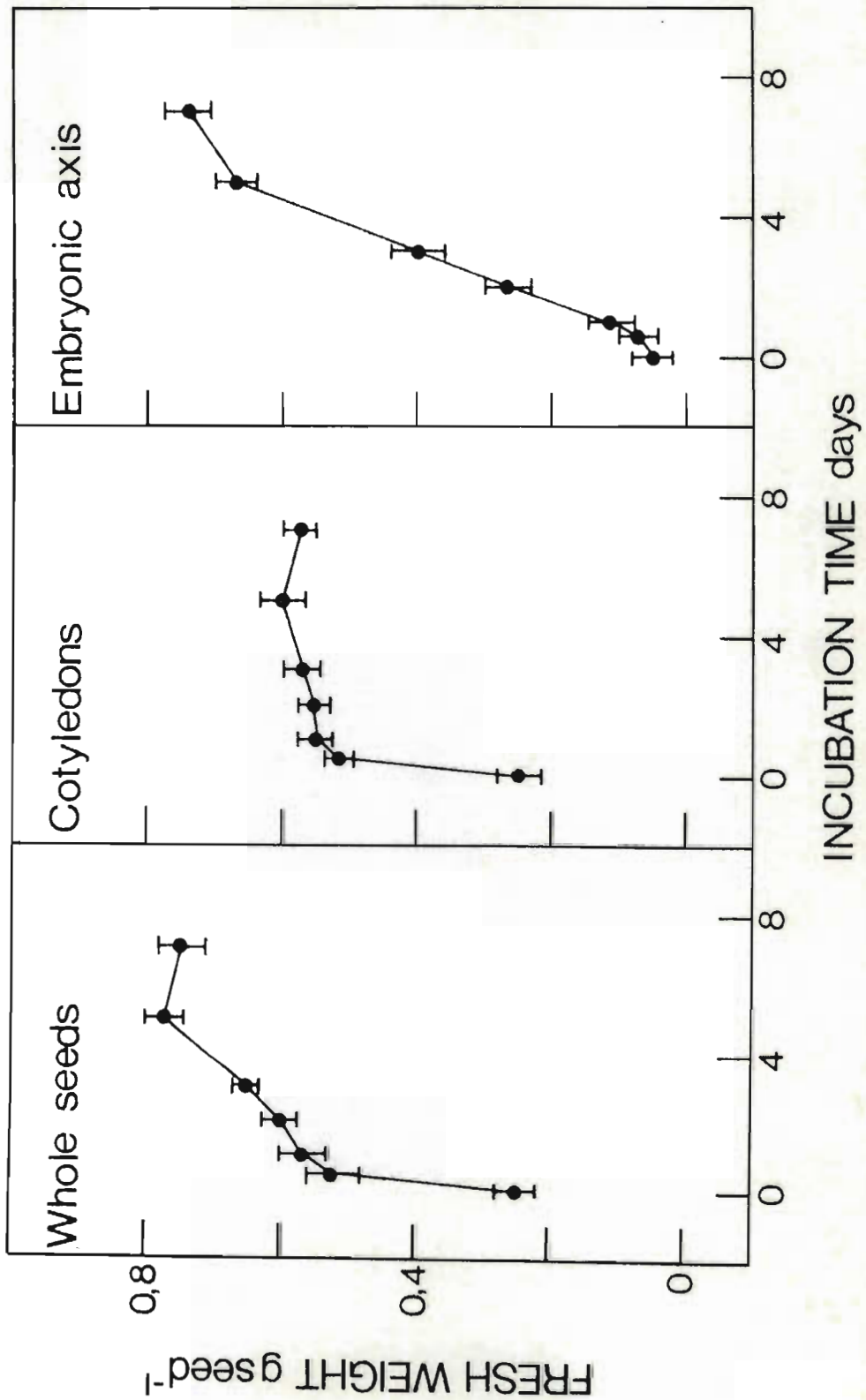
The cytokinin extraction, paper and column chromatography, bioassay and radioassay techniques are all described in detail in the Materials and Methods section. All samples were extracted for cytokinins and stip-loaded onto paper chromatograms. Portions of these chromatograms were used for radioassays and if sufficient material was available for bioassays. Column chromatography was also used to obtain more information about the nature of the radioactive compounds detected. In the soyabean callus bioassays of the material from the endogenous cytokinin experiments, zeatin standards were used rather than kinetin standards, which were used in all the other bioassays.

4.3 Results

4.3.1 Endogenous cytokinin experiments

Phaseolus vulgaris seeds maintained at 20°C imbibed water rapidly during the first 12 hours of incubation. Most of this initial fresh weight increase was due to water uptake by the cotyledons (Figure 4.1). The fresh weight of the embryonic axis only started to increase significantly after the commencement of the experiment.

Figure 4.1 Water uptake of *Phaseolus vulgaris* seeds during incubation. (A) whole seeds; (b) cotyledons; (C) embryonic axis. Standard error of the mean is indicated.


Whole seeds
Cotyledons
Embryonic axis
FRESH WEIGHT g seed-1
0,8
0,4
0
0
4
8
INCUBATION TIME days

The cytokinin content of dry embryos and cotyledons is shown in Figure 4.2. The results obtained indicated that the axis itself contained a considerable amount of endogenous cytokinin, in fact far more than did the cotyledons. Paper chromatography indicated the presence of only one peak of activity, which co-eluted with zeatin and ribosylzeatin. Column chromatography of cotyledons, however, revealed a number of peaks of activity (Figure 4.3), co-eluting with zeatin, ribosylzeatin, dihydrozeatin and possibly zeatin and ribosylzeatin glucosides. Cytokinin activity in the seed components after 12 hours imbibition and 24, 48 and 96 hours germination can be seen in Figure 4.4. Cytokinin activity in the plumules was relatively constant during the 96 hour germination period, whereas cytokinin levels fluctuated in the radicles and cotyledons. The increases and decreases in cytokinin activity of the cotyledons and radicles appeared to complement one another, suggesting an interchange of cytokinins between these organs.

From Figure 4.5, which depicts the cytokinin activity detected in the cotyledons of intact embryos, it can be seen that the levels of extractable cytokinins fluctuated during the seven day incubation period. The fluctuations, the trends of which could be demonstrated more readily by presenting the total activity detected in each extract as nanogramme zeatin equivalents per 12,5 grammes fresh weight, showed that during early germination, when radicle growth was stimulated (between one and two days) the cytokinin

Figure 4.2 Cytokinin activity detected in intact dry seeds (A) and dry cotyledons (B) of *Phaseolus vulgaris*. Each histogram represents the activity that was recorded in the equivalent of 40 seeds. Extracts were purified using Dowex 50 cation exchange resin and the ammonia elutes were separated on paper chromatograms with *iso*-propanol:25 per cent ammonium hydroxide:water (10:1:1 v/v). Z = zeatin; ZG = zeatin glucoside; ZR = ribosylzeatin.

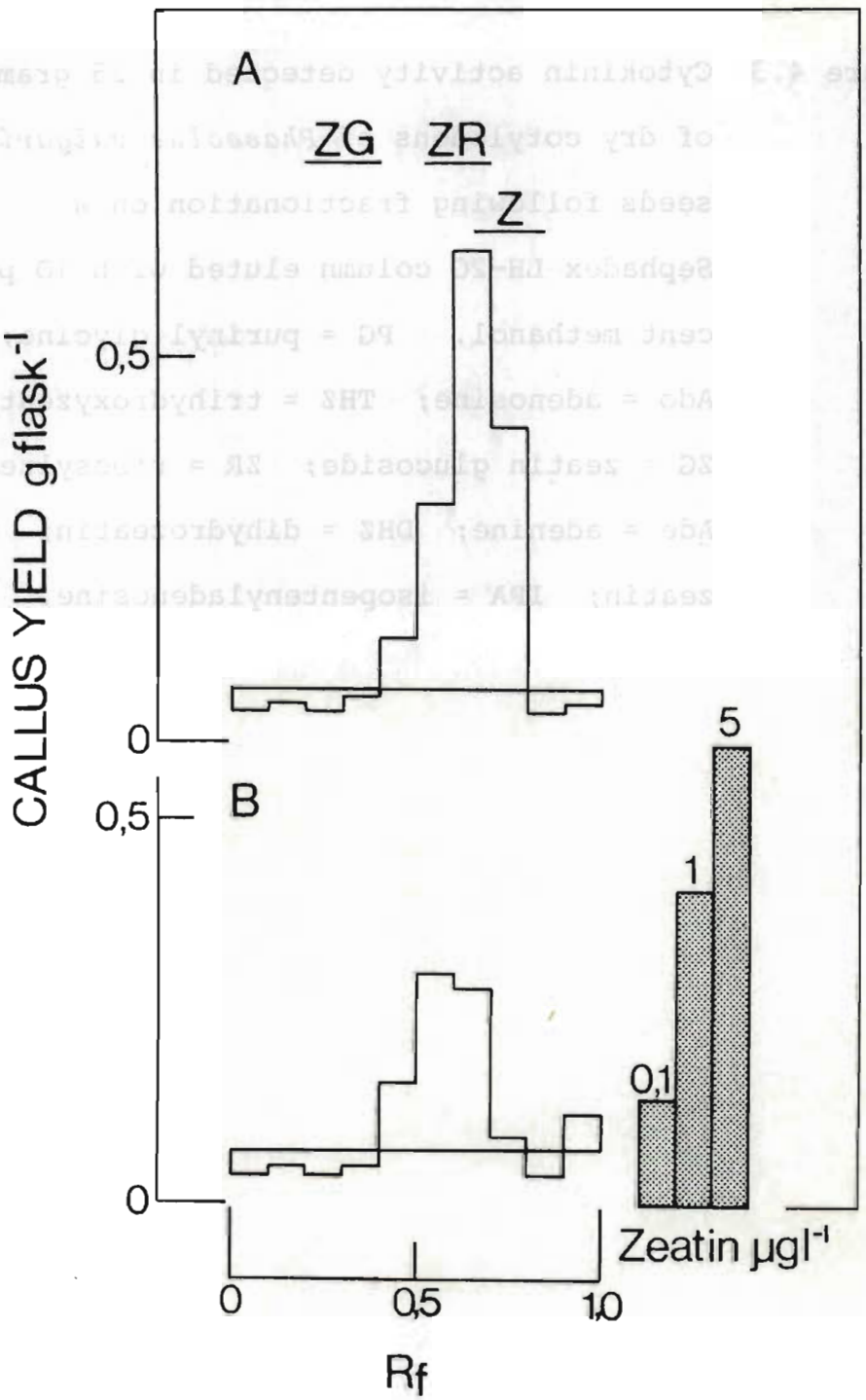
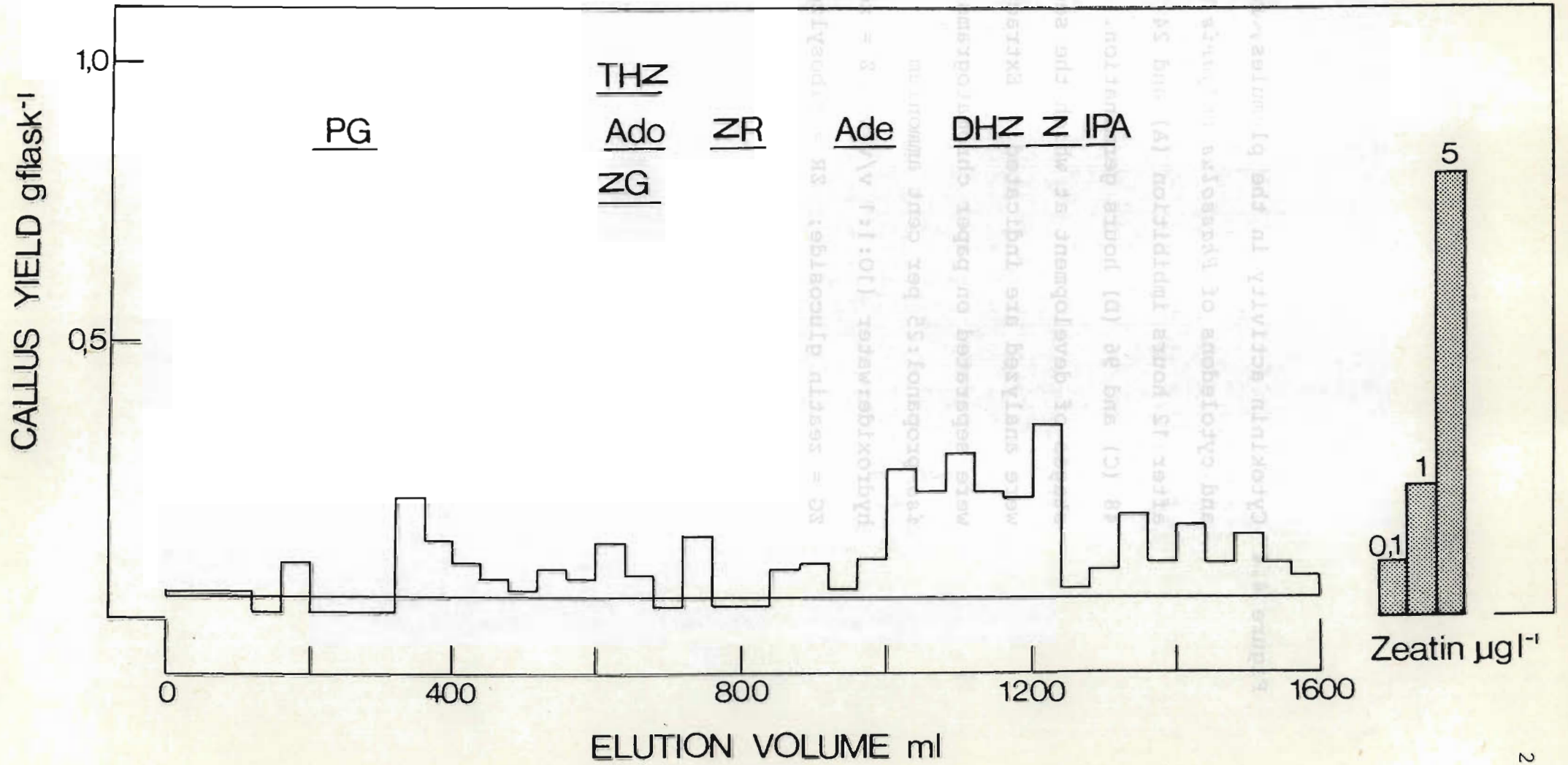

A
ZG
ZR
Z
0,5
0
B
0,5
0
CALLUS YIELD g flask-1
0,1
1
5
Zeatin µgl-1
0
0,5
1,0
Rf

Figure 4.3 Cytokinin activity detected in 25 grammes of dry cotyledons of *Phaseolus vulgaris* seeds following fractionation on a Sephadex LH-20 column eluted with 10 per cent methanol. PG = purinyl glycine; Ado = adenosine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.


CALLUS YIELD gflask-1
1,0
0,5
PG
THZ
Ado
ZG
ZR
Ade
DHZ
Z
IPA
0
400
800
1200
1600
ELUTION VOLUME ml
5
1
0,1
Zeatin µg l-1

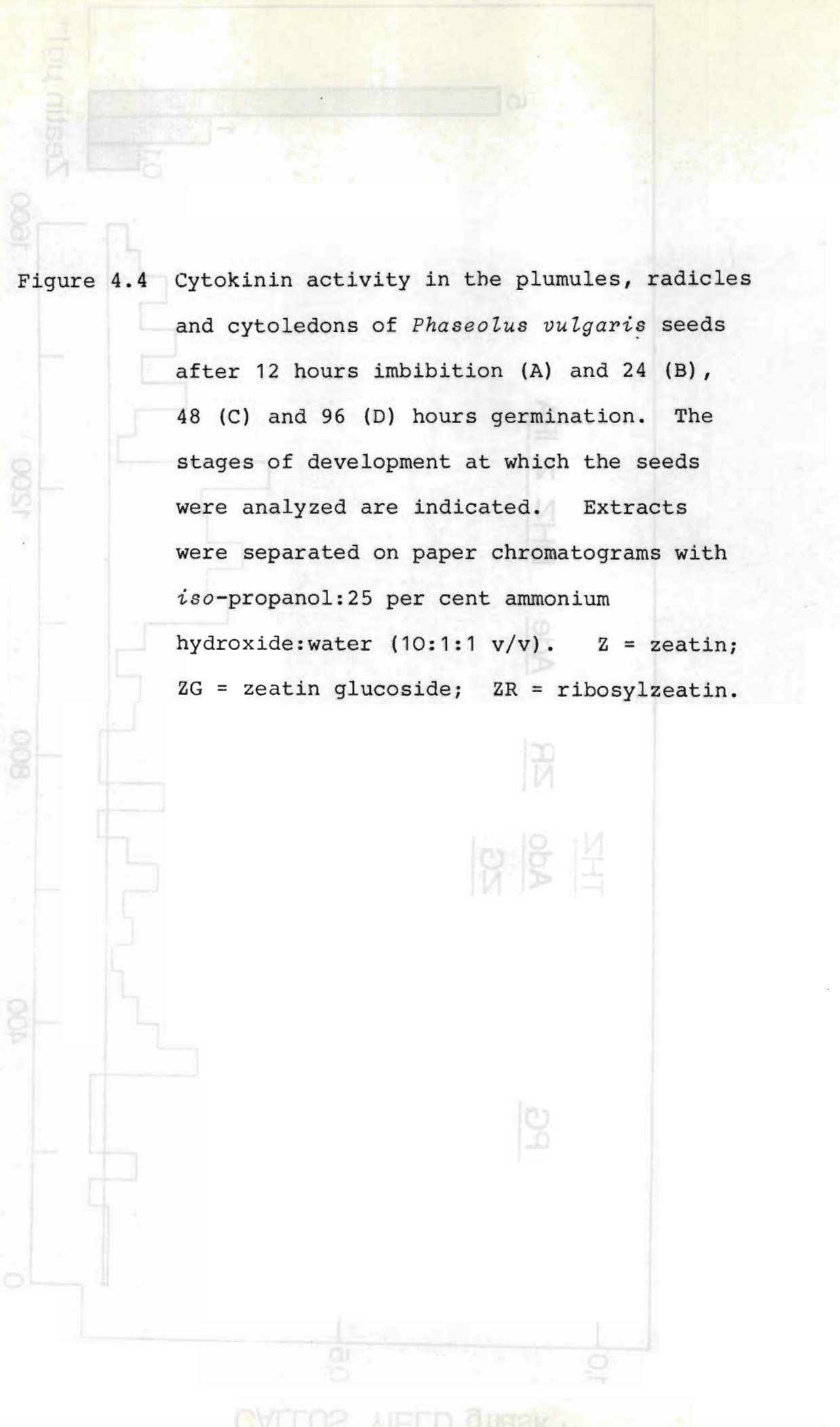


Figure 4.4 Cytokinin activity in the plumules, radicles and cytoledons of *Phaseolus vulgaris* seeds after 12 hours imbibition (A) and 24 (B), 48 (C) and 96 (D) hours germination. The stages of development at which the seeds were analyzed are indicated. Extracts were separated on paper chromatograms with *iso*-propanol:25 per cent ammonium hydroxide:water (10:1:1 v/v). Z = zeatin; ZG = zeatin glucoside; ZR = ribosylzeatin.

