

ABSTRACT OF THESIS

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Title of Thesis Effects of a quaternary ammonium compound and
..... gibberellic acid on the growth of Phaseolus.

The effects of the quaternary ammonium compound (2-chloroethyl) trimethylammonium chloride, CCC, and gibberellic acid, GA₃, on various aspects of growth of light- and dark-grown plants of Phaseolus vulgaris were studied.

For light-grown plants to which CCC was applied at 10⁻²M on day 4 or day 7 after planting, the main effects of treatment were: a marked reduction in internode elongation, stem and root dry weight and total leaf area; an increase in chlorophyll content whether expressed on an area or on a cell basis; to delay unfolding of trifoliate leaves, to change their shape, and reduce cell number, although this last effect is probably indirect due to shading of primordia by the primary leaves; to prolong the period over which growth occurred and usually to reduce the rate of growth. CCC treatment was most effective in reducing growth when applied on day 4. GA₃ applied on day 7 counteracted almost all the effects of CCC treatment.

In dark-grown plants CCC treatment increased fresh and dry weight, and cell number for the first pair of leaves, and if applied early enough reduced the rate of loss of cotyledon weight. CCC effects on primary leaves were similar to effects of red or white light; light and CCC effects were additive. The CCC effects were not reversed by far-red radiation, nor were red light effects counteracted by GA₃, although this compound counteracted effects due to CCC.

CCC could always be detected in treated plants except

in immature seeds from which it was absent. When effects of CCC on the gibberellin content of treated plants were studied, it was found that CCC treatment was associated with a low level of gibberellins.

germination
A hypothesis is proposed to explain the effects of CCC on light- and dark-grown plants. The morphological changes following CCC treatment result from an effect of CCC on naturally occurring gibberellins. It is proposed that CCC treatment has two main effects: (1) it inhibits the synthesis of natural gibberellins, (2) it retards the liberation of the gibberellins believed to be in bound form in the cotyledons of germinating seedlings. The counteraction of CCC effects by GA₃ is seen to be the result of applied GA₃ substituting for endogenous gibberellins whose synthesis is inhibited by CCC, and this action constitutes the so-called interaction discussed by other workers.

EFFECTS OF A QUATERNARY AMMONIUM COMPOUND AND
GIBBERELIC ACID ON THE GROWTH OF PHASEOLUS

by

Gil Martins Felipe

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SUMMARY

The effects of the quaternary ammonium compound (2-chloroethyl) trimethyl ammonium chloride, CCC, and gibberellic acid, GA₃, on various aspects of growth of light- and dark-grown plants of Phaseolus vulgaris were studied.

For light-grown plants to which CCC was applied at 10^{-2} M on day 4 or day 7 after planting, the main effects of treatment were: a marked reduction in internode elongation, stem and root dry weight and total leaf area; an increase in chlorophyll content whether expressed on an area or on a cell basis; to delay unfolding of trifoliolate leaves, to change their shape, and reduce cell number, although this last effect is probably indirect due to shading of primordia by the primary leaves; to prolong the period over which growth occurred and usually to reduce the rate of growth. CCC treatment was most effective in reducing growth when applied on day 4. GA₃ applied on day 7 counteracted almost all the effects of CCC treatment.

In dark-grown plants CCC treatment increased fresh and dry weight, and cell number for the first pair of leaves, and if applied early enough reduced the rate of loss of cotyledon weight. CCC effects on primary leaves were similar to effects of red or white light; light and CCC effects were additive. The CCC effects were not reversed by far-red radiation, nor were

red light effects counteracted by GA₃, although this compound counteracted effects due to CCC.

CCC could always be detected in treated plants except in immature seeds from which it was absent. When effects of CCC on the gibberellin content of treated plants were studied, it was found that CCC treatment was associated with a low level of gibberellins.

A hypothesis is proposed to explain the effects of CCC on light and dark-grown plants. The morphological changes following CCC treatment result from an effect of CCC on naturally occurring gibberellins. It is proposed that CCC treatment has two main effects: (1) it inhibits the synthesis of natural gibberellins; (2) it retards the liberation of the gibberellins believed to be in bound form in the cotyledons of germinating seedlings. The counteraction of CCC effects by GA₃ is seen to be the result of applied GA₃ substituting for endogenous gibberellins whose synthesis is inhibited by CCC, and this action constitutes the so-called interaction discussed by other workers.

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I- INTRODUCTION

A. General

Growth retardants are defined by Cathey (1964) as "chemicals which slow down the processes of cell division and cell elongation in shoot tissues and regulate plant height physiologically without formative effects".

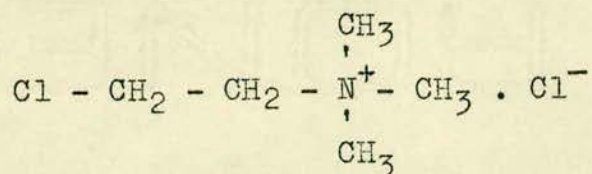
The first group of growth retardants were reported in 1949 by Mitchell, Wirwille and Weil and consisted of nicotinium compounds. These were characterized by their marked effects in retarding stem elongation (see Cathey, 1964).

Wirwille and Mitchell (1950) showed that a group of quaternary ammonium carbamates also retarded growth, without causing malformation in the plants. The most active of these compounds was found to be 4-hydroxyl-5-isopropyl-2-methyl-phenyltrimethyl ammonium chloride, 1-piperidine carboxylate (AMO-1618). Application of AMO-1618 brought about considerable suppression of stem growth in Chrysanthemum (Cathey, 1959).

Later, the growth retarding effects of phosphonium compounds were reported, in 1955 (see Cathey, 1964). The most active of this family of compounds is 2,4-dichloro-benzyl tributyl phosphonium chloride (Phosfon).

Tolbert (1960 a, b, c; 1961) reported the growth

retardant effects of a new group of quaternary ammonium compounds, the most active of which was (2-chloroethyl) trimethyl ammonium chloride also known as Cycocel or CCC (chloro-choline-chloride). The formula of this substance is as follows:



The substance is a white crystalline compound readily soluble in water.

The work described in this thesis is concerned principally with this compound.

B. The effects of CCC on plants

Tolbert (1960 a, b, c) compared the chemical structure of CCC with that of related compounds showing growth retarding activity. From his studies he pointed out that:

- (1) the trimethyl ammonium cation in the molecule was essential for activity,
- (2) for optimal activity, the length of the C chain which contains the covalent attached halide should be two carbons,
- (3) the Cl^- could be replaced by Br^- .

In these papers Tolbert concluded by saying that the mode of action of CCC was unknown and that the compound could act by attachment to a specific binding

site. This site could be thought of as a protein surface with two binding positions for the molecule: one position could be specific for the trimethyl ammonium cation, the second position would be located at a distance about equal to the length of the ethyl carbon chain away from the first site. Tolbert concluded that perhaps binding of this type could change metabolism in such a way as to lead to retardation of stem growth. Tolbert (1960 b) showed that CCC treatment caused shorter and thicker stems and darker and broader leaves in wheat, and that the compound was effective in a range from 10^{-6} to 10^{-2} M. Tolbert's work opened a new field for the investigation of growth and growth substances, and many papers dealing with the effects of CCC have appeared over the last seven years.

CCC does not appear to be a substance naturally found in plants. Mayr and Presoly (1961) found by chromatography of extracts of tomatoes and wheat a substance which they supposed to be CCC, but this claim has not been substantiated (Cathey, 1964).

Tolbert (1960 b) showed that CCC was not broken down in plant tissues and Zeevaart (1966) showed that CCC was accumulated in the seeds of treated plants, so that effects appeared in plants of the next generation. Once it has been accumulated by plants CCC appears to exert a long lasting effect. However CCC does not remain in the soil for long periods as it is decomposed fairly rapidly (Linser, Kühn and Bohring, 1964).

It was supposed that CCC exerted an effect either on cell division or on cell expansion. Cell division and cell expansion were inhibited in the longitudinal plane in the sub-apical region of stems of Chrysanthemum, but cell division and cell expansion in the transverse plane of the sub-apical regions of the stem were both stimulated (Sachs and Kofranek, 1963) thus resulting in shorter but thicker stems. In Pharbitis nil cell division in stems was decreased by CCC (Zeevaart, 1964), while Sachs and Wohlers (1964) showed that cell division was inhibited in callus cultures of stem tissue of geranium, Chrysanthemum and carrot. CCC treatment also inhibited the growth of cell suspensions of Digitalis (Lamba and Staba, 1963). Growth of callus of stem tissue of tobacco was found by Murashige (1965) to be inhibited by CCC treatment although no apparent effect was observed by Sachs and Wohlers (1964). However, Kelley and Postlethwait (1962) showed that in Pteridium gametophytes CCC stimulated the rate of cell division.

CCC usually reduces overall growth of plants, but this is not general and some variation occurs according to the species concerned. In Mentha piperita (Lamba and Andries, 1965), winter wheat (Michniewicz, 1965) and in plants of Pharbitis nil maintained in long days (Zeevaart, 1964), the overall growth was reduced or even completely suppressed. However, the growth of Cleome and Petunia plants (Cathey, 1964) was

not affected by CCC treatment. CCC treatment was also found to reduce the rate of dry weight increase for several species (Cathey and Stuart, 1961), but for tobacco plants growing in water culture, treatment with CCC increased dry matter content (Humphries, 1963a).

Of the effects of CCC on plant parts a great number of data are available for stem and leaves, but few are available concerning the growth of roots. Growth of roots was increased by CCC treatment for wheat (Sturm and Jung, 1964 a) and beans (Plaut, Halevy and Shmueli, 1964), but treatment reduced root fresh and dry weights for Datura stramonium (Dall'Olio, 1964a).

The usual effect of CCC on stems is to reduce or completely inhibit extension growth. This effect has been observed in a large number of plants, e.g.: strawberry (Guttridge, 1964), wheat (Tolbert, 1960a; Humphries, Welbank and Witts, 1965; Sturm and Jung, 1964b), pear (Modlibowska, 1965), Bryophyllum daigremontianum (Zeevaart and Lang, 1963), Chrysanthemum (Lindstrom and Tolbert, 1960; Sachs and Kofranek, 1963), squash and cucumber (see Cathey, 1964), sugar-beet (Humphries and French, 1965), sunflower (Jones and Phillips, 1967), Samolus parviflorus in long days (Baldev and Lang, 1965), mustard (Humphries, 1963a), Datura stramonium (Dall'Olio, 1964a; Lamba and Andries, 1966), Poinsettia (Kofranek, Sciaroni and Byrne, 1962; Joiner and Sheenan, 1964), and mung beans (Nwachuku and Lockhart, 1964). The only exception to this list of plants

showing decreased stem growth following treatment is snapdragon (Halevy and Wittwer, 1965a) in which it was found that the growth of the stem was increased both in length and dry weight. In this case CCC was applied as a leaf spray and it is known that spray treatment with CCC damages the foliage (Guttridge, Anderson and Stewart, 1966).

The stem of treated plants may be thicker than that of control as observed by Tolbert (1960a) for wheat and by Wittwer and Tolbert (1960a) for tomatoes. Treatment with CCC increased the number of vascular bundles in the stem of wheat (Mayr and Presoly, 1963). As a result of the decrease in stem growth following treatment, the ratio of roots/tops was increased for beans and tomatoes (Tognoni, Halevy and Wittwer, 1967), but no effect was noted for wheat (El Damaty, Kühn and Linser, 1965). Cathey and Stuart (1961) showed that at $10^{-2}M$ CCC was moderately active in suppressing or reducing stem elongation in darkness for several species. In mustard and mung-beans, CCC treatment had no effect on the photoreaction controlling stem length by means of red and far-red radiation (Nwachuku and Lockhart, 1964).

Thus, in general, total plant growth and growth of stems are decreased by CCC treatment but root growth may be increased.

A large number of papers present data about effects of CCC on leaf growth. Total leaf area was

found to be increased by CCC treatment for Chrysanthemum (Lindstrom and Tolbert, 1960) and mustard (Humphries, 1963a), but was decreased for sugar-beet (Humphries and French, 1965). Not all authors distinguish between effects on leaf number and on area of individual leaves, however there are reports of individual leaf area being increased (Humphries, 1963b) and decreased (Dyson, 1965). The total number of leaves produced was increased by treatment in sugar-beet (Humphries and French, 1965) but was not altered for wheat (Tolbert, 1960a). The fresh weight of leaves was increased by treatment in Datura stramonium (Dall'Olio, 1964a), and leaf dry weight was increased in sunflower (Humphries, 1963b), Datura stramonium (Dall'Olio, 1964a) and snapdragon (Halevy and Wittwer, 1965a). No effect of CCC treatment on initiation of leaves in Chrysanthemum (Sachs and Kofranek, 1963) was found, but the time of appearance of new leaves was delayed in Lolium temulentum (Stoddart, 1965) and wheat (Tolbert, 1960b). Leaf longevity was found to be increased in plants of Cannabis sativa treated with CCC (Dall'Olio, 1964b). Wheeler (1962) found no effect of CCC on growth of leaf disks of Phaseolus grown in darkness although CCC treatment suppressed the promotive effect of gibberellic acid, GA₃.

In general it has been found that plants treated with CCC have darker leaves than control plants, (e.g. Tolbert, 1960a; Lindstrom and Tolbert, 1960; Wittwer

and Tolbert, 1960a). Where chlorophyll content of leaves was measured, increases have been reported following treatment (Humphries, 1963a; Stoddart, 1965). Laborie (1963) observed that in tomatoes and wheat, leaves on treated plants were darker than the control, and if chlorophyll content was calculated on an area basis, the content of chlorophyll a and b was increased by CCC treatment, but if the content was expressed on a fresh weight basis, the content was only slightly increased. Mme. Laborie studied the effect of CCC on several leaf pigments including β -carotene, luteine and chlorophylls a and b, and observed that only the level of chlorophyll a seemed to be sensitive to treatment. It would appear that the darker colour of leaves on treated plants may be due to either a real increase in total chlorophyll content, or to an increase in chlorophyll per unit area in those cases where CCC causes a decrease in leaf area.

Senescence can be retarded by CCC application. Beevers and Guernsey (1967), using the loss of protein, chlorophyll and RNA as a measurement of senescence, observed that CCC delayed senescence in Tropaeolum leaf disks; it is of interest that GA₃ was more effective than CCC in delaying senescence of these disks. Retardation of senescence has been reported for leaves of beans (Halevy and Wittwer, 1965b), brocolli and tomatoes (Halevy, Dilley and Wittwer, 1966). Lettuce given post-harvest treatment with CCC also showed retarded senescence although post-harvest treatment of brocolli

(Halevy, Dilley and Wittwer, 1966) was ineffective.

A large variety of effects of CCC on flowering have been reported. Thus, flower number can be increased, reduced or not affected by treatment (e.g. Poole, 1963; Baldev and Lang, 1965; Humphries and French, 1965). CCC effects on the photoperiodic response have been examined in detail for Pharbitis nil, a short day plant, by Zeevaart (1964), who found the compound could either inhibit or increase flower production according to time of application in long or short days.

The number of fruit buds in pear (Modlibowska, 1965) and of berries set in Vitis vinifera (Coombe, 1965) were increased by CCC application. The growth of seeds of Pharbitis was not affected by CCC (Zeevaart, 1966). The yield of wheat was increased by CCC treatment (Tolbert, 1960b; Sturm and Jung, 1964b; Pinthus and Halevy, 1965; Humphries, Welbank and Witts, 1965) whilst in Phleum pratense CCC treatment increased the number of heads per plant (Stoddart, 1964). The amount of crude protein in wheat grains was not altered by CCC (Sturm and Jung, 1964a).

Using methods of growth analysis it was found that net assimilation rate was decreased by CCC treatment for mustard (Humphries, 1963a) but was not affected for sugar-beet (Humphries and French, 1965) and for beans and tomatoes growing in water culture, although relative growth rate and leaf area ratio were decreased if CCC was applied (Tognoni, Halevy and Wittwer, 1967).

The effects of CCC on the physiology and biochemistry of plants has also been extensively studied. The respiration of roots from etiolated pea seedlings was stimulated by CCC (Heatherbell, Howard and Wicken, 1966). Transpiration rate of slash pine was reduced by treatment (Asher, 1963), although CCC had no effect on the transpiration coefficient in wheat (El Damaty, Kühn and Linser, 1965). CCC has been claimed to increase frost resistance in pear (Modlibowska, 1965) and to give increased drought resistance in beans (Halevy and Kessler, 1963) and in Chrysanthemum (Lindstrom and Tolbert, 1960). CCC may also affect the vernalization process. For example, in winter wheat CCC treatment reduced the effectiveness of the low temperature treatments (Michniewicz, 1965). In Oenothera biennis only the highest dose of CCC (750µg) retarded the elongation of the floral stem when the plant had been vernalized, although smaller doses stimulated the growth of the floral stem of vernalized plants (Picard, 1967). Germination of lettuce was inhibited (Wittwer and Tolbert, 1960b) and retarded in kale (Knypl, 1967) by CCC treatment.

Van Emden (1964) suggested that CCC causes large changes in plant composition. In Lolium temulentum (Stoddart, 1965), CCC caused an accumulation of carbohydrates and the content of amino-acids was increased over that of control plants provided that plants also received nitrogen as fertilizer. The content of nitrogen per leaf was increased with CCC treatment in sun-

flower (Humphries, 1963b) and tobacco, although nitrogen content was decreased for stems of tobacco (Humphries, 1963a). The pool of extractable soluble N was not affected by CCC in wheat plants. The content of proline, methionine, lysine, threonine, alanine, valine and leucine was increased, while arginine and hydroxy-proline levels were reduced (Linser, Neumann and El Damaty, 1965).

The foregoing account illustrates the wide and diverse effects that have been found to result from CCC treatment. A number of suggested explanations for the effects of CCC have been advanced and it is now appropriate to discuss some of these.

Because of the large effects on stem growth a number of authors have looked for effects of CCC treatment on indole acetic acid metabolism. Halevy (1963) showed for cucumber seedlings that CCC treatment increased the activity of peroxidase and of IAA-oxidase and suggested that CCC affects auxin synthesis. At about the same time Kuraishi and Muir (1963) showed that IAA treatment could enhance growth of stem segments cut from pea plants previously treated with CCC, whereas GA₃ treatment had little effect. Using sunflowers, Knypl (1964) showed that longitudinal extension of the hypocotyl was retarded by CCC, and that this effect was counteracted by IAA. It has also been claimed that IAA inhibited seed germination and root elongation for lettuce (Khan and Tolbert, 1966b) and that CCC counteracted both these effects, although CCC applied alone was ineffective;

CCC did not reverse the effects of indole-3-propionic acid and of indole-3-butyric acid. The reports might seem to favour the idea that CCC acts through effects on IAA metabolism, however the inhibition of growth of tobacco stem calluses by CCC is not reversed by IAA (Mura-shige, 1965) nor is the inhibition of cell division and expansion found by Sachs and Wohlers (1964) for callus cultures of various species.

A very substantial body of data exists to show that effects of CCC are often counteracted by application of GA₃; because of this, several authors (e.g. Lockhart, 1962) have considered CCC to be an anti-gibberellin. Much of the early work on the counteraction of CCC effects by GA₃ is reviewed by Cathey (1964) and is not discussed further here. The view has developed that a close relationship exists between CCC and gibberellin metabolism and that perhaps CCC acts by influencing the levels of natural gibberellins. It seemed reasonable to examine the effects of CCC on synthesis of natural gibberellins and using Fusarium moniliforme, Kende, Ninnemann and Lang (1963), Ninnemann, Zeevaart, Kende and Lang (1964) and Harada and Lang, (1965) proved that CCC treatment did inhibit the synthesis of gibberellins by the fungus, without destroying gibberellins already formed. CCC inhibition of gibberellin synthesis was also shown in Pharbitis nil (Zeevaart, 1966), and it has been claimed that CCC treatment inhibits the formation of gibberellin-like substance in embryo of barley

(Yomo and Iinuma, 1966). Other reports show the amount of extractable gibberellins to be reduced in treated plants, e.g. potato (Humphries and Wheeler, 1962), Avena sativa (Simpson, 1966), Impatiens glandulifera and Pisum (Reid and Carr, 1967) and in sunflower (Jones and Phillips, 1967).

How, and at what point CCC stops the synthesis of gibberellins is still unknown; the only approach to this so far appears to be that by Harada and Lang (1965) who fed kaurene, kaurenol and steviol to cultures of Fusarium and found that no conversion to gibberellins occurred, if CCC was also given, and Dennis, Upper and West (1965) believe that the inhibition by CCC of the gibberellin synthesis occurs beyond the stage of formation of (-) kaurene.

The evidence that CCC inhibits gibberellin synthesis, has been used to explain the observed effects which are held to result from a shortage of gibberellins within treated plants; and application of GA₃ has been thought to counteract these effects, by unblocking the inhibition of synthesis of natural gibberellins, although of course it could equally be that GA₃ substitutes for natural gibberellins. However some data do not support fully these ideas. Sachs and Wohlers (1964) found that inhibitory effects of CCC on stem tissue cultures could not be counteracted by GA₃ application, while Paleg, Kende, Ninnemann and Lang (1965) found that the GA₃-mediated release of sugars from barley

endosperm could not be counteracted by CCC. This finding does not mean that CCC does not affect synthesis of gibberellins, since the compounds might already be present in the endosperm tissues, and CCC does not destroy gibberellins already formed. Beevers and Guernsey (1967) using Tropaeolum leaf disks found that GA₃ and CCC exerted similar effects in delaying senescence of the tissues.

That CCC effects are far from simple is clear from the complex interactions between this compound and other growth promoting or retarding compounds such as coumarin, kinetin and maleic hydrazide. Knypl (1964) observed that the retardation by CCC of longitudinal extension of the hypocotyl of sunflower seedlings was counteracted by coumarin. For kale Knypl (1967) showed that CCC retarded seed germination and that this effect was counteracted by kinetin and by GA₃. Coumarin also retarded germination and this effect was counteracted by kinetin but not by GA₃. If CCC and coumarin were applied together, then germination was stopped, and only GA₃ plus kinetin could counteract this effect. Because of these facts Knypl believes that CCC and coumarin block two different metabolic pathways. Khan and Tolbert (1966a) also observed that coumarin, like IAA, inhibited lettuce seed germination, and the effect of coumarin was counteracted by CCC in red light (there was no effect in far-red light or darkness), but not by IAA or GA₃. The whole picture has become even more complex with recent findings of Mishra and Paul (1967). B-Nine

(N-dimethyl amino succinamic acid) and MH (maleic hydrazide) are two well-known chemical substances which retard or inhibit growth. Mishra and Paul (1967) showed that CCC or MH reduced the early shoot growth of rice, while B-Nine alone was ineffective, although it counteracted the effect of either CCC or MH. MH reduced markedly the early shoot growth of rice, and also counteracted the effect of CCC. It would appear that these substances all act on different parts of the metabolic pathway which leads to growth.

From this it can be seen that while information about CCC effects is extensive, results and interpretations are also controversial and incomplete. More detailed data are still needed to clarify an understanding of how this compound acts on plants.

C. The nature of the investigation

In the work to be described, attention has been directed at determining in detail, effects of CCC on growth and form of plants of Phaseolus vulgaris grown in conditions of light and darkness. Especial emphasis has been made on effects of CCC on leaf characters since, from literature, it is clear that our knowledge in this field is inadequate. At the same time a basic objective behind the work has been to try to discover more about the mode of action of CCC, and to do this it has been necessary to study the effects of GA₃ on growth of con-

trol plants and plants treated with CCC. The work has also led to the measurement of levels of gibberellins and gibberellin-like substances in Phaseolus plants. The results from the growth studies and from the work involving applied and endogenous gibberellins, has led to the formation of a hypothesis to explain the mode of action of CCC on Phaseolus vulgaris.

It may be mentioned here that Phaseolus vulgaris was chosen as test material for a number of reasons; firstly, plants of this species grow rapidly and traverse their life cycle within 2-3 months; secondly, French bean plants have been shown to respond to CCC by other workers and it is known that response to applied gibberellins is large; thirdly, seeds of this species are large and known to contain considerable amounts of gibberellins during development; fourthly, considerable experience of growing Phaseolus plants under controlled environmental conditions has been accumulated in the Botany Department at Edinburgh, and a large body of data on leaf growth in this species is available.

II- MATERIAL AND METHODS

A. Material

This study was conducted using plants of French bean. Phaseolus vulgaris (L.) Savi. cultivar Sutton's Selected Canadian Wonder.

Plants were grown from seed which varied in weight between 0.4 and 0.6g, except in one experiment where smaller seeds were used.

B. Methods

1- General

Seeds were planted in washed river sand (Levensat number 13) in 4.5 inch plastic pots. Each pot received 100ml of Hoagland's number 1 solution (Hoagland and Arnon, 1938) at planting. After emergence above the sand, which occurred 6 - 7 days after planting, material was watered on alternate days with Hoagland's solution or distilled water.

Experiments on light-grown plants were performed in Growth-rooms in which temperature, light intensity and day-length were controlled (Dale, 1964a). Throughout, the cycle length was 24 hours, and day-length was 12 hours with a light intensity of 850f.c. from 5ft. 80^{watt} warm white fluorescent tubes (Phillips). Tungsten light was not used in any experiment. Two seeds per

pot were sown at planting and on day 6 one seedling was removed from each pot. Temperature was 22.5°C ($\pm 1^{\circ}\text{C}$).

Experiments on dark-grown plants were performed in a completely darkened room in which temperature was controlled at 25°C ($\pm 1^{\circ}\text{C}$). When plant material had to be handled a dim green safe light was used. Light from a 15w. tungsten bulb passed through a Kodak Clear Light Green Safelight (Wratten series 12). This generates light between wavelengths of 500 and 560 nm. Miss D. Murray (1967) shows that this irradiation has no effect on either fresh weight or cell number of primary leaves of Phaseolus vulgaris.

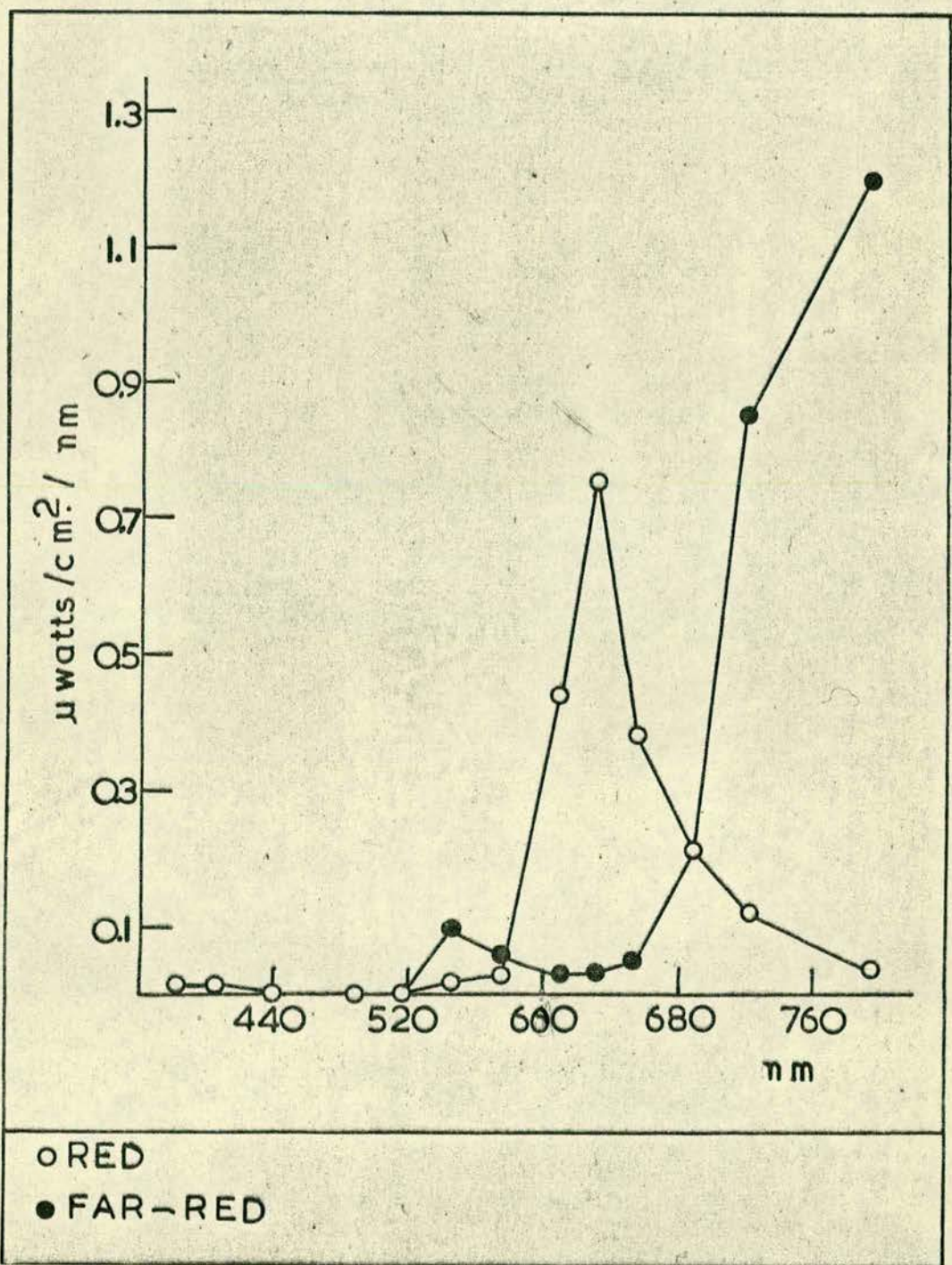
In some experiments dark-grown seedlings were treated for some minutes with light. The light source consisted of a rack holding three 5ft. 80w. coolwhite fluorescent tubes and 20, 25watt tungsten bulbs. These illuminated a shelf 20 inches below, on which plant material was placed. The fluorescent tubes and the tungsten bulbs were wired independently and could be used separately or together. This arrangement was used for irradiation with white light and also for irradiations using red and far-red light. White light treatment: dark-grown plants were transferred to the illumination rack and received 10 minutes of white light. Only fluorescent tubes were used. The light intensity was about 600 f.c. at plant level.

Red and Far-red treatments: irradiation of dark-grown material with red and far-red was carried out in a

Figure 1 - Light quality for red and far-red irradiations.

Red light: light from coolwhite fluorescent tubes passed through a single sheet of red cellophane paper.

Far-red: light from 25w tungsten bulbs passed through a sheet of red cellophane paper and a blue glass plate (see Methods).



specially designed box which was placed on the shelf below the light battery during illumination. The box was constructed with two square windows on the top surface. They held glass filters in grooved channels. Apart from light passing through the filters, the box was light tight. Red light was generated by filtering the light from cool-white fluorescent tubes through one sheet of red cellophane paper (Dennison Manufacturing Co. Ltd.). The red cellophane transmitted no light below 516 nm, thus no blue light was transmitted; while the coolwhite fluorescent tubes emitted small quantities of far-red light. The use of this filter and light source produced a transmission curve as seen in Figure 1. 10 minutes of red light were given to the treated plants. Far-red light was generated by passing light through red cellophane paper and a blue glass plate. The light source used was 25 watt tungsten bulbs which are rich in far-red light. The red cellophane prevented transmission in the blue and the blue glass plate cut down transmission in the red region. The transmission curve for far-red is also shown in Figure 1. Plants treated with far-red received 10 minutes of this radiation. The transmission curves presented in Figure 1 were provided by Miss D. Murray.

CCC treatments are discussed in the next section. Where gibberellic acid, GA₃ (BDH, Poole) was applied to plants, this was done on Day 7 after planting. A drop of 10 μ l in volume was applied to the apex with a micro-

pipette. GA₃ was made up in ethanolic solution in a range of concentrations from 1 to 1000ppm. Final concentration of ethanol in the solution was 0.2% or less according to the dilution.

2- Harvest

At many harvests leaf area and dry weight of plant parts were measured, as interest centred on the classical parameters of growth analysis. In addition, measurements of stem and leaf length and leaf shape were sometimes made.

For each treatment five plants were usually harvested and in every experiment suitable controls were grown and measured.

Leaf Area: this was measured using a commercial leaf area meter (Evans Electroselenium Ltd.), for which calibration curves have been prepared using pieces of known area of either black paper or bean leaf tissue (Hurd and Rees, 1966).

Dry Weight: at harvest each plant was divided into first pair of leaves, other leaves, stem and roots. Before weighing, the plant parts were dried for 24 hours in an oven at about 80°C.

Derived data: Relative Growth Rate (R) and Net Assimilation Rate (E) were calculated according to Williams (1946). Thus,

$$R = \frac{\ln W_2 - \ln W_1}{t_2 - t_1} \text{ g/g/day}$$

$$E = \frac{\ln A_2 - \ln A_1}{A_2 - A_1} \times \frac{W_2 - W_1}{t_2 - t_1}$$

where A is a leaf factor and can be either leaf dry weight or leaf area. In this work A refers to leaf area. W is the total dry weight and $t_2 - t_1$ is the time interval between two harvests.

Leaf Area Ratio (L.A.R.) can be calculated either for the term $\bar{L}a = R/E$ in which case it represents a mean value for a stated interval $t_2 - t_1$, or it may be calculated as an instantaneous value for the time of harvest from the term Area/Dry weight. In this work the latter term is used.

Stem Lengths: these were measured in cm. using a transparent plastic ruler.

Leaf length: each leaf of the first pair was measured along the midrib at daily intervals from day 6 to day 11. Subsequent measurements were made at two-day intervals.

Measurements for trifoliate leaves were made daily. In plants treated with CCC the trifoliate leaves unfolded later than in the control and a straight comparison of lengths was impossible. It was therefore decided to use an artificial starting point for the measurements so that the trifoliate leaves of all treatments could be compared on the same basis. Measurements were begun when the median leaflet reached a length equal to or greater than three cm., this being the approximate length of control leaflets on unfolding. Thus the time

scale used is not chronological age but what may be called modified leaf plastochron age (c.f. Erickson and Michelini, 1957) in which day 1 represents the day on which the stated trifoliate leaves of the plants in all treatments are three cm. long.

Leaf Shape: this was compared using the method of Melville (1937). For this the leaf length was divided in 100 units and a grid of ten unit squares was super-imposed on a tracing of the leaf. Leaf width was then measured in grid units at intervals of one unit along the length of the lamina. (See figure 2). Using this method comparisons could be made of the shape of leaves of different sizes. In the data for leaf shape the ratio of leaf width for treated and control leaves is shown as $\sqrt[4]{\quad}$ of the original ratio. Using this term a difference in ratio of 0.05 is equivalent to a 25% difference in absolute terms and has been assumed to indicate a real difference.

3- Analytical

Cell Number: cell counts were made for leaf material macerated in 5% chromic acid (Brown and Rickless, 1949). It was not always possible to count, immediately, cells in the large number of samples collected in many experiments. Use was therefore made of the method of Dale (1964c) for counting cells in dried material. This meant that cell counts could be made at leisure after harvesting material. Fresh material was always used

Figure 2 - Method for the determination of leaf shape.

The leaf length was divided in 100 units and a grid of ten unit squares was super-imposed on a tracing of the leaf. The denominator of the ratio shows the longitudinal position along the midrib at ten unit intervals from base (0) to apex (100) of the leaf. The numerator is the horizontal value for these positions in terms of grid units. To compare a treated leaf with control the numerator of the treated leaf is divided by the numerator of the control leaf for the same denominator (see Methods).

for cell counts on dark-grown plants.

Chlorophyll Content: as a rule 25cm² of leaf was used and extracted in 60ml of boiling 80% acetone and optical density of the solution measured at 645 and 663 nm in a spectrophotometer (Unicam SP 100). Chlorophyll was estimated using the constants listed by Arnon (1949).

Gibberellin Content: gibberellins were extracted from 4 and 11 day old plants and immature and dry seeds, according to the method of Zeevaart (1966). Five seeds were extracted several times with methanol at 2°C and subsequently twice at room temperature. The combined extracts were evaporated to dryness. Five complete plants were frozen in liquid nitrogen and lyophilized. The dry material was extracted with methanol at 2°C. The extract was then made up to 80% methanol and lipid material was removed by mixing with petroleum ether. Methanol was evaporated from the aqueous extract under reduced pressure. Potassium phosphate buffer (pH 8.2) was added to the water residue which was then partitioned against ethyl acetate to give a basic fraction. The water residue was adjusted to pH 2.5 with 6N HCl, and partitioned against ethyl acetate to give an acidic fraction. Both fractions were evaporated to dryness and the residues were analyzed by thin layer chromatography. The residues were taken up in a small volume of methanol and applied as a one cm. wide band to the starting line on 10x20cm glass plates covered with a layer of silica-gel G (Merck) at a thickness of 400 μ . The solvent

system used was a mixture of chloroform: ethyl acetate: acetic acid (60:40:5) (Zeevaart, 1966). After running, the plates were left for 4 days until all the solvent had evaporated. The plates were then divided in 10 equal zones and the silica gel from each of these was scraped off into small petri dishes (5 cm diameter). Three ml. distilled water was then added to the silica, and the slurry covered with a filter paper, ready for bioassay.

Gibberellin Bioassay: the bioassay used was the hypocotyl extension of Grand Rapids Lettuce (Frankland and Wareing, 1960). According to Brian (1966) this is one of the few tests for gibberellins which gives a response to practically all the known gibberellins.

Every time that an extract was assayed a standard series made from 0 to 10 μ g of gibberellic acid (GA₃) was used for comparison. Three ml of solution were put in a petri dish. A blank thin layer chromatogram (without extract) received the same treatment as the ones with extracts. The plate was divided in 10 equal zones and the silica scraped off and put in the petri dishes of the standard series. The silica was then covered with filter paper. Lettuce seeds were germinated in complete darkness at 25 $^{\circ}$ C for 2 days. Then six seedlings were put in the petri dishes on top of the filter paper. The dishes were incubated in continuous light in a Growth-room at 26 $^{\circ}$ C. After 5 days the hypocotyls were measured.

The amount of gibberellins is given as μ g equi-

valent of gibberellic acid and were deduced by extrapolation using the response curve of hypocotyl length for the standard gibberellic acid series.

Identification of CCC: the seed extract and the basic fraction of the plant extract used for gibberellin bioassay were also used for identification of CCC. Extracts were applied to paper chromatograms. The solvent system used was butanol: acetic acid: water (4:1:1.8) and descending chromatography on Whatmann 3MM paper was used (Zeevaart, 1966). After drying, presence of CCC on the chromatogram was detected by dipping in modified Dragendorff reagent (Thies and Reuther, 1954).

III PRELIMINARY EXPERIMENTS

Before the main experiments could be performed it was necessary to establish two points of technique. These were firstly the concentrations of CCC to be used and secondly the time at which CCC was to be applied to be effective.

