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ENDOGENOUS LEVELS OF CYTOKININS IN AGEING CUCUMBER COTYLEDONS

A Thesis

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ABSTRACT

ENDOGENOUS LEVELS OF CYTOKININS IN AGEING CUCUMBER COTYLEDONS

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There are several procedures for isolating cytokinins which involve a number of steps including extraction, solvent fractionation and column or paper chromatography. Various combinations of these procedures were tried in an attempt to isolate cytokinins from cucumber cotyledons. It was found that with this tissue it was preferable to omit a few steps to minimize the loss of cytokinins activity.

The endogenous cytokinin levels in etiolated cucumber cotyledons of different ages were estimated and compared with the capacity of the cotyledons to produce chlorophyll after exposure to light. There was a parallel relationship between cytokinin levels and chlorophyll production. The cytokinin levels were highest in 8 day old cotyledons, at which time the production of chlorophyll was also maximal. It is proposed that cytokinins have an important role in chlorophyll production.

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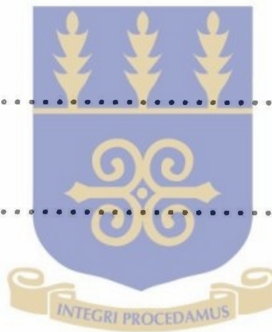

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INTRODUCTION

Plants appear to be almost entirely dependent on hormone-based mechanisms for coordination of growth and development. A plant hormone is generally regarded as an organic compound which is produced naturally in plants and which is active in minute amounts in controlling growth and other functions at a site remote from its place of production. There are three main groups of natural chemical regulators or plant hormones - the auxins, cytokinins and gibberellins. These three types of hormones each brings about a great many growth and physiological responses; each type induces a spectrum of effects and the three spectra overlap to some extent. For example, all three types of hormones can induce both cell division and cell enlargement. All three types of hormones can also cause formation of parthenocarpic fruit. Although the three types of plant hormones have some common biological activities, each group also induces distinctive responses on which specific bioassays are based.

Studies of endogenous growth regulators provide an important basis for the understanding of the hormonal control mechanisms in plant tissue. Most studies have been focused on the auxins, gibberellins, ethylene and abscisic acid (ABA), while only a few have been carried out on cytokinins. Cytokinin-like substances produced by the root may regulate senescence of leaves on intact plants (Sitton et al., 1967; Wareing and Seth, 1967). Although the application of kinetin had little or no effect on leaf senescence on intact tobacco plants (Engelbrecht, 1964), Fletcher (1969) has demonstrated that N^6-benzyladenine (BA) could retard leaf senescence on intact bean plants under normal growth conditions. This has

been confirmed by Jacoby and Dagan (1970). The levels of endogenous growth regulators are determined by environment, time and genetic potential, and senescence may be determined by the balance between senescence retardants (cytokinins, gibberellins and auxins) and senescence accelerators (ethylene, abscisic acid and unidentified factors) (Carr et al., 1972). The effectiveness of any one of the senescence retardants in delaying senescence is dependent on the species and plant age. The senescence retardants maintain the synthesis of such molecules as chlorophyll, protein and nucleic acids. On the other hand, a rise in the levels of senescence accelerators and/or a decline in senescence retardants would result in a decrease in the synthesis or an increase in the degradation of chlorophyll, protein and nucleic acids. These changes, accompanied by a loss of membrane integrity, would lead to senescence, and finally death.

In addition to retardation of senescence, cytokinins influence chloroplast differentiation (Stetler and Laetsch, 1965) and chlorophyll production (Fletcher and McCullagh, 1971a) and this latter effect is dependent on the age of the cotyledons. Therefore in the present study an attempt was made to measure changes in endogenous levels of cytokinins during ageing of etiolated cucumber cotyledons and to correlate these changes with the capacity of the cotyledons to produce chlorophyll.

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REVIEW OF LITERATURE

Cytokinins have been detected in a wide range of plants and they are considered to play an important and diverse role in many aspects of plant growth and development (Letham, 1967; Skoog and Armstrong, 1970; Kende, 1971). Cytokinins and other growth regulators have been demonstrated to retard senescence of plants. Research has mainly dealt with the effect of exogenous application of cytokinins on senescence of various detached plant organs, including leaves, flowers, fruits (Ballantyne, 1965; Gilbart and Dedolph, 1965; Heide and Oydvin, 1969; Maclean and Dedolph, 1962); their effect upon the metabolism of protein and nucleic acid (Helgeson, 1968); respiration; and photosynthesis (Gilbart and Dedolph, 1965; Halevy *et al*., 1966). It has been known for some time that a normal feature of senescing leaves, whether intact or detached from the plant, is the breakdown of chlorophyll and protein, leading to a steady decline in the content of these substances (Fletcher, 1969). When the senescence of detached leaves is delayed by the application of hormones, the levels of chlorophyll, protein and RNA are maintained, and the incorporation of radioactive precursors into protein and RNA is enhanced (Osborne, 1962; Wollgiehn, 1967). It has been suggested by some (Fletcher and Osborne, 1966) that the hormonal action is mediated through a regulation of DNA-dependent RNA synthesis, while others have suggested that it is by prevention of protein degradation (Tavares and Kende, 1970). Others contend that localized retardation of senescence in detached leaves is associated with mobilization of metabolites from untreated zones (Mothes and Engelbrecht, 1961; Gunning and Barkley, 1963; Muller and Leopold, 1966). Cytokinin treatment is also known to prevent a rise in

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nuclease and protease activities in senescing leaf tissue (Anderson and Rowan, 1966; Srivastava, 1965, 1968), and thus presumably prevents degradation as well as stimulates synthesis of macromolecules.

