CYTOKININS IN *ECKLONIA MAXIMA*

AND THE EFFECT OF SEAWEED

CONCENTRATE ON PLANT

GROWTH

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.

Bryan Charles Featonby-Smith

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ABSTRACT

The endogenous cytokinin levels in the brown alga *Ecklonia maxima* (OSBECK) PAPENF., and the effect of applications of the seaweed concentrate (Kelpak 66) prepared from this alga, on the growth and yield of various plants was investigated.

Tentative identification of the cytokinins present in *Ecklonia maxima* using High Performance Liquid Chromatography revealed the presence of *cis* and *trans*-ribosylzeatin, *trans*-zeatin, dihydrozeatin and isopentenyladenosine.

Seasonal and lunar variations in the endogenous cytokinin levels in fresh and processed *Ecklonia maxima* material were investigated. Lamina, stipe and holdfast regions of one, two and three metre plants harvested from February 1981 until January 1982 together with samples of processed material from the normal production run, collected over the same period were used in this investigation. Analysis revealed both qualitative and quantitative changes in the cytokinin levels which were closely correlated to the seasonal patterns of growth of *Ecklonia maxima*. During summer zeatin, ribosylzeatin and their dihydroderivatives were responsible for most of the detected activity. The cytokinin glucosides increased above the levels of free cytokinins during winter. The lunar cycle study of material harvested on a daily basis during April – May 1983 revealed marked fluctuations in the cytokinin levels in the various tissues of two metre plants which were closely correlated with the phases of the moon.

Greenhouse trials were conducted to determine the effects of the commercially available seaweed concentrate (Kelpak 66) on the growth of *Lycopersicon esculentum* MILL. plants in nematode infested soil. Kelpak 66 at a dilution of 1 : 500 improved the growth of treated plants significantly,

irrespective of whether it was applied as a foliar spray at regular intervals, or whether the soil in which the plants were grown was flushed once with the diluted seaweed concentrate. Root growth was significantly improved whenever the seaweed concentrate was applied. Associated with this improved root growth was a reduction in the infestation of *Meloidogyne incognita* (KOIFORD and WHITE) CHITWOOD.

Finally, the effect of seaweed concentrate and fertilizer applications on the growth and endogenous cytokinin content of *Beta vulgaris* L. and *Phaseolus vulgaris* L. plants was investigated. Seaweed concentrate at a dilution of 1 : 500 applied as a foliar spray improved the growth of treated plants significantly, irrespective of whether it was applied on its own or together with a chemical fertilizer. Root growth and the endogenous cytokinin content of these roots increased with seaweed concentrate application. Increases were also detected in the cytokinin content of fruits of *Phaseolus vulgaris* plants treated with seaweed concentrate. Associated with this increase in the cytokinin content was an increase in the dry mass of the fruit from treated plants.

The significance of these findings and the possible relationship between the endogenous cytokinins present in *Ecklonia maxima* and the effect of the seaweed concentrate on plant growth is discussed.

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INTRODUCTION

The use of marine algal extracts as plant growth stimulants is not a recent phenomenon. Seaweeds particularly in Europe, have been used for their manural value by coastal farmers for centuries, and extracts prepared from marine algae became available in the 1950's. It is only recently, however, that comprehensive research into the use of seaweeds in agriculture has begun. This has been initiated as a result of man's need to establish more efficient and economical methods of crop production, and the necessity for a better understanding of plant growth and development in order to achieve greater production. The most commonly used seaweeds in the manufacture of commercial extracts and concentrates are *Macrocystis pyrifera* (L.) C. AGARDH, *Ascophyllum nodosum* (L.) LE JOLIS and *Ecklonia maxima* (OSBECK) PAPENF.

The reasons why seaweed extracts and/or concentrates are beneficial to plant growth are still unclear. The presence of trace elements has been put forward as a possible explanation for seaweeds beneficial effects on plant growth. It would, however appear as if such an explanation is not totally adequate, as the amount of seaweed applied to crops would contain too little of these elements to elicit the growth responses which have been observed (BLUNDEN, 1977).

Reports that seaweed releases unavailable minerals from the soil have been made. FRANKI (1960) established that leaves of *Lycopersicon esculentum* L. (tomato) treated with a seaweed extract contained more manganese than was present in the seaweed itself. Concluding that seaweed had released unavailable

manganese from the soil. OFFERMANS (1968) found iron availability to be greatest in soil to which seaweed had been applied. AITKEN and SENN (1965) reported that seaweed extract used in nutrient deficient culture solutions eliminated or lessened deficiencies of magnesium, manganese, zinc and boron. LYNN (1972) studied the chelating properties of *Ascophyllum nodosum* by adding the seaweed extract to mineral deficient solutions used 'on *Capsicum annum* L. (green pepper). He showed improved plant utilization of boron, copper, iron, manganese and zinc.

QUASTEL and WEBLEY (1947) showed that alginic acid which is present in seaweed in large quantities is an important soil conditioner. Alginates were shown to improve the crumb structure and moisture retaining characteristics of light soils and ameliorated the sticky nature of clay soils.

That plant hormones, and in particular cytokinins, may be involved was suggested by BOOTH (1966). This conclusion was reached because most of the responses obtained with the use of seaweed extracts were similar to those observed when cytokinins were applied to plants. BLUNDEN and WILDGOOSE (1977) demonstrated close correlations between the results obtained from the use of kinetin and commercial seaweed extracts of equivalent cytokinin activities in field trials conducted on *Solanum tuberosum* L. (potato). These authors suggested that the effects of the seaweed extracts were due to their cytokinin content. Similar results were obtained when the shelf-life of *Citrus aurantiifolia* (CHRISTM.) SWINGLE (lime) was increased after post-harvest immersion of the fruit in kinetin and seaweed extracts of known cytokinin activity (BLUNDEN, JONES and PASSAM, 1978). Further circumstantial evidence supporting this hypothesis was the detection of cytokinin-like activity in a number of marine algae (HUSSAIN and BONEY, 1969; JENNINGS, 1969; KENTZER, SYNAK, BURKIEWICZ and BANAS, 1980; KINGMAN and MOORE, 1982). It is also well established that these hormones are present in commercial extracts prepared from marine algae (BRAIN,

CHALOPIN, TURNER, BLUNDEN and WILDGOOSE, 1973; BLUNDEN, 1977; BLUNDEN and WILDGOOSE, 1977). Thus, although there is no direct evidence to suggest that the cytokinins present in seaweed extracts are responsible for the beneficial results obtained with the use of these extracts, it would seem likely that these compounds are one of the major active constituents of seaweed extracts. This concept is supported by the nature of cytokinins, their effect on plant growth and the relatively high levels found in seaweed extracts (BLUNDEN and WILDGOOSE, 1977).

Having implicated cytokinins in the various responses of plants to seaweed extract application, it is important to consider whether any seasonal variation in the cytokinin content of the extracts exists. Variation in both quality and quantity of cytokinins present in batches of seaweed extract manufactured at different times of the year, could account for some of the erratic results obtained with the use of seaweed extracts. In order to establish whether such variation does exist the present study was undertaken. The following aspects were investigated.

1. A study to determine the presence and identity of the cytokinins in *Ecklonia maxima*.

2. A study to determine the level of cytokinin activity in processed *Ecklonia maxima* material and in the lamina, stipe and holdfast regions of *Ecklonia maxima* plants over a seasonal and lunar cycle.

3. A study to determine the effect of the aqueous seaweed concentrate (Kelpak 66) on the growth of various crops.

CHAPTER I

LITERATURE REVIEW

The Use of Seaweeds in Agriculture

The earliest recorded use of seaweeds can be found in the writings of Shen Nung (3000 B.C.), a Chinese physician who told of their medicinal value. Later during the time of Confucius (800 – 600 B.C.), it was reported that housewives were using seaweed as a foodstuff (GLICKSMAN, 1969). The use of seaweed in agriculture is a practice which has been carried out for centuries. It was common for farmers in coastal areas to apply seaweed as a soil conditioner. With the advent of chemical fertilizers in the late 1800's the use of seaweed declined in popularity. In recent years, however, when it was discovered that the addition of chemicals to the soil had an adverse effect on the environment, natural sources of fertilizers or fertilizer supplements became a popular alternative. Commercial seaweed extracts became available in the 1950's. The most commonly used seaweed being *Ascophyllum nodosum* of the order Fucales. During the last 30 years seaweed research in agriculture has increased sharply and several species have been studied for use as a supplemental fertilizer and soil conditioner. The responses of plants to seaweed extract application have been many and varied and include, higher yields; longer shelf-life; increased nutrient uptake and changes in plant tissue composition; increased resistance to frost, fungal diseases and insect attack; and better seed germination. For the purpose of this review each of these responses of plants to seaweed extracts will be dealt with separately.

The application of seaweed to improve the growth and yield of terrestrial

plants is fast becoming an accepted practice. It is however, only relatively recently that the effects of seaweed treatments have been documented. The earliest records on the beneficial effects of seaweed on crop yields was reported by AITKEN, SENN and MARTIN (1961). They demonstrated that extracts of *Ascophyllum nodosum* increased the respiratory metabolism of various citrus seedlings. AITKEN (1964) found that the application of seaweed extract to nutrient deficient cultures lessened and/or nullified the deficiency effect, resulting in the improved growth of *Citrus sinensis* L. OSB. (grapefruit) seedlings. Seaweed extracts have also been found to increase the yield of various other crops (AITKEN and SENN, 1965). STEPHENSON (1966) found significant increases in yield in both *Fragaria x ananassa* L. (Strawberry) and *Brassica campestris* L. (turnips) with seaweed application.

BOOTH (1966) recorded a 20 per cent increase in the yield of potatoes with the application of a seaweed extract. "Algifert" the Norwegian seaweed extract produced from the brown algae *Ascophyllum nodosum* resulted in significant increases in the yields of three *Cucumis sativus* L. (cucumber) varieties (POVOLNY, 1971). Further work by POVOLNY (1974) on *Lycopersicon esculentum* showed that by dipping the pots containing these plants in a one per cent solution of "Algifert" the overall dry mass of the plants increased by 36 per cent over the control plants.

BLUNDEN (1972) conducted trials on a number of crops using a seaweed extract prepared from species of the Laminariaceae and Fucaceae (S.M.3.). Foliar application to *Musa acuminata* L. (banana) plants decreased the time to shooting and increased the average bunch weight of the fruits. Soil applications of the seaweed extract in trials conducted on *Gladiolus* spp. L. (gladioli) increased the average corm weight significantly. Increased crop yields were obtained in commercial trials with *Solanum tuberosum, Zea mays* L. (sweet corn), *Capsicum annum, Lycopersicon esculentum* and *Citrus arantium* L. (orange). BLUNDEN

and WILDGOOSE (1977) carried out further trials on the effect of foliar applications of seaweed extract (S.M.3) on *Solanum tuberosum*. They found that seaweed application six weeks after shoot emergence to plants of the variety King Edward gave a statistically significant increase in yield. However, no significant increase in yield was found for the cultivar Pentland Dell. Foliar application of an aqueous seaweed extract to *Beta vulgaris* L. (sugar beet), (BLUNDEN, WILDGOOSE and NICHOLSON, 1979) produced significant increases in the root weight, root sugar content and in clarified juice purity by reducing the concentration of amino-nitrogen and potassium.

KOTZE and JOUBERT (1980) found that applications of seaweed concentrate (Kelpak 66) to *Secale cereale* L. (rye) plants resulted in significant increases in growth and nutrient uptake and that these effects were dependant on the concentration at which the seaweed concentrate was applied. NELSON and VAN STADEN (1984) found in trials conducted on *Triticum aestivum* L. (wheat) that seaweed concentrate applied either as a root drench, or as a foliar spray resulted in a marked increase in culm diameter due to increased cell size, especially of the vascular bundles and a significant increase in grain yield over the control.

DRIGGERS and MARUCCI (1964) found that the fruit of *Prunus persica* L. BATSCH. (peach) sprayed with seaweed extract before harvest had a longer shelf-life than did untreated fruit. Sixteen days after harvest 32,2 per cent of the controls were rotten, compared to 14,7 per cent of the fruit from treated trees. Studies by SENN and SKELTON (1966) showed that the shelf-life of fruits of *Prunus persica* were significantly increased by the application of pre-harvest sprays of seaweed extract. They showed that the percentage of unmarketable fruit was greatly reduced. SKELTON and SENN (1969) found that the time of application and the number of seaweed treatments was important. Earlier applications beginning with full bloom gave better results than did sprays later

on in the growing season. The optimal number of treatments was found to be three. In trials conducted on *Malus pumila* MILL. (apple) trees, POVOLNY (1969), found that two foliar applications of seaweed extract, four and two weeks before harvesting, significantly increased the shelf-life of the fruit. Seaweed extract application resulted in a 4,3 per cent loss after storing compared to the 39,2 per cent loss in the untreated fruit of the control. POVOLNY (1972) in trials conducted on *Prunus persica* and *Prunus armeniaca* L. (apricot) found that seaweed treatment prior to harvest reduced the storage losses of fruits by 35 per cent and 30 per cent respectively. Further work conducted by POVOLNY (1976) on *Lycopersicon esculentum* showed that the application of seaweed extracts prior to harvest decreased fruit losses after four weeks of storage by up to 45 per cent when compared to the controls.

BLUNDEN, JONES and PASSAM (1978) investigated the effects of post-harvest dipping of fruits in seaweed extract solutions. They found no significant effect on ripening time as a result of dipping *Solanum melongeria* L. (aubergines), *Persia gratissima* GAERTN. (avocado) and *Pyrus communis* L. (pear). However, *Musa acuminata* and *Mangifera indica* L. (mango) fruits showed a significant increase in the rate of ripening after being dipped in diluted seaweed extract. In trials conducted on *Citrus aurantiifolia* there was a significant reduction in the rate of "degreening" in fruits treated with seaweed extract.

Increased nutrient uptake by plants treated with seaweed concentrate was first reported by AITKEN and SENN (1965). They found in trials conducted on *Cucumis melo* L. (melon) that seaweed extract application increased the uptake of magnesium, nitrogen and calcium. In two commerical trials conducted by BOOTH (1966), seaweed extract increased the nitrogen, phosphorus, potassium, calcium, magnesium and iron content of *Solanum tuberosum* plants and the sugar-content of *Beta vulgaris* (sugar beet). BLUNDEN (1977) in trials conducted on various

pastures found that seaweed extract had no effect on fresh or dry mass production. However the crude protien content of the treated plants was higher than that of the controls.

Increased frost resistance of plants treated with seaweed extracts has been reported by SENN, MARTIN, CRAWFORD and DERTING (1961). They found, that *Lycopersicon esculentum* plants treated with seaweed extract withstood temperatures of 29°F which resulted in the death of untreated controls. In another trial, they found that while the first autumnal frost killed the untreated controls, the seaweed treated plants survived.

Increased resistance of plants to fungal disease was reported by SENN, MARTIN, CRAWFORD and DERTING (1961) who observed the mildew infestation on *Cucumis melo* plants was reduced with the application of a seaweed extract. BOOTH (1966) reported similar results with *Brassica campestris* L. (turnip) as well as a 50 per cent reduction in damping off in *Lycopersicon esculentum* plants and a reduction in *Botrytis* infection in *Fragaria x ananassa*. DRIGGERS and MARUCCI (1964) and AITKEN and SENN (1965) found that seaweed extract reduced the incidence of brown rot in *Prunus persica*. STEPHENSON (1966) confirmed the findings of BOOTH (1966) in trials conducted on both *Brassica campestris* and *Fragaria x ananassa*. She found that seaweed extract application reduced the incidence of powdery mildew on *Brassica campestris* from 85 per cent of control plants to only 15 per cent of treated plants. In trials conducted on *Fragaria x ananassa* 1,7 per cent of the fruit on treated plants became infected with *Botrytis* compared to 12,9 per cent of the fruit on the untreated plants.

Reduced incidence of insect attack on plants treated with seaweed extracts has been observed on numerous occasions in the field. There is however, very little scientific evidence available supporting these observations. BOOTH (1964)

showed that aphid reproduction was reduced on seaweed treated plants. It was suggested that behavioural, rather than nutritional, factors were responsible for the lowered rate of reproduction. STEPHENSON (1966) in trials conducted on *Beta vulgaris* (sugar beet), found that on the leaves of plants treated with seaweed extract there was a marked reduction in aphid numbers when compared to untreated control plants. In experiments conducted on *Malus pumila* trees the occurence of red spider mite was reduced with seaweed application and in trials conducted on *Chrysanthemum* L. plants both aphid and red spider mite populations were significantly reduced with seaweed extract application.

BUTTON and NOYES (1964) established that the percentage germination of *Festica rubra* L. (red fiscue) could be increased by soaking the seed in solutions of seaweed extract. They reported that drying the seed after soaking in seaweed extract negated the beneficial effects. AITKEN and SENN (1965) in trials conducted on the seeds of various species found that seaweed treatment resulted in an increase in the respiratory rate and germination percentage of treated seeds. At high concentrations of seaweed extract germination was found to be inhibited. The optimum concentration was found to vary between species. SENN and SKELTON (1969) confirmed these findings. GOH (1971) reported that seaweed extract stimulated the rate of germination of *Trifolium repens* L. (white clover) seed in a mineral pasture soil of low fertility.

It seems reasonable to conclude that seaweed extracts do have some beneficial effects on plant growth and could conceivably play a useful role in horticulture and agriculture in the future. The active compound/s are as yet unknown, although cytokinins seem to be implicated in some of the responses of plants to seaweed extracts. Consideration must, however also be given to other compounds present in the seaweed extracts as some evidence suggests, that these may also have a role to play.

Cytokinins – Isolation, identification, biosynthesis and metabolism

A cytokinin may be defined as a N^6- substituted adenine, which can be extracted from plant tissues, which promotes growth in cultured callus cells, or is responsible for a particular biological response within plant tissues (VAN STADEN and DAVEY, 1979).

The existence of cytokinins, which were originally regarded as specific cell division compounds, was recognised 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, VONSALTZA and STRONG, 1955; 1956). Kinetin was originally extracted from autoclaved herring sperm and was termed kinetin because of its ability to bring about cytokinesis (MILLER, 1961). Kinetin, however, is not regarded as a natural compound, but rather as an experimental artifact. LETHAM (1963) isolated the first naturally occurring cytokinin, 6-(4- hydroxy – 3-methyl -*trans* -2-butenyl-amino) purine (zeatin) from immature *Zea mays*. Since the isolation of zeatin, many other naturally occurring cytokinins, including ribosylzeatin, dihydrozeatin, dihydroribosylzeatin and their glucosylated derivatives have been isolated from most higher plant organs and tissues, bacteria, fungi and algae, as well as some insects (LETHAM, 1978). Although the level or site at which cytokinins are active within the cell is not known, they appear to exert their effect on plant metabolism as mediators, promotors or inhibitors of growth at a level close to, although not necessarily at the genome (BURROWS, 1975). It is generally accepted that cytokinins are involved in cell division (MILLER, 1961; FOSKET, VOLK and GOLDSMITH, 1977); that they can retard senescence (RICHMOND and LANG, 1957); or that they bring about nutrient mobilization within plant tissues (MOTHES, ENGELBRECHT and KULAJEWA,

1959; MOTHES and ENGELBRECHT, 1961; MOTHES, ENGELBRECHT and SCHUTTE, 1961).

Techniques for the isolation, purification and identification of cytokinins have markedly improved in recent years. It appears that bioassays will remain an integral part of the assessment and identification process although numerous reports in the literature state that the technique is inaccurate and an approximation (LETHAM, 1967a, 1978; DEKHUIJZEN and GEVERS, 1975; HORGAN, PALNI, SCOTT and MCGAW, 1981). The physical techniques of mass spectrometry, gas liquid chromatography and high-pressure liquid chromatogrpahy (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 regarded as more accurate methods of cytokinin identification. Although these sensitive techniques are essential tools in any modern day hormonal analysis, the bioassays will always be an important and fundamental part of cytokinin research, essential in determining the biological activity of separated compounds. Of the many bioassays available, those using callus derived from, the stem pith of tobacco (*Nicotiana tabacum* L. cv Wisconsin 38) (MURASHIGE and SKOOG, 1962) and the cotyledon of soybean (*Glycine max* L. cv. Acme) (MILLER, 1963, 1965) remain the most reliable and specific (LETHAM, 1967 a, b). Other cytokinin bioassays which are in use include, the carrot phloem (*Daucus corota* L.) tissue culture bioassay (LETHAM, 1967b), the oat leaf senescence (*Avena sativa* L.) bioassay (VARGA and BRUINSMA, 1973) as well as the *Amaranthus caudatus* L. (amaranthus) betacyanin (BIDDINGTON and THOMAS, 1973), lettuce (*Lactuca sativa* L.) seed germination (MILLER, 1958) and the soybean hypocotyl (MANOS and GOLDTHWAITE, 1975) assays. Although tissue culture bioassays are time consuming (21 - 28 days) and there is the risk of high rates of contamination, they are considered to be the most sensitive and reliable of the cytokinin bioassays because of their specificity, their wide linear response range

and that microbial growth is eliminated. An added advantage is that no endogenous cytokinins have been detected in soybean callus maintained on kinetin (VAN STADEN and DAVEY, 1977).

Cytokinins occur in plants as both free cytokinins and as bases adjacent to the anticodon triplet in specific tRNA species. The latter type occurs in tRNA of micro-organisms, plants and animals, while free cytokinins appear to be restricted to higher plants (CHEN and MELITZ, 1979). 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 (MURAI and HALL, 1973; BURROWS, 1975) as it has been established that isopentenyladenosine is only active once it has been released from tRNA. These differences between tRNA and the free cytokinins suggests that they arise from different biosynthetic pathways and that they have different functions.

The cytokinin isopentenyladenosine, was identified as a constitutent of tRNA in brewers yeast (ZACHAU, DUTTING and FELDMAN 1966). HALL, CSONKA, DAVID and MCLENNAN (1967) identified isopentenyladenosine in crude yeast and calf liver tRNA, which resulted in DNA and RNA being regarded as possible sources of cytokinins. Other cytokinins which have been extracted from tRNA include isopentenyladenine, 2- methylthio – isopentenyladenosine, 2- methylthio – isopentenyladenine and *cis*-ribosylzeatin (MURAI, ARMSTRONG and SKOOG, 1975; BURROWS, 1978a). The biosynthesis of tRNA cytokinins takes place at the polymer level during post-transcriptional processing (LETHAM and PALNI, 1983). Δ^2-Isopentenyl pyrophosphate is the immediate precursor of the Δ^2-isopentenyl sidechain of N^6-(Δ^2-isopentenyl) adenosine in tRNA. N^6-(Δ^2-isopentenyl) adenosine then serves as the substrate for further oxidation and substitution (BURROWS, 1978a). Mevalonic acid serves as the precursor for

isopentenyl pyrophosphate in tRNA.

In contrast to our understanding of the biosynthesis of tRNA cytokinins, very limited progress has been made in elucidating the mechanisms and rate of biosynthesis of free cytokinins. This lack of knowledge is as a result of the extremely low levels of endogenous cytokinins in plant tissue and the central role of the most likely precursors (adenine, its nucleoside or nucleotides) in cellular metabolism (LETHAM and PALNI, 1983). There exists two schools of thought as to the synthesis of free cytokinins. BURROWS (1978a, b); STUCHBURY, PALNI, HORGAN and WAREING (1979); NISHINARI and SYONO (1980a, b) and CHEN (1981); advocate the *de novo* biosynthesis of free cytokinins from adenine monomers. Whereas KLEMEN and KLÄMBT (1974); MAAS and KLÄMBT (1981a, b) and HELBACH and KLÄMBT (1981) favour the release of free cytokinins as intact by-products of tRNA degradation. It has been suggested (SHELDRAKE, 1973) that the hydrolysis of tRNA to mononucleotides may result in the release of free cytokinins in dying, autolyzing cells of differentiating vascular tissue. HAHN, HEITMAN and BLUMBACH (1976) calculated that sufficient cytokinin was released from tRNA in the bacterium, *Agrobacterium tumefaciens* (SMITH and TOWNSHEND) CONN. to account for all the free cytokinins detected. Similarly a hypothesis has been advanced that free cytokinin production could be accounted for by the turnover of cytokinin containing tRNAs in *Lactobacillus acidophilus* (KLEMEN and KLÄMBT, 1974) and primary roots of *Zea mays* (LEINEWEBER and KLÄMBT, 1974) and by degradation of oligonucleotides in intact *Phaseolus vulgaris* L. (bean) roots (MAAS and KLÄMBT, 1981a).

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 unlikely to be such that it would supply the cytokinin levels detected in tissues (SHORT and TORREY, 1972; MUIRA and HALL, 1973;

LETHAM, 1978; STUCHBURY, PALNI, HORGAN and WAREING, 1979). SHORT
and TORREY (1972) found that the activity of free cytokinins in pea roots is 27
times the cytokinin activity in tRNA, so unless a very rapid turnover of tRNA
occurs in meristematic tissue, these results indicate that tRNA is not the principal
source of free cytokinins. Further evidence in support of an independant
biosynthetic pathway of free cytokinins was presented by BURROWS and FEUL
(1981) who suggested that the tRNA contribution to free cytokinins is not likely
to exceed two per cent in *Solanum tuberosum* cells. BARNES, TIEN and GRAY
(1980) stated that tRNA degradation was 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 released by tRNA is only a small
fraction of the total. Conclusive evidence for the *de novo* synthesis of free
cytokinins was provided by CHEN and ECKERT (1976); BURROWS (1978b);
NISHINARI and SYONO (1980a, b); HORGAN, PALNI, SCOTT and MCGAW (1981),
who demonstrated the incorporation of labelled adenine and adenosine into
cytokinins of the *trans* form, that is, free cytokinins. CHEN and MELITZ (1979)
isolated an enzyme from a higher plant which was capable of catalyzing the
synthesis of cytokinin nucleotides thus demonstrating an independent synthetic
pathway to that of tRNA degradation.

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 metabolised in three 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 glucosylation (O-glucosides) (PARKER, LETHAM, WILSON, JENKINS, MACLEOD and SUMMONS, 1975), reduction (dihydrozeatin) (LETHAM, PARKER, DUKE, SUMMONS and MACLEOD, 1976) and oxidation (6-(2,3,4-trihydroxy- 3-methylbutylamino) purine) (VAN STADEN, DREWES and HUTTON, 1982) and

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

Cytokinins in marine algae

The occurrence of cytokinins in marine algae has been the subject of several studies. These hormones have been detected in extracts of the unicellular marine algae *Gymnodininum splendens* LEBOUR. and *Phoeodactylum tricornutum* BOHLIN. (BENTLY-MOWAT and REID, 1968). The cytokinins were extracted and purified (LOEFFER and VAN OVERBEEK, 1964) and the activity of the cytokinin-like compounds estimated by means of the radish leaf bioassay (KURAISHI, 1959). Results showed variations from 0,1 to 10 µg Kg^{-1} of fresh algal material. HUSSAIN and BONEY (1969) demonstrated the presence of a single cytokinin-like substance in the stipe and two in the holdfast of the macroscopic brown algae *Laminaria digitata* (HUDS.) LAMOUR. The cytokinins were extracted in methanol, purified on Dowex 50 cation exchange resin and separated using paper chromatography. Cytokinin-like activity was estimated using the radish leaf senescence bioassay (KURAISHI, 1959). HUSSAIN and BONEY (1971) demonstrated the presence of cytokinins in the microscopic algae *Cricosphaera elongata* (DROOP) BRAARUD and *Cricosphaera carterae* (BRAARUD and FAGERLAND) BRAARUD. using the oat leaf senescence bioassay. The level of cytokinins in cells in the motile phase were found to be present in larger quantities than in the non-motile forms. JENNINGS (1969) detected the presence of cytokinins in two marine algae. The extraction procedure was based on methods used by LETHAM and BOLLARD (1961) for the detection of cytokinins in the fruits of some higher plants, and which led to the isolation of zeatin by LETHAM (1963). The activity of the cytokinin-like compounds was estimated by means of the barley leaf senescence bioassay (KENDE, 1964). It was found that *Ecklonia radiata* (TURN) J. AGARDH. (Phaeophyta) appeared to contain two free cytokinins and *Hypnea musciformis* (WULFEN) LAMOUR. (Rhodophyta) to have at least one. The cytokinins resembled those found in higher plants in being basic and polar, as indicated by their partitioning properties in ethyl acetate and their ion exchange and chromatographic

properties. AUGIER (1972, 1974) and AUGIER and HARADA (1972, 1973) detected the presence of cytokinins in a number of marine algae using various techniques. Cytokinins were extracted in methanol purified on a Sephadex LH-20 column eluted with 35 per cent ethanol and separated using thin layer chromatography. Cytokinin-like activity was estimated using the tobacco callus and oat leaf senescence bioassays. Utilization of the above techniques resulted in the detection of cytokinin-like substances in the algae, *Enteromorpha linza* (L.) J. AGARDH. (Chlorophyceae), *Cystoseira discors* C. AGARDH. (Phaeophyceae) and *Gymnogongrus norvegicus* (GUNN) J. AGARDH. (Rhodophyceae). VAN STADEN and BREEN (1973) established the presence of cytokinins in three species of colonial green algae. Cytokinin-like compounds were extracted from *Pandorina morum* BORY., *Eudorina elegans* EHRENBERG and *Volvox carteri* STEIN in 80 per cent ethanol, separated on Whatman No. 1 chromatography paper and assayed by means of the soybean callus bioassay.

PEDERSEN and FRIDBORG (1972) demonstrated the presence of cytokinin-like activity in sea water taken from the Fucus-Ascophyllum zone and PEDERSEN (1973) using combined gas chromatography and mass spectrometry quantitatively estimated and identified the cytokinin present in sea water in the Fucus-Ascophyllum zone as 6-(3 methyl -2- butenylamino) purine. KENTZER, SYNAK, BURKIEWICZ and BANAS (1980) demonstrated cytokinin-like activity in sea water taken from the *Fucus vesiculosus* L. zone. The cytokinin-like substance showed properties typical of 6-(3 methyl -2- butenylamino) purine and was also present in extracts taken from the thallus of *Fucus visiculosis*.

Cytokinins and algal growth

Exogenous applications of kinetin at varying concentrations has been found to either promote or inhibit cell division and elongation of a wide spectrum of both multicellular and unicellular algae.

The first report of increased algal growth with the exogenous application of kinetin was presented by MOEWUS (1959a, b). Kinetin at the concentration 0,06 mg ℓ^{-1} was found to increase the level of mitotic activity in the algae *Acetabularia mediterrania* LAMOUR.

Since 1955, there have been numerous reports of kinetin stimulating algal growth in members of the Chlorophyceae viz. *Chlorella pyrenoides* CHICK and *Oedogonium cardiacum* (HASS) WITTR. (KIM 1961; 1962); *Acetabularia mediterrania* LAMOUR (DE VITRY, 1962); *Acetabularia crenulata* LAMOUR (SPENCER, 1968); *Caulerpa prolifera* (FORSSK.) LAMOUR and *Fritschiella tuberosa* IYENG (MELKONIAN and WEBER, 1975); Ulvaphyceae (ROBERTS, SLUIMAN, STEWART and MATTOX, 1981) viz. *Ulva lactuca* L. (PROVASOLI, 1957; 1958a, b), *Ulva rigida* C. AGARDH (AUGIER, 1972); *Enteromorpha compressa* (L.) GREV. (AUGIER, 1972); Euglenophyceae viz. *Euglena gracilis* KLEBS (SUPNIEWSKI, KRUPINSKA, SUPNIEWSKA and WACLAW, 1957); Xanthophyceae viz. *Vaucheri sessilus* (VAUCH.) DE CAND. (KIM, 1961; 1962); Cyanophyceae viz. *Anabaena variabilis* KÜTZ (KIM, 1961; 1962;); *Nostoc punctiforme* (KÜTZ.) HARIOT (FERNANDEZ, BALLONI and MATERASSI, 1968); and *Anacystis nidulans* (LYNGB.) DROUET and DAILY (AHMAD and WINTER, 1968); Charophyceae viz. *Spirogyra longata* VAUCHER (OLSZEWSKA, 1958); and *Chara zeylanica* (WILLD.) KL. (IMAHORI and IWASA, 1965); Eustigmatophyceae (ANTIA, BISALPUTRA, CHENG and KALLEY, 1975) viz. *Nannochlorus oculata* DROOP (BENTLEY-MOWAT, 1967). In the Phaeophyte, kinetin was found to stimulate the growth of *Pilayella littoralis*

(L.) KJELLM. and *Ectocarpus fasiculatus* HARV. (PEDERSEN, 1968; FRIES, 1971);
and *Stypocaulon scroparium* KÜTZ. (AUGIER, 1972).

Kinetin applications have been found to inhibit growth and development
in members of the Chlorophyceae viz. *Chlorella vulgaris* BEIJ, *Protococcus viridus*
C. AGARDH, *Scendesmus obliquus* (TURP.) KÜTZ and species of the genus
Chlorococcum MENEGHINI (TAMIYA and MORIMURA, 1960; FERNANDEZ,
BALLONI and MATERASSI, 1968); *Caulerpa prolifera* (FORSSK.) LAMOUR.
(AUGIER, 1972); and *Bryopsis mucosa* LAMOUR. (AUGIER, 1972); Ulvaphyceae
viz. *Enteromorpha compressa* (L.) GREV (AUGIER, 1972); Cyanophyceae viz.
Chlorogloea fritschii MITRA (AHMAD and WINTER, 1968); Charophyceae viz.
Nitella hookeri A.BR. (STARLING, CHAPMAN and BROWN, 1974); Rhodophyceae
viz. *Petroglossum nicaeense* DUBY. (PERRONI and FELICINI, 1974). Amongst
the Phaeophyceae tested only *Lithosiphin pusillus* HARV. was found to show a
negative response to kinetin application (STARLING, CHAPMAN and BROWN,
1974).