A. The effect of different concentrations of CCC

From the work of Tolbert (1960b) it is apparent that CCC is effective on different plants at a range of concentrations from 10^{-2} to 10^{-6} M. The first experiment was therefore designed to investigate the effect of three levels of CCC on growth of bean plants. ²/₆

CCC* was made up in aqueous solution at 10^{-6} M and 100ml of solution was applied as a soil drench to each pot on the fourth day after planting. Measurements were made of leaf area, plant dry weight and internode length on day 22, i.e. 18 days after treatment.

*CCC obtained from BDH, Poole (technical grade). Recrystallisation from acetone did not result in greater activity of the compound.

Leaf area (Table 1)

TABLE 1

Area (cm²) of primary and trifoliolate leaves of plants treated with three concentrations of CCC and measured 22 days after planting.

	C C C			
	CONTROL	10 ⁻⁶ M	10 ⁻⁴ M	10 ⁻² M
One leaf of 1st pair	132.8	114.5	114.0	70.8
Trifoliates				
1	178.0	173.2	169.2	51.0
2	191.0	188.8	168.2	54.0
3	195.0	182.4	158.0	44.6

CCC at 10⁻²M markedly reduced the leaf area of the first pair of leaves, and at the other two concentrations values were slightly smaller than those of the control. CCC at 10⁻²M also reduced leaf area of the trifoliolate leaves. The slight reduction caused by CCC at 10⁻⁴M in the second and third trifoliolate leaves and by CCC at 10⁻⁶M in the third trifoliolate leaf did not occur when this experiment was repeated.

Dry weight (Table 2)

Total plant dry weight was markedly reduced by CCC at 10⁻²M and a slight effect was also noted for CCC at 10⁻⁴M. The lowest concentration had no effect on total dry weight. With CCC at 10⁻²M root and leaf dry weights were both reduced by approximately 50%. A rather

larger effect was found for dry weights for stem plus petiole which were reduced by about 70% and this is correlated with the large effects on internode length.

Length of the internodes (Table 3)

All concentrations of CCC tested reduced the length of the 1st, 2nd and 3rd internodes dependent on the concentrations used. The effect ranged from a reduction of over 90% at $10^{-2}M$ to over 60% at $10^{-4}M$ and 30% at $10^{-6}M$.

From this experiment it was concluded that although CCC at $10^{-6}M$ was effective in reducing internode length, the most spectacular effects on leaf growth, as measured by area and dry weight, were obtained with CCC at $10^{-2}M$ and this concentration was therefore chosen as the standard for further experiments. In subsequent experiments treatment with CCC involved the application of 100ml of $10^{-2}M$ CCC as a soil drench.

B. The effect of time of application of CCC

Since a major objective in the work was to determine the effect of CCC on leaf growth, and since the primary leaves which contribute a large proportion of the leaf area are mature by day 16, it was felt that early application of CCC was necessary if growth of these leaves was to be affected. In this experiment CCC was applied on Days 1, 4, or 7 after planting; these treatments are designated CCC1, CCC4 and CCC7,

and this convention for indicating days of treatment is used throughout.

The results for leaf area (Table 4) and dry weight (Table 5) showed that all treatments had large but similar effects, although the effect on primary leaf area of CCC4 compared with CCC7 was confirmed in later experiments.

It was concluded that time of application of the chemical was not critical on light-grown plants (but see later account for dark-grown plants). The times of application were therefore chosen arbitrarily for convenience to be Day 4 and Day 7.

TABLE 2

Dry weight (g) of plants treated with three concentrations of CCC and measured 22 days after planting.

	C C C			
	CONTROL	$10^{-6}M$	$10^{-4}M$	$10^{-2}M$
All leaves	1.60	1.65	1.45	0.72
Stem and Petiole	0.78	0.79	0.58	0.24
Roots	0.60	0.57	0.54	0.28
Total dry weight	2.98	3.01	2.57	1.24

TABLE 3

Internode length (as % of the control) for plants treated with three concentrations of CCC and measured 22 days after planting.

	C C C		
	$10^{-6}M$	$10^{-4}M$	$10^{-2}M$
1st internode	74.3	43.5	7.6
2nd internode	68.5	34.2	8.6
3rd internode	72.1	31.6	6.3

TABLE 4

Area (cm²) of primary and trifoliolate leaves of plants treated with CCC on Days 1, 4 or 7 after planting and measured 26 days after planting

	CONTROL	CCC1	CCC4	CCC7
One leaf of 1st pair	114.0	82.2	72.7	92.0
Trifoliates				
1	167.4	61.8	69.8	49.4
2	168.4	91.4	85.2	73.4
3	196.0	158.4	110.4	129.6

TABLE 5

Dry weight (g) of plants treated with CCC on Days 1, 4 or 7 after planting and measured 26 days after planting

	CONTROL	CCC1	CCC4	CCC7
Total Leaves	2.19	1.06	0.94	1.05
Stem	1.22	0.39	0.34	0.41
Roots	1.00	0.48	0.40	0.42
Total Weight	4.41	1.93	1.68	1.88

IV- RESULTSA. Effects of CCC and GA₃ on light-grown plants

The object of the experiments described in this section has been to investigate in detail effects of CCC on light-grown Phaseolus plants, and to ascertain how these effects may be modified by concurrent or later application of gibberellic acid.

1- Counteraction of CCC effects by GA₃

The counteraction of effects of CCC and other growth retardants by gibberellic acid has already been referred to (see Introduction). The first experiment was designed to investigate the effects of five levels of GA₃ (0, 0.01, 0.1 and 10.0ug per plant) on the response of plants treated with CCC on day 4 or day 7. Plants were harvested 24 days after planting.

There was no significant effect of GA₃ alone on primary leaf area (Table 6) and this was confirmed in later experiments. A decrease in leaf area was found with CCC (as in the preliminary experiments); the reduction was greatest when CCC was applied on day 4. This effect was counteracted by GA₃ and the extent of counteraction was dependent on the concentration of GA₃. Thus GA₃ alone had no effect on primary leaf area but had large effects when applied to CCC treated plants.

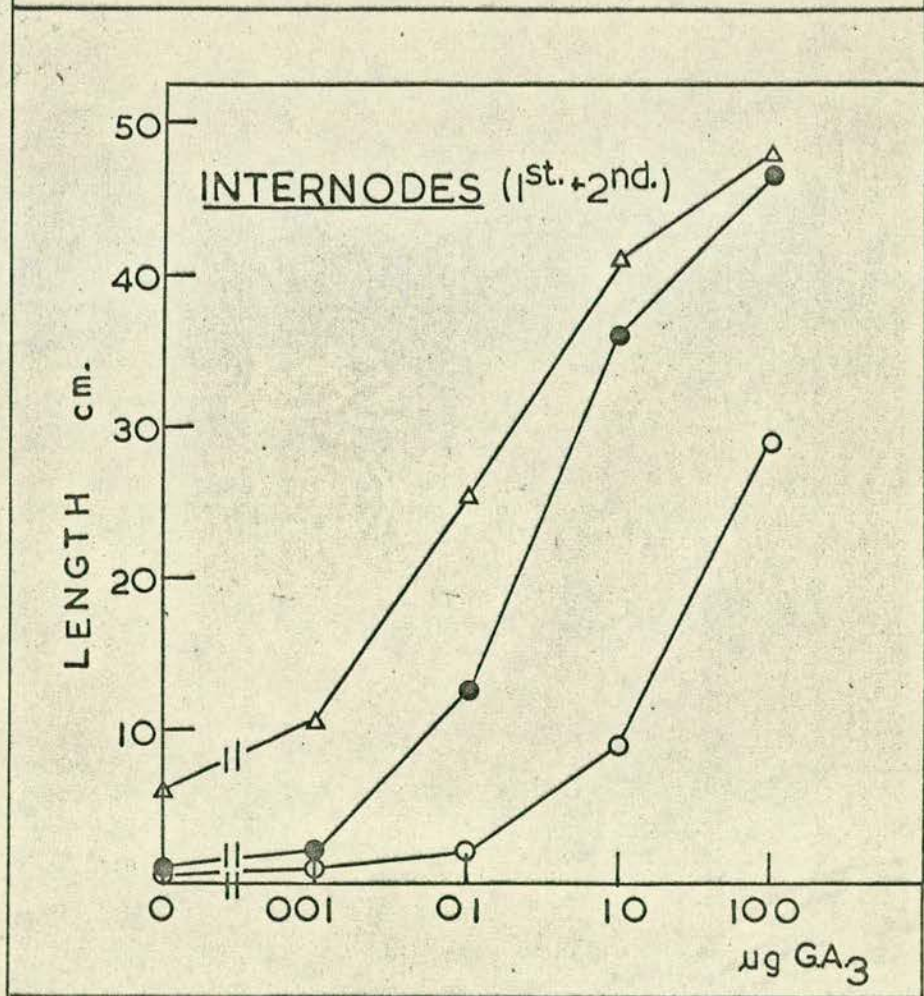
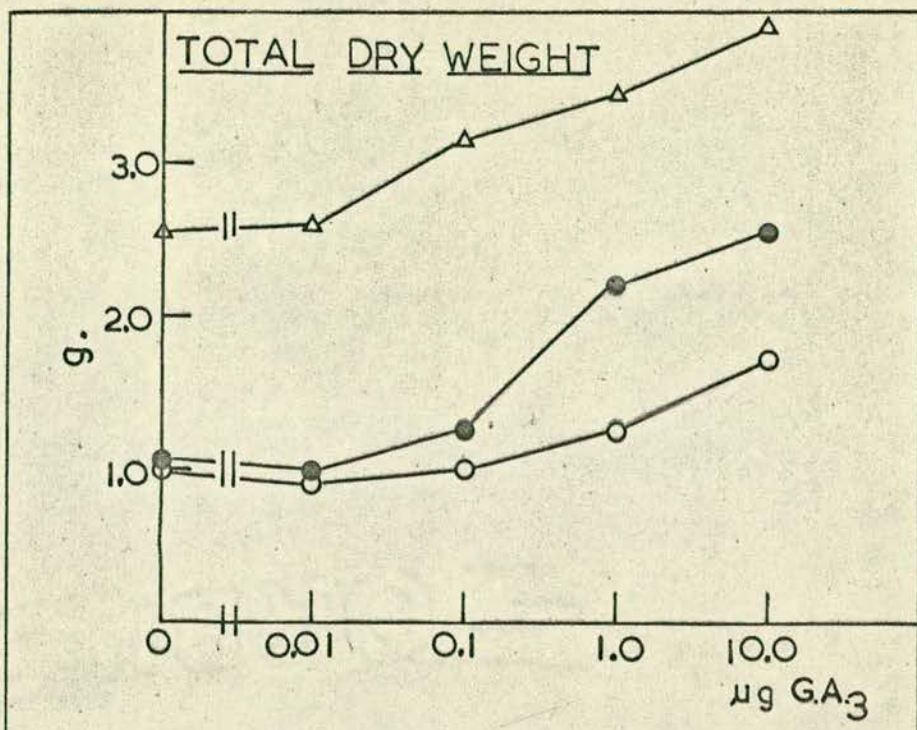
TABLE 6

Leaf area (cm^2) of plants treated with CCC on day 4 or day 7 after planting and five levels of GA₃ on day 7 after planting. Measured 24 days after planting.

		0 GA ₃	0.01 μg GA ₃	0.1 μg GA ₃	1.0 μg GA ₃	10.0 μg GA ₃
One leaf of the 1st. pair	No CCC	135.8	124.5	119.1	122.9	126.0
	CCC day 4	68.6	55.1	69.5	89.2	107.0
	CCC day 7	83.8	85.9	91.3	100.4	101.6
Total area per plant	No CCC	704.4	703.0	721.8	713.8	738.8
	CCC day 4	230.0	173.2	221.0	326.7	374.7
	CCC day 7	262.0	280.0	317.0	473.8	520.4

Figure 3 - Effects of CCC and GA₃ on light-grown plants.

Figure shows total dry weight (above) and the length of internodes of plants treated with CCC on day 4 or 7 after planting and a range of GA₃ concentrations on day 7. Plants were harvested 24 days after planting.



Δ:Control

○:CCC(4) ●:CCC(7)

A slight increase in total area per plant was found for plants treated with the highest concentrations of GA₃. CCC reduced the total area per plant by between 60 and 70 percent. This effect was counteracted by applying concentrations of GA₃ of 1.0 and 10.0ug per plant but not with lower concentrations. There were some indications that GA₃ was more effective when applied to the CCC7 series.

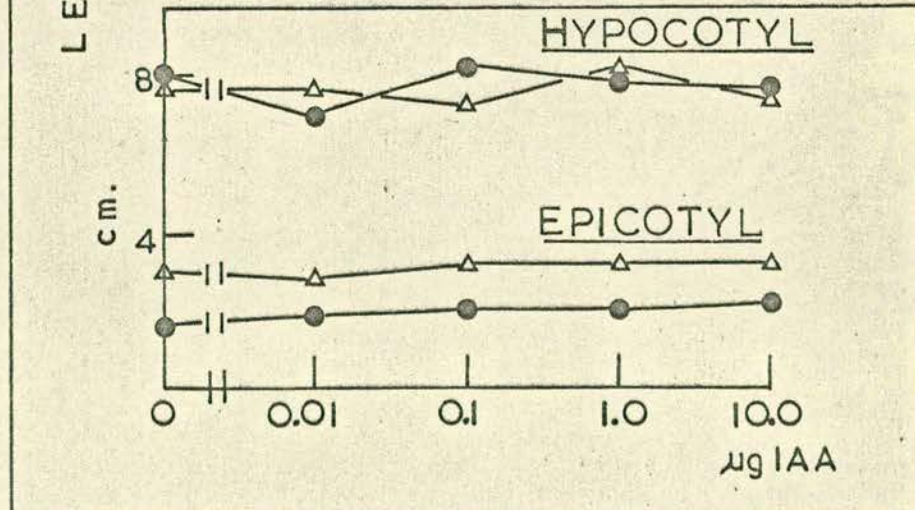
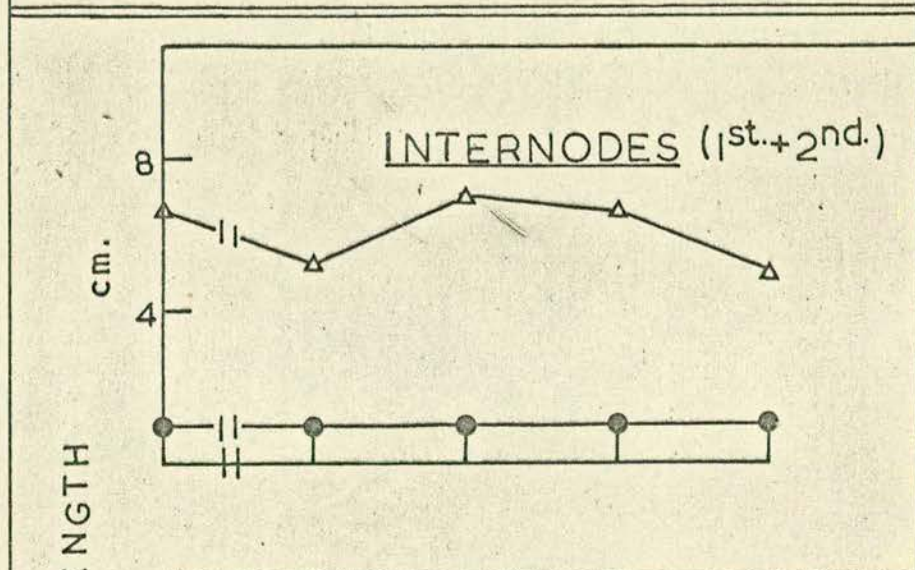
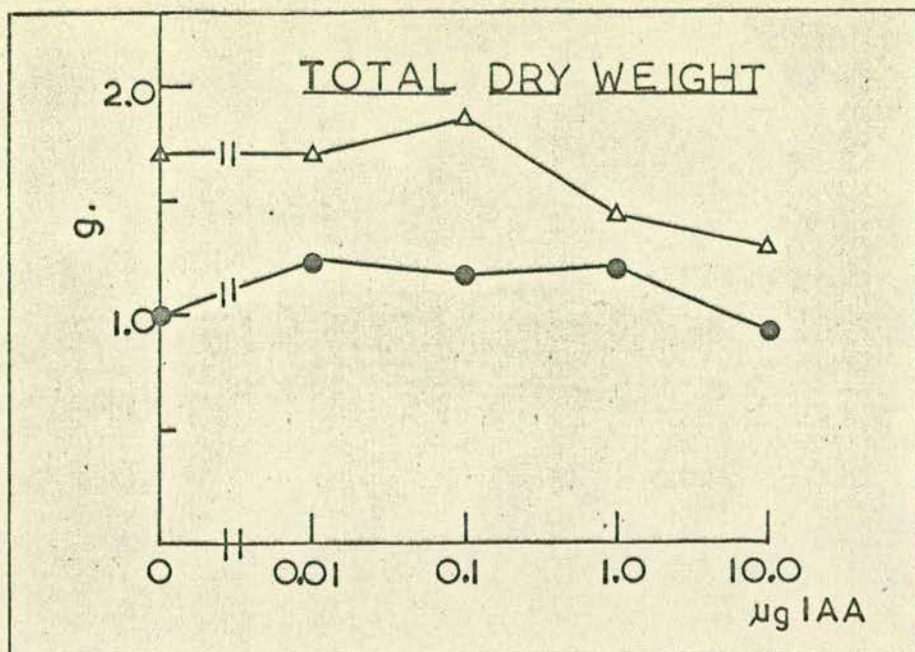
Dry weight data for the whole plant are shown in Figure 3 and show that a marked increase results from GA₃ treatment on control plants. Increasing levels of GA₃ also tend to counteract the marked dry weight reductions found for CCC applications at both day 4 and 7. Throughout, the lowest level of GA₃ (0.01ug per plant) was ineffective and the highest concentration most effective.

The preliminary experiments indicated clearly the very marked retardation of internode elongation resulting from CCC treatment. The present experiment showed (Figure 3) that these effects could be completely counteracted for the first two internodes by GA₃ at 10ug per plant for plants treated with CCC on day 7. For plants treated with CCC on day 4 counteraction by GA₃ was incomplete, although still considerable. Even GA₃ at 0.01ug showed an effect on internode elongation although this was greatest when plants were not treated with CCC.

The large effects of GA₃ on internode elongation

Figure 4 - Effects of IAA on light-grown plants
treated with CCC on day 7.

Figure shows data for total dry weight and the length of the hypocotyl, epicotyl and the first two internodes. Plants were harvested when 19 days old. The highest concentration of IAA had a retarding effect and caused malformation in control and plants treated with CCC.



Δ: Control

●: +CCC (day 7)

of plants treated with CCC led to a further experiment in which the possibility that indolyl acetic acid might act in a similar manner to GA₃ was investigated. Results for lengths of the first two internodes of plants treated with IAA applied to the apex at the same range of concentrations as GA₃ showed that this compound had no effect on plants treated or untreated with CCC (Figure 4).

2- Detailed investigation of the effect of CCC and GA₃ on growth rate and leaf characters

The experiment now to be described is the main one in this section and represents a large scale repetition and extension of a number of smaller experiments in which the effects of CCC and GA₃ on growth of plants and in particular of leaves, were investigated.

Six treatments were used in the experiment. These were no CCC, CCC applied at day 4 or day 7, with or without application of GA₃ at 10ug per plant. The design and schedule of harvests were as shown on table 7.

TABLE 7

Experimental treatments			
	no CCC	CCC on Day 4	CCC on Day 7
no GA ₃	A	B	C
with GA ₃	D	E	F

Harvesting schedule							
Day	Treatment						
	A	B	C	D	E	F	
Number of plants harvested							
4	3	-	-	-	-	-	
7	3	3	-	-	-	-	
11	3	3	3	3	3	3	
15	3	3	3	3	3	3	
19	3	3	3	3	3	3	
23	3	3	3	3	3	3	
27	3	3	3	3	3	3	
31	3	3	3	3	3	3	
	24	21	18	18	18	18	

Figures are the number of plants harvested on the indicated days; only certain of the results for 31 day old plants are reported below. The number of plants in this experiment was the maximum possible, having in mind space limitations in the growth-room and the need to avoid shading between adjacent plants.

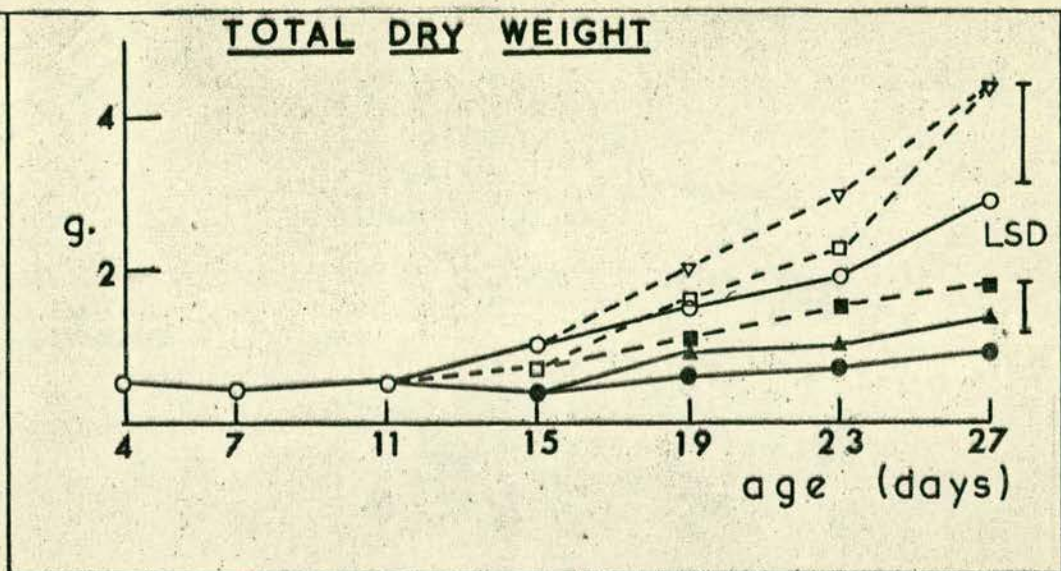
a- General growth of plants and plant parts

Growth of plants as assessed by dry weight changes are given on Figure 5.

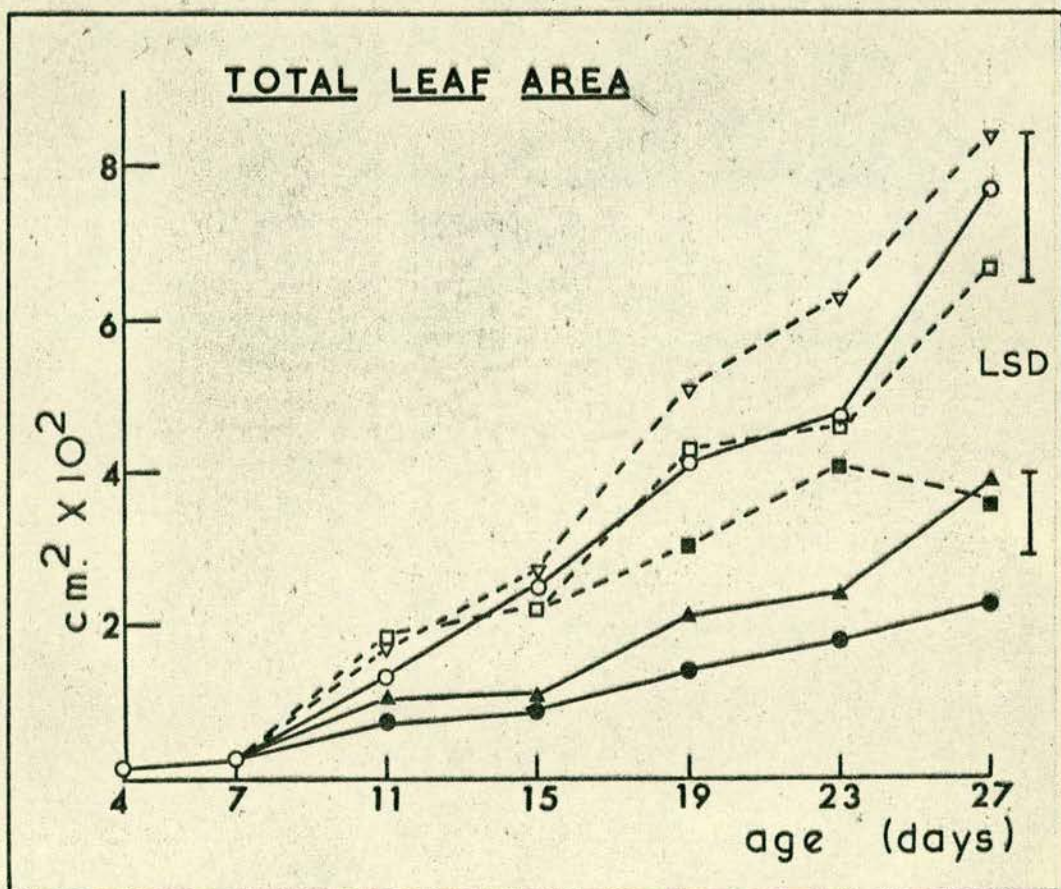
For untreated plants dry weight increased throughout the experiment after day 11, but for plants treated with GA₃ with or without CCC on day 7 (D and F) dry weight increased at a slightly faster rate. Both treatments with CCC (B and C) reduced the total plant dry weight, and although GA₃ counteracted the effect of CCC applied on day 7 it was not as effective when CCC was applied on day 4; the values for dry weight were slightly higher than those for CCC applied on day 4, but counteraction was not complete. Thus, treatment with GA₃ tended to increase total dry weight and to counteract the effect of CCC when the latter was applied on day 7. As already reported CCC reduced total dry weight. For most of this experiment the results fall into two groups (indicated by solid and open symbols in the graphs); one group consists of the CCC treatments and the CCC4 with GA₃ set and these show lower values than the remaining group of three treatments which include Control, GA₃ and the CCC7 with GA₃ sets.

Figure 5 - Effects of CCC on growth of light-grown plants as measured by total leaf area and total dry weight.

Total dry weight is shown (above) and total leaf area for plants treated with CCC with or without GA₃. Least significant differences are given for day 27. Each point is the average value for three plants.



I LSD



○: control plants ●: CCC(4) □: CCC(day 7)+GA₃
 ▽: GA₃ treated ▲: CCC(7) ■: CCC(day 4)+GA₃

This was true also for total leaf area per plant (Figure 5).

The total leaf area increased for all treatments up to day 27. This continued increase was the result of expansion of leaves on the main stem as well as of production of leaves on lateral branches in control and GA₃ treatments. Development of lateral branches in the CCC series contributed very little to the leaf area. The fastest rates of increase and hence the largest area were found for GA₃ plants with the control set being slightly smaller and not significantly different from the CCC7 with GA₃ set. CCC treated plants showed a very much slower rate of increase, with early application retarding growth slightly more than application on day 7. GA₃ applied to plants treated with CCC on day 4 counteracted the effect of the retardant in part but not completely and values for area were always less than for the control series. Thus the total leaf area was markedly reduced by application of CCC. GA₃ was fully effective in counteracting this effect in the CCC7 series. That is to say early application of CCC apparently causes an irreversible effect which becomes fixed before day 7 when GA₃ is applied.

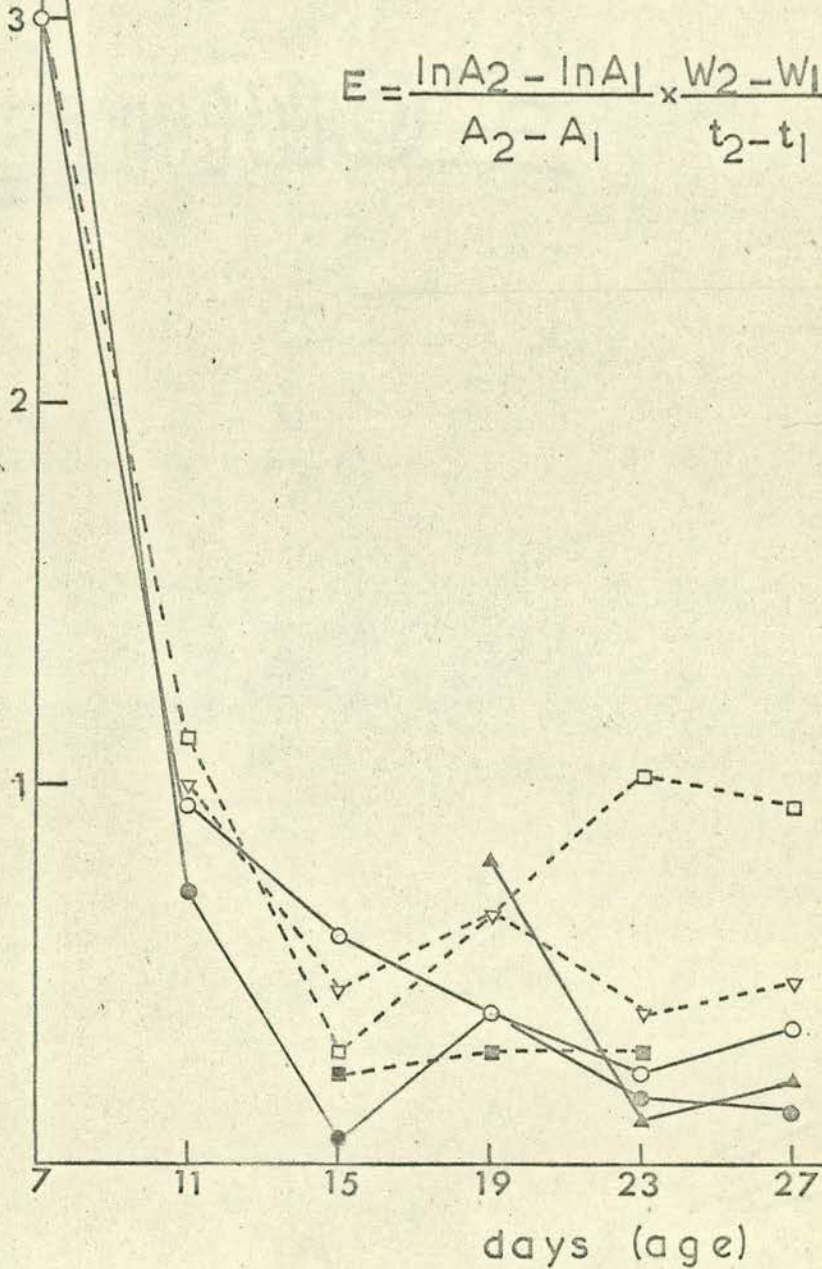
Using these values of dry weight and leaf area, relative growth rates and net assimilation rates were calculated. Great variation occurred in these parameters of all treatments, probably due to the small sample size. For all treatments R decreased from day

Figure 6 - Net assimilation rate of light-grown plants.

NET
ASSIMILATION (E)
RATE

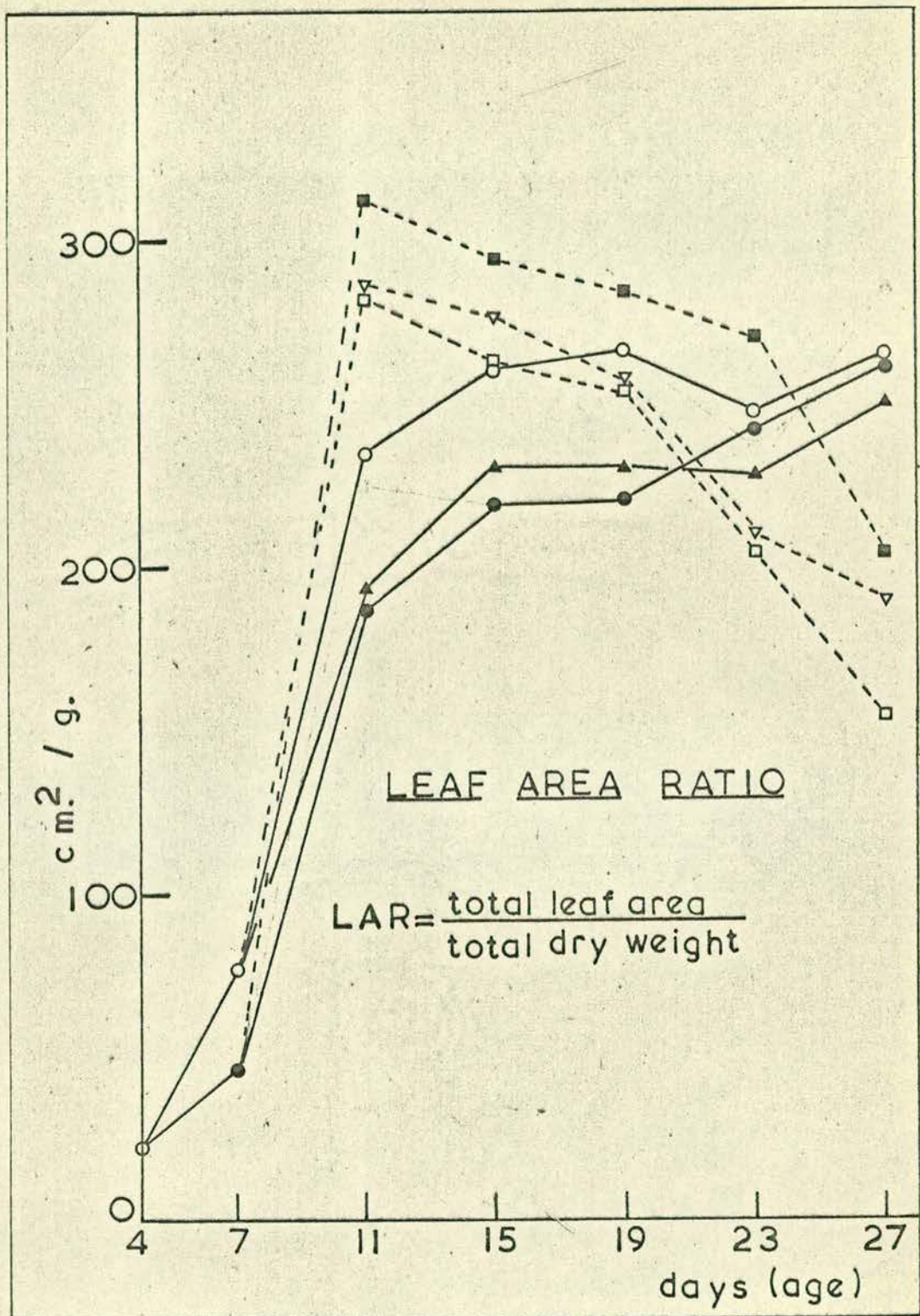
$$E = \frac{\ln A_2 - \ln A_1}{A_2 - A_1} \times \frac{W_2 - W_1}{t_2 - t_1}$$

(g/cm²/day) x 10³



- :control ●:CCC (4) ◻:CCC (day 7)+GA₃
 ▽:GA₃ ▲:CCC (7) ◻:CCC (day 4)+GA₃

Figure 7 - Leaf area ratio.



○: control	▲: CCC (day 7)
▽: GA ₃	■: CCC (day 4) + GA ₃
●: CCC (day 4)	□: CCC (day 7) + GA ₃

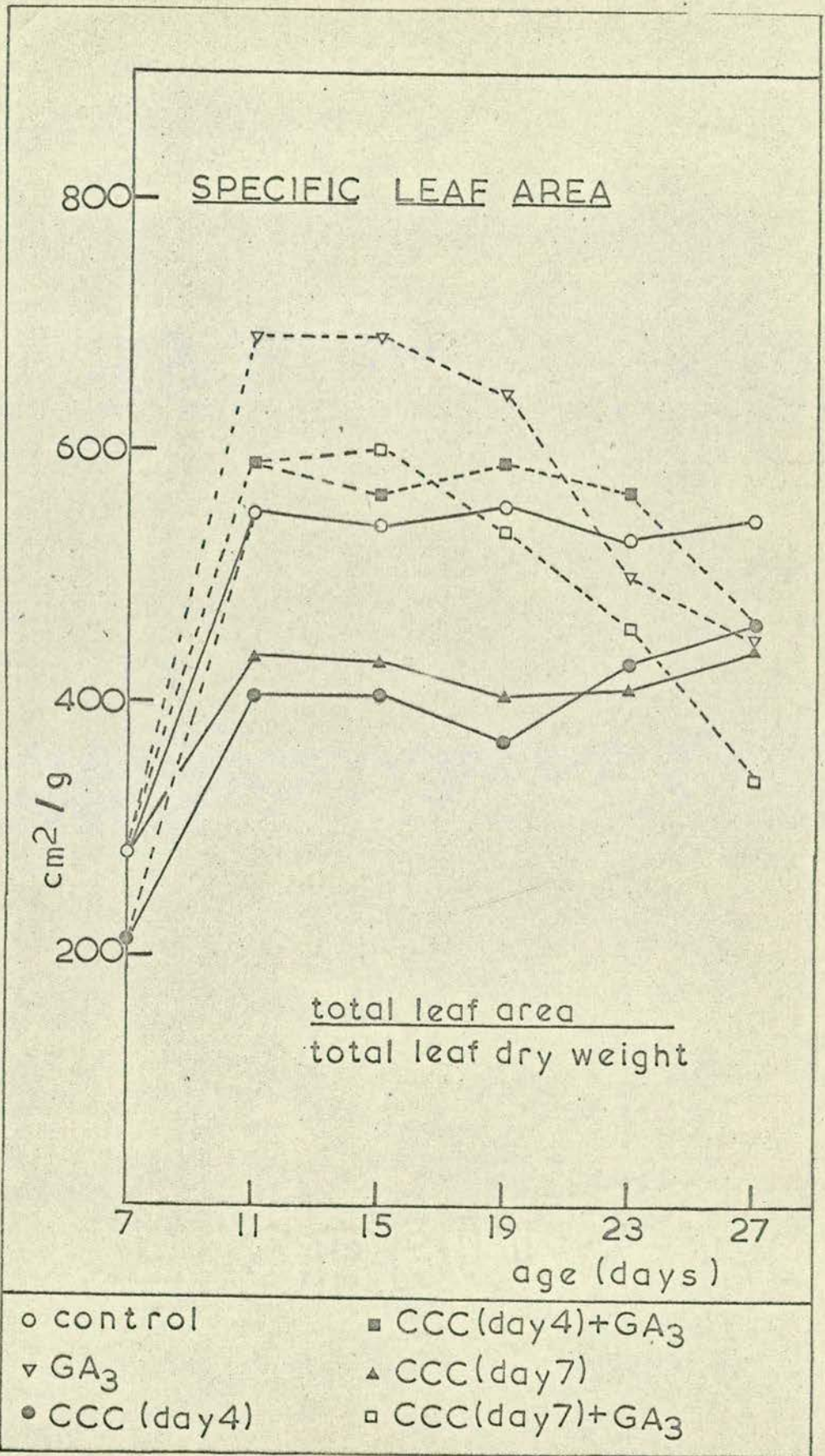
4 till 27 and it was not possible to distinguish statistically significant effects of the different treatments, although treatments involving CCC showed values which were usually lower than those for the control series.