Much of the work concerning leaf senescence has been done using detached leaves or leaf discs floated on water. It is generally assumed that the senescence of leaf discs is similar to that of attached leaves except greatly accelerated. The losses of protein and chlorophyll, symptoms of senescence, are usually extensive after 5 to 8 days detachment for discs of Taraxacum officinale (Back and Richmond, 1971; Fletcher, 1966), Xanthium (Osborne, 1962; Abrams and Pratt, 1966), Nasturtium (Back and Richmond, 1971; Beavers, 1966; Mizrahi et al., 1970), Rumex (Back and Richmond, 1971; Goldthwaite and Laetsch, 1968), brussels sprouts (Dennis et al., 1967), beans (Goldthwaite and Laetsch, 1967; Phillips et al., 1969), and most other plant leaves studied (Atkin and Srivastava, 1969). These losses are more extensive if the discs are maintained in darkness (Dale, 1967; Goldthwaite and Laetsch, 1967; Lewington and Simon, 1969; Mizrahi et al., 1970; Simon, 1967). Leaf age somewhat affects the rate of disc senescence. Discs from young leaves senesce more slowly than discs from old leaves (Dale, 1967; Dennis et al., 1967; Goldthwaite and Laetsch, 1967; Wollgiehn, 1967). The ability of plant growth regulators to retard senescence is less on discs from young leaves than from old leaves (Back and Richmond, 1971; Dennis et al., 1967; Abrams and Pratt, 1966). Some workers have reported differences in the senescence pattern of attached leaves compared to leaf discs (Lewington and Simon, 1969; Lewington et al., 1967); Miller and Leopold, 1966; Simon, 1967; Abrams and Pratt, 1966; Wollgiehn, 1967). Dennis et al. (1967) found different changes in the chloroplast ultrastructure

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during senescence of attached old leaves and detached aged leaves. Lewington et al. (1967) concluded that senescence of detached leaves was not the same process as senescence of attached leaves. Some differences are to be expected, since a leaf disc, as such, is not connected to the transport system of the whole plant and can neither receive nor lose metabolites and hormones (Muller and Leopold, 1966).

Literature on natural growth regulators in relation to senescence is still very meager. Senescence-delaying compounds and kinin-like substances have been detached in a variety of plant materials and some of them have been shown to be purines or purine derivatives. Letham (1966) isolated such substances from plum fruitlets and maize kernels. One of the active factors from plums had a UV spectrum similar to 6-N-substituted adenines. Letham (1963) found that this material and kinetin could be separated from each other by paper chromatography. Because of the low concentration of the active materials in plum fruitlets and the limited availability of the source materials, Letham (1963) has also used immature corn in his further work. He isolated a crystalline substance which he named zeatin. It was more active than kinetin and appeared to be identical with the factor from plum fruitlets. Beaucheshe (1961) has reported an active component from corn which has an ultraviolet spectrum quite similar to adenine. The factor was more active than kinetin in stimulating cell division and in promoting bud formation in tobacco tissue cultures. Miller (1962) obtained a highly active material from corn, of which concentrations down to 11 μg/liter could be detected by growth promoting activity in soybean tissue cultures.

The presence of cytokinins in seeds has been reported in lettuce (Barzili and Mayer, 1964); sunflower (Miller and Witham, 1964); watermelon

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(Maheshwari and Prakash, 1967); pumpkin (Gupta and Maheshwari, 1970) and lupin (Koshimizu et al., 1967). It is notable that endosperm, young fruitlets, and seeds are rich in cytokinins. There is no rigorous evidence concerning the sites of cytokinin biosynthesis in plants. Because of high cytokinin concentration in young fruits and seeds, it is generally assumed that cytokinins are formed in these organs. While this is indeed probable, the alternative still exists that cytokinins are transported to and accumulated in developing fruits.

Recently, cytokinins have been obtained at relatively high concentrations from roots and from root exudates. Cytokinins have been found in root exudate of tobacco (Kulaeva, 1962), of grapes (Loeffler and Van Overbeek, 1964), of maple (Nitsch and Nitsch, 1965). Seth and Wareing (1965) isolated cytokinin-like substances from bean roots and Biswas (1964) demonstrated the presence of kinetin-like substance in Laxton's Progress peas. Based on circumstantial evidence, it has been proposed that cytokinins are also synthesized in the meristematic region of roots. Weiss and Vaadia (1965) found that the cytokinin content of sunflower roots was almost entirely restricted to the youngest portion of the root tip. Furthermore, the cytokinin concentration in the root exudate of sunflower plants did not decrease even when the tops were removed for 4 days (Kende, 1965). Also, excision of the root causes physiological symptoms in the shoot that are typical for cytokinin deficiency (Mothes, 1966; Kende and Sitton, 1967). Kulaeva (1962) was the first to test the hypothesis that roots supply a hormone to the leaves, which is involved in the regulation of senescence. She studied the ability of crude xylem exudate of tobacco plants to delay ageing of detached tobacco leaves and reported positive results. The presence of cytokinins in the xylem sap

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was proved by showing that two chromatographically purified fractions of the root exudate of sunflowers contained factors that stimulated cell division in the soybean callus assay (Kende, 1965). Similar results have been obtained by several investigators using different plants, and it now appears that cytokinins fulfil the role of Chibnall's (1939) hypothetical root hormone. Sitton *et al*. (1967) demonstrated that cytokinin concentration in xylem sap increased during the growth phase of sunflowers and that it decreased almost ten fold when growth ceased and flowering started. Similarly, Fletcher (1969) observed in attached bean leaves that biochemical symptoms of senescence, such as decline in protein and RNA levels, became evident at the onset of flowering and that cytokinin treatment during the period delayed senescence of the leaf. Application of a cytokinin had little effect on protein and RNA content prior to flower formation and maturation (Fletcher, 1969). One of the causes of senescence then appears to be a reduction of cytokinin level in the leaves. When the cytokinin level in the leaves declines, a new centre of cytokinin synthesis is formed in fruitlets. This shift of the cytokinin distribution within the plant may further enhance ageing of the shoot by lowering the capacity of the leaves to retain metabolites (Mothes *et al*., 1959; Mothes, 1960) and by directing the flow of assimilates to the cytokinin-rich, growing fruits (Leopold and Kawase, 1964; Seth and Wareing, 1967).