Cytokinins in extracts of marine algae

There has been much speculation as to the amount and type of growth regulating substances, especially plant growth hormones, which exist in seaweed materials used as plant growth supplements (KINGMAN and MOORE, 1982). This speculation arose due to the fact that when bioassays were performed to determine the presence of plant hormone-like substances, positive responses from seaweed materials were obtained. In recent years it has been hypothesized that the presence of growth regulators in seaweed products which have been applied to the planting media or as foliar applications to crops, might play an important role in certain plant physiological responses to the seaweed extract. BRAIN, CHALOPIN, TURNER, BLUNDEN and WILDGOOSE (1973) demonstrated the presence of cytokinin-like compounds in a commercial aqueous seaweed extract (S.M.3.) prepared from species of Laminariaceae and Fucaceae. Cytokinins were detected by means of the promotion of growth in vivo of carrot explants (LETHAM, 1967b) and by the radish leaf disc expansion assay (KURAISHI, 1959). BLUNDEN and WILDGOOSE (1977) using the radish leaf bioassay found the cytokinin-like activity of the seaweed extract (S.M.3.) to be equivalent to 125 mg ℓ^{-1} kinetin in aqueous solution. In 1978, BLUNDEN, JONES and PASSAM detected the presence of cytokinin-like sustances in three commercial seaweed extracts (S.M.3.; Marinure; Algistim). KINGMAN and MOORE (1982) revealed the presence of cytokinin-like compounds in extracts of a commercial preparation of Ascophyllum nodosum after extraction in ethyl acetate followed by gas liquid chromatography (G.L.C.).

Ecklonia maxima (Osbeck) Papenf.   Distribution and Life Cycle

Ecklonia maxima a member of the Alariaceae (Laminariales) was known for a long time as *Ecklonia buccinalis*. In 1940, however, PAPENFUSS established that the specific name *maxima* had priority.

The sporophyte of *Ecklonia maxima* is locally abundant between Cape Agulhas, the southern most point of Africa, and somewhere on the Namibian coast, in all about 1 200 kilometres of shore (SIMONS and JARMAN, 1981). This distribution is not continuous however, for the species is absent, or is represented by occasional stunted individuals, along the northern and the greater part of the western shores of False bay. The absence or scarcity of *Ecklonia maxima* in this region is owing no doubt to sea temperatures which prevail there during the summer months. ISAAC (1938) who studied the temperature conditions of the South African coastal waters, found that for normal growth of *Ecklonia maxima* the mean annual temperature must not exceed 14,6°C. The high temperature conditions that eliminate *Ecklonia maxima* from the flora of the northern and western parts of False bay are apparently less marked or are not attained along the central and southern parts of the eastern shore of this bay. This enables the species to reappear at certain sites along this coast, starting at the mouth of the Steenbras river, and extending eastwards beyond False bay as far as Papenkuilsfontein near Cape Agulhas (PAPENFUSS, 1942). Where *Ecklonia maxima* does not dominate it's niche it is replaced by *Laminaria pallida* (GREVILLE) J. AGARDH on the Cape peninsula and eastwards, and by *Laminaria schinzii* FOSLIE along the west coast (SIMONS and JARMAN, 1981).

Sporophyte development of *Ecklonia maxima* begins with fertilization of the egg. A wall then forms about the zygote and elongation occurs with the zygote ultimately dividing transversely. Further divisions take place in transverse

and longitudinal planes giving rise to a somewhat elongated monostromatic (thallus composed of a single layer of cells) sporophyte. The rhizoids which develop are nonseptate and usually unbranched. The young sporophyte becomes distromatic at a comparatively early stage. The first cells to divide periclinally are those in the basal region, the part destined to form the stipe.

In the juvenile state, the thallus of *Ecklonia maxima* consists of a simple, somewhat elongated, blade borne on a stipe. In slightly older stages pinnae develop along both margins of the blade. (Figure 1.1). The pinnae are initiated in the meristematic region at the base of primary blade and are progressively shifted upward by elongation of the primary blade. Later the distal portion of the primary blade is worn away and the pinnae become aggregated in a dense cluster immediately above the transition region, the older pinnae continually wearing away and new ones being initiated. In older plants, the stipe becomes hollow and its terminal portion becomes inflated, forming a large float. Mature plants may attain a length of seven meters or more from the base of the stipe to the tips of the longest pinnae. (Figure 1.2).

In 1977 the State, for the first time granted permission for the direct exploitation of the standing stock of *Ecklonia maxima* beds off Kommetjie on the Cape west coast. This concession was granted to Kelp Products (Pty) Limited, South Africa for the processing of fresh seaweed into a concentrate (Kelpak 66) for use in agriculture and horticulture as a plant growth stimulant.

As a result of the possible impact exploitation of this natural resource could have on the environment, SIMONS and JARMAN (1981) initiated a survey into the exploitability of the kelp beds. Reproductive sporophylls were found on *Ecklonia maxima* throughout the study period, thus expectations of *de novo* recolonization were, at all times, high. It was found, however, that cyclic

Figure 1.1   (a)   Juvenile   thallus   of   *Ecklonia   maxima*
consisting of a simple elongated blade borne
on a stipe.

(b)   Older plant with pinnae developing along both
margins of the blade.

(a)   x 1

(b)   x 1
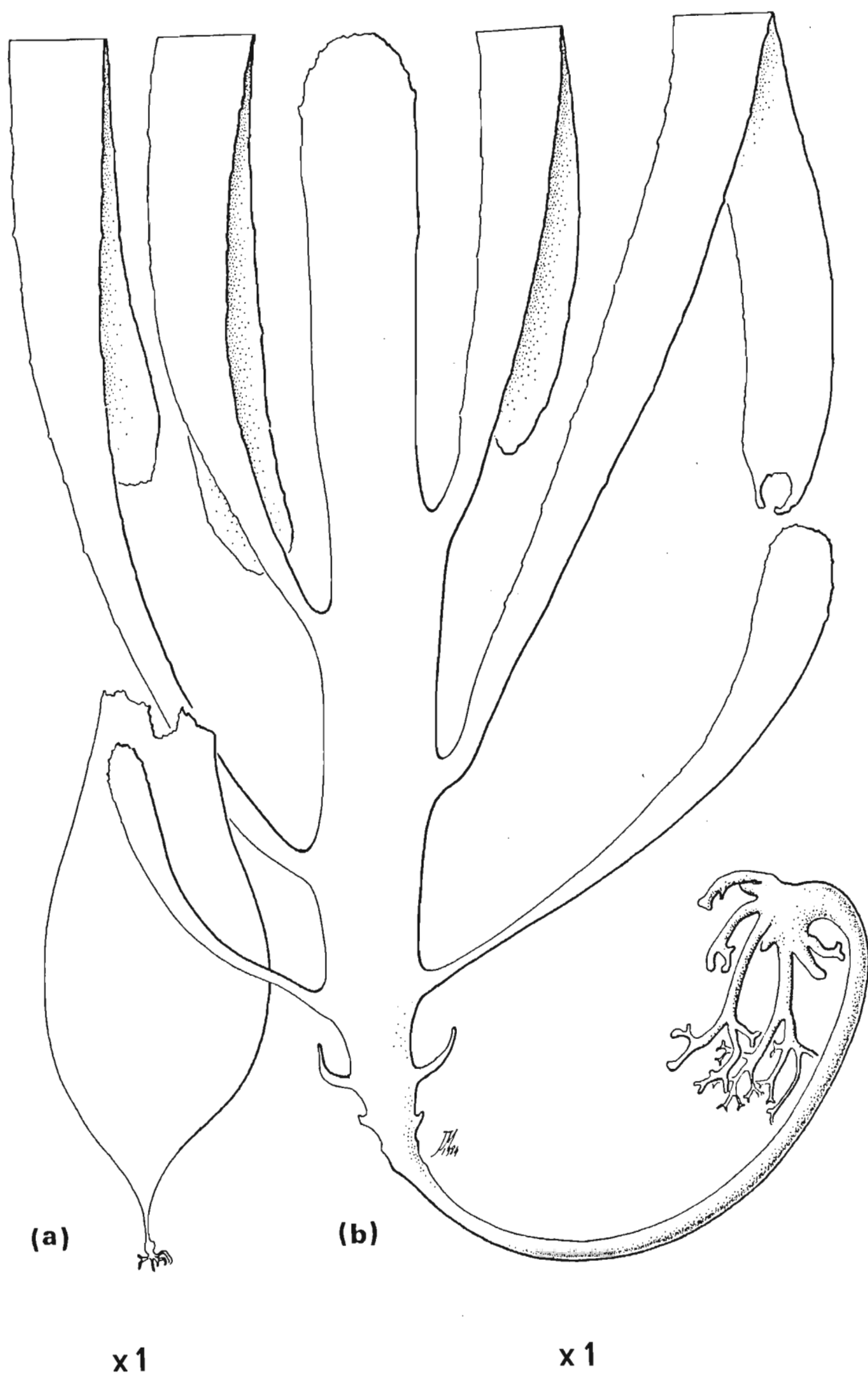
Figure 1.2   An adult plant of *Ecklonia maxima* to illustrate overall
morphology.

LAMINA

HOLDFAST

STIPE
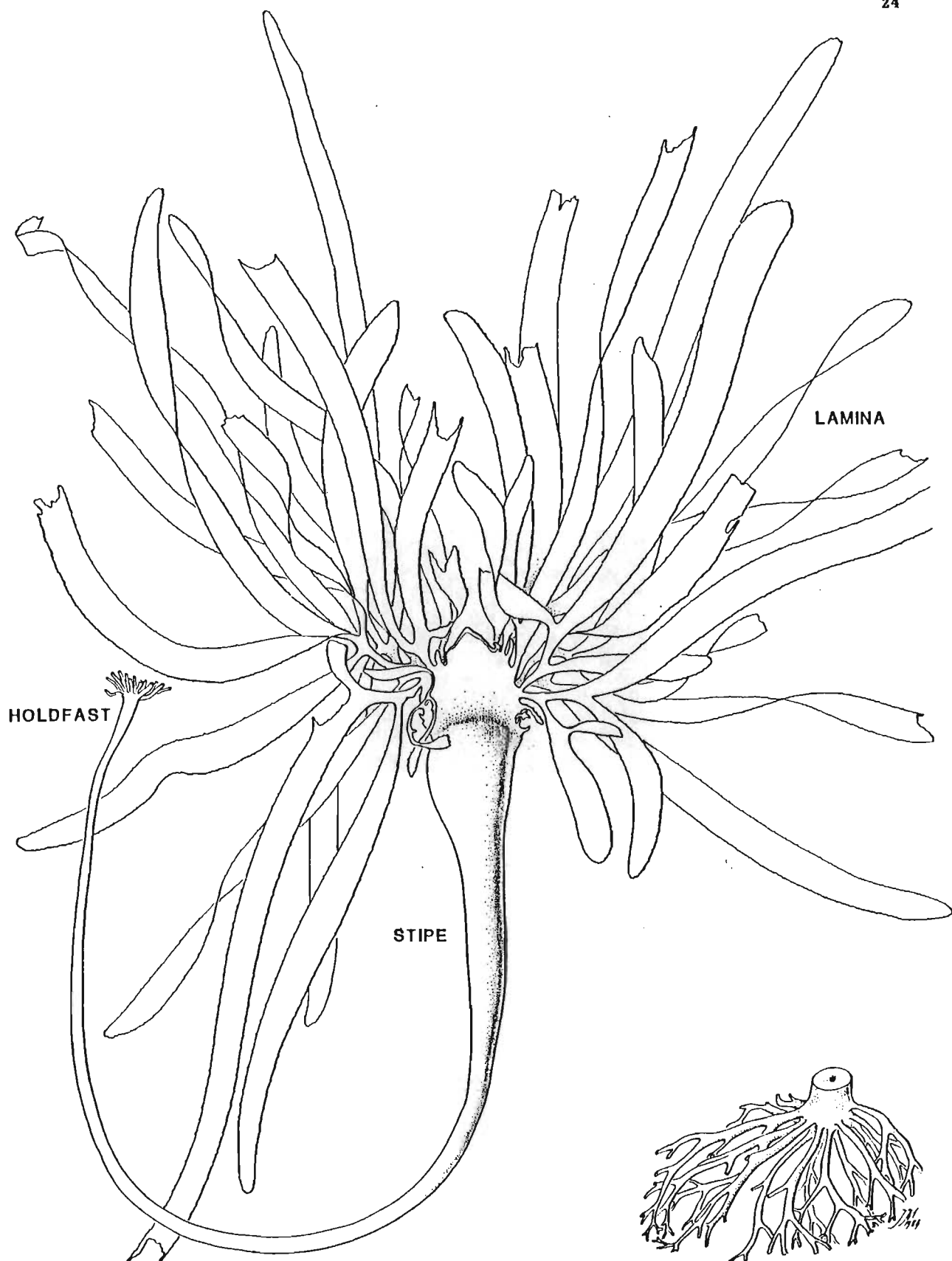
x 0,05
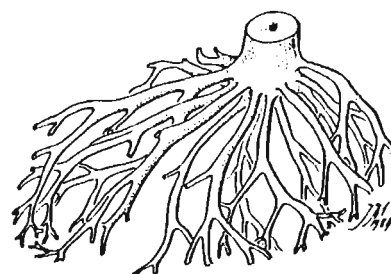
x 0,2

reproduction in a harvested area proved of less importance to the recovery of the kelp-stock than did a remnant stand of young subcanopy sporophytes, for it was found that non-kelp species dominated on completely cleaned surfaces for at least 12 months, while good kelp-recruitment followed when plants with stipes of 250 millimetres or less were left intact.

Maximum commercial harvest in this project was 3 000 tons (fresh mass) per annum of *Ecklonia maxima* stipes, at which rate the 250 hectare *Ecklonia maxima* field at Kommetjie would be worked once ever 5,5 years. This would allow sufficient scope for the 3 year cropping rotation scheme. SIMONS and JARMAN (1981) concluded that although the cropping periodicity may result in a 12 per cent annual production loss, this would not effect the environment. At present Kelp Products (Pty) Limited is harvesting two tons of *Ecklonia maxima* per day from a standing stock of about five to ten kilograms metre^{-2}.

The seaweed concentrate (Kelpak 66) manufactured by Kelp Products, is prepared from the stipes of *Ecklonia maxima* by a cell burst process which does not involve the use of heat, chemicals or dehydration which could denature certain of the valuable components present in the seaweed. Selected *Ecklonia maxima* plants are harvested at low tide by excising the plant at the base of the stipe above the holdfast. The excised plants are then allowed to wash up onto the shore from where they are collected and transported to the factory where they are hand washed. The stipes are then progressively reduced in particle size. These reduced particles finally pass under extremely high pressure into a low pressure chamber wherein the energy introduced into the particles is released causing them to shear and disintegrate. The diluted concentrate (40 : 60 per cent, seaweed : water) is then bottled and marketed under the name Kelpak 66[R].

CHAPTER II

MATERIALS AND METHODS

General

The seasonal variation in both the quantity and quality of cytokinins present in fresh and processed *Ecklonia maxima* (OSBECK) PAPENF. material was examined over a period of 12 months from February 1981 until January 1982. In general terms it can be said that plant material was extracted for cytokinins to determine the amount and type of compounds present. After paper and column chromatography, the extracts were subjected to the soybean callus bioassay in order to determine the cytokinin-like activity associated with the extract. It must be noted that although extracts were subjected to High Performance Liquid Chromatography (H.P.L.C), and co-elution with authentic cytokinin markers was achieved, identification can only be regarded as tentative, as mass spectrometric techniques were not employed, such facilities not being available.

In addition to cytokinin extraction and identification, processed *Ecklonia maxima* (Kelpak 66) was applied to various horticultural and agricultural crops in order to determine its effect on plant growth and development. For these studies Kelpak 66 was used at a dilution of 1:500 throughout, either on its own or together with a liquid fertilizer.

Harvesting of plant material

Plants of *Ecklonia maxima* (OSBECK) PAPENF. used in the seasonal

study were harvested at low tide during the first week of each month for the period February 1981 until January 1982. Plants of three different size classes, that is, one, two and three metre high plants were collected. As far as possible plants of similar size and age were included in each group. The plants were immediately frozen and transported by air to Pietermaritzburg. In the laboratory plants were divided into three parts : lamina, stipe and holdfast (Figure 2.1). Twenty gramme samples were taken from each plant part and these were then stored at -20°C until required for analysis.

All plant material harvested for the lunar cycle study was harvested at noon, over the period 12 April 1983 until 12 May 1983 as indicated in Table 2.1. Whole plants were removed, deep frozen and transported to Pietermaritzburg. In the laboratory plants were divided into lamina, stipe and holdfast sections, 20 gramme samples from each section were then stored at -20°C until required for analysis.

Material for use in the seasonal study of processed seaweed concentrate was collected at monthly intervals from February 1981 to January 1982, and a five kilogramme sample collected in November 1982 was used for the identification study. After harvesting the material was processed and deep frozen until required for analysis.

Figure 2.1   An adult plant of *Ecklonia maxima* to illustrate overall
             morphology. H, holdfast; S, stipe; L, lamina.

LAMINA
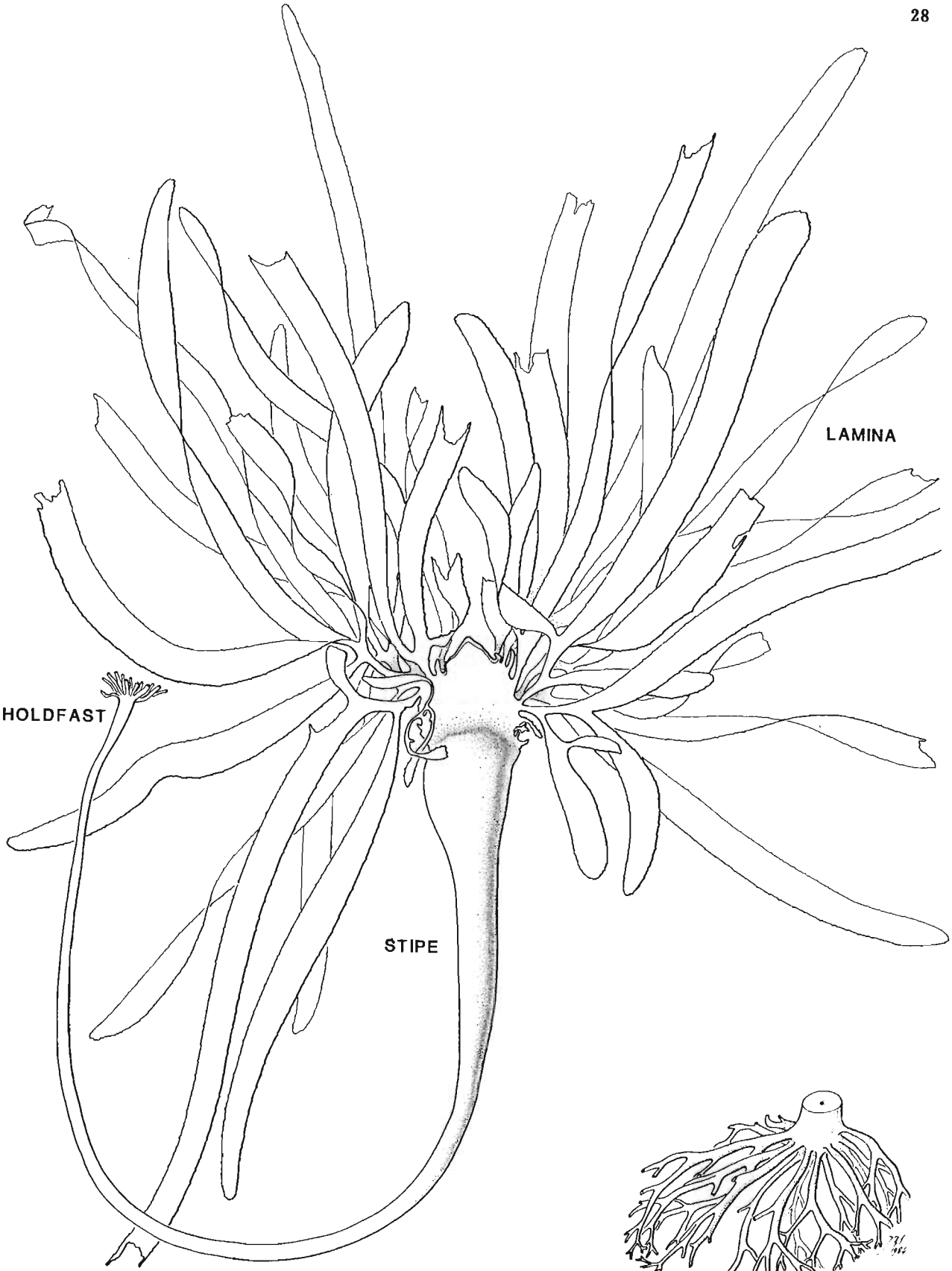
HOLDFAST

STIPE

x 0,05
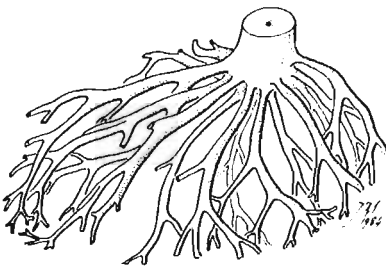
x 0,2

Table 2.1 Material of *Ecklonia maxima* harvested for the lunar
cycle over the period 12 April 1983 until 12 May 1983.
All material was collected at 12 noon.

Plant part collected

Date of Collection	Lamina	Stipe	Holdfast
12 April	x	x	x
13 April	x	x	x
14 April	x	x	x
17 April	x	x	x
18 April	x	x	–
20 April	x	x	x
21 April	x	x	x
23 April	x	x	x
26 April	x	x	x
27 April	x	x	x
28 April	x	x	x
1 May	x	x	–
4 May	x	x	x
5 May	x	x	x
8 May	x	x	x
10 May	x	x	x
12 May	x	x	x

Extraction and purification of plant material for cytokinins

Cytokinins were extracted from the plant material by homogenising 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 100 millilitres of 80 per cent ethanol and the pH of the ethanolic extracts was adjusted to 2,5 with dilute hydrochloric acid. Twenty grammes (gramme for gramme fresh mass) 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), were shaken in the extracts for one hour. The extracts were then passed through the exchange resin at a flow rate of one millilitre per minute. The columns were washed with 100 millilitres of 80 per cent ethanol. The ethanolic eluate and wash constituted the aqueous fraction. In those samples where the aqueous fraction was analysed, 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 three millilitres of 80 per cent ethanol and strip loaded onto Whatman No. 1 chromatography paper. The cytokinins were eluted off 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 three millilites of 80 per cent ethanol. These ethanolic fractions were then strip loaded onto Whatman No. 1 chromatography paper.

In order to tentatively identify the cytokinins present in *Ecklonia maxima*, five kilogrammes fresh mass of processed *Ecklonia maxima* stipes was extracted for 24h at 5°C in 5 000 millilitres of 80 per cent ethanol. The extract was then filtered through Whatman No. 1 filter paper and the residues washed

with a further 1 000 millilitres of 80 per cent ethanol. The combined ethanolic extracts were then taken to dryness *in vacuo* at 35°C and the residues resuspended in 500 millilitres of 80 per cent ethanol. The pH of the extract was adjusted to 2,5 with dilute hydrochloric acid. The acidified extract was then passed through 2,5 kilogrammes 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) at a flow rate of 50 millilitres per hour. The column was washed with a further 2 000 millilitres of 80 per cent ethanol. The combined eluate and the wash constituted the aqueous phase, the pH of which was adjusted to 7,0 with dilute sodium hydroxide, the fraction was then concentrated to dryness under vacuum. The residue was resuspended in 20 millilitres of 80 per cent ethanol and strip-loaded onto five sheets (46 x 57 cm) of Whatman No. 1 chromatography paper. The cytokinins were eluted from the cation exchange resin with 5 000 millilitres of 5N ammonium hydroxide. The ammonia eluates were concentrated to dryness *in vacuo* at 35°C and the residues taken up in 20 millilitres of 80 per cent ethanol and strip loaded onto five sheets of Whatman No. 1 chromatography paper (46 x 57cm). A portion (equivalent to 20 grammes fresh mass) of the chromatograms of both the aqueous and ammonia fractions were analysed for cytokinin activity using the soybean callus bioassay.

The technique of using cation exchange resin for the purification and recovery of cytokinins from ethanolic plant extracts has been employed extensively (ENGLEBRECHT, 1971; HEWETT and WAREING, 1973b; LORENZI, HORGAN and WAREING, 1975; VAN STADEN, 1976a, b; WANG, THOMSON and HORGAN, 1977). Most of the known cytokinins may be recovered from acidified plant extracts when Dowex 50W-X8 cation exchange resin is used (TEGELY, WITMAN and KRASNUK, 1971; DEKHUIJZEN and GEVERS, 1975). LETHAM (1978), however, suggested that there is a danger of hydrolyzing the active compounds using these resins and suggested that strongly acidic resins

were not necessary for cytokinin purification and that cellulose phosphate may be preferable. MILLER (1965), however, confirmed the validity of Dowex 50 and VAN STADEN (1976c) demonstrated that zeatin, ribosylzeatin and their glucosylated derivatives could be efficiently purified from plant material by means of Dowex 50 cation exchange resin.

Chromatographic Techniques

Paper Chromatography

Extracts were strip loaded, in a one centimetre strip, onto sheets of Whatman No. 1 chromatography paper. The chromatograms were then developed with *iso*-propanol: 25 per cent ammonium hydroxide: water (10:1:1 v/v) (P.A.W.) in a descending manner until the solvent front was approximately 30 centimetres from the origin. The chromatograms were then oven dryed at 30°C for 24 hours. The chromatograms were subsequently divided into ten equal Rf zones and stored at -20°C for further anaylsis. For estimation of cytokinin activity, the strip of chromatography paper corresponding to each Rf zone was placed into a 25 millilitre erlenmeyer flask. This was subsequently assayed for cell division activity using the soybean callus bioassay (MILLER, 1965).

Column Chromatography

Column chromatography was used to fractionate extracts so that the cytokinins could be tentatively identified on a basis of co-elution with authentic cytokinin markers. Column chromatographic techniques are most frequently used

to purify extracts prior to bioassay and the physical techniques of cytokinin identification such as high performance liquid chromatography, gas-liquid chromatography and mass spectrometry.

The technique used was based on that of ARMSTRONG, BURROWS, EVANS and SKOOG (1969). The column (90 x 2,5 centimetres) was packed with Sephadex LH-20 and eluted with 35 per cent ethanol at a flow rate of 15 millilitres an hour at 20°C. As can be seen from Figure 2.2, the authentic cytokinin markers, glucosylzeatin, ribosylzeatin, zeatin, isopentenyladenosine and isopentenyladenine can all be separated, with distinct troughs between them, using this system. This efficiency of separation was maintained when separating a plant extract. The histogram in Figure 2.3, represents the cytokinin activity detected by means of the soybean callus bioassay, in a stipe extract of *Ecklonia maxima* which, had been purified by means of Dowex 50 and paper chromatography, prior to being separated on a Sephadex LH-20 column eluted with 35 per cent ethanol.

Bioassays of paper chromatograms indicated the presence of active cell division components 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. The extract was then concentrated to near dryness and taken up in one millilitre of 35 per cent ethanol and applied to the column.

After elution through the columns, the fractions were combined in 40 millilitre fractions in erlenmeyer flasks. In extracts to be used in high performance liquid chromatography, an aliquot of each fraction was used for bioassay and the remainder was dried on a hot plate (30°C) in a stream of air and stored at -20°C until required for further analysis. In all other extracts, the 40 millilitre fractions were dried and assayed for cytokinin activity using the

Figure 2.2   The separation of authentic cytokinins achieved using

a Sephadex LH-20 column eluted with 35 per cent ethanol,

as detected by ultra violet absorbance (254 nanometres).

ZG = glucosylzeatin;   ZR = ribosylzeatin;   Z = zeatin;

IPA = isopentenyladenosine;   2iP = isopentenyladenine.
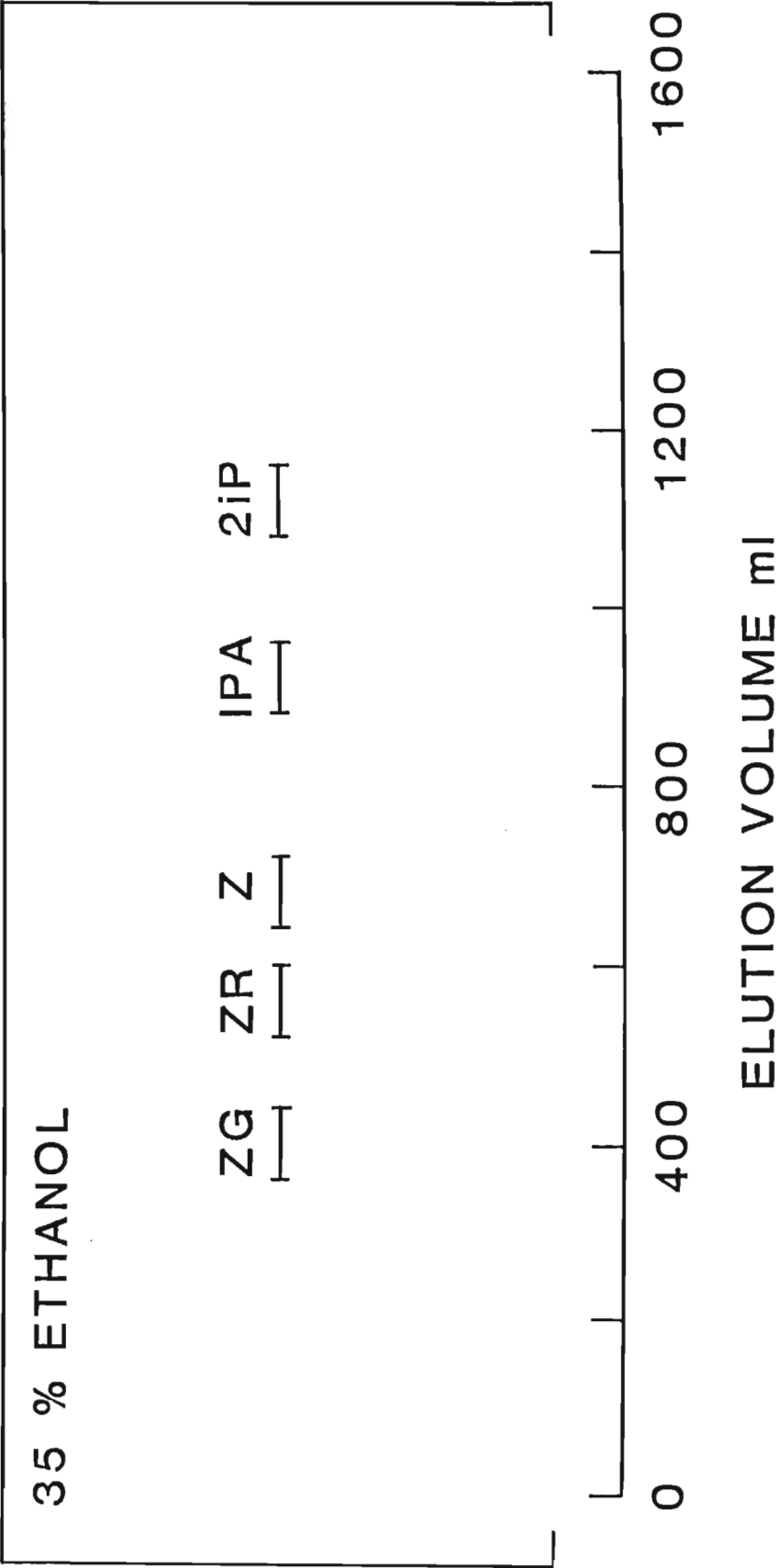
Figure 2.3   The cytokinin activity detected in stipe material of
Ecklonia maxima fractionated on a Sephadex LH-20
column eluted with 35 per cent ethanol. Prior to column
chromatography the extract was purified on paper. ZG
= glucosylzeatin; ZR = ribosylzeatin; Z = zeatin; 1PA
= isopentenyladenosine; 2iP = isopentenyladenine.

ELUTION VOLUME ml

CALLUS YIELD g flask^{-1}

KINETIN µgl^{-1}
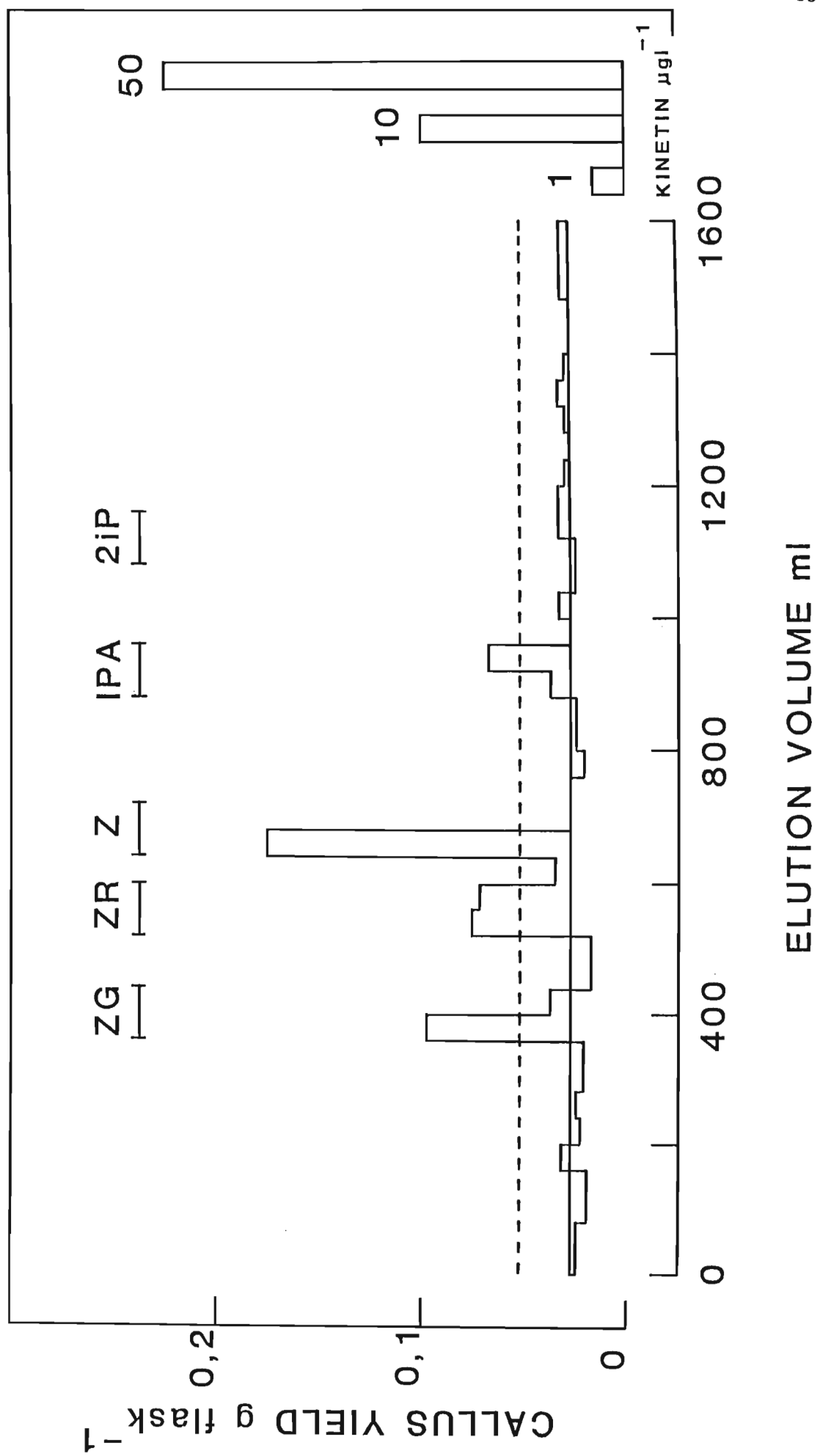
soybean callus bioassay (MILLER, 1963; 1965).

