An Investigation of Ethylene Inhibition of Growth in Etiolated *Avena sativa* Coleoptiles

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B R O C K U N I V E R S I T Y

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This thesis is dedicated to all those individuals who helped make life worthwhile during the past seven years, ... and two degrees - and especially to the the one and only Ms. Peppermint Patti.

ABSTRACT

Growth rates of etiolated Avena sativa coleoptiles in pH 7.0 buffered medium are stimulated in a synergistic manner by IAA and 320 µl/l carbon dioxide. The suggestion that carbon dioxide stimulated growth involves dark fixation is supported by the ability of 1 mM malate to replace carbon dioxide, with neither factor able to stimulate growth in the presence of the other (Bown, Dymock and Aung, 1974).

The regulation of Avena coleoptile growth by ethylene has been investigated in the light of this data and the well documented antagonism between carbon dioxide and ethylene in the regulation of developmental processes. The influence of various permutations of ethylene, IAA, carbon dioxide and malate on the rates of growth, ^{14}C-bicarbonate incorporation, ^{14}C-bicarbonate fixation, and malate decarboxylation have been investigated.

In the presence of 320 µl/l carbon dioxide, 10.8 µl/l ethylene inhibited growth both in the absence and presence of 20 µM IAA with inhibition times, of 8-10 and 12-13 minutes respectively. In contrast ethylene inhibition of growth was not significant in the absence of growth stimulation by CO_2 or 1 mM malate, and the normal growth increases in response to CO_2 and malate were blocked by the simultaneous application of ethylene. The rates of incorporation and dark fixation of ^{14}C-bicarbonate were not measurably influenced by ethylene, IAA or malate, either prior to or during the changes in growth rates induced by these agents.

The data does not support the hypothesis that ethylene inhibition of growth results from an inhibition of dark fixation, but suggests that ethylene may inhibit a process which is subsequent to fixation.

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1. INTRODUCTION

1 - 1 Functional Relationships of Plant Hormones.

Considerable information has been accumulated about hormone systems and chemical control mechanisms as they pertain to the study of plants. It is well known that whereas most animal hormones have relatively specific types of physiological and biochemical functions, plant growth hormones have widely overlapping functions. Each hormone is distinctive with respect to its chemical structure and its ability to elicit characteristic growth responses, but each of the five major classes of growth regulators is capable of altering most aspects of development, including cell division, cell enlargement, differentiation and differential growth phenomenon. The five major classes of plant growth hormones, and their diversity of effects are summarized in Table I.

With overlapping functions in development, it becomes increasingly difficult to identify one substance as being responsible for the control of cell elongation, another for cell division, another for root growth and so forth. The processes of growth and differentiation appear more and more to be regulated by concerted interactions between the various growth regulators. It appears that the simultaneous interactions of two or more of the five major plant hormones is a major feature in the regulation of plant growth. Furthermore, the various classes of plant growth regulators are more readily distinguished through molecular structure and their activities in certain specific bioassays than by general areas of physiological and biochemical control.

With respect to the ideas of biochemical control of plant growth, Leopold and Kriedemann (1975) state the following in their introduction

Table I The diversity of effects of the five major groups of plant hormones.

Developmental process	Known regulatory activities for:				
	Auxin	Gibberellin	Cytokinin	Abscisic acid	Ethylene
Dormancy		x	x	x	x
Juvenility	x	x			
Growth rate	x	x	x	x	x
Flower initiation	x	x	x	x	x
Sex determination	x	x	x		x
Fruit set	x	x	x		x
Fruit growth	x	x	x	x	x
Fruit ripening	x	x	x		x
Tuberization	x	x	x	x	x
Abscission	x	x	x	x	x
Rooting	x	x	x		x
Senescence	x	x	x	x	x

This table illustrates the diversity of effects of the five known groups of plant hormones for 12 different growth and developmental processes. The existence of a known effect of each hormone on a developmental process is indicated by an X; the absence of such a mark does not imply no hormonal effect on that developmental process but rather the absence of such a report in the literature (from Chapter 18, 'Chemical Regulation of Growth and Development', in Plant Growth and Development by Leopold and Kreidemann, McGraw-Hill, New York, 1975)

to the topic of plant growth regulation:

> "The evolution of higher plants with their complex regulatory systems can be viewed as the manifestation of increasingly elaborate adaptations of the chemical regulatory systems to achieve a finer adjustment of internal controls and the tuning of these controls for finer sensing and response of the plant to its environment".

The various plant growth regulators undoubtedly act as part of these fine controls to maintain the proper balance of functions within the plant.

When one examines the problem of how a hormone at very low concentrations can exert very large physiological effects, it is clear that any mechanism of action of the hormone must involve a large amplification effect. The hormone molecules can be expected to exert an influence on some process that alters a large number of other molecules, often resulting in a cascade effect on numerous cellular processes. A key to determining the mechanism of action of the plant growth regulators then, would be to determine what initial process the hormone was acting upon which resulted in the subsequent chain of events that occurred after that initial action.