Great variation also occurred in E and all treatments followed the same pattern in showing a marked decline after day 7. There was no significant difference in the net assimilation rate in the various treatments and the control series (Figure 6), although CCC treatments were lower than the control series and later values for the GA₃ treated sets tended to be higher than those for control.

The expansion of the leaf surface in relation to plant dry weight is given by the leaf area ratio and from Figure 7 it can be seen that after a large initial rise in this parameter, treatments with GA₃ showed a gradual but continued fall after day 11. In contrast the CCC4 and CCC7 treatments showed a continued slow increase after day 11 and from day 15 the control values remained constant. GA₃ thus caused an initial increase in lamina expansion per unit plant dry weight whereas CCC tended to have the opposite effect.

This analysis can be extended by estimating specific leaf area (i.e. the ratio total leaf area/leaf dry weight). This is shown in Figure 8 and it is again apparent that leaves treated with CCC alone showed a lower value than control leaves, and were thus thicker.

Figure 8 - Specific leaf area.



Treatment with GA₃ increased the value initially (that is, leaves became thinner) but subsequently the values fell until by day 27 they were close to those for the CCC series and slightly lower than the control values. This suggests that the effects of GA₃ on the first formed leaves were not exerted to the same extent on later formed leaves.

Data for distribution of dry matter in roots and stems and the ratio for the two are shown in Figure 9 and Table 8.

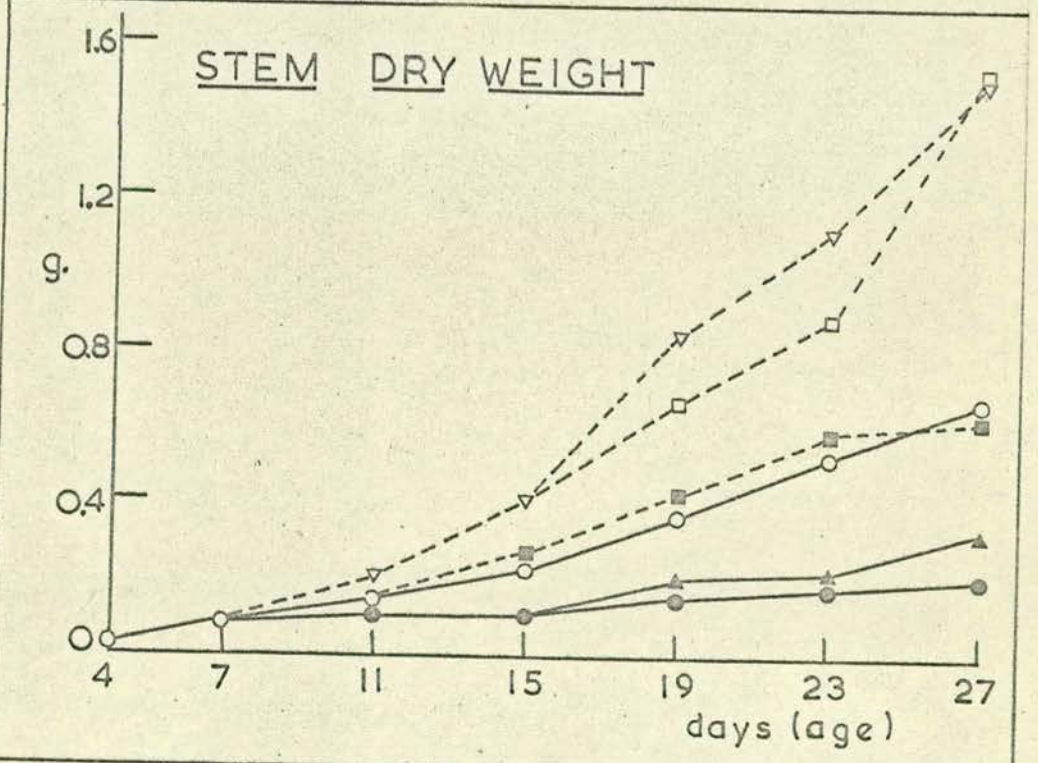
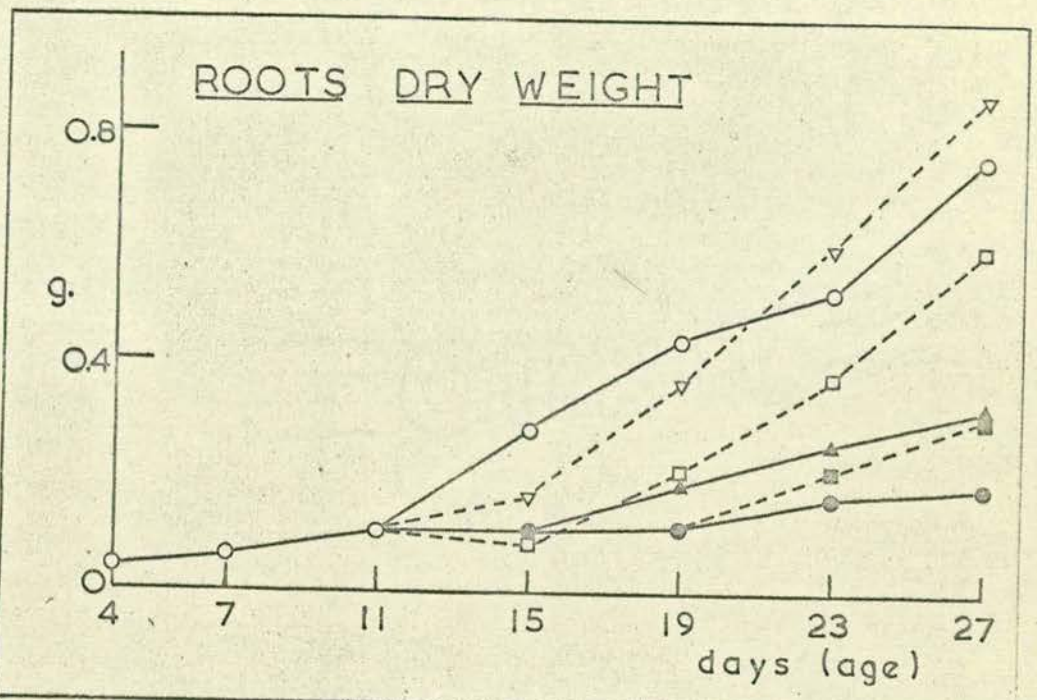
Growth of the roots was very markedly reduced by CCC treatments and was only partially counteracted by GA₃ application for the CCC7 treatment, and not affected for the CCC4 set. For control and the GA₃ treatment root dry weight increased with time at a similar rate.

Very large effects of GA₃ were noted for stem dry weight. For example at day 27 GA₃ treated plants had a mean value for stem dry weight of 1.51g against 0.66g for the control, and 0.19g for the CCC4 treatment which was slightly less than the value for the CCC7 set. GA₃ fully counteracted the effect of CCC given on day 4 and these plants had a similar stem dry weight to the control. A much larger effect was found for GA₃ applied to plants treated with CCC on day 7 and these values were close to those for the GA₃ set. Thus GA₃ increased the stem dry weight considerably, and CCC decreased it.

The large effects of GA₃ on stem dry weight also

Figure 9 - Dry weight of light-grown plants.

Root dry weight (above) and stem dry weight. Each point is the average value for three plants.



○ Control	▲ CCC (day 7)	■ CCC (4) + GA ₃
● CCC (day 4)	▼ GA ₃	□ CCC (7) + GA ₃

affected the ratio of root dry weight/stem dry weight. For all treatments which include GA₃ the ratio has a value ranging from 0.9 to 0.2. In contrast, CCC treatment and the control series showed values for this ratio

TABLE 8

Ratio of root dry weight to stem dry weight

Age (days)	11	15	19	23	27
Control	0.9	1.3	1.2	1.0	1.1
GA ₃	0.8	0.4	0.4	0.5	0.6
CCC4	1.1	1.1	0.7	0.9	0.9
CCC7	0.8	0.9	0.9	1.2	1.0
CCC4GA ₃	0.9	0.3	0.3	0.4	0.5
CCC7GA ₃	0.4	0.2	0.3	0.4	0.4

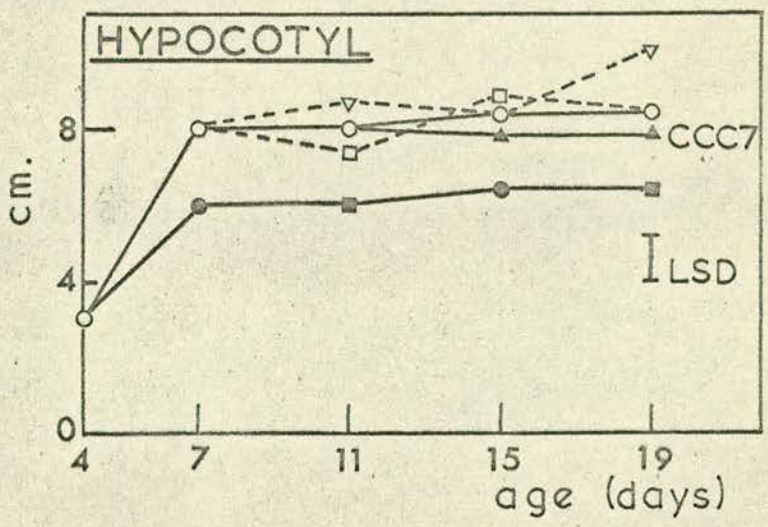
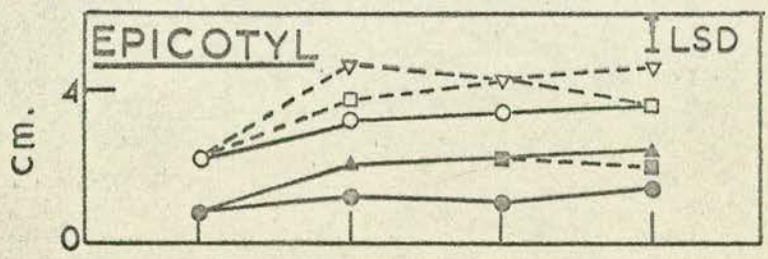
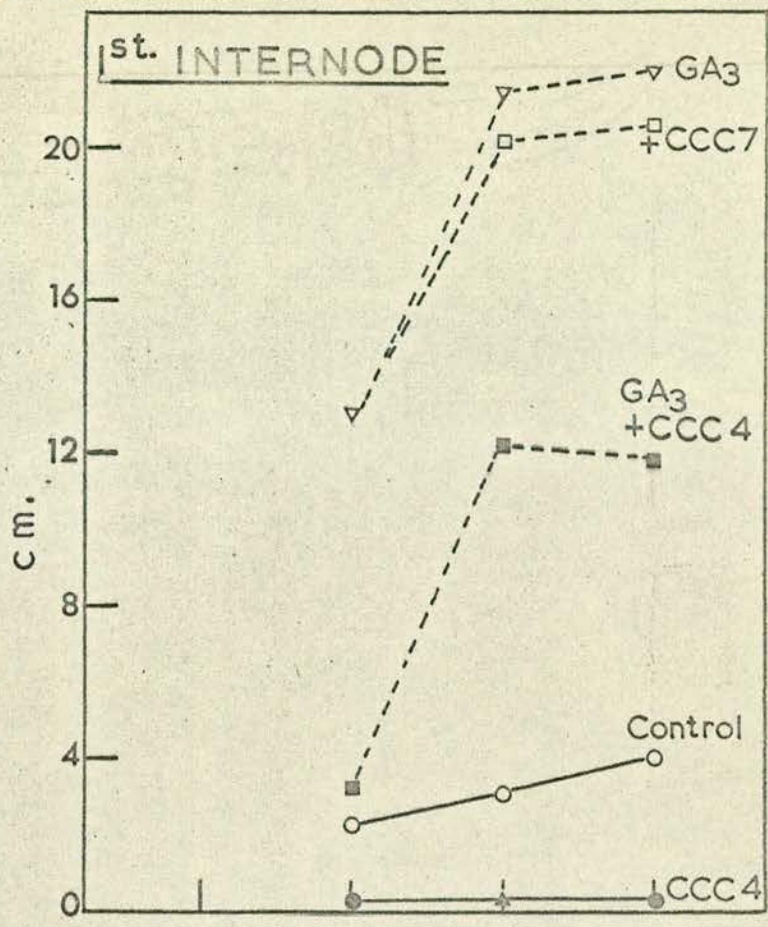
close to unity. A major effect of GA₃ appears to be to divert dry matter preferentially to the stems while having little effect on roots. CCC treatment leads to a reduction of dry matter in the plant as a whole, but does not affect the distribution between roots and stems.

Measurements were also made of length of the hypocotyl, the epicotyl and the first internode for all treatments (Figure 10).

Figure 10 - Length of hypocotyl, epicotyl and first internode of light-grown plants treated with CCC and GA₃.

Least significant differences are shown for day 19 for the hypocotyl and epicotyl lengths. Measurements were also made for the second and third internodes, with results similar to the first internode and for this reason they are not presented.

LENGTH



Examination of data for the control plants showed that hypocotyl elongation was complete by day 7, hence it might be expected that application of GA₃ and also of CCC on day 7 would not have any effect on this organ, and this was so. Application of CCC on day 4 reduced hypocotyl length by about 25%.

For epicotyls and first internode, growth continued after day 7 and all treatments had an effect. CCC applied either on day 4 or on day 7 reduced the length of both epicotyl and first internode. GA₃ counteracted this effect for the first internode but was not completely effective for epicotyls on plants treated on day 4 with CCC. Presumably growth had nearly ceased and GA₃ was applied too late to be fully effective in counteracting the retardation due to CCC. Treatment with GA₃ resulted in considerable elongation of the main stem and plants showed a tendency to climb while it was also observed that apical dominance was much less marked and lateral branches developed freely.

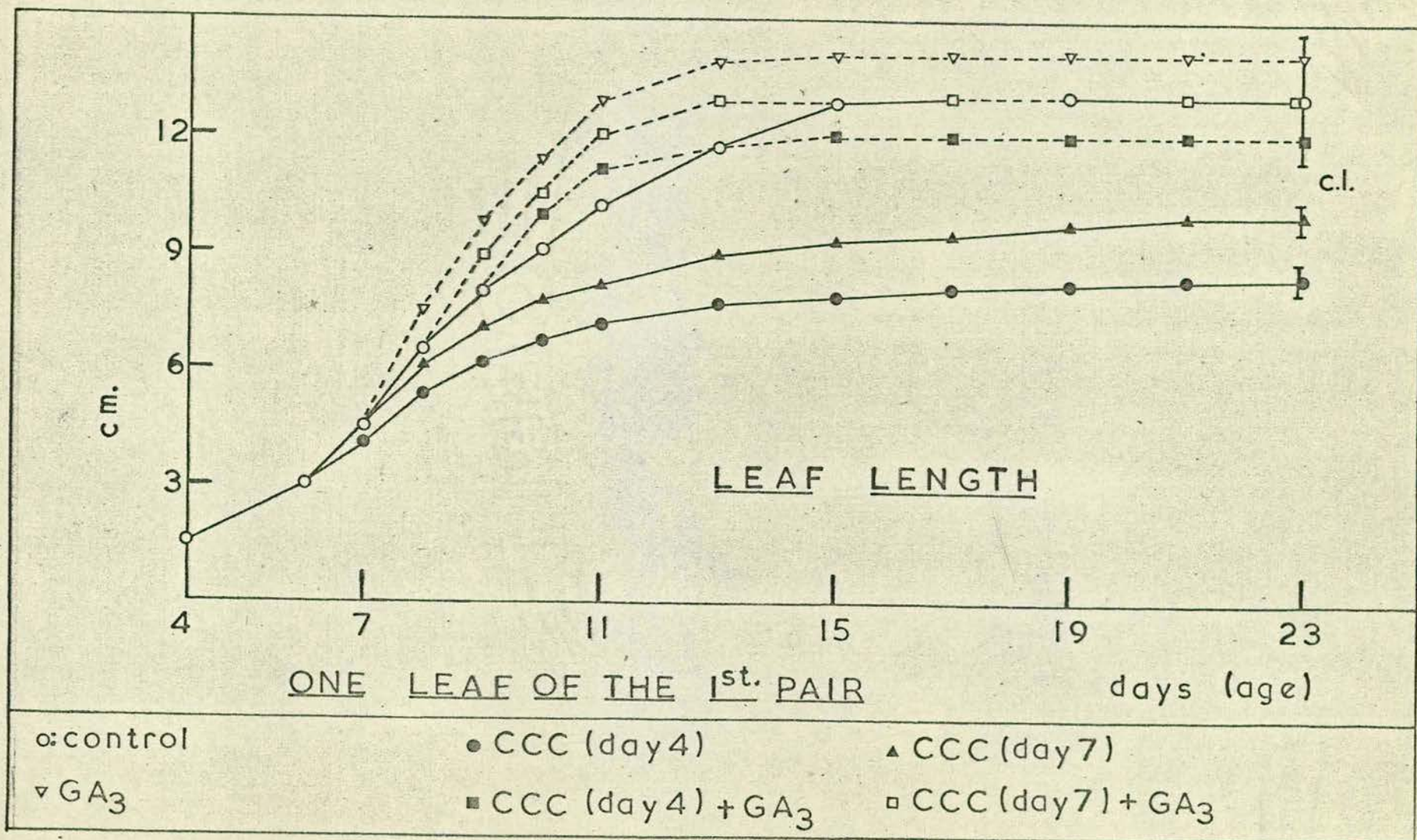
b- Growth and characteristics of leaves

Leaf growth rates

A harvest of three plants was made on day 4 and length of the primary leaves measured. Since this involved uprooting the seedlings they were discarded after the measurements had been made. Leaf length of a group of five plants was measured daily between day 6 and day 11 and thereafter at intervals of two days; the same group of plants was used throughout. The trifoliolate

Figure 11 - Leaf length.

Measurements were made along the midrib. Each point is the average value for five light-grown plants (10 leaves) and the same set of plants was measured from day 7 until day 23. Confidence limits are shown for day 23.



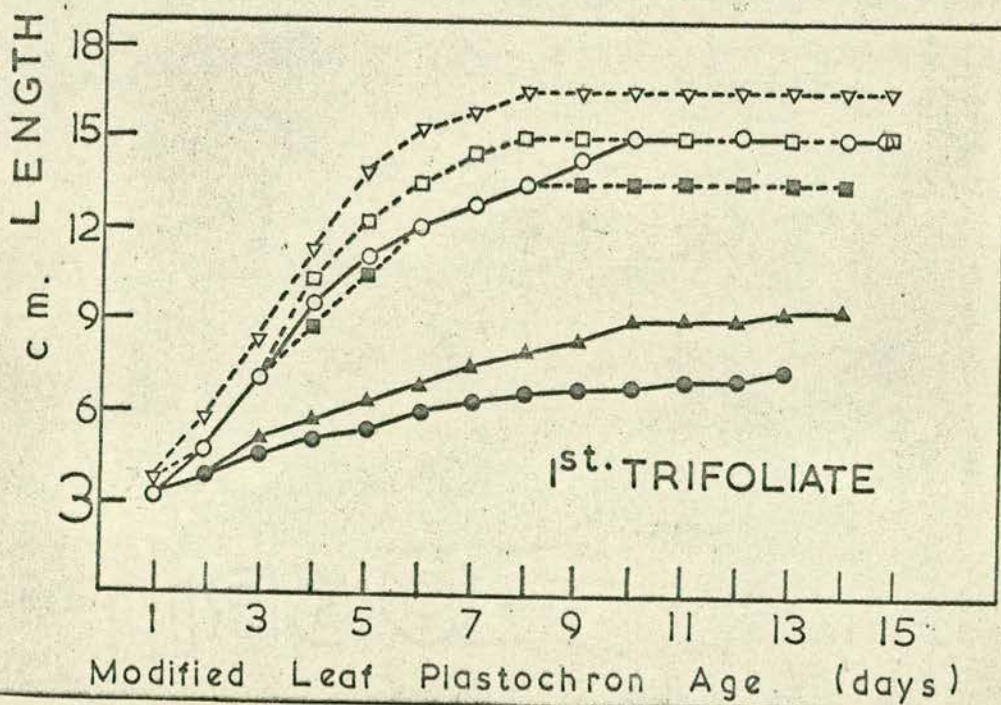
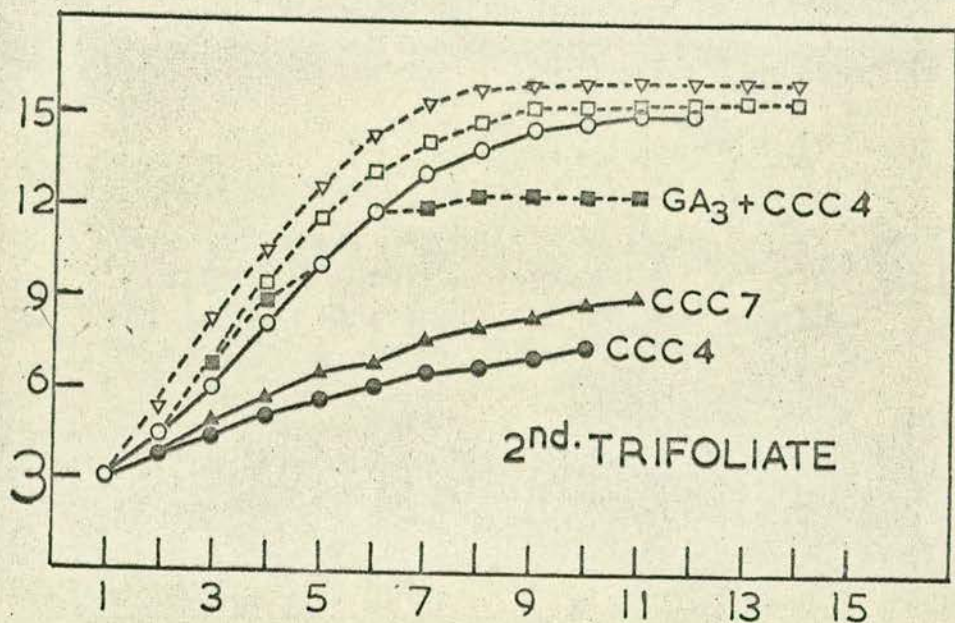
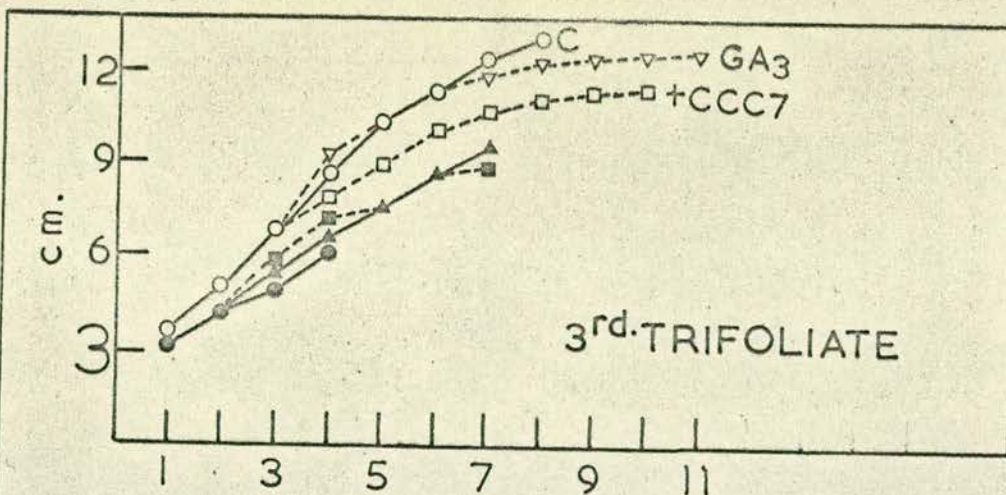
leaves were measured daily (Figures 11 and 12).

Differences in length between primary leaves of the control and plants treated with CCC on day 4 began to appear by day 7. In the control plants increase in length was rapid until about day 15 and ceased thereafter. Final length of leaves in CCC7 plants was greater than for the CCC4 treatment but the changes in length followed the same pattern for both sets of plants, being, even at an early age, much slower than that for the control. In the CCC treatments length increased slowly until day 21. At this stage, length was still significantly less than that of control plants. Thus CCC increased the period over which growth in length occurred but retarded the rate of growth when compared with the control. In plants treated with GA₃ the rate of growth in length was higher than that for the control initially, but from day 11 the rate began to decrease, and from day 15 onwards no further increase in length occurred. When GA₃ was applied to plants treated with CCC the same pattern of response was observed, i.e., the rate of increase of length was initially greater than that for the control plants. The rate of increase for CCC4GA₃ was less than that for CCC7GA₃, which in turn was less than that for GA₃ alone. No significant differences were found between any of the treatments involving GA₃ and the control.

In the trifoliate leaves measurements were commenced on the day on which leaflets reached a length of

Figure 12 - Leaf length for the first three trifoliate
leaves.

Age is given as modified leaf plastochron age
(see Methods).



Modified Leaf Plastochron Age (days)

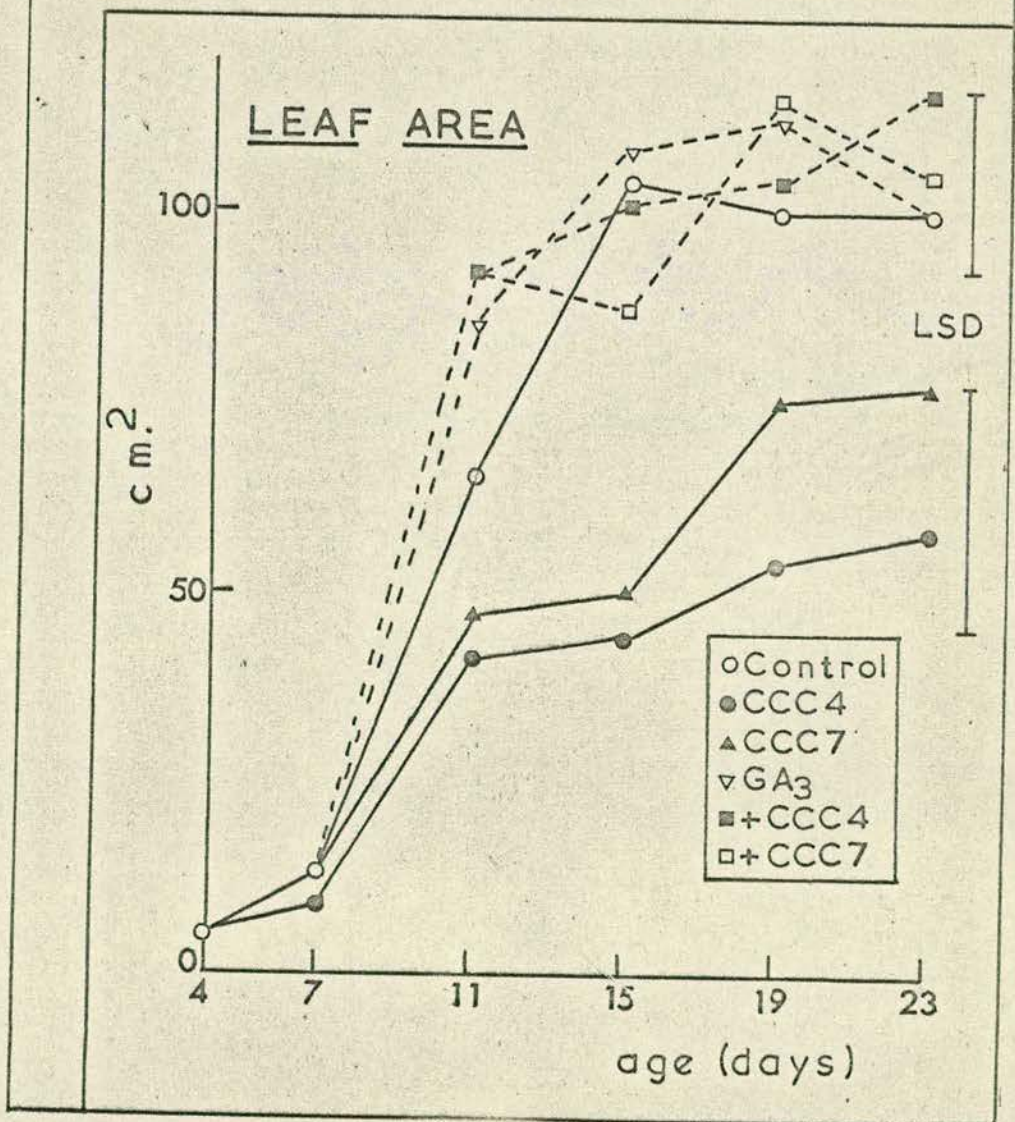
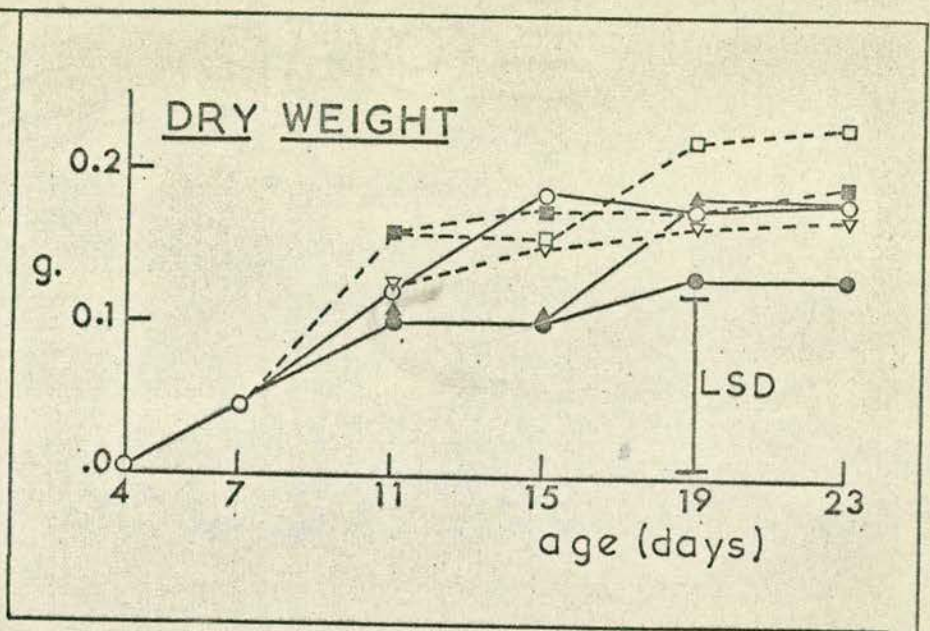
three cm. This overcame the problem which arose from different rates of unfolding of these leaves. It means that the time scale on Figure 12 is not chronological time, but rather time from an arbitrary starting point when the leaflets reached the 3 cm. length.

For the first trifoliate leaf differences between treatments became apparent early on. At day 2 there was a hint of two distinct groups of curves: one group consisting of CCC4 and CCC7 plants and a second group containing control, GA₃, CCC4GA₃ and CCC7GA₃ treated plants. The growth rates for this leaf and for the second trifoliate leaf were qualitatively similar to those shown for the first pair of leaves. Plants treated with CCC alone showed lower rates and smaller final length than control and GA₃ treated plants. Again CCC treatment appeared to prolong the period over which growth in length occurred. For CCC treated plants it is of interest that the third trifoliate leaves show a different kind of response from the two previous leaves: the rate of growth in length was higher initially than in the first and second trifoliate leaves, although rates were still less than those for GA₃ and control plants.

The curves in Figure 12 also give an indication of differences in the time of unfolding of the trifoliate leaves in the different treatments. This can be seen if the last points of each curve are examined. From the method of measurement, leaves that unfold early will be measured a larger number of times than leaves

Figure 13 - Area and dry weight of the primary leaves
of light-grown plants.

Least significant differences are shown for day 19
(dry weight) and for day 23 (area).



which unfold late. It is then apparent, for example, that first and second trifoliate leaves in treatments involving CCC and CCC on day 4 followed by GA₃ unfolded up to two days later than leaves on control plants. GA₃ did not affect the rate of unfolding of the first trifoliate leaf but accelerated (compared with control) the rate of unfolding of the second and third trifoliate leaves which commenced unfolding up to four days before those leaves in the CCC-treated series. Application of CCC on day 4 had a somewhat greater effect on rate of unfolding than application on day 7, and the effects of early application could be only partially counteracted by addition of GA₃.

The results of these measurements can be summarized thus: treatment with CCC caused a slower rate of unfolding of the trifoliate leaves from the apex, and on unfolding, trifoliate and primary leaves grew in length at a slower rate and over a larger period than leaves in control plants; GA₃ treatments tended to bring about earlier unfolding and more rapid growth in length than for control leaves, although final length was similar to that for the control.

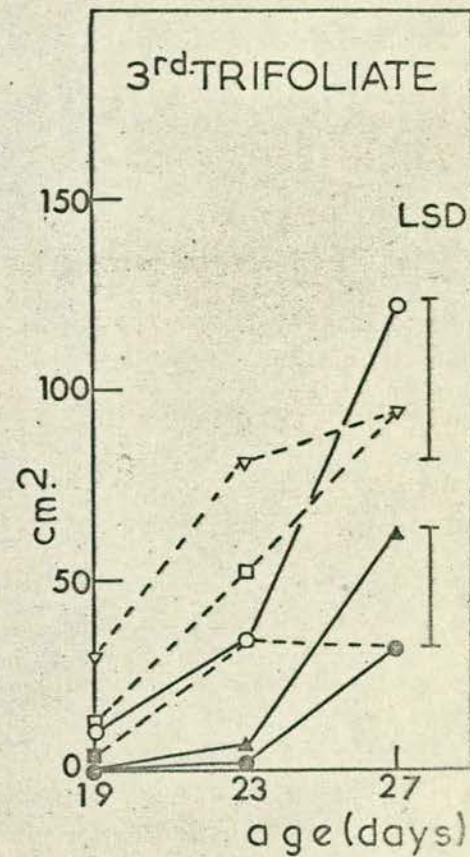
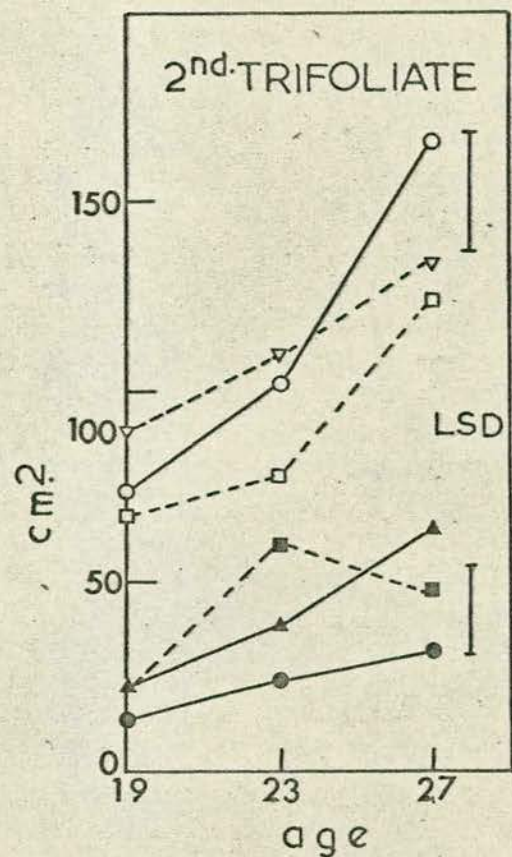
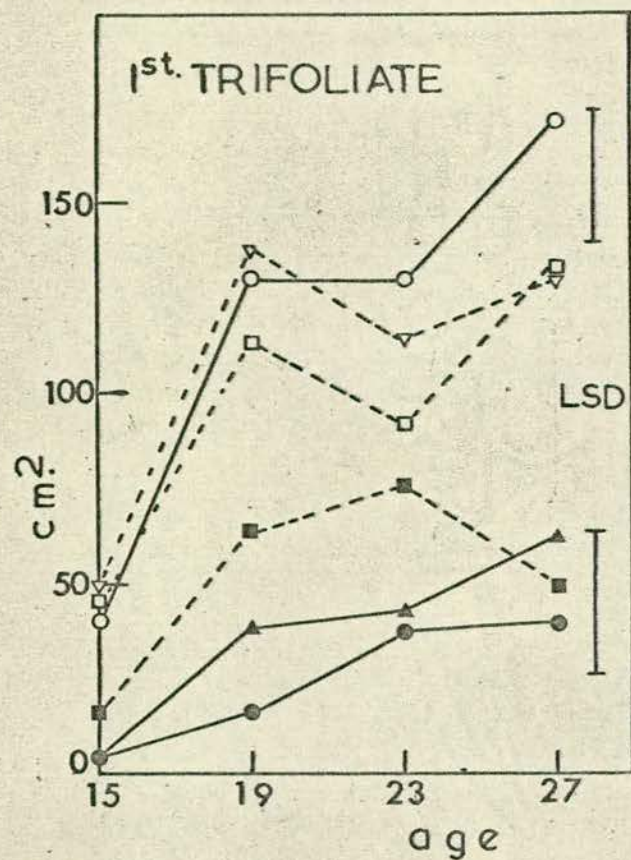
Leaf area and dry weight

Curves for growth in leaf area of the primary and first two trifoliate leaves (Figures 13 and 14) fell into two groups of which the lower consisted of the CCC treatments, with CCC applied on day 4 being always lower than the curve for CCC applied on day 7.

Figure 14 - Area of trifoliolate leaves of light-grown
plants.

Least significant differences are shown for day 27.