High Performance Liquid Chromatography

Separation of authentic cytokinins and cytokinin extracts was achieved by reversed phase high performance liquid chromatography (H.P.L.C.). The column used was a Hypersil 25 centimetre O.D.S. (5 micrometre, C18 bonded, 250 x 4 millimetre l.D.) with a flow rate of 1,5 millimetres per minute maintained by a 3 500 p.s.i. single piston reciprocating pump. Absorbance was recorded with a Varian variable wavelength monitor at 265 nanometres which was fitted with a 8 micrometre flow-through cell. Separation was achieved using a Varian 5 000 liquid chromatogram and the data output recorded using a Vista 4 000 data system. Partially purified extracts obtained after Sephadex LH-20 chromatography were redissolved in methanol (100 per cent) and 10 microlitre aliquots were injected into the chromatograph. At the start of the programme the mobile phase consisted of acetonitrile:water (10:90), this ran isocratically for 10 minutes, to 18 per cent acetonitrile in five minutes, isocratically for five minutes, to 20 per cent acetonitrile in five minutes. Aliquots of 1,5 millilitres from each sample run were collected, air dried, and then assayed for cell division activity.

In order to obtain the elution times of the various endogenous cytokinins occuring in seaweed, authentic cytokinin standards were run through the same column using the same programme.

Soybean Callus Bioassay

Tissue culture bioassays are regarded as being the most sensitive

of the cytokinin assays. The soybean *(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 in use (VAN STADEN and DAVEY, 1979). The advantages of this bioassay as previously mentioned are that microbial growth is eliminated and also that no natural cytokinins have been detected in soybean callus maintained on kinetin (VAN STADEN and DAVEY, 1977). The only disadvantage is that it is time consuming. This assay system was used to determine the cytokinin activity of fractions from paper, column and high performance liquid chromatography.

Callus was obtained from the cotyledons of soybeans according to the procedure described by MILLER (1963; 1965) and was maintained by three weekly subculture. Four stock solutions were prepared and the nutrient medium was made up as outlined in Table 2.2. Either 15 or 20 millilitres of medium was added to 25 or 50 millilitre erlenmeyer flasks which contained 0,15 and 0,30 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 1,05 bars for 20 minutes before being transferred to a sterile transfer chamber.

Once the agar had set, three pieces of soybean stock callus, each weighing approximately 10 milligrammes, were placed on the medium in each flask. The flasks were then incubated in a growth room where a constant termperature (25°C $\pm$ 2°C) and continuous low light intensity (cool white flourescent 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. Activity which was significantly different from the control at the 0,01 per cent level was calculated and is indicated on the histograms as a dotted line, while the control value is represented by a solid line. Kinetin

TABLE 2.2.

BASAL MEDIUM FOR SOYBEAN CALLUS BIOASSAY

(Adapted from MILLER, 1963; 1965)

Stock solution	Chemical	g ℓ^{-1} Stock solution	Millilitres of stock solution per litre of medium.
	$KH_2 PO_4$	3,0	
	KNO_3	10,0	
	$NH_4 NO_3$	10,0	
Stock 1	$Ca(NO_3)_2. 4H_2O$	5,0	100
	$MgSO_4. 7H_2O$	0,715	
	KCL	0,65	
	$MnSO_4. 4H_2O$	0,14	
	NaFeEDTA	1,32	
	$ZnSO_4. 7H_2O$	0,38	
Stock 2	H_3BO_3	0,16	10
	KI	0,08	
	$Cu(NO_3)_2. 3H_2O$	0,035	
	$(NH_4)_6 Mo_7 O_{24}. 4H_2O$	0,01	
	Myo-inositol	10,0	
	Nicotinic acid	0,2	
Stock 3	Pyridoxine HCL	0,08	10
	Thiamine HCL	0,08	
Stock 4	NAA	0,2	10
Additional	Sucrose		30 g ℓ^{-1} medium
	Agar		10 g ℓ^{-1} medium

pH adjusted to 5,8 with Na OH

standards were included with each bioassay. To estimate gross levels of cytokinin activity, results were expressed as kinetin equivalents.

Nematode Counts

The method used for extracting nematodes from soil was the decanting plus sieving plus Baermann tray technique (S.B.T.) described by CHRISTIE and PERRY (1951). SPAULL and BRAITHWAITE (1979) found, with a few exceptions, that the S.B.T. was the most suitable method for extraction of nematodes from sandy soils.

Soil samples were passed through a four millimetre aperture sieve to remove course debris and then coned and quartered six times (SPAULL and BRAITHWAITE, 1979). One hundred millilitres were then removed, soaked in water for one hour and the clay fraction dispersed with a vibro mixer for five minutes. The slurry was then washed with 4 000 millilitres of water through a one millimetre aperture sieve into a jug and thoroughly mixed. The slurry was allowed to settle for 30 seconds, then decanted through a 53 micrometre aperture sieve over a 38 micrometre sieve. A further 4 000 millilitres of water was added to the remaining soils and the process repeated. The nematodes were then separated from the residue on sieves by the Baermann tray method (WHITEHEAD and HEMMING, 1965). Fifty millilitres of soil were spread on double thickness two-ply Kleenex tissues supported on a 140 millimetre diameter wide-mesh plastic screen in a plastic tray. Water was added until the soil was just wet. The screen was removed after 24 hours and the nematode suspension condensed by sedimentation. First in a 100 millilitre measuring cylinder for at least four hours and then in a 25 millilitre test tube. All but 3 millilitres was siphoned off and one third of the nematodes counted in a Sedgwick Rafter counting slide. All counts

were expressed as the number of nematode larvae present in 100 millilitres of soil.

To determine the number of nematodes in root samples, the roots were washed and excess moisture removed with absorbent paper. The roots were then cut into approximately 10 millimetre lengths, mixed and a five gramme sub-sample incubated in a sealed polythene bag containing 10 millilites of 3,3 per cent of 130 volume hydrogen peroxide. The treatment was replicated three times. After six days, the contents of each bag was extracted using the Baermann tray method described above and the number of nematodes counted, using a Sedgwick Rafter counting slide.

Chlorophyll Extraction

Leaf discs were punched from mature leaves. The discs were then extracted for chlorophyll in 100 per cent methanol for 24 hours (TALLING and DRIVER, 1963). A sample from each of the treatments was scanned from 350 nanometres to 700 nanometres on a DMS 90 double beam (UV-Vis) spectrophotometer to determine the chlorophyll absorbance peaks. An example is presented in Figure 2.4. Two absorbance peaks were found, one at 440 nanometres and the other at 660 nanometres. The remaining five chlorophyll extracted replicates from each treatment were subsequently tested for absorbance at these two wavelengths.

Figure 2.4   Absorbance curve for chlorophyll extracted

from the leaves of the plants analysed.
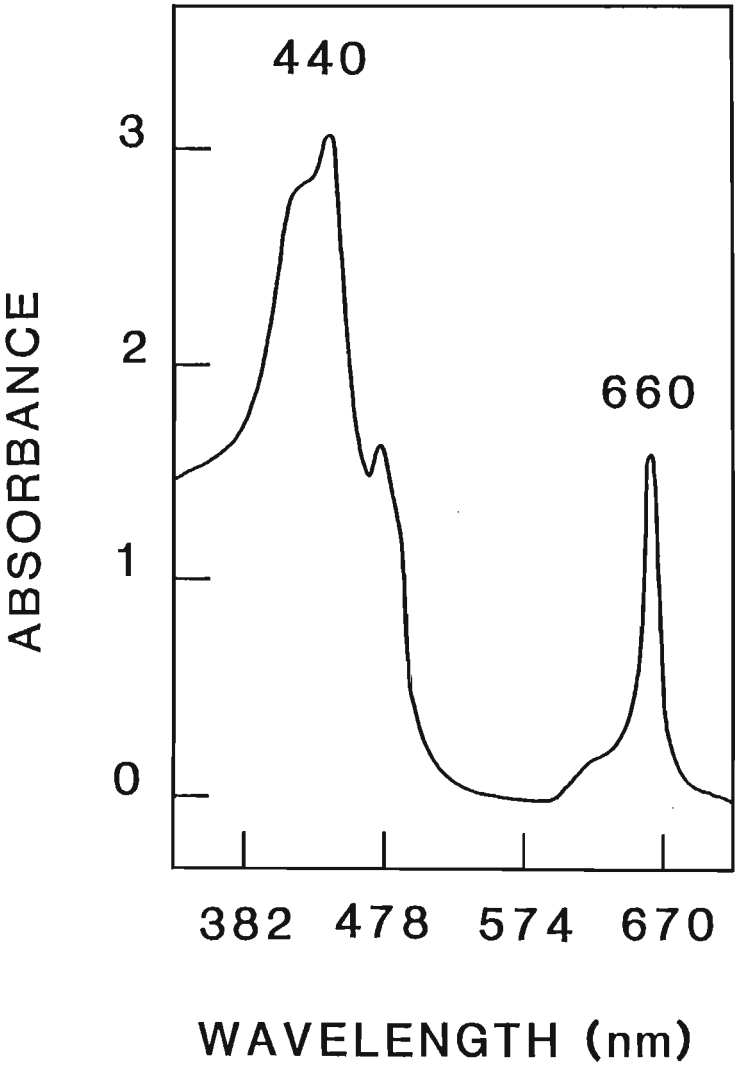
CHAPTER III

TENTATIVE IDENTIFICATION OF THE CYTOKININS PRESENT IN *ECKLONIA MAXIMA*

Introduction

The endogenous hormones and in particular cytokinins present in terrestrial plants have been extensively studied. There has however, been very little research conducted to establish the presence and identity of the cytokinins found in marine plants. JENNINGS (1969) detected cytokinin-like compounds in extracts of *Ecklonia radiata* and *Hypnea musciformis* and found that these algal cytokinins resembled, chemically, the cytokinins usually extracted from higher plants. Kinetin-like compounds have also been detected in *Laminaria digitata* (HUSSAIN and BONEY, 1969), *Fucus vesiculosis* (KENTZER, SYNAK, BURKIEWICZ and BANAS, 1980), *Ascophyllum nodosum* (KINGMAN and MOORE, 1982) and in sea water from the *Fucus Ascophyllum* zone (PEDERSEN and FRIDBORG, 1972). There has however, been very little attempt at positively identifying the cytokinin-like compounds present in marine algae. PEDERSEN (1973) however, using combined gas chromatography and mass spectrometry succeeded in quantitatively estimating and identifying the cytokinin 6-(-3 methyl -2- butenylamino) purine in sea water from the *Fucus Ascophyllum* zone.

In these experiments extracts taken from the stipes of *Ecklonia maxima* material were subjected to the commonly used techniques of ion exchange chromatography and Sephadex LH-20 column chromatography in an attempt to assess the type of cytokinins present in *Ecklonia maxima*. Tentative identification of these compounds was then achieved using reversed phase high performance

liquid chromatography.

Experimental procedure and results

Five kilogrammes of processed *Ecklonia maxima* stipe material harvested in December 1982 was extracted and purified as described in the Materials and Methods section. After separation using paper chromatography, the equivalent of twenty grammes of stipe material taken from both the aqueous and ammonia fractions was analysed for cytokinin activity using the soybean callus bioassay according to the procedures outlined in the Materials and Methods section (Figure 3.1).

High levels of cytokinin-like activity were detected in the ammonia fraction between Rf 0,5 - 0,9. No significant levels of cytokinin-like activity were detected in the aqueous fraction (Figure 3.1) thus for the remainder of this study this fraction was ignored. The compounds present in the ammonia fraction co-chromatographed with ribosylzeatin, zeatin and their dihydro derivatives (Figure 3.1).

The areas of activity detected on paper were then eluted off the respective zones of the remainder of the paper chromatograms with 2 000 millilitres of 80 per cent ethanol. The eluates were filtered, concentrated, and fractionated on a Sephadex LH-20 column eluted with 35 per cent ethanol (ARMSTRONG, BURROWS, EVANS and SKOOG, 1969) as previously described in the Materials and Methods section. Forty millilitre fractions were collected and a one millilitre aliquot of each, representing approximately 20 grammes of plant material, was used for bioassay purposes (Figure 3.2). The remainder of each fraction was dried on a hot plate (30°C) in a stream of air and then stored at -20°C.

Figure 3.1   Cytokinin activity detected in (A) the ammonia fraction and (B) the aqueous fraction of an extract equivalent to 20 grammes of processed *Ecklonia maxima* stipe material harvested in November 1982. The extract was purified using Dowex 50 cation exchange resin and the eluates separated on paper chromatogrpahy with *iso*-propanol : 25 per cent ammonium hydroxide : water (10:1:1 v/v). The region above the dotted line is significantly different from the control at the level P = 0,01. ZR = ribosylzeatin; Z = zeatin.
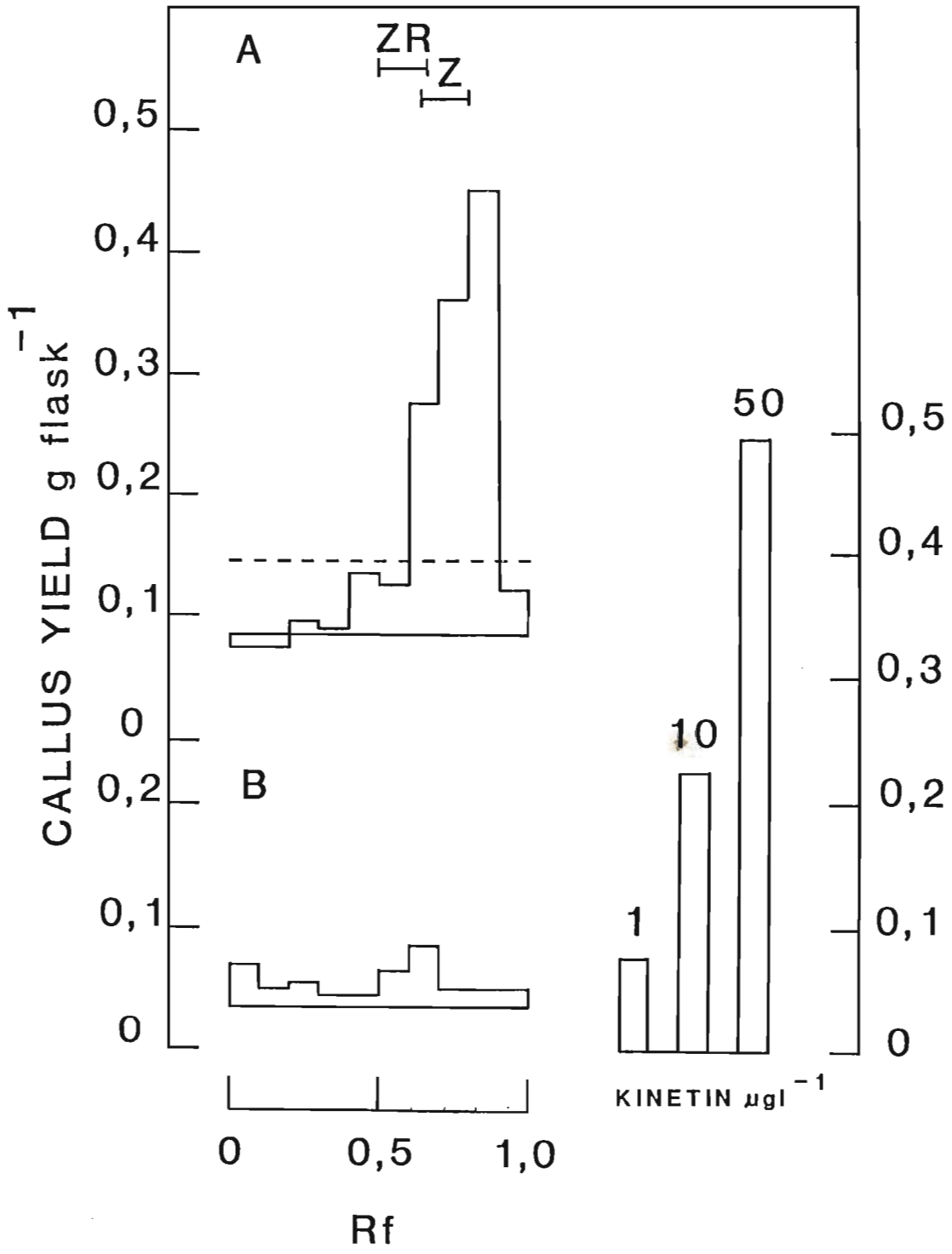
Figure 3.2   Cytokinin activity detected in the equivalent of 20 grammes of processed *Ecklonia maxima* stipe material harvested in November 1982. After paper chromatography the biologically active region (Rf 0,7 - 0,9, Figure. 3.1) was eluted off paper, concentrated and fractionated on a Sephadex LH-20 column eluted with 35 per cent ethanol. The region above the dotted line is significantly different from the control at the level P = 0,01. ZG = glucosylzeatin; ZR = ribosylzeatin; Z = zeatin; IPA = isopentenyladenosine; 2iP = isopentenyladenine.

CALLUS YIELD g flask⁻¹

0,3 0,2 0,1 0

ELUTION VOLUME ml

0   400   800   1200   1600

ZG   ZR   Z   IPA   2iP

KINETIN µg l⁻¹

50

10

1
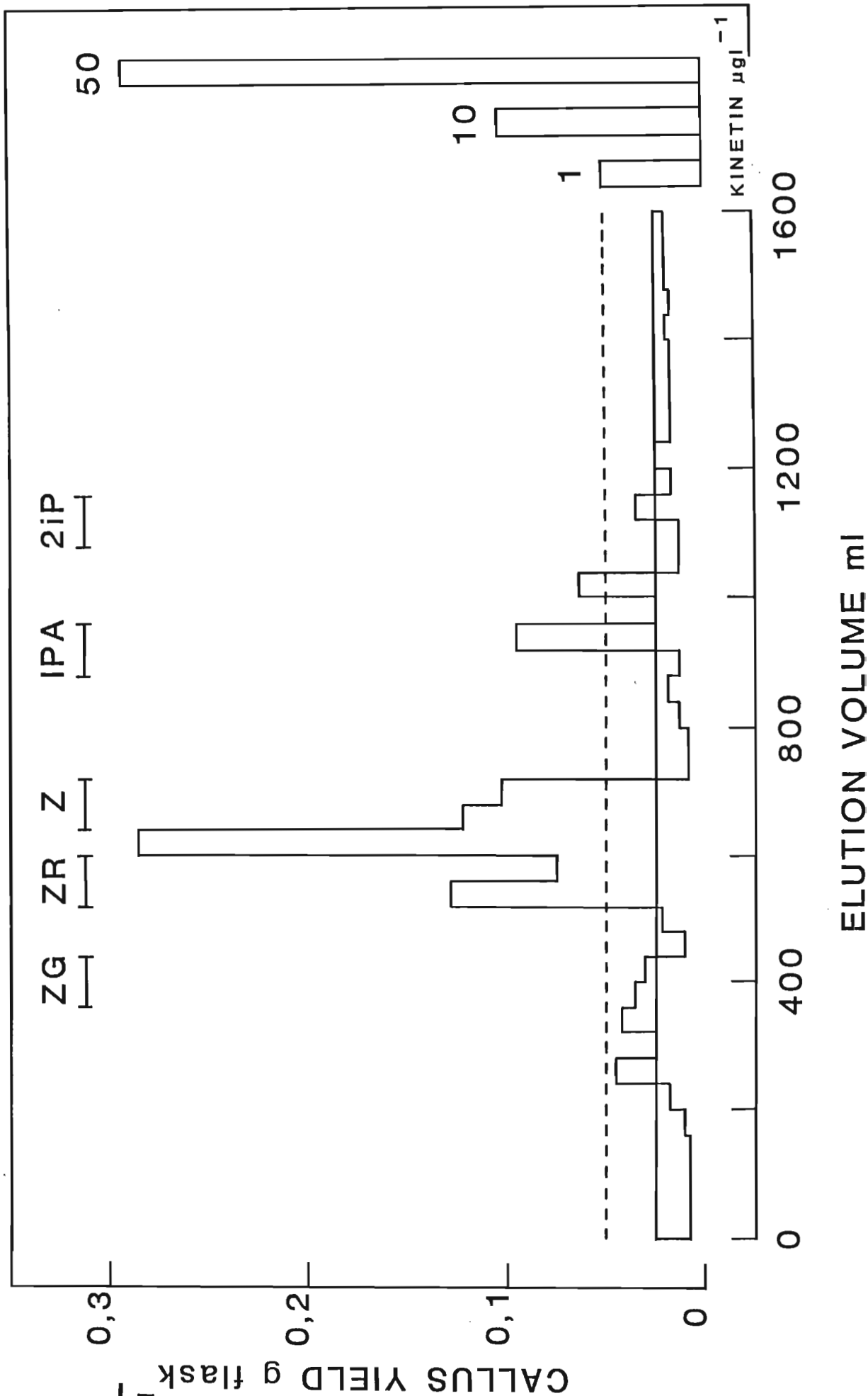
Four distinct peaks of biological activity were detected in the Sephadex column eluate of the five kilogrammes of material harvested in December 1982 (Figure 3.2). The first occurred at an elution volume of 520 - 600 millilitres and co-eluted with ribosylzeatin, the second had an elution volume of 600 - 720 millilitres and co-eluted with zeatin, and the third had an elution volume of 920 - 960 millilitres and co-eluted with isopentenyladenosine, the fourth peak did not co-elute with any of the authentic markers used.

The remainder of the residues from the 40 millilitre fractions , corresponding with the beginning, middle and end of the peaks of biological activity detected after Sephadex LH-20 fractionation were redisolved in one millilitre of 100 per cent methanol and subjected to high performance liquid chromatography. Ten microlitre aliquots from each sample were injected into the column through a 10 microlitre external loop injection valve (Varian 5 000 series), the reciprocating pump controlled by a solvent programmer delivered the mobile phase which, at the beginning of each programme consisted of acetonitrile:water (10:90). Sample separation was achieved using the programme as outlined in the Materials and Methods section. Each sample run was replicated three times and eluates from each run were collected in thirty fractions (one fraction per minute). These fractions were then assayed for cell division inducing activity using the soybean callus bioassay.

In order to establish profiles of the peaks recorded after Sephadex LH-20 separation, aliquots of authentic cytokinins were subjected to high performance liquid chromatography to determine the retention times of the various compounds (Figure 3.3.). The profile of a ten microlitre aliquot of the biologically active peak which co-eluted with ribosylzeatin (Figure 3.4) revealed two UV-absorbing peaks which co-chromatographed with the *cis* (17,3 minutes) and *trans* (16,0 minutes)

Figure 3.3   Separation of authentic cytokinin markers by reversed

phase H.P.L.C. tZR = *trans*-ribosylzeatin; cZR =

cis-ribosylzeatin; tZ = *trans*-zeatin; cZ = *cis*-zeatin;

DHZ = dihydrozeatin; IPA = isopentenyladenosine.
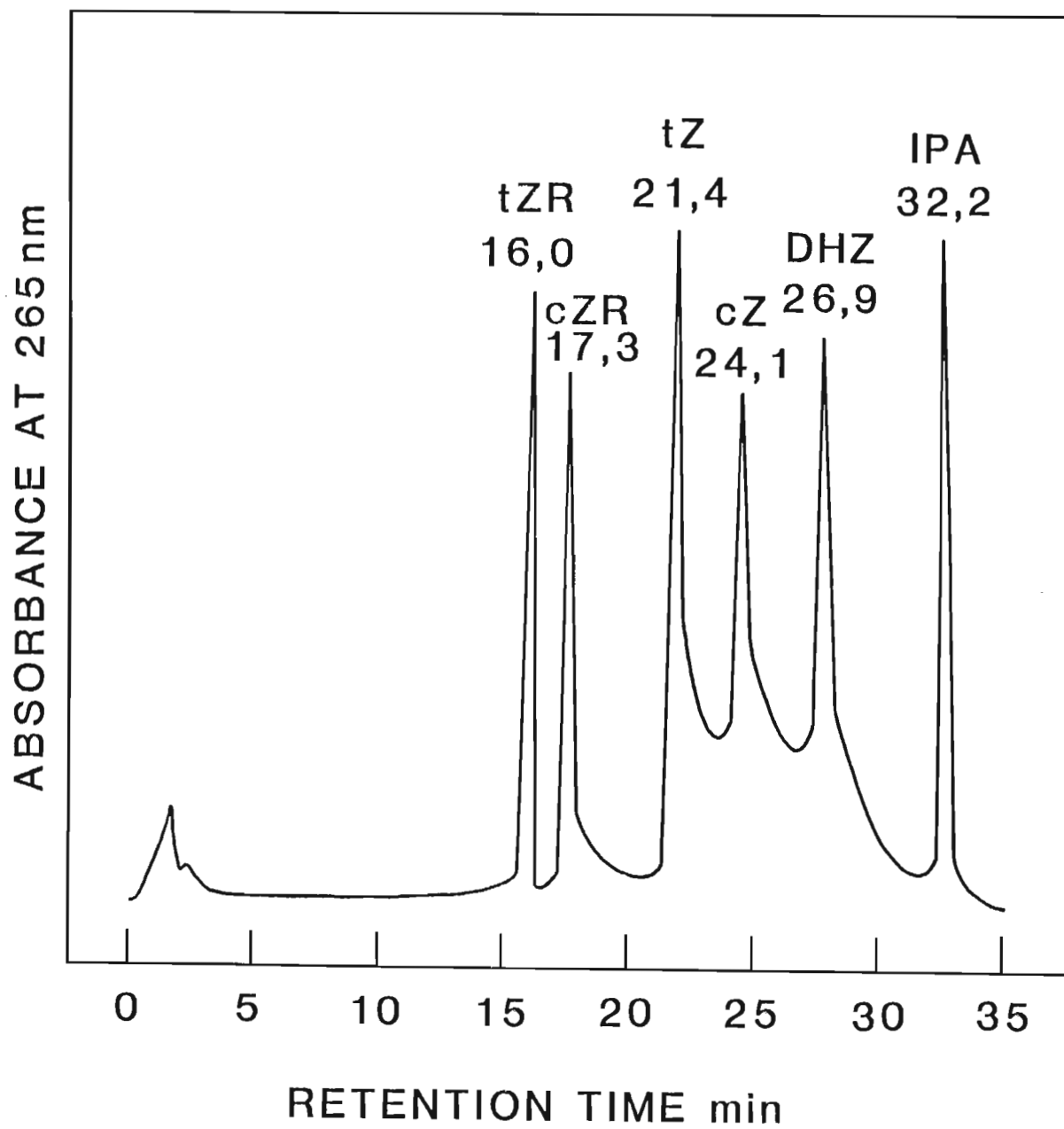
Figure 3.4   Separation by reversed phase H.P.L.C. of the biologically active peak which co-eluted with ribosylzeatin after Sephadex LH-20 column chromatography (520 – 600 millilitres, Figure 3.2). The histogram represents the biological activity associated with the H.P.L.C. fraction collected. tZR = *trans*-ribosylzeatin; cZR = *cis*-ribosylzeatin.
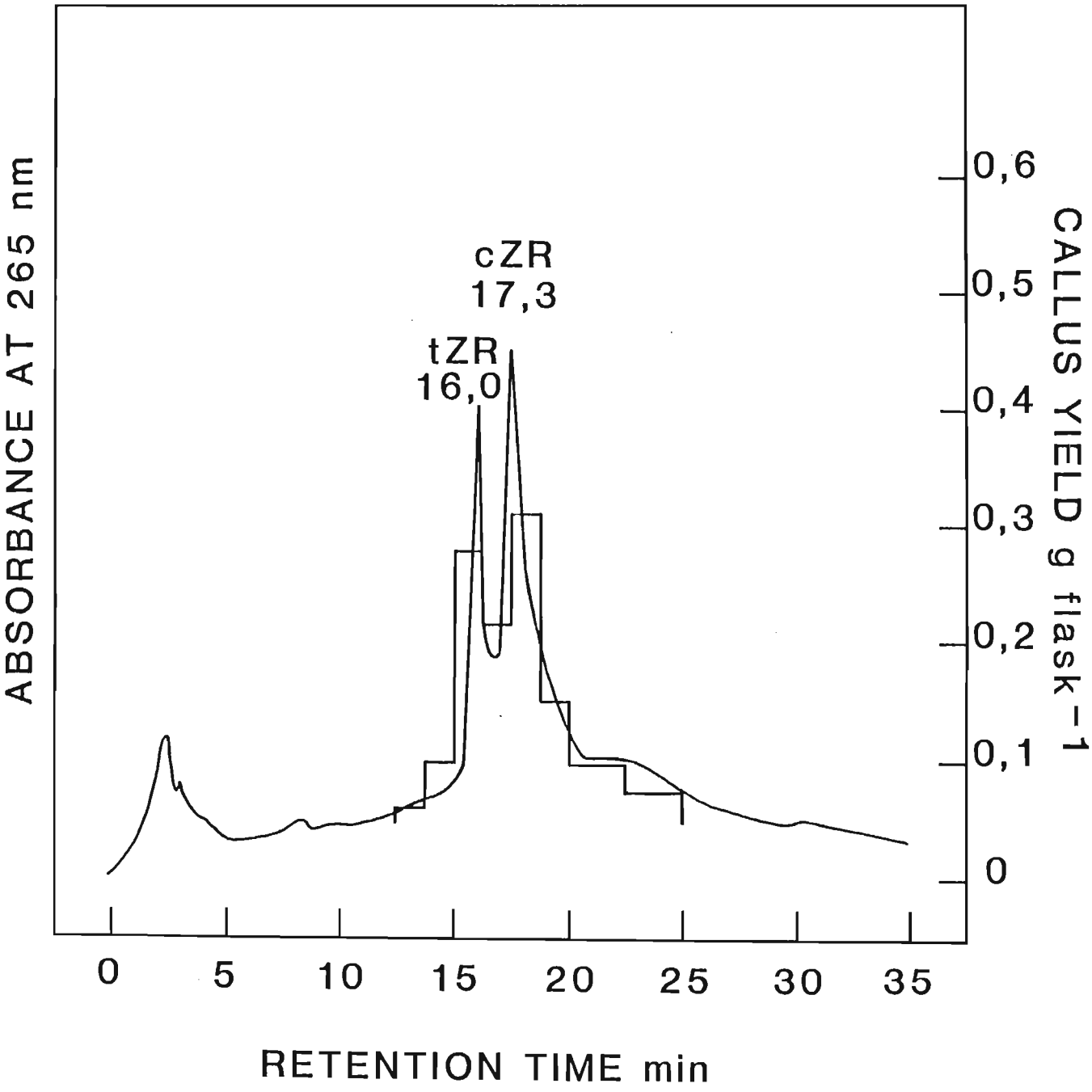
Figure 3.5   Separation by reversed phase H.P.L.C. of the biologically active peak which co-eluted with zeatin after Sephadex LH-20 column chromatography (600 - 720 millilitres, Figure 3.2). The histogram represents the biological activity associated with the H.P.L.C. fraction collected. tZ = *trans*-zeatin; DHZ = dihydrozeatin.
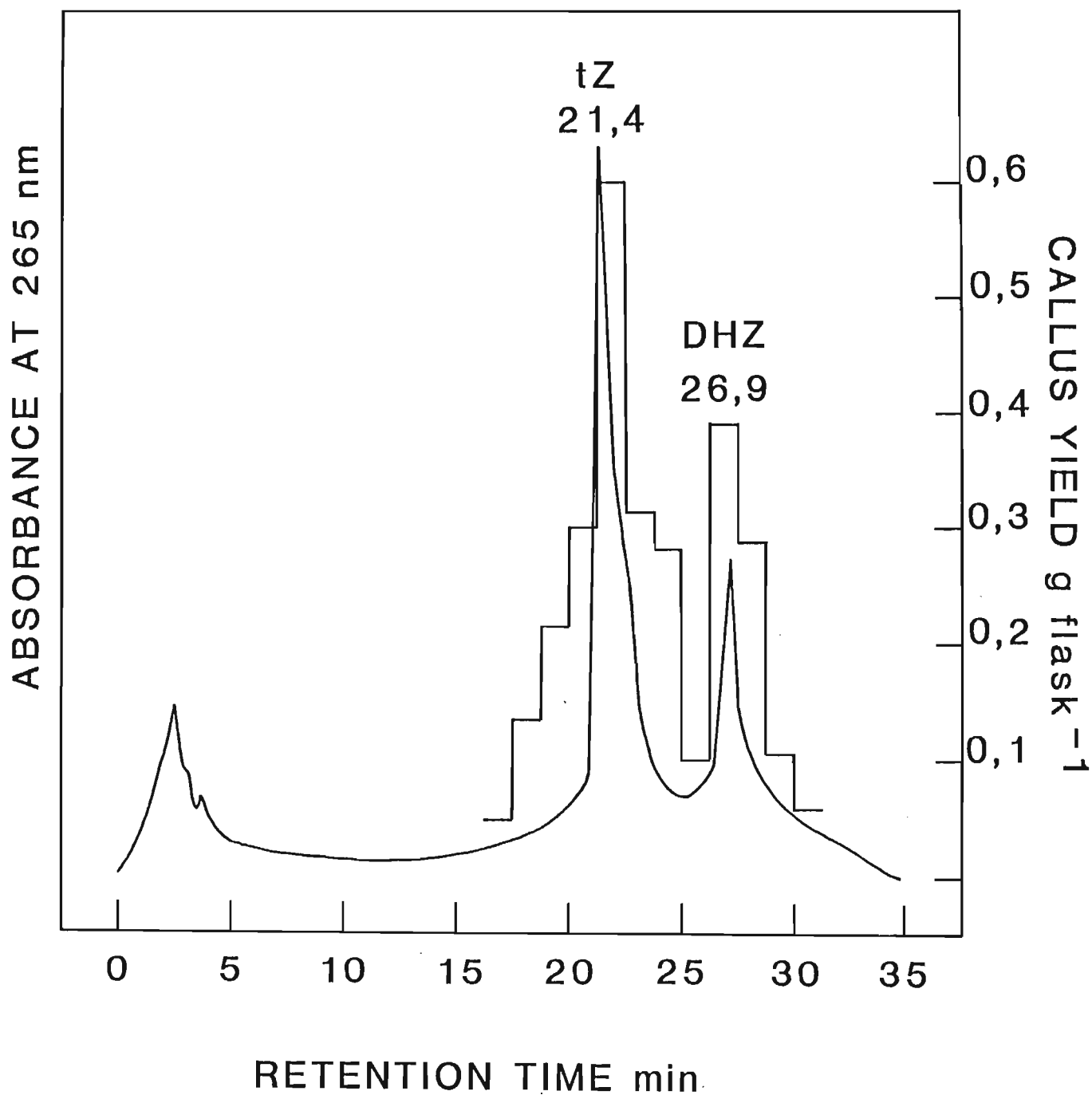
Figure 3.6   Separation by reversed phase H.P.L.C. of the
              biologically active peak which co-eluted with
              isopentenyladenosine after Sephadex LH-20 column
              chromatography (920 - 960 millilitres, Figure 3.2).
              The histogram represents the biological activity
              associated with the H.P.L.C. fraction collected.
              IPA = isopentenyladenosine.
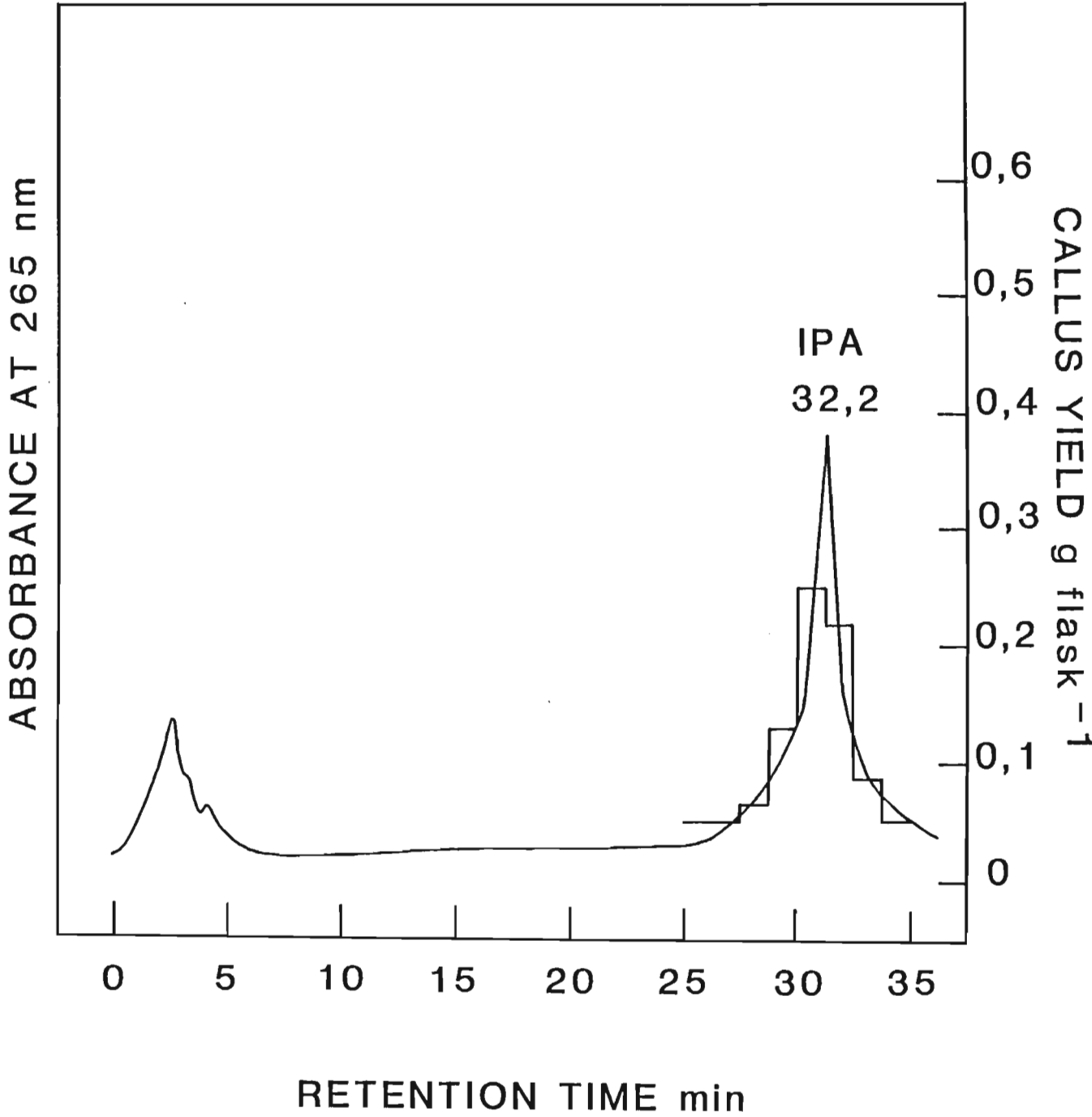
isomers of ribosylzeatin. The peak which co-eluted with zeatin is shown in Figure 3.5. The major UV-absorbing peak co-chromatographed with *trans*-zeatin (21,4 minutes) and a second smaller peak co-chromatographed with dihydrozeatin (26,9 minutes). The third peak detected after Sephadex LH-20 fractionation co-chromatographed with isopentenyladenosine (32,2 minutes) (Figure 3.6).