Until recently the usual approach to the study of hormone action on a particular phenomenon such as growth had been to measure its rate or magnitude before and after treatment with hormone. This approach has provided much information on such matters as the concentration dependence of hormone action, sensitivity to inhibitors, etc., but it largely ignored one of the most important aspects of hormone action, the latent period (or lag phase) between application of the hormone and manifestation of the response. This is the period during which

hormone-induced changes in cellular processes are taking place before the physiological response to the hormone is evident. Information on the timing of responses to plant hormones is particularly useful in attempts to establish the mechanism responsible for observed cause-effect relationships in hormone responses. The recent review of Evans (1974) on rapid responses to plant hormones provides a summary of most of the available kinetic information to date, especially with respect to data pertaining to auxin action.

Visible growth in plants, such as in stems and coleoptiles, is mainly growth by cell enlargement, while the formation of roots or fruits rests in the first stages on a greater stimulation of cell division, which only later is followed by cell enlargement. Growth of stems and coleoptiles would include then, all processes that are involved in the subsequent elongation of preformed cells and is readily expressed as elongation per unit time.

1 - 2 Important Features of Several Plant Growth Regulators in Relation to Coleoptile Growth

It is evident from Table I that all five classes of plant hormones have some effect on growth rate in many plant systems. The observed effects are many and varied, depending on the hormone applied, and the type of plant that it is applied to.

Evans (1974) summarizes the effect of auxin application on the growth rate and wall extensibility of a variety of plant tissues. When auxin is added to coleoptile or stem segments there is a latent period usually of 9 - 15 minutes during which no increase in growth rate occurs, and this is followed by a short period during which the growth rate increases

to a higher steady rate. Rapid effects of other hormones have also been reported by Evans (1974) but the majority of the work on increased growth promotion by plant hormones has centered on the auxins.

Ethylene has been shown to produce rapid inhibition of elongation growth in a variety of systems (Abeles, 1973) and a recent report by Bown and Aung (1974) indicated that ethylene inhibited the growth of Avena coleoptile sections after 10 - 15 minutes exposure to the gas. Ethylene also has many other profound regulatory activities in many plant systems (Table I) and like auxins, is formed endogenously in plants in sufficient quantities to bring about regulatory effects. It is therefore considered a plant hormone, even though it is a gas and is transferred by gaseous diffusion within the plant. Such characteristics support the inclusion of ethylene within the limits of the definition of a plant hormone as originally proposed by Went and Thimann (1937). They defined a plant hormone as:

> "....a substance which being produced in any part of an organism, is transferred to another part and influences a specific physiological process".

The growth promotion by auxin and the growth inhibition by ethylene have surprisingly similar latent periods suggestive but not necessarily indicative of some common site of action. Several non-hormonal compounds have also been shown to have rapid effects on cell elongation. Carbon dioxide has been shown to have two types of growth stimulatory effects on Avena coleoptiles (Bown, Dymock and Aung, 1974) and it is significant to note that atmospheric levels of carbon dioxide (300µl/l) significantly increase the growth rate of coleoptile sections with a

lag period of 12 - 15 minutes. Of further significance is the fact that IAA and carbon dioxide together result in a synergistic stimulation of Avena coleoptile growth (Bown, et al, 1974). Thimann (1940) and Thimann and Bonner (1948) reported that several organic acids, most notably malate and fumarate, were able to promote coleoptile elongation both in the absence and presence of IAA. Bown et al (1974) provided further evidence that malate and carbon dioxide were interchangeable in their ability to stimulate coleoptile growth to the same magnitude and that the latent period prior to the increase in growth was similar. It has also been suggested that growth stimulation by low levels of carbon dioxide is a result of dark carbon dioxide fixation, with the fixation process resulting in the production of four-carbon acids which are required to replace Kreb's acids lost to the Kreb's cycle during the biosynthesis of amino acids and proteins (Bown and Aung, 1974; Splitt-stoesser, 1966). It has been further demonstrated that the major dark fixation enzyme phosphoenolpyruvate carboxylase (EC 4·1·1·31) has been detected in Avena coleoptiles and that malate and aspartate are the first detectable products of ^{14}C-bicarbonate fixation (Bown and Lampman 1971).

Of further significance are the reports of competitive or antagonistic effects of ethylene and carbon dioxide in the regulation of various growth responses noted by Burg and Burg (1967b) and Kang, Yokum, Burg and Ray (1967) for a variety of plant tissues. The reports suggest a carbon dioxide-mediated reversal of an ethylene response and that competition for a common site of action of these molecules might be a possible explanation of the observed data.