TRIFOLIATE LEAVES: AREA



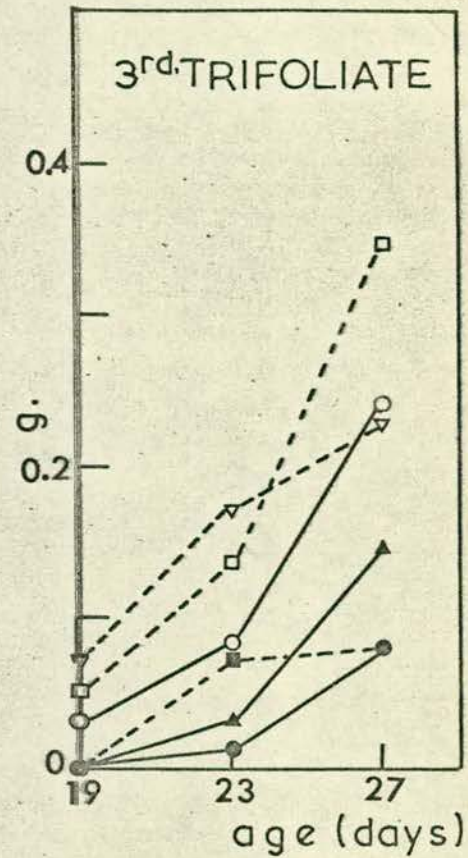
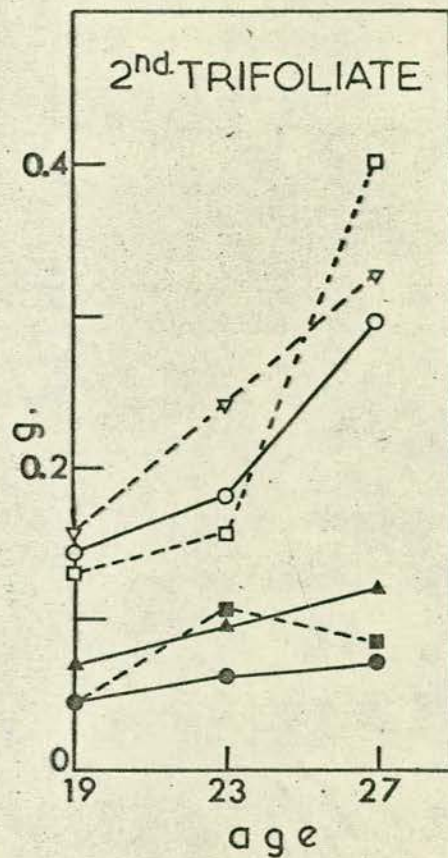
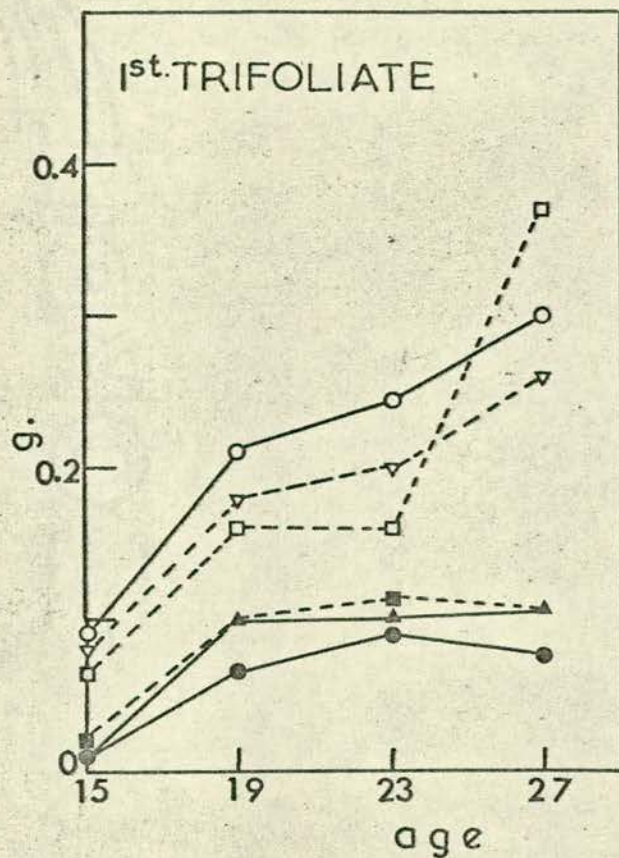
○:control
▽:GA₃

●:CCC (day 4)
▲:CCC (day 7)

■:CCC (day 4) + GA₃
□:CCC (day 7) + GA₃

Figure 15 - Dry weight of trifoliate leaves of
light-grown plants.

TRIFOLIATE LEAVES: DRY WEIGHT



control
GA₃

CCC (day 4)
CCC (day 7)

CCC (day 4) GA₃
CCC (day 7) GA₃

The second group of curves included the control and GA₃ series as well as the curve for plants given both CCC and GA₃ on day 7. For the first pair of leaves GA₃ completely counteracted the effects of CCC applied on day 4, but this was not so for the first two trifoliate leaves, where early application of CCC had larger effects, although GA₃ did cause a slight increase in area. This treatment (i.e. CCC4GA₃) therefore resembled most closely the CCC series when considered for the trifoliate leaves. Least significant differences confirm that the curves are of two families, with treatments within a family being not significantly different from each other. For the third trifoliate leaf the effects of CCC are much less marked because expansion in the control and GA₃ series was incomplete, and this is confirmed by the leaf length data (Figure 12). The continued slow expansion of the primary leaves in the CCC series is confirmation of what was reported about length (Figure 11) for these leaves. It should be noted that the somewhat irregular nature of some of these curves reflects the small sample size.

The dry weight (Figure 13) of the first pair of leaves was not much affected either by treatment with CCC or with GA₃. The smaller dry weight shown by treatment with CCC4 was not significantly different from that of the other treatments. Thus the effect of CCC in strongly reducing the leaf area of the first pair of leaves was not paralleled by a similar effect on dry weight.

Figure 16 - Cell number of primary leaves of light-grown plants.

Results for one leaf of the first pair are shown. Dry material was used, and each point is the average value for three leaves. Least significant difference is shown for values on day 19.

○: Control

▽: GA₃

●: CCC (day 4)

▲: CCC (day 7)

■: CCC (day 4) + GA₃

□: CCC (day 7) + GA₃

ONE LEAF OF THE
FIRST PAIR

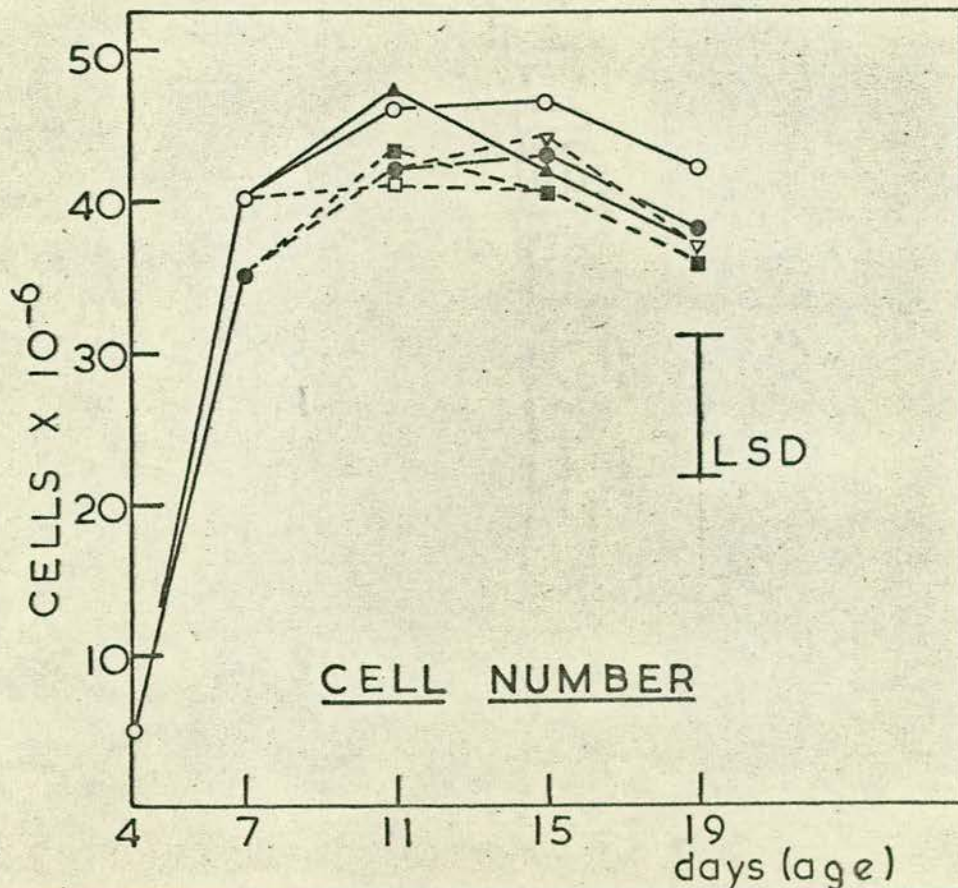
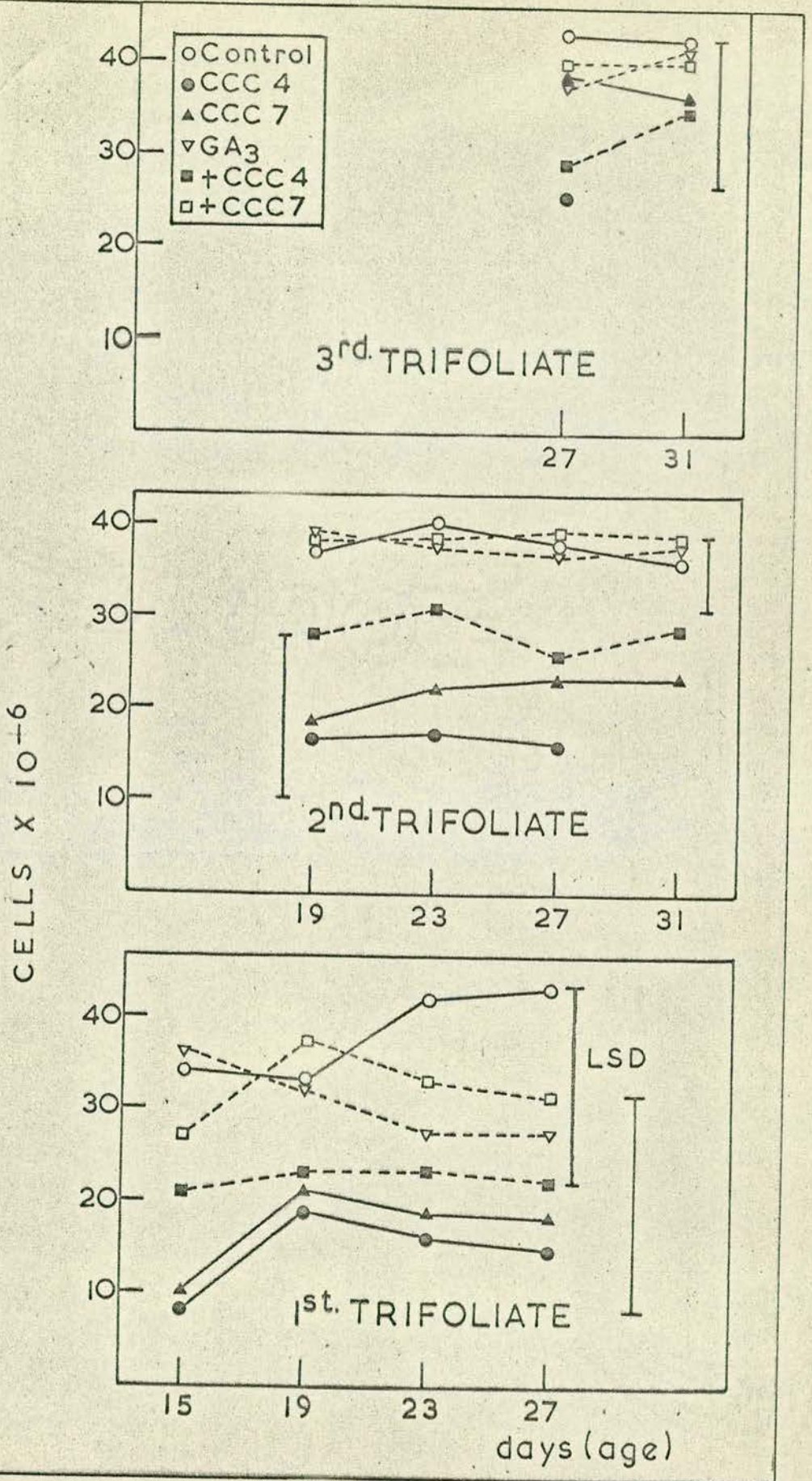


Figure 17 - Cell number of trifoliolate leaves of
light-grown plants.

Cell number of the median leaflet of each of the first three trifoliolate leaves is shown. Dry material was used. Least significant differences are shown. Each point is the average value for three leaves.



For the trifoliolate leaves (Figure 15) the pattern of change for dry weight was very similar to that for leaf area (Figure 14). That is to say that CCC and GA₃ showed similar effects upon both leaf area and dry weight of the trifoliolate leaves.

Cell number and cell volume

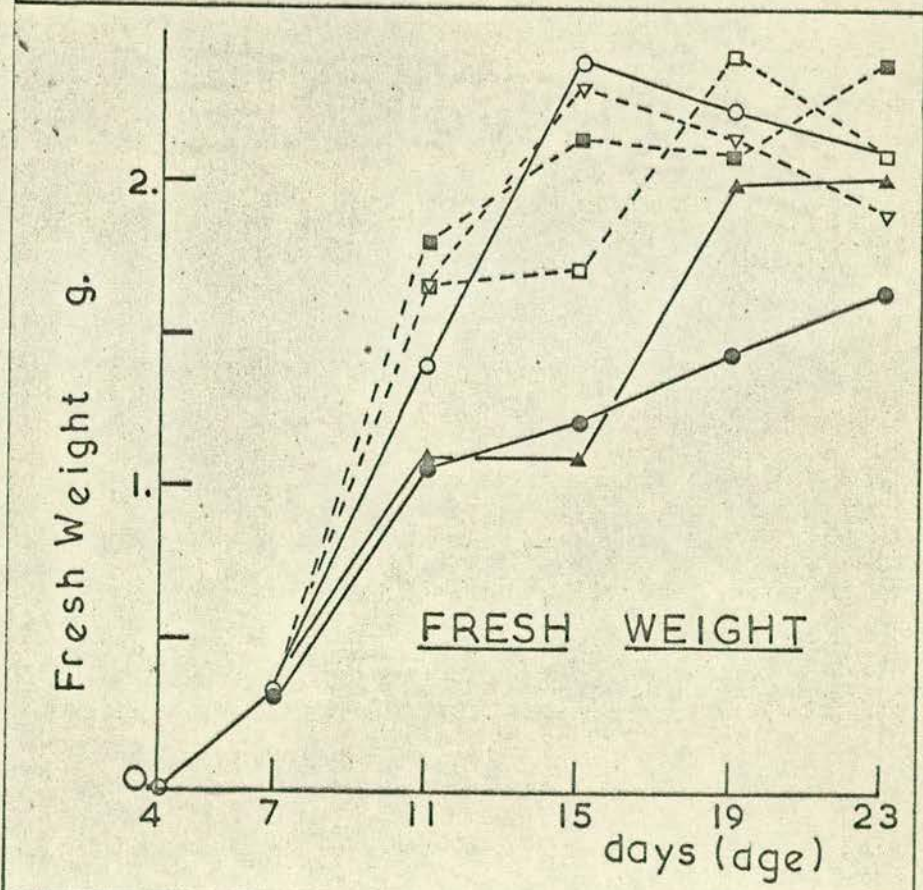
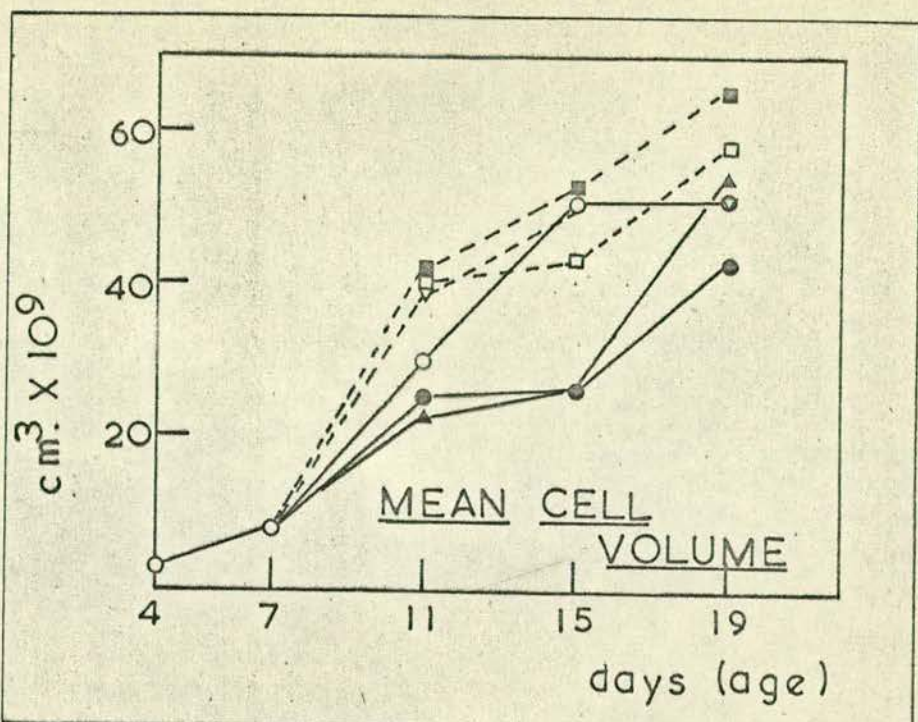
All cell counts in this experiment were made using dried material.

For the first pair of leaves cell number was not affected by any treatment (Figure 16). The cell number increased between day 4 and day 7, from a value of about five million cells to about forty million per leaf with some further increase between day 7 and 11. Final cell number was between forty and forty-five million if the reduction in values for harvest on day 19 is ignored. Reductions of this sort have been reported for other material (e.g. lupin and sunflower - Sunderland, 1960; cucumber - Milthorpe and Newton, 1963) and have been attributed to breakdown of the older cells. It seems odd that a similar decline should be noted for material which due to drying has already a large proportion of damaged cells.

In the trifoliolate leaves the situation was different and large effects of treatment were found (Figure 17). From all the data it would appear that cell divisions had ceased by the time of first harvest of the trifoliolate leaves, since large and consistent increases in cell number were not found in any treatment. For the

Figure 18 - Mean cell volume and fresh weight of,
primary leaves.

Cell volume was derived from the values for fresh
weight and cell number.



ONE LEAF OF THE 1st. PAIR

- control
- CCC (day 4)
- ▽ G.A.₃
- ▲ CCC (day 7)
- CCC (day 4) + G.A.₃
- CCC (day 7) + G.A.₃

first two trifoliolate leaves CCC had an effect in reducing cell number to about 50% of the control value. CCC on day 4 had a larger effect than CCC on day 7 but this difference was not statistically significant. GA₃ alone had no effect in altering cell number for any of the leaves and counteracted completely the effect of CCC applied on day 7 and partially counteracted the effect of CCC on day 4. For the third trifoliolate leaf the effect of CCC applied on day 7 was negligible, but from the one value available for the CCC4 treatment, this appears to have caused a large reduction in cell number. Cell number in the control series was similar for all the first three trifoliolate leaves.

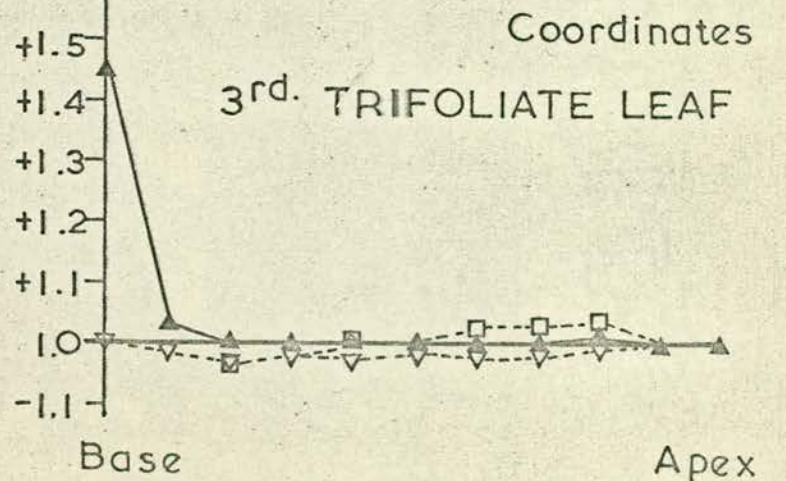
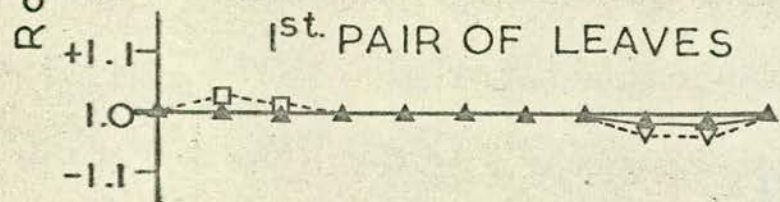
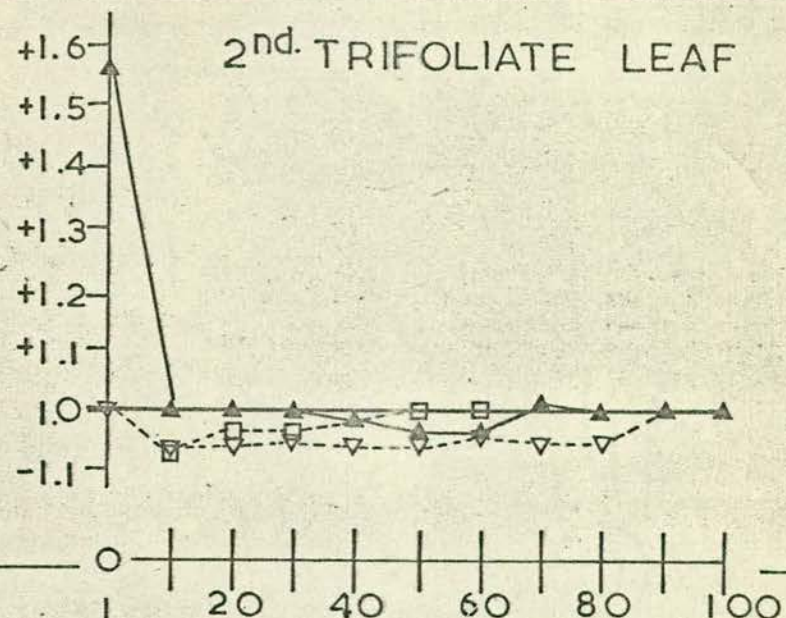
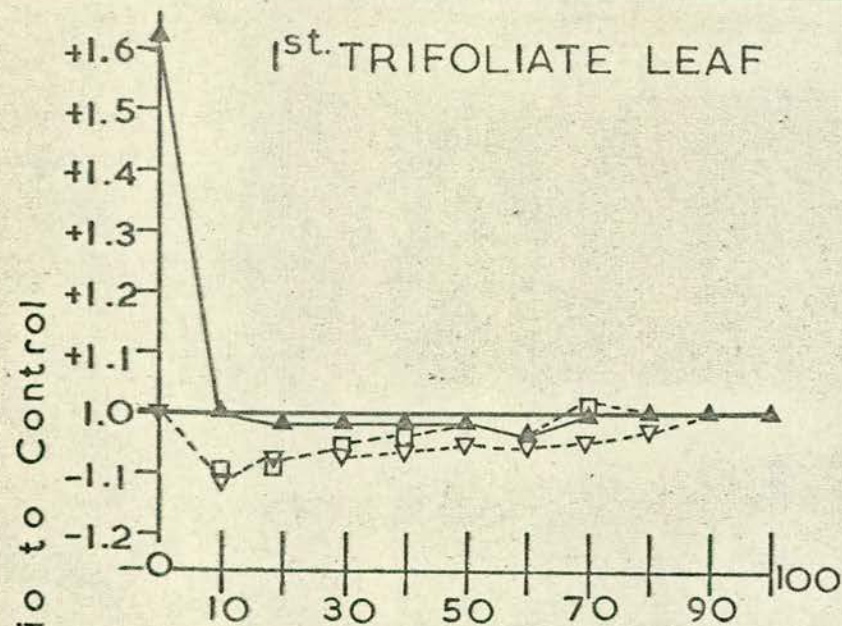
Since fresh weight was measured for the first pair of leaves, it was possible to determine cell volume. The fresh weight data (Figure 18) which resemble in form the curves for leaf area (Figure 13) show that CCC brought about a reduction in fresh weight and the final cell volumes for the CCC series were also slightly reduced. (It is not possible to calculate significant differences for the volume data which are mean values and unreplicated).

Leaf shape

Measurements of leaf shape were made at every harvest except the first three. Figure 19 shows the results of the plants when 31 days old. The results for all the other harvests were similar to those shown in the figure. The values for plants treated with CCC

Figure 19 - Leaf shape of light-grown plants.

Leaf shape was calculated according to Melville (1937).



▲ CCC (day 7)

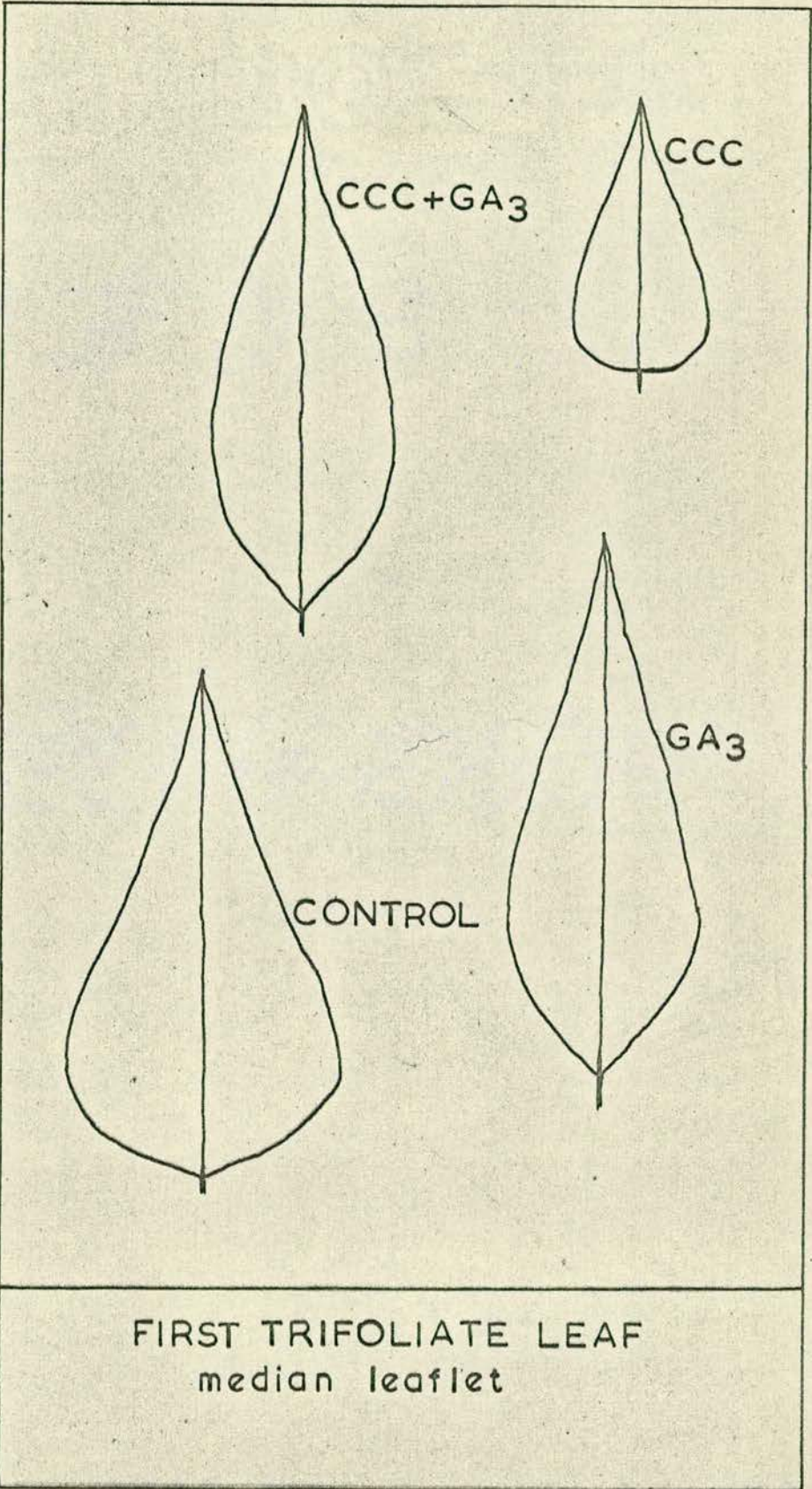
▼ GA₃

Leaf Shape

◻ CCC (day 7) + GA₃

Figure 20 - Leaf shape of light-grown plants.

The figure shows the actual shape of the median leaflet of the first trifoliate leaf given different treatments. The CCC-treated leaflet shows a truncate base and the other leaflets a cuneate base.



on day 4 or day 7 were similar as were the values for all CCC-treated plants given GA₃. Thus only the CCC-treatments involving application on day 7 with or without GA₃ are shown in the figure. All comparisons are made using untreated control leaves as the reference standard.

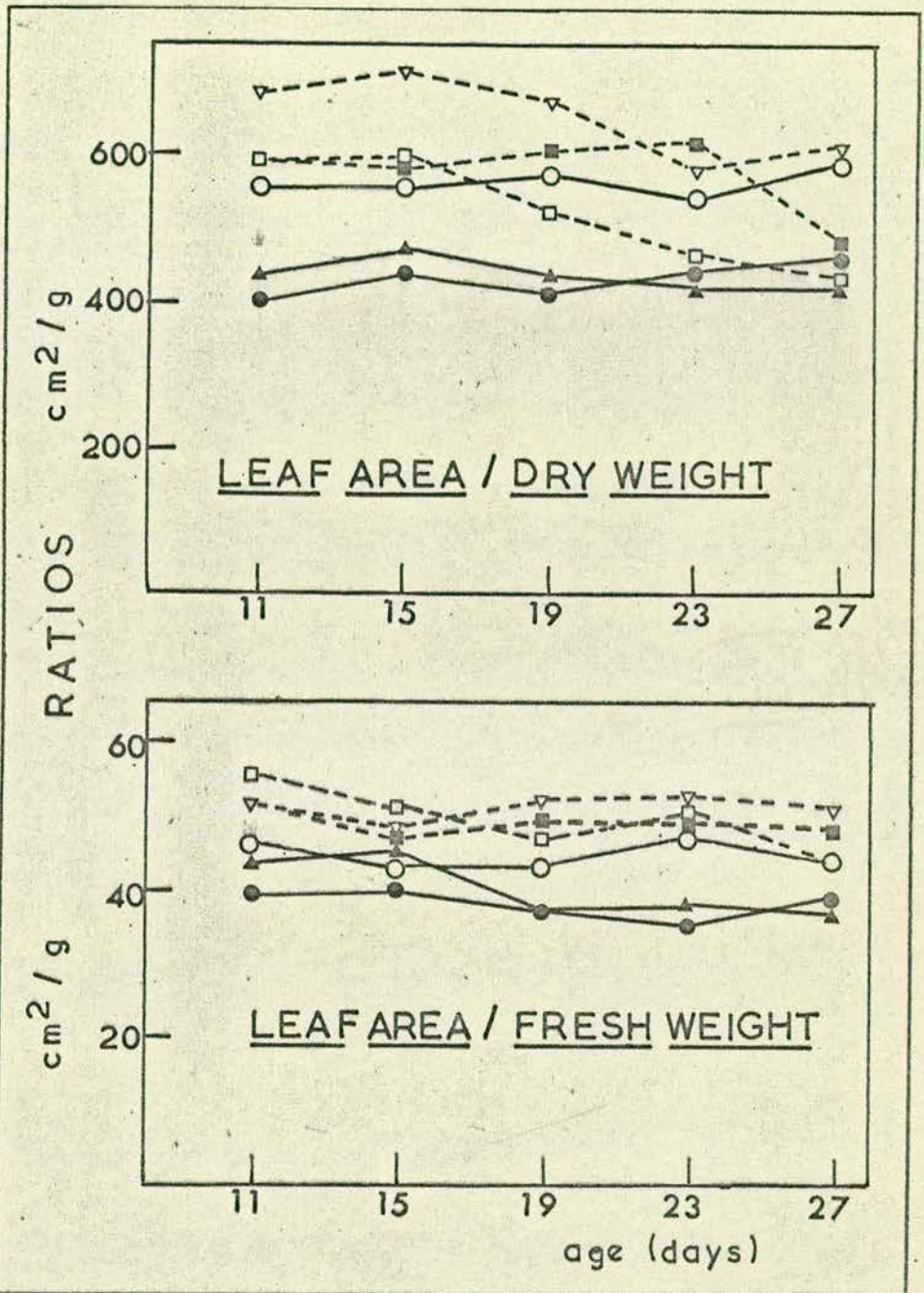
There was no difference in shape of the first pair of leaves in any of the treatments, and the ratio of breadth to that of the control series was very close to 1.00. Thus, neither GA₃ nor CCC affected the shape of the first pair of leaves.

The median leaflet of the trifoliate leaves on control plants and plants treated with GA₃ with or without CCC were compared. These leaflets had a cuneate base while for plants treated with CCC alone a truncate base was found. Apart from the effect on the base of the leaflet CCC did not alter the shape of the lamina. For the first and second leaves GA₃ completely counteracted the effects of CCC and brought about relative reduction in leaf breadth, so that leaflets on GA₃-treated plants appeared longer and narrower (Figure 20). On the third trifoliate leaf the effect of CCC was still apparent but the effects of GA₃ in causing reduction in lamina breadth were much less marked.

Leaf shape is generally taken to mean shape of the leaf in the horizontal plane. However, differences in shape in the vertical plane (thickness) were also noted in this experiment. Leaf thickness can be measured

Figure 21 - Ratio of area and weight for the primary leaves.

The ratio leaf area/dry weight (above) and ratio leaf area/fresh weight are given for one leaf of the first pair of leaves of light-grown plants.



ONE LEAF OF THE 1st. PAIR

○:control ▲:CCC (day 7)

▽:GA₃ ■:CCC (day 4) + GA₃

●:CCC (day 4) ◻:CCC (day 7) + GA₃

either directly or indirectly as the ratio of area fresh or dry weight of leaves. In this experiment the latter approach was used and data are shown in Figure 21. Leaves treated with CCC showed a lower area per unit weight than control leaves, which in turn showed a lower value than GA₃- treated leaves. Thus leaves treated with CCC were thicker than leaves treated with GA₃. The same general trends were shown for area/dry weight ratio and for area/fresh weight ratio although the contrasts between treatments were less marked in the ratio area/fresh weight. The main finding was confirmed by direct measurements made in another experiment (Table 9) which showed that CCC-treated leaves were almost double the thickness of leaves treated with GA₃.

TABLE 9

Thickness of the first pair of leaves (in μ) measured 23 days after planting. Fresh material. L S D : 50.

Position	control	CCC7	GA ₃	CCCGA ₃
Apex	166	234	124	134
Middle	184	244	131	127
Base	177	245	138	136
Average	176	241	131	132

Content of chlorophyll in the first pair of leaves

It was always observed in our experiments that CCC-treated plants had **darker** leaves. Also, the leaf area on CCC-treated plants was always smaller than that for the control plants. Thus, the CCC-treated leaves could either have the same amount of chlorophyll as the control and be darker because the leaf area was **smaller**, or the content of chlorophyll in CCC-treated plants could be greater than that for the control. It was not possible to check this point in the main experiment but data from a subsequent run are included here because of their relevance. CCC was applied on day 7 after planting. Plants were harvested when 10, 26, and 38 days old and the content of chlorophyll in the first pair of leaves was determined.

Table 10 shows the content of chlorophyll on an area basis (mg/100cm² of leaves).

There was no change in the total chlorophyll content in control plants between days 10 and 26 but subsequently a decrease was noted by the time that the plants were 38 days old. For plants treated with CCC there was a considerable increase in total chlorophyll from day 10 until day 38, at which time the leaves did not show any sign of senescence.



TABLE 10

Content of chlorophyll of the first pair of leaves
on an area basis (mg/100cm²)

Treatment	10 days old	26 days old	38 days old
CCC 7	3.14	4.62	5.11
Control	2.36	2.32	1.98
GA ₃	2.01	1.20	1.59
CCC GA ₃	1.82	2.33	1.78

TABLE 11

Content of chlorophyll of the first pair of leaves
on a cell basis (mg/10⁷ cells)

Treatment	10 days old	26 days old	38 days old
CCC 7	0.32	0.94	1.04
Control	0.33	0.55	0.47
GA ₃	0.41	0.36	0.48
CCC GA ₃	0.41	0.74	0.57

TABLE 12

Content of chlorophylls a and b of the first pair of leaves on a cell basis (mg/10⁷ cells)

Treat- ment	chloro- phyll	10days old	26 days old	38 days old
CCC 7	a	0.20	0.63	0.73
	b	0.11	0.31	0.31
Control	a	0.21	0.36	0.33
	b	0.13	0.19	0.14

Treatment with GA₃ counteracted the effects of CCC treatment and reduced total chlorophyll to levels less than in the control leaves.

Since similar areas contain different numbers of cells, total chlorophyll per 10⁷ cells was calculated. The data in table 11 show that chlorophyll per 10⁷ cell increased in the CCC series, but remained more or less constant for control and GA₃-treated plants. Thus, on both a cell number and an area basis, treatment with CCC increased the chlorophyll content of the first pair of leaves above the level in control leaves and cells. In contrast chlorophyll per cell remained similar to the control values for plants treated with GA₃. GA₃ counteracted completely the effect of CCC.

Separate analyses of the content of chlorophyll a and b expressed on a cell basis (mg/10⁷ cells) showed

that in CCC-treated plants the increase in total chlorophyll was due to increase in both chlorophyll a and chlorophyll b (Table 12).

3- Summary and general comments

From these results the following conclusions were drawn:

1) Leaf area and dry weight per plant were markedly reduced by CCC treatment. These effects were counteracted completely by GA₃ if the plants were treated with CCC on day 7 but to a smaller extent if CCC was applied on day 4.

2) There was a suggestion that values of R and E were smaller for CCC treatments than for the control series.

3) Dry weights of stem and roots were reduced by CCC, but the effect on stem was counteracted completely by GA₃, irrespective of the time of application of CCC. The effect of CCC on roots was only partially counteracted when CCC was applied on day 7, and not at all when CCC was applied on day 4.

4) For the primary leaves, CCC and GA₃ had no effect on dry weight, cell number and leaf shape, but leaf area, leaf length, fresh weight were all markedly reduced by CCC and mean cell volume was slightly reduced. GA₃ counteracted fully these effects when CCC was given on day 7 and to a lesser extent when application of CCC was made on day 4. CCC was effective in

increasing the thickness of leaves, an effect which was also counteracted by GA₃.

5) The chlorophyll content of the primary leaves both on an area basis and on a cell basis was increased for plants treated with CCC; this effect was counteracted by application of GA₃.