In order to substantiate the results, the biological activity present in each one minute fraction collected during each sample run was superimposed upon the corresponding UV-absorbance trace of each sample run.

Discussion

Tentative identification of the cytokinins present in *Ecklonia maxima* harvested in November 1982 has shown that they resemble closely those found in terrestrial plants (MUROFUSHI, INOUE, WATANABE, OTA and TAKAHASHI, 1983). Analysis showed that the *cis* and *trans* isomers of ribosylzeatin, *trans*-zeatin, dihydrozeatin and isopentenyladenosine, appear to be the major cytokinins present in the stipes of *Ecklonia maxima* harvested at this time of the year. ENTSCH, LETHAM, PARKER, SUMMONS and GOLLNOW (1979) put the number of endogenous cytokinins extracted from higher plants at 25, the most frequently identified being zeatin and its derivatives (LETHAM, 1963; HORGAN, HEWETT, PURSE, HORGAN and WAREING, 1973; VAN STADEN and DREWES, 1975; PURSE, HORGAN, HORGAN and WAREING, 1976; VAN STADEN, 1978; VAN STADEN and DAVEY, 1979). It has been established however, that this is not always the case in lower plant orders. PEDERSEN and FRIDBORG, 1972 and PEDERSEN, 1973 identified isopentenyl-aminopurine as the major cytokinin occurring in sea water from the *Fucus Ascophyllum* zone. KENTZER, SYNAK, BURKIEWICZ and BANAS (1980) demonstrated the presence of a cytokinin-like substance showing properties typical

of isopentenylaminopurine in extracts taken from the thallus of *Fucus vesiculosis*. Isopentenylaminopurine and its derivatives have also been detected in bacteria (HELGESON and LEONARD, 1966; UPPER, HELGESON, KEMP and SCHMIDT, 1970; RATHBONE and HALL, 1972) and in the bryophytes (BEUTLEMAN and BAUER, 1977; WANG, HORGAN and COVE, 1981). Although isopentenylaminopurine was not identified in the stipes of *Ecklonia maxima* in this study, Sephadex LH-20 fractionation of extracts taken from the stipes of material harvested during the seasonal cycle (Chapter IV) apparently did contain activity which co-chromatographed with this compound. Zeatin and its derivatives were, however, always present in greater quantities than isopentenylaminopurine and its derivatives.

There have been numerous hypothesis put forward as to the "active" compound/s present in plant tissue. Zeatin and its derivatives are often regarded as being active *per se* because they are active in cytokinin bioassays. HECHT, FRYE, WERNER, HAWRELAK, SKOOG and SCHMITZ (1975) suggested that the free base cytokinins (zeatin and ribosylzeatin) would 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 deribosylation of cytokinin ribosides in concert with other cytokinin metabolic enzymes. More recently VAN STADEN, DREWES and HUTTON (1982) suggested that trihydroxyzeatin, a biologically active intermediate of zeatin oxidation may in fact be an active cytokinin compound. 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.

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 being dependent on the spatial arrangement, as well as the type of atoms in the N^6-substituent (HECHT, 1979). MATSUBARA (1980) 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 steriochemistry of the substituents attached to the double bond as an important feature of the side chain. Of the compounds which fulfill these structural requirements zeatin has been found to be the most active (SCHMITZ, SKOOG, PLAYTIS and LEONARD, 1972; LETHAM, 1972).

In a previous chapter reference was made to the possible involvement of cytokinins in the various growth responses observed in plants treated with seaweed extracts or concentrates. If cytokinins are indeed involved in bringing about these responses, the type of cytokinin present in the extract or concentrate is of great importance. It is significant therefore, that of the cytokinins identified in *Ecklonia maxima* stipes harvested in November 1982, the compounds with chromatographic properties similar to the zeatin group of cytokinins accounted for most of the detected activity.

CHAPTER IV

SEASONAL VARIATION IN THE CYTOKININ CONTENT
OF BOTH FRESH AND PROCESSED *ECKLONIA MAXIMA*

Introduction

Higher plants undergo an orderly sequence of events during their life cycle, which must involve a complex control system to ensure integration and co-ordination of these events (WAREING, 1977). It is now accepted that a group of plant growth regulators, the cytokinins, form part of this control system which regulates plant growth and development (WAREING, 1977; LETHAM, 1978).

It is widely assumed that the control of growth and morphogenesis in eukaryotic algae is mediated, at least in part, by the same group of endogenous growth regulators as occurs in higher plants. Although there is no evidence against this assumption (THIMANN and BETH, 1959;) it is noteworthy that very few of these regulatory substances have been isolated and definitively identified from algae.

This study was undertaken in order to determine the quantitative and qualitative changes in cytokinins during vegetative growth of *Ecklonia maxima* plants over a twelve month period, from February 1981 until January 1982. In addition to establishing the total cytokinin activity present in the plant at a particular time during the study period, it was hoped that this investigation would yield information as to the type of cytokinin present in the algae, and the distribution of these cytokinins within the plant body.

Experimental procedures

Analysis for cytokinins present in fresh *Ecklonia maxima* material was conducted on plants belonging to three different size classes, viz. one metre, two metres and three metres in length. The plants were harvested at monthly intervals for the period February 1981 until January 1982. In addition to the monthly samples two metre plants were harvested for the lunar cycle study on a daily basis during April – May 1983 as indicated in the Materials and Methods section. Samples of processed material used for cytokinin determination were taken from the normal production run over the same period. Material for analysis was taken from processed material and lamina, stipe and holdfast regions of whole plants, weighed into 20 gramme lots, and extracted and purified according to the procedures outlined in the Materials and Methods section. After separation using paper chromatography, the extracts were analysed for cytokinin activity using the soybean callus bioassay. In order to obtain more information as to the nature of the cytokinin-like activity recorded on paper chromatograms, 20 gramme (fresh mass) samples were extracted, as described in the Materials and Methods section, and loaded on to a Sephadex LH-20 column which was eluted with 35 per cent ethanol.

Results

Throughout the entire study period the water temperatures at Ouderkraal ± 2 kilometres from Kommetjie were obtained (Figure 4.1). Ocean temperatures have been found to be of considerable importance in determining the overall growth and vigour of *Ecklonia maxima*, with normal growth being achieved where the mean annual temperature does not exceed 14,6°C (PAPENFUSS, 1942). During

Figure 4.1   Ocean temperatures recorded at Oudekraal ± 2 km
              from the harvest site at Kommetjie for the period
              February 1981 until January 1982. Mean   monthly
              temperatures were supplied by the Marine Development
              branch of the Department of Environmental Affairs
              (personal communication).
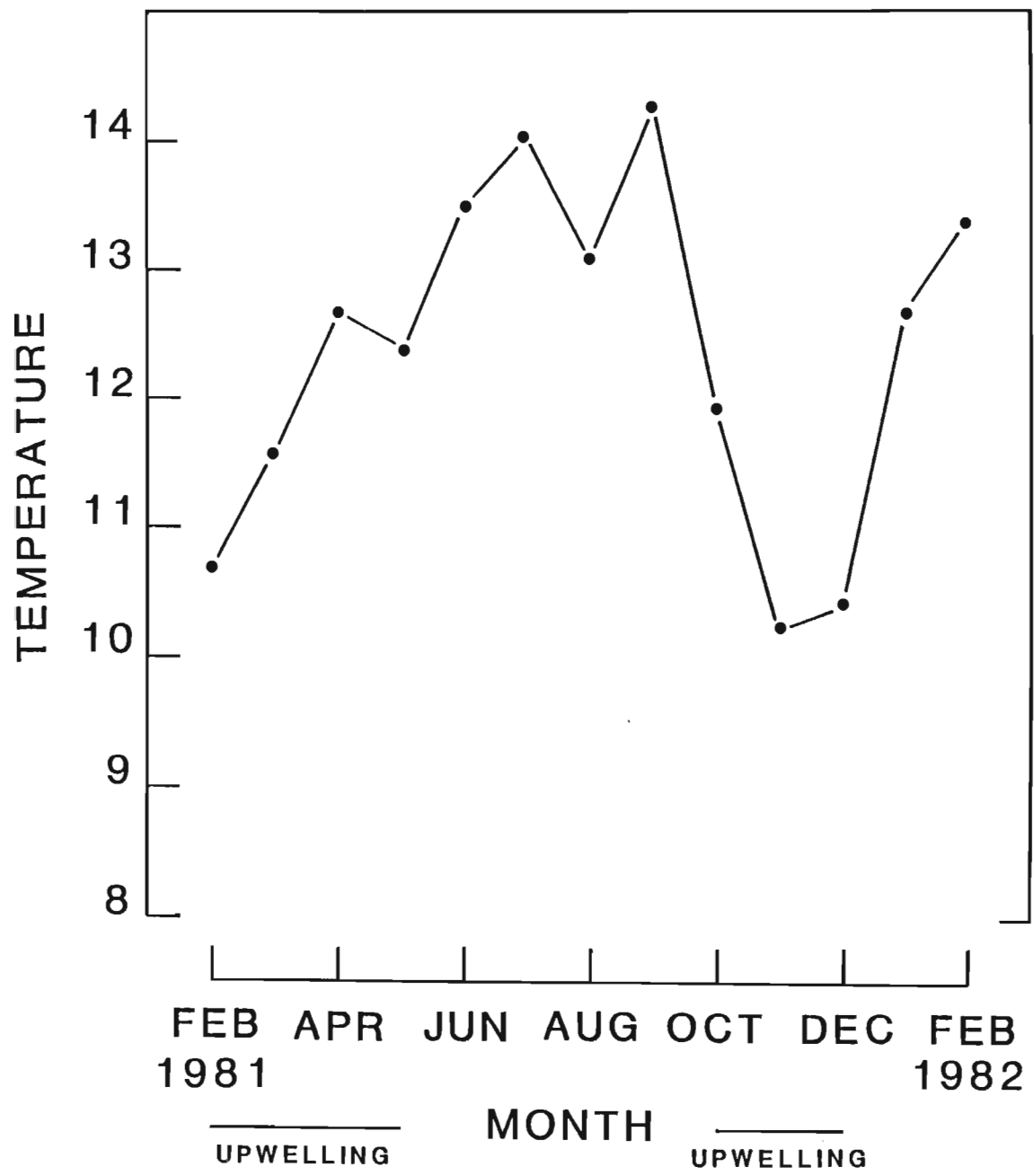
the sampling period the mean water temperature was 12,4°C with a mean summer temperature of 11,4°C and that of winter 13,1°C. The reason for the low summer temperatues (Figure 4.1) was the occurance of off-shore south-easterly winds at this time of the year. These winds result in the upwelling of cold nutrient rich waters of the Antartic being brought up to the surface and into the kelp beds. The introduction of an increased nutrient supply together with the increased light conditions experienced during summer, result in this being the most active growing season of *Ecklonia maxima*.

The seasonal variation in the growth pattern of *Ecklonia maxima* was accompanied by significant changes in the detectable levels of cytokinin in the three different regions of the plant tested. As the peaks of activity in the plants of the different size classes and processed material did not always coincide it was decided to examine each of these separately.

Processed material

It is evident from the results of paper chromatography of extracts of processed *Ecklonia maxima* material that both quantitative and qualitative variation existed in the cytokinin activity present during the study period (Figure 4.2).

Cytokinin activity in February, March, April and May occurred at Rf 0,6 - 0,9 and co-chromatographed with ribosylzeatin and zeatin. Activity during June, July and August was detected mainly at Rf 0,2 - 0,4 and co-chromatographed with glucosylzeatin. During this period the activity at Rf 0,6 - 0,9 declined. The level of activity at Rf 0,6 - 0,9 increased again in September and remained relatively constant for the remainder of the study period. Over the same period the level of activity at Rf 0,2 - 0,4 declined. In order to obtain more information

Figure 4.2   The endogenous cytokinin activity present in extracts
of processed *Ecklonia maxima* material expressed
per 20 grammes fresh mass. 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). The region
above the dotted line is significantly different from
the control at the level $P = 0,01$. ZG = glucosylzeatin;
ZR = ribosylzeatin; Z = zeatin.
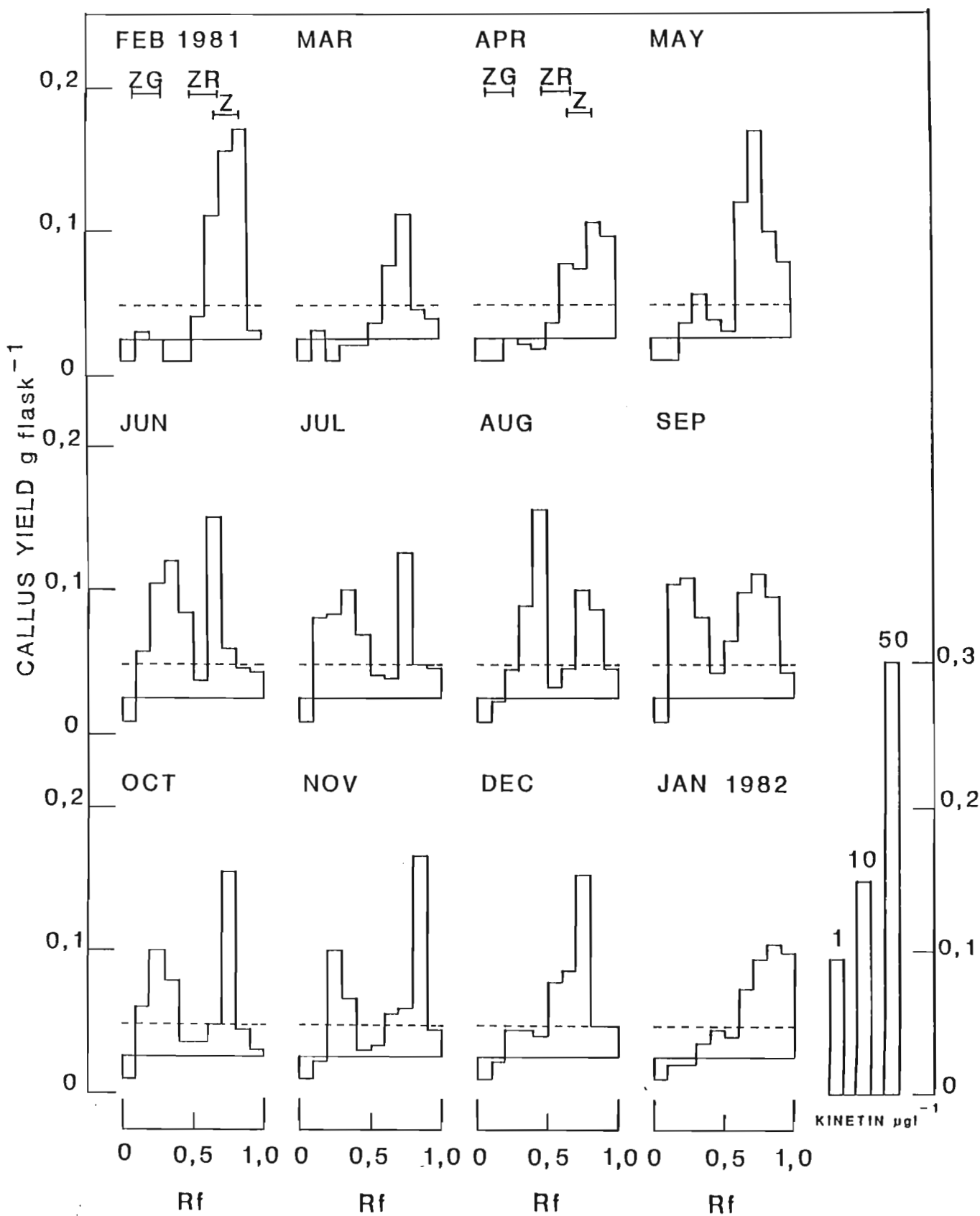
as to the nature of the cytokinin activity present in processed *Ecklonia maxima* material, paper chromatograms of material harvested in February and June were applied to Sephadex LH-20 columns and eluted with 35 per cent ethanol. Fractionation of the February sample revealed the presence of cytokinin activity co-eluting with ribosylzeatin, zeatin, isopentenyladenosine and isopentenyladenine (Figure 4.3). Activity in the June sample, however, co-eluted mainly with glucosylzeatin, smaller peaks of ribosylzeatin and zeatin were also detected (Figure 4.4.).

To obtain an overall picture of the quantitative and qualitative seasonal variation in the cytokinin activity present in processed *Ecklonia maxima* material, the activity recorded in Figure 4.2 which was significantly different from the controls was expressed as ng kinetin equivalents $20g^{-1}$ fresh material. The information is expressed graphically in Figure 4.5. From the results it appears that the quantitative and qualitative variation which does exist coincides with the seasonal periodicity of growth and development of this alga. High levels of glucosylzeatin occurred in June, July and August, coinciding with a reduction in growth of the alga. During summer, the most active period of growth of *Ecklonia maxima*, the level of cytokinin activity co-chromatographing with glucosylzeatin declined and the majority of the activity co-chromatographed with ribosylzeatin and zeatin.

One metre plants

The cytokinin activity recorded on paper chromatograms of lamina, stipe and holdfast sections of one metre *Ecklonia maxima* plants, which was significantly different from the controls, was expressed as ng kinetin equivalents $20g^{-1}$ fresh material (Figure 4.6). From the results it is evident that there are pronounced

Figure 4.3   The cytokinin activity in 20 grammes of processed
              Ecklonia maxima material harvested in February
              1981 following fractionation on a Sephadex LH-
              20 column eluted with 35 per cent ethanol. The
              extract was initially purified by paper
              chromatography. The region above the dotted line
              is significantly different from the control at the
              level P = 0,01. ZG = glucosylzeatin; ZR =
              ribosylzeatin; Z = zeatin; IPA = isopentenyladenosine;
              2iP = isopentenyladenine.
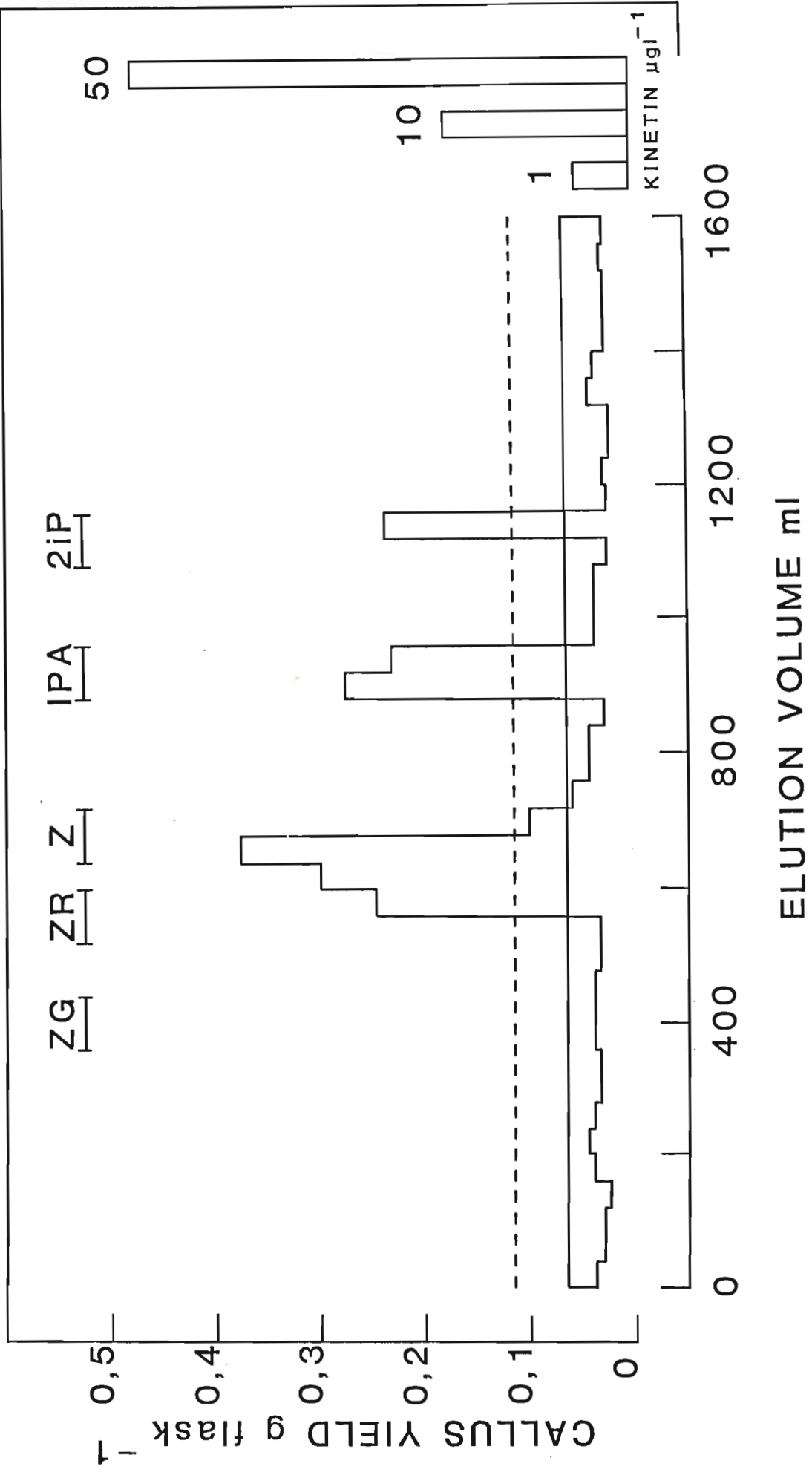
Figure 4.4   The cytokinin activity in 20 grammes of processed
              Ecklonia maxima material harvested in June 1981
              following fractionation on a Sephadex LH-20 column
              eluted with 35 per cent ethanol. The extract was
              initially purified by paper chromatography. The
              region above the dotted line is significantly different
              from the control at the level $P = 0,01$. ZG =
              glucosylzeatin; ZR = ribosylzeatin; Z = zeatin;
              IPA = isopentenyladenosine; 2iP = isopentenyladenine.
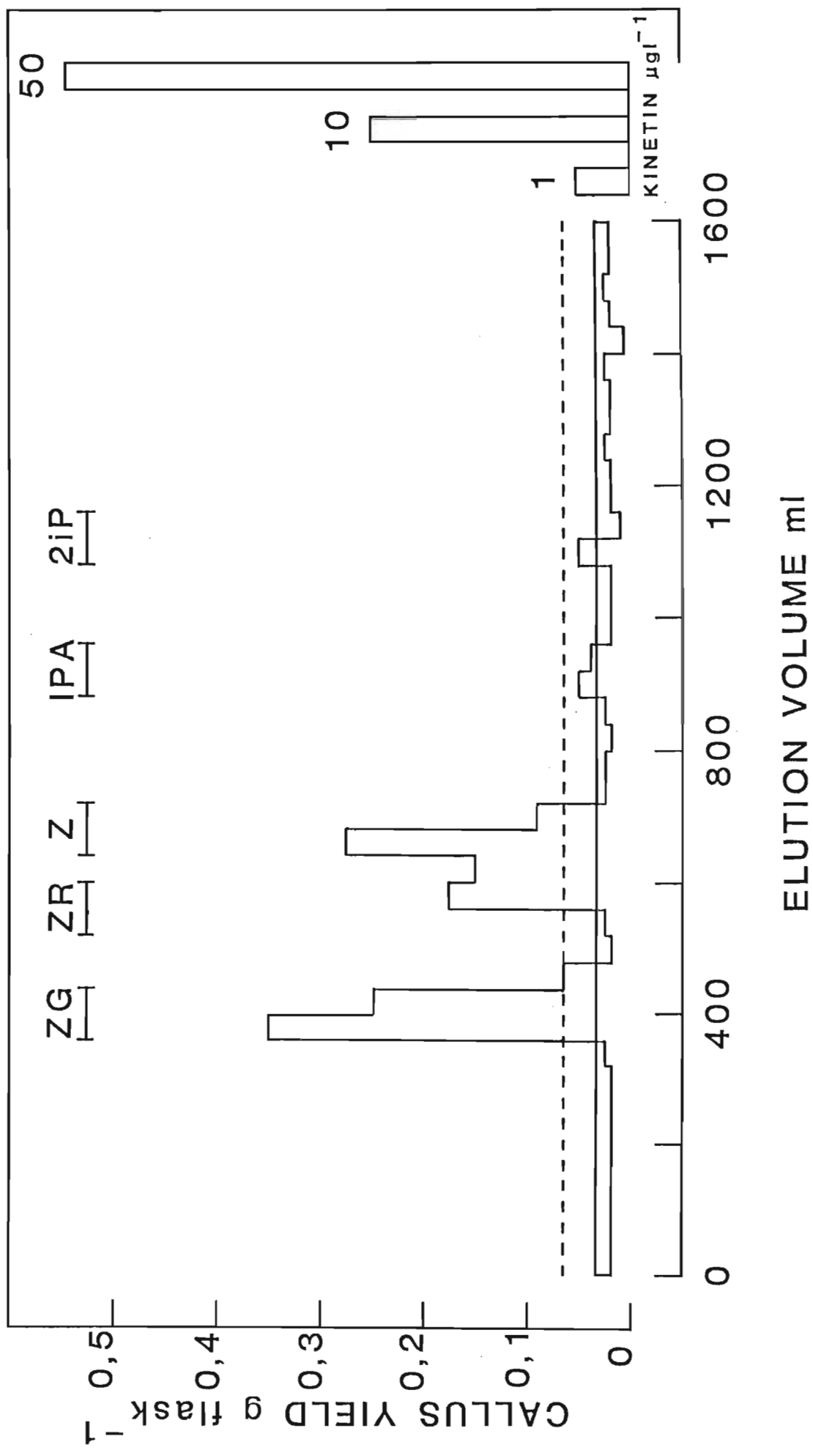
Figure 4.5   Combined zeatin and ribosylzeatin activity (●–●)
               and glucosylzeatin activity (o–o) in processed *Ecklonia*
               maxima material harvested at monthly intervals
               from February 1981 until January 1982. Activity
               detected after paper chromatography which was
               significantly different from the controls is expressed
               as kinetin equivalents.