Several things are now evident. First of all, there is a similarity in the 10 - 15 minute latent period prior to growth promotion by auxins, atmospheric levels of carbon dioxide and malate, or the growth inhibition by ethylene. This might be suggestive of a common site of action in the cell for each of these components in growth regulation. There are concerted interactions reported in the literature for various combinations of these growth regulatory compounds that have been briefly mentioned thus far. The biochemical events that are occurring within the cell during the latent period prior to the growth responses to auxins and ethylene are not clear as will be seen.

Evidence indicates that dark carbon dioxide fixation is occurring prior to the growth stimulation by atmospheric levels of carbon dioxide and indeed mediates the response in conjunction with the respiratory mechanisms within the cell. It might be reasonable then to propose that some concerted interaction between auxins, carbon dioxide and malate, and ethylene might exist in the regulation of the growth of Avena coleoptiles, and that this might involve the processes of dark carbon dioxide fixation and respiration.

It becomes essential then that concise answers are obtained to the questions regarding growth regulatory mechanisms involved in Avena coleoptile tissues with respect to the involvement of auxins, carbon dioxide and malate, and ethylene.

The understanding of the mechanisms of action of the various endogenous plant growth regulators is the key to solving the problems in understanding plant growth. Of immediate interest is the study of auxins and ethylene, particularly with respect to their growth

influences on each other, and on other factors which also regulate the growth processes, namely carbon dioxide and malate.

A review of the literature involving: (a) a brief historical overview of the study of plant hormones; (b) a review of the possible mechanisms of action of auxins and ethylene; (c) a closer examination of the interrelationships between auxins, ethylene, carbon dioxide and malate in controlling coleoptile growth; and (d) the involvement of dark carbon dioxide fixation and respiration in the process of coleoptile growth would be appropriate. Then consideration may be given to a possible mechanism of growth regulation for Avena coleoptiles that involves a concerted interaction between these various growth regulatory compounds.

1 - 3 Review of Literature

A. Historical Background - A Brief Perspective

In 1880, Charles Darwin published his book "The Power of Movement in Plants". This manuscript detailed Darwin's extensive studies on the relationships involved in plant growth with particular emphasis on the types of movement involved. His work sparked a tremendous period of scientific examination into the mechanisms of plant growth regulation. Pickard, in her preface to the 1966 reprint edition of the book stated that it was probably best remembered for entrenching in the physiological literature a particular experimental organ - the Graminaceous coleoptile - and for Darwin's observation that the phototropism of the coleoptile depends upon ".....some matter in the upper part which is acted on by light, and which transmits its effects to the lower part".

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2 - Theories of Location

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(Darwin, 1880). This statement lead other researchers to pursue the nature of the stimulus involved.

Boysen Jensen cut off coleoptile tips and stuck them on again with gelatin [1]. He observed that phototropism proceeded normally in these plants, though stumps alone did not bend at all, making clear that the bending stimulus did not require a continuity of living cells for its passage. Paàl repeated these experiments in 1919 and showed additionally that the stimulus could not pass various physical barriers he employed. He also found that placing the barriers assymetrically resulted in the bending of the coleoptiles. Similarly, he balanced tips on one side of the stumps and observed that the plants curved away from that side. He therefore proposed that the tip controls the growth of the coleoptile at all times by continually producing a regulatory substance which moves symmetrically toward the base. Under unilateral illumination, an asymetry of the substance develops in the tip and is translated as it migrates downward into asymetric growth. Thus Paàl showed that phototropic bending was mediated by a diffusible substance, and that this substance controlled the growth of the coleoptile at all times.

Soding measured growth rates and was able to show that decapitated coleoptiles stopped growing, whereas recapitated plants resumed growth at a more slightly reduced growth rate than normal. Stark in 1921, Neilsen in 1924 and Seubert in 1925 all tried but failed to isolate the

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[1] References to the original papers by all workers discussed in this and the following paragraph may be found in "Phytohormones", by F.W. Went and K.V. Thimann (The MacMillan Co., New York), 1937.

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growth substance from tissue extracts. Experiments reported by Went in 1926 and 1928 outlined his success in obtaining the growth factor. He set excised tips onto gelatin blocks and permitted the diffusate to collect. When he balanced the blocks assymetrically on coleoptile stumps the latter bent away strongly. The magnitude of the curvature was proportional to the number of tips which had been set on the blocks. Cholodny carried out similar work with geotropically responding roots. It was then proposed that phototropic and geotropic curvatures were mediated by the lateral movement of the growth substance. This became known as the Cholodny-Went Theory and resulted in the development of sensitive biological assays for the growth stimulating substance.

The growth regulatory substance was subsequently identified as indoleacetic acid and by 1930, plant physiologists had begun research upon the growth regulation by this hormone. From 1930 to 1950, knowledge of the growth regulation by auxins became more precise. Each of the following two decades resulted in the discovery of several more classes of plant hormones, these being the gibberellins, the cytokinins the inhibitor group that included abscisic acid and some phenol compounds; as well as the apparent rediscovery of ethylene gas.