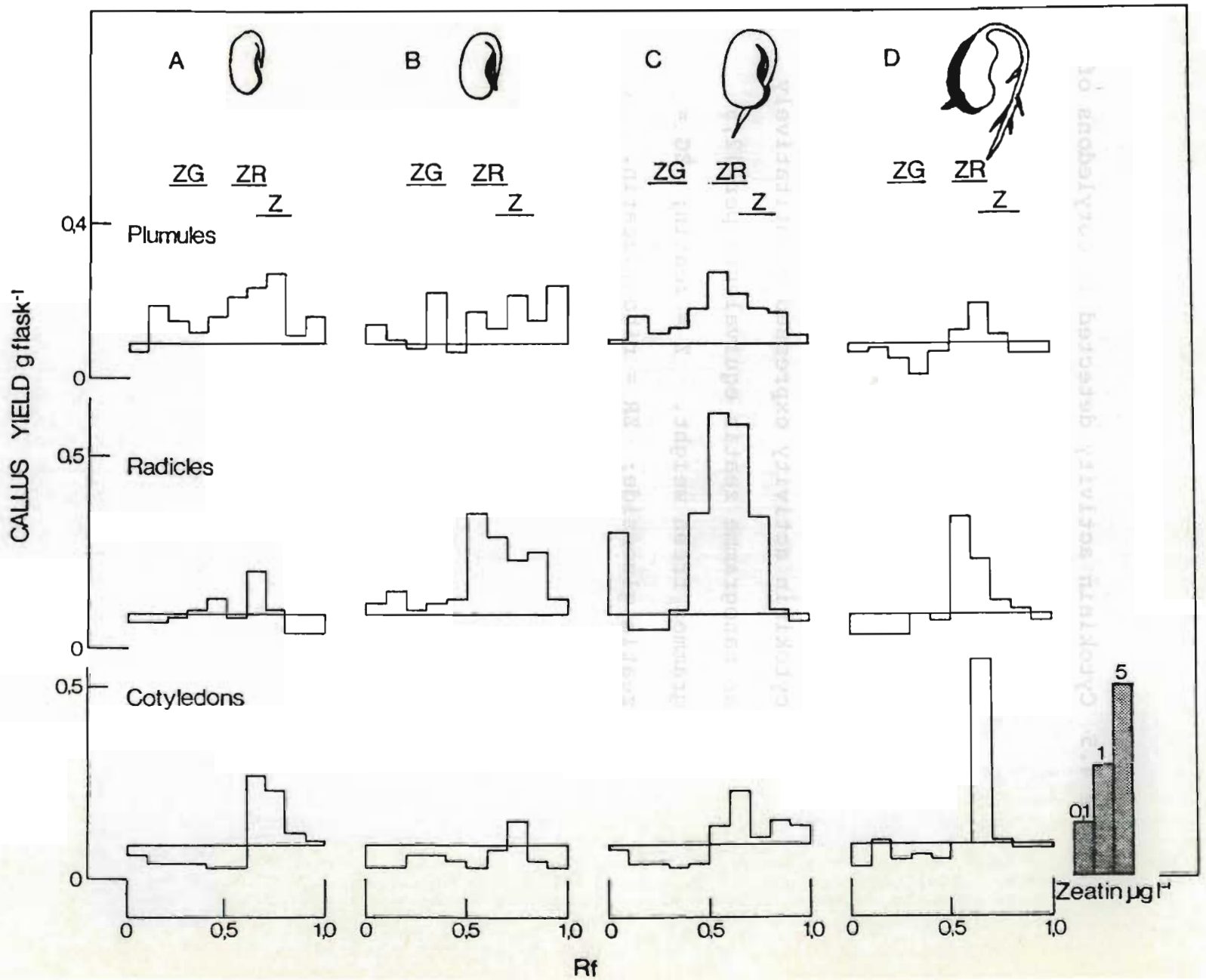
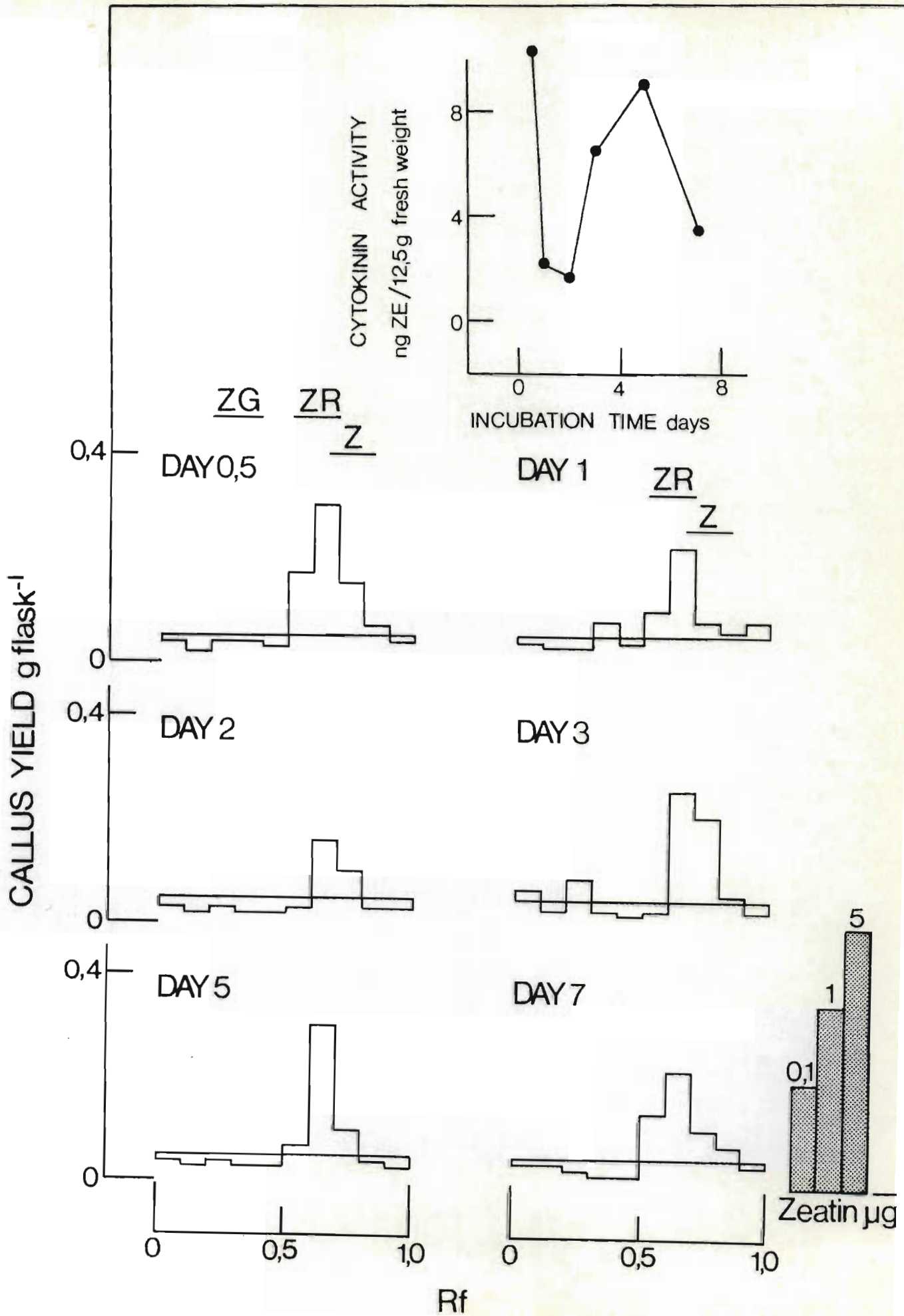

A
B
C
D
ZG
ZR
Z
Plumules
Radicles
Cotyledons
0,4
0,5
0,5
0
0
0
CALLUS YIELD g flask-1
0
0,5
1,0
Rf
0,1
1
5
Zeatin µg l-1

Figure 4.5 Cytokinin activity detected in cotyledons of intact *Phaseolus vulgaris* seeds incubated for seven days. Extracts were separated on paper chromatograms with *iso*-propanol: 25 per cent ammonium hydroxide:water (10:1:1 v/v). Insert represents the total cytokinin activity expressed quantitatively as nanogramme zeatin equivalents per 12,5 grammes fresh weight. Z = zeatin; ZG = zeatin glucoside; ZR = ribosylzeatin.


CYTOKININ ACTIVITY
ng ZE/12,5g fresh weight
8
4
0
0
4
8
INCUBATION TIME days
ZG
ZR
Z
0,4
DAY 0,5
0
DAY 1
ZR
Z
CALLUS YIELD g flask-1
0,4
DAY 2
0
DAY 3
0,4
DAY 5
0
DAY 7
5
1
0,1
Zeatin µg
0
0,5
1,0
0
0,5
1,0
Rf

content of the cotyledons decreased and reached its lowest level. This decrease was reversed between days three and five, when lateral root formation occurred. After seven days, when the plumule development became more significant, the cytokinin levels of the cotyledons again declined. When the embryonic axis was removed and the isolated cotyledons incubated on their own, their cytokinin content decreased steadily (Figure 4.6). Removal of the embryonic axis at different times during the incubation period, indicated that the cytokinin content of the cotyledons could be related to the time that the embryonic axis was attached to them (Table 4.1).

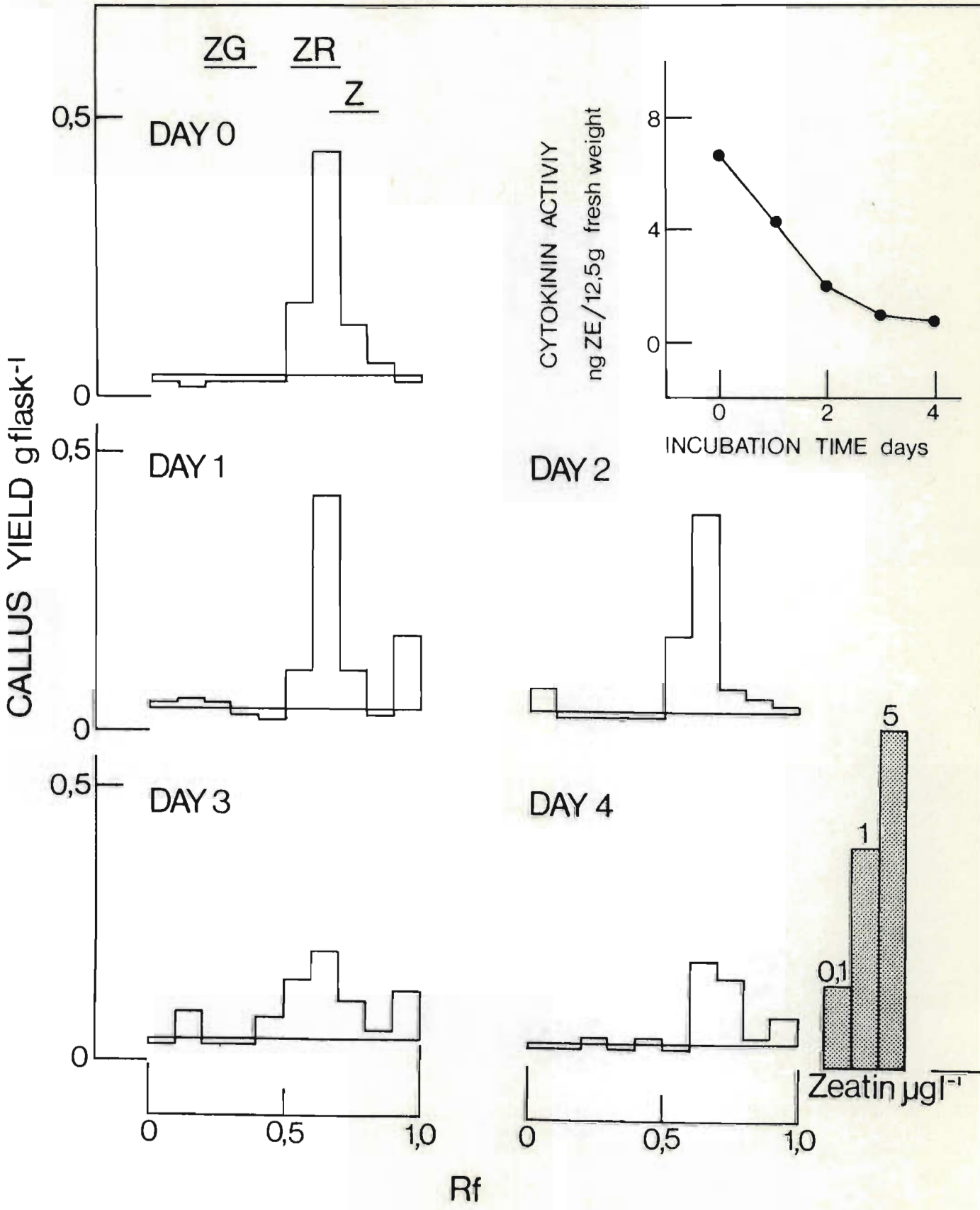
Table 4.1 The effect of the presence of the embryonic axis on the cytokinin content of *Phaseolus vulgaris* cotyledons. Cytokinin activity is expressed quantitatively in terms of nanogramme zeatin equivalents per 12,5 grammes fresh weight.

	Embryonic Axis Present (Days)		
	0	2	4
Cytokinin content of cotyledons (ng ZE per 12,5 grammes fresh weight)	0,9	1,9	6,9

4.3.2 Transport and metabolism of 8(^{14}C) *t*-zeatin applied to the radicle of germinating seeds

The aqueous fractions from the extracts in these experiments were relatively clean in comparison to the aqueous fractions obtained from leaf material in earlier experiments. It was thus decided to determine the radioactivity occurring

Figure 4.6 Cytokinin activity detected in excised cotyledons of *Phaseolus vulgaris* seeds incubated for four days. Extracts were separated on paper chromatograms with *iso*-propanol:25 per cent ammonium hydroxide:water (10:1:1 v/v). Insert represents the total cytokinin activity expressed quantitatively as nanogramme zeatin equivalents per 12,5 grammes fresh weight. Z = zeatin; ZG = zeatin glucoside; ZR = ribosylzeatin.


ZG
ZR
Z
DAY 0
DAY 1
DAY 2
DAY 3
DAY 4
0,5
0
CALLUS YIELD g flask⁻¹
CYTOKININ ACTIVIY
ng ZE/12,5g fresh weight
8
4
0
0
2
4
INCUBATION TIME days
5
1
0,1
Zeatin µgl⁻¹
0
0,5
1,0
Rf

in these fractions in order to assess its effect on the distribution of the radioactivity recovered from the seeds. Radioactivity was detected in both the Dowex 50 and aqueous fractions of extractions obtained from the radicles, plumules and cotyledons (Table 4.2). Most of the detected activity was recovered from the Dowex 50 fraction of each extract. Although a relatively large percentage of radioactivity was detected in the aqueous fraction of the extracts obtained from the radicles, in overall terms, this activity did not have a great impact on the distribution of the radioactivity recovered from the seeds as a whole (Table 4.3). For this reason, and because the aqueous extracts were difficult to purify, only the Dowex 50 fractions were subsequently analyzed in detail.

Table 4.2 The percentage radioactivity (% dpm) recovered in the Dowex 50 and aqueous fractions of extracts of *Phaseolus vulgaris* seeds which had been incubated for 24 and 48 hours following the application of 8(^{14}C)*t*-zeatin to the radicle tip.

Incubation Period (Hours)	Fraction Analyzed	Radioactivity (% dpm/Seed Component)		
		Radicles	Plumules	Cotyledons
24	Dowex 50	58,2	83,6	71,0
	Aqueous	41,8	16,4	29,0
48	Dowex	50,3	68,7	68,4
	Aqueous	49,7	31,3	31,6

Table 4.3 The distribution of radioactivity (A expressed as a percentage of the total activity recovered and B expressed as a percentage of the activity recovered in the Dowex 50 fraction only) in seeds of *Phaseolus vulgaris* seeds, 24 and 48 hours after applying 8(^{14}C)*t*-zeatin to the radicle tip.

	Incubation Period (Hours)	Radioactivity (% dpm/Seed Component)		
		Radicles	Plumules	Cotyledons
A	24	29,4	1,2	69,4
	48	24,5	1,5	74,0
B	24	25,4	1,4	73,2
	48	19,3	1,6	79,1

Expressing the radioactivity recovered in the Dowex 50 fractions of the seeds per component or per one gramme fresh weight, did not affect the overall distribution pattern (Table 4.4). It did, however, affect the percentage radioactivity in each component, especially in the plumules. The amount of radioactivity in the radicle decreased with time and increased in the cotyledons and plumules (Figure 4.7A). After 24 hours incubation, 62,5 per cent of the radioactivity exported from the treated radicle was recovered in the plumules and 37,5 per cent in the cotyledons. After 48 hours, only 46,4 per cent was recovered in the plumules and 57,4 per cent in the cotyledons. The distribution pattern was different when the results were expressed per seed component. After 24 and 48 hours incubation, 98,1 and 98,0 per cent, respectively, of the radioactivity transported from the radicle was re-

Table 4.4 The distribution of radioactivity (A expressed as % dpm/ seed component and B expressed as % dpm/one gramme fresh weight) in the Dowex 50 fraction of the components of *Phaseolus vulgaris* seeds, 24 and 48 hours after applying 8(^{14}C)*t*-zeatin to the radicle tip.

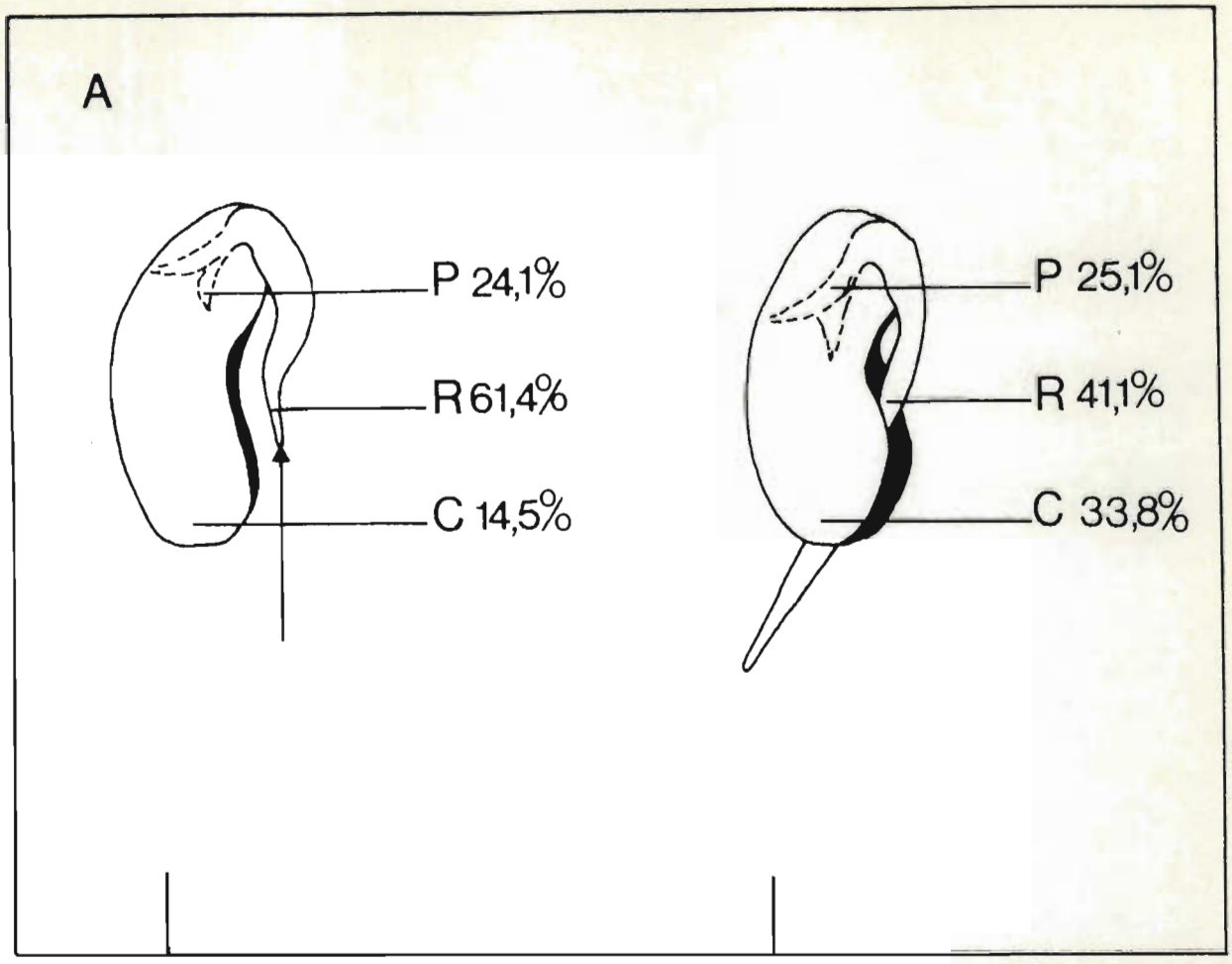
Seed Components	Radioactivity (% dpm)			
	24 Hours		48 Hours	
	A	B	A	B
Radicle	25,4	61,4	19,3	41,1
Plumule	1,4	24,1	1,6	25,1
Cotyledons	73,2	14,5	79,1	33,8

covered in the cotyledons. However, as in the previous experiments, the results expressed per one gramme were considered more significant and were the results which were discussed.