By comparison, there are few reports on the cytokinin content of leaf and stem tissue. Possibly because of difficulties in extraction and purification, little attention has been paid to cytokinins in leaves of plants. Engelbrecht (1971) reported seasonal changes in buds and leaves of *Populus tremulu* and *Acer platanoides*; she suggested that zeatin, its riboside and ribotide are the main cytokinins present. Skene (1972)

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detected cytokinin activity in grape leaves. Hewett and Wareing (1973) reported at least seven cytokinins in mature leaves of Populus robusta Schneid. Two of these have similar elution volumes to zeatin and zeatin riboside on sephadex LH-20. They observed seasonal changes in cytokinin activity in mature leaves and xylem sap with total cytokinin activity and diversity being at a maximum in expanding leaves. As leaves age, the amount and number of cytokinins decrease with yellow senescent leaves having only one detectable cytokinin, thought to be a glucoside (Hewett and Wareing, 1973). The cytokinin levels of proximal portions of streptocarpus leaves showed very little change during the summer and early autumn months, whereas those of the distal portions changed markedly. It is postulated that the increase in cytokinins during January may induce abscission, while the decrease in February appears to be directly related to senescence of the distal portions of the leaves (Staden, 1973). Bornman (1969), who worked with unifoliate species of streptocarpus found a marked decline in the cytokinin levels of the leaf as the leaf approached abscission. It was suggested that abscission is induced if the ratio of cytokinin to ABA falls below a critical level. However, the changes in endogenous cytokinin levels occurring in these plants prior to the development of an abscission layer are unknown. Mayak and Halevy (1970) studied changes in the level of cytokinins during maturation and senescence of rose petals. The content of endogenous cytokinins in petals of short-lived variety (Golden Klave) was lower than in a long-lived variety (Lovitan). Application of the cytokinin, BA, increased the longevity of the short-lived variety and postulated that cytokinins participate in the endogenous regulation of senescence in rose petals.

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It was suggested (Heide, 1964) that the environmental factors affect the levels of endogenous auxins and cytokinins, and this hypothesis was supported by the responses of the plants to exogenous auxins and cytokinin under various temperature regimes (Heide, 1965). Stress conditions, either atmospheric or in the soil, result in changes in endogenous abscisic acid, and cytokinin levels (Livne and Vaadia, 1972) as well as ethylene concentration (El-Beltagy and Hall, 1973). Gibberellin and cytokinin levels can be altered by different photoperiodic regimes (Lang, 1960; Van Staden and Wareing, 1972). Exposure of seeds (Van Staden et al., 1972) and vegetable buds (Hewett and Wareing, 1973) to low temperature stratification or chilling result in increases in cytokinins. Van Staden and Wareing (1972) reported that red light increased cytokinin levels in light sensitive Rumex seeds, and that this was phytochrome controlled. However, Carr et al. (1972) could detect no change in cytokinin levels in etiolated barley leaves, even though kinetin could stimulate leaf unrolling.

Cytokinins seem to be involved in promoting the synthesis of chlorophyll as well as development of the chloroplasts (Fletcher and McCullagh, 1971a). Etiolated barley seedlings lose the ability to produce chlorophyll and soluble proteins with increasing age. Kinetin retards this process (Stobart and Shewry, 1972). In order to claim that a particular hormone participates in the regulation of naturally occurring processes, it is essential to show its correlative change with the process studied, as well as its influence via exogenous applications. Fletcher and McCullagh (1971a) found that etiolated cucumber cotyledons pretreated with synthetic cytokinins (BA) and exposed to light for 3 hours had up to 450% more chlorophyll than the water controls. They also found that

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the lag phase in chlorophyll synthesis exhibited by etiolated cotyledons of all ages after exposure to light could be eliminated by treatment with cytokinins. They proposed that cytokinins have an important role in chlorophyll production. They also observed that in cucumber cotyledons the capacity to produce chlorophyll decreased with increasing age and it was suggested that this might be due to a decline in the levels of endogenous cytokinins.

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MATERIALS AND METHODS

Cucumber (Cucumus sativus L. cv. Chicago Pickling) seeds were purchased from W. Atlee Burpee Co., benzyladenine (BA) from Calbiochem Co., and alkaline phosphatase from Sigma Ltd.