The original discovery of ethylene gas as a plant hormone took place early in the 1900's and research on it was carried out during much the same time period as that for auxins. In his summary of the historical background of ethylene research, Abeles (1973) noted that by the middle of the 1930's most of the physiological effects of ethylene had been described. For reasons not entirely clear, research in

ethylene physiology slowed in the late 1930's through the late 1950's although a number of workers in the field did continue with active contributions to the knowledge accumulated thus far. The difficulty of using bioassays and other crude chemical techniques to measure ethylene production by plants was probably a major factor in the decline in ethylene research. The introduction of gas chromatography as an analytical technique in 1959 provided a valuable research tool to measure ethylene production, and interest in ethylene research again was renewed with vigor.

Although extensive research has been done on both auxins and ethylene, there is little conclusive evidence to explain the mechanism of action of either of these compounds, either in their individual effects on a wide variety of growth phenomena (see Table I) or with respect to their growth influence on each other and on other factors which also regulate growth. Auxins have profound growth stimulatory effects on stems and coleoptile growth. Ethylene on the other hand, has very distinct inhibitory effects on elongation phenomena. A review of the literature pertaining to the mechanism of action of auxins and ethylene is appropriate before further consideration is given to the relationships that exist between auxins, ethylene and atmospheric levels of carbon dioxide in the total growth process.

B. Mechanism of Action of Auxin

Current hypotheses concerning the initial auxin action suggest three functions through which large amplification effects could be obtained. The three major categories are:

1. An initial action of auxin on the molecular processes concerned with RNA or protein metabolism leading to increased protein synthesis.
2. An initial action on some preformed enzyme or protein leading to altered activities of this enzyme or protein.
3. An initial action on some cellular membrane leading to the changed compartmentation of growth related compounds.

These three theories are largely exclusive of each other and will be discussed individually with evidence for and against each of them presented in turn.

The hormonal regulation of nucleic acid synthesis and thus of protein synthesis has been a prime possibility for the mechanism of action of auxin. This has been well summarized by Evans and Ray (1969) in their discussion of the gene activation hypothesis and was further considered by Evans (1974) in his review of rapid responses to plant growth hormones. From an historical standpoint, it was observed by Silberger and Skoog (1953) that the growth of tobacco pith tissue induced by IAA was preceded by a proportional increase in RNA and that this increase was maximal at the concentration of auxin which would produce maximal growth.

With the introduction of inhibitors of protein synthesis, it was found that the inhibition of incorporation of leucine into protein by chloramphenicol was closely correlated with the inhibition of growth (Nooden and Thimann, 1965). Using the incorporation of $^{32}PO_4$ into RNA as a measure of RNA synthesis it was found that the application of IAA to pea stems resulted in enhanced RNA synthesis and that a phenol extractable (buffer insoluble) fraction of RNA showed a two phase curve,

or two levels of response to increasing IAA concentrations, which was characteristic of growth responses to auxins (Roychoudhury and Sen, 1964). Key and Shannon (1964) found that the incorporation of labelled nucleotides into the nucleic acids was stimulated by both IAA and 2,4-D.[2] Key (1964) concluded that these experiments implied that the regulation of growth by auxins might involve a regulation of RNA synthesis and ultimately that of RNA-directed protein synthesis.

The use of methylated-albumin-kieselguhr (MAK) columns to separate the various fractions of nucleic acids facilitates the examination of the incorporation of label (either as $^{32}PO_4$ or labelled nucleotides) into soluble RNA (sRNA), DNA, light and heavy ribosomal RNA, and a messenger RNA (mRNA) fraction. Key, Barnett and Lin (1967) found that auxin stimulated increases in incorporation into all these nucleic acid fractions, especially into the ribosomal component. Key and Ingle, (1964) found that 5-fluorouracil could bring about a sixty percent inhibition of RNA synthesis without interfering with auxin-stimulated growth in their studies on the relative effectiveness of various types of nucleic acid inhibitors. They found that 5-fluorouracil inhibited each of the RNA fractions separable on MAK columns with the single exception of the mRNA-like fraction. Thus the inhibition of growth by nucleic acid inhibitors appeared to be the result of the suppression of synthesis of some component found in the mRNA fraction.

Masuda and Tanimoto (1967) determined that the auxin-stimulated synthesis of RNA could be antagonized by an antiauxin in a manner similar to the antagonism of growth. O'Brien, Jarvis, Cherry and Hanson (1968) postulated that auxin-regulation of DNA-directed RNA synthesis

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[2] 2,4 - dichlorophenoxyacetic acid

might occur through an increase in DNA template activity, or possibly through an increase in the effectiveness of RNA polymerase. In studies of 2,4-D-stimulated RNA synthesis in soybean tissue, O'Brien et al (1968) found that the addition of RNA polymerase to the chromatin from auxin-treated soybeans yielded about the same maximum RNA synthesis as was found when RNA polymerase was added to chromatin from untreated controls. This suggested that a major effect of auxin on RNA synthesis could be through an enhancement of RNA polymerase activity.