6) For the trifoliate leaves, length, area, dry weight, and cell number were markedly reduced by CCC especially for the first and second trifoliate leaves. GA₃ counteracted all these effects. For the third trifoliate leaf the effects of CCC were less marked, and in the case of cell number no effect was found when CCC was applied on day 7. The rate of unfolding of the trifoliate leaves was delayed by CCC application but was enhanced by GA₃, which also counteracted the effects of CCC. CCC-treated leaves always showed a truncate base and this effect on shape was also counteracted by GA₃.

7) GA₃ alone was effective in increasing total dry weight of the plant and internode length and in bringing about an earlier unfolding of the trifoliate leaves.

8) CCC applied on day 4 was always more effective in reducing growth than when applied on day 7. In general, treatment with CCC appeared to prolong the period over which growth of the various organs occurred.

While leaf area was reduced by CCC, dry weight was not affected. It would seem that even with smaller

leaf area than the control, the primary leaves in the CCC-treated plants, being the main photosynthesising organs in the plant, are able to retain much of the material produced since growth elsewhere is reduced, and hence demand for nutrients from other parts of the plant is reduced.

In contrast to the primary leaves which were well developed when CCC was applied, the trifoliolate leaves were present only as comparatively undeveloped primordia when CCC treatment was made, and it might be expected that the effects of the chemical could be greater in these circumstances.

The curves for cell number show no increase from the first harvest. This could be because the first harvest was made at a time when cell number was at its final value. This would agree with the data presented by Humphries and Wheeler (1964) for the first trifoliolate leaf of French beans. These authors show cell counts for much earlier developmental stages, but the final values agree with our results for control leaves, which could be plotted as a continuation of their curve.

CCC was most effective in modifying growth in the first two trifoliolate leaves, but the effects were less marked on the third leaf. Because of the growth habit of a CCC-treated plant (i.e., very short internodes) the first and second trifoliolate leaves develop in the shadow of the primary leaves which cover them,

Figure 22 - Shading effects on the trifoliate leaves.

The primary leaves on CCC-treated plants were taped down to the sides of the pots with cellotape and shading from these leaves on the first two trifoliate leaves was reduced. A group of control plants received the same treatment.

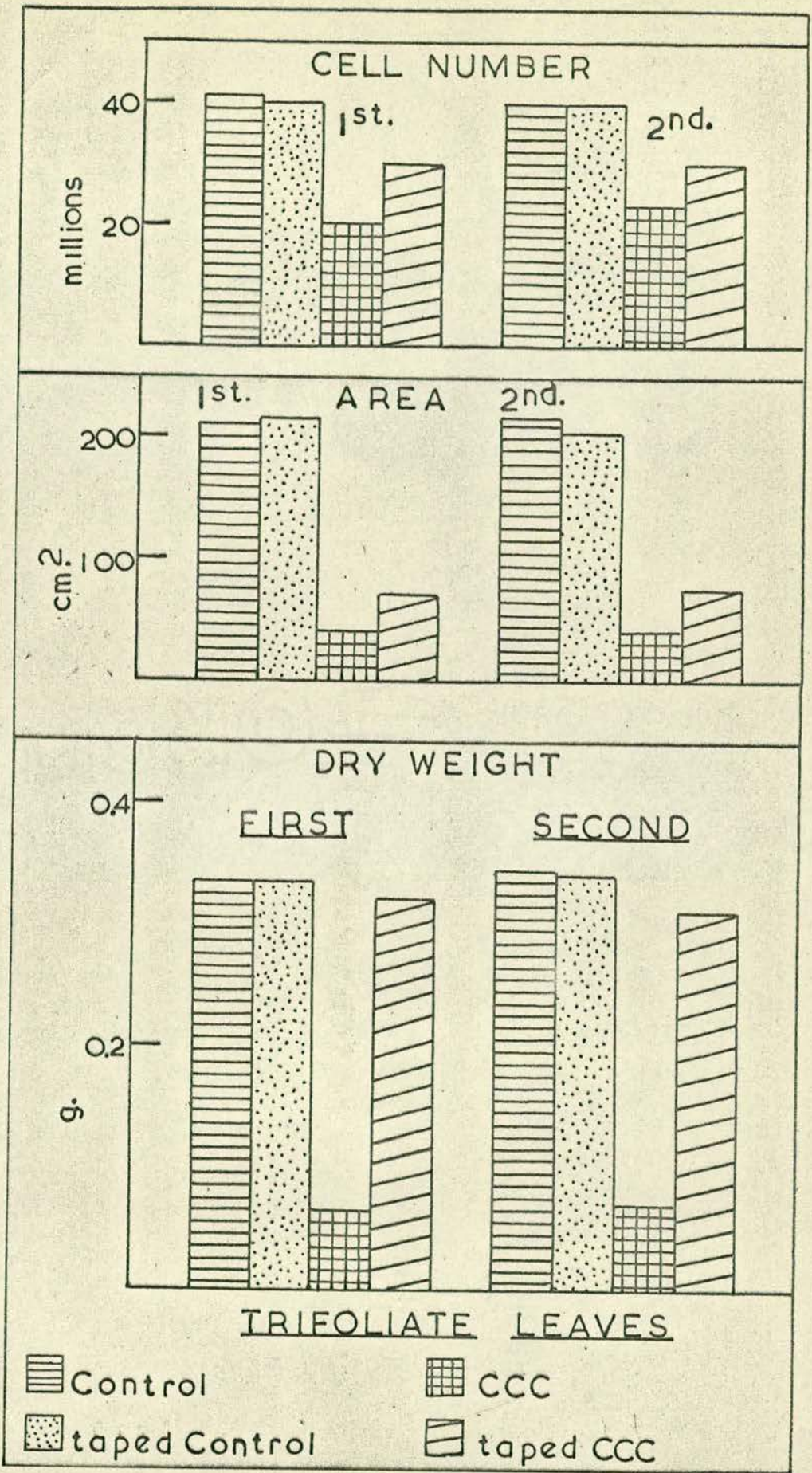
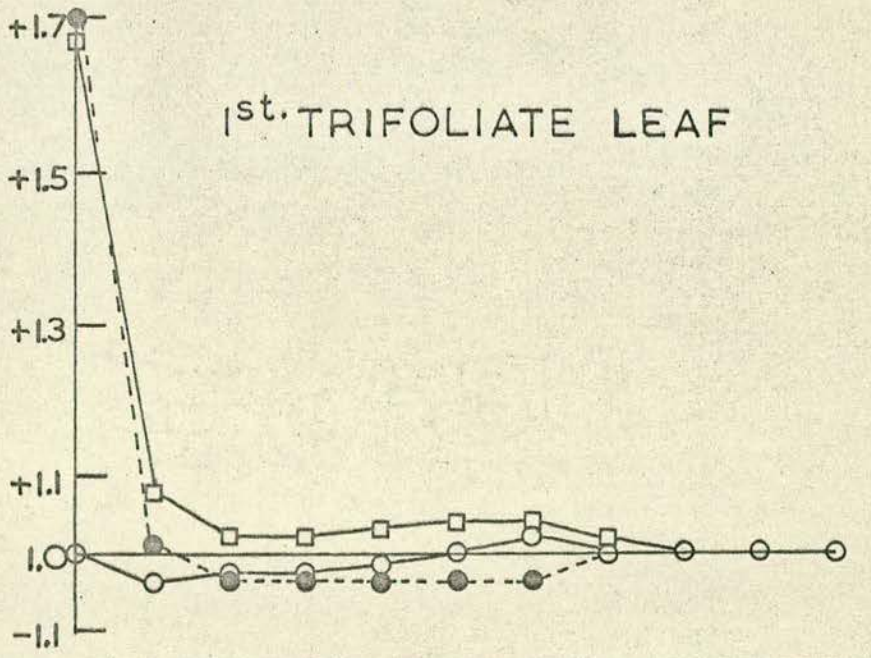
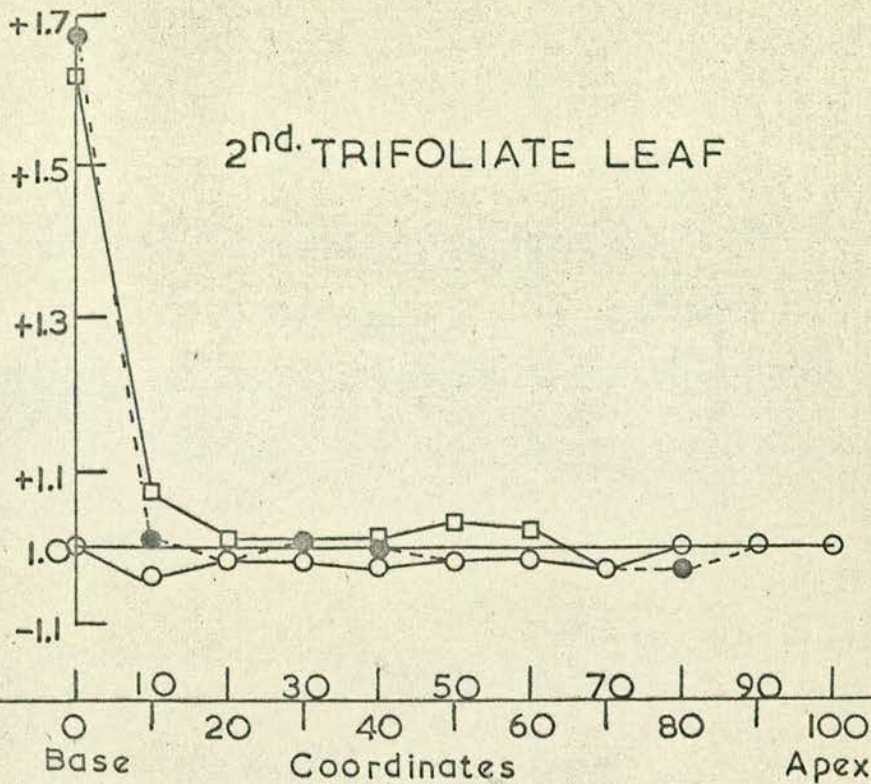


Figure 23 - Shading effects on shape of trifoliate
leaves.

Plants were treated as described for Figure 22.



○ taped Control
 ● CCC
 □ taped CCC

while the third trifoliate leaf is normally carried above the first pair and therefore receives full illumination. Thus CCC by acting on the stem and reducing expansion could have exerted an indirect and secondary effect on the first trifoliate leaves, the main effect being due to lack of light. To check this an experiment was performed in which the first pair of leaves was tied down to the sides of the pots using sellotape so that the trifoliate leaves would receive more light (even so some shadowing from the primary leaf basis and the third trifoliate still occurred). Control plants received the same treatment. Data for leaf characters are shown in Figures 22 and 23. Values for cell number and leaf area were slightly increased by the taping treatment but nevertheless some reduction was still found (see Discussion). This is not so for dry weight. The reduction brought about by CCC was completely reversed for leaves exposed to the light, showing that in CCC-treated plants the first two trifoliate have a smaller dry weight because of the shadowing effect of the primary leaves and not as a direct effect of CCC. Leaf shape was not altered by full exposure to light and it may be concluded that the truncate base of these leaves is a real result of CCC treatment.

B- Effects of CCC on dark-grown plants

In view of the very different pattern of growth of dark-grown plants from that of plants grown in light conditions, some experiments were performed with CCC on material grown in complete darkness. This section describes some of the results.

1- The effect of different concentrations of CCC

This experiment involved application at planting of CCC at 10^{-6} , 10^{-4} and 10^{-2} M. Plants were grown for 15 days in complete darkness and at harvest estimates of plant dry weight, stem length, and cell number in the first pair of leaves were made.

None of the concentrations of CCC used had any effect on total dry weight of the plants at day 15, (Table 13) although CCC at 10^{-2} M reduced stem dry weight by a significant amount and increased leaf weight. No effect of CCC on root dry weight was found.

TABLE 13

Application of CCC at 10^{-6} , 10^{-4} and $10^{-2}M$ at planting. Dry weight (g) of plants when 15 days old. Standard errors are shown for control.

	CONTROL	CCC $10^{-6}M$	CCC $10^{-4}M$	CCC $10^{-2}M$
1st pair of leaves	0.03±0.01	0.03	0.03	0.07
Stem	0.23±0.01	0.24	0.25	0.14
Roots	0.08±0.01	0.09	0.09	0.09
Total weight	0.34±0.03	0.36	0.37	0.30

CCC at $10^{-2}M$ also caused a pronounced reduction in length of the epicotyl and hypocotyl, although lower concentrations had no effects (Table 14).

TABLE 14

Application of CCC at 10^{-6} , 10^{-4} and $10^{-2}M$ at planting. The length (cm.) was measured when the plants were 15 days old.

	CONTROL	CCC $10^{-6}M$	CCC $10^{-4}M$	CCC $10^{-2}M$
Hypocotyl	25.3	25.0	23.1	10.6
Epicotyl	16.4	16.0	14.8	3.2

The hypocotyl of plants treated with CCC was thicker than that of the control plants and the number of vascular bundles in plants treated with CCC at $10^{-2}M$ was examined in a supplementary experiment.

The vascular bundles were counted in sections of the hypocotyl of three treated and three control plants. Since the length of the hypocotyl of treated and control plants was very dissimilar, every hypocotyl was divided in five equal portions and from each segment three sections were cut by hand. Thus, it was possible to compare the same region of the hypocotyl of control and treated plants. The sections which were made from 13-day old plants were stained with a 10% aqueous solution of aniline hydro-chloride, filtered before use.

In the control plants the hypocotyl was long (Table 14), thin, and hollow. In transverse section the central cavity of the hypocotyl had a very wide diameter. Plants treated with CCC showed a very thick, short hypocotyl (Table 14). In transverse section the central cavity of the hypocotyl had a very narrow diameter.

The number of vascular bundles at different positions in the hypocotyl is given in Table 15 and was found to be constant for all positions in control hypocotyls with a mean value of 15.

TABLE 15

Number of vascular bundles in the hypocotyl of plants treated with CCC. Dark-grown plants harvested when 13 days old.

Position	no CCC	CCC
1 (base)	16	19
2	15	21
3	15	23
4	15	21
5 (apex)	16	20
\bar{x}	15	20

In CCC-treated plants, the number of bundles was more variable and there was a slight suggestion that the central part of the hypocotyl (positions 2, 3 and 4) had more vascular bundles than the rest. On average the CCC-treated plants had 20 bundles as against 15 in the control - an increase of 33%. (c.f. Mayr and Presoly, 1963).

Returning now to the experiment involving different concentrations of CCC it was found that the most spectacular effects of treatment occurred on the primary leaves (Table 16).

TABLE 16

The effect of different concentrations of CCC applied at planting. Cell number (in millions) and the fresh weight (in g.) were measured when the plants were 15 days old. Data are for first pair of leaves.

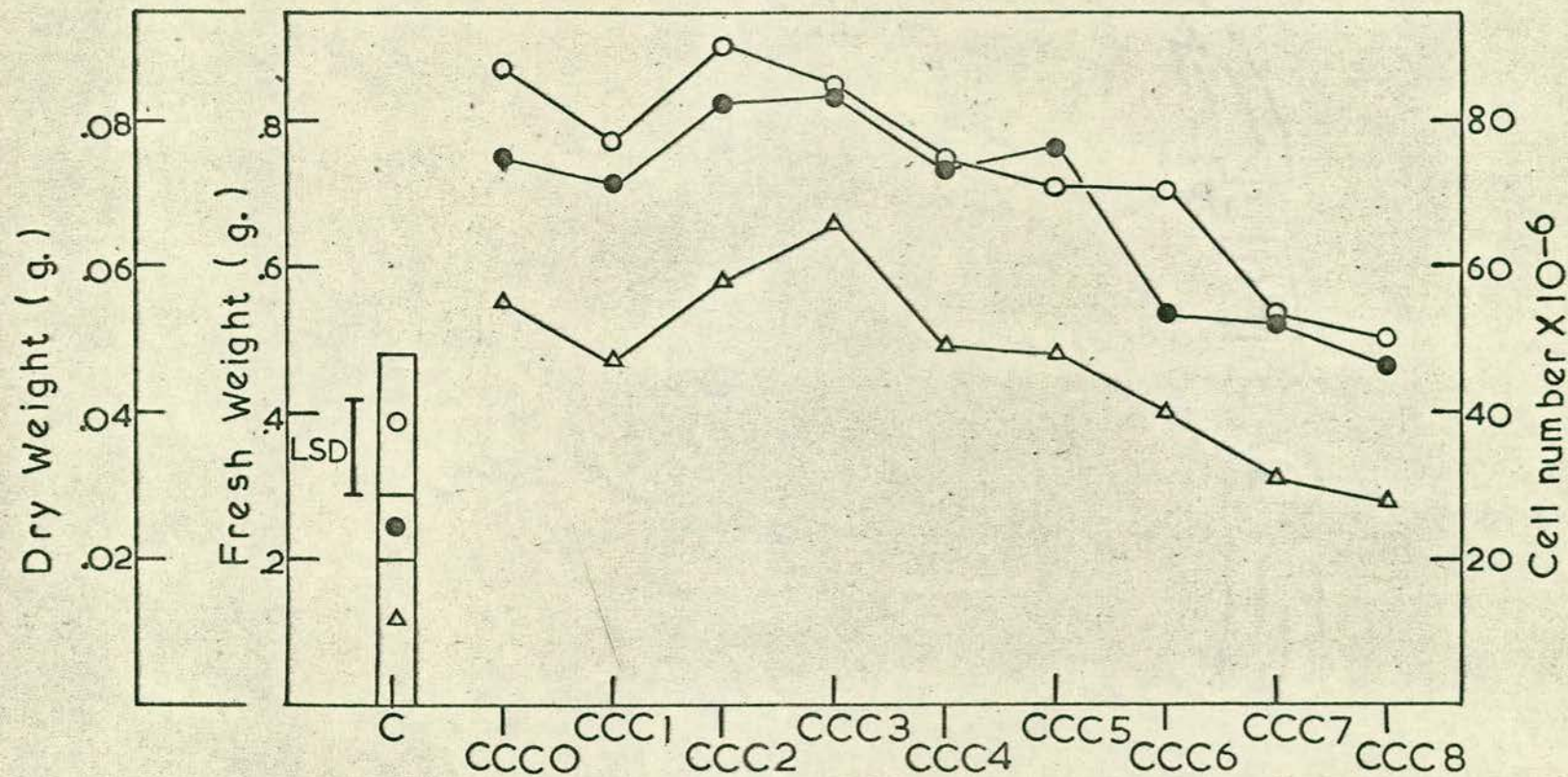
	CONTROL	CCC $10^{-6}M$	CCC $10^{-4}M$	CCC $10^{-2}M$
Cell number	48	50	51	87
Fresh weight	0.20	0.22	0.24	0.55

Once again the lowest concentrations of CCC had no effect on leaf characteristics, but at $10^{-2}M$ the compound caused a very large increase in both cell number and fresh weight (also in dry weight as shown in table 13). The cell number was close to that of light-grown leaves of control plants and fresh weight was nearly three times that of the dark control.

Thus CCC also showed very large effects when applied to dark-grown plants at a concentration of $10^{-2}M$, although lower concentrations were ineffective. Dark-grown plants treated with CCC showed reduced internode length and dry weight of the stem and this was similar to the effects of CCC on light-grown plants. The effects of CCC on the first pair of leaves were completely different from the effects of the compound on these organs when light-grown, in that the cell number and fresh and dry weights of dark-grown leaves were increased. On light-grown plants CCC had

Figure 24 - Effects of CCC on primary leaves of dark-grown plants.

Plants were harvested when 14 days old. For this experiment the temperature was 25°C for 4 days and 21°C afterwards. Least significant differences are given for the fresh weight values.



CCC: days of application

O: cell number
 Δ: fresh weight
 ●: dry weight

DARK GROWN PLANTS

plants 14 days old

FIRST PAIR OF LEAVES

no effect on cell number and dry weight of primary leaves.

In the remaining experiments only CCC at $10^{-2}M$ was used.

2- The effect of different times of application of CCC

CCC had large effect on dark-grown plants when applied at planting. The effect of CCC when applied later on was next examined.

CCC was applied to dark-grown plants in the normal way on day 0, 1, 2, 3, 4, 5, 6, 7, or 8 and plants were harvested when they were 15 days old. Cell number, and fresh and dry weights of leaves were measured, and dry weight of other organs was also examined. In this experiment the temperature was $25^{\circ}C$ during the first four days of the experiment but subsequently had to be reduced to $21^{\circ}C$. The whole experiment was later repeated at a temperature of $25^{\circ}C$ throughout, and all other experiments with dark-grown plants were performed at this temperature (except experiment 6 of this section, Figure 32).

Treatment with CCC increased the cell number of the first pair of leaves. CCC applied from planting to day 4 was very effective and the cell number of treated leaves was double that of the untreated control (Figure 24). Application of CCC on day 7 or 8 had no significant effect on cell number.

The curves for the fresh and dry weights of

leaves followed the same pattern as the curve for the cell number (Figure 24). CCC had large effects in increasing the values for these parameters when applied up to day 4 after planting.

By dividing fresh weight by cell number an estimate of mean cell volume can be obtained. It was found that CCC treatment increased cell volume by only a small amount so that a correlation must exist between cell number and leaf fresh weight. For this experiment this was calculated to have a correlation coefficient, r , of 0.932 for 7 d.f. and the regression equation was:

$$X = 23.99 + 104.92Y$$

where X = cell number in millions

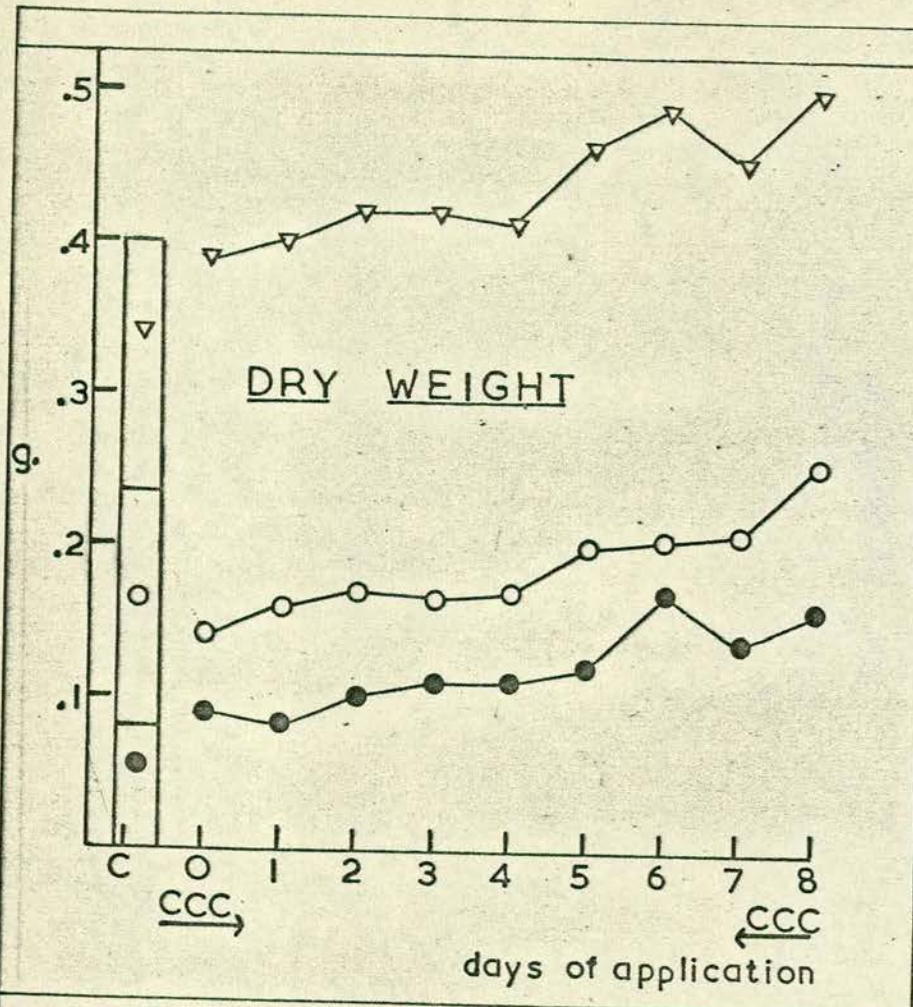
and Y = leaf fresh weight in g.

This relationship made it possible to measure effects using leaf fresh weight as a suitable parameter, and meant that the precision of experiments could be increased, since many more determinations could be made of fresh weight than of cell number.

In the repetition of this experiment the temperature was kept at 25°C throughout. The results for fresh and dry weights only (Table 17) showed that CCC began to be less effective when applied on day 4, but caused large increases in the fresh and the dry weights when applied between day 0 and day 4. When applied later than day 4 there was progressively less effect and the plants that received CCC on day 7 had similar fresh and dry weights to the control plants.

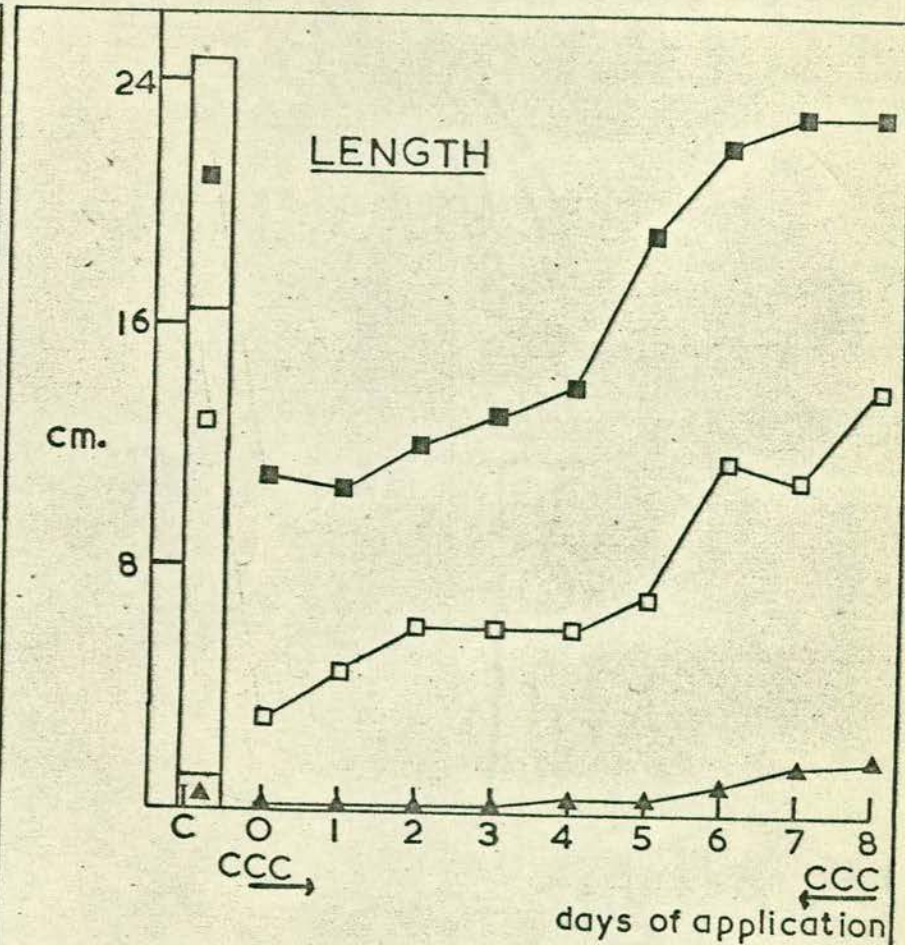
Figure 25 - Effects of CCC on dry weight and stem
length of dark-grown plants.

Plants were harvested when 14 days old. Temperature
as in Figure 24.



∇: total
 ○: stem
 ●: roots

DARK GROWN



PLANTS

■: hypocotyl
 □: epicotyl
 ▲: 1st internode

TABLE 17

Different times of application of CCC. Temperature of 25°C throughout. Plants harvested when 15 days old. Fresh and dry weights (in g.) of the first pair of leaves.

	Fresh Weight	Dry Weight
CONTROL	0.210	0.035
Time of CCC application (days)		
0	0.550	0.082
1	0.490	0.075
2	0.512	0.073
3	0.505	0.076
4	0.459	0.070
5	0.394	0.062
6	0.262	0.045
7	0.201	0.036

Thus, CCC was very effective in increasing the cell number, and fresh and dry weights of the first pair of leaves of dark-grown plants when applied early, i.e., between planting day and day 4. The period during which a response to CCC treatment occurs appears to be slightly prolonged if the temperature is reduced as in the preceding experiment (Figure 24).

When stem dry weights were considered it was found that CCC applied at planting had most effect in decreasing this parameter (Figure 25). The

decrease in the stem dry weight depended upon the day of CCC application, and earlier applications decreased it more than later applications. CCC applied on day 8 had no effect at all on stem dry weight.

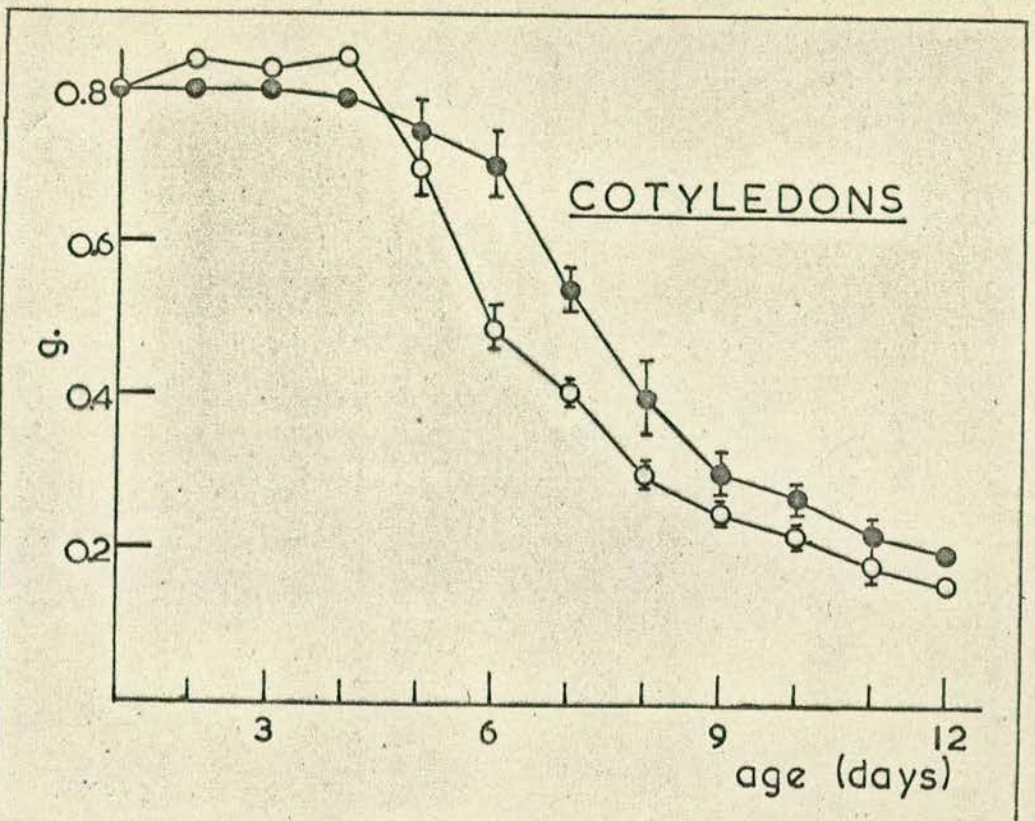
Some CCC treatments increased the root dry weight. The earlier applications (CCC on day 0 up to day 2) had small effects which were not significant, but later application led to marked increases in the root dry weight, and in plants treated on day 8 the dry weight was double that of the untreated control plants. Thus, while later applications of CCC (after day 4) were not very effective on the growth of leaves and the stem, such treatments were still very effective in increasing the root dry weight (contrast with light-grown plants, Figure 9).

The total dry weight of plants that received CCC on day 0 to day 4 was very similar to the control; the root dry weight was slightly higher, and the leaf dry weight much higher than for the control, but this was counterbalanced by the reduction in the stem dry weight. For the later applications, the total plant dry weight was higher than in the control (Figure 25).

Since growth in length of epicotyl and hypocotyl is almost complete by day 8 it might be anticipated that delay in application of CCC would reduce the extent to which these organs respond to the chemical. This was confirmed (Figure 25) and it will be seen that early application caused a pronounced decrease in length of the epicotyl and hypocotyl but that this effect was re-

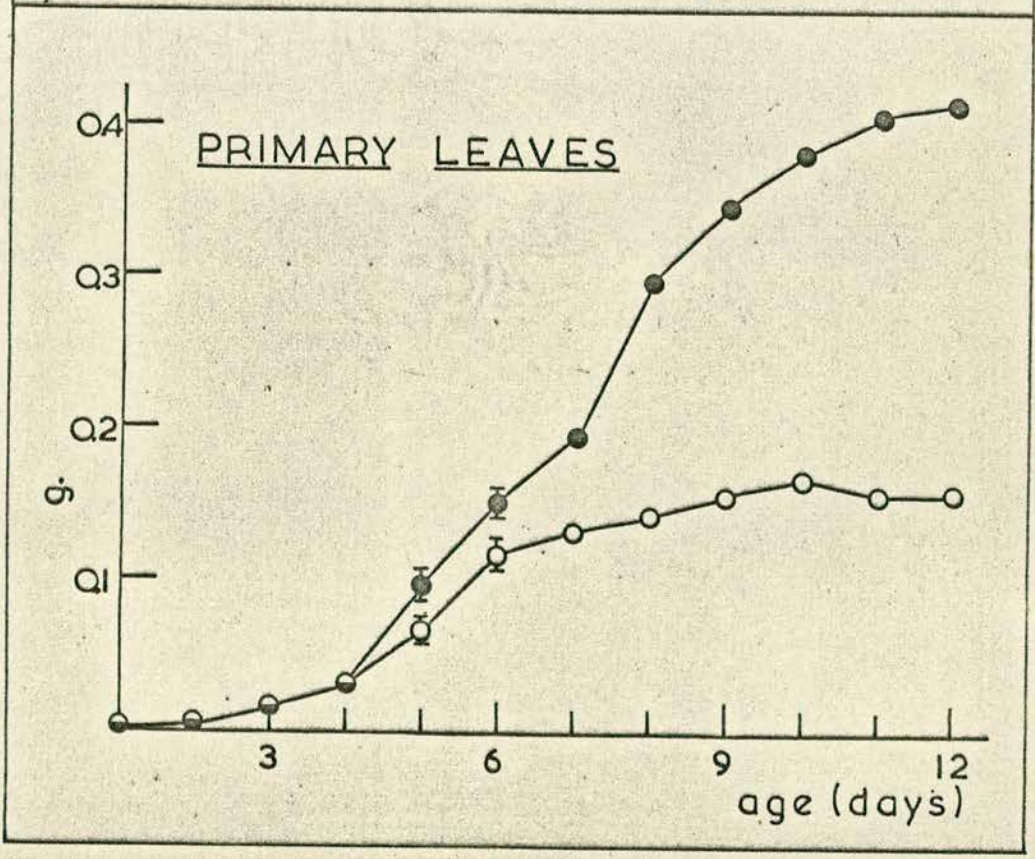
Figure 26 - Changes in fresh weight in the primary leaves and pair of cotyledons of dark-grown plants treated with CCC at planting.

Confidence limits are shown. Each point is the average value for 15 plants.



Dark-grown plants
 ○ Control
 ● CCC

FRESH WEIGHT



duced as CCC application was delayed. Although data for the first internode were also obtained these are not informative since plant-to-plant variation is high and the lengths when measured were small.

3- Further effects of CCC applied on day 0 on growth of the first pair of leaves and cotyledons

From examining plants of last experiment it seemed that the plants receiving early application of CCC had larger and heavier cotyledons than the control plants. This was investigated and at the same time the time course of development of leaves on CCC-treated plants was followed, since in the previous experiments measurements were made only when the plants were fully developed.

For this experiment CCC was applied at planting and plants were harvested daily. Fifteen replicates were used at each harvest and to reduce variation as far as possible, only seeds weighing between 0.3 and 0.4g were used. Fresh weight of the first pair of leaves and of the pair of cotyledons was measured daily.

Data (Figure 26) show that from day 1 until day 4 after planting there was an increase in the fresh weight of the pair of leaves, but no difference was found between plants treated or untreated with CCC. Difference between treatments appeared on day 5, and confidence limits show that at that stage leaves on CCC-treated plants were significantly larger than the control. Fresh weight of control leaves increased sharply from day 4 until day 6, and a slight increase continued

until day 9. For plants treated with CCC, the fresh weight of leaves increased strongly from day 4 until the end of the experiment and the difference between control and treated leaves became bigger and bigger.

Fresh weight of the cotyledons (Figure 26) had not begun to decline by day 4, but between day 4 and day 5 differences began to appear, and the cotyledons of the control plants lost fresh weight more rapidly than those of the CCC-treated plants. Between day 5 and day 11 the difference between treatments was statistically significant. Thus, on the same day in which differences appeared between control and CCC-treated leaves, the difference between control and CCC-treated cotyledons also appeared. The cotyledon differences could indicate that perhaps CCC treatment reduces export or breakdown of material from the cotyledons. This material could be nutrients, hormones or both. The suggestion (Heatherbell, Howard and Wicken, 1966) that CCC acts by uncoupling respiratory phosphorylation reactions seems unlikely as this would lead to a more rapid drop in cotyledon weight.

4- Effects of CCC and of light on leaf and stem development of dark-grown plants

When applied to light-grown plants CCC has very little effect on cell number of the first pair of leaves. In dark-grown plants CCC treatment results in increases in leaf cell number.