Paper chromatography of the Dowex 50 fractions of the seed extracts indicated the presence of three radioactive peaks which were referred to as radioactive peaks 1, 2 and 3. Radioactive peaks 1 (R_f 0,0-0,2) and 2 (R_f 0,2-0,5) were probably zeatin metabolites and radioactive peak 3 (R_f 0,5-1,0) probably contained the original radioactive zeatin. The percentage radioactivity associated with these three peaks is shown in Table 4.5. Radioactive peak 2 was the major peak in the radicles and cotyledons. The radioactivity associated with this peak increased with time in these organs. Radioactive peak 1 (R_f 0,0-0,2) was the major peak in the plumules.

Figure 4.7A The percentage radioactivity detected in the components of germinating *Phaseolus vulgaris* seeds 24 and 48 hours after applying radioactive zeatin to the radicle tip of these seeds. P = plumule; R = radicle; C = cotyledon; ⟶ = site of application.

B The percentage radioactivity detected in the components of germinating *Phaseolus vulgaris* seeds 24 and 48 hours after applying radioactive zeatin to cotyledons of these seeds. P = plumule; R = radicle; TC = treated cotyledons; UC = untreated cotyledons; ⟶ = site of zeatin application. The percentage radioactivity is expressed per one gramme fresh weight.


A
P 24,1%
R 61,4%
C 14,5%
P 25,1%
R 41,1%
C 33,8%

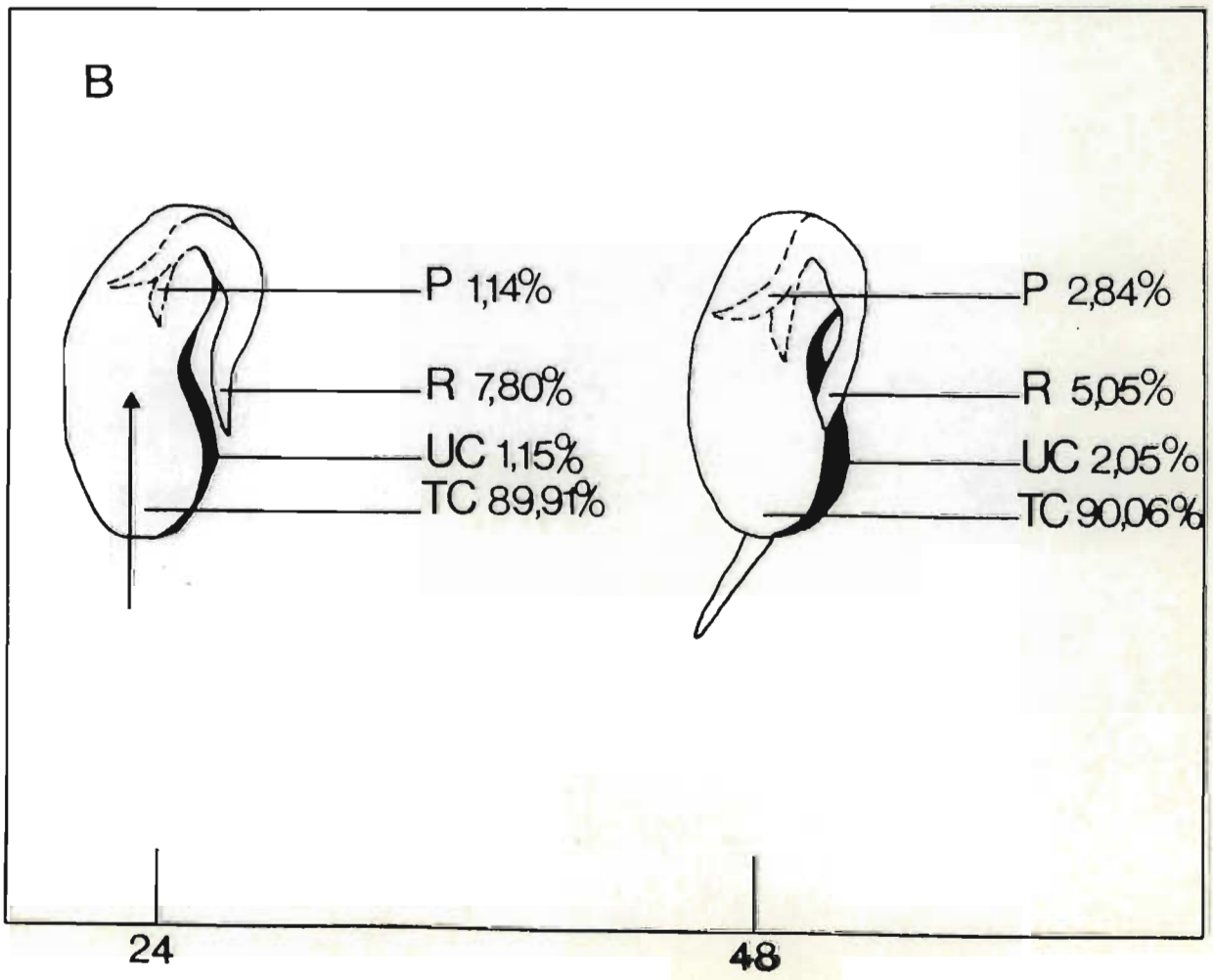
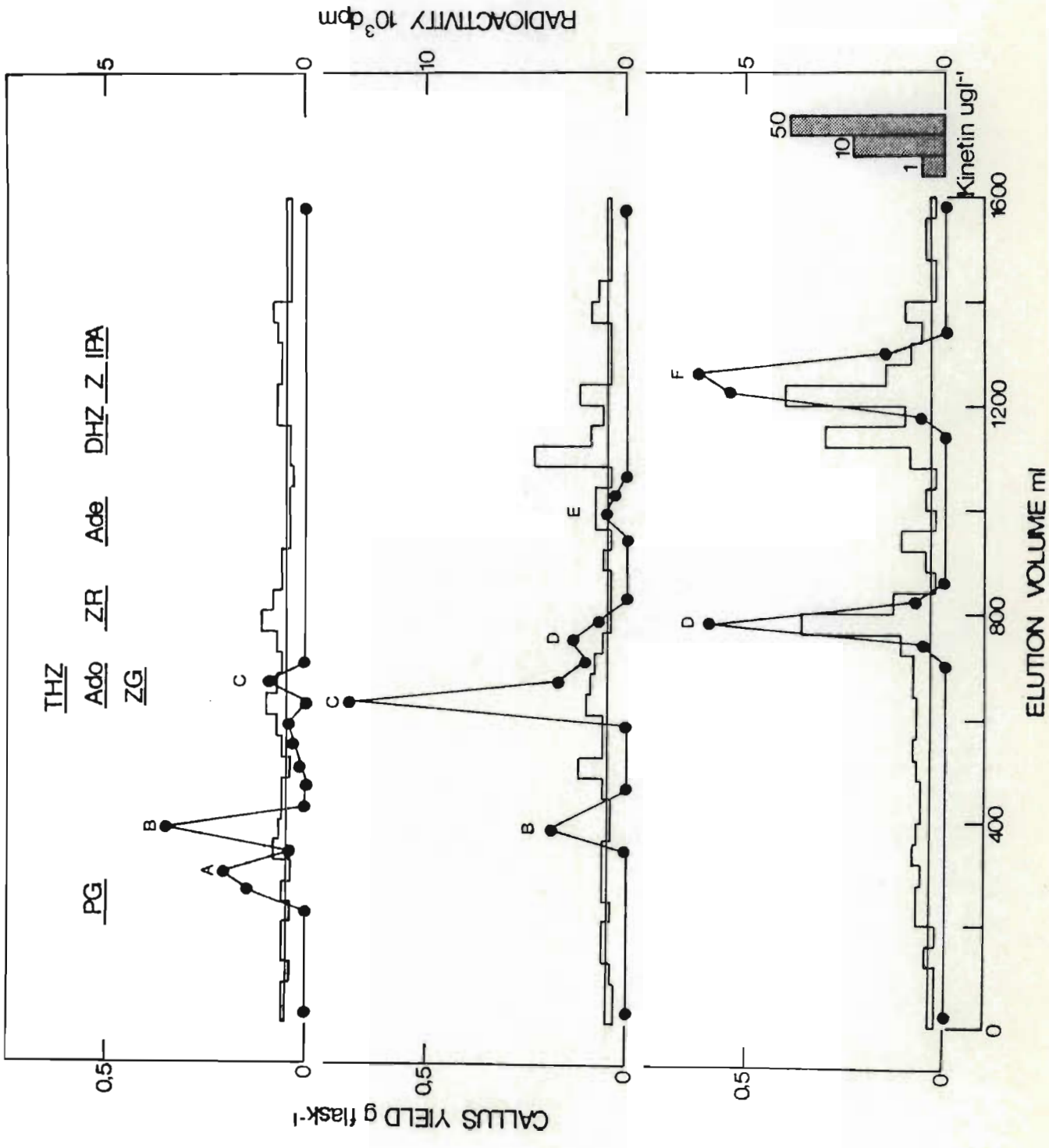

B
P 1,14%
R 7,80%
UC 1,15%
TC 89,91%
P 2,84%
R 5,05%
UC 2,05%
TC 90,06%
24
48

Table 4.5 The percentage of radioactivity in radioactive peaks 1 (R_f 0,0-0,2), 2 (R_f 0,2-0,5) and 3 (R_f 0,5-1,0) in the components of *Phaseolus vulgaris* seeds 24 and 48 hours after applying 8(^{14}C)*t*-zeatin to the radicle.

Incubation Period (Hours)	Seed Components	Radioactivity (% dpm/Radioactive Peak)		
		Radioactive Peak 1 (R_f 0,0-0,2)	Radioactive Peak 2 (R_f 0,2-0,5)	Radioactive Peak 3 (R_f 0,5-1,0)
24	Radicles	27,9	58,6	13,5
	Plumules	50,1	44,7	5,2
	Cotyledons	39,7	51,3	9,0
48	Radicles	27,2	60,4	12,4
	Plumules	53,7	43,0	3,3
	Cotyledons	24,7	71,2	4,1

To obtain more information about the radioactive metabolites formed in the germinating seeds, radioactive peaks 1, 2 and 3 were eluted individually from paper chromatograms and fractionated on Sephadex LH-20 columns. There was, however, only sufficient cotyledonary material for column chromatography. The same three radioactive peaks were detected in all components and fractionation of the cotyledons was thus regarded as representative of the radioactive peaks in all components. The metabolically more active embryonic axis could, however, have affected the percentage and number of metabolites formed. From Figure 4.8 it can be seen that six radioactive peaks were detected

Figure 4.8 Radioactivity (●——●) and biological activity (histograms) detected after fractionation of radioactive peaks 1 (R_f 0,0-0,2), 2 (R_f 0,2-0,5) and 3 (R_f 0,5-1,0) obtained from paper chromatograms, on a Sephadex LH-20 column eluted with 10 per cent methanol. The activity was that which was extracted from the cotyledons 24 hours after applying 8(^{14}C)*t*-zeatin to the radicle tips of germinating *Phaseolus vulgaris* seeds. Radioactive peaks A, B, C, D, E and F are indicated in the figures. PG = purinyl glycine; Ado = adenosine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.


CALLUS YIELD g flask-1
RADIOACTIVITY 10³dpm
ELUTION VOLUME ml
Kinetin ugl-1
PG
THZ
Ado
ZG
ZR
Ade
DHZ_Z_IPA
A
B
C
D
E
F
0,5
0
5
10
50
400
800
1200
1600

following column chromatography. These peaks were referred to as radioactive peaks A, B, C, D, E and F. Poor separation by paper chromatography probably resulted in the overlap of peaks detected in radioactive peaks 1, 2 and 3. Radioactive peaks A and B were primarily associated with R_f 0,0-0,2 (radioactive peak 1). The chromatographic properties of these peaks were not affected by alkaline phosphatase treatment. Radioactive peak A co-eluted with purinyl glycine, but peak B did not co-elute with any cytokinin standards. Radioactive peaks C and E appeared to be associated with radioactive peak 2 (R_f 0,2-0,5). Radioactive peak C, which co-eluted with trihydroxyzeatin, zeatin glucoside and adenosine, was unaffected by β-glucosidase treatment. This implies that cytokinin glucosides were not a component of this peak. Peak E did not co-elute with any cytokinin standards, but its elution volume was similar to that of an unidentified compound formed following zeatin oxidation (Figure 1.19). Relatively little biological activity was associated with the radioactive peaks A, B, C and E. Peaks D and F were biologically very active and co-eluted with ribisylzeatin and zeatin respectively. Potassium permanganate oxidation of radioactive peak F showed that dihydrozeatin was not a component of this peak.

4.3.3 Transport and metabolism of 8(^{14}C)*t*-zeatin applied to the cotyledons of germinating seeds

The radioactivity associated with the aqueous fractions of the extracts was also determined in this experiment. From

Table 4.6 it can be seen that most of the radioactivity recovered from the germinating seeds was associated with the Dowex 50 fractions of the extracts. For this reason, only the data obtained for the Dowex 50 fractions were used for further discussion. Most of the radioactivity recovered was found in the cotyledons to which the labelled zeatin was applied. This distribution pattern remained the same whether the results were expressed per seed component or per one gramme fresh weight (Table 4.7). The percentage radioactivity recovered in the seed components expressed per one gramme fresh weight is shown in Figure 4.7B. Of the radioactivity exported out of the treated cotyledons, the highest percentage was recorded in the radicles. The amount of radioactivity being transported to the radicles did, however, decrease with time, while that being transported to the plumule and untreated cotyledons increased after 48 hours.

Table 4.6 The distribution of radioactivity (A expressed as a percentage of the activity recovered in both the Dowex 50 and aqueous fractions and B expressed as a percentage of the activity recovered in the Dowex 50 fraction only) in seeds of *Phaseolus vulgaris* 24 and 48 hours after applying 8(^{14}C)*t*-zeatin to one of the cotyledons.

	Incubation Period (Hours)	Radioactivity (% dpm/Seed Component)			
		Treated Cotyledons	Untreated Cotyledons	Radicles	Plumules
A	24	96,60	1,30	2,04	0,06
	48	92,60	1,90	5,20	0,30
B	24	97,30	1,24	1,42	0,03
	48	93,20	2,18	4,42	0,16

Table 4.7 The distribution of radioactivity (A expressed as % dpm/seed component and B expressed as % dpm/one gramme fresh weight) in the Dowex 50 fractions of the components of *Phaseolus vulgaris* seeds 24 and 48 hours after applying 8(^{14}C)*t*-zeatin to one of the cotyledons.

Seed Component	Radioactivity (% dpm)			
	24 Hours		48 Hours	
	A	B	A	B
Treated cotyledons	97,30	89,91	93,20	90,06
Untreated cotyledons	1,24	1,15	2,18	2,05
Radicles	1,42	7,80	4,42	5,05
Plumules	0,03	1,14	0,16	2,84

Paper chromatographic separation of the Dowex 50 fractions into their respective constituents indicated the presence of three peaks of radioactivity. These radioactive peaks had the same chromatographic properties as those detected in the components of seeds to which labelled zeatin was applied to the radicle tip. The percentage radioactivity recovered at radioactive peaks 1, 2 and 3 in the components of the seeds is shown in Table 4.8. Radioactive peak 2 was the major peak in all components. Radioactive peak 3 decreased over time, with only 6,9 per cent of the radioactivity in the treated cotyledons being associated with this peak after 48 hours. This implies that the radioactive zeatin was rapidly metabolized. The results obtained from the plumules indicated that the labelled compounds associated with radioactive peak 2 (R_f 0,2-0,5) were formed first and that with time, these metabolites were

Table 4.8 The distribution of radioactive peaks 1 (R_f 0,0-0,2), 2 (R_f 0,2-0,5) and 3 (R_f 0,5-1,0) in the components of germinating *Phaseolus vulgaris* seeds 24 and 48 hours after applying radioactive zeatin to one cotyledon. Radioactivity is expressed as % dpm recovered/radioactive peak.

Incubation Period (Hours)	Seed Components	Radioactivity (% dpm/Radioactive Peak)		
		Radioactive Peak 1 (R_f 0,0-0,2)	Radioactive Peak 2 (R_f 0,2-0,5)	Radioactive Peak 3 (R_f 0,5-1,0)
24	Treated cotyledons	30,0	57,7	12,3
	Untreated cotyledons	31,2	60,3	8,5
	Radicles	32,1	55,0	12,9
	Plumules	0	100	0
48	Treated cotyledons	20,7	72,4	6,9
	Untreated cotyledons	16,7	76,1	7,2
	Radicles	41,5	56,5	2,0
	Plumules	46,5	53,5	0

changed to compounds that were very polar and were detected at R_f 0,0-0,2 (radioactive peak 1). As in *Ginkgo biloba* leaves, the metabolic pathway of the labelled zeatin appeared to be zeatin → radioactive peak 2 → radioactive peak 1.

Radioactive peaks 1, 2 and 3 were not separated by column chromatography, as their chromatographic properties were similar to those of radioactive peaks 1, 2 and 3 formed

following the application of zeatin to the radicle. In order to establish more about the nature of these peaks and their rates of metabolism, radioactive peaks 1 and 2 were re-applied to cotyledons of germinating *Phaseolus vulgaris* seeds. Radioactive peak 3 was not re-applied as it mainly contained residual labelled zeatin (Figure 4.8).

4.3.4 Transport and metabolism of radioactive peaks 1 and 2 re-applied to germinating seeds

Radioactive peaks 1 (R_f 0,0-0,2) and 2 (R_f 0,2-0,5), obtained after applying labelled zeatin to *Phaseolus vulgaris* cotyledons, were re-applied to the cotyledons of 12 hour imbibed seeds. The distribution of radioactivity following the re-application of radioactive peak 1 is shown in Table 4.9. Expressing the results per one gramme fresh weight and per seed component, affected the percentage radioactivity recovered in each seed component, but the overall distribution pattern was, however, essentially similar. That is, the amount of activity recorded in the cotyledons decreased, while the radioactivity in the plumules increased during the 48 hour incubation period. The highest percentage radioactivity was recorded in the plumules. Following the application of zeatin to the cotyledons, the highest percentage radioactivity was recovered in the radicles. A greater percentage radioactivity was transported out of the cotyledons following the application of radioactive peak 1 than radioactive zeatin. This suggests that radioactive peak 1 was a more readily translocatable form than zeatin.