Preliminary Methods

In the preliminary studies various methods for purifying extracts containing cytokinins were tried. Essentially the methods were not very much different from the final method used in the experiment. In all trials 75 grams tissue were used and cytokinins were extracted using the methods of Van Staden, Webb and Wareing (1972). The 75 grams of cotyledons were homogenized with 500 ml of 80% methanol. The filtrates were brought to the aqueous state under vacuum at 35°C. In the first trial the aqueous extract was adjusted to pH 9.0, and partitioned with petroleum ether (b.p. 60-80°C). The aqueous extract was adjusted to pH 2.5, partitioned with ethyl acetate, then readjusted to pH 7.0 and partitioned with water-saturated n-butanol. The remaining aqueous extract was reduced to a small volume and treated with alkaline phosphatase (1 mg enzyme/2 ml extract) for 24 hours at 30°C at pH 8.2 in 0.1 M Tris buffer and 0.01 M $MgCl_2$. This was then partitioned with water-saturated n-butanol, and the remaining aqueous extract discarded. This butanol fraction after enzyme treatment is hereafter called the aqueous fraction. Two standardized columnsystems were used in the trial experiments, celite and sephadex LH-20. Celite No. 545 was made into a slurry with 3N HCl and poured into a column. The celite was washed with 3N HCl acid until a visible yellow band had passed down the length of the column. The celite was then washed with

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water until the effluent was neutral. The water slurry from the column was filtered, and the material was spread in thin layers in trays and dried in the oven overnight. The partition columns were constructed and developed as described by Hall (1962). Each column used in the experimental isolations was identical to a standardized column (2 cm diameter precision bore) packed with 40 grams of Celite 545 coated with 10 ml of the lower phase of ethanol acetate: 1-propanol: H_2O, 4:1:2 v/v. The coated Celite was packed into the column in layers by using a Teflon plunger on the end of a steel rod. Samples (butanol and aqueous fractions) were dissolved in 4 ml of the lower phase of the solvent mixed well with 8 grams of Celite-545, and packed on top of the column. The column was developed by passage of the upper phase of this solvent at room temperature; flow rate was 30 ml/hr. The first 110 ml was collected. Armstrong et al. (1969) described the use of Sephadex LH-20 column chromatography as an analytical and preparative tool for the isolation and identification of cytokinins. Samples were then separated on a Sephadex LH-20 column (3 cm x 90 cm) using the technique of Armstrong et al. (1969). The column was eluted with 1 litre of 35% ethanol at a flow rate of 25 ml/hr. After discarding the void volume fractions were evaporated to dryness at 35°C and assayed for cytokinin activity.

In the second trial, petroleum ether and Celite column were omitted.

In the third trial, paper chromatography was included in the methods and again petroleum ether and Celite column omitted. Paper chromatography was carried out on Whatman No. 1 paper 45 - 57 cm. The extract was streaked onto a line 10 cm from the end of Whatman No. 1 paper. The papers were developed in descending fashion in butanol: acetic

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acid: water (12:3:5 v/v) until the front was about 32 cm from the origin. After drying, the chromatograms were divided into 10 equal strips and each strip cut into small pieces. These pieces were eluted with 80% ethanol overnight and assayed for cytokinin activity with cucumber cotyledons. The solvent system was tested for cytokinin activity with this assay. The solvent system had no cytokinin activity.

The final method used in the experiment is described below.

Methods

Cucumber plants were germinated in the dark at 28 ± 1°C for 0, 2, 4, 6, 8, 10 and 12 days. At the completion of the treatments the cotyledons were removed from the seedlings and extracted for cytokinins using methods of Van Staden, Webb and Wareing (1972). Cytokinin levels were expressed as BA equivalents calculated from BA standards (Figure 1).

Seventy-five grams of cotyledons were homogenized with 500 ml 80% methanol. The homogenate was extracted for 24 hours at 4°C and then filtered. The residue was washed with a further 500 ml of methanol and then discarded. The combined filtrates were concentrated to the aqueous phase under vacuum at 35°C and left at -20°C overnight. Samples were thawed and then centrifuged at 10,000 g for 30 minutes. The precipitate was washed twice with water, recentrifuged and discarded. The combined supernatants were brought to 100 ml, adjusted to pH 2.5 with HCl and extracted as follows:

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Figure 1. Increase in chlorophyll levels in etiolated cucumber cotyledons treated with various concentrations of benzyl-adenine, and exposed to light for 1½ h. Chlorophyll content expressed as absorbancy, λ, at 665 mμ of acetone extracts.