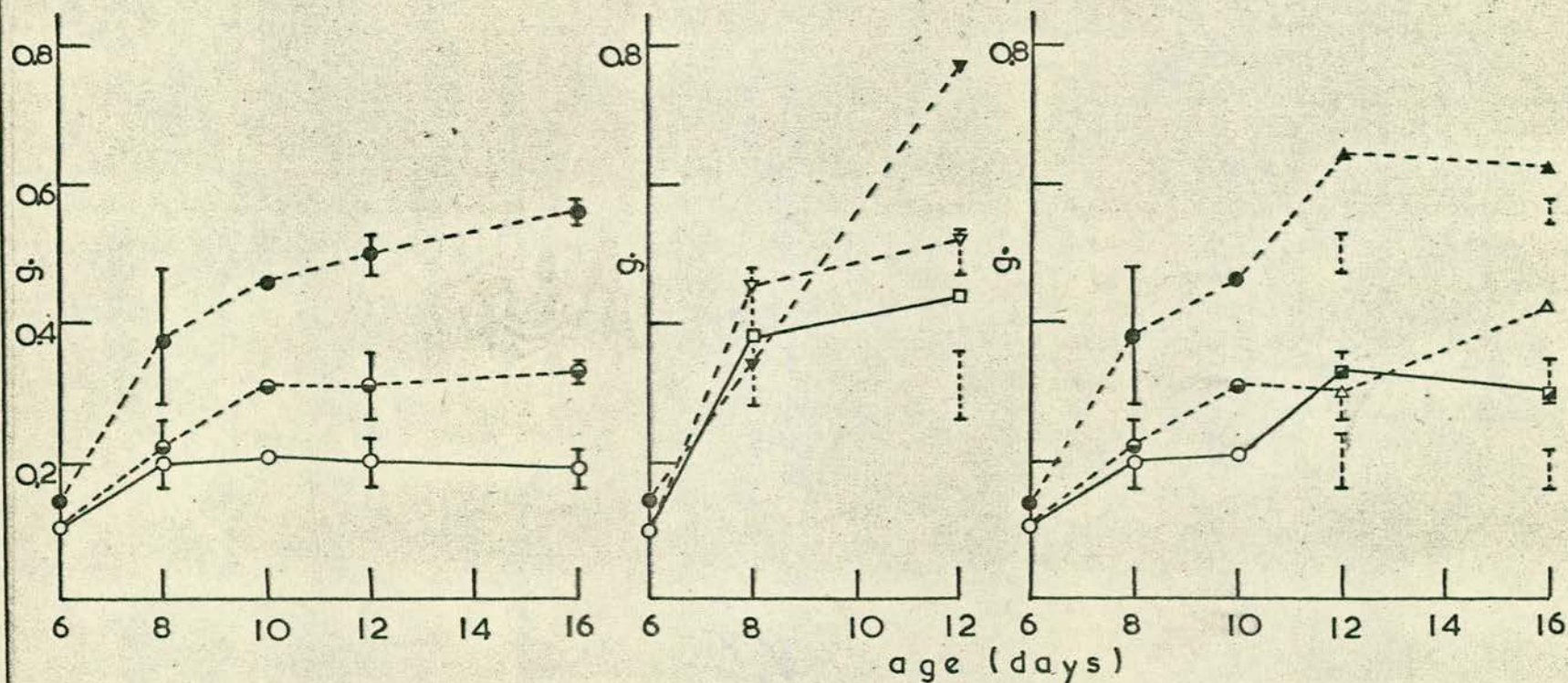
Miss D. Murray (1967) has shown that short periods of light increase the cell number and fresh weight

Figure 27 - Effects of CCC and white light on primary
leaves.

Confidence limits are shown and each point is the
average of ten replicates.

PRIMARY LEAVES - FRESH WEIGHT

Dark-grown plants



○ Control
 ● CCC (day 0)
 ◉ CCC (day 6)

□ light (day 6)
 ▼ " " + CCC (0)
 ▽ " " + CCC (6)

■ light (day 10)
 ▲ " " + CCC (0)
 △ " " + CCC (6)

of primary leaves of dark-grown Phaseolus plants, and that this effect involves the red:far-red reaction.

Whether the light and CCC treatments act on the same mechanism was now examined in an experiment in which both CCC and light treatments were combined.

CCC was applied to the plants on day 0 or on day 6. Ten minutes white light were given to plants on day 6 or on day 10 after planting. After treatment, plants were returned to dark conditions. The light-treated plants were harvested 2 and 6 days after treatment. The dark-grown control, and the CCC treatments were harvested on days 6, 8, 10, 12 and 16 after planting. The fresh weight of leaves and cotyledons and the length of hypocotyl were measured for 10 replicates of each treatment.

Results for the fresh weight of the leaves are given in Figure 27. For dark-control plants untreated with light or CCC the increase in leaf fresh weight stopped by day 8 but for the plants treated with CCC at planting, fresh weight was still increasing between day 12 and 16, and at day 8 the difference between values for control and CCC series was already significant.

For CCC applied on day 6, the increase in fresh weight continued until day 10.

Light treatment on day 6 resulted in an increase in fresh weight which continued after day 8, and this increase was much greater than that for control plants and also greater than for plants which received CCC on day 6, but smaller than that for plants treated with CCC on day 0.

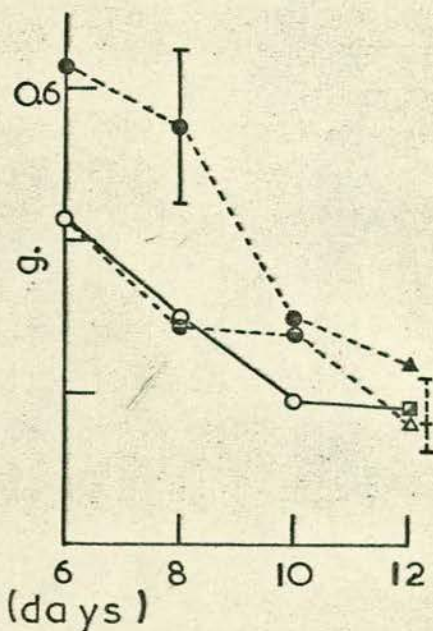
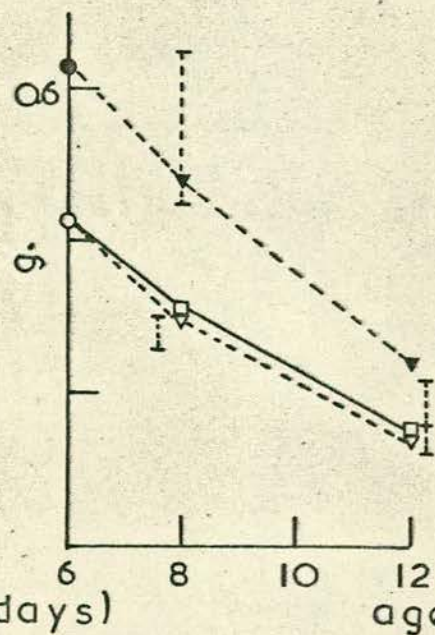
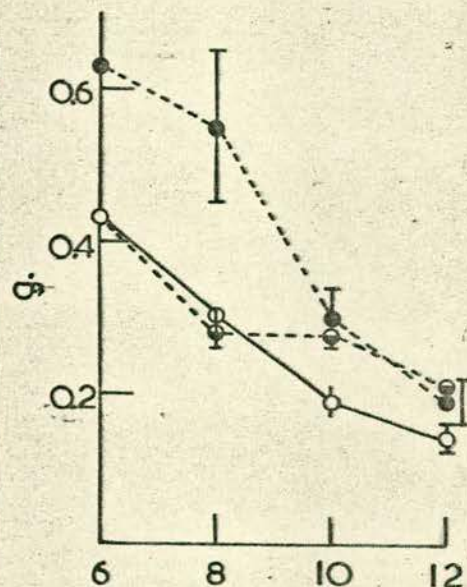
Figure 28 - Changes in cotyledon fresh weight.

Confidence limits are shown.

COTYLEDONS

Dark-grown plants

FRESH WEIGHT



○ Control

● CCC (day 0)

● CCC (day 6)

□ Light (day 6)

▽ Light (6) + CCC (0)

▽ Light (6) + CCC (6)

■ Light (day 10)

▲ Light (10) + CCC (0)

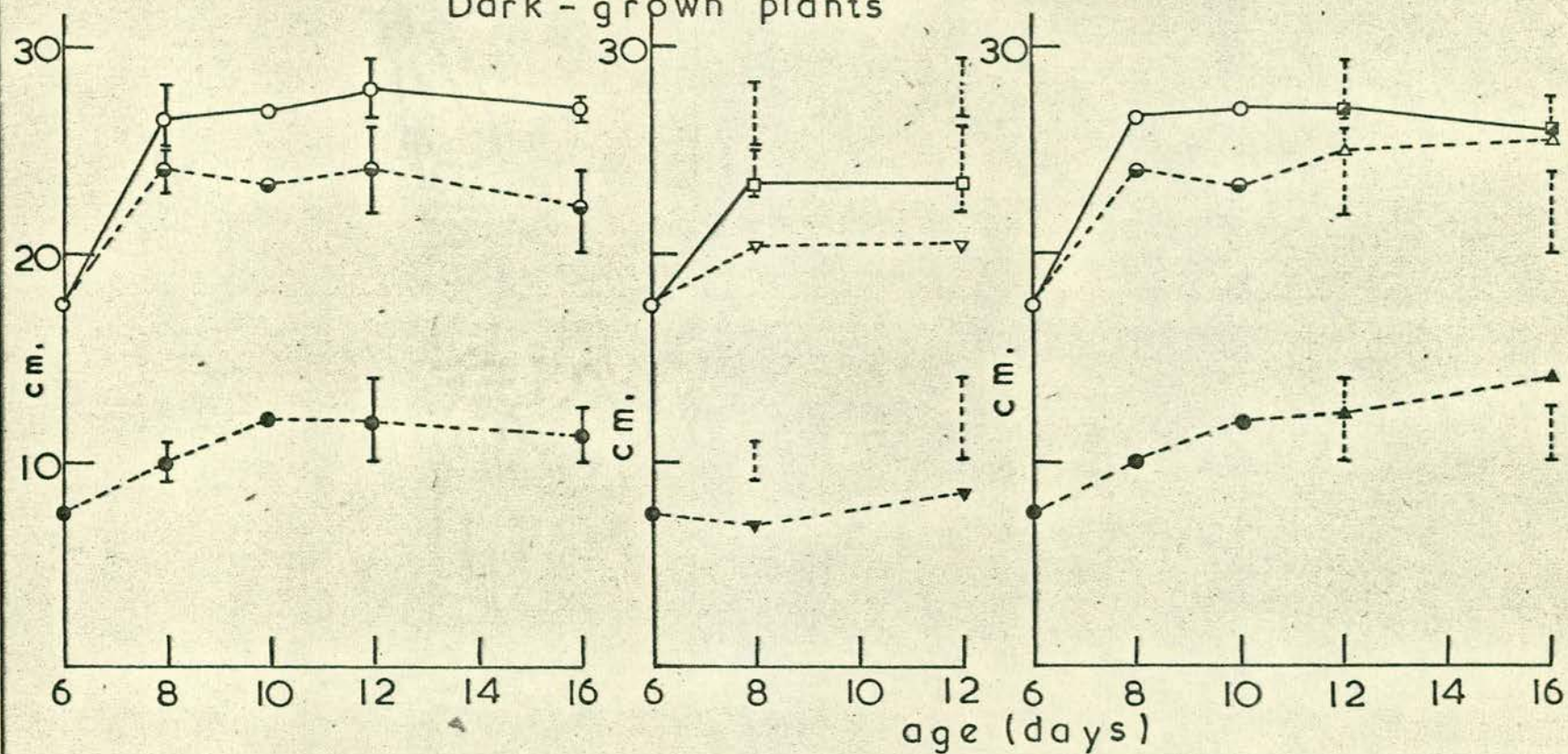
△ Light (10) + CCC (6)

Figure 29 - Changes in hypocotyl length.

Confidence limits are shown.

HYPOCOTYL LENGTH

Dark - grown plants



○ Control
● CCC (day 0)
● CCC (day 6)

□ Light (day 6)
▼ Light (6) + CCC (0)
▼ Light (6) + CCC (6)

■ Light (day 10)
▲ Light (10) + CCC (0)
▲ Light (10) + CCC (6)

Light applied on day 6 had additive effects with both CCC treatments. The values for the early CCC and light treatments are much higher at day 12 than those for either CCC or light treatment alone and represent the sum of the CCC and light effects over the dark-control.

Application of light on day 10 increased the fresh weight of the primary leaves, whose growth had stopped by day 8. Like light applied on day 6, this treatment had an additive effect upon CCC treatments.

Figure 28 shows values for fresh weight of cotyledons. The cotyledons of plants treated with CCC on planting day lost weight much slower^{ly} than the control ones, regardless of whether light was also applied or not. CCC applied on day 6 was much less effective in retarding the loss of fresh weight of the cotyledons probably because the processes leading to loss of weight were already far advanced.

CCC applied at planting markedly reduced hypocotyl elongation, and CCC applied on day 6 also had a significant effect. It was also found that light treatment on day 6 also suppressed hypocotyl growth and again, as for primary leaves, light and CCC had additive effects. Application of light on day 10 had no effect since hypocotyl elongation was by then complete (Figure 29).

Thus, CCC and light increase fresh weight (and cell number) of the first pair of leaves of dark-grown plants. As already mentioned the light effect is mediated through the phytochrome system. Whether the CCC

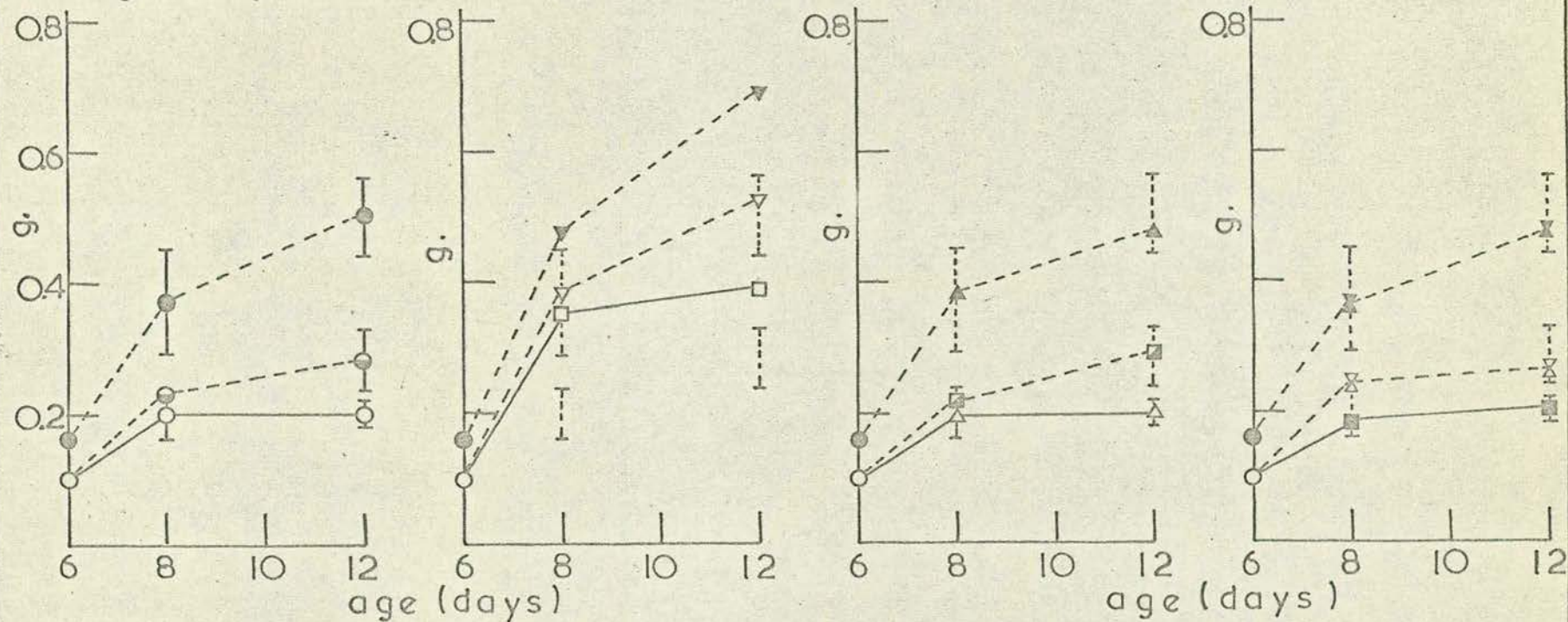
Figure 30 - Effects of CCC and light on the primary
leaves of dark-grown plants.

Confidence limits are shown. Each point is the average
value for ten plants.

1st. PAIR OF LEAVES

FRESH WEIGHT

Dark-grown plants



○ Control	□ Red	■ Far-Red	■ Red:Far-Red
● CCC (day 6)	▼ " + CCC (0)	▲ " + CCC (0)	× " + CCC (0)
● CCC (day 0)	▽ " + CCC (6)	△ " + CCC (6)	× " + CCC (6)

response is also associated with the phytochrome system was now investigated.

5- The role of CCC and the red:far-red reaction in controlling growth of dark-grown plants

CCC was applied as before on day 0 and on day 6. Ten minutes red and far-red irradiations were given on day 6. Where both light qualities were used the far-red irradiation was given immediately after the red irradiation. Ten replicates of each treatment were harvested at 6, 8, and 12 days after planting.

Figure 30 shows the results for the fresh weight of the leaves. The dark-grown control and the CCC treatments showed similar results to the previous experiment (Figure 27). The effect of red light was to increase markedly fresh weight and this effect was still apparent at day 12. Far-red radiation had no effect and the fresh weight of the leaf pair was the same as for the controls. Far-red treatment reversed the effects of red, so that the values of fresh weight were equivalent to those of dark-grown control.

Red light given to plants treated with CCC increased the fresh weight above the values for CCC or red light alone. Thus, red light and CCC have an additive effect. In the treatment with CCC and both red and far-red light, far-red reversed the effects of red light, but had no effect in the increment of growth due to CCC and the values for these treatments were equivalent to those for CCC given alone.

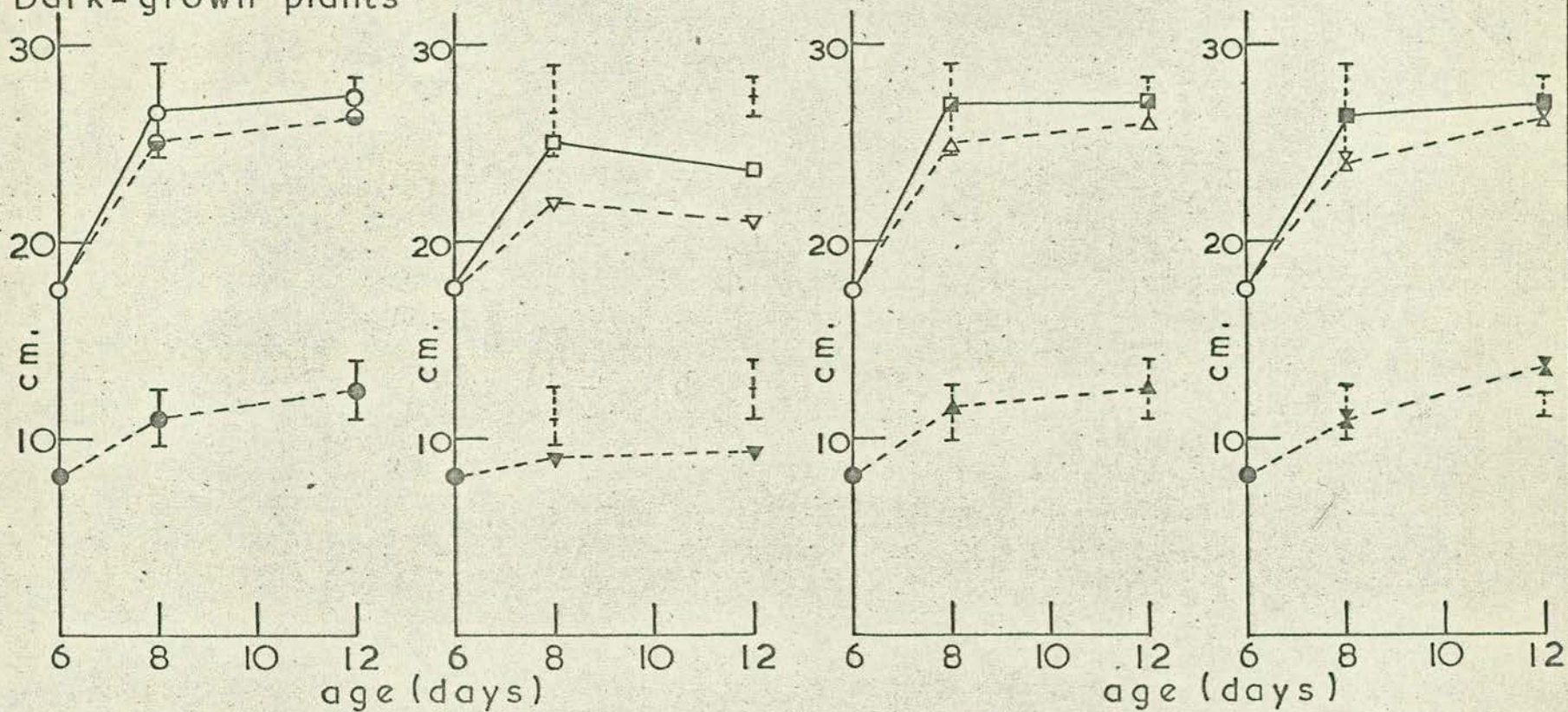
Figure 31 - Effects of CCC and light on hypocotyl
length of dark-grown plants.

Confidence limits are shown.

Dark-grown plants

HYPOCOTYL

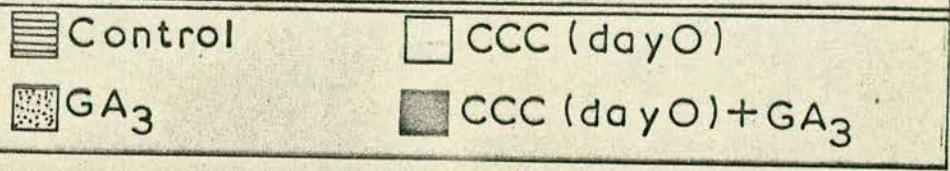
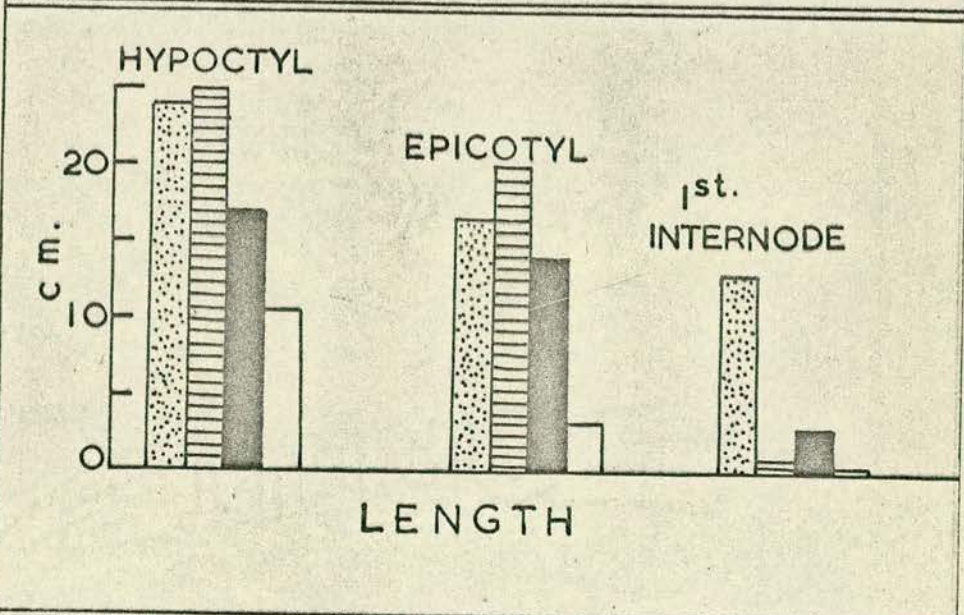
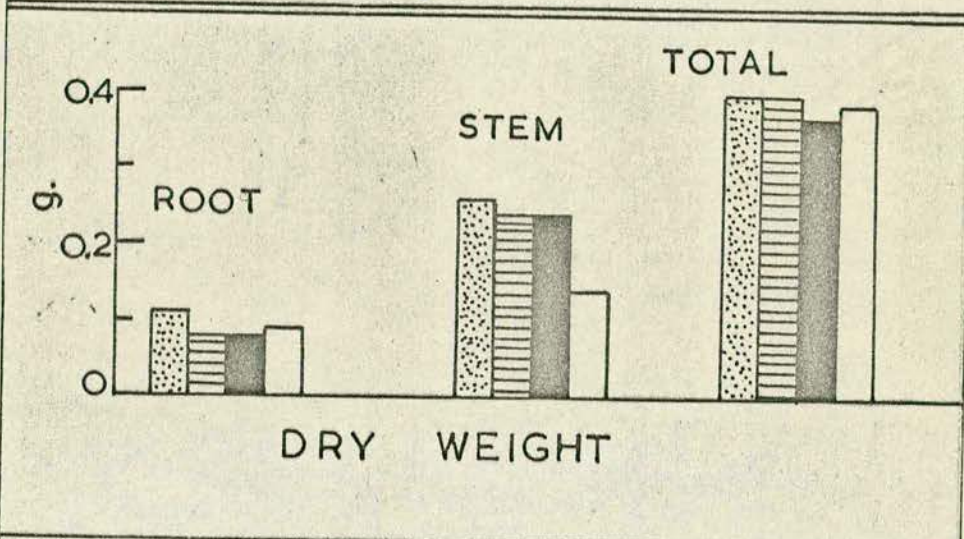
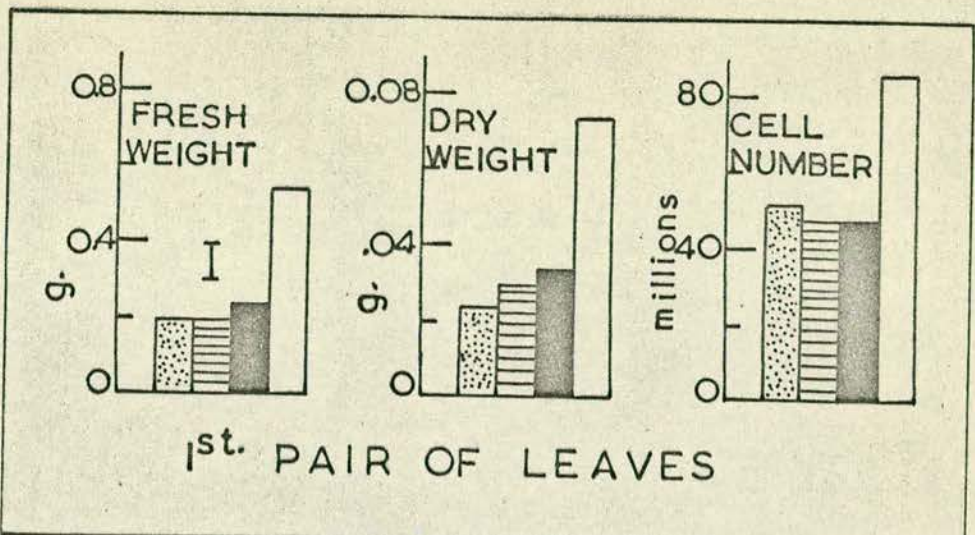
LENGTH



○ Control	□ Red	■ Far-Red	■ Red:Far-Red
● CCC (day 0)	▼ " + CCC (0)	▲ " + CCC (0)	× " + CCC (0)
○ CCC (day 6)	▽ " + CCC (6)	△ " + CCC (6)	⊗ " + CCC (6)

Figure 32 - Effects of CCC and GA₃ on dark-grown plants.

The plants were harvested when 14 days old. In this experiment the temperature was 25°C for 4 days and 21°C afterwards.



Red light and CCC also have additive effects on the length of the hypocotyl (cf. results with white light, Figure 29) as shown in Figure 31. Far-red radiation did not reverse the effects of CCC, but again reversed only the effect of red light, so that where all treatments were given together, (i.e., CCC + red light + far-red light) the results resembled those for the treatment with CCC alone.

Thus, far-red radiation reversed the increase in fresh weight of primary leaves brought about by treatment with red light, but did not reverse the effects of CCC. This seems to indicate **that** CCC acts independently of the photomorphogenic reaction involving phytochrome.

6- Effects of CCC and GA₃ on dark-grown plants

It has been shown that GA₃ counteracts most of the effects of CCC on light-grown plants. The effects of GA₃ on dark-grown plants were now examined to determine whether the large responses to CCC could again be counteracted.

CCC was applied on day 0 and plants treated with GA₃ received 10 μ g of this compound applied to the apex seven days after planting. Plants were harvested when 15 days old.

Cell number of the first pair of leaves was increased by CCC and this effect was counteracted by application of GA₃, although GA₃ applied alone (Figure 32)

did not affect the number of cells. The curves for fresh weight and dry weight of the first pair of leaves follow the same pattern as that for the cell number (Figure 32). CCC treatment resulted in a large increase in fresh and dry weights. GA₃ had no effect applied on its own, but did counteract the effects of this early application of CCC.

As before, stem dry weight was reduced by application of CCC and was not affected by GA₃ applied alone although GA₃ counteracted the effects of CCC, so that values were similar to those of the control series.

Root dry weight, also shown in Figure 32, was not affected by treatment with either CCC or GA₃ alone or in combination, nor was total dry weight affected by the compounds.

The effect of treatment on length of the hypocotyl, epicotyl and first internode was also examined (Figure 32). GA₃ treatment had no effect upon either the hypocotyl or the epicotyl. CCC treatment reduced the length of both structures, and GA₃ counteracted these effects, as was found for light-grown plants. The first internode was the only part of the dark-grown plants which responded to application of GA₃ alone, but great variation occurred with this treatment.

7- Effects of GA₃ and light on dark-grown plants

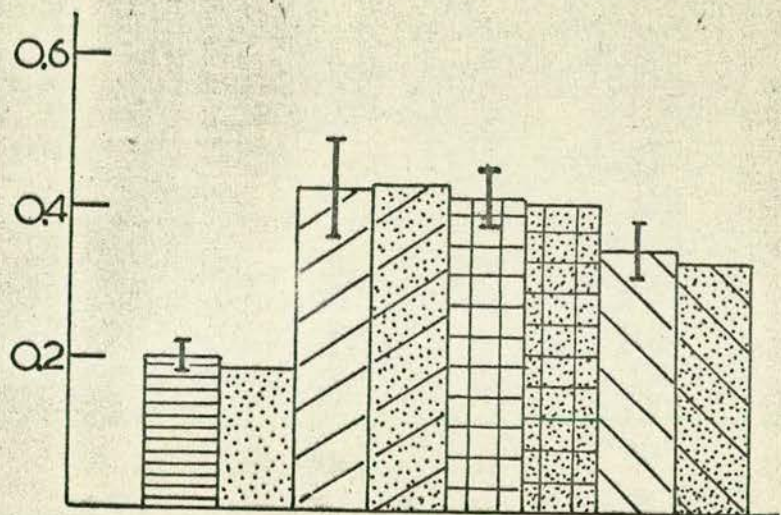
Previous experiments showed that treatment with CCC and light gave similar end results for dark-grown



Figure 33 - Effects of GA₃ and light on primary
leaves of dark-grown plants.



Confidence limits are shown. Replication was ten-
fold.



1st. PAIR OF LEAVES

FRESH WEIGHT



 Control  GA₃

 Light (day 6)  Light (6)+GA₃

 Light (day 7)  Light (7)+GA₃

 Light (day 8)  Light (8)+GA₃

plants, although probably operating by different mechanisms. The light effect appears to involve the phytochrome system with the promotive effect of red light being reversed by far-red radiation. Far-red did not reverse the CCC effects, but GA₃ could counteract the CCC response. The question now arises as to whether GA₃ could modify the effects of light.

Dark-grown plants were treated with 10 μ g of GA₃ on day 7 after planting. Treatments with light consisted of ten minutes white light given to the plants on either day 6, 7 or 8. Thus, in one treatment, light was given before GA₃, in the second, light and GA₃ were given on the same day, and in the third, light was given after GA₃ treatment. Plants were harvested when 12 days old. The fresh weight of the first pair of leaves was measured.

Light applied on day 6, 7 or 8 increased the fresh weight of the first pair of leaves, and this effect was not counteracted by application of GA₃ before, together, or after the light treatment (Figure 33). This then is additional evidence that the light and CCC mechanisms operating on leaves are independent.

8- Summary and general comments

Summarizing the results for dark-grown plants, we have:

1) Plants respond in several ways to CCC given at a concentration of 10^{-2} M.

2) CCC treatment increases fresh weight and dry weight and cell number for the first pair of leaves.

3) When CCC is applied at day 0, changes in the size of the leaves and cotyledons which results from treatment begin to appear at the same time.

4) Effects of CCC are most marked when the compound is applied before day 4.

5) Effects of CCC treatment are similar to effects of white or red light and these effects are additive.

6) CCC effects are not counteracted by far-red radiation.

7) Light effects are counteracted by far-red treatment and involve a red:far-red photomorphogenic reaction.

8) Effects of CCC are counteracted by application of GA₃, whereas

9) Light effects are not counteracted by application of GA₃.

Thus, CCC and light acting upon dark-grown plants give similar end results. But since CCC effects are not reversed by far-red treatment and light effects are not reversed by GA₃ applications and since the light and CCC effects are additive, we must assume that the two stimuli act through two different mechanisms which at a certain stage of the chain of reactions become common, and which lead to an increase in size of the first pair of leaves and at the same time bring about a decrease in the hypocotyl length.

Comparing the results of light and dark-grown

plants we have the following:

Major similarities:

1) In both light and dark-grown plants CCC brings about a decrease in stem length and in stem dry weight.

2) Concentrations of CCC at 10^{-4} and 10^{-6} M are ineffective, except that in light-grown plants there is some effect of both these concentrations on stem length.

Major differences:

1) The dry weight, cell number and area of the first pair of leaves are markedly increased by CCC in dark-grown plants. No such effect on cell number and dry weight is found for light-grown plants, where reduction in area is found.

2) Root dry weight of dark-grown plants is either not affected or is increased by CCC, whereas in light-grown plants the root dry weight is decreased by CCC treatment.

3) Total dry weight is unaffected by CCC treatment in dark-grown plants, but is strongly reduced by treatment in light-grown ones.

4) Application of CCC on day 7 is ineffective for dark-grown plants, but has a marked effect on light-grown plants.

C-Determination of CCC and GA₃ in treated plants

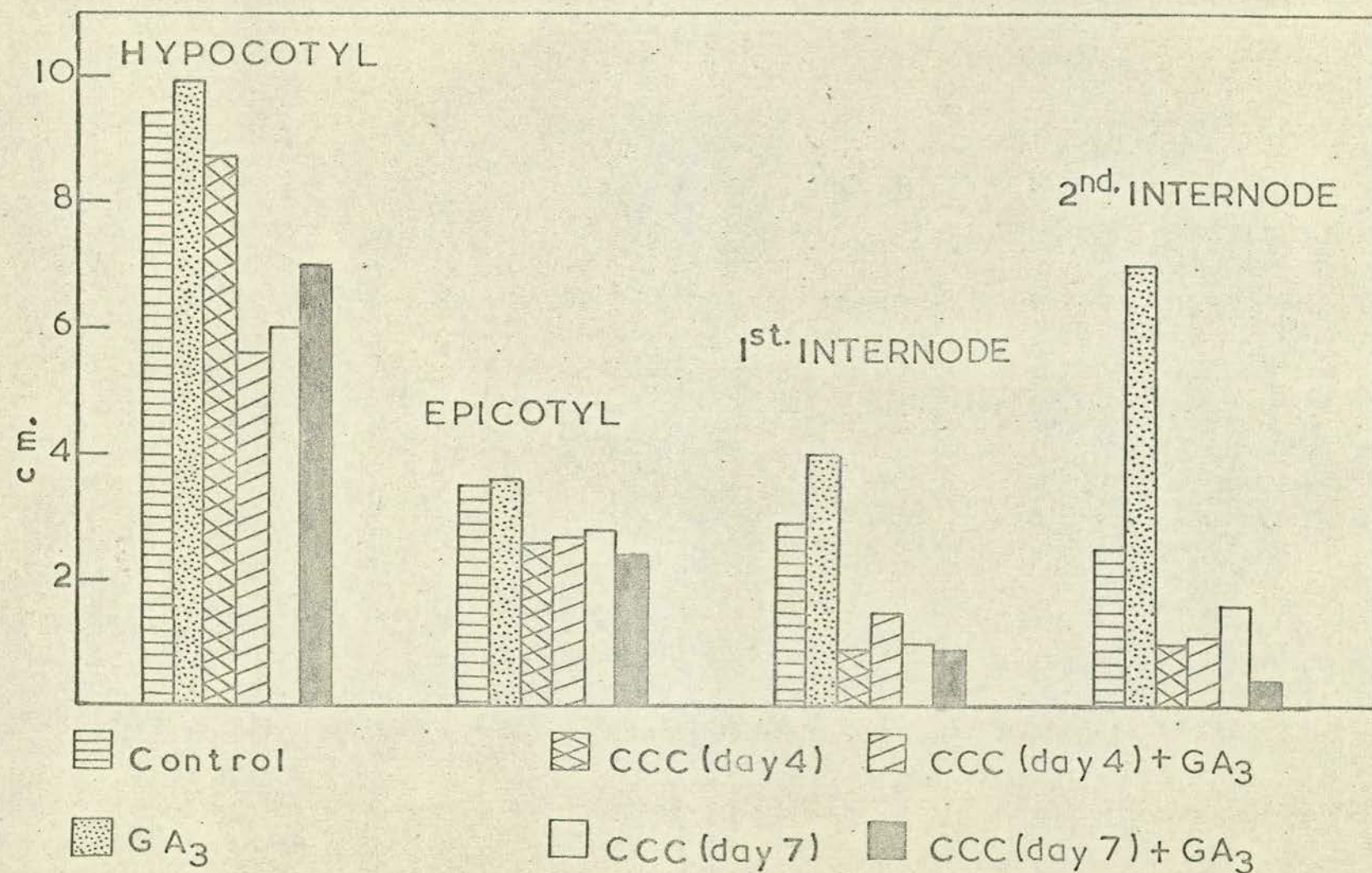
Work described in this section was performed to determine whether CCC can be detected throughout the life of treated plants, and whether the compound is passed into developing seeds and hence into a second generation of plants. At the same time it was felt desirable to examine gibberellin content of plants treated with CCC, especially as there is growing evidence that the growth retardant can inhibit synthesis of gibberellins in a number of species (Kende, Ninnemann and Lang, 1963; Zeevaart, 1966) and since GA₃ counteracts many of the growth effects of CCC. The experiments in this section are of two types; firstly those in which morphological characteristics of plants enable some assessment of CCC and gibberellin content to be made, and secondly biochemical studies in which estimates of gibberellins are made.

1- After effects of CCC and GA₃ and carry-over into seeds

In this experiment plants treated with CCC on day 4 or 7 and GA₃ on day 7 were grown for several weeks for production of seeds. The seeds were collected and planted to study the after-effects of these two compounds on the second generation. Plants grown from the seeds were harvested when 25 days old and length of internodes was determined as well as area and shape of primary and trifoliate leaves.

Figure 34 - After-effects of CCC and GA₃.

Plants were harvested when 25 days old.