The auxin stimulus could involve an enhancement of more RNA, or of qualitatively different RNA species. Thompson and Cleland (1971) compared the competitive characteristics of RNA for binding to DNA. They found that the IAA-stimulated RNA was not measurably different from RNA of control coleoptiles.

An important consideration on mechanisms of action of auxins must be mentioned at this point. The majority of reports involving RNA and protein synthesis involve long periods of exposure to auxin prior to measurement of some parameter such as incorporation of leucine into protein, or before growth measurements were made.

The long term effects of auxin may well involve nucleic acid-directed protein synthesis, but the short term effects, particularly the response times for auxin stimulation of growth that are summarized by Evans (1974) occur within a 10 - 15 minute period. This time period is generally considered too short for RNA and protein metabolism to be involved in the initial stages prior to growth stimulation by auxin.

Kinetic experiments in which the timing of the growth response to auxin was measured, have provided important clues to the type of

mechanism that might be involved in auxin action. Ray and Ruesink (1962) reported that the first parameter that is of note in kinetic experiments is the lag time between auxin application and the first detectable response manifested as an increase in growth rates. Ray and Ruesink reported lag times ranging from 10 to 15 minutes for coleoptile sections and similar values were subsequently reported for numerous other tissue types and are summarized by Evans (1974).

Evans and Ray (1969) found that pretreatment of Avena coleoptile sections with inhibitors of RNA and protein synthesis did not extend the latent period in response to auxin, even though cyanide and/or low temperature did. Kinetic analysis of their data indicated that the results were consistent with the gene activation hypothesis of auxin action only under the improbable assumption that the half-life of auxin-induced RNA and protein was about 2 or 3 minutes. Barkley and Evans (1970) and Penny (1971) have also shown that the latent period in the response of other tissues to auxin was insensitive to antibiotics.

Further evidence that the primary action of auxin was not at the level of protein synthesis has been provided by Pope and Black (1972) and Penny (1971), who reported that auxin could cause a sizeable promotion of elongation in antibiotic-treated tissue in which protein synthesis had been essentially eliminated.

Nissl and Zenk (1969) also provided evidence against the mediation of protein synthesis during the initial stimulation of elongation by auxins. Using elevated concentrations of auxins and by performing their experiments at elevated temperatures they could reduce the lag

period essentially to zero. This they stated was an indication that auxin was able to bring about growth responses almost immediately upon entry of the auxin into the cell, and probably then without involvement of protein synthesis.

It should be pointed out, however, that Nissl and Zenk used a temperature of 40°C and a very high auxin concentration. These growth conditions could not be considered physiological and the sections stopped growing after 3 minutes, and began to shrink after 6 minutes. The experiments were carried out at a pH 4.7 and it is well known that the pH optimum for acid-promoted cell elongation in Avena coleoptiles is between 3 and 4. Evans (1974) suggested that perhaps auxins rapidly modified the cuticle to allow access of hydrogen ions which are responsible for the observed growth response. If the immediate stimulation of elongation by high concentrations of IAA at 40°C as reported by Nissl and Zenk (1969) and Durand and Zenk (1972) were in fact a normal auxin response, then it should be possible to eliminate it using a high concentration of antiauxin or using various metabolic inhibitors. Unfortunately this has not been tested.

Ray and Ruesink (1969) also studied the aftereffect, or the time following the withdrawal of the auxin supply until the stimulated growth rate began to revert to lower endogenous levels. Their estimate for the aftereffect was 40 minutes. Evans and Hokanson (1969) obtained shorter aftereffects by employing lower auxin concentrations, indicating that the time required for the auxin to be transported out of the sections was important. Dela Fuente and Leopold (1970) were able to correct for the transport time and arrived at an estimate of 30 minutes

needed for a 50 percent decline in growth rate.

Collectively, the kinetic experiments indicate that auxin rapidly stimulates growth upon entry into the cell, and that stimulation decays rapidly upon auxin exit. Since the nucleic acid-directed protein synthesis should take longer than a few minutes, and since the lag phase prior to auxin-stimulated growth is 10 - 15 minutes, it would seem unlikely that the initial action of auxin is mediated by an effect on RNA or protein metabolism.

The second hypothesis proposes that the initial auxin action is on some preformed enzyme or protein that leads to alteration of the activities of this enzyme or protein. Evans and Ray (1969) suggested that some growth-limiting protein (GLP) or other limiting substrate might be used up in the growth process as part of the rapid response to auxin. Nissl and Zenk (1969) suggested further that the auxin effect might be on a preformed system which regulated cell wall synthesis or on some growth limiting substances already present in the cell wall that could have a fairly direct influence on the cell wall loosening process, with subsequent growth resulting from turgor pressure.