Table 4.9 The percentage radioactivity (A expressed per seed component and B expressed per one gramme fresh weight) in the Dowex 50 fractions of extracts of *Phaseolus vulgaris* seeds which had been incubated for 24 and 48 hours following the re-application of radioactive peak 1 to the cotyledons.

Incubation Period (Hours)	Radioactivity (% dpm)					
	Cotyledons		Radicles		Plumules	
	A	B	A	B	A	B
24	96,7	37,9	2,0	11,0	1,3	51,1
48	34,8	2,5	11,7	3,9	53,8	93,6

Table 4.10 shows that part of the applied radioactive peak 1 was converted to compounds which occurred at R_f 0,2-0,5 and R_f 0,5-1,0 respectively. These peaks, therefore, had the same R_f values as radioactive peaks 1, 2 and 3 recorded following the application of labelled zeatin to the seeds.

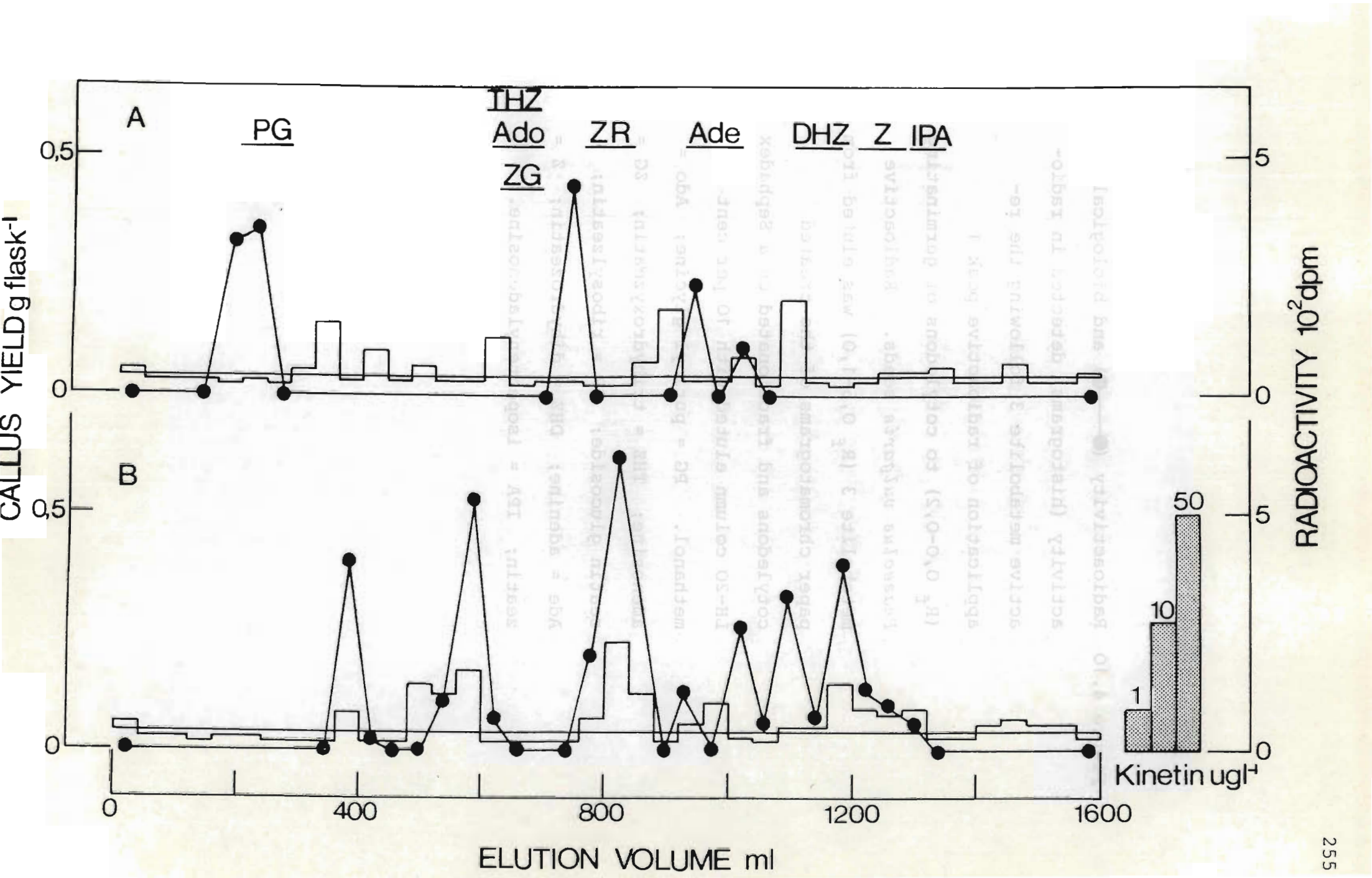
Table 4.10 The distribution of radioactive metabolites 1 (R_f 0,0-0,2), 2 (R_f 0,2-0,5) and 3 (R_f 0,5-1,0) in the components of germinating *Phaseolus vulgaris* seeds 24 and 48 hours after applying radioactive peak 1 (R_f 0,0-0,2) to the cotyledons. Radioactivity is expressed as % dpm recovered/radioactive metabolite.

Incubation Period (Hours)	Seed Components	Radioactivity (% dpm/Radioactive Metabolite)		
		Metabolite 1 (R_f 0,0-0,2)	Metabolite 2 (R_f 0,2-0,5)	Metabolite 3 (R_f 0,5-1,0)
24	Cotyledons	2,8	30,5	66,7
	Radicles	18,0	26,0	56,0
	Plumules	9,3	14,4	76,3
48	Cotyledons	76,0	24,0	0
	Radicles	0	4,8	95,2
	Plumules	12,7	22,0	65,3

Column chromatography (Figures 4.9 and 4.10) revealed that these peaks were not exactly the same as radioactive peaks 1, 2 and 3 (Figure 4.8), and they were subsequently referred to as radioactive metabolites 1 (R_f 0,0-0,2), 2 (R_f 0,2-0,5) and 3 (R_f 0,5-1,0). From Table 4.10 it can be seen that except for the cotyledons, after 48 hours most activity was found at R_f 0,5-1,0, that is, radioactive metabolite 3 which co-chromatographs with zeatin and ribosylzeatin. Column chromatography of this metabolite,from cotyledonary material (Figure 4.10),revealed that zeatin and ribosylzeatin were, in fact, components of this metabolite. Another radioactive peak, which had a similar elution volume to one of the peaks detected following fractionation of radioactive metabolite 1(Figure 4.9A) was also detected. The occurrence of this peak was probably due to poor separation by paper chromatography. Radioactive peak 1 therefore appeared to be relatively unstable and was readily converted back to zeatin and ribosylzeatin. The radioactivity which was not exported out of the cotyledons by 48 hours appeared to be converted back to radioactive peak 1.

The distribution of radioactivity following the reapplication of radioactive peak 2 (R_f 0,2-0,5) is shown in Table 4.11. The radioactivity in the cotyledons and radicles decreased and the radioactivity in the plumules increased during the 48 hour incubation period. Radioactive peak 2 also appeared to be more translocatable than radioactive zeatin applied to the cotyledons.

Figure 4.9 Radioactivity (●——●) and biological activity (histograms) detected in radioactive metabolites 1 and 2 following the re-application of radioactive peak 1 (R_f 0,0-0,2) to cotyledons of germinating *Phaseolus vulgaris* seeds. Radioactive metabolite 1 (R_f 0,0-0,2) (A) and radioactive metabolite 2 (R_f 0,2-0,5) (B) were eluted from paper chromatograms of the treated cotyledons and fractionated on Sephadex LH-20 columns eluted with 10 per cent methanol. PG = purinyl glycine; Ado = adenosine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.


A
B
PG
THZ
Ado
ZG
ZR
Ade
DHZ
Z
IPA
0,5
0
5
0
CALLUS YIELD g flask⁻¹
RADIOACTIVITY 10²dpm
1
10
50
Kinetin ugl⁻¹
0
400
800
1200
1600
ELUTION VOLUME ml

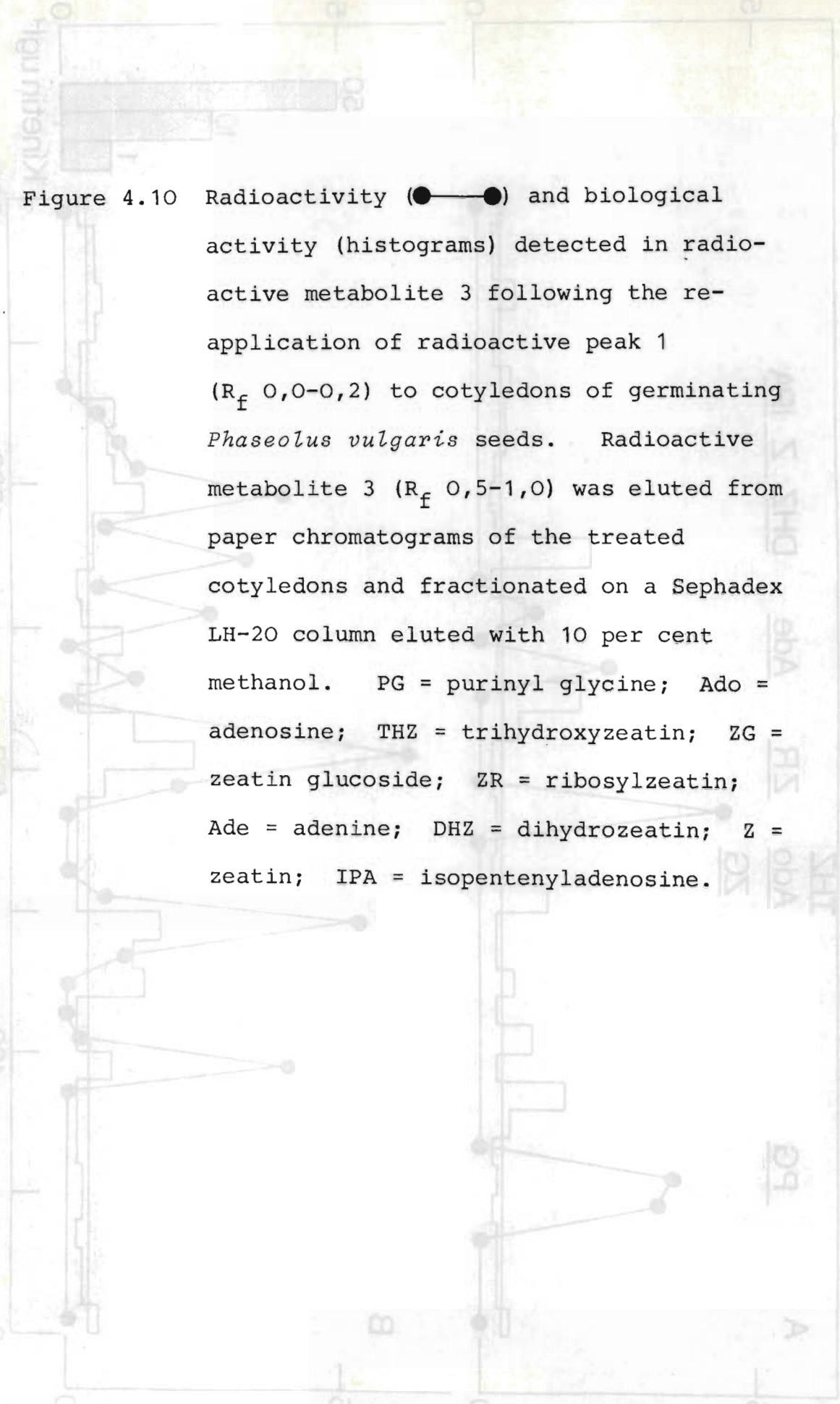


Figure 4.10 Radioactivity (●——●) and biological activity (histograms) detected in radioactive metabolite 3 following the re-application of radioactive peak 1 (R_f 0,0-0,2) to cotyledons of germinating *Phaseolus vulgaris* seeds. Radioactive metabolite 3 (R_f 0,5-1,0) was eluted from paper chromatograms of the treated cotyledons and fractionated on a Sephadex LH-20 column eluted with 10 per cent methanol. PG = purinyl glycine; Ado = adenosine; THZ = trihydroxyzeatin; ZG = zeatin glucoside; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = isopentenyladenosine.

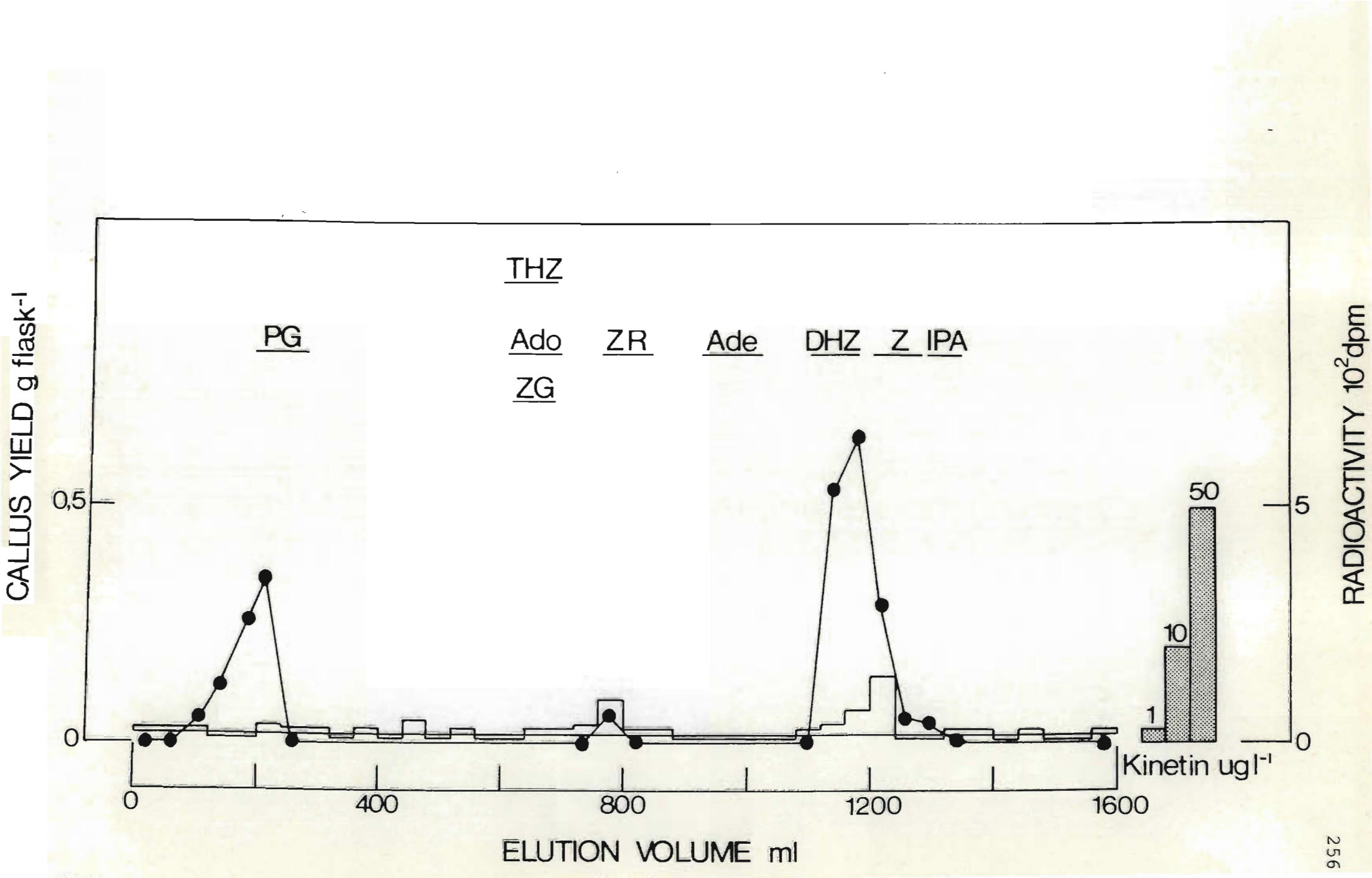

CALLUS YIELD g flask⁻¹
RADIOACTIVITY 10²dpm
THZ
PG
Ado
ZR
Ade
DHZ
Z
IPA
ZG
0,5
0
5
0
50
10
1
Kinetin ug l⁻¹
0
400
800
1200
1600
ELUTION VOLUME ml

Table 4.11 The percentage radioactivity (A expressed per seed component and B expressed per one gramme fresh weight) in the Dowex 50 fractions of *Phaseolus vulgaris* seeds which had been incubated for 24 and 48 hours following the re-application of radioactive peak 2 to the cotyledons.

Incubation Period (Hours)	Radioactivity (% dpm)					
	Cotyledons		Radicles		Plumules	
	A	B	A	B	A	B
24	85,0	10,6	9,0	21,6	6,0	67,8
48	80,0	8,4	2,5	1,6	17,0	90,0

Three radioactive peaks were also detected on paper chromatographs following the re-application of radioactive peak 2. These peaks were also referred to as radioactive metabolite 1 (R_f 0,0-0,2), 2 (R_f 0,2-0,5) and 3 (R_f 0,5-1,0). The distribution of these metabolites is shown in Table 4.12. From this Table it can be seen that radioactive peak 2 appeared to be relatively stable within these organs. This peak was not extensively metabolized as most of the recovered radioactivity was still detected at R_f 0,2-0,5. When exported to the radicles, and in particular to the plumules, a large proportion of the recovered radioactivity was detected at R_f 0,5-0,8 which co-chromatographs with zeatin and ribosylzeatin.

The purity of these re-applied radioactive peaks must, however, be taken into account when considering their transport and metabolism, especially as the separation achieved by paper chromatography was not always complete.

Table 4.12 The distribution of radioactive metabolites 1 (R_f 0,0-0,2), 2 (R_f 0,2-0,5) and 3 (R_f 0,5-1,0) in the components of germinating *Phaseolus vulgaris* seeds 24 and 48 hours afer re-applying radioactive peak 2 (R_f 0,2-0,5) to one of the cotyledons. Radioactivity is expressed as % dpm recovered/ radioactive metabolite.

Incubation Period (Hours)	Seed Components	Radioactivity (% dpm/Radioactive Metabolite)		
		Metabolite 1 (R_f 0,0-0,2)	Metabolite 2 (R_f 0,2-0,5)	Metabolite 3 (R_f 0,5-1,0)
24	Cotyledons	31,0	69,0	0
	Radicles	6,0	53,0	41,0
	Plumules	0	48,0	52,0
48	Cotyledons	16,0	84,0	0
	Radicles	33,0	67,0	0
	Plumules	14,0	30,0	56,0

4.4 Discussion

The cotyledons and embryonic axes of dry *Phaseolus vulgaris* seeds contained a considerable amount of cytokinin-like activity. The results of the endogenous cytokinin studies indicated that there is a delicate balance between the cotyledonary cytokinins and those found in the embryonic axis. The initial decrease in cytokinin activity in the cotyledons was probably due to utilization and/or export during the early stages of development. Once the radicles had started growing rapidly, this decrease in cotyledonary

cytokinins was reversed, supporting the hypothesis that cytokinins supplied by the root tip move into the cotyledons (LETHAM, 1971; DEI, 1978), which have been reported to be unable to synthesize these hormones (RYBICKA, ENGELBRECHT, MIKULOVICH and KULAEVA, 1977). Further evidence in support of such a relationship was the fact that whenever the embryonic axis was removed, the cytokinin content of the excised cotyledons decreased steadily to very low levels. Similar results were reported by RYBICKA, ENGELBRECHT, MIKULOVICH and KULAEVA (1977) and VAN ONCKELEN, CAUBERGS and DE GREEF (1977). The decrease in cytokinin activity in isolated cotyledons confirms that these organs are not capable of synthesizing cytokinins.