CYTOKININ ACTIVITY ng KE /20g fresh mass

MONTH

1981   1982

FEB MAR APR MAY JUN JUL AUG SEP OCT NOV DEC JAN
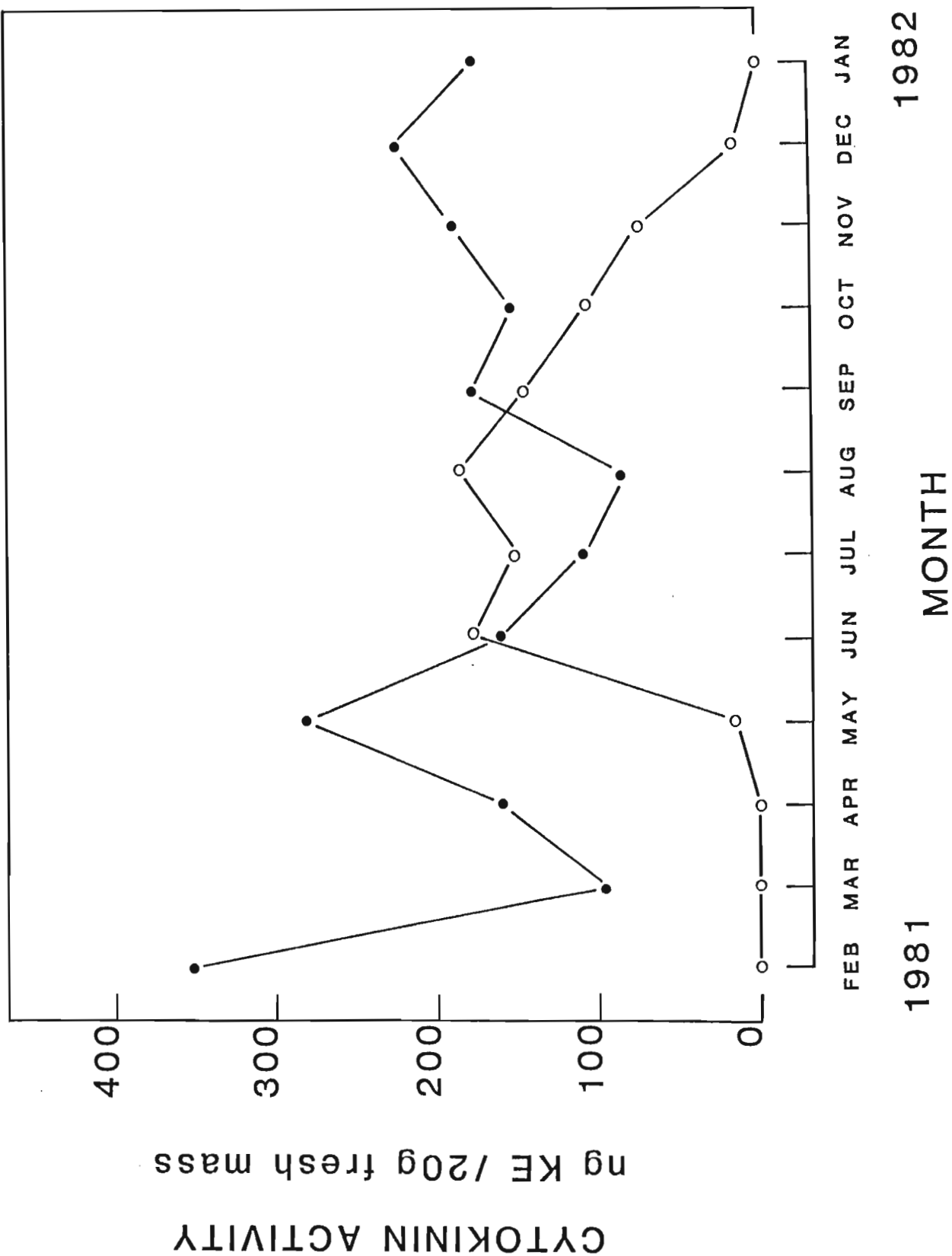
Figure 4.6   The total cytokinin activity in 20 grammes of lamina
(▲-▲), stipe (o-o) and holdfast (●-●) regions of one
metre *Ecklonia maxima* plants harvested at monthly
intervals from February 1981 until January 1982.
Activity detected after paper chromatography which
was significantly different from the controls is
expressed as kinetin equivalents.
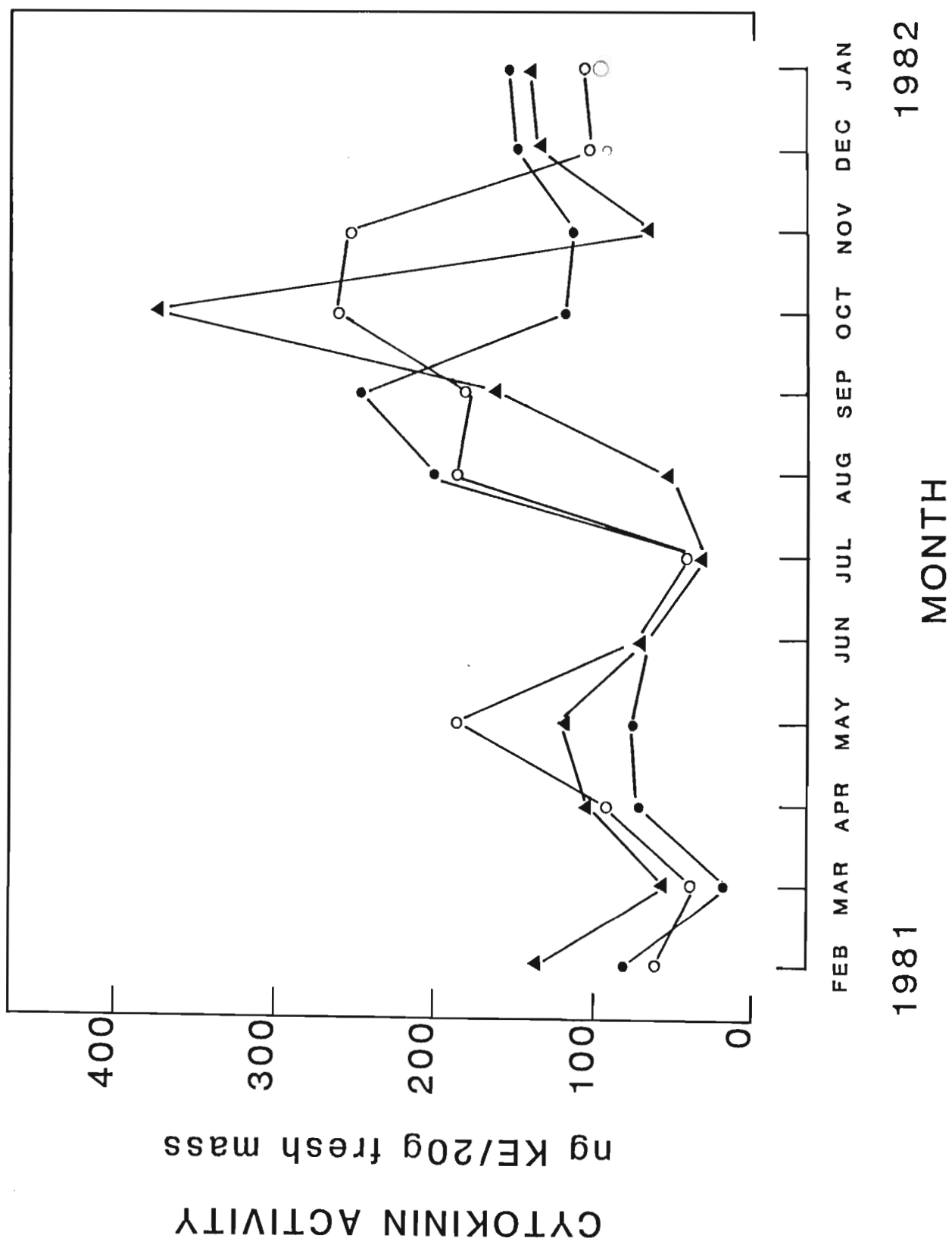
quantitative variations in the cytokinin activity present in the plant tissues analysed. These changes appear to coincide with the seasonal periodicity of growth and development. The highest levels of cytokinin-like activity were detected in August, September, October and November, coinciding with the onset of spring and early summer, the most vigorous growing period of *Ecklonia maxima*. A second smaller peak of activity was detected in all three tissues examined in May just prior to winter. Qualitative differences in the cytokinin activity detected in the tissues examined, occurred when the overall level of cytokinins in the plant increased (Figures 4.7 - 4.9). In order to establish the nature of this variation, paper chromatograms of extracts of lamina and holdfast material harvested in September and stipe material harvested in October were applied to a Sephadex LH-20 column and eluted with 35 per cent ethanol. Fractionation of all three extracts revealed the presence of cytokinin activity co-eluting with ribosylzeatin and zeatin (560 - 600 and 640 - 760 millilitres respectively), while a third peak co-eluting with glucosylzeatin (360 - 440 millilitres) was also detected (Figures 4.10 - 4.12).

Two metre plants

The cytokinin activity recorded in two metre plants, expressed as ng kinetin equivalents $20g^{-1}$ fresh material is depicted graphically in Figure 4.13. High levels of activity were detected in the lamina and stipe tissue during February. This activity declined in March and April. The highest levels of cytokinin activity were detected in May and June in all three tissues examined. The levels of detectable cytokinins decreased in July and remained low for the remainder of the study period. Seasonal variation in the type of cytokinins found in two metre plants followed similar trends to those which occurred in processed material, although the level of glucosylzeatin detected was not as great. Activity detected after paper chromatography of extracts of lamina, stipe and holdfast tissues,

Figure 4.7   Combined zeatin and ribosylzeatin activity (●–●)

and glucosylzeatin activity (o–o) detected in the

lamina of one metre *Ecklonia maxima* plants harvested

at monthly intervals from February 1981 until January

1982. Activity detected after paper chromatography

which was significantly different from the controls

is expressed as kinetin equivalents.

Figure 4.8   Combined zeatin and ribosylzeatin activity (●–●)
and glucosylzeatin activity (o–o) detected in the
stipes of one metre *Ecklonia maxima* plants harvested
at monthly intervals from February 1981 until January
1982. Activity detected after paper chromatography
which was significantly different from the controls
is expressed as kinetin equivalents.

Figure 4.9   Combined zeatin and ribosylzeatin activity (●–●)
and glucosylzeatin activity (o–o) detected in the
holdfasts of one metre *Ecklonia maxima* plants
harvested at monthly intervals from February 1981
until January 1982. Activity detected after paper
chromatography which was significantly different
from the controls is expressed as kinetin equivalents.
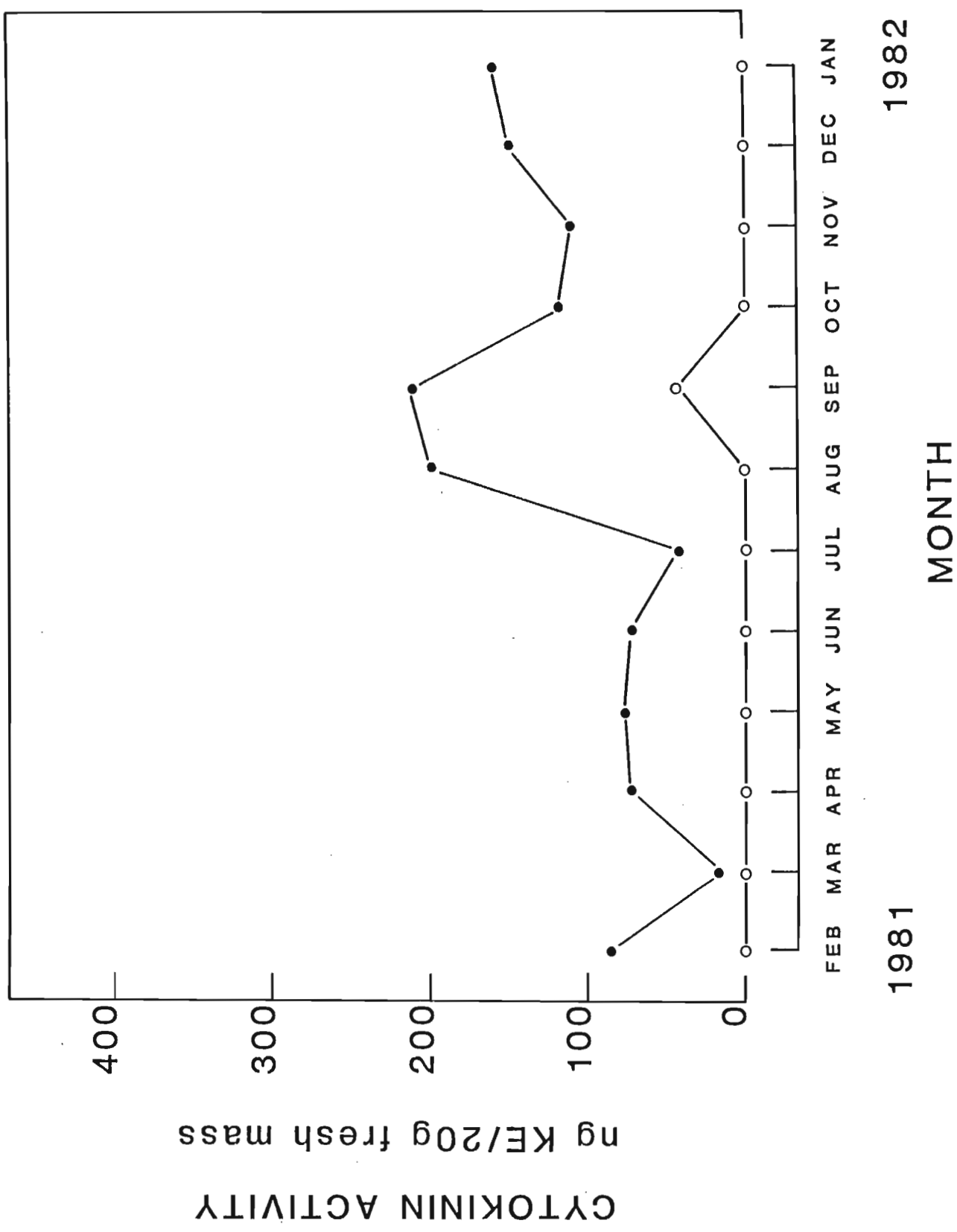
Figure 4.10   The cytokinin activity in 20 grammes of lamina

material of one metre plants harvested in September

1981 following fractionation on a Sephadex LH–

20 column eluted with 35 per cent ethanol. The

extract   was   initially   purified   by   paper

chromatography. The region above the dotted line

is significantly different from the control at the

level P = 0,01.   ZG = glucosylzeatin;   ZR =

ribosylzeatin; Z = zeatin; IPA = isopentenyladenosine;

2iP = isopentenyladenine.

CALLUS YIELD g flask^{-1}

ELUTION VOLUME ml

ZG ZR Z IPA 2iP

KINETIN µg l^{-1}

50

10

1
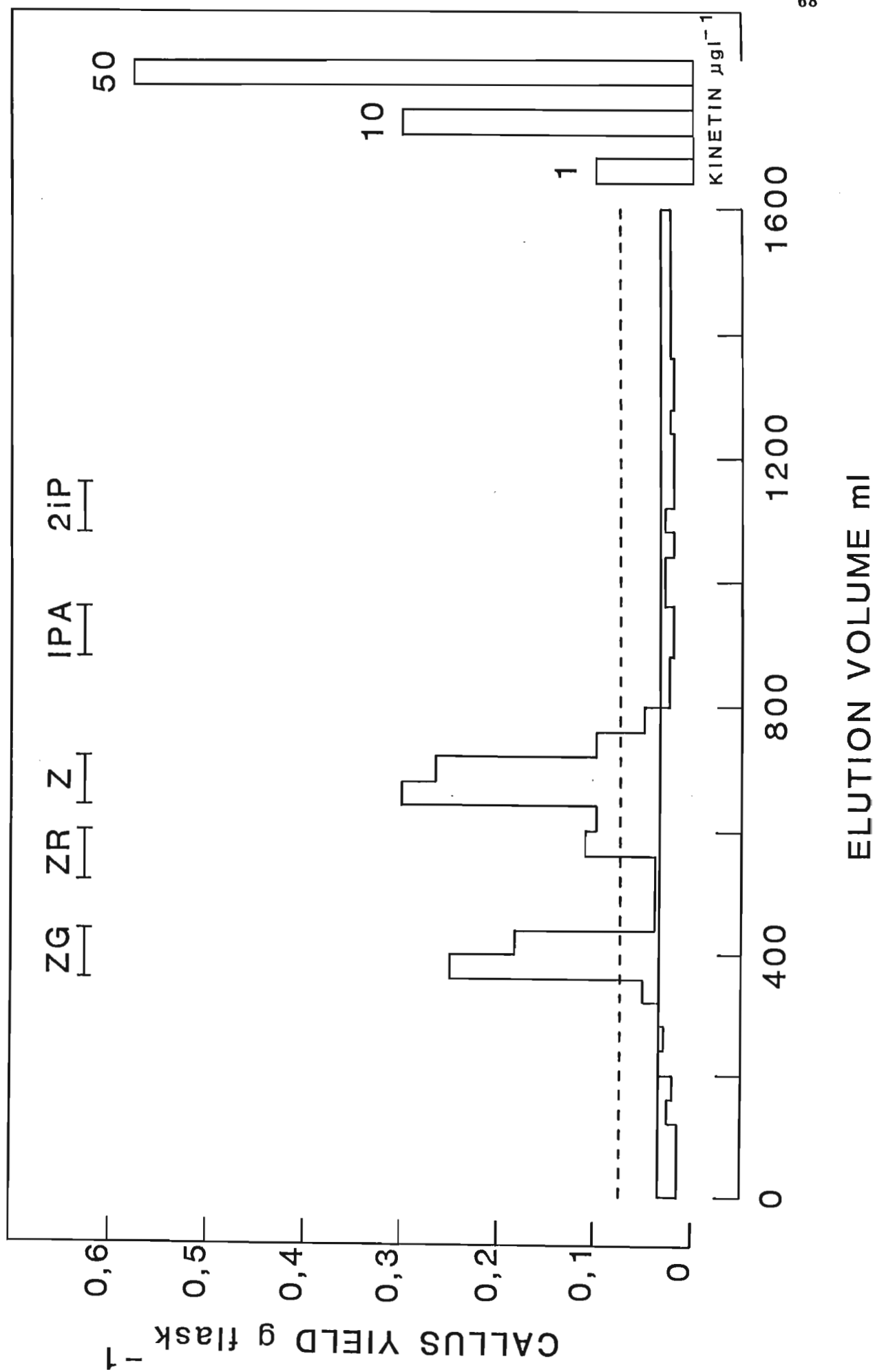
Figure 4.11   The cytokinin activity in 20 grammes of stipe material

of one metre plants harvested in October 1981

following fractionation on a Sephadex LH-20 column

eluted with 35 per cent ethanol. The extract was

initially purified by paper chromatography. The

region above the dotted line is significantly different

from the control at the level $P = 0,01$. ZG =

glucosylzeatin; ZR = ribosylzeatin; Z = zeatin; IPA

= isopentenyladenosine; 2iP = isopentenyladenine.
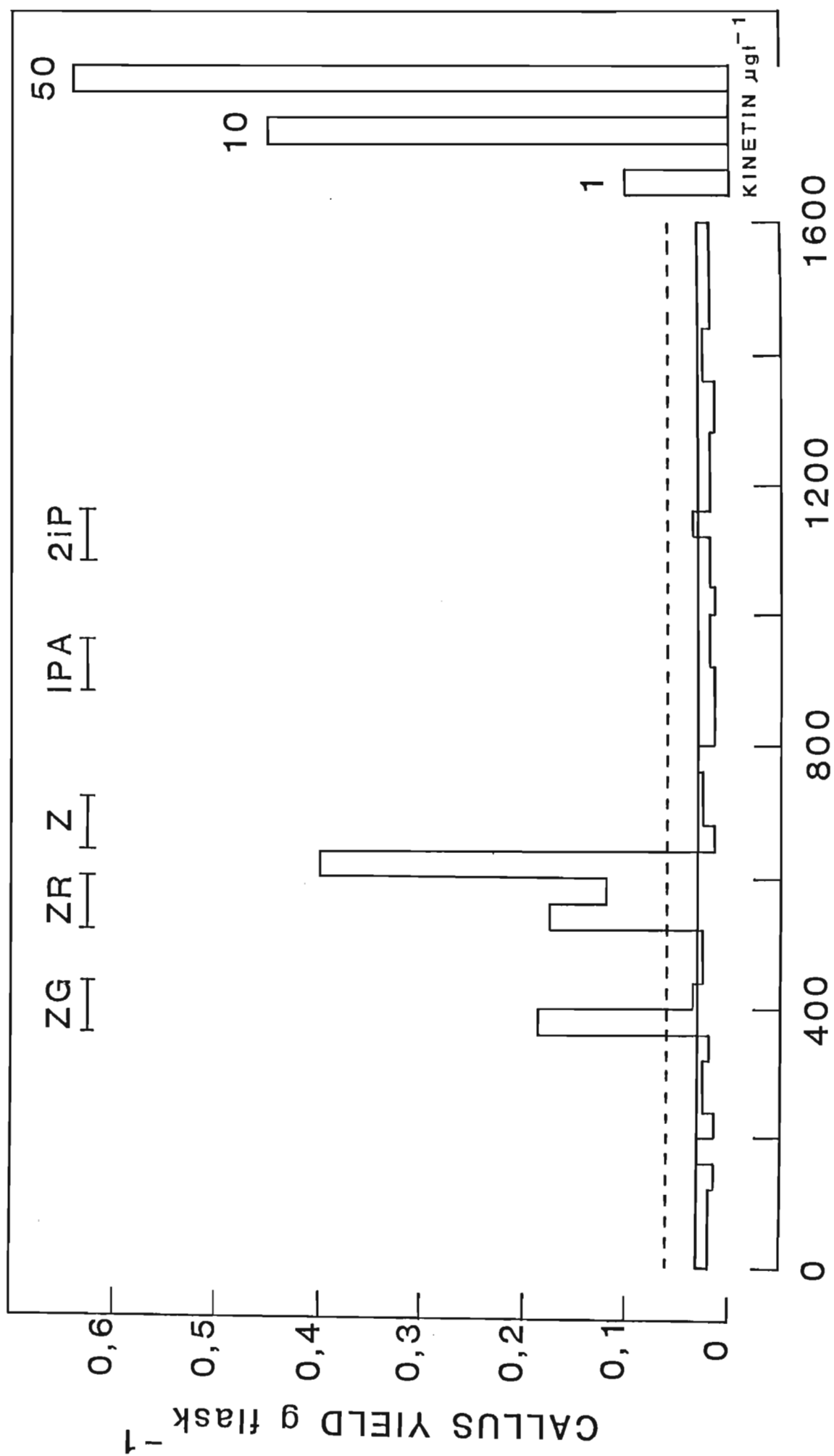
Figure 4.12   The cytokinin activity in 20 grammes of holdfast
               material of one metre plants harvested in September
               1981 following fractionation on a Sephadex LH-
               20 column eluted with 35 per cent ethanol. The
               extract   was   initially   purified   by   paper
               chromatography. The region above the dotted line
               is significantly different from the control at the
               level P = 0,01. ZG = glucosylzeatin; ZR =
               ribosylzeatin; Z = zeatin; IPA = isopentenyladenosine;
               2iP = isopentenyladenine.

CALLUS YIELD g flask^{-1}

ELUTION VOLUME ml

KINETIN µgl^{-1}

ZG ZR Z IPA 2iP

50 10 1

Figure 4.13　The total cytokinin activity in 20 grammes of lamina (▲-▲), stipe (o-o) and holdfast (●-●) regions of two metre *Ecklonia maxima* plants harvested at monthly intervals from February 1981 until January 1982. Activity detected after paper chromatography which was significantly different from the controls is expressed as kinetin equivalents.
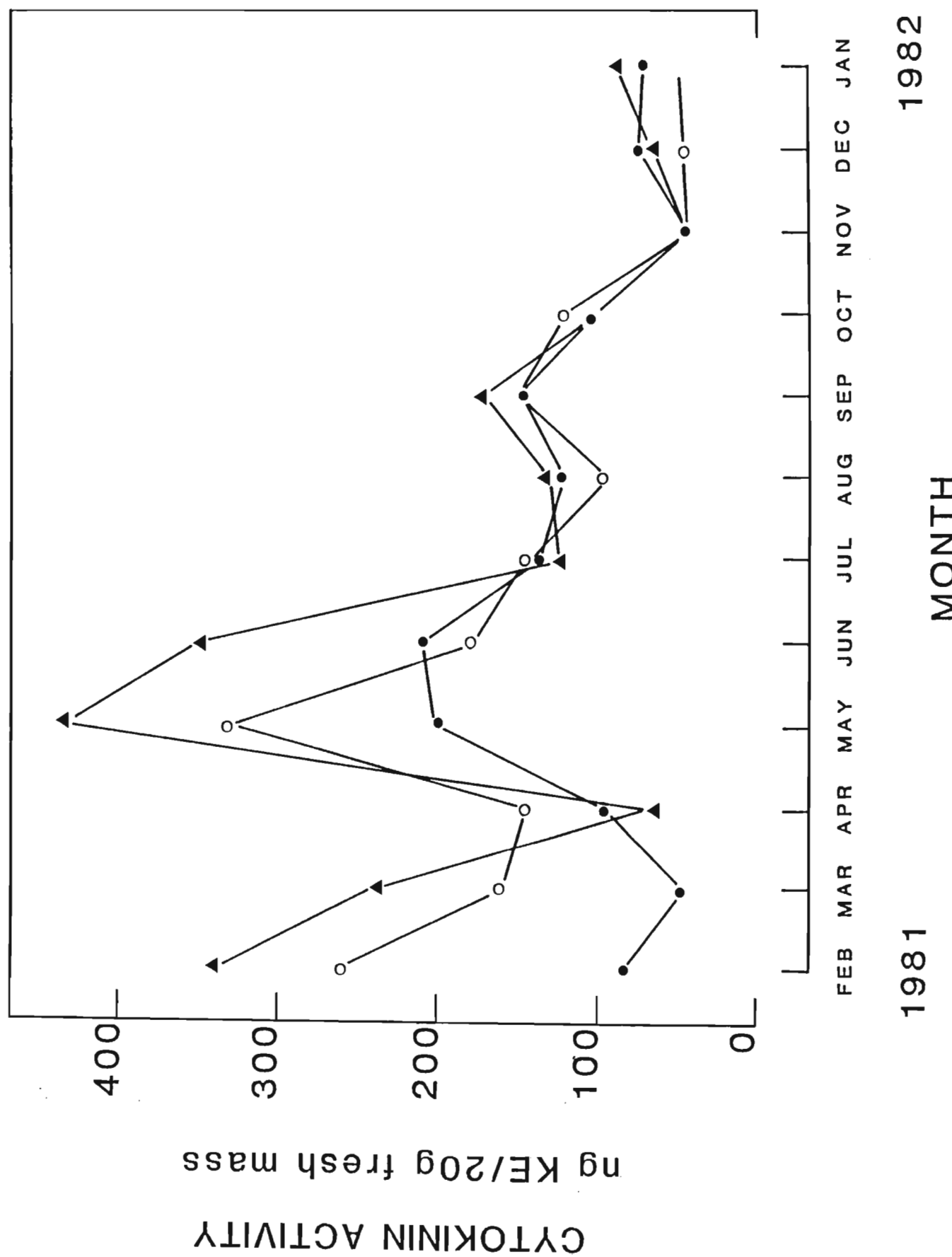
which were significantly different from the controls were expressed as ng kinetin equivalents $20g^{-1}$ fresh material and is presented graphically in Figures 4.14 - 4.16. Detectable levels of cytokinin-like activity co-chromatographing with glucosylzeatin became evident in all three tissues examined in May. High levels of activity co-chromatographing with glucosylzeatin were present in the lamina and holdfasts of plants harvested in June (Figures 4.14 and 4.16 respectively), and in the stipes of plants harvested in June and July (Figure 4.15). Activity co-chromatographing with glucosylzeatin present in the stipes in June and July exceeded the level of activity which co-chromatographed with ribosylzeatin and zeatin (Figure 4.15).

Cytokinin activity detected in the lamina, stipe and holdfast regions of plants harvested over the 30 day period from April - May 1983 co-chromatographed with zeatin, ribosylzeatin and their dihydroderivatives. When the detected activity was expressed as kinetin equivalents (Figures 4.17 - 4.19) it was evident that the fluctuations in the levels of cytokinins in the different regions of the plant were correlated with the phases of the moon. The activity recorded reached a peak at each quarter of the moon, then decreased in the following 24 - 48 hour period. The level of cytokinin glucosides detected in the different regions of the plant were negligible.

Three metre plants

The cytokinin activity detected in lamina, stipe and holdfast regions of three metre plants expressed as ng kinetin equivalents $20g^{-1}$ fresh material is presented graphically in Figure 4.20. High levels of activity were evident in the stipe and holdfast regions of the plant in June. The majority of the cytokinin activity present in the holdfast in June co-chromatographed with glucosylzeatin