Cleland (1971) reported that inhibition of growth followed inhibition of protein synthesis by 20 - 25 minutes regardless of growth rate. This indicated that the growth inhibition was due to inherent instability of the GLP rather than to exhaustion of the pool through growth. His study of the amount and rate of auxin-induced growth which occurred when cycloheximide was added just prior to, or after the auxin indicated that the rate of elongation was determined by the size of the GLP pool, and that it rapidly expanded from a small size in the absence of auxin

to a maximum size 20 - 25 minutes after addition of auxin.

Penny (1971) also proposed the existence of a GLP pool. When she blocked protein synthesis with cycloheximide she was able to estimate that the GLP pool had a half-life of 12 - 17 minutes. She concluded that once the GLP pool was exhausted it was necessary to have new synthesis of GLP before the rapid auxin response could occur again. Evidence was available in other reports to show that protein synthesis was not necessary for the initial rapid response to auxin provided a pool of GLP was present. On addition of auxin the GLP pool increased to a maximum size but became depleted by the inhibition of protein synthesis (Cleland 1971; Pope and Black, 1972). If protein synthesis was inhibited shortly after the addition of auxin, then there would be only a short increase in growth due to auxin. Protein synthesis was necessary to maintain the increased growth rate attributed to auxin.

This leaves several questions unanswered, such as the exact nature of the GLP, although probably protein in nature; the ways in which auxin might result in an increased GLP pool size; and the function of the GLP in auxin stimulation of growth.

Cleland (1961) was unable to obtain any evidence that auxin action was on enzymes of the respiratory systems. A second possibility for auxin action being mediated by preformed enzymes involves those enzymes which might alter the characteristics of the cell wall. Heyn (1931) had demonstrated that the stimulation of growth by auxin could be attributed to a softening of the cell wall. Tagawa and Bonner

(1957) developed a technique by which they could distinguish between plastic and elastic changes in the cell wall. They were able to demonstrate that auxin stimulation of growth was closely associated with changes in plasticity. Bonner (1960) demonstrated that in oat coleoptiles the tissue responded to auxin treatments with an increase in plasticity which was similar in magnitude to the increase in growth.

Primary plant cell walls are composed of cellulose fibrils cemented together by pectins and hemicelluloses. It was thought that auxins might act by removing the calcium ions which cross-link the carboxyl groups of these cementing polymer compounds. Cleland (1960a) however, using radioactive calcium was unable to show a release of calcium from the walls sufficient enough to account for the auxin stimulations of growth. Another consideration was the enzymatic alteration of the degree of methylation of the pectic groups in the cell wall. Schrank (1956) found that ethionine, which strongly inhibits methylation, was a very potent inhibitor of oat coleoptile growth. Cleland (1960b) showed that in the case of corn coleoptiles auxin-stimulation of growth did not require a methylation step.

As an alternate approach, Fan and MacLachlan (1966) found an impressive correlation between auxin stimulation of growth and the associated increase in cellulase in pea stems. In their experiments to determine whether auxins altered the cellulose fibrils they found also that not only did actinomycin D and puromycin inhibit the auxin stimulation of growth, but that increases in cellulase were also prevented. It was also noted that 5-fluorodeoxyuridine had no effect on the increases in cellulase attributed to IAA.

(1957) developed a recording device which can record instantaneous plastic and elastic changes in a leaf well. They were able to demonstrate that decrease in elasticity was closely correlated with changes in plasticity. Bonner (1960) demonstrated that in oat coleoptiles the change responsible for auxin treatment was an increase in plasticity which was similar in magnitude to the increase in growth.

Primary plant cell walls are composed of cellulose fibrils cemented together by pectins and hemicelluloses. It was thought that auxins might act by removing the calcium ions from cross-links between carboxyl groups of these cementing polymers. Cleland (1959), however, using radioactive calcium was unable to show a release of calcium from the walls of artificial enough to account for the auxin stimulation of growth. Another consideration was the enzymatic alteration of the degree of polymerization of the cell wall groups in the cell wall. Bonner (1950) found that pectinase, which according to Cleland (1959) is a very potent inhibitor of the cellulase growth. Cleland (1959) showed that it was one of the cellulases auxin-stimulation of growth did not require protein synthesis.

In an alternate approach, Carr and Weintraub (1960) found an increase cytoplasmic streaming in pea stems. In their experiments in Gerardia vaginata stems also that not only did indoleacetic acid promote the rate of elongation of growth, but it increased only very slightly the viscosity. It was also noted that p-fluorophenylalanine had an effect on the increase in cytoplasmic streaming in pea.