It is well documented that the embryonic axis is necessary for the development of normal hydrolytic enzyme activity in cotyledons during germination (PENNER and ASHTON, 1967; GEPSTEIN and ILAN, 1979; MINAMIKAWA and KOSHIBA, 1979; MOROHASHI, 1980). It has been suggested that cytokinins exported from the embryonic axes of dicotyledons are responsible for initiating hydrolytic enzyme activity in the cotyledons (DAVIES and CHAPMAN, 1979; GEPSTEIN and ILAN, 1979; MOROHASHI, 1980). In these experiments, radioactive zeatin applied to the radicle tip of *Phaseolus vulgaris* seeds appeared to be transported to the cotyledons. Radioactivity was also transported to the plumule, but the amount of radioactivity in these organs depended on how the results were expressed. Expressing radioactivity per component resulted in very little radioactivity being de-

tected in the plumules, which is in agreement with the report that the shoot in germinating bean seeds develops very slowly (METIVIER and PAULILO, 1980a). The finding that labelled zeatin was transported rapidly from the radicle to the cotyledons, correlates well with the reported time of the onset of hydrolytic enzyme activity, and the subsequent transfer of food reserves from the cotyledons to the developing embryonic axis (METIVIER and PAULILO, 1980a,b). This transfer is at its greatest after two days. During the first 20 hours of germination, the radicle apparently utilizes its own reserves, that is, prior to the increase in hydrolytic enzyme activity. The transport of radioactivity to the cotyledons also suggests that these organs do not synthesize cytokinins, but that these hormones are imported from the embryonic axis. It must be borne in mind, however, that cytokinins are not the only factors involved in the stimulation of hydrolytic enzyme activity, particularly as applied cytokinins do not always completely replace the axis, and as the presence of the axis is not always a requirement for normal enzyme development (DAVIES and CHAPMAN, 1980; METIVIER and PAULILO, 1980b).

Contrary to the above results, it has been reported that most of the (^{3}H) zeatin applied to the radicles of radish seedlings was not exported to the rest of the seedling (GORDON, LETHAM and PARKER, 1974). In these experiments, however, the cytokinin was applied to five day-old seedlings in which it would be expected that food mobilization

was well under way and in which transport from the cotyledons towards the embryonic axis predominated. VAN STADEN (1981b) could also find no clear evidence for an embryo-endosperm interaction of cytokinins during the germination of *Zea mays* following the application of 8(^{14}C)*t*-zeatin to the radicle tip. This might, however, have been anticipated as it has previously been reported that gibberellins are the primary hormonal messengers between the embryo and endosperm in monocotyledons (JACOBSEN, HIGGINS and ZWAR, 1979).

The results obtained following the application of labelled zeatin to the cotyledons of germinating seeds, indicated that there is apparently very little export of cytokinins to the embryonic axis during the early stages of seed germination. If expressed per one gramme fresh weight, the radioactivity, which was exported, was mainly transported to the radicle, but it decreased during the incubation period. This could support the fluctuations in endogenous cytokinin activity which suggested that cytokinins could be transported from the cotyledons to the axis during the initial stages of development. If, however, the results are expressed per seed component, an increase in radioactivity was detected in the radicles after 48 hours. METIVIER and PAULILO (1980a) reported that the embryonic axis only becomes the centre of metabolic activity after 24 hours incubation, and that the greatest transfer of food reserves in bean seeds occurs after 48 hours, that is, when an increase in radioactivity

was detected in the radicle. This could suggest that cytokinins were exported passively with the food reserves. The unphysiological concentration of zeatin applied to the cotyledons must also be considered in this respect. It would, thus, seem as if the radicle does not require significant amounts of cytokinin from the cotyledons during its initial stages of development, although the significance of the exported radioactivity is difficult to assess in terms of endogenous cytokinin levels and the large amount of zeatin applied. These results do, however, support earlier suggestions (TZOU, GALSON and SONDHEIMER, 1973; GORDON, LETHAM and PARKER, 1974) that there is a barrier to the transport of cytokinins out of the cotyledons. The possibility does exist, however, that the applied cytokinin was not in a translocatable form. VAN STADEN (1981a) reported that the metabolite formed from the radioactive zeatin was more extensively transported than radioactive zeatin applied to the endosperm of germinating *Zea mays*. Experiments with the major radioactive peaks (metabolites) formed in the cotyledons after zeatin application, indicated that some of these compounds, particularly the most polar ones (Radioactive peak 1, R_f 0,0-0,2) were more readily exported than zeatin when applied to the cotyledons. The exported metabolites were predominantly transported to the plumules.

Both in these experiments, and in earlier work by SONDHEIMER and TZOU (1971), it was found that the labelled

zeatin was metabolized rapidly in embryonic axes and cotyledons of *Phaseolus vulgaris*. Column chromatography of the cotyledons of *Phaseolus vulgaris* seeds, following the application of zeatin to the radicle, resulted in the detection of compounds which co-eluted with purinyl glycine, adenosine, trihydroxyzeatin and zeatin glucoside, ribosylzeatin and zeatin. This suggested that oxidation, ribosylation and possibly glucosylation, were involved in the metabolism of the radioactive zeatin. Only cotyledonary material was separated by column chromatography and it is possible that if sufficient embryonic axis material had been available for column chromatography, more radioactive peaks would have been detected. VAN STADEN (1981b) reported the formation of more radioactive metabolites following the application of radioactive zeatin to the embryonic tissues of *Zea mays*, than when it was applied to the endosperm. He said that this was probably due to the fact that the embryonic tissues are metabolically more active than the endosperm. This could probably also be related to the embryonic axis and cotyledonary tissue of germinating *Phaseolus vulgaris* seeds.

Previous metabolic studies on germinating seeds have resulted in a number of zeatin metabolites being formed, but no common metabolic pathway has been detected. SONDHEIMER and TZOU (1971) detected dihydrozeatin and dihydroribosylzeatin as major metabolites following the application of radioactive zeatin to bean axes. These compounds were not detected in this study. SUMMONS,

ENTSCH, LETHAM, GOLLNOW and MACLEOD (1980) recorded adenine nucleotides, adenosine and adenine as the major radioactive peaks following the application of radioactive zeatin to *Zea mays* kernels. They proposed that side chain cleavage, rather than side chain modification, was the major form of zeatin metabolism. They also suggested that cytokinin oxidase type enzymes (WHITTY and HALL, 1974) are involved in these reactions. VAN STADEN (1981a,b) also reported that oxidation, rather than side chain modification (glucosylation) was the predominant way in which zeatin was metabolized. The results of these experiments indicated that oxidation was probably also a major metabolic pathway for the radioactive zeatin applied to germinating beans, although ribosylation also occurred. That is, modification to the adenine ring was also an important metabolic process. PARKER, LETHAM, GOLLNOW, SUMMONS, DUKE and MACLEOD (1978) have shown that a number of metabolites are formed as a result of ribosylation in nine day-old *Lupinus* seedlings. From their work, and the results of these experiments, it would appear that the number of metabolites formed is related to seedling age and development. It was also observed that while applied zeatin was metabolized readily by reactions which brought about side chain cleavage and/or substitution to the adenine ring, the seed material also seemed to have the capacity to reverse these reactions, particularly as radioactive compounds which co-chromatographed with zeatin and ribosylzeatin were detected when the metabolites occurring at R_f 0,0-0,2 and R_f 0,2-0,5 were re-applied to

the cotyledons. The extent to which compounds co-eluting with purinyl glycine, trihydroxyzeatin, adenosine and adenine, the latter which has been reported to play a part in cytokinin biosynthesis (CHEN and PETSCHOW, 1978a; STUCHBURY, PALNI, HORGAN and WAREING, 1979; VAN STADEN, 1979a), participate in this interconversion is currently unknown.

GENERAL DISCUSSION

In the preceding four chapters, radioactive zeatin was applied to diverse plant systems, that is, leaves, whole plants and seeds, in order to obtain information relating to specific problems of cytokinin transport. In this section, an attempt has been made to link together the transport and metabolism of radioactive zeatin in these systems. Understanding cytokinin transport is essential in assessing the role of these growth regulators in plants. The significance of cytokinin transport can perhaps be questioned if the phenomena, such as hydrolytic enzyme activity and nutrient mobilization, which the transported cytokinins are meant to effect, are not analyzed in conjunction with cytokinin transport. Although it was realized that cytokinin transport is not an isolated process, it was not feasible to analyze a number of parameters during the course of these experiments.

Early studies indicated that exogenous cytokinins were not readily transported away from the site of application (SACHS and THIMANN, 1964). Subsequent experiments have contradicted these earlier reports (OSBORNE and BLACK, 1964; GORDON, LETHAM and PARKER, 1974), and the results of these experiments also confirm that exogenous cytokinins are transported in plants. A number of factors have to be considered when assessing the significance of the transported radioactivity in the various systems. The site of application of the radioactive zeatin could have affected

transport. When cytokinins were supplied to the stem or radicle tip, it could be presumed that they would enter the transpiration stream directly, which would facilitate transport. Cytokinins applied to leaves and cotyledons were applied to a localized area and not directly into the vascular system. This would probably result in transport within these organs, as well as possibly transport out of these organs. The experimental period could thus also be a limiting factor with regard to cytokinin transport. Approximately the same percentage radioactivity was exported out of *Citrus sinesis* leaves after 6, 12 and 18 days, after 5 and 10 days from *Phaseolus vulgaris* leaves, and after 24 and 48 hours from treated *Phaseolus vulgaris* cotyledons. When radioactive zeatin was applied to the radicle tip of germinating *Phaseolus vulgaris* seeds, there was an increase in exported radioactivity after 24 and 48 hours. These results could imply that extending the experimental period would not have affected the results. The results could also imply that radioactivity exported from leaves and cotyledons, was not significant or that the cytokinins which these organs were capable of using, was utilized immediately and the excess applied cytokinin was inactivated. Transport should, therefore, also be considered in terms of the unphysiological cytokinin concentration applied to the plants. The actual cytokinin, that is zeatin, applied to the various systems may also have affected transport, especially from those organs (such as senescing leaves) in which it was not a major endogenous cytokinin. It is interesting to note that the radioactive

metabolites formed from the radioactive zeatin, that is, radioactive peak 1 (R_f 0,0-0,2) and 2 (R_f 0,2-0,5), were more readily transported than zeatin when they were re-applied to mature *Ginkgo biloba* leaves and cotyledons of germinating *Phaseolus vulgaris* seeds. This could imply that zeatin was not readily transported and had to be metabolized prior to transport. In the case of treated leaves, it must also be remembered that the few treated leaves could not only be responsible for cytokinin transport to the rest of the plant and a dilution factor, in terms of the whole plant, must also be taken into account.

Taking the above points into consideration, the radioactivity transported in the various systems, can perhaps be regarded as reflecting normal cytokinin translocation. The amount of radioactivity transported away from the site of application, differed in the various systems. Relatively little radioactivity was exported out of deciduous (*Ginkgo biloba*), evergreen (*Citrus sinesis*) and annual (*Phaseolus vulgaris*) leaves and *Phaseolus vulgaris* cotyledons, whereas a relatively high percentage radioactivity was transported from the radicle tip of germinating *Phaseolus vulgaris* seeds and in the stem of these plants, during their growth and development. From this it might appear as if leaves do not contribute to cytokinins in the rest of the plant, but in terms of endogenous cytokinin levels, the radioactivity exported from these leaves may, in fact, have been significant. This could imply that leaves are potentially capable of exporting cytokinins. Although approximately the same per-

centage radioactivity was exported out of deciduous, evergreen and annual leaves, the distribution pattern of radioactive compounds exported from the evergreen leaves (*Citrus sinesis* leaves) suggested that this exported radioactivity may have been the most significant. The similar percentages of radioactivity exported from expanding, mature and senescing *Ginkgo biloba* leaves suggested that this exported radioactivity was not significant, as greater export would have been anticipated from mature and senescing leaves than expanding leaves. The relatively low percentage radioactivity in the developing fruit of *Phaseolus vulgaris* plants, following the application of radioactive zeatin to the primary leaves, compared with the percentage radioactivity in the fruit following zeatin application to the stem, suggests that the radioactivity exported out of these leaves may not have been very important. The export of radioactivity out of *Citrus sinesis* leaves co-incided with the new flush of growth and with the period of cytokinin utilization suggested by HENDRY (1980). That is, during bud swell, immediately after warmer temperatures were experienced. There is, however, no conclusive evidence that cytokinin transport out of *Citrus sinesis* was more significant than transport out of the other leaves.

In terms of cytokinin glucosides in leaves and the export of radioactive compounds from leaves, it would appear that these compounds essentially fulfil the same function in all leaves, but the fate of these compounds could be different. Glucosylation appears to be a mechanism for regulating leaf

metabolism, in mature and senescing leaves or during periods of slow growth, to allow photosynthates and minerals to be translocated to the non-photosynthetic parts of the plant and the developing reproductive organs. That is, by eliminating zeatin and ribosylzeatin in leaves by inactivation (glucosylation), other organs would be able to compete more effectively for photosynthates and other nutrients. Zeatin and ribosylzeatin are reported to mobilize nutrients, whereas glucosylated cytokinins apparently do not have this capacity (VAN STADEN and DAVEY, 1979). This could imply that other organs cannot compete with the leaves for root-produced cytokinins or that these cytokinins are transported in the transpiration stream to the leaves as suggested by DAVEY and VAN STADEN (1981). Radioactive zeatin applied to the transpiration stream of *Phaseolus vulgaris* was, however, transported to preferential sites. That is, the vegetative apices, buds and flowers appeared to be the strongest cytokinin sinks in vegetative and flowering plants and the leaves, rather than the developing seeds, appeared to be the strongest sinks in fruiting plants. The preferential transport of cytokinins to leaves of fruiting plants could possibly account for the increased glucosylation capacity of mature and senescing leaves. It would appear that in annual and deciduous leaves, a large proportion of cytokinins, which accumulate in mature and senescing leaves, is compartmentalized, as suggested by ENGELBRECHT (1971). Although these cytokinins can potentially be re-utilized, the bulk of these cytokinins are apparently lost upon abscission. Inactivation, rather

than storage, would be the primary function of cytokinin glucosides in these leaves. In evergreen leaves, it appears that some glucosides are probably re-utilized. They are exported out of the leaves and utilized in the new flush of growth, but the extent to which these glucosides are converted to free bases and their ribosides (LORENZI, HORGAN and WAREING, 1975) and/or catabolically metabolized (HENDRY, 1980), could not be determined.

If cytokinins are, in fact, exported from leaves, the results of the metabolic studies indicated that zeatin was not involved in transport. This could imply that cytokinin glucosides are not extensively metabolized in leaves prior to being exported but are metabolized in the stem tissue. This is suggested by the fact that metabolites of zeatin were more extensively transported than zeatin itself and that a compound co-eluting with purinyl glycine appeared to be an important translocatory form arriving in untreated components, rather than cytokinin glucosides.

In view of the radioactivity transported in the transpiration stream of *Phaseolus vulgaris* plants and from the radicle tip of these seeds, compared with the relatively small amount of radioactivity exported from leaves and cotyledons, it would appear that cytokinins produced in the root are mainly involved in cytokinin distribution in plants. The occurrence of cytokinins in the phloem (VAN STADEN and DAVEY, 1978c; WEILER and ZIEGLER, 1981) suggests that cytokinin transport is not, however, a simple root-to-shoot

type transfer (DAVEY, 1978). Although the significance of the radioactivity exported out of leaves is difficult to assess, it does indicate that some of the cytokinins in the phloem could have originated in the leaves. Cytokinins in the phloem could, however, also originate in the roots or be transported laterally from the xylem (VAN STADEN and DAVEY, 1979). Radioactive transport in the phloem of *Phaseolus vulgaris* stems and from *Ginkgo biloba* leaves and *Phaseolus vulgaris* leaves and cotyledons, suggested that cytokinins could have been transported passively in the phloem along with the assimilate stream and would probably be recycled or redistributed to the roots and/or fruits in deciduous trees and fruits of annual plants as suggested by VAN STADEN (1976a,b), or stored in the bark as suggested by VAN STADEN and DAVEY (1981b).

Another problem encountered in assessing the significance of the transported radioactivity in the various systems, was determining how the results should be expressed. That is, per component/organ or per constant weight. Both of these methods have merit, but often result in different distribution patterns. Expressing results per constant weight could place results in perspective with regard to differences in weight and activity expressed per cell, whereas expressing results per organ places results in perspective to size and cell numbers. As mentioned earlier, cytokinin activity per cell is perhaps the ideal parameter for expressing cytokinin activity, and both of these methods have advantages in achieving this aim. Al-

though results expressed per one gramme were discussed in these experiments, the results expressed per organ, perhaps should also be considered.

The significance of the transported radioactivity can also only be assessed in relation to the actual zeatin metabolites involved in transport. Any exogenously applied cytokinin can be regarded as excess in terms of endogenous cytokinin levels and the concentration of the applied cytokinin is usually unphysiological. Both of these facts could have a profound effect on metabolism, but are extremely difficult to overcome. Increasing the sample number and decreasing the amount of cytokinin applied, could alleviate these problems, but may not always be practical. HECHT (1979) is of the opinion that plant tissues are not responsive to exogenous cytokinins as they utilize their own cytokinin supply. This could imply that metabolism of exogenous cytokinins would not reflect metabolism of endogenous cytokinins. FOX, CORNETTE, DELEUZE, DYSON, GIERSAK, NIU, ZAPATA and MCCHESNEY (1973) have, however, also proposed that a plant is not likely to develop a separate system for dealing with exogenously applied zeatin and that it is more likely that exogenous cytokinins follow normal metabolic pathways of cells. The occurrence of radioactive metabolites which did not co-elute with endogenous cytokinins does, however, suggest that the exogenous zeatin may not have been metabolized by the main metabolic pathway normally functioning in the treated tissues, but may have followed a metabolic pathway for in-

activating the large amount of zeatin applied to the plants.

It could also be suggested that wounding, as the result of injecting the radioactive zeatin into the tissues with a microsyringe, affected zeatin metabolism. Wounding has been reported to promote cytokinin synthesis (CARLSON and LARSON, 1977). The same three radioactive peaks were, however, detected on paper chromatograms of the radicle extracts, which were not damaged in any way, as in all the other extracts, which may have been wounded. 8(^{14}C)*t*-Zeatin has also been applied to soyabean callus, which was also not damaged in any way (VAN STADEN and HUTTON, 1982). The same three radioactive peaks were also detected on paper chromatograms of these soyabean callus extracts. Column chromatography of these extracts also resulted in radioactive peaks with similar elution volumes to the radioactive peaks detected following column chromatographic separation of the extracts in these experiments. This implies that radioactive zeatin metabolism was not a wounding response.