Internode length may be considered to be one of the most sensitive tests for presence of CCC or GA₃ in these bean plants. Data in Figure 34 are informative. CCC treatment of the parents led to a marked reduction in internode length in the F₁ generation. That is to say the effects of CCC are carried over from one generation to the next, irrespective of whether CCC treatment was also followed by treatment with GA₃. There is also evidence that plants treated with GA₃ alone produce progeny which have longer internodes than the control series. This carry-over of the effects of GA₃ treatment is very marked in the first two internodes. Plants from parents treated with both CCC and GA₃ show only carry-over effects of CCC.

When leaf area in the F₁ generation was considered, no effects of treatment of the parents were found on either primary or trifoliolate leaves (Table 18).

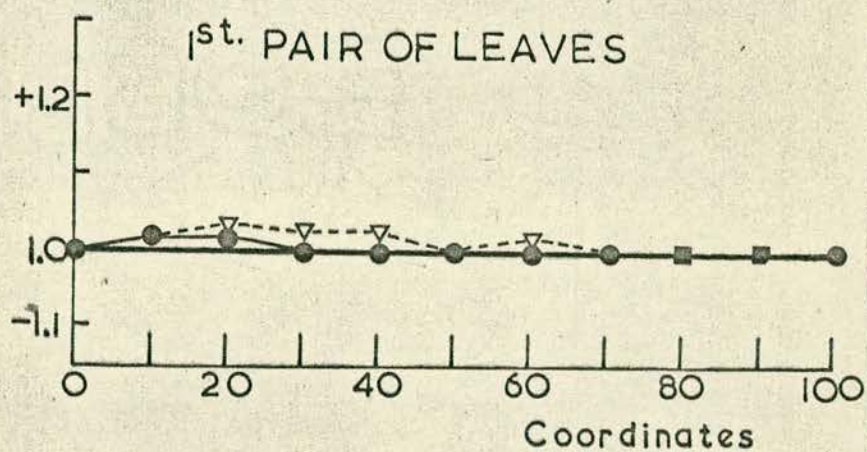
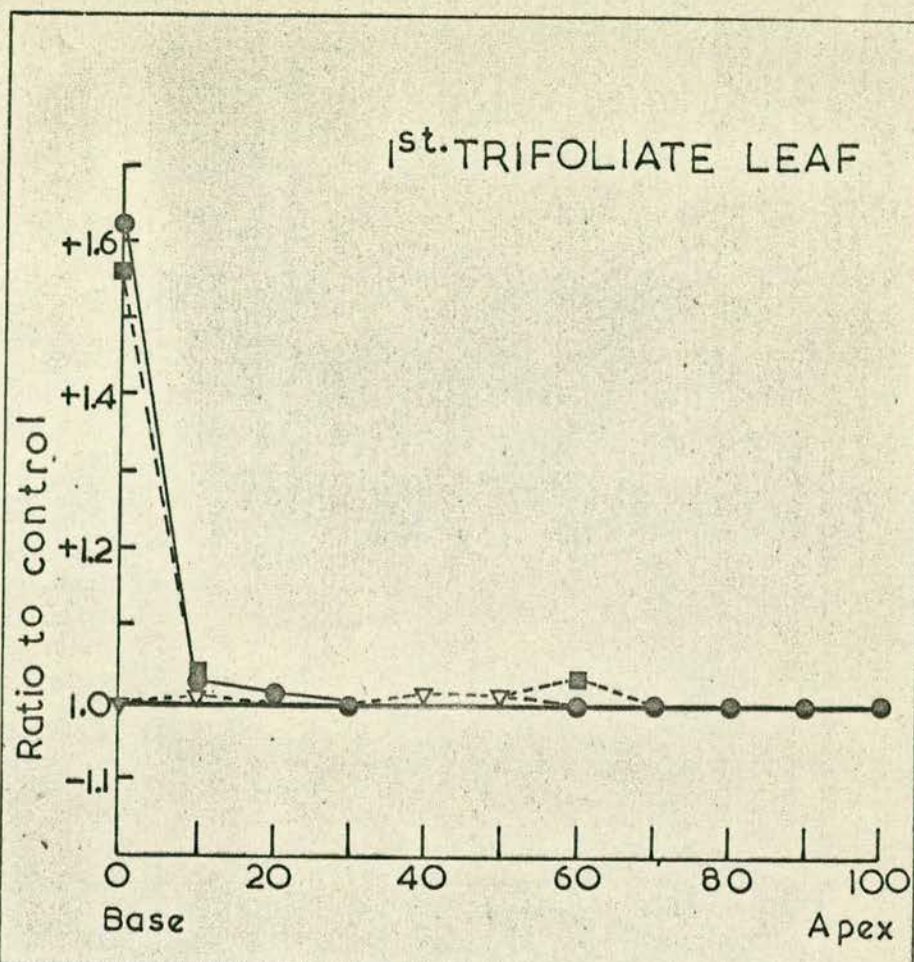
TABLE 18

Leaf area (in cm²) of F₁ generation of plants treated with CCC, with or without GA₃, and of control plants, harvested when 25 days old. Standard errors of mean are given for control series.

	CONTROL	GA ₃	CCC4	CCC4 GA ₃	CCC7	CCC7 GA ₃
One leaf of the 1st pair	91.1±6.4	85.8	107.0	82.5	78.5	73.6
1st trifoliolate leaf	121.7±13.6	146.5	149.5	148.3	123.5	121.1

Figure 35 - After-effects of CCC and GA₃ on leaf
shape of F₁ generation.

Plants harvested when 25 days old.



CCC / C

GA₃ / C

CCCGA₃ / C

Leaf
Shape

Leaf shape measurements are shown in Figure 35. All the progeny showed similar shapes for the primary leaves but treatment of the parents with CCC with or without GA₃ led to the progeny having trifoliate leaves with truncate bases compared with the cuneate base of progeny from control or GA₃ treatments (c.f. Figure 19).

Some important conclusions can be derived from this experiment. Firstly, progeny of plants treated with CCC show the growth reduction and changes in leaf shape, characteristic of CCC treatment. Thus, where GA₃ was applied with CCC, only the effect of CCC is carried over to the F₁ generation, although GA₃ applied by itself does give carry-over effects in the F₁ progeny at least in internode length. Secondly, the lack of effect of CCC on leaf shape of the primary leaves of the parental generation is exactly paralleled by a similar lack of effect in the progeny, even though trifoliate leaves show changes characteristic of CCC treatment. Clearly primary leaves and trifoliate leaves respond differently to CCC and lack of response of primary leaves in the parents may not be solely due to the relative lateness of application of the retardant.

2- The qualitative detection of CCC

The carry-over into the next generation of effects characteristic of CCC could be due to either of two causes. Firstly, CCC could be accumulated within the seeds and thus give rise directly to effects comparable

to those exerted on the parents, or secondly, CCC could cause an indirect effect by modifying morphogenesis in the seeds without actually being accumulated within them, e.g., by modifying the course of, say, gibberellin synthesis. Identification of CCC within the seeds from treated plants would support the first suggestion (without nullifying the second) and attempts were made to extract and identify CCC in such seeds. This part of the investigation was also linked to a systematic study of the presence of CCC in plants of different ages, and in developing fruits.

Plant material of various ages and from various treatments was extracted in methanol and the extracts were applied on paper for chromatography. Results are given in Table 19.

In all plants treated with CCC (with or without GA₃) regardless of age of plant, CCC could be detected, although the compound was never found in immature seeds. However CCC was detected in the wall of the pods bearing these immature seeds. Since in dry seeds CCC could always be detected, even for treatments in which GA₃ was also applied, the retardant must have moved into the seeds late in their development. The data confirm the morphological effects shown previously, and support the first of the alternatives stated above.

A second series of experiments was carried out to check on how long it takes for CCC to enter the seedlings during the process of germination. CCC was applied

at planting day and seeds were removed from the sand, carefully washed to remove CCC from the outside of the testa and extracted at one, six, twelve and twenty-four hours after CCC application. The seeds after six hours had begun to swell, and at twenty-four hours the tip of the radicle had already emerged from the testa.

No CCC spot was found in chromatograms of the seeds after one hour. CCC was detected in treated seeds after six hours and subsequently. Thus, it can be said that CCC applied on day 0 enters the seed very early on in the germination process, at between one and six hours after planting.

From these experiments several points must be made:

- 1) CCC enters the plant soon after application.
- 2) CCC could always be detected throughout the life of the plant.

- 3) Plants treated with CCC and GA_3 which show all the effects of GA_3 counteraction, always showed the presence of CCC when extracted although morphologically resembling control plants. The seeds of these plants always showed the presence of CCC, which explains why in the second generation these plants have characteristics of CCC treatment.

- 4) No CCC could be detected in the immature seeds, and thus early development of the embryo was not directly under the influence of CCC.

TABLE 19

Detection of CCC in plant material. +:CCC spot detected in chromatogram.

Plant material		Treatment	Experiments		
			1	2	3
4 day old plants	light-grown	Control	-	-	
	dark-grown	Control	-	-	
		CCC day 0	+	+	
11 day old plants	light-grown	Control	-	-	
		CCC day 7	+	+	
		GA ₃	-	-	
		CCCGA ₃	+	+	
	dark-grown	Control	-	-	
		CCC day 0	+	+	
Immature seeds	Control	-	-	-	
	CCC day 7	-	-	-	
	GA ₃	-	-		
	CCCGA ₃	-	-		
Fruit wall	Control			-	
	CCC day 7			+	
Mature (dry) seeds	Control	-	-		
	CCC day 7	+	+		
	GA ₃	-	-		
	CCCGA ₃	+	+		

3- Gibberellin content

In considering the gibberellin content of plants in this study there are two problems:

- a) what are the effects of CCC application upon the content of naturally-occurring gibberellins?
- b) what is the fate of gibberellic acid when applied to bean plants?

These two problems have been investigated and are reported separately.

- a) The effect of CCC treatment on naturally-occurring gibberellins.

The naturally-occurring gibberellins of plants treated with CCC were studied at several developmental stages. This was done in a number of separate experiments which for convenience are considered together. Assay was by means of the lettuce hypocotyl test and gibberellin contents are expressed in terms of gibberellic acid equivalents (μg per plant or seed).

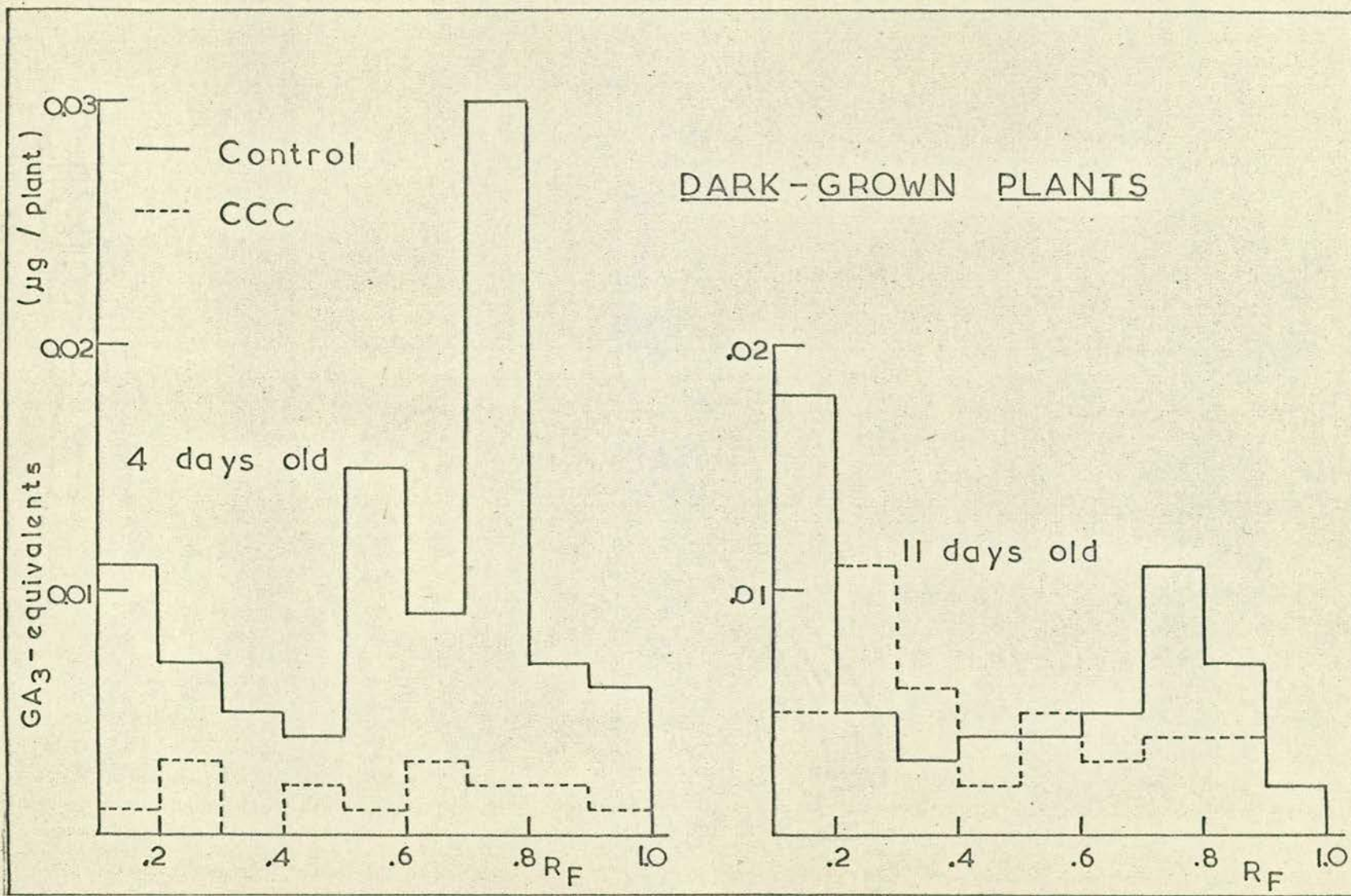
No gibberellins were detected in the dry seeds used in these experiments, and this agrees with observations of Wheeler (1960).

In the first run, seeds were planted in sand in the dark and CCC was applied in the usual way at planting to one set of pots. Control and treated seeds were extracted for gibberellins after 1, 6, 12, and 24 hours and after 4 and 11 days from planting.

At 6 hours the seeds had begun to swell and by 24 hours the tip of the radicle had emerged. No

Figure 36 Gibberellin activity of dark-grown plants.

Gibberellin-like activity for different regions of the chromatogram is shown. As explained in text R_F 0.0 - 0.1 is not included in the figures since no reaction was found in this region.



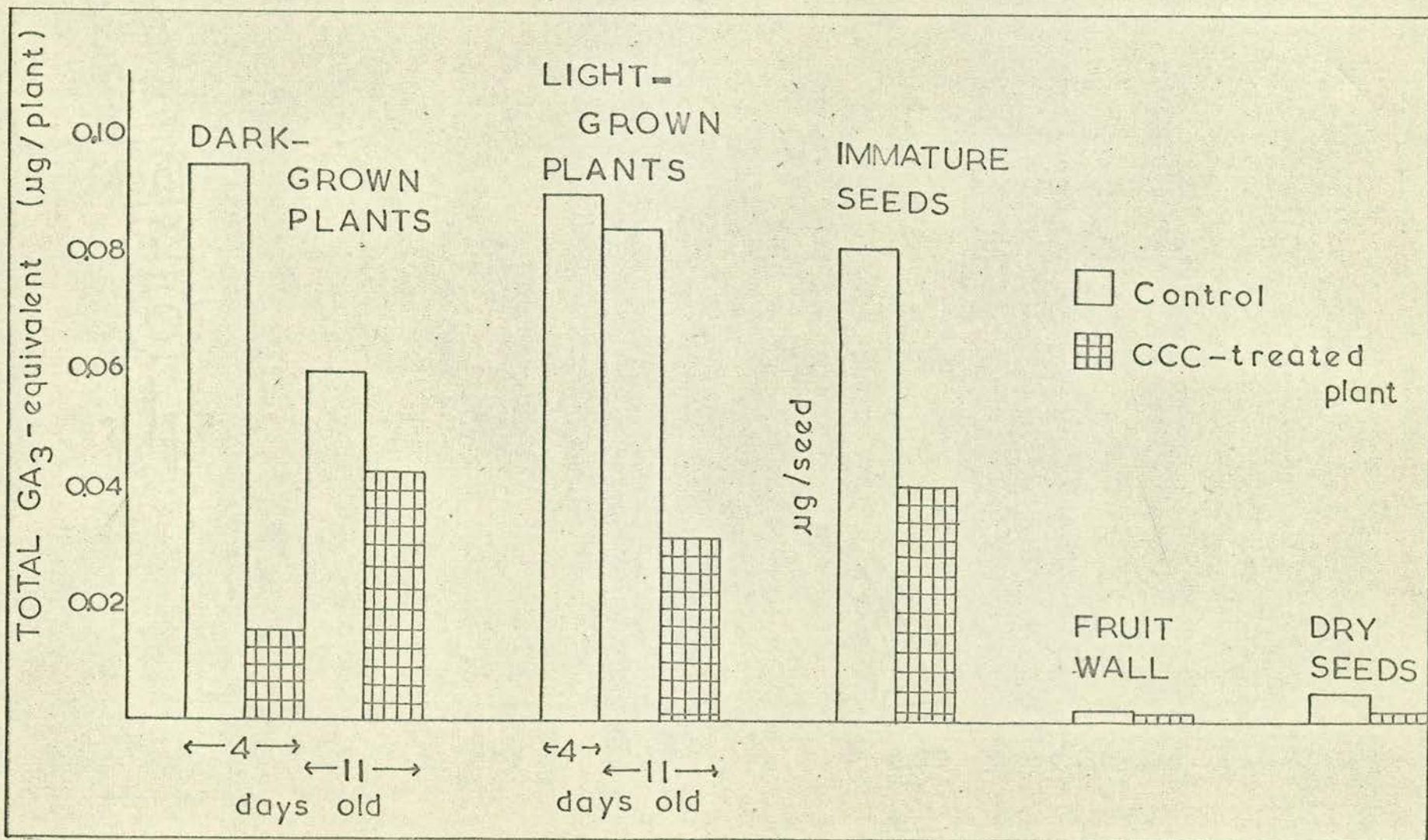
visible difference could be seen between control and CCC-treated seeds and no detectable amount of gibberellin could be found in any of these extracts from either control or CCC-treated seeds. Thus, during the first day of germination no gibberellin could be chemically extracted from the seeds. This could indicate either that no gibberellins were synthesized over this period, or that gibberellins bound within the seed were not liberated in an extractable form.

The results for seedlings 4 days old are completely different. Control seedlings (Figure 36) show three regions of marked growth promotion indicating presence of gibberellins: the first was found in the region of the chromatogram with R_F 0.1 - 0.3, the second was at R_F 0.5, and the third was at R_F 0.8. (In different runs the position of these regions on the chromatograms varied slightly). With all extracts it was impossible to get any growth response of the lettuce seedlings to the region corresponding to the origin of the chromatogram (R_F 0.0 - 0.1), and this region is not shown in the figures. These three regions could not be detected in extracts from plants to which CCC was applied at planting. The total gibberellin content of 4-day old treated plants was very much lower than that of the controls (Figure 37).

For the 11-day old controls and plants treated with CCC, a peak for gibberellins in the region 1 was found, but the second region was not detected.

Figure 37 - Total gibberellin content of plants
treated with CCC.

These data are derived by integrating values obtained
for different regions of the chromatograms.



The total amount is shown in Figure 37. In the 4-day plants the gibberellin level in treated plants was one sixth of that of the control. For the 11-day old plants the amount of gibberellins in the control was much less than that of the 4-day old plants. However, an increase in total gibberellins was noted for the CCC treatment. Thus, it appeared that CCC treatment reduced the level of gibberellins compared with the controls, while in the control plants themselves gibberellin level declined with time, perhaps as the compounds were used in the growth of these plants.

The larger amount of gibberellin in the 11-day old plants treated with CCC compared with the 4-day series is difficult to interpret. One possibility is that gibberellins were lost during the extraction process for day-4 material, but this is difficult to believe because several different extractions were made, and it is unlikely that material was lost in all the samples. Another possibility is that gibberellins continue to be liberated after day 4 even in presence of CCC (see Discussion).

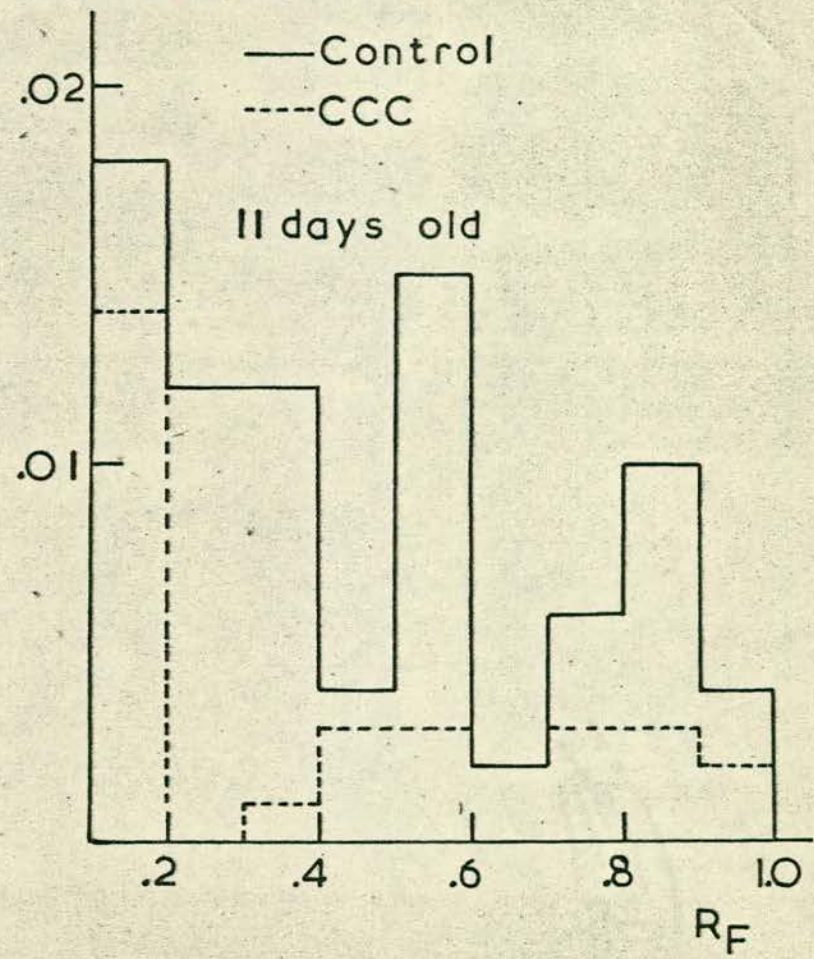
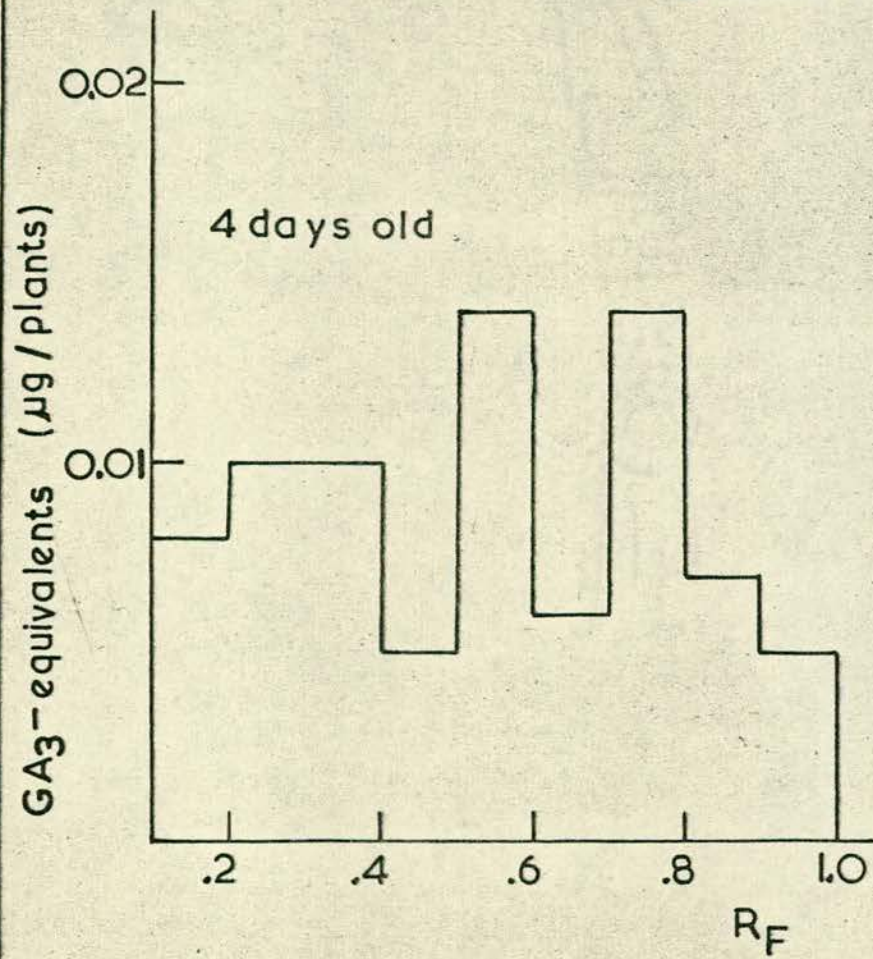
The next experiment involved the use of light-grown plants treated with CCC on day 7. Control material was harvested on day 4 (i.e., before CCC treatment) and harvests were also made of 11-day old plants and of immature and mature seeds.

In extracts of plants 4 days old the three regions of positive response indicating gibberellins were

Figure 38 - Gibberellin activity of light-grown plants.

See also Figure 36.

LIGHT-GROWN PLANTS



also found as for the 4-day old dark-grown control plants. This was to be expected because at this stage in both experiments the seedlings had not emerged above the sand and and the light-grown plants were receiving very little light, if any. In Figure 38, the region 3 is less marked than for the 4-day old dark-grown plants.

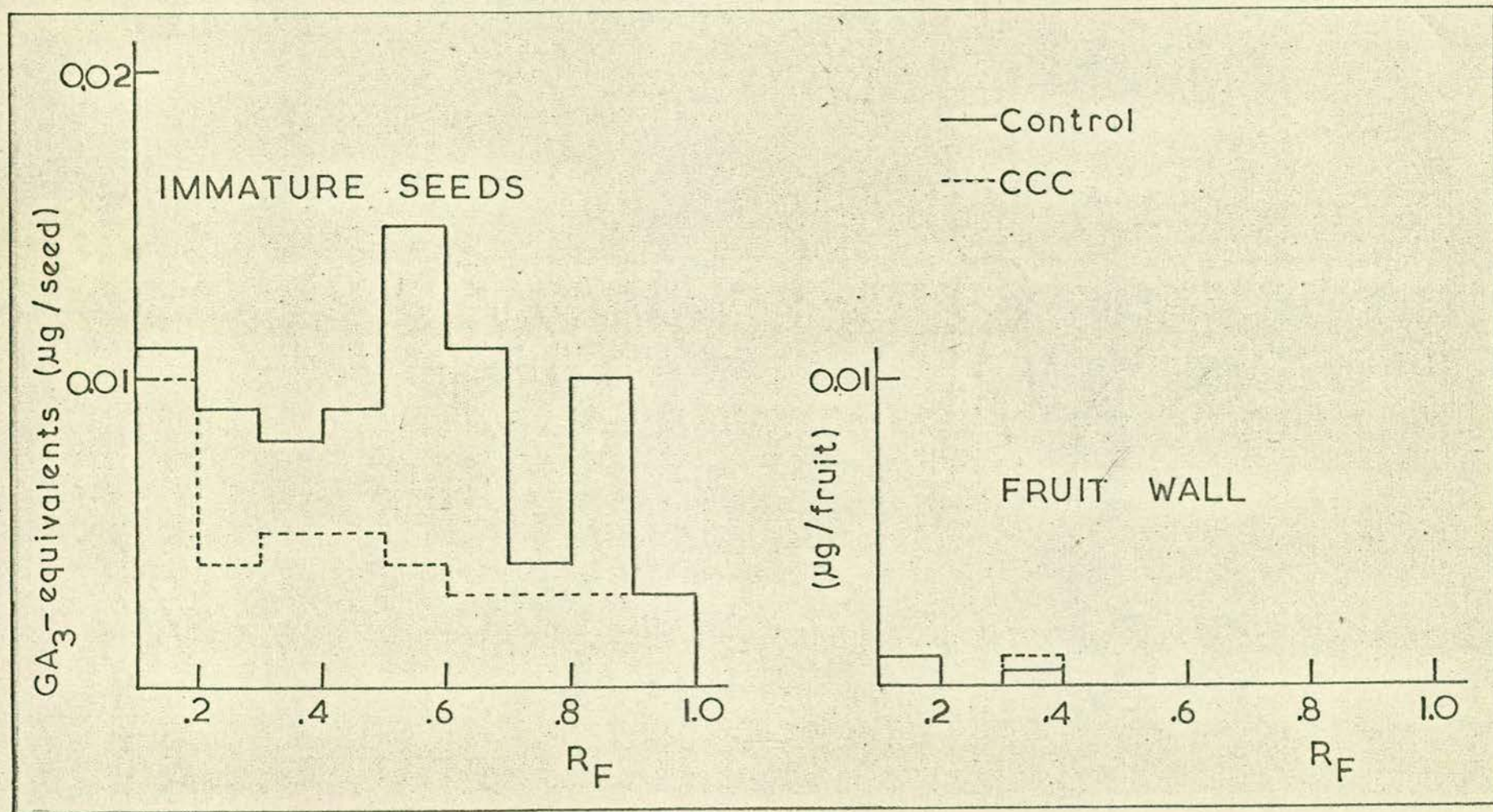
Data for the 11-day old light-grown plants also show (Figure 38) the same kind of response as that for the 4-day old plants; and the distribution of growth activity on the chromatogram is similar for these two groups of plants. For the 11-day old plants treated with CCC the extracts showed little gibberellin activity, except in region 1.

In Figure 37 the total amount of gibberellins for these plants is shown. CCC treatment reduced the amount of the natural gibberellins to less than half of the amount of the control. The control plants 4 and 11 days old show a similar gibberellin content.

Differences between gibberellin levels in light- and dark-grown plants at day 11 are of interest. In the light controls gibberellin level is higher than in the dark series and this could indicate synthesis of gibberellins in the light so that the level remains similar to that of the 4-day old plants. In the dark, synthesis may not occur and gibberellins may be used up in growth, so that the level falls. When CCC is added to the plants, levels of gibberellins in light-grown plants at day 11 is much reduced, perhaps

Figure 39 - Effects of CCC treatment in gibberellin
activity of fruits.

See also Figure 36.



because CCC inhibits synthesis of new gibberellins.

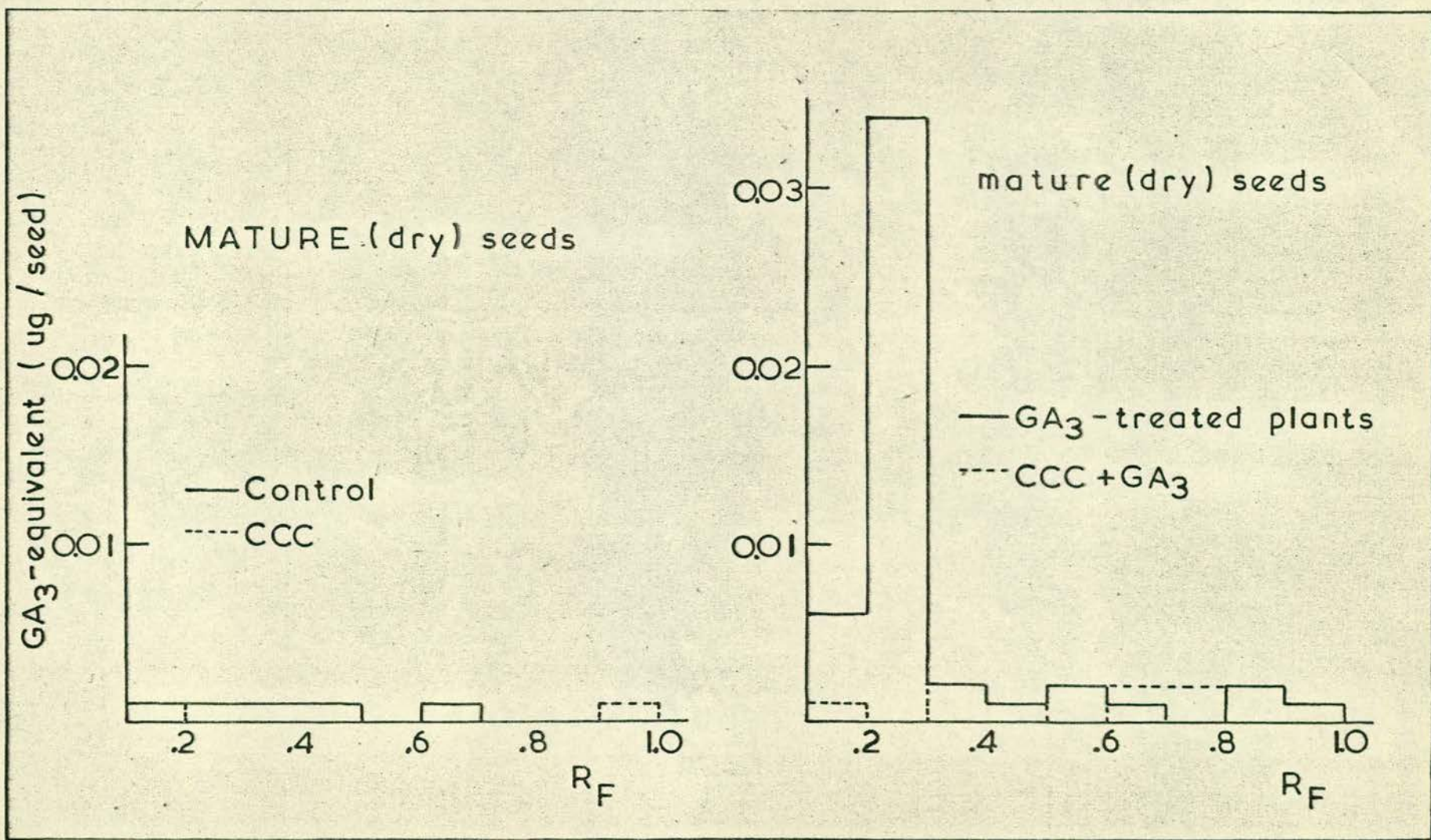
Results for experiments in which immature seeds and the wall of the fruits containing these immature seeds were extracted are shown in Figure 39.

Immature seeds of control plants showed again the three regions of gibberellin activity. CCC treatment decreased the amount of activity. Region 1 and perhaps region 2 can also be detected on the CCC chromatograms. From Figure 37, it can be seen that CCC reduced the total amount of gibberellins. But, since CCC-treated plants produced five seeds, the total amount produced in the seeds would be about 0.2 μ g. Thus, some gibberellin was produced inside these seeds, and CCC cannot have stopped entirely the synthesis of gibberellin in the seeds; the amount is nevertheless smaller than that in control seeds.

Figure 39 shows the data for the wall of the pods of these immature seeds, and no gibberellin was found. Thus, for both treated and control plants, gibberellins are found in the seeds but not in the wall of the fruits. The effects of CCC in this part of the plant are explicable when related to the data of the experiment on CCC content, where it was shown that CCC was detected in the wall of the pods but not inside the seeds. Thus, gibberellins could be synthesized normally in the seeds of CCC-treated plants, because the compound was present only in the wall of the fruits and not in the seeds. The dry seeds contained

Figure 40 - Gibberellin activity of mature seeds.

See also Figure 36.



CCC, and presumably it entered the seeds only after some synthesis of gibberellins had occurred.

In Figures 37 and 40 are shown the data for mature (dry) seeds and no gibberellin activity was found either in control or in plants treated with CCC. Thus, it must be assumed that the gibberellins in the immature seeds are either broken down **or**, as suggested by McComb (1961), are bound to something else in the dry seeds and cannot be extracted as active gibberellins, at least with the extraction method used in this study. When the seeds germinate, then these gibberellins are liberated at certain stages, and growth will occur. This liberation of gibberellins may be inhibited or retarded at least partially when CCC is applied earlier on, since the level in treated 4-day old dark-grown plants is much lower than in the control series (see Discussion).

From this we have:

- 1) CCC appears to inhibit synthesis of natural gibberellins in treated plants and perhaps also retards liberation of gibberellins from germinating seeds.

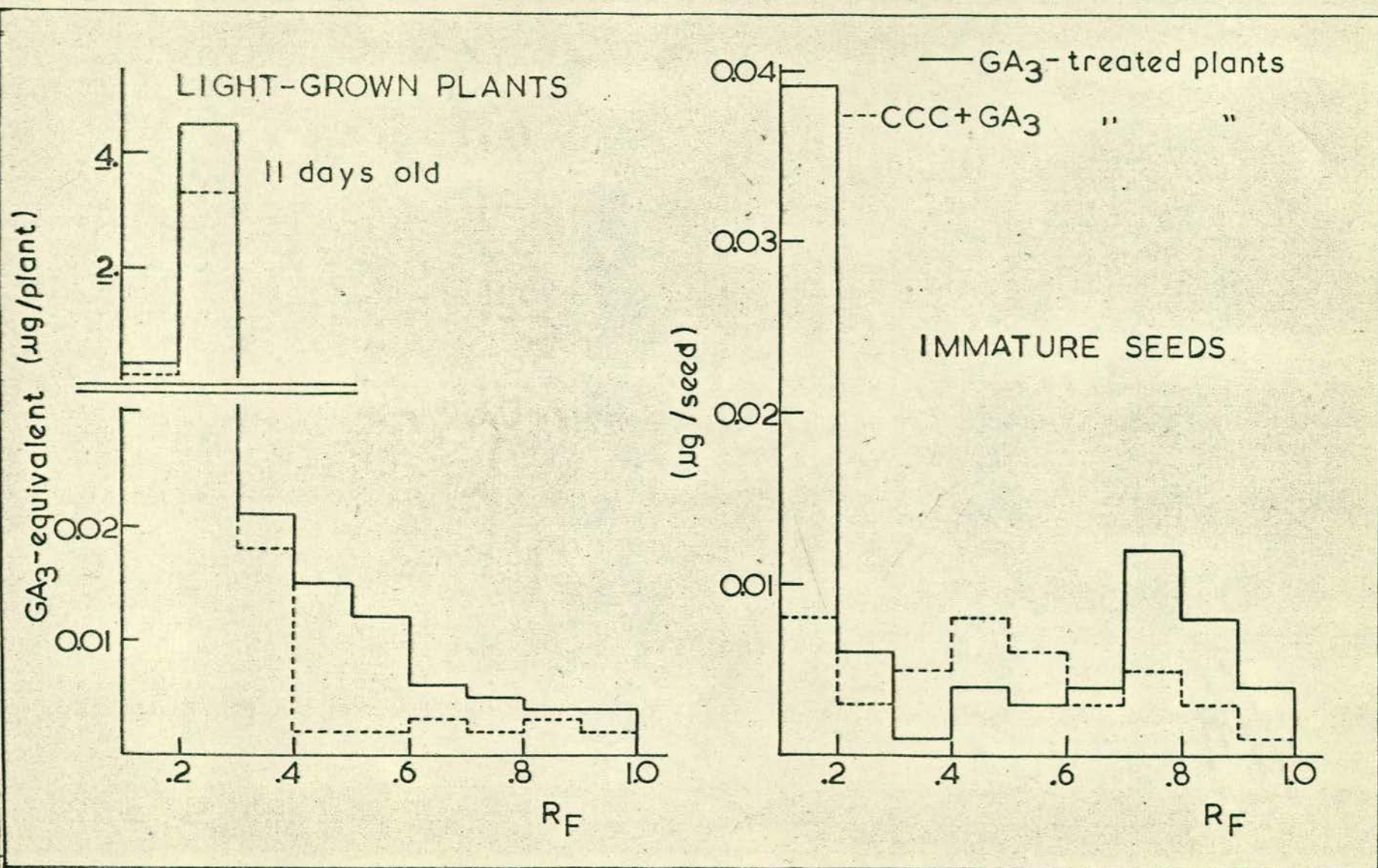
- 2) Synthesis of gibberellins in seeds is only partially inhibited by CCC since the compound does not enter the seeds until late in their development.