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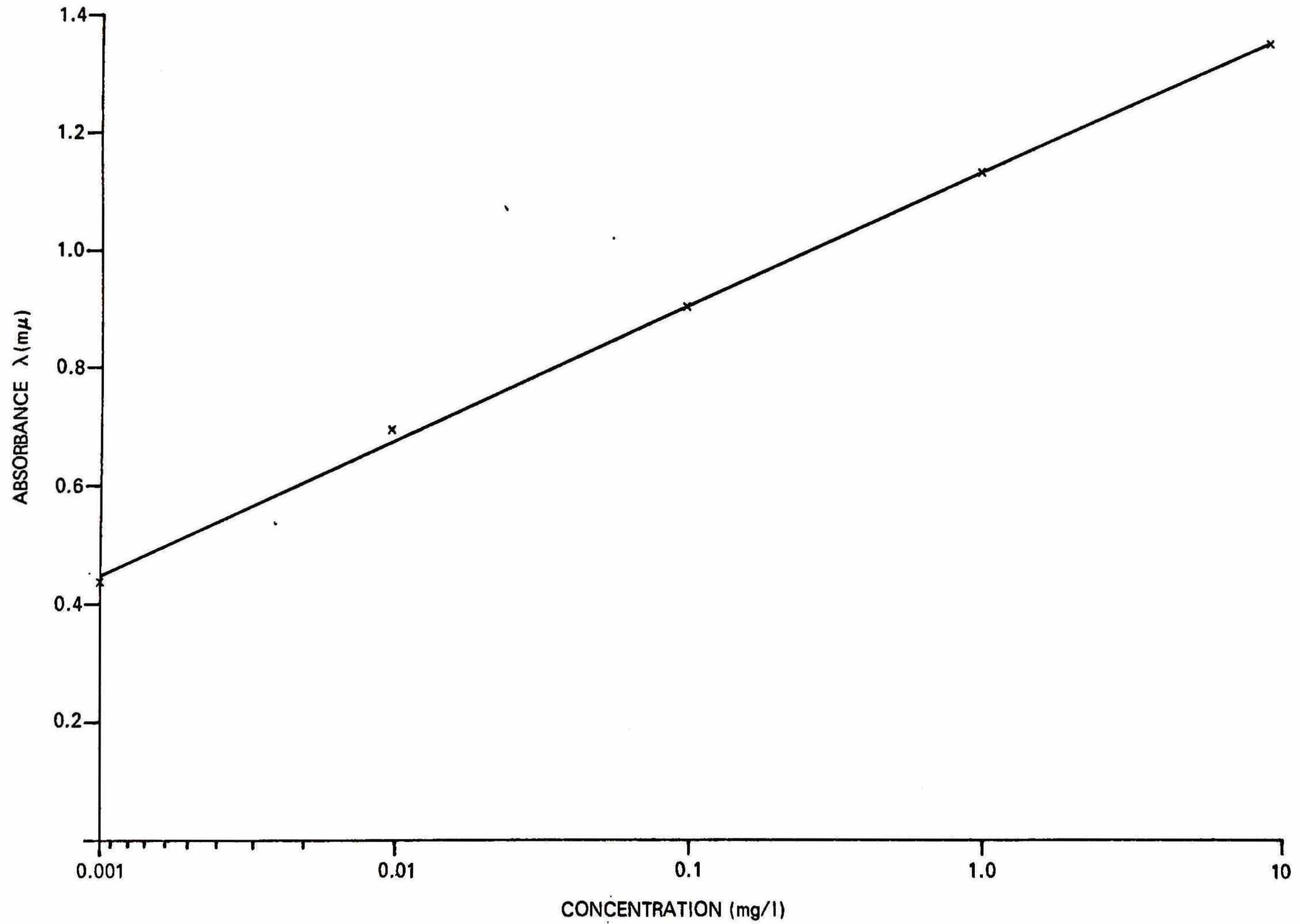

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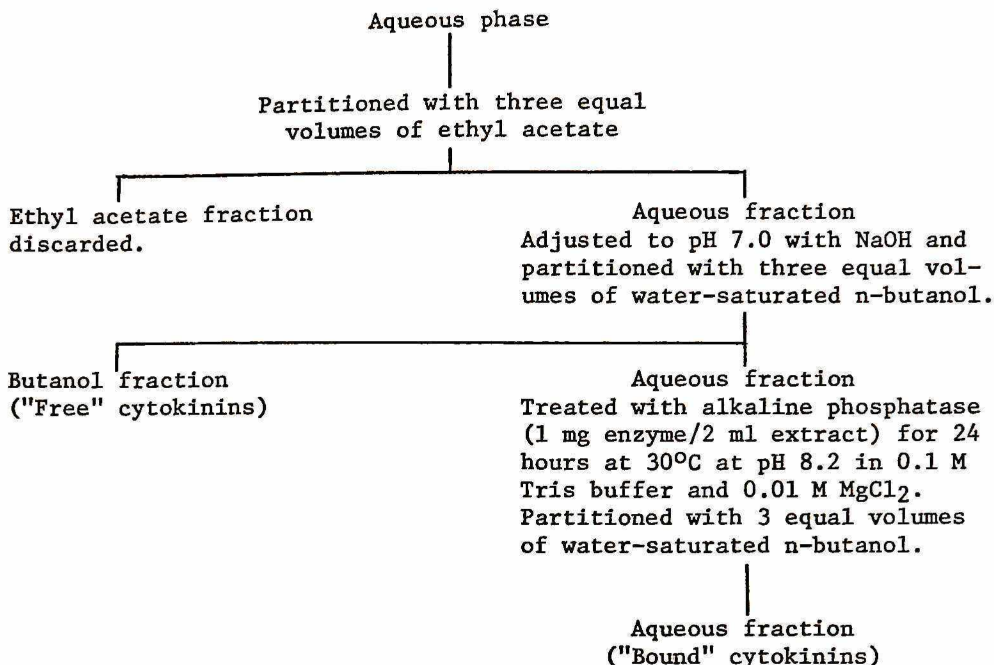

The butanol and aqueous fractions were taken to dryness under vacuum at 35°C, redissolved in 80% ethanol, streaked on Whatman No. 1 chromatography paper, and separated with butanol:acetic acid:water (12:3:5 v/v). The dried chromatograms were divided into 10 equal strips and the portion corresponding to Rf 0.7 - 1.0 was cut into small pieces and eluted with 80% ethanol overnight. The known synthetic and natural cytokinins (zeatin, kinetin, BA and isopentenyl-adenine) were run alongside the plant sample and were found to move to Rf 0.7 - 1.0. Cytokinin activity was determined by using the bioassay based on the chlorophyll formation in cucumber cotyledons (Fletcher and McCullagh, 1971b).

Cucumber plants were germinated in the dark at 28 ± 1°C for 6 days. The cotyledons were excised in dim green light and placed in 5 cm petri dishes containing 3 ml of cytokinin (BA) solutions ranging from 0.001 to

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to 10 mg/1. The dishes were returned to the dark at 28 ± 1°C for 14 hours and then moved into fluorescent light with an intensity of 220 ft-c. After 1½ hours the chlorophyll from 7 cotyledons was extracted with 80% acetone, brought up to a volume of 10 ml and the chlorophyll levels determined by measuring their absorbance at 665 mu.