The actual metabolism of radioactive zeatin appeared to be a complex process, apparently involving ribosylation, glucosylation, oxidation and side chain reduction. Metabolism appeared to be essentially similar in all the treated plant tissues, with oxidation apparently the most important metabolic pathway. The fact that metabolism appeared to be the same in senescing leaves and germinating seeds, could suggest that this metabolism was primarily involved with inactivating the large amounts of exogenously

applied zeatin, rather than the normal metabolism of these tissues. Radioactive peaks 1 (R_f 0,0-0,2), 2 (R_f 0,2-0,5) and 3 (R_f 0,5-1,0) were detected on paper chromatograms of all extracts. Radioactive peak 2, which co-chromatographed with adenosine, trihydroxyzeatin and zeatin glucoside, was usually the major peak. Radioactive peak 1 co-chromatographed with purinyl glycine and appeared to become more important with time and radioactive peak 3, which probably represented residual radioactive zeatin, decreased with time. Metabolic studies indicated that the metabolic pathway of the radioactive zeatin involved, zeatin → radioactive peak 2 → radioactive peak 1. These steps also appeared to be reversible. Column chromatographic separation of these three radioactive peaks in the treated tissues usually resulted in nine radioactive peaks. The percentage of radioactivity associated with each of these peaks appeared to be different in the various treated tissues, possibly reflecting the metabolic capacities of these tissues.

Radioactive peaks 1, 2 and 3 as well as compounds associated with these peaks following column chromatography, have also been reported in *Salix babylonica* cuttings (VAN STADEN and HUTTON, 1981), *Zea mays* kernels (VAN STADEN, 1981a,b), dormant *Platanus occidentalis* L. cuttings (VAN STADEN, 1982b), *Rosa hybrida* leaves (VAN STADEN, 1982a) and soyabean callus (VAN STADEN and HUTTON, 1982). Side chain cleavage, probably brought about by oxidation, was reported to be an important way in which zeatin was metabolized in all of the above systems.

The proposed metabolic pathways for radioactive zeatin in *Ginkgo biloba* leaves (Diagram 1.3, page 125), is probably applicable to all the systems analyzed. This proposed zeatin metabolism is essentially similar to zeatin metabolism described by LETHAM and PALNI (1981) in Diagram 2 (page 47), except for the formation of trihydroxyzeatin and purinyl glycine and possibly adenine. Trihydroxyzeatin and purinyl glycine are oxidation products resulting from the breaking of the double bond in the allyl group in the side chain of zeatin (MILLER, 1965). Trihydroxyzeatin is a biologically active intermediate in this reaction (VAN STADEN, DREWES and HUTTON, 1982) and purinyl glycine is the biologically inactive end-product (MILLER, 1965; LETHAM, SHANNON and MCDONALD, 1967). Trihydroxyzeatin has been isolated from *Zea mays* kernels (LETHAM, 1973). VAN STADEN, DREWES and HUTTON (1982) have proposed that the presence of a compound which resembles N-(purin-6-yl) glycine (purinyl glycine) suggests that the oxidative process, which zeatin undergoes in the plant, could well parallel that which is observed when zeatin is treated with an oxidant, such as potassium permanganate. Oxidation of cold and radioactive zeatin with potassium permanganate (Figure 1.19) did result in a number of peaks which co-eluted with peaks detected in these extracts, some of which co-eluted with purinyl glycine and trihydroxyzeatin.

The significance of these oxidation products in these plant systems is not clear. Compounds co-eluting with purinyl glycine appeared to accumulate in treated tissues and also

appeared to be involved in transport. The re-application and metabolic studies indicated that these compounds may have been converted back to active cytokinins. It is also interesting to note that purinyl glycine has similar chromatographic properties to cytokinin nucleotides (VAN STADEN, DREWES and HUTTON, 1982). VONK and DAVELAAR (1981) have reported that cytokinin nucleotides are involved in transport in the inflorescence stalk of *Yucca flaccida*. Compounds co-eluting with trihydroxyzeatin also appeared to be important oxidation products. Trihydroxyzeatin is biologically very active and co-eluted with adenosine (VAN STADEN, DREWES and HUTTON, 1982), which is often reported as a major zeatin metabolite (HENSON, 1978a; SUMMONS, ENTSCH, LETHAM, GOLLNOW and MACLEOD, 1980; PALMER, SCOTT and HORGAN, 1981) resulting from side chain cleavage by cytokinin oxidase type enzymes (WHITTY and HALL, 1974). Further investigations could reveal that these are actually the same compound. It has been suggested that trihydroxyzeatin could be an active cytokinin form (VAN STADEN, DREWES and HUTTON, 1982). VAN STADEN (1982c) has, however, subsequently shown that trihydroxyzeatin is slowly converted to zeatin by soyabean callus, and he suggested that zeatin itself is responsible for cell division. The formation of all these oxidation products from radioactive zeatin may, therefore, not necessarily be related to the formation of functional metabolites but may be related to storage and inactivation of the applied cytokinin. The apparently minor metabolic pathways, ribosylation and side chain reduction, may, in fact, have been more important than oxidation in

terms of the formation of functional metabolites.

If oxidation is primarily involved in inactivating excess zeatin, the radioactivity which was exported may thus have been important. Although the observation that the transported radioactivity did not always co-elute with biological activity could also imply that these radioactive compounds were not normally involved in cytokinin transport.

REFERENCES

ALVIM, R., HEWETT, E.W. and SAUNDERS, P.F. 1976. Seasonal variation in the hormone content of willow. I. Changes in abscisic acid content and cytokinin activity in the xylem sap. *Plant Physiol.* 57, 474-476.

ARMSTRONG, D.J., BURROWS, W.J., EVANS, P.K. and SKOOG, F. 1969. Isolation of cytokinins from tRNA. *Biochem. Biophys. Res. Commun.* 37, 415-456.

BARNES, M.F., TIEN, C-L. and GRAY, J.S. 1980. Biosynthesis of cytokinins by potato cell cultures. *Phytochemistry* 19, 409-412.

BEEVER, J.E. and WOOLHOUSE, H.W. 1973. Increased cytokinin from roots of *Perilla frutescens* during flower and fruit development. *Nature* 149, 31-32.

BEEVER, J.E. and WOOLHOUSE, H.W. 1974. Increased cytokinin export from the roots of *Perilla frutescens* following disbudding or floral induction. In: *Mechanisms of Regulation of Plant Growth.* Bieleski, R.L., Ferguson, A.R. and Creswell, M.M. (eds.). Bulletin 12, The Royal Soc. N.Z., Wellington. pp. 681-686.

BIDDINGTON, N.L. and THOMAS, T.H. 1973. A modified *Amaranthus* betacyanin bioassay for the rapid determination of cytokinins in plant extracts. *Planta* 111, 183-186.

BIDDINGTON, N.L. and THOMAS, T.H. 1978. Influence of different cytokinins on the transpiration and senescence of oat leaves. *Physiol. Plant.* 42, 369-374.

BLUMENFELD, A. and GAZIT, S. 1970. Cytokinin activity in avocado seeds during fruit development. *Plant Physiol.* 46, 331-333.

BLUMENFELD, A. and GAZIT, S. 1971. Growth of avocado fruit callus and its relation to exogenous and endogenous cytokinin. *Physiol. Plant.* 25, 369-371.

BOROWSKA, B. and RUDNICKI, R.M. 1975. Changes in the levels of cytokinin in apple seeds during stratification. *Fruit Sci. Reports* 2, 1-16.

BROWN, N.A.C. and VAN STADEN, J. 1973. The effect of stratification on the endogenous cytokinin levels of seed of *Protea compacta* and *Leucadendron daphnoides*. *Physiol. Plant.* 28, 388-392.

BURROWS, W.J. 1975. Mechanisms of action of cytokinins. *Curr. Adv. Pl. Sci.* 7, 837-847.

BURROWS, W.J. 1978a. Evidence in support of biosynthesis *de novo* of free cytokinins. *Planta* 138, 53-57.

BURROWS, W.J. 1978b. Incorporation of ^{3}H-adenine into free cytokinins by cytokinin-autonomous tobacco callus tissue. *Biochem. Biophys. Res. Commun.* 84,743-748.

BURROWS, W.J. and CARR, D.J. 1969. Effects of flooding the root system of sunflower plants on the cytokinin content in the xylem sap. *Physiol. Plant.* 22, 1105-1112.

BURROWS, W.J. and CARR, D.J. 1970. Cytokinin content of pea seeds during their growth and development. *Physiol. Plant.* 23, 1064-1070.

BURROWS, W.J. and FUELL, K.J. 1981. Cytokinin biosynthesis in cytokinin-autonomous and bacteria-transformed tobacco callus tissues. In: *Metabolism and Molecular Activities of Cytokinins*. Guern, J. and Péaud-Lenoël, C. (eds.) Springer-Verlag, Berlin. pp. 44-55.

CAPLIN, S.M. and STEWARD, F.C. 1948. Effect of coconut milk on the growth of explants from carrot root. *Science* 108, 655-657.

CARLSON, W.C. and LARSON, M.M. 1977. Changes in auxin and cytokinin activity in roots of red oak, *Quercus rubra* seedlings during lateral root formation. *Physiol. Plant.* 41, 162-166.

CARNES, M.G., BRENNER, M.L. and ANDERSON, C.R. 1975. Comparison of reversed-phase high-pressure liquid chromatography with Sephadex LH-20 for cytokinin analysis of tomato root pressure exudate. *J. Chromatog.* 108, 95-106.

CHEN, C-M. 1981. Biosynthesis and enzyme regulation of the interconversion of cytokinins. In: *Metabolism and molecular activities of cytokinins.* Guern, J. and Péaud-Lenoël, C. (eds.). Springer-Verlag, Berlin. pp. 34-43.

CHEN, C-M. and ECKERT, R.L. 1976. Evidence for the biosynthesis of transfer RNA-free cytokinin. *FEBS Letters* 64, 429-434.

CHEN, C-M. and MELITZ, D.K. 1979. Cytokinin biosynthesis in a cell-free system from cytokinin-autotrophic tobacco tissue cultures. *FEBS Letters* 107, 15-20.

CHEN, C-M. and KRISTOPEIT, S.M. 1981. Metabolism of cytokinin: Deribosylation of cytokinin ribonucleoside by adenosine nucleosidase from wheat germ cells. *Plant Physiol.* 68, 1020-1023.

CHEN, C-M. and PETSCHOW, B. 1978a. Cytokinin biosynthesis in cultured rootless tobacco plants. *Plant Physiol.* 62, 861-865.

CHEN, C-M. and PETSCHOW, B. 1978b. Metabolism of cytokinin. Ribosylation of cytokinin bases by adenosine phosphorylase from wheat germ. *Plant Physiol.* 62, 871-874.

CHINBALL, A.C. 1939. *Protein metabolism in the plant.* Yale University Press, New Haven, Connecticut.

CHINBALL, A.C. 1954. Protein metabolism in rooted runner-bean leaves. *New Phytol.* 53, 31-37.

COLBERT, K.A. and BEEVER, J.E. 1981. Effect of disbudding on root cytokinin export and leaf senescence in tomato and tobacco. *J. Exp. Bot.* 32, 121-127.

COWLEY, D.E., DUKE, C.C., LIEPA, A.J., MACLEOD, J.K. and LETHAM, D.S. 1978. The structure and synthesis of cytokinin metabolites. I. The 7- and 9-β-D-glucofuranosides and pyranosides of zeatin and 6-benzylaminopurine. *Aust. J. Chem.* 31, 1095-1111.

DAVEY, J.E. 1978. Cytokinins in *Lupinus albus* L. *PhD Thesis*, University of Natal, Pietermaritzburg.

DAVEY, J.E. and VAN STADEN, J. 1976. Cytokinin translocation: Changes in zeatin and zeatin-riboside levels in the root exudate of tomato plants during their development. *Planta* 130, 69-72.

DAVEY, J.E. and VAN STADEN, J. 1977. A cytokinin complex in the developing fruits of *Lupinus albus*. *Physiol. Plant.* 39, 221-224.

DAVEY, J.E. and VAN STADEN, J. 1978a. Cytokinin activity in *Lupinus albus*. I. Distribution in vegetative and flowering plants. *Physiol. Plant.* 43, 77-81.

DAVEY, J.E. and VAN STADEN, 1978b. Cytokinin activity in *Lupinus albus*. II. Distribution in fruiting plants. *Physiol. Plant.* 43, 82-86.

DAVEY, J.E. and VAN STADEN, J. 1978c. Cytokinin activity in *Lupinus albus*. III. Distribution in fruits. *Physiol. Plant.* 43, 87-93.

DAVEY, J.E. and VAN STADEN, J. 1981. Cytokinin activity in *Lupinus albus*. V. Translocation and metabolism of 8(^{14}C)*t*-zeatin applied to the xylem of fruiting plants. *Physiol. Plant.* 51, 45-48.

DAVIES, H.V. and CHAPMAN, J.M. 1979. The control of food mobilization in seeds of *Cucumis sativus* L. II. The role of the embryonic axis. *Planta* 146, 585-590.

DAVIES, H.V. and CHAPMAN, J.M. 1980. The control of food mobilization in seeds of *Cucumis sativus* L. III. The control of protein degradation. *Planta* 149, 288-291.

DEI, M. 1978. Inter-organ control of greening in etiolated cucumber cotyledons. *Physiol. Plant.* 43, 94-98.

DEKHUIJZEN, H.M. 1980. The occurrence of free and bound cytokinins in clubroots and *Plasmodiophora brassicae* infected turnip tissue cultures. *Physiol. Plant.* 49, 169-176.

DEKHUIJZEN, H.M. and GEVERS, E. 1975. The recovery of cytokinins during extraction and purification of clubroot tissue. *Physiol. Plant.* 35, 297-302.

DOREE, M. and GUERN, J. 1973. Short-time metabolism of some exogenous cytokinins in *Acer pseudoplatanus* cells. *Biochim. Biophys. Acta* 304, 611-622.

DOREE, M. and SADORGE, P. 1968. Transport, metabolism and biological activity of some cytokinins. In: *Biochemistry and Physiology of Plant Growth Substances*. Wightman, F. and Setterfield, G. (eds.). Runge Press, Ottawa. pp. 1155-1167.

DUKE, C.C., LETHAM, D.S., PARKER, C.W., MACLEOD, J.K. and SUMMONS, R.E. 1979. The complex of O-glucosylzeatin derivatives formed in *Populus* species. *Phytochemistry* 18, 819-824.

DUKE, C.C., MACLEOD, J.K., SUMMONS, R.E., LETHAM, D.S. and PARKER, C.W. 1978. The structure and synthesis of cytokinin metabolites. II. Lupinic acid and O-β-D-glucopyranosylzeatin from *Lupinus angustifolius*. *Aust. J. Chem.* 31, 1291-1301.

DUMBROFF, E.B. and BROWN, D.C.W. 1976. Cytokinin and inhibitor activity in roots and stems of sugar maple seedlings through the dormant season. *Can. J. Bot.* 54, 191-197.

DUMBROFF, E.B. and WALKER, M.A. 1979. The oat-leaf senescence test for cytokinins reconsidered. *Ann. Bot.* 44, 767-769.

ENGELBRECHT, L. 1971. Cytokinins in buds and leaves during growth, maturity and ageing (with a comparison of two bio-assays). *Biochem. Physiol. Pflanzen.* 162, 547-558.

ENGELBRECHT, L. 1972. Cytokinins in leaf cuttings of *Phaseolus vulgaris* L. during their development. *Biochem. Physiol. Pflanzen.* 163, 335-343.

ENTSCH, B. and LETHAM, D.S. 1979. Enzymic glucosylation of the cytokinin, 6-benzylaminopurine. *Plant. Sci. Letters* 14, 205-212.

ENTSCH, B., LETHAM, D.S., PARKER, C.W., SUMMONS, R.E. and GOLLNOW, B.I. 1980. Metabolites of cytokinins. In: *Plant Growth Substances 1979*. Proceedings of the 10th International Conference on Plant Growth Substances, Madison, Wisconsin, July 1979. Skoog, F. (ed.). Springer-Verlag, Berlin. pp. 109-118.

ENTSCH, B., PARKER, C.W., LETHAM, D.S. and SUMMONS, R.E. 1979. Biochim. Biophys. Acta 570, 124. *Cited in* Entsch, B., Letham, D.S., Parker, C.W., Summons, R.E. and Gollnow, B.I. 1980. Metabolites of Cytokinins. In: *Plant Growth Substances 1979*. Skoog, F. (ed.). Springer-Verlag, Berlin. pp. 109-118.

EL SADI, M.T. 1972. Transport and metabolism of Kinetin-8-^{14}C in the mesocotyl and coleoptile of *Zea mays* L. *Ann. Bot.* 36, 99-107.

FEATONBY-SMITH, B. and VAN STADEN, J. 1981. Endogenous cytokinins and rooting of leaf cuttings of *Phaseolus vulgaris* L. *Z. Pflanzenphysiol.* 102, 329-335.

FELDMAN, L.J. 1979. Cytokinin biosynthesis in roots of corn. *Planta* 145, 315-322.

FORSYTH, C. and VAN STADEN, J. 1981. The effects of root decapitation on lateral root formation and cytokinin production in *Pisum sativum*. *Physiol. Plant.* 51, 375-379.

FOX, J.E., CORNETTE, J., DELEUZE, G., DYSON, W., GIERSAK, C., NIU, P., ZAPATA, J. and MCCHESNEY, J. 1973. The formation, isolation and biological activity of a cytokinin 7-glucoside. *Plant Physiol.* 52, 627-632.

GAWER, M., LALOUE, M., TERRINE, C. and GUERN, J. 1977. Metabolism and biological significance of benzyladenine-7-glucoside. *Plant Sci. Letters* 8, 267-274.

GEPSTEIN, S. and ILAN, I. 1979. Cytokinin-induced amylolytic activity in bean cotyledons: Identification of the regulated enzyme. *Plant and Cell Physiol.* 20, 1603-1607.

GEPSTEIN, S. and ILAN, I. 1980. Evidence for the involvement of cytokinins in the regulation of proteolytic activity in cotyledons of germinating beans. *Plant and Cell Physiol.* 21, 57-63.

GOLDACRE, P.L. 1959. Potentiation of lateral root induction by root initials in isolated flax roots. *Aust. J. Bot. Sci.* 12, 388-396.

GOODWIN, P.B., GOLLNOW, B.I. and LETHAM, D.S. 1978. Phytohormones and growth correlations. In: *Phytohormones and related compounds - A Comprehensive Treatise* II. Letham, D.S., Goodwin, P.B. and Higgins, T.J.V. (eds.). Elsevier/North Holland, Amsterdam. pp. 215-243.

GORDON, M.E., LETHAM, D.S. and PARKER, C.W. 1974. The metabolism and translocation of zeatin in intact radish seedlings. *Ann. Bot.* 38, 809-825.

HABERLANDT, G. 1913. Zur physiologie der zellteilung. *Sitz. Ber. K. Preuss Akad. Wissensch.* 16, 318-345.

HAHN, H. 1975. Cytokinins: A rapid extraction and purification method. *Physiol. Plant.* 34, 204-207.

HAHN, H., DE ZACKS, R. and KENDE, H. 1974. Cytokinin formation in pea seeds. *Naturwissenschaften* 61, 170.

HAHN, H., HEITMANN, I. and BLUMBACH, M. 1976. Cytokinins: Production and biogenesis of N^6-(Δ^2-isopentenyl) adenine in cultures of *Agrobacterium tumefaciens* strain B6. *Z. Pflanzenphysiol.* 79, 143-153.

HALL, R.H. 1973. Cytokinins as a probe of developmental processes. *Ann. Rev. Plant Physiol.* 24, 415-444.

HALL, S.M. and BAKER, D.A. 1972. The chemical composition of *Ricinus* phloem exudate. *Planta* 106, 131-140.

HALL, R.H., CSONKA, L. DAVID, H. and MCLENNAN, B. 1967. Cytokinins in the soluble RNA of plant tissues. *Science* 156, 69-71.