Tanimoto and Masuda (1968) found that B-1, 3-glucanase another of the hydrolytic enzymes implicated in the action of auxin on the cell wall, increased in activity during auxin stimulation of growth. Wada, Tanimoto and Masuda (1968) reported that applications of the enzyme directly to the tissue could induce some initial growth responses. Ruesink (1969) noted however that he could not achieve enhancement of growth with application of cellulase to oat coleoptiles even though his physical measurements of the wall indicated that the extensibility had been changed by the enzyme. This did cast some doubt on the cell-wall hydrolyzing enzymes acting as the main mediator of auxin action.

Although some reports indicate that auxin treatments increase the level of some cell wall components such as cellulose and hemicelluloses in pea stems (Christiansen and Thimann, 1950), hemicelluloses and pectins in oat coleoptiles (Bayley and Setterfield, 1957) and some cold and hot water soluble pectic substances (Albersheim and Bonner, 1959), it is doubtful that any of these components could be the GLP since these increases generally occurred after lengthy auxin treatments. Bennet-Clark (1956) and Ordin and Bonner (1957) were unable to determine any cell wall synthesis associated with auxin treatment.

Cleland (1973) determined that cycloheximide inhibited both coleoptile elongation and the H+ ion excretion that resulted from auxin treatment. He suggested that proteins involved in hydrogen ion excretion might be extremely labile and could possibly be the GLP. This remains to be determined as yet.

The third major hypothesis for the mechanism of auxin action involves some action on the cellular membrane leading to a change in

compartmentation of some growth related compound. The first approach to this problem involved a study by Bonner, Bandurski and Millerd (1953) that indicated auxin action might result from a stimulation of a metabolic uptake of water, but more recent evidence suggested that water uptake activities were osmotic in nature and not metabolically driven. Ordin, Cleland and Bonner (1955) reported that increases in osmotic values of the ambient solution resulted in exponential depressions of cell enlargement. Further to this Hackett (1952) had already reported that as growth proceeded there might be an actual dilution of the osmotic components associated with water uptake. This would be expected if growth were initiated by a softening of the wall followed by osmotic influx and turgor driven extension.

As early as 1943, Commoner, Fogel and Muller suggested that auxin stimulation of growth involved a stimulation of cation uptake. Gregory and Cocking (1966) reported that isolated protoplasts taken from tomato fruits and completely removed of any cell wall material were able to respond to auxin by the uptake of water probably resulting from an uptake of solutes. In 1969, Weigl suggested that auxin molecules could become attached to lipid components of membranes and result in greatly altered ion permeability of the membrane. He set up model membranes and demonstrated that auxins could bind to his models, with high specificity for the active auxins. He attributed this binding facility to the spacing of positive and negative charges on the auxin matching the opposite charges of the lecithin component of his membranes.

Related to the membrane phenomenon is the possibility that there may be a membrane bound factor that is released in the presence of

auxin to become an initiator of RNA polymerase. This suggestion has been reported by Hardin, Cherry, Morré and Lembi (1972). Such a factor could well provide a link between a plasmalemma site of auxin attachment and the auxin regulation of nucleic acid synthesis. The binding of the auxin molecule could result in a cascade of events occurring after this initial effect at the plasmalemma.

Rayle and Cleland (1970b) reported that solutions of low pH could result in a rapid loosening of the cell wall and immediate growth stimulation. The suggestion has been made then that the early responses to auxin might be mediated by an auxin effect on a proton pump in the plasmalemma and that the consequent decrease in the pH of the cell wall region might be responsible for the rapid responses (Rayle, 1973). The high H+ ion concentration could result in the cleavage of proposed alkali-stable, acid labile cross-links in the cell wall (Rayle and Cleland, 1970b) or the activation of a hydrolytic enzyme that results in the enzymatic cleavage of critical wall-bonds (Johnson, Daniels, Dowler and Rayle, 1974). The end result of both processes produces the elongation of the cell driven by turgor pressure.

Hagar, Menzel and Krauss (1971) provided evidence for the proton pump theory by demonstrating that metabolism was required to provide energy for the activation of the pump and the lowering of the pH in the wall.

Barkley and Leopold (1973) reported that rapid auxin effects could not be due to lowered pH since some tissues such as light grown pea stem segments would respond to auxins but not to lowered pH values. Cleland and Rayle (1975) dispute this report and state that the thick

cuticle in pea stems protects the cell wall from the low pH. They further reported that the tissue will respond to auxin and low pH in a similar manner if the cuticle is slit to allow for entry of the H+ ions to the cell wall. Cleland (1973) had previously reported that by blocking auxin-stimulated growth with respiratory inhibitors or with cycloheximide, it was possible to show a cessation of the drop in pH attributed to auxin. Thus the lowering of the pH due to auxin showed requirements for metabolism and protein synthesis.

Marre, Lado, Caldogno and Colombo (1973) reported that the extent of the pH drop was proportional to the growth stimulating activity of four different auxins and the fungal toxin fusicoccin. This latter agent has a very dramatic short-termstimulating effect on the growth of a wide variety of plant tissues and always causes a simultaneous drop in pH (Marre, Colombo, Lado and Caldogno, 1974).