In order to determine the levels of chlorophyll produced in cotyledons of different ages, another batch of cucumber seeds were grown for 4, 6, 8 and 10 days under the same growth conditions described above. At the completion of the treatments cotyledons were excised in dim green light and placed in 5 cm petri dishes containing 3 ml of water. The dishes were returned to the dark at 28 ± 1°C for 14 hours and then exposed to fluorescent light with an intensity of 220 ft-c. After various hours of exposure to light, the chlorophyll was extracted with 80% acetone and chlorophyll levels were calculated according to the methods described by Kirk (1968).

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RESULTS

Considerable difficulty was encountered in trying to isolate cytokinins from etiolated cucumber cotyledons. In developing the technique for isolating cytokinins, the methods of Staden et al. (1972) were used. It was found that with cucumber cotyledons the methods according to Staden et al. (1972) needed some modifications. Some of the modifications included addition of two standard columns, namely celite and Sephadex LH-20. An attempt was made to improve the amount of cytokinins obtained at the end of the fractionation and partition steps. In the first preliminary experiment the aqueous extract of 75 grams cucumber cotyledons were extracted for cytokinins using the methods of Staden et al. (1972) and celite and Sephadex LH-20 columns. No cytokinin activity could be detected in the final aqueous phase. It was observed that omission of petroleum ether (b.p. 60-80°C) step seemed to improve the yield of cytokinins.

It is possible that the cytokinins may have partitioned into the petroleum ether phase. Hence the use of petroleum ether to free cytokinins from inhibitory substances did not appear to be a satisfactory procedure. It is however possible that other cytokinins than those encountered in cucumber cotyledons may not be soluble in petroleum ether. The preliminary experiments showed that partitioning of acidic aqueous solutions of cytokinins with ethyl acetate for removal of inhibitors was the most satisfactory method even though some of the cytokinin activity which occurred as nucleosides or nucleotides might have been lost in the ethyl acetate phase. Following this step, further purification of the extract was attempted using celite chromatography. It was

observed with leaf tissue that the celite column removed coloured impurities which were not present in etiolated cucumber cotyledons and hence this step was dropped. In addition to solvent partitioning it was found that paper chromatography improved the resolution of cytokinins. In the preliminary experiments using the solvent system butanol: acetic acid: water (12:3:5 v/v) the highest cytokinin activity was found at Rf 0.7 - 1.0 and all the authentic cytokinins tested, including BA, kinetin, zeatin and isopentenyl adenine, moved to this range. Hence this portion of the paper chromatogram was used in all subsequent experiments. An attempt was made to determine whether the cytokinin activity present at Rf 0.7 - 1.0 of the butanol and aqueous extracts was due to more than one natural compound. The extracts were separated on a Sephadex LH-20 column using the technique of Armstrong et al. (1969). The fractions were collected and there seemed to be cytokinin activity in all fractions. The second fraction co-chromatographed with zeatin. This step, Sephadex LH-20 column separation, was omitted since all the fractions combined together gave more cytokinin activity in both butanol and aqueous fractions. Furthermore, it was observed that reducing fractionation and partitioning steps minimized the loss of cytokinin activity. Therefore, petroleum ether fractionation, Celite and Sephadex LH-20 column chromatography were omitted. After the initial ethyl acetate and 1-butanol fractionations described earlier, followed by paper chromatography the eluates were tested for cytokinin activity.

The levels of cytokinins in ageing etiolated cucumber cotyledons were determined in two different experiments. In both instances the "Free" and the "Bound" cytokinin activity in cucumber cotyledons followed a similar trend. Using the extraction procedures described

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the "Free" form is composed of free bases and/or nucleosides and the "Bound" form are nucleotides. Starting from the dry seed up to 4 days after germination the levels of cytokinin activity in the cotyledons appeared to be constant (Figure 2). At 6 days there was a gradual increase and by 8 days, the levels of cytokinin activity were the highest. Two days later when the cotyledons are probably senescing there was a drastic decline in the level of cytokinin activity.

The production of chlorophyll in etiolated cucumber cotyledons at 0 and 2 days after germination were not determined because of the difficulty in removing the seed coat. After 1½ hours of exposure to light there was a steady increase in chlorophyll levels from 4 to 8 days and by 10 days the capacity to produce chlorophyll had declined (Figure 3). This trend in chlorophyll production was similar to the pattern observed in endogenous levels of cytokinins. At 8 days when the cotyledons had the highest amount of cytokinins (Figure 2) the capacity to produce chlorophyll was also maximum at all time intervals (Table 1).

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Figure 2. Concentrations of "free", "bound", and total cytokinin activity (expressed as BA equivalent) in cotyledons of different ages.