Figure 4.14   Combined zeatin and ribosylzeatin activity ($\bullet - \bullet$)
and glucosylzeatin activity ($\circ - \circ$) detected in the
lamina of two metre *Ecklonia maxima* plants harvested
at monthly intervals from February 1981 until January
1982. Activity detected after paper chromatography
which was significantly different from the controls
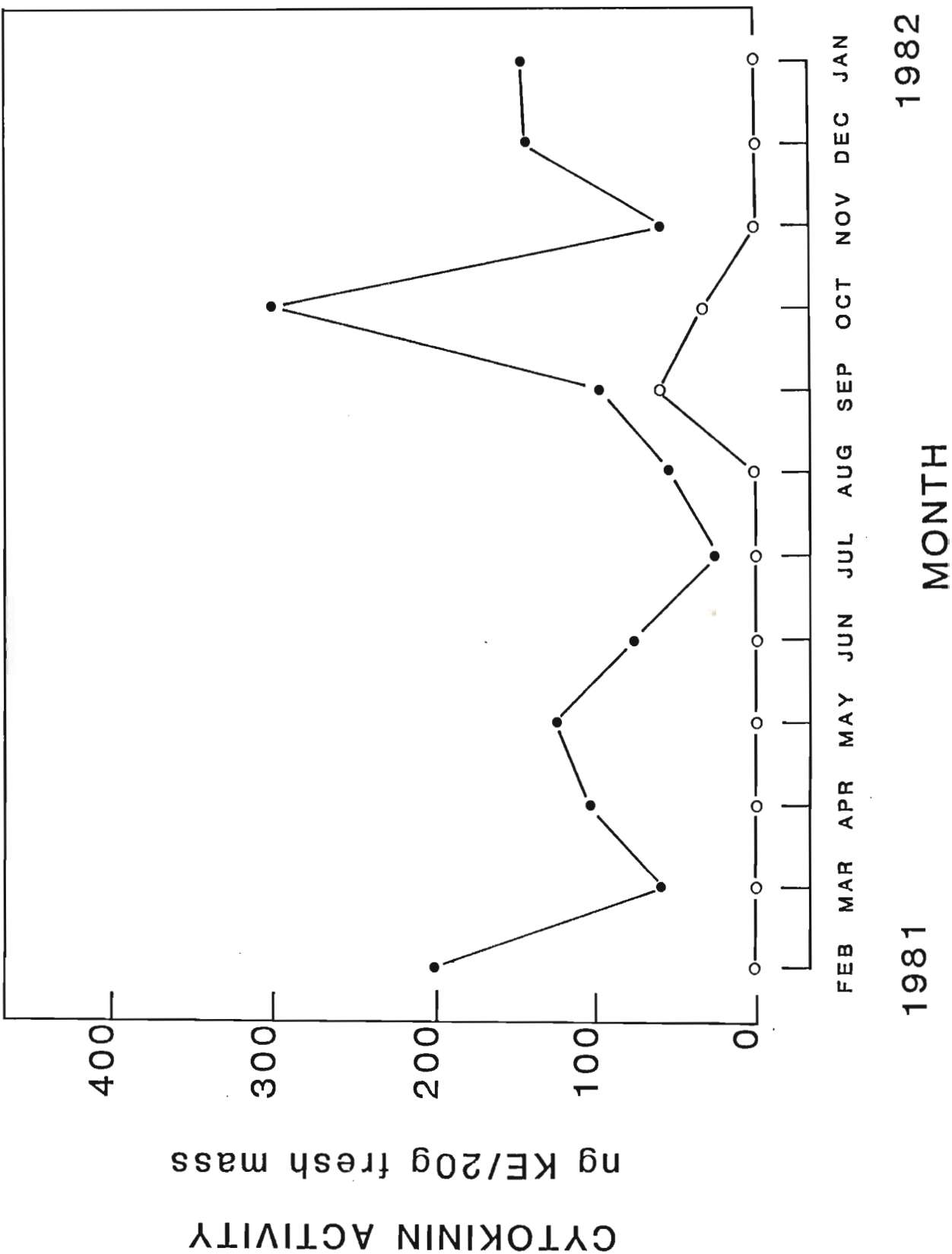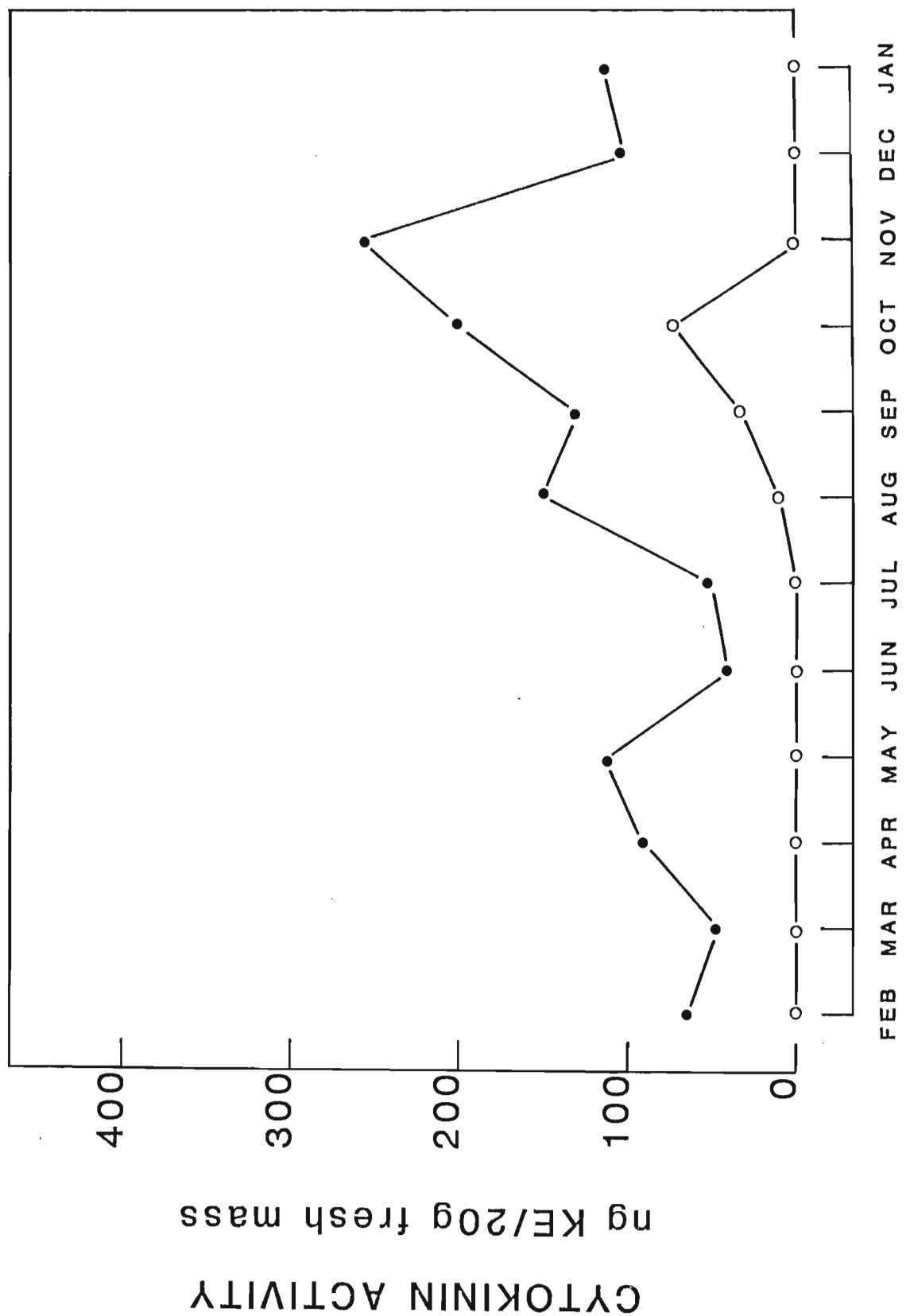is expressed as kinetin equivalents.
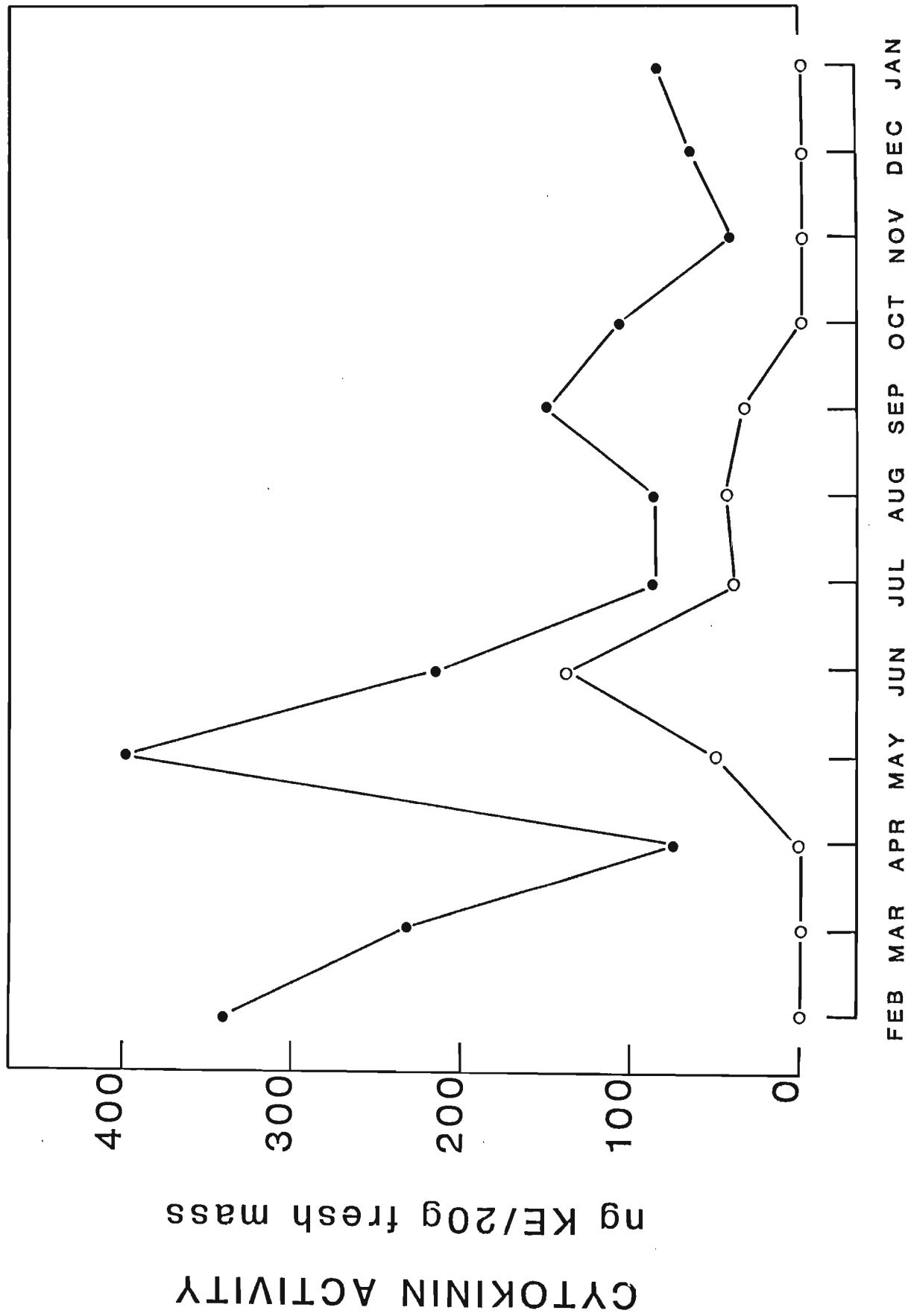
Figure 4.15   Combined zeatin and ribosylzeatin activity (●–●) and glucosylzeatin activity (o–o) detected in the stipes of two metre *Ecklonia maxima* plants harvested at monthly intervals from February 1981 until January 1982. Activity detected after paper chromatography which was significantly different from the controls is expressed as kinetin equivalents.
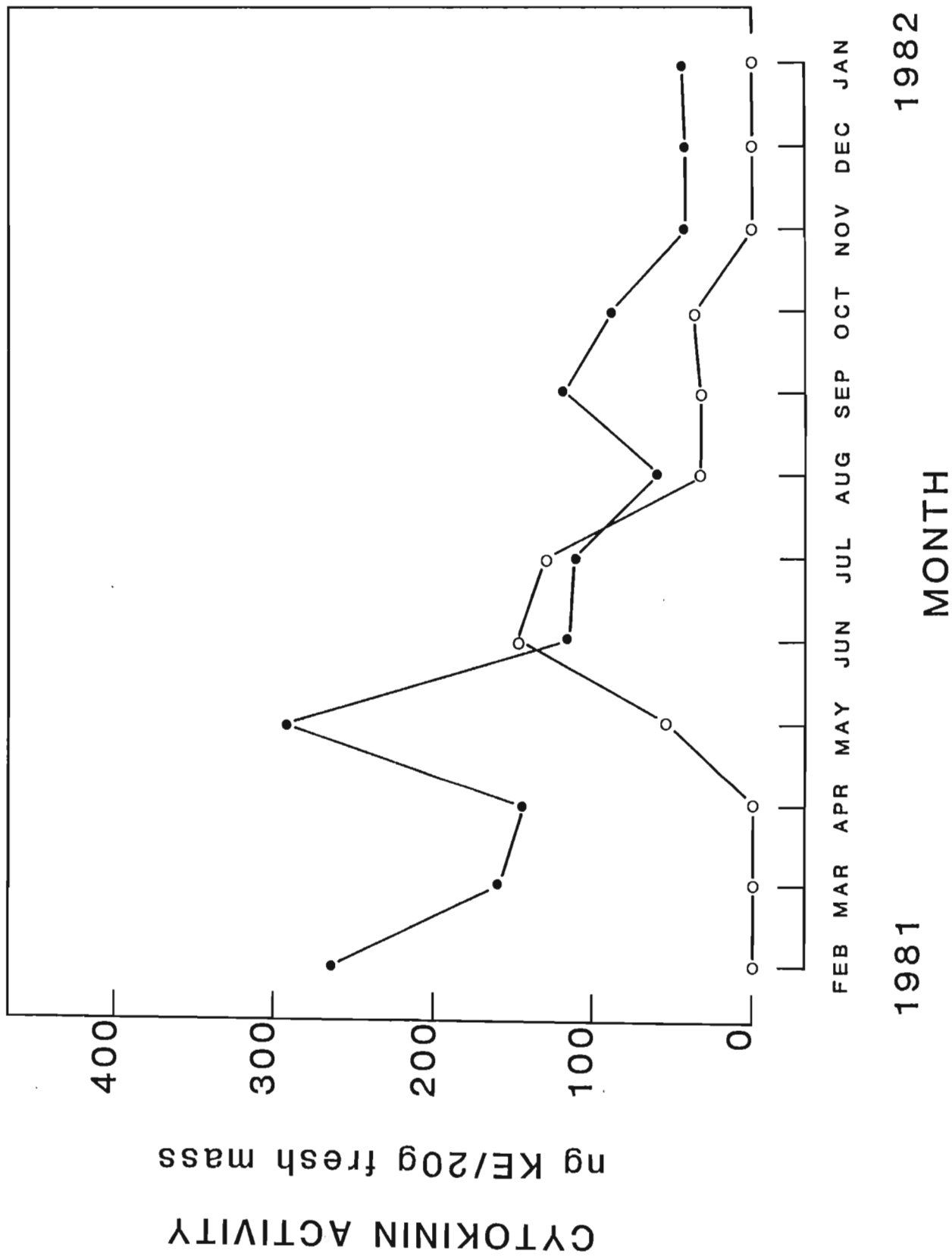
Figure 4.16   Combined zeatin and ribosylzeatin activity (● – ●) and glucosylzeatin activity (o – o) detected in the holdfasts of two metre *Ecklonia maxima* plants harvested at monthly intervals from February 1981 until January 1982. Activity detected after paper chromatography which was significantly different from the controls is expressed as kinetin equivalents.
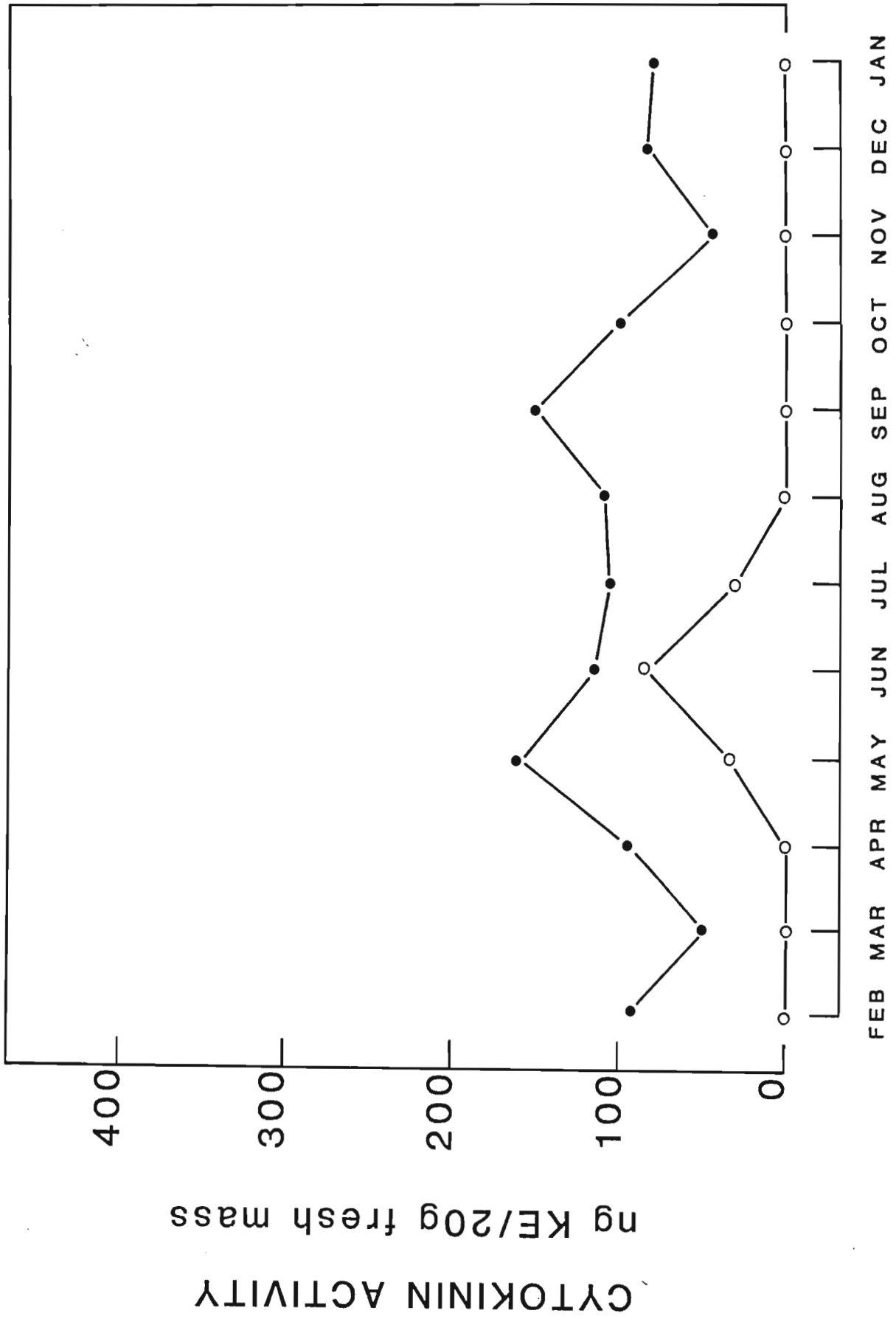
Figure 4.17   Cytokinin activity in the lamina of two metre *Ecklonia maxima* plants during the lunar cycle study. Activity detected after paper chromatography which was significantly different from the controls is expressed as kinetin equivalents.
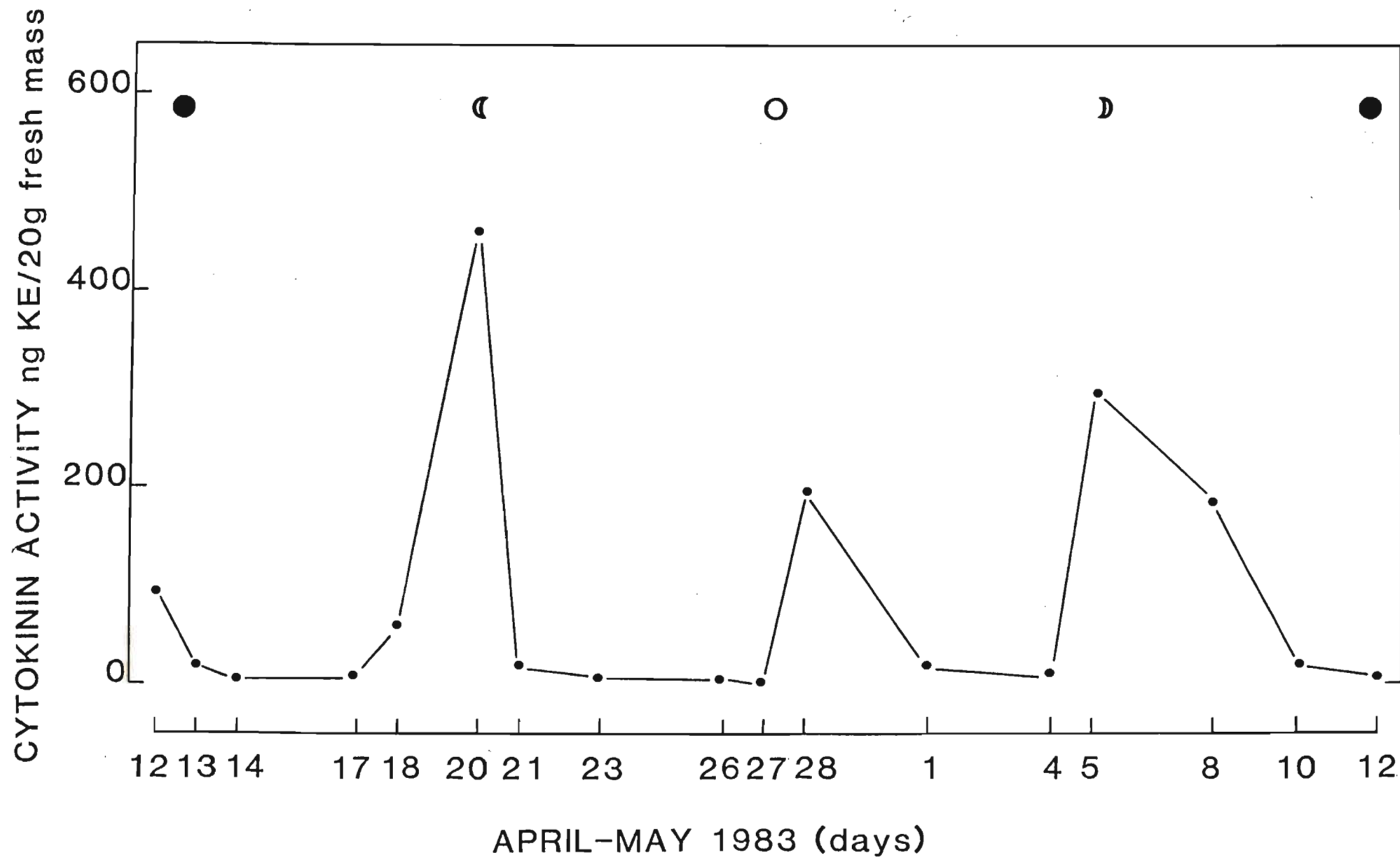
Figure 4.18   Cytokinin activity in the stipes of two metre
               Ecklonia maxima plants during the lunar
               cycle study. Activity detected after paper
               chromatography which was significantly
               different from the controls is expressed as
               kinetin equivalents.
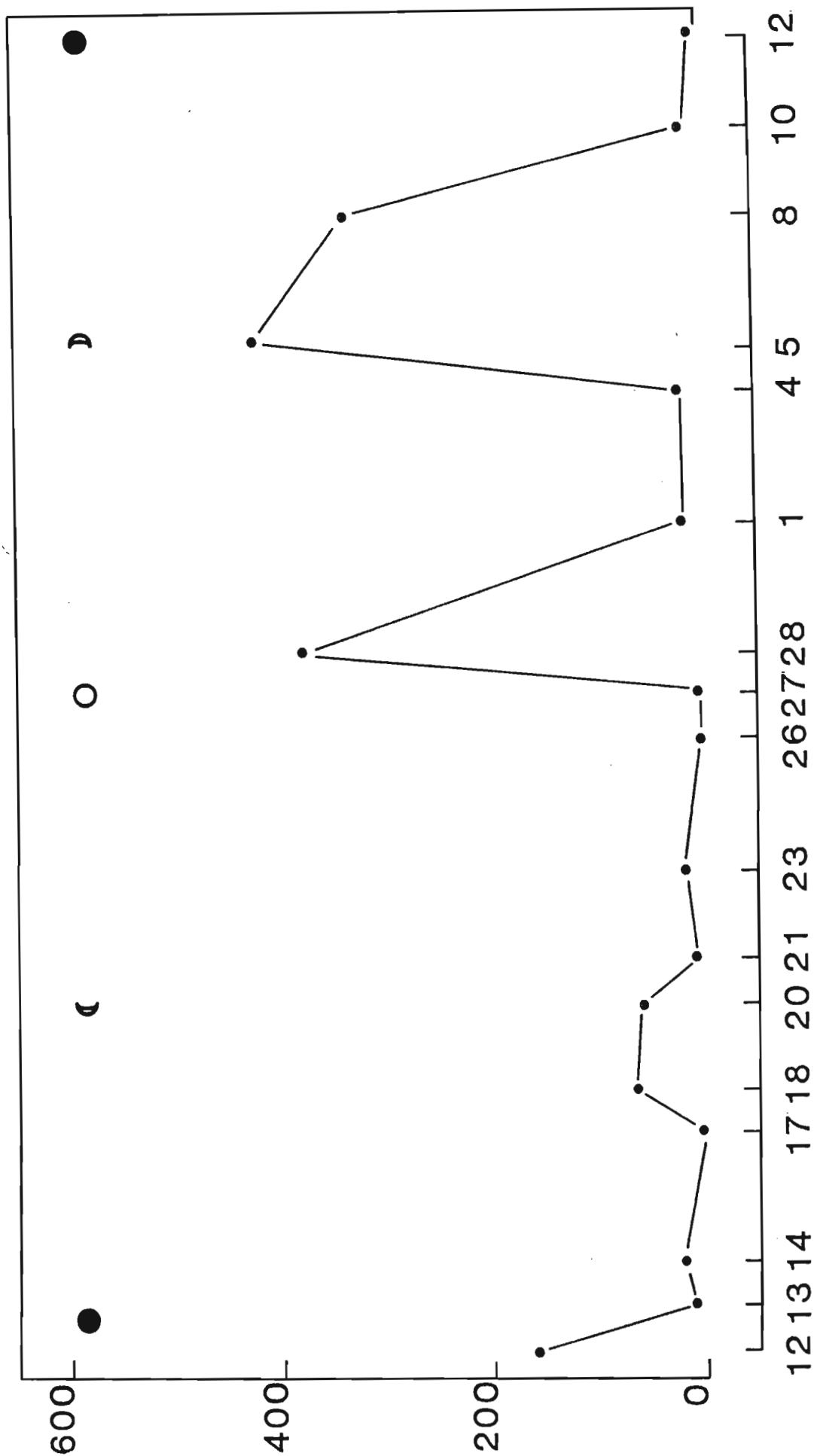
Figure 4.19   Cytokinin activity in the holdfasts of two
               metre *Ecklonia maxima* plants during the
               lunar cycle study. Activity detected after
               paper chromatography which was significantly
               different from the controls is expressed as
               kinetin equivalents.

(Figure 4.21). Cytokinin glucosides were not detected in lamina and stipe extracts. The level of cytokinins present in extracts of lamina material were low throughout the study period (Figure 4.20).

Discussion

There have been numerous reports on seasonal changes in cytokinins of terrestrial plant tissues. Most of these studies have dealt specifically with changes in the endogenous cytokinin levels in leaves (ENGELBRECHT, 1971; HEWETT and WAREING, 1973a,b; VAN STADEN, 1976b and 1977; HENSON, 1978a,b; DAVEY and VAN STADEN, 1978; VAN STADEN and DAVEY, 1978 and 1981; HENDRY, VAN STADEN and ALLEN, 1982). Research has shown that the cytokinin activity of expanding leaves is usually low (VAN STADEN, 1976a; DAVEY and VAN STADEN, 1978; HENSON 1978a; HENDRY, VAN STADEN and ALLAN, 1982) probably due to the rapid utilization of cytokinins in these organs. Zeatin and ribosylzeatin appear to be 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 (VAN STADEN, 1976a, b; HENSON, 1978a; VAN STADEN and DAVEY, 1981) or as conditions unfavourable for growth approach (LORENZI, HORGAN and WAREING, 1975; HENDRY, VAN STADEN and ALLEN, 1982), there is usually an increase in cytokinin activity, as well as a change in the predominant cytokinin form. Cytokinin glucosides appear to be the major cytokinins present during these periods.

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,
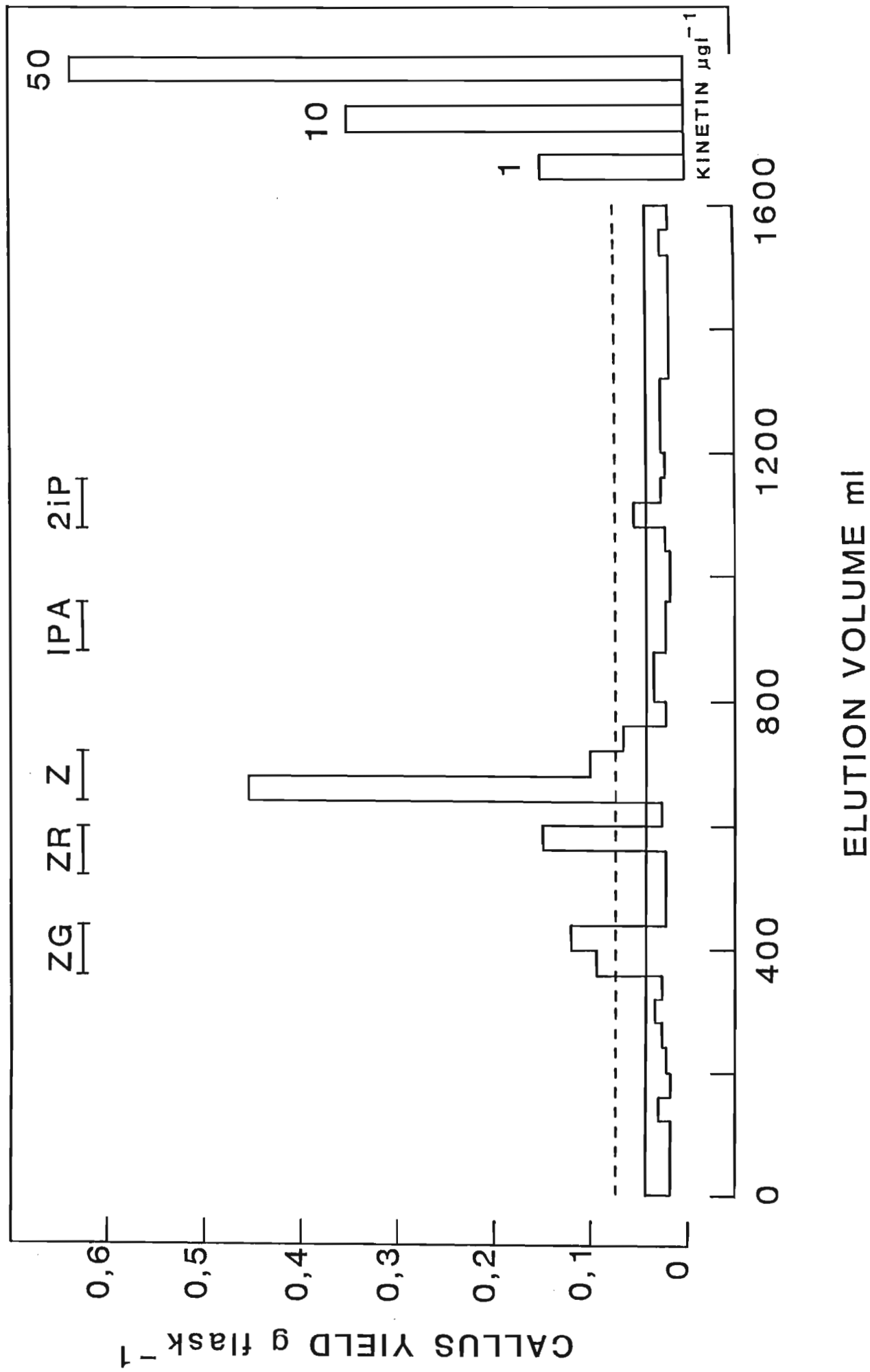
Figure 4.20 The total cytokinin activity in 20 grammes of lamina
(▲–▲), stipe (o–o) and holdfast (●–●) regions of three
metre *Ecklonia maxima* plants harvested at monthly
intervals from February 1981 until January 1982.
Activity detected after paper chromatography which
was significantly different from the controls is
expressed as kinetin equivalents.
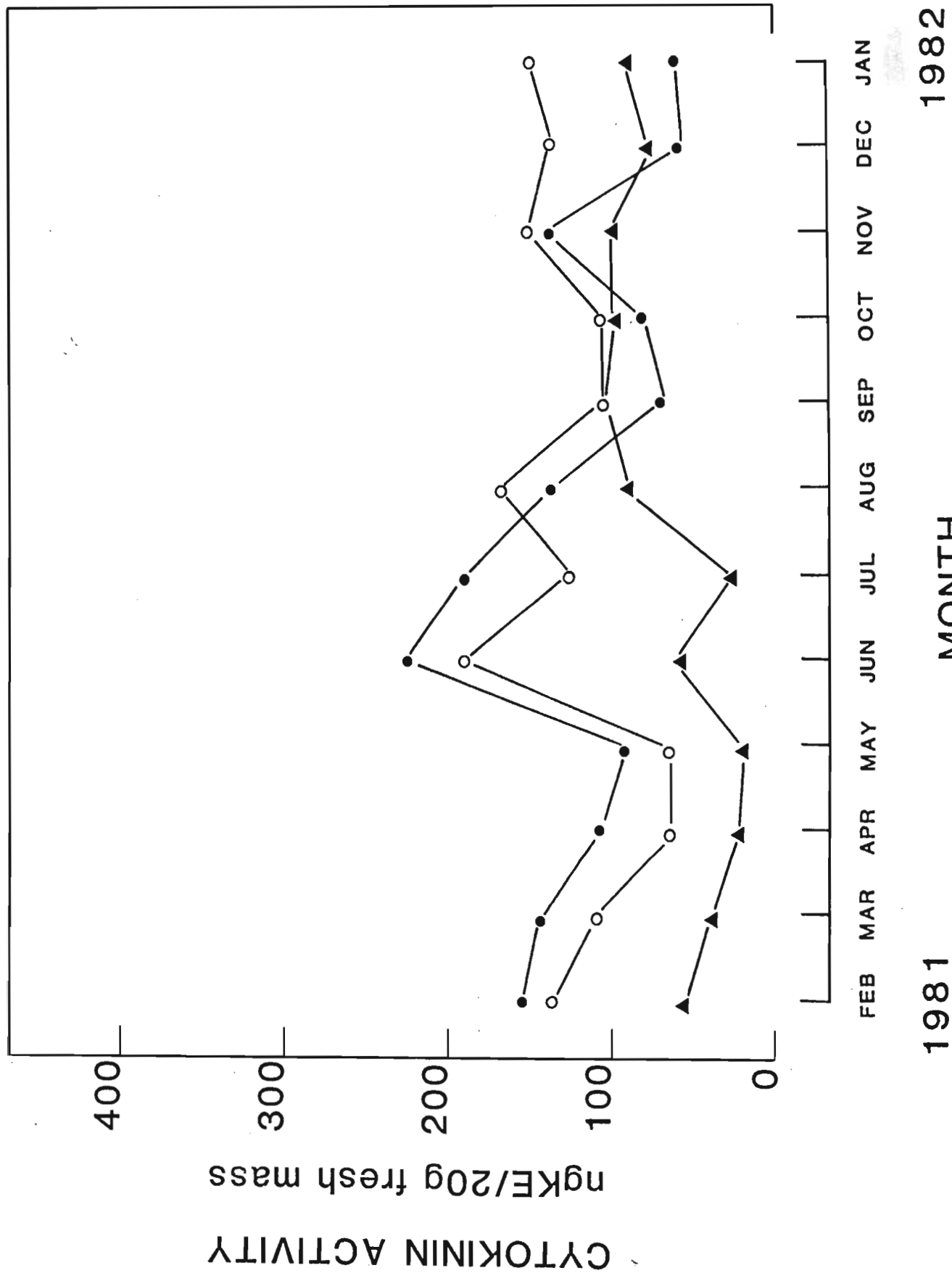
Figure 4.21   Combined zeatin and ribosylzeatin activity (●—●), and glycosylzeatin activity (o–o) detected in the holdfasts of three metre *Ecklonia maxima* plants harvested at monthly intervals from February 1981 until January 1982. Activity detected after paper chromatography which was significantly different from the controls is expressed as kinetin equivalents.
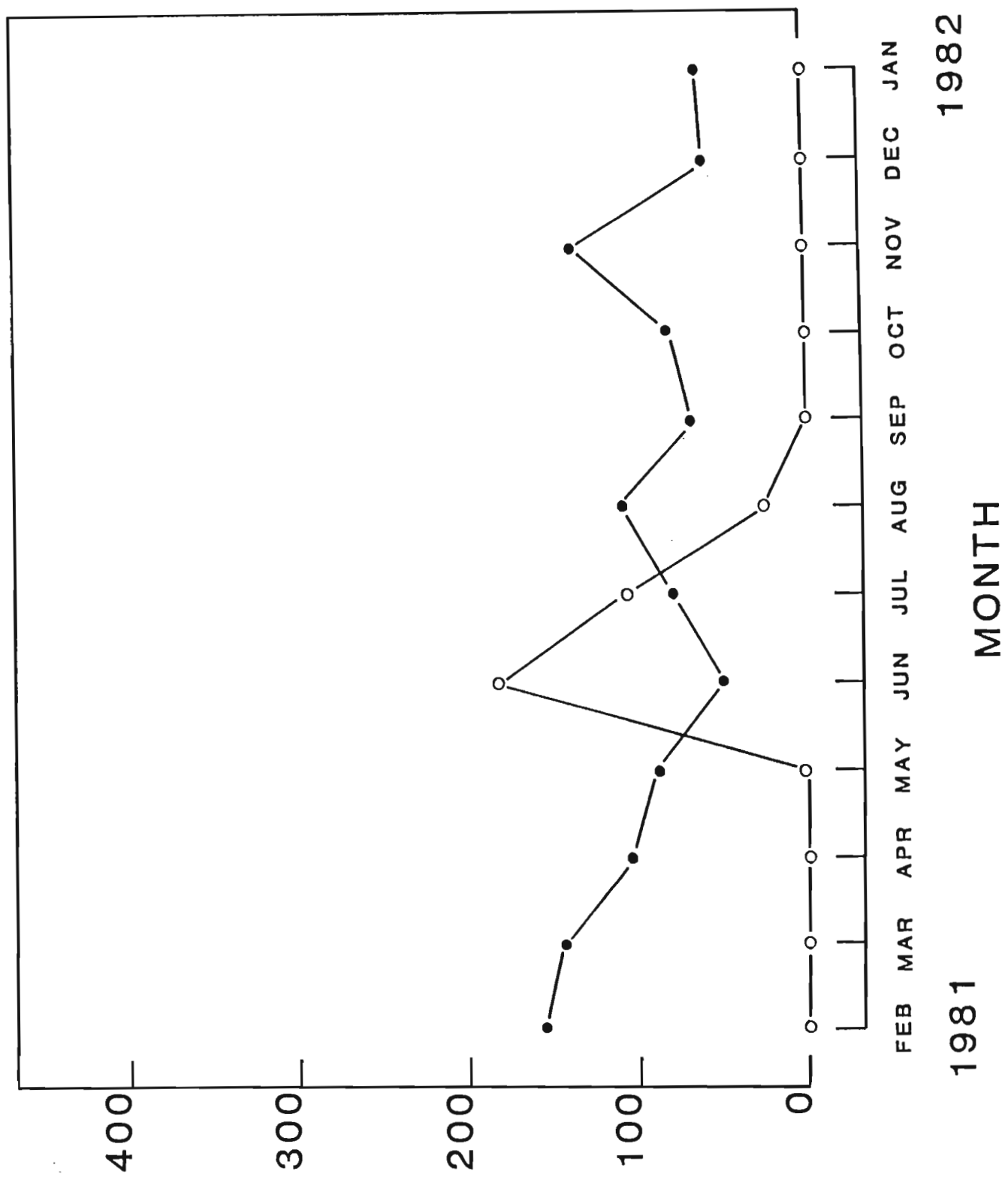
HORGAN, HENSON and DAVIS, 1976; HENSON and WHEELER, 1977; VAN STADEN and DAVEY, 1979; VONK and DAVELAAR, 1981), inactivation or detoxification products (HOAD, LOVEYS and SKENE, 1977; PARKER, LETHAM, GOLLNOW, SUMMONS, DUKE and MACLEOD, 1978), translocatable forms (VAN STADEN, 1976b; VAN STADEN and DIMALLA, 1980), active cytokinin forms (FOX, CORNETTE, DELEUZE, DYSON, GIERSAK, NIV, 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, 1977; PARKER, LETHAM, GOLLNOW, SUMMONS, DUKE and MACLEOD, 1978; VAN STADEN and DIMALLA, 1980; PALMER, SCOTT and HORGAN, 1981). The suggestion of storage compounds used in its widest sense to accommodate inactivation and transport compounds (VAN STADEN and DAVEY, 1979), is the most widely accepted function of cytokinin glucosides.