- 3) Plants in darkness, it seems, may not synthesize gibberellins.

- 4) Detailed experiments to characterize the gibberellins in the three regions were not done. According to list of gibberellins found in Phaseolus vulgaris

Figure 41 - Gibberellin activity of plants and seeds
treated with GA₃.

See also figure 36. Note the difference in scale
shown in the diagram for light-grown plants.



presented by Brian (1966), and by comparison with several gibberellins run in our solvent system, region 1 could contain gibberellin A₈ and region 2 could contain gibberellin A₅. The identity of region 3 is unknown. These suggestions are tentative only.

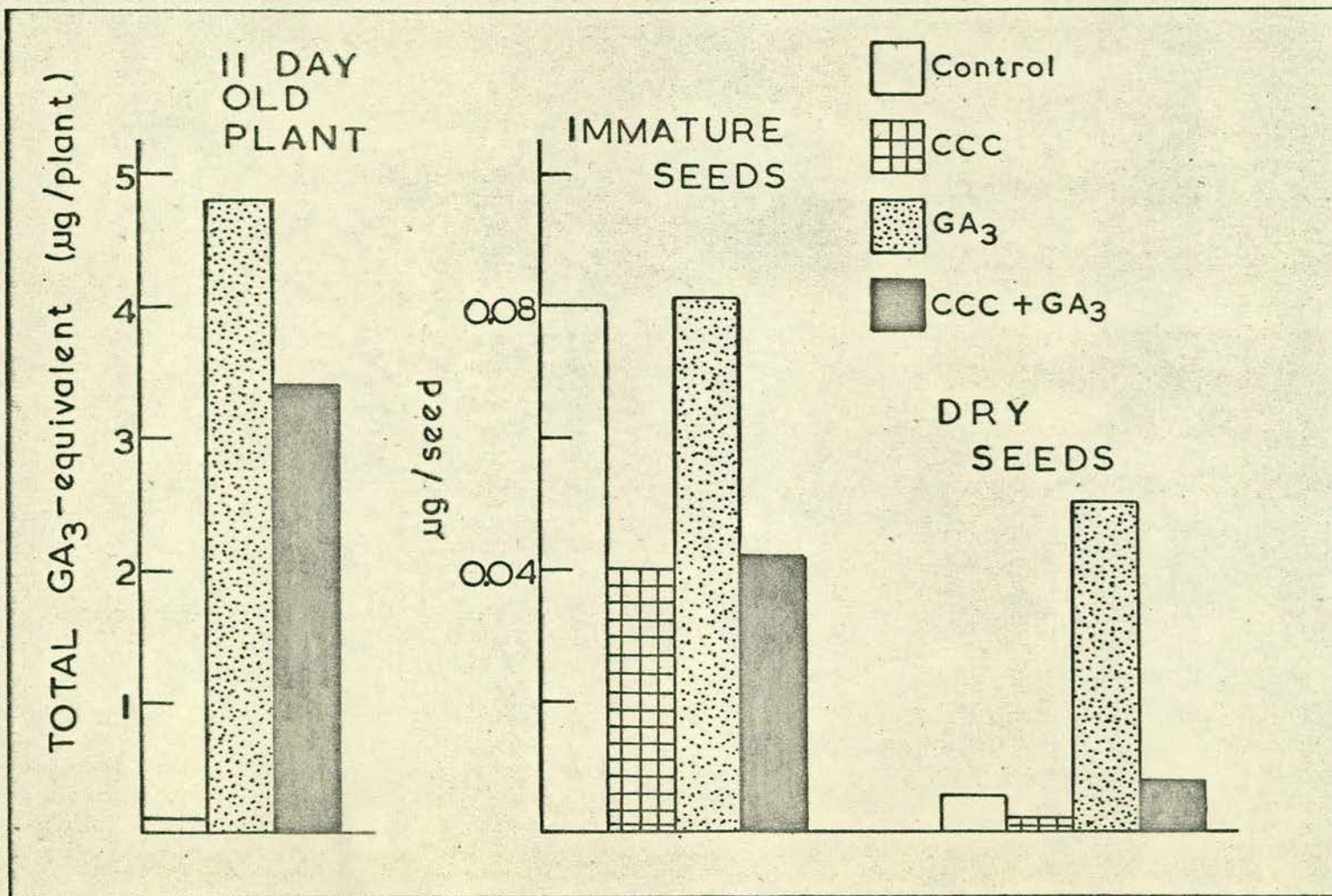
b- The fate of applied gibberellic acid in the plants

Light-grown plants treated on day 7 with gibberellic acid with or without CCC were extracted when 11 days old. Immature and mature seeds were also extracted.

Figure 41 shows the results for 11 day-old plants. A large promoting response appeared in extracts for both the GA₃ and GA₃CCC-treated plants at about R_F 0.3. The chromatogram did not show the other prominent regions shown by the control plants, and it appears that GA₃ application changes the normal balance of gibberellins in the plant. This was also found for the immature seeds (Figure 41) which also show the large promotive effect on growth at the origin of the chromatogram. It was found that GA₃ ran at an R_F of 0.2 - 0.3 in the solvent used in these experiments. On treatment with sulphuric acid, GA₃ shows a characteristic fluorescence in U.V. light (MacMillan and Suter, 1963). The material in the extracts running at the same R_F as GA₃ also showed this fluorescence, and it was concluded that the large peaks represented extracted gibberellic acid. Thus, GA₃ applied to the plants could be extracted and identified. The plants treated with CCC and GA₃ together also showed a great amount of extractable GA₃,

Figure 42 - Gibberellin content of plants and seeds
treated with GA₃, with or without CCC.

See also Figure 37.



but the immature seeds in these plants did not. This could be due to utilisation of GA_3 by the plants for growth (between day 11 and the time of production of the seeds), so that there is less to accumulate in the seeds.

The total amount of gibberellins in plants treated with GA_3 are shown in Figure 42. Small differences were found for the 11-day old plants with or without CCC, but a larger reduction was found in gibberellin in immature seeds of plants treated with both CCC and GA_3 . Also shown in the figure for 11 day-old plants, are the data showing the great amount of GA_3 in relation to the endogenous gibberellins present in untreated controls. In the immature seeds, control and GA_3 -treated plants showed the same amount of gibberellins.

Figure 40 also shows the data for mature (dry) seeds. The chromatograms were similar to those for extracts from the control and CCC-treated plants, and very little gibberellin was detected. But in seeds from GA_3 -treated plants, a large reaction was found at R_F 0.3, and again recognized as being due to GA_3 . Thus, in GA_3 -treated plants, some of the compound was not used and was accumulated in the seeds. Hence, in contrast to natural gibberellins, GA_3 was not bound or transformed in the dry seeds and could be readily extracted.

Thus:

- 1) Light-grown plants treated with CCC and GA_3 show the morphological characteristics of GA_3 -treated plants because although CCC stops the synthesis of

gibberellins, GA₃ substitutes for these gibberellins, and the plants grow as fast or faster than the control plants.

2) In plants treated with both CCC and GA₃ the latter compound is used up, since mature seeds do not yield any GA₃. However, the seeds do accumulate CCC, and this has its effects on morphology of the second generation.

3) F₁ plants from GA₃-treated parents exhibit certain aspects of GA₃-treated plants because this chemical is accumulated in the seeds.

4) Dark-grown plants do not normally react strongly to GA₃. However when applied on day 7 the compound counteracts the effects of CCC applied on day 0, because the gibberellin level is kept at a sub-optimal level by CCC treatment although the plants are still at a developmental stage at which growth could occur if gibberellins are provided.

5) The after-effects: the after-effects of CCC comprise two features, (a) the actual carry-over effects of CCC which are reflected in a low gibberellin content in seeds, and (b) the carry-over of CCC itself when it is passed on to the next generation. In the case of GA₃ treatment, the chemical itself is accumulated in the seed, and because of this, progeny shows characteristics of GA₃ treatment.

V- DISCUSSION

For convenience the results are considered under two main headings; firstly, the effects of CCC on growth and general morphology of the plants, and secondly, the interrelation between CCC and gibberellins and a consideration of the mode of action of CCC.

A- The effects of CCC on morphology

Results show that the growth of Phaseolus vulgaris is markedly affected by application of CCC and that most of the effects of CCC could be counteracted by application of GA₃, although GA₃ applied alone affected only the growth of the stems, the total plant dry weight, and the rate of unfolding and shape of the trifoliate leaves. Reports in the literature show that CCC usually reduces plant growth when measured either as stem length or as total dry weight or total leaf area (Cathey and Stuart, 1961), and light-grown plants of Phaseolus react in this way to CCC, although when plants are maintained in complete darkness rather different results are found.

Some of the most intriguing results in this thesis are those relating to growth of the primary leaves which reacted differently to CCC depending upon whether plants were grown in light or darkness.

For plants grown in light, leaf area was reduced (c.f. Humphries and French, 1965) as was the fresh weight and length of the leaves; leaf dry weight and cell number

were not affected by CCC treatment. Treated leaves were thicker than controls, and the chlorophyll content was greater. It is of interest that not only is the chlorophyll content increased, but chlorophyll synthesis continues over a long period.

Some authors (e.g. Dall'Olio, 1964a) found that CCC treatment increased leaf dry weight but no effect was found on dry weight of primary leaves of treated Phaseolus plants. However since area was reduced, dry weight per unit area was greater and this is correlated with the increase in leaf thickness. The evidence for the trifoliate leaves from the shading experiments suggests strongly that the decrease in the dry weight of these leaves was caused by shading, and is thus a secondary effect of CCC rather than a direct effect of treatment.

When cell number was considered, CCC treatment caused an increase for dark-grown primary leaves to a level comparable to that for the light-grown control, but there was no effect of the compound on cell number in light-grown primary leaves. In the first and second trifoliate leaves which only unfolded in light, CCC treatment led to a smaller cell number, which however was slightly increased when shading from the first pair of leaves was reduced. But some shading still occurred from the primary leaves basis and the third trifoliate leaf, whose cell number was not affected by CCC, and it could be that the decrease in cell number in the first

two trifoliolate leaves is due more to the effect of shading than to an action of CCC itself. It has been reported that trifoliolate leaf cell number is much reduced under low light intensities (Dale, 1965), such as could arise from shading by the other leaves. The lack of effect on the third trifoliolate leaf cannot be due to absence of CCC since it was detected throughout the life of the plant. Thus, the possibility that CCC had disappeared from the plant by the time that the third leaf was formed has to be dismissed. Zeevaart (1964) showed that CCC treatments inhibit cell division in internodes of Pharbitis nil, and CCC treatment also resulted in inhibition of cell division in callus tissue derived from carrot, Pelargonium and Chrysanthemum stems (Sachs and Wohlers, 1964). The absence of effects on leaf tissue suggests that CCC does not act as a general mitotic inhibitor for all plant tissues. It could be that treatment reduced divisions in certain planes of the leaf (thus leading to differences in shape) and that this effect on number is compensated for by more divisions elsewhere. Such an effect on cell numbers in stems of Chrysanthemum was reported by Sachs and Kofranek (1963). The effect of CCC treatment on cell number of the primary leaves of dark-grown plants will be discussed in more detail elsewhere.

Shape of the trifoliolate leaves was changed by CCC treatment but there was no effect on shape of the primary leaves. This fact can be explained if it is

assumed that shape is determined early in the development of these leaves and that hence only very early application of CCC could change the shape. In the case of the trifoliolate leaves such application early in development was made and had effect. But the primary leaves are already well developed in the embryo (Dale, 1964b) and CCC even given at planting was applied too late to have an effect. One has still to explain lack of effect on the shape of those leaves in the F_1 generation for CCC-treated plants. Analyses showed that CCC was not present in the seeds during early development of the embryo, and it seems likely that when CCC entered the seeds the primary leaves were already well formed and again incapable of responding to the chemical by changes in shape.

The large effects of CCC treatment in decreasing the stem growth in Phaseolus both in dark and light-grown plants are in full agreement with results observed by most authors working with this compound.

Root dry weight on light-grown plants was reduced by CCC treatment. Few data are available in the literature about effects on roots, but Dall'Olio (1964a) found that development of roots of Datura stramonium was reduced by CCC. Sturm and Jung (1964a) found that CCC increased root growth in wheat, and Tognoni, Halevy and Wittwer (1967) found increased growth of adventitious roots on the hypocotyls of beans grown in water culture in presence of CCC. For dark-grown beans, response to CCC was dependent on the day of application and could

lead to increased growth of the roots; a decrease in root dry weight was never found. From these results it is clear that CCC treatment has varied effects on root growth, depending on whether the treated plants develop in light or darkness.

A major effect of CCC treatment is to increase the period over which growth occurs compared with controls and this is seen from changes in leaf length, area, etc.. At the same time the rate of growth is often reduced and the extension of the growth period is not sufficient to compensate for this and to allow final size to reach the control level. An exception to this situation is seen for chlorophyll where CCC treatment increases both period over which the pigments are produced and also the rate of production.

B- The mode of action of CCC

Workers using CCC have in the main interpreted their results on two main theories. These are either, that CCC acts by reducing the levels of diffusible (and hence of physiologically active) auxin in treated plants so that growth and in particular stem growth, is reduced, or that CCC interferes in the gibberellin metabolism of treated plants. Our results can be discussed under both these possibilities.

1- CCC and IAA

It could be that CCC acts in Phaseolus by reducing the level of diffusible auxin. The effectiveness of CCC in reducing stem growth is in keeping with this as is the greater growth of roots in dark-grown plants, since if CCC was decreasing the auxin level, this level should reach a point at which increasing root growth would occur. However, application of IAA had no effect upon CCC-treated plants grown in light (Figure 4) or dark (unpublished), whereas the effects of CCC were counteracted by application of GA₃. These points in themselves do not rule out an effect of CCC on IAA levels, for it is a common finding that at physiological concentrations IAA seldom affects stem growth of intact plants although it may have massive effects on isolated stem portions. Also many authors believe that GA₃ increases the level of the diffusible auxin (see Brian, 1966) while other writers consider that GA₃ reduces IAA-oxidase and peroxidase activity thus allowing an increase in auxin levels, while CCC is claimed to do the opposite (Halevy, 1963). Kuraishi and Muir (1963) suggest that the mode of action of CCC is through a lowering of auxin levels in the plant, and that the antagonistic effect of CCC is on IAA metabolism but not on that of GA₃. Knypl (1964) also agrees with this idea and goes so far as to say that CCC should not be regarded as an anti-gibberellin. But evidence supporting these ideas is not at all clear since as Brian (1966) states,

assays for endogenous IAA were either not made, or if they were made, it is uncertain that IAA was the only growth substance determined. In other cases there was failure to detect any decrease in peroxidase or IAA-oxidase activity after application of GA₃. We have performed experiments (unpublished) in which peroxidase activity was determined following the methods of McCune and Galston (1959) and McCune (1961) and we found an increase in activity in the hypocotyl when CCC was applied. However plants treated with GA₃ also showed an increase in the peroxidase activity. Even if the increase in peroxidase activity is a real one, it does not necessarily correlate with IAA levels, since the enzyme is far from being specific. One may conclude that it seems unlikely that CCC has a direct effect on IAA and hence cannot be regarded as an antiauxin as Knypl (1964) has claimed. It is not denied that CCC may indirectly affect IAA levels although we have no data to support this possibility.

2- CCC and gibberellins

In contrast to the lack of effect of IAA, GA₃ counteracted almost all the effects of CCC. Thus: for primary leaves of light-grown plants, CCC treatment reduced area, length and fresh weight, and these reductions were counteracted by GA₃; CCC treatment increased the thickness of these leaves and GA₃ counteracted the effect; chlorophyll content was increased by CCC treatment which also increased the period over which chloro-

phyll was formed, and both these effects were counteracted by GA₃ application; unfolding of trifoliolate leaves was delayed and their rate of growth was reduced in length by CCC application and GA₃ counteracted these effects; on dark-grown plants, CCC treatment increased cell number, fresh and dry weight of the primary leaves, and again GA₃ counteracted all these effects, even though GA₃ application alone had little effect on dark-grown plants. These effects on leaves were paralleled by effects on stems. The massive reduction in internode length in light and dark-grown plants caused by CCC treatment was counteracted by GA₃.

Other reports of GA₃ counteraction of the effects of CCC treatment relate principally to effects on stems e.g. for Pharbitis nil (Zeevaart, 1964), for pears (Modlibowska, 1964), for Chrysanthemum (Sachs and Kofranek, 1963) and for sunflower (Jones and Phillips, 1967).

Our data and the facts in the literature strongly suggest that a close relation must exist between CCC and GA₃ and the idea of true antagonism between these two substances cannot be dismissed easily.

One can envisage the relationship between the two compounds to be of two possible types; firstly, a competitive interaction whereby CCC binds on to metabolic sites thus preventing reactions involved in growth from occurring, while presence of GA₃ allows the reactions to occur, or secondly, that CCC prevents synthesis or liberation of gibberellins which can be

substituted for by GA₃.

The second possibility seems much more reasonable in the light of our experimental results, and the hypothesis that will now be discussed is that the effects of CCC result from inhibition of synthesis of endogenous gibberellins, and from the reduction in availability of such gibberellins as are present in Phaseolus seeds. A corollary is that GA₃ can substitute for endogenous gibberellins. With this hypothesis the large differences in effective concentrations of CCC (10⁻²M) and GA₃ (10-1000ppm) can be interpreted if one postulates that a great amount of CCC is needed to stop the chain of reactions leading to gibberellin formation, while only a small amount of GA₃ is needed to substitute for the endogenous gibberellins. On this hypothesis no relation exists between the effective concentrations of CCC and GA₃, because they are not acting against each other.

For convenience the hypothesis will be discussed under three main headings.

a- Does CCC inhibit synthesis of gibberellins ?

Published evidence that CCC is associated with inhibition of gibberellin synthesis has already been reviewed in the Introduction to this thesis. Basically the evidence is either that CCC directly inhibits synthesis, as has been shown for Fusarium (Kende, Ninnemann and Lang, 1963; Ninnemann, Zeevaart, Kende and Lang, 1964; Harada and Lang, 1965) and Pharbitis nil (Zeevaart, 1966), or that CCC treatment leads to lower

levels of extractable gibberellins as in potatoes (Humphries and Wheeler, 1962) and in sunflower (Jones and Phillips, 1967).

Our experiments show that CCC treatment inhibits the synthesis of gibberellin in light-grown plants markedly, since when 11 days old, treated plants showed much lower levels of gibberellin than the control plants. The light-grown control plants at 4 and 11 days show the same content of gibberellins. Thus, if between day 4 and day 11 some gibberellin was used up, synthesis must have occurred to maintain the gibberellin at the same level. On the other hand, the level of gibberellin in dark-grown plants fell from day 4 until day 11, and if the gibberellin was used up it was not replaced by newly synthesized gibberellin, suggesting either that synthesis of new gibberellin does not occur in Phaseolus kept in darkness or that synthesis occurs at a very low level. If the former suggestion is the right one, when CCC is applied, it will inhibit synthesis of gibberellins only in light-grown plants (Figure 37), and the effect of CCC on dark-grown plants must be explained in another way. If on the other hand some synthesis does occur in the dark, since CCC has no effect when applied on day 7, this is evidence that synthesis does not occur after that time.

CCC treatment reduced the level of gibberellin in immature seeds below that found for control seeds. On a per seed basis, treated seeds contained almost

half the gibberellin content of control seeds, and thus a considerable synthesis of gibberellins must occur in both sets of seeds. The yield of gibberellins in the immature seeds was large, and in the control, levels of 320 μ g of GA₃ equivalent per kilo of fresh weight of seeds were found (these values are higher than the figure of 250 μ g of GA₃ equivalent per kilo fresh weight quoted by Radley (1958) for Phaseolus).

The experiments on detection of CCC showed that the compound remained present throughout the life of the plant, and was transferred to the seeds. However it was also shown that for a long period CCC remained in the wall of the fruits and only entered the seeds late in their development. Thus, in immature seeds, the action of CCC may not be exerted since it is not present in the embryo or cotyledons, and gibberellin synthesis can occur at least for some period. This escape from the inhibiting effects of CCC can explain the comparatively high level of gibberellins in young seeds of treated plants. As stated, the results showed that the amount of gibberellin in these seeds is lower than in the control and it could be that the CCC present in the wall of the pods inhibited the synthesis of gibberellins in the testa of the immature seeds. (According to Radley (1958) the testa of the seeds of Phaseolus has a content in gibberellin similar to that of the cotyledons). Thus, the evidence is that where CCC is present, levels of gibberellins are low and that where CCC is absent, as in the

embryo and cotyledons of immature seeds, gibberellin content is high. Correlations of this type do not unequivocally show that CCC inhibits gibberellin synthesis, but taken with data in the literature must be very suggestive.

b- Does CCC affect destruction of gibberellin or liberation of bound gibberellin ?

We have no clear data to prove that CCC does not influence destruction of gibberellins, but the fact that, in dark-grown plants treated with CCC, gibberellin level rises between days 4 and 11 suggests that unless synthesis outpaces destruction, which from the preceding section seems unlikely, destruction does not occur. No destruction of gibberellins in presence of CCC occurred in cultures of Fusarium (Ninnemann, Zeevaart, Kende and Lang, 1964). If destruction of gibberellins is unlikely, what are the effects of CCC treatment on liberation of bound gibberellin ?

As already stated gibberellins were detected in the immature seeds, but could not be detected on maturation. It has been suggested that, during maturation, gibberellins become bound to something else, like protein, and cannot be easily extracted (McComb, 1961; Brian, 1966). If this is so then the appearance of gibberellins during germination could be due to liberation from the bound form, as well as to possible synthesis of new gibberellin. In our experiments, free gibberellins could not be extracted during the first 24 hours of germination but by day 4 a large amount of extractable

gibberellins was found for control plants and a much smaller amount for treated plants. In darkness, over the next seven days gibberellin level in control plants fell, but increased in treated plants. The decline in level of gibberellins in control plants indicates either breakdown or utilisation, but does not indicate whether dark synthesis occurs. The fact that CCC has no effect when applied to dark-grown plants seven days old or older, in spite of the fact that such plants are still growing and can respond to a short irradiation with light, is further evidence that synthesis of gibberellins does not occur in the dark. For treated plants, if synthesis is prevented, the rise in gibberellins between days 4 and 11 must come either from continued liberation from a bound form, or from a reduction in the rate of gibberellin breakdown to a lower level than in control plants. The fact that a rise occurs, as opposed to the level remaining constant, suggests that the first alternative is the correct one and that CCC treatment reduces the rate of liberation of bound gibberellin.

Miss D. Murray (1967) has shown that CCC is only effective on dark-grown plants if the cotyledons are present, and in this work it is shown that CCC retards the loss in weight of the cotyledons during germination. This involvement of cotyledons in the response to CCC as well as the slower loss in cotyledon weight for treated plants could be correlated with a reduced rate of liberation of gibberellin from the

cotyledons , which are known to contain extractable gibberellins (Wheeler, 1960). It has been shown from our experiments that CCC enters germinating seeds soon after application and has the same effect on dark-grown plants whether it is applied at planting or at day 3, but is less effective after day 4, and completely ineffective after day 7. Since no differences exist for treatments on day 0 or day 3, it is clear that CCC exerts its effect on some process occurring at or before day 4. It is suggested that this process is the liberation of gibberellins from the cotyledons. We know that no liberation occurs during the first 24 hours of germination. Thus, the initial phases of the liberation should occur between days 1 and 4 from planting. Wheeler (1960) also shows that the content of the growth substance in the cotyledons of Phaseolus reaches a maximum value between day 2 and day 7. Since CCC still has a slight effect on dark-grown plants when applied on day 6, if it is accepted that in dark-grown plants synthesis of gibberellins does not occur, then liberation of bound gibberellins goes on up until day 6.

To summarize the situation, it seems that until day 4 both dark and light-grown plants are in rather the same situation, and for both, CCC treatment results in a reduction in the liberation of gibberellins. After about day 5, plants emerge above sand and the situation changes. For dark-grown plants liberation of gibberellin from the cotyledons continues and CCC can act if

there is still some gibberellin to be liberated. In the light-grown plants the liberation of gibberellins also continues, but synthesis of new gibberellins begins to occur. Thus, in light-grown plants CCC can act in two ways; retarding the liberation of gibberellins from the seeds (during early phases of the development) and also inhibiting the synthesis of new gibberellin throughout the life of the plant. Because of this, CCC can be applied at a late stage to light-grown plants and still be effective through the inhibition of gibberellin synthesis.

c- The effect of GA₃ in substituting for endogenous gibberellins.

The experiments in which GA₃ was given to plants and the gibberellin content assayed show a number of interesting points.

GA₃ could always be extracted from plants treated only with GA₃ and even mature seeds from such plants yielded the compound, showing that it was not stored in the seeds in a bound form as were the naturally-occurring gibberellins. For plants treated with CCC and GA₃ together the situation is different. The 11 day old plants show a large peak for GA₃ but this is not present for the immature seeds. Thus GA₃ is accumulated in the immature seeds in absence of CCC but not in its presence. This could be explained if GA₃ is being utilized in those plants treated with CCC, so that less of the compound is available for accumulation in the seeds. It must be remembered that plants treated with both compounds show a slightly faster growth rate than that of the control

plants. It is suggested that when CCC treatment stops synthesis of natural gibberellins, GA₃ can substitute for these compounds so that the counteraction of CCC at the morphological level is the result of GA₃ and not of resumed synthesis of endogenous gibberellins.

In view of the fact that GA₃ may substitute for endogenous gibberellins the word 'counteract' has been used rather than 'reverse', to describe the effect of GA₃ on CCC-treated plants. This seems more appropriate since GA₃ does not reverse the effects of CCC, and allows gibberellin synthesis to occur, but only counteracts the effects of lack of natural gibberellins. What other workers have termed a reversal by GA₃ of CCC action on morphological grounds may well be counteraction in the sense that we define it.

At this point it is relevant to comment on the suggestion by Lockhart (1962) that CCC and GA₃ interact in determining stem length in Phaseolus. On Lockhart's interpretation it is suggested that the two compounds influence similar metabolic activities, but in an opposite manner. Such an interpretation is not in agreement with our suggestions that CCC acts by suppressing synthesis and liberation of endogenous gibberellins, and that GA₃ only acts in the place of these natural gibberellins.

3- Explanation of observed effects

It is now possible to discuss the observed effects of CCC and GA₃ in terms of the hypothesis ad-

vanced above.

The reduction in growth is seen not to be due to a direct effect of CCC, but to an effect of the compound on gibberellic metabolism, leading to low levels of gibberellins which result in a reduced growth rate. This interpretation is similar to that of Baldev, Lang and Agatep (1965) who postulated that in pea plants treated with AMO-1618 the lowered gibberellin level is the cause and not the consequence of the growth retardation.

An explanation for the different effects of CCC applied on day 4 and day 7 has already been discussed for dark-grown plants. For light-grown plants the results always show that CCC was more effective when applied at day 4 compared with application on day 7. This can be explained, because in the case of CCC applied to plants up to 4 days old, gibberellin liberation was uniformly affected but CCC stopped the synthesis of gibberellins as occurs on emergence into light. Hence plants treated when 7-day old were able to synthesize gibberellins up to day 7 and thus had more gibberellin than the plants treated on day 4.

An increase in the period over which growth occurs has been shown for dark- and light-grown plants treated with CCC. If some of the early processes of growth are regulated by the rate of liberation of bound gibberellins and CCC retards this liberation, then an increase in the period over which liberation occurs could well result in the growth processes continuing

over a longer period of time but at a lower rate.

The observation that cotyledon weight was conserved following CCC treatment is relevant here. Paleg (1960) showed that GA_3 increases amylase activity in barley endosperm. It could well be, and we intend to investigate this, that amylase activity in Phaseolus cotyledons is induced by gibberellins. The action of CCC in inhibiting gibberellin liberation could thus have an effect on amylase induction and starch breakdown, so that cotyledon weight declines more slowly than for controls. The slow liberation of gibberellin will lead to a slow starch breakdown to give carbon skeletons at a reduced rate but over a longer period. The retardation of liberation of bound gibberellins could also be involved in the conservation of cotyledons weight. McComb (1961) showed in Phaseolus seeds that hydrolysis with ficin increased the gibberellin activity, and concluded that the gibberellins were bound to protein. When liberation occurs the protein could suffer metabolic changes and be transferred from the cotyledons. If the liberation of gibberellin is retarded then the breakdown of the protein may also be retarded. Thus, the conservation of weight of the cotyledons could be due to either slow starch breakdown, or slow protein breakdown, or possibly both processes.

The effects of CCC treatment on the primary leaves needs more detailed consideration.

The basic facts are that for light-grown plants

CCC did not affect the cell number, whereas for dark-grown plants treatment with CCC increased cell number to a level similar to that for untreated plants grown in light. It was also shown that the effects of light on cell number were additive. Also the effects of CCC treatment were counteracted by GA₃.

It has been shown (Murray, 1967) that in dark-grown plants, cell number of primary leaves increases from a value of about half a million to about 20 millions per leaf, over the period until day 8, after which the cell number does not increase. This increase in cell number occurs irrespective of treatment with CCC or exposure to light. For plants emerging into light on about day 5-6 a considerable increase in cell number occurs over the period day 6 to day 8, brought about by a mechanism involving the phytochrome system. This is apparently a pathway to division which is independent of the background pathway operating in darkness. Treatment with CCC leads to a large increase in cell number over the period day 6 to day 8, and the evidence presented supports the idea that light and CCC act independently. There are thus at least three pathways which lead to cell division and these are shown in Figure 43. The scheme incorporates the observations that division does not occur in the absence of roots or cotyledons, and the suggestion that ability of cells to divide may be lost due to ageing changes in the cells themselves.

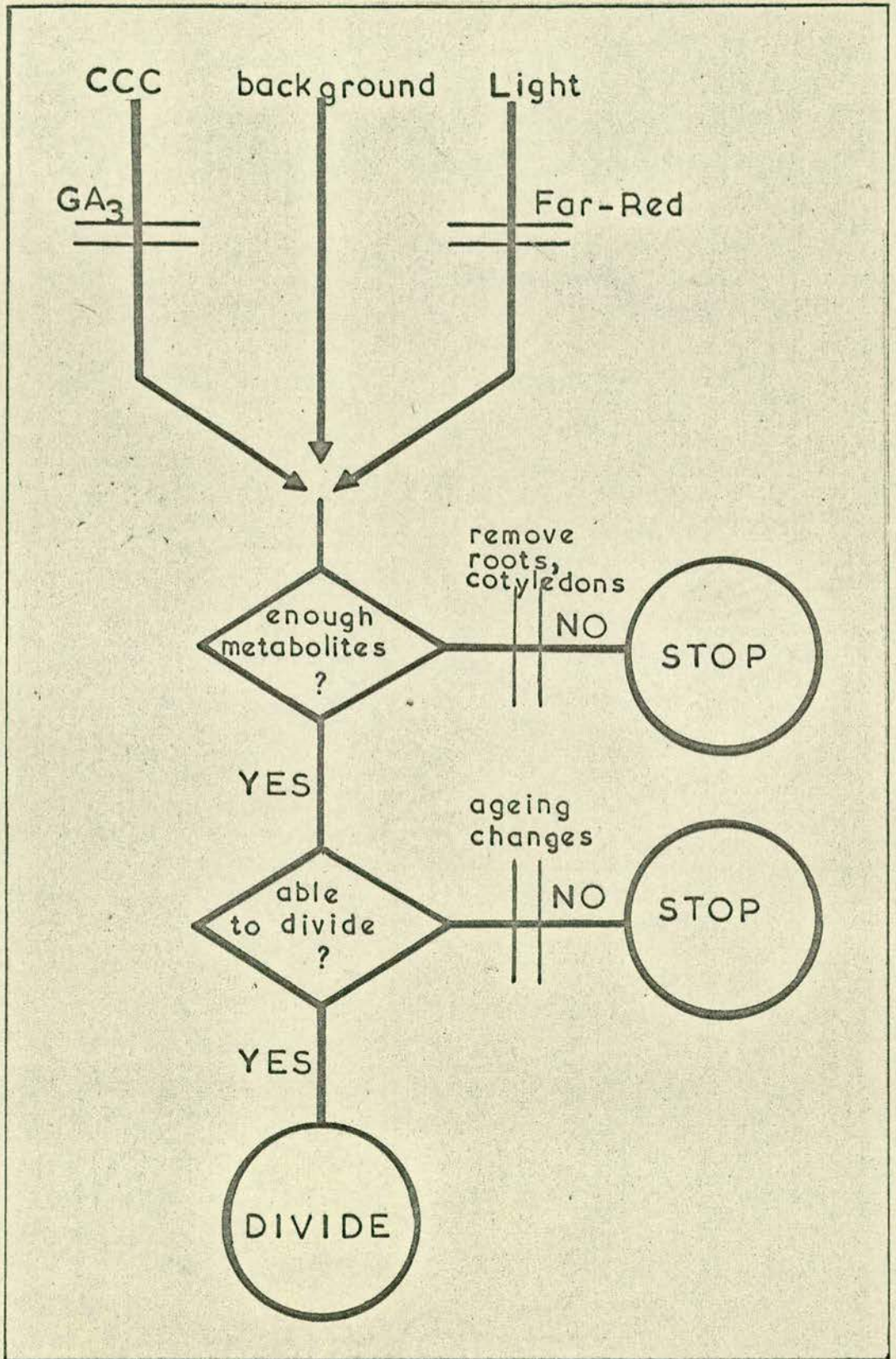
One may ask how it is that CCC treatment brings

about the increase in cell number, and how this effect can be counteracted by GA₃ application. CCC could conceivably act either through a mechanism which does not involve gibberellins or through the gibberellin liberation. The first alternative is unlikely since all the other effects of CCC can be explained using the hypothesis involving gibberellin, and since GA₃ can again counteract CCC effects. Thus it seems likely that the increase in cell number is brought about through a system involving the reduced rate of liberation of bound gibberellin. On this interpretation it follows that low levels of gibberellin allow cell division to proceed at a fast rate whereas high levels of gibberellin, endogenous or applied, reduce rate of division. This action of gibberellins as a valve controlling cell number increase is independent of the background pathway in darkness since GA₃ alone applied to dark-grown plants has no effect on division.

In control plants, high levels of endogenous gibberellin are liberated by about day 4 and these levels preclude cell number increase via the CCC mediated pathway. Yet divisions involving this pathway are most numerous around day 7 and can be prevented by GA₃ application at this time. It could be therefore that the effect of endogenous gibberellins is not an immediate one, since if this were so it would occur as early as day 5; this is not observed because GA₃ applied on day 7 counteracts the effect of CCC. Maybe the effect is

Figure 43 - Proposed scheme for the effects of CCC
and light on cell divisions in leaves.

The scheme shows the pathway proposed for the action
of CCC upon cell division in leaves as well as the
background and light pathways. Explanation of the
scheme is given in the discussion.



on particular cell divisions. By day 7 the majority of divisions in primary leaves are in the palisade layer (Dale, 1964b) and it could be that gibberellin level, if low, increases the number of divisions in this layer in darkness. The finding that division is low when gibberellin concentration is high is at variance with results from disks from dark (Wheeler, 1962) and light-grown (Dale, 1966) leaves, where it was shown that gibberellic acid was necessary for divisions to occur. Sachs and Kofranek (1963) showed that for Chrysanthemum, treatment with CCC while decreasing divisions on the longitudinal plane, increased division on the transverse plane in the sub-apical region of the stem. This suggests that a direct involvement of gibberellins in the division process may be unlikely and that the effects are indirect through another mechanism. The discrepancy in the effects of gibberellic acid on division rates for disks and intact leaves of Phaseolus reflects this, as well as reflecting the basic differences between intact and detached organs.

In light-grown primary leaves CCC does not give an increase in cell number over that of the control plants. On the scheme in Figure 43 one can postulate that all of the pathways shown may be involved (but see below). The lack of additiveness of CCC and light effects under these conditions could be due to some restriction placed on cell number, and it is of interest that Dale (1965) has shown that in a wide range

of temperatures, day lengths and light intensities, cell number in primary leaves is constant at about 40 - 45 millions per leaf. Dark-grown leaves treated with CCC do not show larger cell number values than this either. It could be therefore that the supply of metabolites essential for the division process is sufficient only to allow for a given number of divisions to occur.

In the case of the trifoliate leaves a rather different explanation must be advanced. Reference has already been made to the fact that the reduction in cell number in treated plants may be due more to shading from the primary leaves than to the effects of CCC. Clearly if the light and CCC pathways both operate for these leaves, low levels of light should be compensated by enhanced activity through the CCC pathway to give similar levels of cell number in treated and control plants. This is not so and suggests that either the CCC pathway may not operate in these leaves or that it may not operate in the light. While one may not rule out the second possibility, the postulation^e that CCC acts on cell division in primary leaves via the liberation of bound gibberellins, which occurs only in the young seedlings, leads to the suggestion that there is a genuine difference in the response of primary and trifoliate leaves to CCC, rather than a different mechanism operating in light and dark.

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