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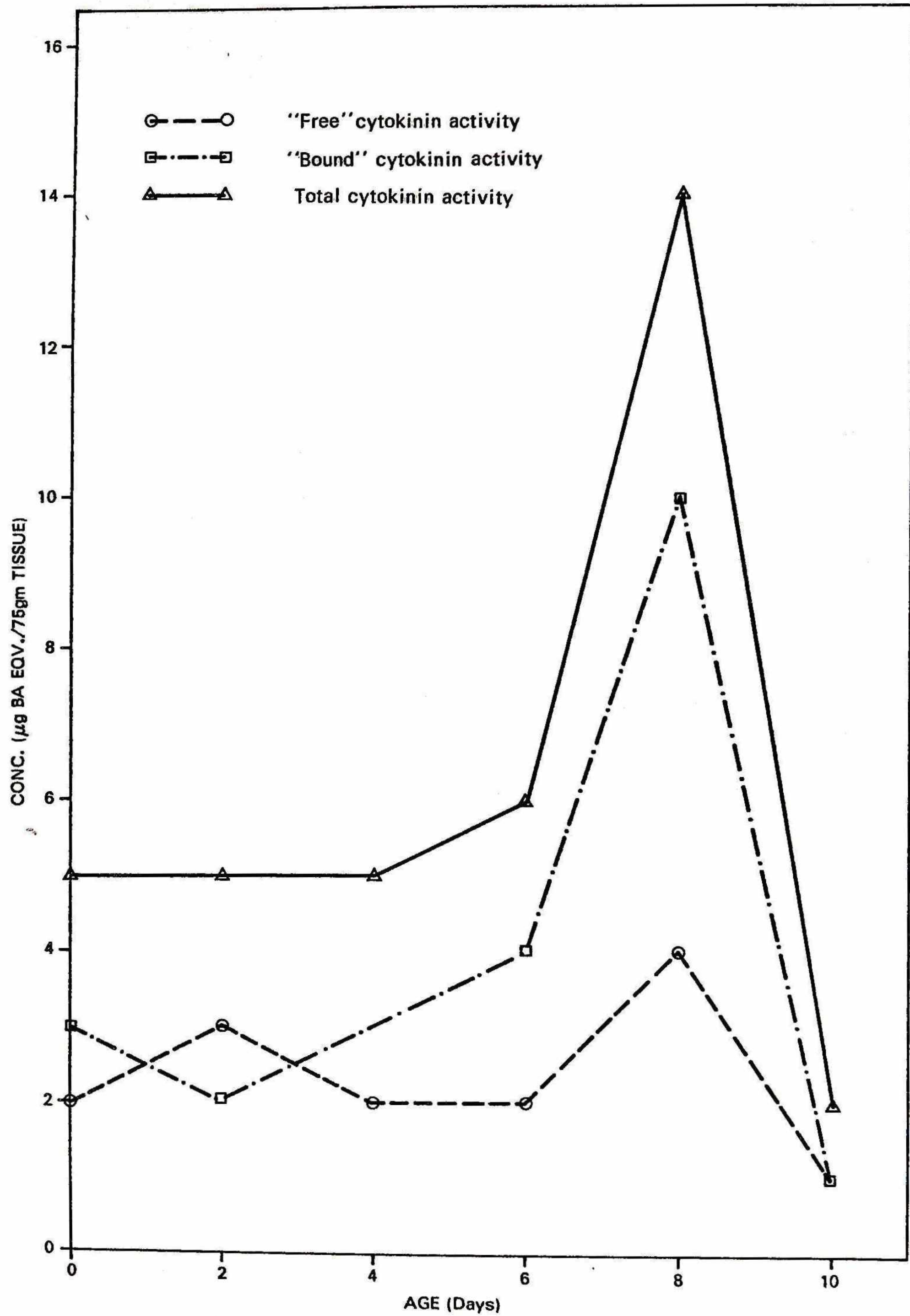

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Figure 3. Total cytokinin activity in cucumber cotyledons of different ages compared to chlorophyll (a + b) levels after 1½ h exposure to light.

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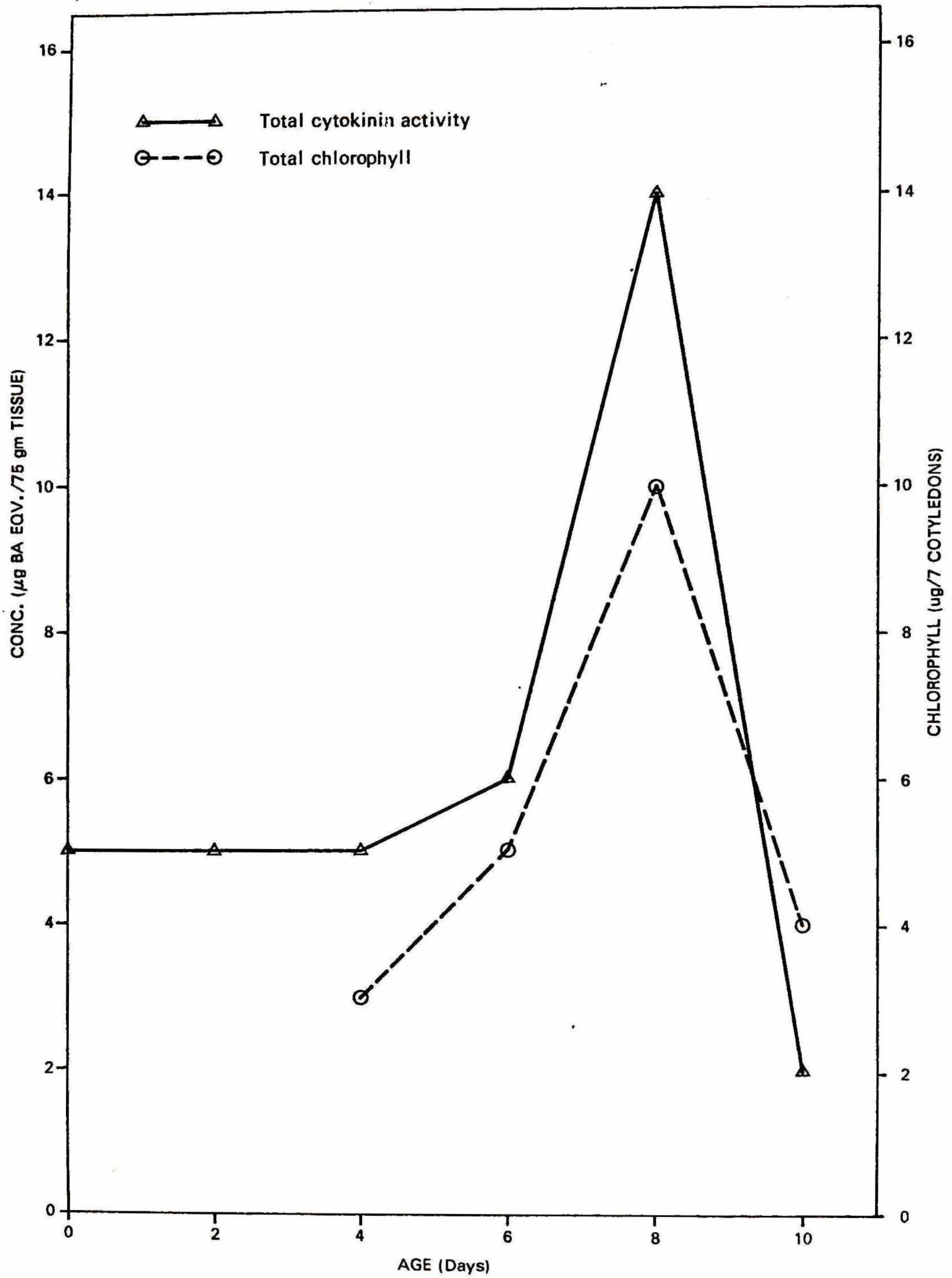