Based upon results obtained from the lamina, stipe and holdfast regions of two metre plants and extracts of processed *Ecklonia maxima* material, it would seem that the most likely function of the activity co-chromatographing with the cytokinin glucosides detected in these tissues is one of storage. The build up of cytokinin-like activity in autumn which had the same chromatographic properties as the cytokinin glucosides was accompanied by a drop in the level of free, active cytokinin bases and their ribosides. PALMER, HORGAN and WAREING (1981a,b) and VAN STADEN and DAVEY (1981) showed that when plant tissues accumulate cytokinin activity, much of the increase in activity appears to be due to O-glucoside metabolites, the level of which may rise much more markedly than that of cytokinin bases and/or ribosides. Further circumstantial evidence in support of the role of cytokinin glucosides as storage metabolites was presented by SUMMONS, ENTSCH, LETHAM, GOLLNOW and MACLEOD (1980) who showed that when cytokinins were no longer required, the excess was metabolised by processes which involved either side chain modification, such as glucosylation and/or side chain

cleavage. With the resumption of growth in spring there is a decline in the level of cytokinin glucosides accompanied by an increase in the level of free cytokinins. These results are similar to those of VAN STADEN and DIMALLA (1978) who found that the decline in the level of glucoside activity following the breaking of dormancy and apical bud growth in potato tubers was accompanied by a rise in the level of cytokinins which co-chromatographed with ribosylzeatin. HENDRY, VAN STADEN and ALLEN (1982) provided more evidence in support of the reutilization of cytokinin glucosides. They found that the high levels of cytokinin glucosides in evergreen leaves of Citrus 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 of *Ecklonia maxima* are hydrolyzed to active cytokinins during the periods of rapid growth.

Three metre plants exhibited similar seasonal trends in cytokinin activity to that which was recorded for two metre plants. That is low levels of activity were recorded in the lamina, stipe and holdfast tissues from February to May and from September to January. This corresponded with the period of most active growth of *Ecklonia maxima*. Cytokinin levels increased in the stipe and holdfast regions of the plant in autumn (June and July), corresponding with a reduction in growth during winter. It was significant that in three metre plants the level of cytokinin-like activity in the lamina remained low throughout the study period. The low levels of detectable cytokinin-like activity in the lamina could have been brought about in one of two ways, either by :

1)   a redistribution of cytokinins from the shoots (in particular the lamina) to the holdfast where they are metabolised and stored, principally in the form of glucosides, or

2) The low levels of activity in the lamina may be accounted for by the metabolism of cytokinins within the tissue itself.

Based upon the results of extracts of holdfast material the first hypothesis would appear to be the most feasible, since the reduced levels of cytokinins in the lamina during the inactive growth period (June to August) is accompanied by an increase in the level of cytokinins co-chromatographing with glucosylzeatin in holdfast material harvested at this time. Unfortunately, even if it is assumed that the low levels of cytokinins present in the lamina are as a result of the redistribution of cytokinins to the holdfast, it remains unknown whether the high levels of cytokinins present in the holdfast were synthesized in the lamina or in the holdfasts themselves.

In the lamina, stipe and holdfast tissues of one metre plants the highest levels of cytokinin-like activity were detected in material harvested in spring (September and October). These results are similar to those of LORENZI, HORGAN and WAREING (1975) and HENDRY, VAN STADEN and ALLEN (1982), who established that in evergreen species, cytokinin activity increases during the start of the growing season with zeatin and ribosylzeatin predominating during spring and summer. The presence of cytokinin activity co-chromatographing with glucosylzeatin was found to coincide with peaks of activity recorded in September and October in all three tissues examined. VAN STADEN and DAVEY (1979) suggested that the occurence of cytokinin glucosides in plant tissue could be a means of regulating cytokinin levels by inactivating excess free cytokinins. This assumption was supported by the work of CHEN (1981) who proposed that glucosylation could be a means of regulating cytokinin levels as supra-optimal cytokinin concentrations were shown to be inhibitory.

From the results of extracts of one metre plants it is evident that the highest level of detectable cytokinins at the start of the growing season were present in the lamina. It has been suggested that high concentrations of cytokinins found in a particular organ may be necessary for the creation of a strong physiological sink capable of competing with the remainder of the plant for nutrients (LUCKWILL, 1977). MOTHES and ENGELBRECHT (1961) demonstrated that sugars and amino acids can be transported preferentially to regions of high cytokinin activity. Therefore the presence of high cytokinin levels in the lamina may have created an active sink for translocated nutrients within the plant.

As has been previously mentioned in the Materials and Methods section, Kelp Products (Pty) Limited harvest two tons of *Ecklonia maxima* per day from a standing stock of about five to ten kilograms metre^{-2} for use in the manufacture of the seaweed concentrate Kelpak 66. It is essential in a venture of this nature to establish the best tissue to use in the manufacture of Kelpak 66, and the best time of the year to harvest this material. Cytokinin analysis of fresh and processed *Ecklonia maxima* material revealed both qualitative and quantitative changes in the levels present in the various tissues. These changes were closely correlated to the seasonal patterns of growth of *Ecklonia maxima*. The highest levels of activity being associated with the lamina and stipe regions of one and two metre plants harvested during autumn and spring. Analysis of material harvested over a lunar cycle from April - May 1983, revealed that the time of the month at which material is harvested is also an important factor to be considered in order to obtain material with a high cytokinin content. The highest levels of activity were recorded in the stipes of plants harvested at new moon and first quarter.

Of the three different size classes anaylsed for cytokinins, plants of approximately two metres in length form the bulk of the material harvested by

Kelp Products for the manufacture of seaweed concentrate. Plants of one metre and less are left intact as it has been demonstrated (SIMONS and JARMAN, 1981), that it is essential to leave a remnant stand of young subcanopy sporophytes for good kelp-recruitment to occur in a harvested area. Plants of three metres or more in length have a reduced cytokinin content compared to two metre plants as well as bearing numerous epiphytes. Difficulty in removal of these epiphytes before processing reduces the desirability of these plants for Kelpak 66 manufacture.

The fact that plants of approximatley two metres in length are the most suitable for use in the production of Kelpak 66 is born out in the results of cytokinin analyses of both two metre plants and processed material. A comparison of these results reveals similar trends in the cytokinin levels over the harvesting period.

CHAPTER V

THE EFFECT OF SEAWEED CONCENTRATE ON THE GROWTH

OF *LYCOPERSICON ESCULENTUM* PLANTS IN

NEMATODE INFESTED SOIL

Introduction

The application of seaweed to improve the growth of terrestrial plants is fast becoming an accepted practice. It is however, only recently that the effects of seaweed treatments have been documented. As mentioned in Chapter I the reported beneficial effects of seaweed include, improved overall plant vigour, improved yield quality and quantity, improved ability of plants to withstand adverse environmental conditions, and improved resistance of plants to disease attack. While the principal active component of seaweed is unknown, it is likely that a number of factors each play an important role in bringing about the responses of plants to seaweed application. Of these factors, cytokinins have been singled out as being of particular importance and have been shown to be present in relatively large amounts in commercial extracts prepared from marine algae (BRAIN, CHALOPIN, TURNER, BLUNDEN and WILDGOOSE, 1973; BLUNDEN, 1977).

An aspect worth considering is that seaweed extracts, in view of their cytokinin content, may effect the resistance of plants to disease. While not eliminating the infestation itself, the applied cytokinins apparently allows the plant to increase its resistance to the disease. Applied kinetin inhibited powdery mildew on *Cucumis sativus* plants (DEKKER, 1963) and decreased the occurrence of stem rust in *Triticum aestivum* (WANG, HAO and WAYWOOD, 1961). In the case of root-knot

nematode infestation, it has been shown that high concentrations of kinetin not only decreased larval penetration into the roots of *Lycopersicon esculentum* plants, but also inhibited the development of those which did enter (DROPKIN, HELGESON and UPPER, 1969). BRUESKE and BERGESON (1972) reported that infestation of the roots with *Meloidogyne incognita* (KOFOID and WHITE) CHITWOOD resulted in decreased cytokinin levels in both the roots and the root exudate of *Lycopersicon esculentum* plants. This decrease in cytokinins may be responsible for the reduced shoot growth associated with nematode infestation. It is in counter-acting this imbalance that the effect of seaweed concentrate may be reflected.

In this study, the effect of seaweed concentrate on the growth of *Lycopersicon esculentum* plants in nematode infested soil was investigated.

Experimental procedure and results.

Plants of *Lycopersicon esculentum* MILL. (cultivar "Moneymaker") were used. Plants were grown in two seasons (winter 1981 and summer 1981/82) under greenhouse conditions. Seeds were germinated in vermiculite and seedlings of approximately 10 centimeters in height were transplanted into a medium (sand : loam : peat moss, 1 : 2 : 1) which was heavily infested with *Meloidogyne incognita* and subsequently treated with seaweed concentrate as indicated in Table 5.1. The seaweed concentrate (Kelpak 66) used in this investigation has been previously described in the Materials and Methods section.

The experimental plants were watered regularly with tap water and were not fertilized during the course of the experiment. No fungicides or pesticides were applied, but the control plants did receive one application of an insecticide when the red spider mite, *Tetranychus cinnabarinus* (BOISDUVAL), was observed. Ten plants

TABLE 5.1

Outline of treatments used to assess the effect of foliar and soil applications of seaweed concentrate on the growth of *Lycopersicon esculentum* plants.

	Control	Foliar Sprays					Soil Flush
		1	2	3	4	5	
Number of seaweed concentrate (Kelpak 66) applications	0	1	2	3	4	5	1
Volume of seaweed concentrate (Kelpak 66) (1:500) applied per plant (cm^3)	0	10	20	30	40	50	500

were used for each treatment. The first foliar application and soil flush were at transplanting, subsequent foliar applications were made every 15 days. The plants grown in the winter of 1981 were harvested after 2,5 months and those of summer, 1981/82 after 3 months. After harvesting, the fresh mass of the various plant parts was determined, the material was then dried for 72 hours at 60°C and the dry mass obtained. Least significant differences (where P<0,05) for all data were calculated after performing an analysis of variance.

Processed seaweed concentrate, which was harvested in February 1981, and which was applied to the plants as indicated in Table 5.1, was used for cytokinin extraction and determination. The cytokinins were extracted and purified as described in the Materials and Methods section and the activity of the cytokinin-like compounds was estimated by means of the soybean callus bioassay (MILLER, 1965). In order to obtain more information as to the nature of the cytokinin-like activity recorded on paper chromatograms, 20 grammes of fresh material was extracted using Dowex 50 and the Dowex 50 extract loaded onto a Sephadex LH-20 column eluted with 35 per cent ethanol as described in the Materials and Methods section. Forty-millilitre fractions were collected, dried in air and each fraction assayed for cell division activity.

In order to determine the effects of seaweed concentrate on the nematode population within a soil, a soil medium (sand : loam : peat moss 1 : 2 : 1) infested with *Meloidogyne incognita* was prepared and subsequently flushed twice, at weekly intervals with seaweed concentrate at a dilution of 1 : 500. Nematode counts were conducted on both the treated and untreated soils prior to, 2 days after, 1 week after and 2 weeks after seaweed application. This experiment was repeated on soil in pots in which *Lycopersicon esculentum* plants had been established (10 centimetres in height). Nematode counts were conducted on soil and root samples from both the treated and untreated controls prior to, and 16 days after treatment. Each treatment

consisted of 4 replicates and the nematodes were extracted and counted as described in the Materials and Methods section. Least significant differences (where P<0,05) for all data were calculated after performing an analysis of variance.

From the results in Figure 5.1 it is evident that the seaweed concentrate, collected in February 1981, contained considerable cytokinin activity. Most of the activity co-chromatographed with zeatin and ribosylzeatin. Some activity was also detected at Rf 0.1 – 0.2 and co-chromatographed with glucosylzeatin.

Sephadex LH-20 fractionation of a similar Dowex 50 extract to that used for paper chromatography showed that the activity recorded on paper chromatograms was due to 2 components (Figure 5.2). The first occurred at an elution volume of 600 – 640 millilitres and co-eluted with zeatin, the second had an elution volume of 1120 – 1160 millilitres and co-eluted with isopentenyladenine.

With the application of seaweed concentrate to *Lycopersicon esculentum* plants similar results were obtained in both the summer and winter experiments, therefore only the summer results are presented. The overall appearance of the plants treated with seaweed concentrate was better than that of the control plants. Maximum growth was obtained with those plants which received the greatest number of foliar applications. Significant increases were detected in root fresh mass, stem fresh mass, leaf fresh mass and fruit fresh mass (Table 5.2).

This increased growth was also reflected in the results of the dry mass (Table 5.3). Five foliar applications of seaweed concentrate significantly improved the yield of treated plants over that of the control. The greatest yield increase, however, was obtained when, instead of regular foliar applications, the soil was flushed once with diluted seaweed concentrate (1 : 500) at the time of transplanting the seedlings (Tables 5.2 and 5.3).

Figure 5.1 Cytokinin activity detected in an extract of 20 grammes of seaweed concentrate harvested during February 1981. Dowex 50 purified extracts were separated on paper using *iso*-propanol : 25 per cent ammonium hydroxide : water (10 : 1 : 1 v/v). The region above the dotted line is significantly different from the control at the level P=0,01. ZR = ribosylzeatin; Z = zeatin; ZG = glucosylzeatin.
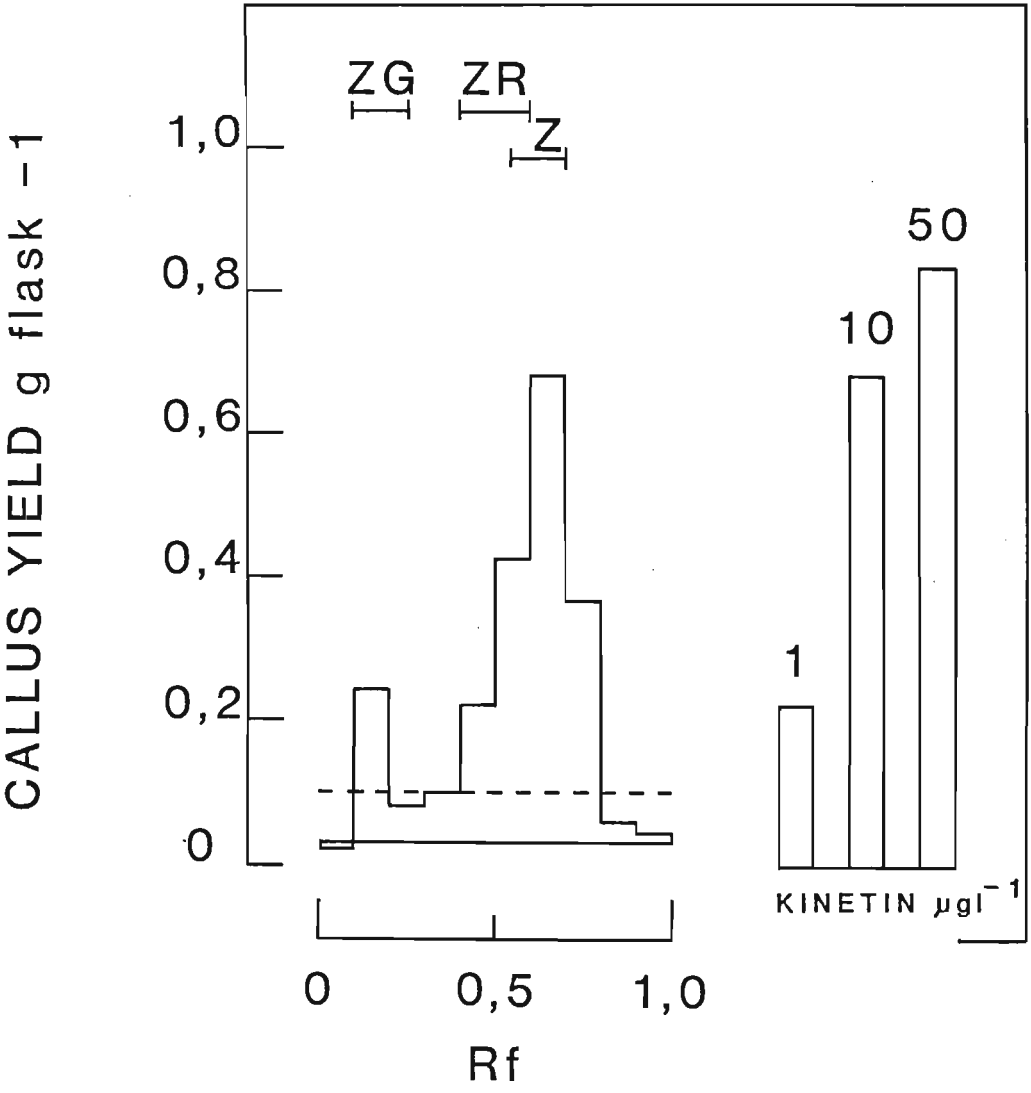
Figure 5.2 Cytokinin activity detected in 20 grammes of seaweed concentrate harvested during February 1981. The Dowex 50 purified extract was fractionated on a Sephadex LH-20 column eluted with 35 per cent ethanol. The region above the dotted line is significantly different from the control at the level $P=0,01$. ZR = ribosylzeatin; Z = zeatin; IPA = isopentenyladenosine, 2iP = isopentenyladenine.
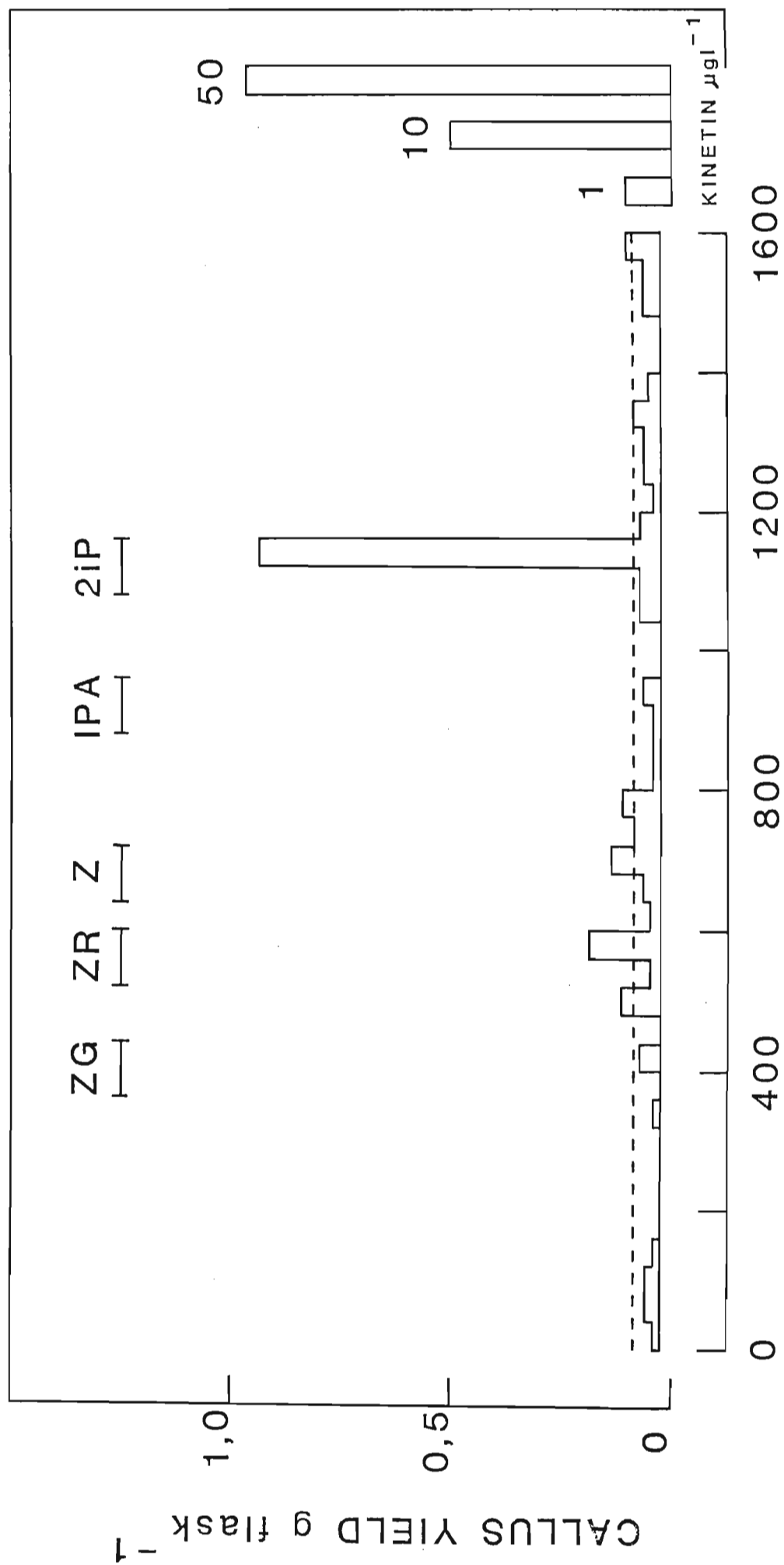
TABLE 5.2

The effect of seaweed treatment on the fresh mass (grammes) of *Lycopersicon esculentum* fruits, leaves, stems and roots grown under summer conditions. Treatments 1 – 5 represent the number of foliar sprays as indicated in Table 5.1. Means for the parameter measured followed by the same letter do not differ significantly (P < 0,05)

Treatment	Parameter measured				
	Fruits	Leaves	Stems	Roots	Total
Control	1.1a	17.5a	16.8a	5a	40.1a
1	2.9a	17.2a	13.9a	4a	40.2a
2	6.9ab	20.3a	19.8b	4.2a	55.3b
3	3.9a	23.7b	20.2b	7.1b	56.8b
4	13.9bc	30.6cd	22.3bc	7.5b	75.1c
5	16.8cd	28.9c	22.6cd	8b	76.5c
One Soil flush	26.1d	33.8d	25.4d	9.4c	96d
Total $\bar{x} \pm$ SE	10.2± 3.7	24.6± 1.8	20.2 ± 1.1	6.4 ± 0.5	62.8 ± 4.8

TABLE 5.3

The effect of seaweed treatment on the dry mass of *Lycopersicon esculentum* fruits, leaves, stems and roots grown under summer conditions. Treatments 1 – 5 represent the number of foliar sprays as indicated in Table 5.1. Means for the parameter measured followed by the same letter do not differ significantly (P < 0,05).

Treatment	Parameter measured				
	Fruits	Leaves	Stems	Roots	Total
Control	0.1a	1.9a	2.3b	1.1a	5.4a
1	0.3ab	1.9a	1.6a	0.8a	4.6a
2	0.7bc	2.3a	2.4b	1.1a	6.6b
3	0.4ab	3.0ab	3.0c	1.6b	7.9c
4	1.0c	3.5cd	2.9c	1.5b	8.9cd
5	1.2c	3.3bc	3.0c	2.0c	9.4de
One Soil flush	1.9d	3.8d	3.6d	2.1c	11.0e
Total $\bar{x}$ + SE	0.8 + 0.3	2.8 + 0.2	2.7 + 0.2	1.5 + 0.1	7.7 + 0.5

The roots of the control plants were severly infested with root-knot nematodes and their overall growth was significantly reduced when compared to the roots of plants which received 5 foliar applications of seaweed concentrate (Figure 5.3).

From the results in Table 5.4, it is evident that seaweed concentrate application to nematode-infested soil did not have any significant effect on the nematode population in the soil within the first 2 days after application. Although the number of nematodes declined during the course of the experiment, on days 9 and 16, the nematode counts were higher in the soils flushed with seaweed concentrate than those recorded for the control. In the second experiment, in which *Lycopersicon esculentum* seedlings were established in nematode infested soil, the nematode counts were significantly higher in the soil flushed twice with seaweed concentrate than those recorded for the control. Analysis of root material however, revealed that the number of nematodes which could be extracted from the roots of plants grown in seaweed concentrate treated soil were significantly lower than that of the control (Table 5.5).

Discussion

As was found with other seaweed extracts (BRAIN,CHALOPIN, TURNER, BLUNDEN and WILDGOOSE, 1973; BLUNDEN, 1977) the commercially available aqueous seaweed concentrate Kelpak 66 harvested in February 1981 contained considerable cytokinin activity. It has been suggested that the beneficial responses obtained with the use of seaweed extracts are similar to those observed when cytokinins are applied to plants (BOOTH, 1966). BLUNDEN and WILDGOOSE (1977) demonstrated close correlations between the results obtained from the use of kinetin and commercial seaweed extracts of equivalent cytokinin activites in field trials
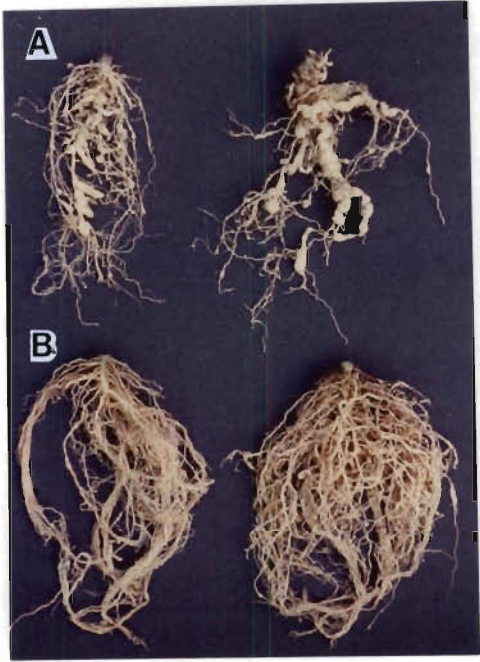
Figure 5.3   The effect of seaweed concentrate application on growth and nematode infestation of the roots of *Lycopersicon esculentum* plants.   A = control;   B = 5 foliar applications of seaweed concentrate.

TABLE 5.4

Number of nematodes detected in 100 millilitres of soil 0, 2,
9 and 16 days after being flushed with either distilled H_2O
(control) or seaweed concentrate (1 : 500). Means for the
parameter measured followed by the same letter do not differ
significantly ($P < 0,05$).

	Time after application (days)			
	0	2	9	16
Control	553	440a	48a	33a
Seaweed flush	553	425a	88b	60b
Total $\bar{x} \pm$ SE		432±20	68±11	46±17

TABLE 5.5

Number of nematodes extracted per 100 millilitres soil and 5 grammes root material, 16 days after the soil was flushed with either distilled H_2O (control) or seaweed concentrate (1 : 500). Means for the parameter measured followed by the same letter do not differ significantly (P < 0,05).

	Soil	Roots
Control	45a	58a
Seaweed flush	158b	35b
Total $\bar{x} \pm$ SE	101 ± 22	46 ± 10

conducted on *Solanum tuberosum* plants. They suggested that the effects of the seaweed extracts were due to their cytokinin content. Similar results were obtained when the shelf-life of *Citrus aurantiifolia* fruits was increased after post-harvest immersion of the fruit in kinetin and seaweed extracts of known cytokinin activity (BLUNDEN, JONES and PASSAM, 1978). The beneficial effects recorded for crops treated with aqueous seaweed extracts have included increased yields (SENN, MARTIN, CRAWFORD and DERTING, 1961; BOOTH, 1966; STEPHENSON, 1966; POVOLNY, 1971; BLUNDEN, 1972; BLUNDEN and WILDGOOSE, 1977; BLUNDEN, WILDGOOSE and NICHOLSON, 1979; KOTZE and JOUBERT, 1980; NELSON and VAN STADEN, 1983). In the present investigation, seaweed concentrate at a dilution of 1 : 500 improved the growth of *Lycopersicon esculentum* significantly irrespective of whether it was applied as a foliar spray at regular intervals or whether the soil medium was flushed once with diluted seaweed concentrate at transplanting.

It was significant that root growth was much improved whenever seaweed concentrate was applied and, in particular, that root-knot nematode infestation was visibly reduced in all cases where seaweed concentrate was applied. This undoubtedly resulted in improved root development, and thus more efficient moisture and nutrient utilization by the plants (WIDDOWSON, YEATES and HEALY, 1973). Results show that although the number of nematodes increased in the soil after the application of seaweed concentrate, the numbers which had established themselves in the roots were reduced when compared to the control. STEPHENSON (1968) reported on the beneficial effects of seaweed applications on the yield of *Lycopersicon esculentum* plants grown in nematode infested soils. Seaweed application was also found to reduce the requirements for the nematocides, dichloropropane and dichloropropylene. TARJAN (1977) found that seaweed applied either as a foliar application or directly to the soil, increased plant weight and decreased nematode infestation in *Citrus medica* L. (lemon) seedlings 17 weeks after application. He found, however, that seaweed application to old, established trees had no effect on yield or nematode

infestation within the roots of treated plants.

There exists in the literature numerous reports on the role of hormones, and in particular cytokinins, in nematode infestion and development in the roots of susceptible hosts (DROPKIN, HELGESON and UPPER, 1969; KOCHBA and SAMISH, 1971; SAWHNEY and WEBSTER, 1975). DROPKIN, HELGESON and UPPER (1969) found that high concentrations of kinetin inhibited both larval penetration and development in the roots of *Lycopersicon esculentum* plants. The levels of cytokinin found in seaweed concentrate may have had a similar effect on nematode infestation and development. BRUESKE and BERGESON (1972) found that infestation of the roots with *Meloidogyne incognita* resulted in decreased cytokinin levels in the root exudate of *Lycopersicon esculentum* plants. This decrease in cytokinin translocation to the shoots may be responsible for the reduced shoot growth associated with nematode infestation. The application of cytokinins found in seaweed concentrate may have been instrumental in overcoming this imbalance.

Apart from the influence of growth regulators, in particular cytokinins, on nematode infestation in plants, WALLACE (1970) found significant interactions between high nematode numbers and low soil fertility in pot trials conducted on *Lycopersicon esculentum* plants. He found that in a well fertilised soil a plant may tolerate a particular nematode population, but, in an infertile soil the same population might cause marked reductions in growth and yield. WALLACE (1970) postulated that there could be an interaction between nematode numbers and soil fertility on plant growth. The levels of inorganic nutrients present in seaweed concentrate may have been instrumental in allowing the treated plants to tolerate the nematode population present in the soil. Untreated control plants were, however, deprived of a nutrient source and were thus susceptible to nematode infestation and suffered marked reductions in growth and yield.

CHAPTER VI

THE EFFECT OF SEAWEED CONCENTRATE AND FERTILIZER

ON GROWTH AND THE CYTOKININ CONTENT OF

BETA VULGARIS AND *PHASEOLUS VULGARIS* PLANTS

Introduction

The beneficial effect of seaweed concentrate on the growth and yield of field crops (BLUNDEN and WILDGOOSE, 1977; BLUNDEN, WILDGOOSE and NICHOLSON, 1979) and the possible involvement of hormones, in particular cytokinins (BOOTH, 1966; BLUNDEN and WILDGOOSE, 1977), is now well established. There has however been little research conducted to determine the effect of seaweed concentrate, which is known to have a high cytokinin content (Chapter IV), on the endogenous levels of cytokinins within vegetative and reproductive organs of treated plants.

It is well documented that cytokinins are involved in nutrient mobilization in vegetative plant organs (MOTHES and ENGELBRECHT, 1961; TURREY and PATRICK, 1979; GERSANI and KENDE, 1982). It has also been suggested that the presence of high levels of cytokinins in reproductive organs (LETHAM, 1973; DAVEY and VAN STADEN, 1977; SUMMONS, ENTSCH, LETHAM, GOLLNOW and MACLEOD, 1980) may be associated with the mobilization process (WAREING and SETH, 1967). VARGA and BRUINSMA (1974) reported that seeds and fruits or newly developing and morphologically changing organs have the potential to act as stronger sinks for cytokinins. This would mean that at certain stages of plant development the distribution of endogenous cytokinins, apparently synthesized in the roots, could be monopolised by a specific organ, thus creating preferential transport within the

developing plant (HUTTON and VAN STADEN, 1984).

In an attempt to establish the effect of seaweed concentrate on the growth and endogenous cytokinin levels within *Beta vulgaris* and *Phaseolus vulgaris* plants, Kelpak 66, was applied as a foliar spray with and without applications of a chemical fertilizer. These plants were used as their endogenous cytokinin levels have been well studied (WANG, THOMPSON and HORGAN, 1977; WANG and HORGAN, 1978; VREMAN, THOMAS and CORSE, 1978; PALMER, SCOTT and HORGAN, 1981).

Experimental procedure and results

Plants of *Beta vulgaris* L. (cultivar "Fordhook Giant") and *Phaseolus vulgaris* L. (cultivar "Wintergreen") were grown under greenhouse conditions. Seeds of *Beta vulgaris* were germinated in vermiculite and subsequently transplanted into a soil medium (sand : loam : peat moss, 1:2:1) when the seedlings were approximately 10 centimetres in height. *Phaseolus vulgaris* seeds were germinated directly in the soil medium. The experimental plants were watered daily with tap water interspersed with applications of the seaweed concentrate (Kelpak 66) and a liquid fertilizer (Liquinure) as indicated in Table 6.1. The seaweed concentrate used in this investigation has been previously described in the Materials and Methods section. The chemical fertilizer used in this investigation is manufactured by Fisons Agrochemicals (Pty) Limited, Johannesburg, South Africa. The fertilizer contains 11 per cent nitrogen, 7,3 per cent phosphorus and 3,7 per cent potassium. The first foliar application of the seaweed concentrate and soil application of the chemical fertilizer were made to *Beta vulgaris* plants at transplanting and to *Phaseolus vulgaris* plants when the seedlings were approximately 14 days old, subsequent applications were made every 15 days. The experiment was terminated after 2,5 months. After harvesting, the fresh mass of the various plants was determined, the material was then dried for 72 hours at 60°C and the dry mass obtained. Least significant differences (where P<0,05) for all data were

TABLE 6.1

Outline of treatments used to assess the effect of foliar applications of seaweed concentrate and soil applications of a liquid fertilizer on the growth of *Beta vulgaris* and *Phaseolus vulgaris* plants.