HECHT, S.M. 1979. Probing the cytokinin receptor site(s). In: *Plant Growth Substances 1979.* Proceedings of the 10th International Conference on Plant Growth Substances, Madison, Wisconsin, July 1979. Skoog, F. (ed.). Springer-Verlag, Berlin. pp. 144-158.

HECHT, S.M., FRYE, R.B., WERNER, D., HAWRELAK, D.S., SKOOG, F. and SCHMITZ, R. 1975. On the "activation" of cytokinins. *J. Biol. Chem.* 250, 7343-7351.

HELBACH, M. and KLAMBT, D. 1981. On the biogenesis of cytokinins in *Lactobacillus acidophilus* ATCC 4963. *Physiol. Plant.* 52, 136-140.

HENDRY, N.S. 1980. Growth and cytokinin studies in valencia orange (*Citrus sinensis* (L.) Osbeck). *MSc Thesis*, University of Natal, Pietermaritzburg.

HENDRY, N.S., VAN STADEN, J. and ALLAN, P. 1982. Cytokinins in Citrus. I. Fluctuations in the leaves during seasonal and developmental changes. *Scientia Hort.* 16, 9-16.

HENSON, I.E. 1978a. Types, formation and metabolism of cytokinins in leaves of *Alnus glutinosa* (L.) Gaertn. *J. Exp. Bot.* 29, 935-951.

HENSON, I.E. 1978b. Cytokinins and their metabolism in leaves of *Alnus glutinosa*(L.)Gaertn. Effects of leaf development. *Z. Pflanzenphysiol.* 86, 363-369.

HENSON, I.E. and WAREING, P.F. 1976. Cytokinins in *Xanthium strumarium* L.: Distribution in the plant and production in the root system. *J. Exp. Bot.* 27, 1268-1278.

HENSON, I.E. and WAREING, P.F. 1977a. Cytokinins in *Xanthium strumarium* L.: The metabolism of cytokinins in detached leaves and buds in relation to photoperiod. *New Phytol.* 78, 27-33.

HENSON, I.E. and WAREING, P.F. 1977b. Cytokinins in *Xanthium strumarium* L.: Some aspects of the photoperiodic control of endogenous levels. *New Phytol.* 78, 35-45.

HENSON, I.E. and WHEELER, C.T. 1977a. Hormones in plants bearing nitrogen-fixing root nodules: Metabolism of 8(^{14}C)*t*-zeatin in root nodules of *Alnus glutinosa* (L.) Gaertn. *J. Exp. Bot.* 28, 1087-1096.

HENSON, I.E. and WHEELER, C.T. 1977b. Hormones in plants bearing nitrogen-fixing root nodules: Cytokinin transport from the root nodules of *Alnus glutinosa* (L.) Gaertn. *J. Exp. Bot.* 28, 1099-1110.

HEWETT, E.W. and WAREING, P.F. 1973a. Cytokinins in *Populus* x *robusta*: Changes during chilling and bud burst. *Physiol. Plant.* 28, 393-399.

HEWETT, E.W. and WAREING, P.F. 1973b. Cytokinins in *Populus* x *robusta:* Qualitative changes during development. *Physiol. Plant.* 29, 386-389.

HEWETT, E.W. and WAREING, P.F. 1973c. Cytokinins in *Populus* x *robusta* Schneid: A complex in leaves. *Planta* 112, 225-233.

HOAD, G.V., LOVEYS, B.R. and SKENE, K.G.M. 1977. The effect of fruit removal on cytokinins and gibberellin-like substances in grape leaves. *Planta* 136, 25-30.

HOCKING, P.J. 1980. Redistribution of nutrient elements from cotyledons of two species of annual legumes during germination and seedling growth. *Ann. Bot.* 45, 383-396.

HORGAN, R. 1975. A new cytokinin metabolite. *Biochem. Biophys. Res. Commun.* 65, 358-363.

HORGAN, R., HEWETT, E.W., HORGAN, J.M., PURSE, J. and WAREING, P.F. 1975. A new cytokinin from *Populus* x *robusta*. *Phytochemistry* 14, 1005-1008.

HORGAN, R., PALNI, L.M.S., SCOTT, I. and MCGAW, B. 1981. Cytokinin biosynthesis and metabolism in *Vinca rosea* crown gall tissue. In: *Metabolism and Molecular Activities of Cytokinins*. Guern, J. and Péaud-Lenoël, C. (eds.). Springer-Verlag, Berlin.

HUFF, A.K. and ROSS, C.W. 1975. Promotion of radish cotyledon enlargement and reducing sugar content by zeatin and red light. *Plant Physiol.* 56, 429-433.

HUTTON, M.J. and VAN STADEN, J. 1981. An efficient column chromatographic method for separating cytokinins. *Ann. Bot.* 47, 527-529.

HUTTON, M.J., VAN STADEN, J. and DAVEY, J. 1982. Cytokinins in germinating seeds of *Phaseolus vulgaris* L. I. Changes in endogenous levels within the cotyledons. *Ann. Bot.* 49, 685-691.

HUTTON, M.J. and VAN STADEN, J. 1982. Cytokinins in germinating seeds of *Phaseolus vulgaris* L. II. Transport and metabolism of 8(^{14}C)*t*-zeatin applied to the radicle. *Ann. Bot.* 49, 693-699.

ILAN, I. and GEPSTEIN, S. 1980. Hormonal regulation of food reserve breakdown in germinating dicotyledonous seeds. *Isr. J. Bot.* 29, 193-206.

ILAN, I. and GOREN, R. 1979. Cytokinins and senescence in lemon leaves. *Physiol. Plant.* 45, 93-95.

ITAI, C., RICHMOND, A. and VAADIA, Y. 1968. The role of cytokinins during water and salinity stress. *Isr. J. Bot.* 17, 187-195.

ITAI, C. and VAADIA, Y. 1965. Kinetin-like activity in root exudate of water-stressed sunflower plants. *Physiol. Plant.* 18, 941-944.

ITAI, C. and VAADIA, Y. 1971. Cytokinin activity in water-stressed shoots. *Plant Physiol.* 47, 87-90.

JABLONSKI, J.R. and SKOOG, F. 1954. Cell enlargement and cell division in excised tobacco pith tissue. *Physiol. Plant.* 7, 16-24.

JACOBSEN, J.V., HIGGINS, T.J.V., and ZWAR, J.A. 1979. Hormonal control of endosperm function during germination. In: *The Plant Seed: Development, Preservation and Germination.* Rubenstein, I., Phillips, R.L., Green, C.E. and Gengenbach, B.G. (eds.). Academic Press, New York. pp. 241-262.

KANNANGARA, T. and BOOTH, A. 1974. Diffusible cytokinins in shoot apices of *Dahlia variabilis*. *J. Exp. Bot.* 25, 459-467.

KENDE, H. 1965. Kinetin-like factors in the root exudate of sunflowers. *Proc. Natl. Acad. Sci. U.S.A.* 53, 1302-1307.

KENDE, H. 1971. The cytokinins. *Int. Rev. Cytology* 31, 301-338.

KENDE, H. and SITTON, D. 1967. The physiological significance of kinetin and gibberellin-like root hormones. *Ann. N.Y. Acad. Sci.* 144, 235-243.

KLEMEN, F. and KLAMBT, D. 1974. Half life of sRNA from primary roots of *Zea mays*. A contribution to the cytokinin production. *Physiol. Plant.* 31, 186-188.

KRECHTING, H.C.J.M., VARGA, A. and BRUINSMA, J. 1978. Absence of cytokinin biosynthesis in pea seeds developing *in vitro*. *Z. Pflanzenphysiol.* 87, 91-93.

LALOUE, M. 1978. Functions of cytokinins. In: *The Biochemical Functions of terpenoids in plants.* Phil. Trans. R. Soc. 284, 449-458.

LEE, P.K., KESSLER, B. and THIMANN, K.V. 1974. The effect of hadacidin on bud development and its implications for apical dominance. *Physiol. Plant.* 31, 11-14.

LEE, T.S., PURSE, J.G., PRYCE, R.J., HORGAN, R. and WAREING, P.F. 1981. Dihydroconiferyl alcohol - a cell division factor from *Acer* species. *Planta* 152, 571-577.

LETHAM, D.S. 1963. Zeatin a factor inducing cell division from *Zea mays*. *Life Sci.* 2, 569-573.

LETHAM, D.S. 1967a. Chemistry and physiology of kinetin-like compounds. *Ann. Rev. Plant Physiol.* 18, 349-364.

LETHAM, D.S. 1967b. Regulators of cell division in plant tissues. V. A comparison of the activities of zeatin and other cytokinins in five bioassays. *Planta* 74, 228-242.

LETHAM, D.S. 1971. Regulators of cell division in plant tissues. XII. A cytokinin bioassay using excised radish cotyledons. *Physiol. Plant.* 25, 391-396.

LETHAM, D.S. 1973. Cytokinins from *Zea mays*. *Phytochemistry* 12, 2445-2455.

LETHAM, D.S. 1974. Regulators of cell division in plant tissues. XX. The cytokinins of coconut milk. *Physiol. Plant.* 32, 66-70.

LETHAM, D.S. 1978. Cytokinins. In: *Phytohormones and Related Compounds - A Comprehensive Treatise 1.* Letham, D.S., Goodwin, P.B. and Higgins, T.J.V. (eds.). Elsevier/North Holland, Amsterdam. pp. 205-251.

LETHAM, D.S. and PALNI, L.M.S. 1981. Cytokinin metabolism and biosynthesis. In: XIII *International Botanical Congress* Sydney. Abstracts.

LETHAM, D.S., PARKER, C.W., DUKE, C.C., SUMMONS, R.E. and MACLEOD, J.K. 1976. O-glucosylzeatin and related compounds - a new group of cytokinin metabolites. *Ann. Bot.* 41, 261-263.

LETHAM, D.S., SHANNON, J.S. and MCDONALD, I.R.C. 1967. Regulators of cell division in plant tissues. III. The identity of zeatin. *Tetrahedon* 23, 479-486.

LETHAM, D.S. and WILLIAMS, M.W. 1969. Regulators of cell division in plant tissues. VIII. The cytokinins of apple fruit. *Physiol. Plant.* 22, 925-936.

LETHAM, D.S., WILSON, M.M., PARKER, C.W., JENKINS, I.D., MACLEOD, J.K. and SUMMONS, R.E. 1975. Regulators of cell division in plant tissues. XXIII. The identity of an unusual metabolite of 6-benzylaminopurine. *Biochim. Biophys. Acta* 399, 61-70.

LORENZI, R., HORGAN, R. and WAREING, P.F. 1975. Cytokinins in *Picea sitchensis* Carriere: Identification and relation to growth. *Biochem. Physiol. Pflanzen.* 168, 333-339.

LUCKWILL, L.C. and WHYTE, P. 1968. Hormones in the xylem sap of apple trees. *S.C.I. Monograph* No. 31, 87-101.

MAASS, H. and KLÄMBT, D. 1981a. On the biogenesis of cytokinins in roots of *Phaseolus vulgaris*. *Planta* 151, 353-358.

MAASS, H. and KLÄMBT, D. 1981b. Cytokinin biosynthesis in higher plants. In: *Metabolism and Molecular Activities of Cytokinins*. Guern, J. and Péaud-Lenoël, C. (eds.). Springer-Verlag, Berlin. pp. 27-33.

MANOS, P.J. and GOLDTHWAITE, J. 1975. A kinetic analysis of the effects of gibberellic acid, zeatin and abscisic acid on leaf tissue senescence in *Rumex*. *Plant Physiol.* 55, 192-198.

MATSUBARA, S. 1980. Structure-activity relationships of cytokinins. *Phytochemistry* 19, 2239-2253.

MCCALLA, D.R., MOORE, D.J. and OSBORNE, D.J. 1962. The metabolism of a kinin, benzyladenine. *Biochim. Biophys. Acta* 55, 522-528.

METIVIER, J. and PAULILO, M.T. 1980a. The utilization of cotyledonary reserves in *Phaseolus vulgaris* L. cv. Carioca. I. Changes in total amylolytic and proteolytic activity and the effects of 6-benzyl-adenine and gibberellic acid upon whole seed-lings. *J. Exp. Bot.* 31, 1257-1270.

METIVIER, J. and PAULILO, M.T. 1980b. The utilization of cotyledonary reserves in *Phaseolus vulgaris* L. cv. Carioca. II. The effects of 6-benzyladenine and gibberellic acid upon embryonated and de-tached cotyledons. *J. Exp. Bot.* 31, 1271-1282.

MIERNYK, J.A. and BLAYDES, D.F. 1977. Short-term metabolism of radioactive kinetin during lettuce seed ger-mination. *Physiol. Plant.* 39, 4-8.

MILLER, C.O. 1958. The relationship of the kinetin and red-light promotions of lettuce seed germination. *Plant Physiol.* 33, 115-117.

MILLER, C.O. 1961. Kinetin and related compounds in plant growth. *Ann. Rev. Plant. Physiol.* 12, 395-408.

MILLER, C.O. 1963. Kinetin and kinetin-like compounds. In: *Modern Methods of Plant Analysis 6*. Peach, K. and Tracey, M.V. (eds.). Springer-Verlag, Berlin. pp. 277-291.

MILLER, C.O. 1965. Evidence for the natural occurrence of zeatin and derivatives: Compounds from maize which promote cell division. *Proc. Natl. Adad. Sci. U.S.A.* 54, 1052-1058.

MILLER, C.O. 1975. Revised methods for purification of ribosyl-*trans*-zeatin from *Vinca rosea* L. crown gall tumor tissue. *Plant Physiol.* 55, 448-449.

MILLER, C.O., SKOOG, F., OKUMURA, F.S., VON SALTZA, M.H. and STRONG, F.M. 1955. Structure and synthesis of kinetin. *J. Amer. Chem. Soc.* 77, 2662-2663.

MILLER, C.O., SKOOG, F., OKUMURA, F.S., VON SALTZA, M.H. and STRONG, F.M. 1956. Isolation, structure and synthesis of kinetin, a substance promoting cell division. *J. Amer. Chem. Soc.* 78, 1375-1380.

MINAMIKAWA, T. and KOSHIBA, T. 1979. Histochemical studies on mobilization of storage components in cotyledons of germinating *Phaseolus mungo* seeds. *Bot. Mag. Tokyo.* 92, 325-332.

MOLISH, H. 1938. *The Longevity of Plants.* Science Press, Lancaster. pp. 226.

MOROHASHI, Y. 1980. Development of mitochondrial activity in pea cotyledons following imbibition. Influence of the embryonic axis. *J. Exp. Bot.* 31, 805-812.

MOTHES, K. and ENGELBRECHT, L. 1961. Kinetin-induced directed transport of substances in excised leaves in the dark. *Phytochemistry* 1, 58-62.

MURAI, H., ARMSTRONG, D.J. and SKOOG, F. 1975. Incorporation of mevalonic acid into ribosylzeatin in tobacco callus ribonucleic acid preparations. *Plant Physiol.* 55, 853-858.

MUIRA, G.A. and HALL, R.H. 1973. *trans*-Ribosylzeatin. Its biosynthesis in *Zea mays* endosperm and the mycorrhizal fungus *Rhizopogen voseolus*. *Plant Physiol.* 51, 563-569.

MUIRA, G.A. and MILLER, C.O. 1969. Cytokinins from a variant strain of cultured soybean cells. *Plant Physiol.* 44, 1035-1039.

MURASHIGE, T. and SKOOG. F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15, 473-497.

MURRAY, E.W. and ADAMS, R.E. 1980. Embryonic control of isocitrate lyase activity in the megagametophyte of ponderosa pine seeds. *Physiol. Plant.* 49, 21-26.

NAGAR, P.K., INDIRA IYER, R. and SIRCAR, P.K. 1982. Cytokinins in developing fruit of *Moringa pterigosperma* Gaertn. *Physiol. Plant.* 55, 45-50.

NAGAR, P.K. and RAJA RAO, T. 1981. Studies on endogenous cytokinins in guava (*Psidium guajava* L.). *Ann. Bot. 845-852.*

NAITO, K., TSUJI, H. and HATAKEYAMA, I. 1978. Effect of benzyladenine on DNA, RNA, protein and chlorophyll contents in intact bean leaves. Differential responses to benzyladenine according to leaf age. *Physiol. Plant.* 43, 367-371.

NETTING, A.C. 1979. Cytokinin metabolism of the level of the whole plant. *The Tenth International Conference on Plant Growth Substances.* Abstracts.

NISHINARI, N. and SYONO, K. 1980a. Biosynthesis of cytokinins by tobacco cell cultures. *Plant and Cell Physiol.* 21, 1143-1150.

NISHINARI, N. and SYONO, K. 1980b. Cell-free biosynthesis of cytokinins in cultured tobacco cells. *Z. Pflanzenphysiol.* 99, 383-392.

NOEL, A. and VAN STADEN, J. 1976. Seed coat structure and germination in *Podocarpus henkelii. Z. Pflanzenphysiol.* 77, 174-186.

NOODEN, L.D. and LEOPOLD, A.C. 1978. Phytohormones and the endogenous regulation of senescence and abscission. In: *Phytohormones and Related Compounds - A Comprehensive Treatise.* I. Letham, D.S., Gollnow, P.B. and Higgins, T.J.V. (eds.). Elsevier/North Holland, Amsterdam. pp. 329-369.

OSBORNE, D.J. and BLACK, M.K. 1964. Polar transport of a kinin, benzyladenine. *Nature*. 201, 97.

PALMER, M.V., HORGAN, R. and WAREING, P.F. 1981a. Cytokinin metabolism in *Phaseolus vulgaris* L. I. Variations in cytokinin levels in leaves of decapitated plants in relation to lateral bud outgrowth. *J. Exp. Bot.* 32, 1231-1241.

PALMER, M.V., HORGAN, R. and WAREING, P.F. 1981b. Cytokinin metabolism in *Phaseolus vulgaris* L. Identification of endogenous cytokinins and metabolism of (8-^{14}C) dihydrozeatin in stems of decapitated plants. *Planta* 153, 297-302.

PALMER, M.V., SCOTT, I.M. and HORGAN, R. 1981. Cytokinin metabolism in *Phaseolus vulgaris* L. II. Comparative metabolism of exogenous cytokinins by detached leaves. *Plant Sci. Letters* 22, 187-195.

PALNI, L.M.S., TAO, G.Q. and LETHAM, D.S. 1982. Cytokinin metabolism in excised oat leaves. *Eleventh International Conference on Plant Growth Substances.* Abstracts. Aberystwyth July 1982.

PARKER, C.W. and LETHAM, D.S. 1973. Regulators of cell division in plant tissues. XVI. Metabolism of zeatin by radish cotyledons and hypocotyls. *Planta* 114, 199-218.

PARKER, C.W. and LETHAM, D.S. 1974. Regulators of cell division in plant tissues. XVIII. Metabolism of *Zea mays* seedlings. *Planta* 115, 337-344.

PARKER, C.W., LETHAM, D.S., WILSON, M.M., JENKINS, J.D., MACLEOD, J.K. and SUMMONS, R.E. 1975. The identity of two new cytokinin metabolites. *Ann. Bot.* 39, 375-376.

PARKER, C.W., LETHAM, D.S., GOLLNOW, B.I., SUMMONS, R.E., DUKE, C.C. and MACLEOD, J.K. 1978. Regulators of cell division in plant tissues. XXV. Metabolism of zeatin by lupin seedlings. *Planta* 142, 239-251.

PARKER, C.W., WILSON, M.M., LETHAM, D.S., COWLEY, D.E. and MACLEOD, J.K. 1973. The glucosylation of cytokinins. *Biochem. Biophys. Res. Commun.* 55, 1370-1376.