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Table 1. Chlorophyll (a + b) levels (μg/7 cotyledons) in cucumber cotyledons excised from etiolated plants of different ages and exposed to light for various durations.

Time of exposure to light, hours	Age of plant, days			
	4	6	8	10
0	3	4	11	3
½	3	5	7	4
1½	3	5	10	4
2½	6	9	13	8
3½	11	11	23	13
5	20	22	30	23
7	30	30	41	35

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DISCUSSION

There are a few investigations in which endogenous changes in cytokinin levels have been correlated with plant senescence. Sitton et al. (1967) have found that the level of cytokinins in root exudates of sunflowers decreased with the onset of shoot senescence. They, however, did not study the effect of exogenous application of cytokinins on senescence of this plant. Thomas (1968) determined cytokinin activity in Brussels sprouts by using the barley leaf section assay. Though this test seems reasonable for showing antisenescence factors, it is not specific for cytokinins (Letham, 1967; Halevy and Wittwer, 1965). Mayak and Halevy (1970) demonstrated a decline in cytokinin activity in senescing rose petals and showed also that exogenous applications of cytokinin delayed the senescence of petals of the short-lived variety. This may show that cytokinins participate in the regulation of senescence in rose petals. In the present study the cytokinin activity in cucumber cotyledons at the initial stages of growth may represent stored cytokinins in the seeds. Root-synthesized cytokinins may have contributed significantly to the high cytokinin activity recorded at 8 days. The sites of cytokinin biosynthesis within plants have not been unequivocally determined. There is strong circumstantial evidence that certain cytokinins are synthesized in root tips and transported in the xylem to the leaves where they have an important function in regulating metabolism and development (Weiss and Vaadia, 1965; Kende, 1971; Woolley and Wareing, 1972). It is therefore possible that the increase in cytokinins in 8 day old cucumber cotyledons resulted from either enhanced synthesis within the cotyledons or enhanced import of

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cytokinins from the roots. The drop in endogenous cytokinin level at 10 days is more likely related to the senescence of the tissue. That senescence is closely related to a drop in endogenous cytokinin levels in plants has been shown previously (Mayak and Halevy, 1970; Engelbrecht, 1971).

The results of chlorophyll studies showed that the capacity for chlorophyll production by dark-grown cotyledons changed during seedling development. This variation presumably reflects differences in the speed with which development proceeds in the dark at different ages of cotyledons. Similar results were obtained by Moore et al. (1972). They found that chlorophyll production was maximum after 8 days of dark-germination in french bean, runner bean, and lupin and after 5 days in mustard. Chlorophyll formation was reduced when the cotyledons were detached earlier or later than this time. Fletcher and McCullagh (1971b) had shown that exogenous applications of cytokinin (BA) influenced chlorophyll production in etiolated cucumber cotyledons. It was suggested that this effect was associated with either maintenance or an enhancement of chlorophyll synthesis (Fletcher, 1969; Adedipe et al., 1971). It has also been observed (Stetler and Laetsch, 1965) that cytokinins are essential for chloroplast differentiation in tobacco tissue. Fletcher and McCullagh (1971a) proposed that the effect of cytokinins in chlorophyll production is the result of induction of rate limiting proteins, and suggested that cytokinins influence both chlorophyll biosynthesis and chloroplast differentiation. The formation of δ-aminolevulinic acid (ALA) has been suggested (Nadler and Granick, 1970) to be the rate limiting product in both heme and chlorophyll production. Fletcher and McCullagh (1971a) have found that when etiolated cucumber cotyledons

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are exposed to light there is a lag phase in both ALA and chlorophyll production. If the cotyledons are pretreated with cytokinins, this lag phase is eliminated. In the present study with cucumber cotyledons of different ages there was a parallel relationship between cytokinin levels and chlorophyll production (Figure 3). When the cytokinin levels were highest in 8 day old cotyledons the production of chlorophyll was also maximum. This finding supports the hypothesis (Fletcher and McCullagh, 1971b) that cytokinins have an important role in chlorophyll production.

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