	Treatments			
	Control	Seaweed concentrate (1:500)	Fertilizer (1:250)	Seaweed concentrate (1:500) & Fertilizer (1:250)
Number of times seaweed concentrate was applied	0	5	0	5
Number of times fertilizer was applied	0	0	5	5
Volume of seaweed concentrate & fertilizer applied cm^3 plant treatment $^{-1}$	0	20	10	20 + 10

calculated after performing an analysis of variance.

Samples of 10 grammes of fresh material taken from the leaves and roots of *Beta vulgaris* plants and the fruits, leaves, stems and roots of *Phaseolus vulgaris* plants were used for cytokinin extraction and determination. The cytokinins were extracted and purified as described in the Materials and Methods section and the activity of the cytokinin-like compounds estimated by means of the soybean callus bioassay (MILLER, 1965). In order to obtain more information as to the nature of the cytokinin-like activity recorded on paper chromatograms, 20 grammes of fresh material was extracted using Dowex 50 and the Dowex 50 extract loaded onto a Sephadex LH-20 column eluted with 35 per cent ethanol as described in the Materials and Methods section. Forty millilitre fractions were collected, dried in air and each fraction assayed for cell division activity.

The effect of seaweed concentrate and fertilizer applications on the chlorophyll content of treated plants was investigated. Leaf disc samples were taken from fully expanded leaves of plants from each treatment. Five leaf discs from a single leaf were placed into vials to which 10 millilitres of methanol (100 per cent) was added and allowed to extract for 24 hours. Each extraction was replicated 5 times. An analysis of variance was performed on all data and the least significant differences (where $P < 0,05$) calculated.

Total leaf area for each treatment was determined using a planimeter. The least significant differences (where $P < 0,05$) were calculated after performing an analysis of variance.

Similar results were obtained with the application of seaweed concentrate and fertilizer to both *Beta vulgaris* and *Phaseolus vulgaris* plants. The overall appearance of plants treated with seaweed concentrate was better than that of the

controls with maximum growth being achieved in those plants which received both seaweed concentrate and fertilizer applications (Figure 6.1).

Seaweed concentrate applied as a foliar spray increased the total yield (roots and leaves) of *Beta vulgaris* plants by 111 per cent over the control. This yield increase manifested itself in both the root and leaf dry mass (Table 6.2). Maximum growth was obtained in those plants which received both seaweed concentrate and fertilizer applications. Total yield (roots and leaves) was 305 per cent greater than that achieved for the control plants (Table 6.2).

Seaweed concentrate increased the total dry mass of *Phaseolus vulgaris* plants by 24 per cent over the control. This dry mass increase manifested itself mainly as increased root growth and higher fruit yield (Table 6.3). With the application of a chemical fertilizer total dry mass increase over the controls was 38 per cent with the effect being predominantly due to the increase in the dry mass of the fruits, leaves and stems (Table 6.3). Maximum growth was again achieved with those plants which received both seaweed concentrate and fertilizer applications. Total yield was 59 per cent greater than that achieved for the control plants (Table 6.3), with significant increases in dry mass being recorded in the fruits, leaves and stems.

The increase in dry mass of the leaves of both *Beta vulgaris* (Table 6.2) and *Phaseolus vulgaris* (Table 6.3) plants was reflected in that both the number of leaves per plant and the leaf area per plant increased. An increase in chlorophyll content of the treated leaves was also recorded, this increase being highest in the leaves of plants treated with seaweed concentrate and fertilizer (Table 6.4 and 6.5). Chlorophyll extracts from the leaves of all treatments showed normal absorption spectra.

Seaweed concentrate and fertilizer applications had a pronounced effect

Figure 6.1   The effect of seaweed concentrate and fertilizer

applications on the growth of 1 = *Beta vulgaris*,

2 = *Phaseolus vulgaris* plants. A = control, B = 5

foliar applications of seaweed concentrate, C =

5 soil applications of fertilizer, D = 5 foliar

applications of seaweed concentrate + 5 soil

applications of fertilizer.

TABLE 6.2

The effect of seaweed concentrate and fertilizer applications on the dry mass (grammes) of *Beta vulgaris* plants. Means for the parameter measured followed by the same letter do not differ significantly (P<0,05). Figures in brackets = percentage increase over the control.

Treatment	Dry mass (grammes)		
	Leaves	Roots	Total
Control	2,5a	1,1a	3,6a
Seaweed concentrate	5,7b (116)	2,3b (100)	8,0b (111)
Fertilizer	8,7c (252)	2,9b (163)	11,6c (225)
Seaweed concentrate and fertilizer	10,7d (340)	4,8c (318)	15,5d (305)
Total $\bar{x} \pm$ SE	6,9 $\pm$ 0,5	2,8 $\pm$ 0,3	9,6 $\pm$ 1,1

TABLE 6.3

The effect of seaweed concentrate and fertilizer applications on the dry mass (grammes) of *Phaseolus vulgaris* plants. Means for the parameter measured followed by the same letter do not differ significantly (P<0,05). Figures in brackets = percentage increase over the control.

Treatment	Dry mass (grammes)				
	Fruits	Leaves	Stems	Roots	Total
Control	2,1a	1,4a	1,6a	0,30a	5,40a
Seaweed concentrate	2,8b (33)	1,7b (21)	1,8a (13)	0,43b (43)	6,73b (24)
Fertilizer	3,0b (42)	1,9bc (35)	2,2b (38)	0,37b (23)	7,45c (38)
Seaweed concentrate and fertilizer	3,2b (57)	2,3c (64)	2,7c (69)	0,40b (33)	8,61d (59)
Total $\bar{x}$ ± SE	2,7 ± 0,19	1,8 ± 0,11	2,0 ± 0,13	0,37 ± 0,03	7,02 ± 0,2

TABLE 6.4

The effects of the various treatments on the number of leaves, leaf area and chlorophyll content of leaves of *Beta vulgaris*. Means for the parameter measured followed by the same letter do not differ significantly (P<0,05).

	Parameter measured		
	Number of leaves plant^{-1}	Total leaf area (cm^2)	Chlorophyll content absorbance (660 nm)
Control	4,7a	304a	0,78a
Seaweed concentrate	7,4b	685b	1,01b
Fertilizer	8,3b	1147c	1,39c
Seaweed concentrate and fertilizer	10,0c	1638d	1,75d
Total $\bar{x}$ ± SE	7,6 ± 1,2	944,1 ± 113,5	1,23 ± 0,3

TABLE 6.5

The effects of the various treatments on the number of leaves, leaf area and chlorophyll content of leaves of *Phaseolus vulgaris*. Means for the parameter measured followed by the same letter do not differ significantly (P<0,05).

	Parameter measured		
	Number of leaves plant^{-1}	Total leaf area (cm^2)	Chlorophyll content absorbance (660 nm)
Control	26a	658a	2,5a
Seaweed concentrate	37b	698a	3,5b
Fertilizer	39b	975b	3,8c
Seaweed concentrate and fertilizer	44c	1120c	4,5d
Total $\bar{x} \pm$ SE	36 $\pm$ 4	863 $\pm$ 55	3,5 $\pm$ 0,09

on the endogenous cytokinin levels of both *Beta vulgaris* and *Phaseolus vulgaris* plants.

At the time of harvesting a great deal of quantitative variation existed in the cytokinin content of *Beta vulgaris* plants. There was, however, very little qualitative variation. On paper only one peak of activity was detected in both the roots and the leaves. This peak co-chromatographed with zeatin and ribosylzeatin (Figure 6.2). Sephadex LH-20 fractionation of a similar Dowex 50 extract as that used for paper chromatography showed that the activity recorded on paper chromatograms was due to at least three components (Figure 6.3). The first occurred at an elution volume of 600 - 680 millilitres and co-eluted with zeatin, the second had an elution volume of 800 - 880 millilitres and co-eluted with isopentenyladenosine, and the third had an elution volume of 1160 - 1200 millilitres and co-eluted with isopentenyladenine.

In Table 6.6 it can be seen that the results obtained by means of paper chromatography show a great deal of variation in the amount of cytokinin present in the plants of the various treatments. The total cytokinin-like activity recorded was highest in the control and lowest in the plants treated with seaweed concentrate and fertilizer. The highest level of cytokinin activity in the roots was detected in those plants treated with seaweed concentrate, and in the leaves of those plants which served as the control. The lowest levels of cytokinin activity in both the roots and leaves was detected in those plants which were treated with both seaweed concentrate and fertilizer.

Analysis of *Phaseolus vulgaris* material taken from fruits, leaves, stems and roots of the various treatments revealed that most of the cytokinin-like activity was detected as a single peak which co-chromatographed with zeatin, ribosylzeatin and their respective dihydroderivatives. Cytokinin glucosides were only detected in plant material taken from the fruits (Figure 6.4). Seaweed concentrate application resulted in higher levels of cytokinin being present in all tissues, particularly the fruits

Figure 6.2   Cytokinin activity detected in 10 grammes of
                fresh *Beta vulgaris* root material taken from
                plants treated with seaweed concentrate. Dowex
                50 purified extracts were separated on paper using
                iso-propanol : 25 per cent ammonium hydroxide
                : water (10:1:1 v/v). ZR = ribosylzeatin; Z =
                zeatin. The region above the dotted line is
                significantly different from the control at the
                level P = 0,01.
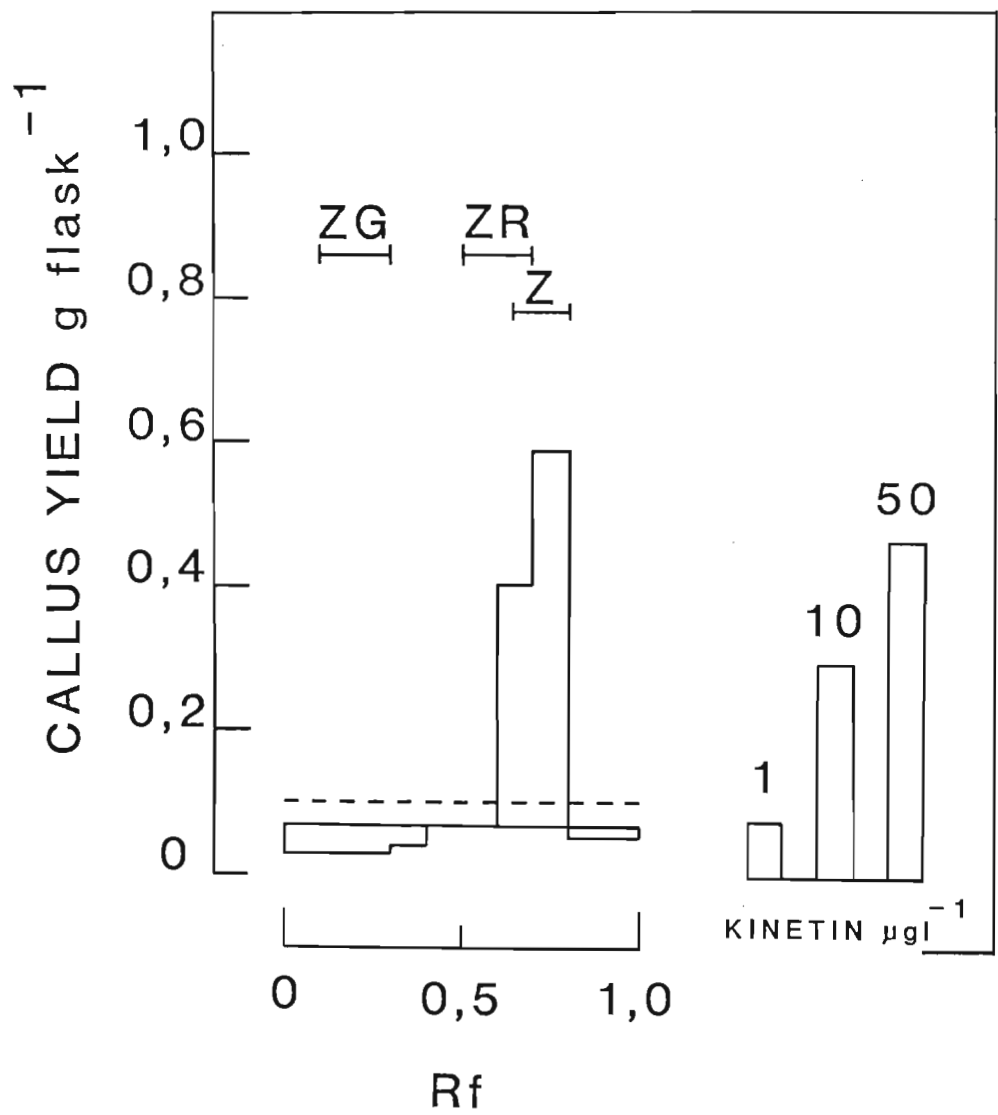
Figure 6.3   Cytokinin activity detected in 10 grammes of root

material taken from *Beta vulgaris* plants treated

with seaweed concentrate. The Dowex 50 purified

extract was fractionated on a Sephadex LH-20

column eluted with 35 per cent ethanol. ZR =

ribosylzeatin;   Z   =   zeatin;   IPA   =

isopentenyladenosine;   2iP = isopentenyladenine.

The region above the dotted line is signifcantly

different from the control at the level P = 0,01.
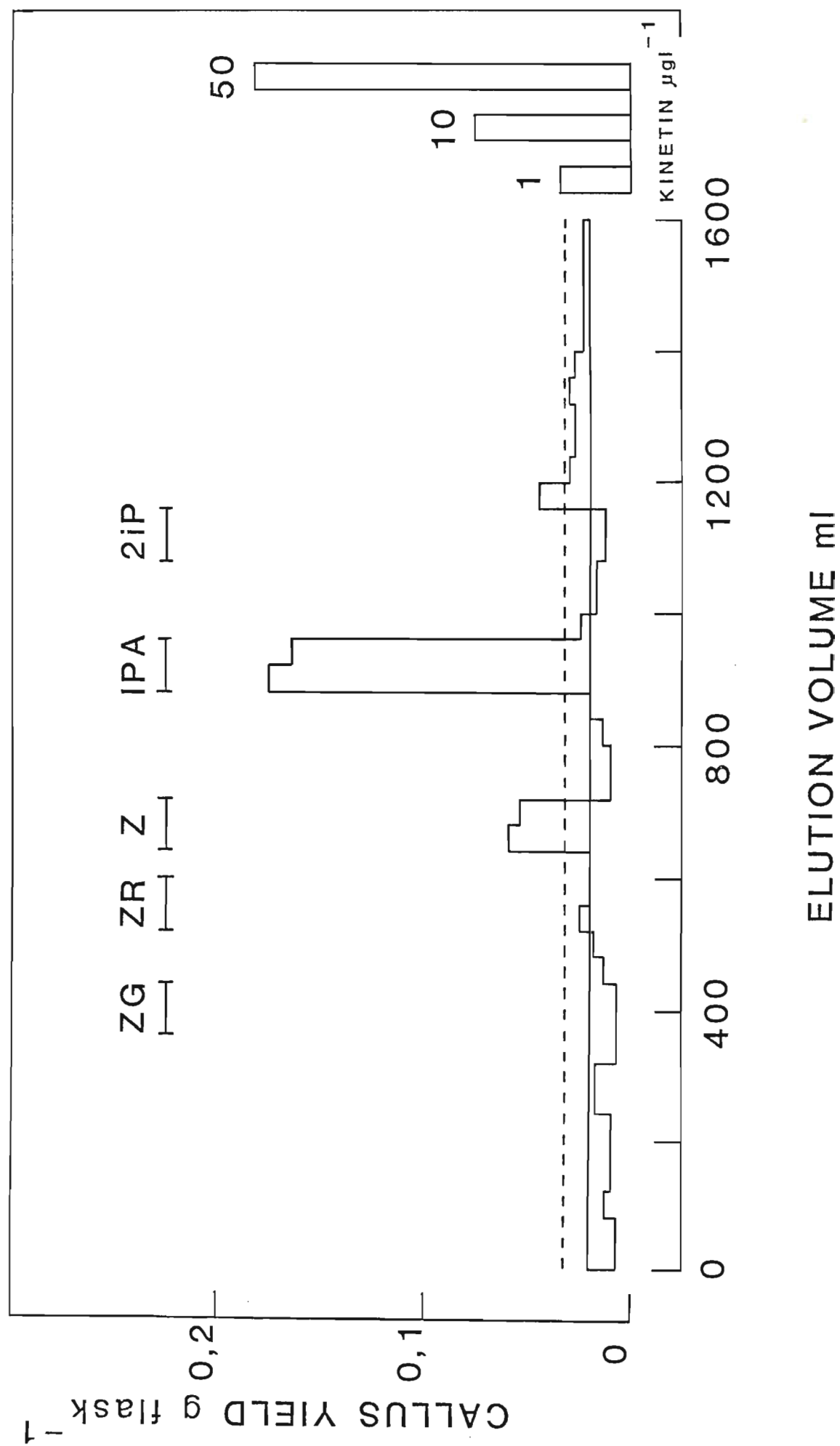
Figure 6.4   Cytokinin activity detected in 10 grammes of fresh
                  Phaseolus vulgaris fruit material taken from plants
                  treated with seaweed concentrate plus a chemical
                  fertilizer.   Dowex   50   purified   extracts   were
                  separated   using   *iso*-propanol :   25   per   cent
                  ammonium hydroxide : water (10:1:1 v/v). ZR =
                  ribosylzeatin; Z = zeatin; ZG = glucosylzeatin. The
                  region   above   the   dotted   line   is   significantly
   different from the control at the level $P = 0,1$.
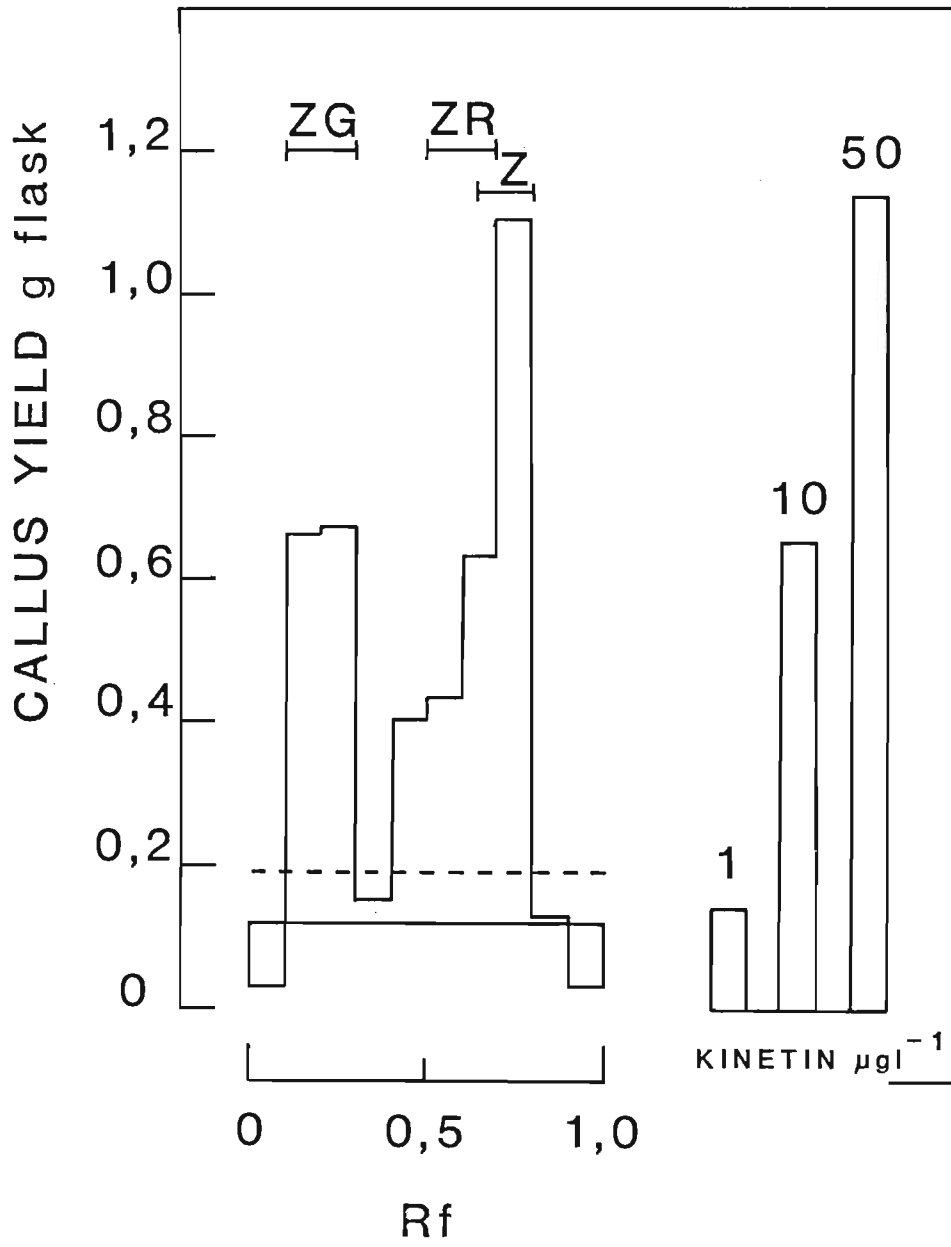
(Table 6.7). Treatment of plants with both seaweed concentrate and fertilizer resulted in most of the cytokinin-like activity being detected in the rest of the plant. Cytokinin glucosides were responsible for most of the activity detected in the fruits, except in seaweed treated plants where the reverse was true (Table 6.8).

Discussion

In this investigation seaweed concentrate at a dilution of 1:500 applied as a foliar spray improved the growth of *Beta vulgaris* and *Phaseolus vulgaris* plants significantly, irrespective of whether it was applied on its own or together with a chemical fertilizer.

Root growth and the endogenous cytokinin content of these roots increased significantly with seaweed concentrate applications. The high level of cytokinin-like activity in the roots of plants treated with seaweed concentrate seems to indicate a build up of cytokinins in the roots and/or a decrease in translocation to the shoots. There are numerous reports in the literature on the role of nutrients and in particular nitrogen in cytokinin translocation (WAGNER and MICHAEL, 1971; GÖRING and MARDANOV, 1976). SALAMA and WAREING (1979) found that the rate of cytokinin export from the roots is lower at suboptimal than at optimal levels of nitrogen in the root environment. The results obtained here show that the application of seaweed concentrate together with a nitrogen source in the form of a chemical fertilizer resulted in a marked decrease in the cytokinin content of the roots. Thus in agreement with SALAMA and WAREING (1979) it would seem that the provision of a nitrogen source resulted in an increase in the export of cytokinins from the roots to the shoots. SHARIFF and DALE (1980) found that under conditions of mineral nutrient stress assimilate supply to the shoot is reduced thus restricting the metabolites available for growth as well as the cytokinin supply from the roots. They showed that tiller bud growth in barley was increased with the application of exogenous cytokinins

TABLE 6.6.

The effects of the various treatments on the total cytokinin levels of *Beta vulgaris*. Activity is expressed as kinetin equivalents in nanogrammes gramme^{-1} fresh material.

Treatment	Cytokinin levels (kinetin equivalents in nanogrammes gramme $^{-1}$ fresh material.)		
	Leaves	Roots	Total
Control	205	147	352
Seaweed concentrate	11	301	312
Fertilizer	17	216	233
Seaweed concentrate and fertilizer	9	74	83

TABLE 6.7

The effects of the various treatments on the total cytokinin levels of *Phaseolus vulgaris*. Activity is expressed as kinetin equivalents in nanogrammes gramme $^{-1}$ fresh material.

Treatment	Cytokinin levels (kinetin equivalents in nanogrammes gramme $^{-1}$ fresh material.)				
	Fruits	Leaves	Stems	Roots	Total
Control	35	8	12	20	75
Seaweed concentrate	328	92	124	113	657
Fertilizer	206	57	108	63	434
Seaweed concentrate and Fertilizer	502	3	27	8	540

TABLE 6.8

The effects of the various treatments on the quality and quantity of cytokinins found in the fruits of *Phaseolus vulgaris* plants. Activity is expressed as kinetin equivalents in nanogrammes gramme^{-1} fresh material.

	Cytokinin activity of the fruits expressed as kinetin equivalents, co-chromatographing with :	
	Zeatin & ribosylzeatin Rf 0,6 - 0,9	Glucosylzeatin Rf 0,1 - 0,4
Control	9	26
Seaweed concentrate	216	112
Fertilizer	40	166
Seaweed concentrate and fertilizer	73	429

to the roots of plants under nutrient stress and that this increase was greater when plants were supplied with both cytokinins and mineral nutrients.

It has been demonstrated that low levels of cytokinins are present in leaves during periods of active leaf expansion (ENGELBRECHT, 1972; VAN STADEN, 1976b; VAN STADEN and DAVEY, 1978). The increase in fresh mass, dry mass, leaf area and chlorophyll content and the corresponding low levels of cytokinins in the leaves of plants treated with seaweed concentrate and a chemical fertilizer suggests that these compounds are rapidly utilized during periods of active growth.

The high levels of cytokinin found in the developing fruits of *Phaseolus vulgaris* plants treated with seaweed concentrate indicates either, an increase in the translocation of cytokinins from the roots (DAVEY and VAN STADEN, 1978; VONK, 1979), or the production of cytokinins within the fruits themselves (HAHN, DE ZACKS and KENDE, 1974). It has been suggested that high concentrations of cytokinins found in the fruits may be necessary for the creation of a strong physiological sink capable of competing with the remainder of the plant for nutrients (LUCKWILL, 1977). MOTHES and ENGELBRECHT (1961) found that sugars and amino acids could be transported preferentially to regions of high cytokinin activity. The results of this investigation show that high concentrations of cytokinins within the fruits of treated *Phaseolus vulgaris* plants are associated with an increase in the dry mass of those fruits. In all treatments except the one which received only seaweed concentrate application, the level of cytokinin glucosides in the fruits exceeded that of the free base cytokinins and their ribosides. Research has shown that when plant tissues accumulate cytokinin-like compounds, much of the increase in activity appears to be due to O-glucoside metabolites, the level of which may rise much more markedly than that of the cytokinin bases and their ribosides (PALMER, HORGAN and WAREING, 1981a; VAN STADEN and DAVEY 1981). Further evidence in support of the role of cytokinin glucosides as storage metabolites was presented by SUMMONS, ENTSCH,

LETHAM, GOLLNOW and MACLEOD (1980). They showed that when cytokinins were no longer required, the excess was metabolised by processes which involved either side chain modification, such as glucosylation and/or side chain cleavage.

The significant increase in root growth resulting from the application of seaweed concentrate in this investigation is similar to that which was previously reported on for *Lycopersicon esculentum* plants (Chapter V). The increase was reflected in the dry mass as well as an elevation in the cytokinin content of the roots. Improved root growth would probably result in more efficient moisture and nutrient utilization by the plants (WIDDOWSON, YEATES and HEALY, 1973). This could explain the overall beneficial growth of those plants treated with seaweed concentrate.

GENERAL CONCLUSION

There may be many cause and effect relationships involved in the response of plants to seaweed concentrate application. It was an interest in plant growth regulators, in particular cytokinins, and the possible involvement of this group of hormones in bringing about some of the responses of plants to seaweed treatment which motivated the current research.

A review of the literature on the presence and possible role of cytokinins in extracts and or concentrates prepared from marine algae indicated that three aspects of research needed attention. These were the chemical identification of the cytokinins present in algal tissues, the determination of the seasonal and lunar variations of the cytokinin levels in *Ecklonia maxima* (OSBECK) PAPENF. and the effect of seaweed concentrate application on the growth and yield of various field crops. *Ecklonia maxima* the large brown alga which is used in the preparation of the seaweed concentrate Kelpak 66, was investigated.

Using Sephadex LH-20 and Reversed Phase High Performance Liquid Chromatography cytokinin-like compounds were detected in the stipe tissue of *Ecklonia maxima*. These compounds had chromatographic properties on paper and Sephadex LH-20 similar to authentic zeatin, ribosylzeatin and isopentenyladenosine. Reversed Phase High Performance Liquid Chromatography revealed the presence of the *cis* and *trans* isomers of ribosylzeatin, *trans*-zeatin, dihydrozeatin and isopentenyladenosine.

Although the literature pertaining to the presence and identity of cytokinins in marine algae is scarce, most of the studies which have been carried out have identified the isopentenyl group of cytokinins as being the most common of these growth regulators detected in algae (PEDERSEN and FRIDBORG, 1972;

PEDERSEN, 1973; KENTZER, SYNAK, BURKIEWICZ and BANAS, 1980). Isopentenyladenosine and its derivatives have also been detected in other lower orders, for example the bacteria (HELGESON and LEONARD, 1966; UPPER, HELGESON, KEMP and SCHMIDT, 1970; RATHBONE and HALL, 1972) and the bryophytes (BEUTELMAN and BAUER, 1977; WANG, HORGAN and COVE, 1981). Zeatin and its derivatives accounted for most of the detected activity in *Ecklonia maxima* and resembled closely the cytokinins found in terrestrial plants (MUROFUSHI, INOUE, WATANABE, OTA and TAKAHASHI, 1983). JENNINGS (1969), in agreement with the findings of this investigation established that the cytokinins extracted from the algae *Ecklonia radiata* (TURN) J. AGARDH. and *Hypnea musciformis* (WULFEN) LAMOUR. resembled chemically, the cytokinins usually extracted from higher plants.

In terms of the seasonal variation in the quality and quantity of cytokinins present in both fresh and processed *Ecklonia maxima* material, it would appear that these compounds essentially fulfil the same function as in terrestrial plants. Research has shown that the cytokinin activity of expanding leaves of terrestrial plants is usually low (VAN STADEN, 1976a; DAVEY and VAN STADEN, 1978; HENSON, 1978a; HENDRY, VAN STADEN and ALLEN, 1982) probably due to the rapid utilization of cytokinins in these organs. As leaves mature (VAN STADEN, 1976a) or as conditions unfavourable for growth approach (HENDRY, VAN STADEN and ALLEN, 1982) there is usually an increase in cytokinin activity, mainly in the form of cytokinin glucosides. The levels of cytokinin activity in *Ecklonia maxima* followed similar trends to those mentioned above. That is low levels of activity were recorded in the lamina, stipe and holdfast tissue from February to May and from September to January, corresponding to the period of most active growth of *Ecklonia maxima*. Cytokinin levels increased in the stipe and holdfast regions of the plant in autumn (June and July), corresponding with a reduction in growth during winter. The only anomaly in this trend was

detected in one metre plants where the highest levels of cytokinin-like activity were detected in material harvested in spring (September and October). These results are similar to those of LORENZI, HORGAN and WAREING (1975) and HENDRY, VAN STADEN and ALLEN (1982), who established that in evergreen species, cytokinin activity increased during the start of the growing season with zeatin and ribosylzeatin predominating during spring and summer.

The lunar cycle study of lamina, stipe and holdfast material from two metre plants harvested on a daily basis during April – May 1983, revealed fluctuations in the levels of cytokinins which were closely correlated with the phases of the moon. High levels of activity co-chromatographing with zeatin and ribosylzeatin were detected in the tissue at new moon, first quarter, full moon and last quarter. From the results of this study and those of the seasonal investigation, it would appear that the erratic results often obtained from the use of commercially available seaweed preparations may stem from variations in cytokinin levels within the algae over a seasonal cycle. The levels varying according to the month in which the algae were harvested. The time during the lunar cycle at which harvesting was carried out may also alter the effectiveness of the product.

The liquid seaweed concentrate (Kelpak 66) is relatively new on the farming scene in South Africa and many people are uncertain as to whether it can play a usefull role in agriculture and horticulture. Scientists themselves hold conflicting opinions as to the effectiveness of the product. In this investigation an attempt was made to substantiate some of the claims made for Kelpak 66 and its effectiveness on plant growth.

Greenhouse trials were conducted on *Lycopersicon esculentum* MILL. plants grown in nematode infested soil. Kelpak 66 at a dilution of 1 : 500 improved

the overall growth and yield of treated plants significantly. The mean fruit yield per plant was : controls 1,2 grammes, five foliar applications of seaweed concentrate 16,8 grammes, one soil application of seaweed concentrate 26,1 grammes. Associated with this yield increase was a significant increase in root growth and a reduction in root-knot nematode infestation whenever seaweed concentrate was applied.

Further pot trials were conducted on *Beta vulgaris* L. and *Phaseolus vulgaris* L. plants to determine the effect of seaweed concentrate on the growth and endogenous cytokinin levels within treated plants. Kelpak 66 was applied as a foliar spray with and without applications of a chemical fertilizer. Seaweed concentrate applied to *Beta vulgaris* plants increased the total yield (roots and leaves) by 111 per cent over the control, with the increase manifesting itself in both the root and leaf fresh mass. Seaweed concentrate application to *Phaseolus vulgaris* plants increased the total fresh mass of the plants by 41 per cent over the control. This fresh mass increase manifested itself mainly as increased root growth (46 per cent increase over the control) and higher fruit yield (51 per cent increase over the control). The endogenous cytokinin content in the roots of plants treated with seaweed concentrate increased significantly over the control in both *Beta vulgaris* and *Phaseolus vulgaris* plants. The application of seaweed concentrate together with a nitrogen source in the form of a chemical fertilizer resulted in a marked reduction in the cytokinin content of the roots. These results are similar to those of SALAMA and WAREING (1979) who suggested that the provision of a nitrogen source resulted in an increase in the export of cytokinins from the roots to the shoots. The increase in fresh mass, dry mass, leaf area, and chlorophyll content and the corresponding low levels of cytokinins in the leaves of plants treated with seaweed concentrate and a chemical fertilizer suggests that these compounds are rapidly utilized during periods of active growth (ENGELBRECHT, 1972; VAN STADEN, 1976a; VAN STADEN and DAVEY 1978).

Although the seaweed concentrate used in this investigation is characterized by its high cytokinin activity, and close correlations have been found to exist between the results obtained from the use of a synthetic cytokinin, kinetin and a seaweed extract of equivalent cytokinin activity (BLUNDEN and WILDGOOSE, 1977), there remains no direct evidence to suggest that this group of plant growth regulators is solely responsible for the improved results obtained with the use of seaweed extracts and or concentrates. It does, however, seem probable that the beneficial results obtained, and the cytokinin content of the seaweed are related.

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