PATE, J.S. and FARRINGTON, P. 1981. Fruit set in *Lupinus angustifolius* cv. Unicrop. II. Assimilate flow during flowering and early fruiting. *Austr. J. Plant Physiol.* 8, 307-318.

PENNER, D. and ASHTON, F.M. 1967. Hormonal control of isocitrate lyase synthesis. *Biochim. Biophys. Acta* 148, 481-485.

PETERSON, C.A. and FLETCHER, R.A. 1973. Formation of fruits on rootless plants. *Can. J. Bot.* 51, 1899-1905.

PETERSON, J.B. and MILLER, C.O. 1977. Glucosylzeatin and glucosyl ribosyl zeatin from *Vinca rosea* L. crown gall tumor tissue. *Plant Physiol.* 59, 1026-1028.

PINFIELD, N.J. and STOBART, A.K. 1972. Hormonal regulation of germination and early seedling development in *Acer pseudoplatanus*. *Planta* 104, 134-145.

PROCHÁZKA, S. 1981. Transport of benzyl-8-^{14}C-adenine in pea seedlings in relation to stem apical dominance. *Biologia Plant.* 68-71.

PROCHÁZKA, S., SCHRAUDOLF, H. and ŠONKA, J. 1977. Transport of ^{14}C-kinetin in intact and decapitated pea seedlings (*Pisum sativum* L.). *Z. Pflanzenphysiol.* 81, 308-313.

PURSE, J.G., HORGAN, R., HORGAN, J.M. and WAREING, P.F. 1976. Cytokinins of sycamore spring sap. *Planta* 132, 1-8.

RADIN, J.W. and LOOMIS, R.S. 1971. Changes in the cytokinins of radish roots during maturation. *Physiol. Plant.* 25, 240-244.

RAMINA, A. 1979. Aspects of (8-^{14}C) benzylaminopurine metabolism in *Phaseolus vulgaris*. *Plant Physiol.* 63, 298-300.

RAMINA, A., PIMPINI, F., BONIOLO, A. and BERGAMASCO, F. 1979. (8-^{14}C) Benzylaminopurine translocation in *Phaseolus vulgaris*. *Plant Physiol.* 63, 294-297.

REUTHER, W. 1977. Citrus. In: *Ecophysiology of tropical crops*. Alvim, P. and Kozlowski, T. (eds.). Academic Press, New York. pp. 409-438.

REYNOLDS, E.S. 1963. The use of lead citrate and high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* 17, 208-212.

RICHMOND, A.E. and LANG, A. 1957. Effect of kinetin on the protein content and survival of detached *Xanthium* leaves. *Science* 125, 650-651.

RYBICKA, H., ENGELBRECHT, L., MIKULOVICH, T.P. and KULAEVA, O.N. 1977. Investigation of endogenous substances with cytokinin activity in pumpkin cotyledons in connection with characteristics of the action of exogenous cytokinins on them. *Soviet Plant Physiol.* 24, 292-299.

SACHS, T. and THIMANN, K.V. 1964. Release of lateral buds from apical dominance. *Nature* 201, 939-940.

SETH, A.K. and WAREING, P.F. 1967. Hormone-directed transport of metabolites and its possible role in plant senescence. *J. Exp. Bot.* 18, 65-77.

SHORT, K.C. and TORREY, J.G. 1972. Cytokinins in seedling roots of pea. *Plant Physiol.* 49, 155-160.

SKENE, K.G.M. 1972a. Changes in the xylem sap of grape vine canes: Changes in activity during cold storage. *Planta* 104, 89-92.

SKENE, K.G.M. 1972b. The effect of ringing on cytokinin activity in shoots of the grape vine. *J. Exp. Bot.* 23, 768-774.

SKOOG, F. and ARMSTRONG, D.J. 1970. Cytokinins. *Ann. Rev. Plant Physiol.* 21, 359-383.

SKOOG, F., STRONG, F.M. and MILLER, C.O. 1965. Cytokinins. *Science* 148, 532-533.

SMITH, A.R. 1977. The involvement of endogenous cytokinins in germination and seedling development of *Zea mays* L. *MSc Thesis*, University of Natal, Pietermaritzburg.

SMITH, A.R. and VAN STADEN, J. 1978. Changes in endogenous cytokinin levels in kernels of *Zea mays* L. during imbibition and germination. *J. Exp. Bot.* 29, 1067-1075.

SMITH, A.R. and VAN STADEN, J. 1979. Cytokinins in excised embryos and endosperm of *Zea mays* L. grown under aseptic conditions. *Z. Pflanzenphysiol.* 93, 95-103.

SONDHEIMER, E. and TZOU, D-S. 1971. The metabolism of hormones during seed germination and dormancy. II. The metabolism of 8-^{14}C zeatin in bean axes. *Plant Physiol.* 47, 516-520.

STEWARD, F.C. and CAPLIN, S.M. 1952. Investigations on growth and metabolism of plant cells. IV. Evidence on the role of the coconut milk factor in development. *Ann. Bot.* 16, 491-504.

STUCHBURY, T., PALNI, L.M., HORGAN, R. and WAREING, P.F. 1979. The biosynthesis of cytokinins in crown gall tissue of *Vinca rosea*. *Planta* 147, 97-102.

SUMMONS, R.E., ENTSCH, B., LETHAM, D.S., GOLLNOW, B.I. and MACLEOD, J.K. 1980. Regulators of cell division in plant tissues. XXVIII. Metabolites of zeatin in sweet-corn kernels: Purifications and identifications using high-performance liquid chromatography and chemical-ionization mass spectrometry. *Planta* 147, 422-434.

SUMMONS, R.E., LETHAM, D.S., GOLLNOW, B.I., PARKER, C.W., ENTSCH, B., JOHNSON, L.P., MACLEOD, J.K. and ROLFE, B.G. 1981. Cytokinin metabolism and physiological responses in plant or bacterial systems. In: *Metabolism and Molecular Activities of Cytokinins.* Guern, J. and Péaud-Lenoël, C. (eds.). Springer-Verlag, Berlin. pp. 69-79.

TAYA, Y., TANAKA, Y. and NISHIMURA, S. 1978. 5´AMP is a direct precursor of cytokinin in *Dictyostelium discoideum*. *Nature* 271, 545-547.

TEGELY, J.R., WITHAM, F.H. and KRASNUK, M. 1971. Chromatographic analysis of a cytokinin from tissues of crown gall. *Plant Physiol.* 47, 581-585.

THIMANN, K.V. 1977. *Hormone action in the whole life of plants*. University of Massachusetts Press.

THIMANN, K.V., SHIBAOKA, H. and MARTIN, C. 1970. On the nature of senescence in oat leaves. In: *Plant Growth Substances*. Carr, D.J. (ed.). Springer-Verlag, Berlin. pp. 561-570.

THOMAS, H. 1975. Regulation of alanine aminotransferase in leaves of *Lolium temulentum* during senescence. *Z. Pflanzenphysiol.* 74, 208-218.

THOMAS, H. and STODDART, J.L. 1980. Leaf senescence. *Ann. Rev. Plant Physiol.* 31, 83-111.

TORREY, J.G. and LOOMIS, R.S. 1967. Auxin-cytokinin control of secondary vascular tissue formation in isolated roots of *Raphanus*. *Am. J. Bot.* 54, 1098-1106.

TURVEY, P.M. and PATRICK, J.W. 1979. Kinetin-promoted transport of assimilates in stems of *Phaseolus vulgaris* L. Localized versus remote site(s) of action. *Planta* 147, 151-155.

TZOU, D-S., GALSON, E.C. and SONDHEIMER, E. 1973. The metabolism of hormones during seed germination and release from dormancy. III. The effects and metabolism of zeatin in dormant and non-dormant ash embryos. *Plant Physiol.* 51, 894-897.

UHEDA, E. and KURAISHI, B. 1977. Increase of cytokinin activity in detached etiolated cotyledons of squash after illumination. *Plant and Cell Physiol.* 18, 481-483.

VAN ONCKELEN, H.A., CAUBERGS, R. and DE GREEF, J.A. 1977. Effect of light treatment and endogenous growth hormones on α and β-amylase activities in cotyledons of *Phaseolus vulgaris* L. *Plant and Cell Physiol.* 18, 1029-1040.

VAN STADEN, J. 1976a. Occurrence of a cytokinin glucoside in the leaves and in honeydew of *Salix babylonica*. *Physiol. Plant.* 36, 225-228.

VAN STADEN, J. 1976b. Seasonal changes in the cytokinin content of *Ginkgo biloba* leaves. *Physiol. Plant.* 38, 1-5.

VAN STADEN, J. 1976c. Extraction and recovery of cytokinin glucosides by means of a cation exchange resin. *Physiol. Plant.* 38, 240-242.

VAN STADEN, J. 1976d. The identification of zeatin glucoside from coconut milk. *Physiol. Plant.* 36, 123-126.

VAN STADEN, J. 1977. Seasonal changes in the cytokinin content of the leaves of *Salix babylonica*. *Physiol. Plant.* 40, 296-299.

VAN STADEN, J. 1978. A comparison of the endogenous cytokinins in the leaves of four gymnosperms. *Bot. Gaz.* 139, 32-35.

VAN STADEN, J. 1979a. The effect of adenine and mevalonic acid on the endogenous cytokinins of a cytokinin-dependent strain of soya bean callus. *Ann. Bot.* 44, 671-675.

VAN STADEN, J. 1979b. The metabolism of zeatin by cell-free plant extracts. *Z. Pflanzenphysiol.* 92, 399-405.

VAN STADEN, J. 1979c. Changes in the endogenous cytokinin levels of excised buds of *Salix babylonica* L., cultured aseptically. *Bot. Gaz.* 140, 138-141.

VAN STADEN, J. 1981a. Cytokinins in germinating maize caryopses. I. Transport and metabolism of 8(^{14}C)*t*-zeatin applied to the endosperm. *Physiol. Plant.* 53, 269-274.

VAN STADEN, J. 1981b. Cytokinins in germinating maize caryopses. II. Transport and metabolism of 8(^{14}C)*t*-zeatin applied to the embryonic axis. *Physiol. Plant.* 53, 275-278.

VAN STADEN, J. 1982a. Transport of 8(^{14}C)*t*-zeatin from mature rose leaves after shoot decapitation. *Bot. Gaz.* In press.

VAN STADEN, J. 1982b. Transport and metabolism of 8(^{14}C)*t*-zeatin in dormant plane cuttings during cold storage. *Z. Pflanzenphysiol.* 105, 351-357.

VAN STADEN, J. 1982c. Metabolism of trihydroxyzeatin applied to soybean callus. In Press.

VAN STADEN, J. and BROWN, N.A.C. 1977. The effect of ringing on cytokinin distribution in *Salix babylonica*. *Physiol. Plant.* 39, 266-270.

VAN STADEN, J. and BROWN, N.A.C. 1978. Changes in the endogenous cytokinins of bark and buds of *Salix babylonica* as a result of stem girdling. *Physiol. Plant.* 43, 148-153.

VAN STADEN, J. and BUTTON, J. 1978. The cytokinin content of aseptically cultured pea fruits. *Z. Pflanzenphysiol.* 87, 129-135.

VAN STADEN, J. and CHOVEAUX, N.A. 1980. Cytokinins in internodal segments of *Salix babylonica*. *Z. Pflanzenphysiol.* 96, 153-161.

VAN STADEN, J. and CHOVEAUX, N.A. 1981. Adenine is not incorporated into the cytokinins of developing fruits of *Lupinus albus*. *Z. Pflanzenphysiol.* 104, 395-399.

VAN STADEN, J., CHOVEAUX, N.A. and DIMALLA, G. 1980. The effect of the antibiotic hadacidin on the growth and cytokinin levels of stem explants. *Z. Pflanzenphysiol.* 100, 291-298.

VAN STADEN, J. and DAVEY, J.E. 1976. Cytokinin translocation in xylem sap of herbaceous plants. *Z. Pflanzenphysiol.* 77, 377-382.

VAN STADEN, J. and DAVEY, 1977. The metabolism of zeatin and zeatin riboside by soya bean callus. *Ann. Bot.* 41, 1041-1048.

VAN STADEN, J. and DAVEY, J.E. 1979. The synthesis, transport and metabolism of endogenous cytokinins. *Plant, Cell and Environ.* 2, 93-106.

VAN STADEN, J. and DAVEY, J.E. 1981a. Cytokinin activity in *Lupinus albus*. VI. Translocation and metabolism of 8(^{14}C)*t*-zeatin applied to the leaves and fruits of fruiting plants. *Physiol. Plant.* 51, 49-52.

VAN STADEN, J. and DAVEY, J.E. 1981b. Seasonal changes in the levels of endogenous cytokinins in the willow *Salix babylonica* L. *Z. Pflanzenphysiol.* 104, 53-59.

VAN STADEN, J. and DIMALLA, G.G. 1977. A comparison of the endogenous cytokinins in the roots and xylem exudate of nematode-resistant and susceptible tomato cultivars. *J. Exp. Bot.* 28, 1351-1356.

VAN STADEN, J. and DIMALLA, G.G. 1978. Endogenous cytokinins and the breaking of dormancy and apical dominance in potato tubers. *J. Exp. Bot.* 29, 1077-1084.

VAN STADEN, J. and DIMALLA, G.G. 1980. Endogenous cytokinins in *Bougainvillea* 'San Diego Red'. I. Occurrence of cytokinin glucosides in the root sap. *Plant Physiol.* 65, 852-854.

VAN STADEN, J. and DIMALLA, G.G. 1981. The production and utilization of cytokinins in rootless, dormant almond shoots maintained at low temperature. *Z. Pflanzenphysiol.* 103, 121-129.

VAN STADEN, J. and DREWES, S.E. 1982. 6-(2,3,4-trihydroxy-3-methylbutylamino) purine a cytokinin with high biological activity. *Phytochemistry.* In Press.

VAN STADEN, J., DREWES, S.E. and HUTTON, M.J. 1982. Biological activity of 6-(2,3,4-trihydroxy-3-methylbutylamino) purine, an oxidation product of zeatin. *Physiol. Plant.* 55, 143-148.

VAN STADEN, J. and HUTTON, M.J. 1981. Transport of labelled zeatin in willow tree cuttings stored in the cold. *S.A.J. Sci.* 77, 476-477.

VAN STADEN, J. and HUTTON, M.J. 1982. Metabolism of 8(^{14}C)*t*-zeatin in soybean callus. In Press.

VAN STADEN, J. and MENARY, R.C. 1976. Identification of cytokinins in the xylem sap of tomato. *Z. Pflanzenphysiol.* 78, 262-265.

VAN STADEN, J. and PAPAPHILIPPOU, A.P. 1977. Biological activity of O-β-D-glucopyranosylzeatin. *Plant Physiol.* 60, 649-650.

VAN STADEN, J. and SMITH, A.R. 1978. The synthesis of cytokinins in excised roots of maize and tomato under aseptic conditions. *Ann. Bot.* 42, 751-753.

VAN STADEN, J., SPIEGELSTEIN, H., ZIESLIN, N. and HALEVY, A.H. 1981. Endogenous cytokinins and lateral bud growth in roses. *Bot. Gaz.* 142, 177-182.

VAN STADEN, J. and WAREING, P.F. 1972. The effect of photoperiod on the levels of endogenous cytokinins in *Xanthium strumarium*. *Physiol. Plant.* 27, 331-337.

VARGA, A. and BRUINSMA, J. 1973. Effects of different cytokinins on the senescence of detached oat leaves. *Planta* 111, 91-93.

VARGA, A. and BRUINSMA, J. 1974. The growth and ripening of tomato fruits at different levels of endogenous cytokinins. *J. Hort. Sci.* 49, 135-142.

VARNER, J.E., BALCE, L.V. and HAUNG, R.C. 1963. Senescence of cotyledons of germinating peas. Influence of axis tissue. *Plant Physiol.* 38, 89-92.

VONK, C.R. 1979. Origin of cytokinins transported in the phloem. *Physiol. Plant.* 46, 235-240.

VONK, C.R. and DAVELAAR, E. 1981. 8(^{14}C)*t*-Zeatin metabolites and their transport from leaf to phloem exudate of *Yucca*. *Physiol. Plant.* 52, 101-107.

WANG, T.L. 1977. *PhD Thesis*, University of Wales. *Cited in* Palmer, M.V., Horgan, R. and Wareing, P.F. 1981. Cytokinin metabolism in *Phaseolus vulgaris* L. *J. Exp. Bot.* 32, 1231-1241.

WANG, T.L., BEUTELMANN, P. and COVE, D.J. 1981. Cytokinin biosynthesis in mutants of the moss *Physcomitrella patens*. *Plant Physiol.* 68, 739-744.

WANG, T.L. and HORGAN, R. 1978. Dihydrozeatin riboside, a minor metabolite from the leaves of *Phaseolus vulgaris* L. *Planta* 140, 151-153.

WANG, T.L., THOMPSON, A.G. and HORGAN, R. 1977. A cytokinin glucoside from the leaves of *Phaseolus vulgaris* L. *Planta* 135, 285-288.

WAREING, P.F. 1977. Growth substances and integration in the whole plant. In: *Symposium XXXI. Integration of Activity in the Higher Plant.* Jennings, D.H. (ed.). Cambridge University Press, London. pp. 337-365.

WAREING, P.F., HORGAN, R., HENSON, I.E. and DAVIS, W. 1976. Cytokinin relations in the whole plant. In: *Plant Growth Regulation.* Pilet, P.E. (ed.). Proc. 9th International Conference on Plant Growth Substances, Lausanne. Springer-Verlag, Berlin. pp. 147-153.

WAREING, P.F. and SETH, A.K., 1967. Ageing and senescence in the whole plant. *Symp. Soc. Exp. Biol.* 21, 543-548.

WEBB, D.P., VAN STADEN, J. and WAREING, P.F. 1973. Seed dormancy in *Acer*. Changes in endogenous cytokinins, gibberellins and germination inhibitors during the breaking of dormancy in *Acer saccharum* Marsh. *J. Exp. Bot.* 24, 105-116.

WEILER, E.W. and ZIEGLER, H. 1981. Determination of phytohormones in phloem exudate from tree species by radioimmunoassay. *Planta* 152, 168-170.

WEISS, C. and VAADIA, Y. 1965. Kinetin-like activity in root apices of sunflower plants. *Life Sci.* 4, 1323-1326.

WHITTY, C.D. and HALL, R.H. 1974. A cytokinin oxidase in *Zea mays*. *Can. J. Bot.* 52, 789-799.

WILSON, M.M., GORDON, M.E., LETHAM, D.S. and PARKER, C.W. 1974. Regulators of cell division in plant tissues. XIX. The metabolism of 6-benzylaminopurine in radish cotyledons and seedlings. *J. Exp. Bot.* 25, 725-732.

WOOLLEY, D.J. and WAREING, P.F. 1972. The role of roots, cytokinins and apical dominance in the control of lateral shoot form in *Solanum andigena*. *Planta* 105, 33-42.

YOKOTA, T., UEDA, J. and TAKAHASHI, N. 1981. Cytokinins in immature seeds of *Dolichos lablab*. *Phytochemistry* 20, 683-686.

YOSHIDA, R. and ORITANI, T. 1972. Cytokinin glucoside in roots of the rice plant. *Plant and Cell Physiol.* 13, 337-343.

ZACHAU, H.G., DÜTTING, D. and FELDMAN, H. 1966. Nucleotidsequenzen zweier Sevingspezifischer Transferribonucleinsauren. *Angewandte Chemie* 78, 392-393.