In concluding this review on the mechanism of action of auxin it should be mentioned again that it would seem likely that the initial rapid responses observed with auxins are too rapid to be a result of RNA-directed protein synthesis. There may be a definite relationship between auxin action and extrusion of H+ ions through the plasmalemma. The involvement of the GLP is of pertinent interest but the role that it plays and how it might be activated are still not clear. Therefore it can be concluded that much remains to be clarified before the initial mechanism of auxin action in the growth stimulation of plant tissues is understood.

C. Mechanism of Action of Ethylene

As with the other plant hormones, the list of regulatory roles for ethylene encompasses the full spectrum of growth and developmental functions (Table I). Foremost among the functions which may be regulated by ethylene are its inhibitory effects on growth. This is, of course, of primary interest to this discussion. The participation of ethylene in the endogenous control of growth rates appears to be widespread in higher plants (Abeles, 1973) and includes not only the inhibitions of elongation growth (Burg and Burg, 1968) but also in certain instances the stimulation of lateral growth (Radin and Loomis, 1969).

The intriguing question that remains unanswered is similar for auxin and ethylene and concerns the mechanism of growth regulation. Again, as with the case of auxins, three theoretical lines of approach have been suggested:

1. Ethylene becomes attached to some metalloprotein site in the cell which can serve in some regulatory manner.
2. Ethylene becomes attached to membrane layers, altering their function in some manner.
3. Ethylene serves to regulate plant processes through an alteration of RNA and resultant protein synthesis.

The metal-adsorption theory of Burg and Burg (1967b) is based on two principal lines of evidence: (1) the changes in biological activity with molecular structure they reported are similar to the changes in adsorption of the gas onto a heavy metal such as silver; (2) the attachment of ethylene to a heavy metal is inhibited by carbon dioxide in a manner similar to the inhibition of many biological responses to ethylene. The competitive nature of CO_2 interference with ethylene

action was reported by Burg and Burg (1967a). They plotted the inverse of the ethylene concentration against the inverse of growth inhibition, in their pea-seedling curvature test, and the plots with and without CO_2 showed the same intercept on the ordinate, which is consistent with a competitive-inhibitor interpretation. Such CO_2 interference with ethylene responses have been reported for many ethylene systems such as the stimulation of abscission (Abeles and Gahagan, 1968), epicotyl hook formation in etiolated seedlings (Kang, Yokum, Burg and Ray, 1967) inhibition of root growth (Chadwick and Burg, 1967) and the stimulation of fruit ripening (Burg and Burg, 1965a,b). In many cases the CO_2 reversal has become a standard test for ethylene-stimulated actions.

Burg and Burg (1967b) have shown an interaction of ethylene and oxygen. They established that depressed oxygen levels inhibit ethylene action. They suggest that the oxygen is necessary for oxidation of the metal-receptor site to which ethylene becomes attached. Abeles and Gahagan (1968) found that the depressed ethylene response at low oxygen levels could be accounted for on the basis of a simple depression of respiration and was therefore in disagreement with the concept of oxidation of a site of attachment. The oxygen requirement is consistent with the greater storage life of fruit when controlled atmospheres of low oxygen and high CO_2 levels are employed.

The nature of the binding of ethylene onto various sites in the plant has been examined by supplying deuterated ethylene and examining it after exposure to the plant for a deuterium displacement by hydrogen atoms (expected for a covalent attachment) or an alteration of the arrangement of the specific deuterium atoms in the molecule (expected

for a hydrogen-bonding attachment). Abeles, Ruth, Forrence and Leather (1972) and Beyer (1972) agreed that the recovered ethylene was unaltered, which is consistent with an attachment neither by covalent nor by hydrogen bonding. The presence of deuterium would be expected to result in enhanced affinity of the ethylene for a heavy metal such as silver, and the lack of a difference in biological activity between the deuterated and the hydrogen-containing ethylene did not support the hypothesis of a metallo-protein attachment site.

Burg and Burg (1967b) suggested zinc as the metal site for ethylene attachment, and Leopold (1972) reported that the zinc containing carbonic anhydrase did in fact adsorb ethylene but that the measured effectiveness of the enzyme was not affected by the adsorption.

The second theory for the mechanism of ethylene action purports that ethylene might have its active site on a membrane surface where it could be attached to a lipid layer. This was tested by Lyons and Pratt (1964) using the swelling of isolated mitochondria as a measure of increased membrane permeability. They observed a more rapid swelling in the presence of ethylene and concluded that the gas was altering membrane permeability. It was later concluded by Mehard and Lyons (1970) that the permeation effects were not specific for ethylene since they tried several other unsaturated gases as well and achieved similar results.

Mehard, Lyons and Kumamoto (1970) tested the membrane alteration idea further using another approach. They studied the effects of a series of alkenes and alkanes on surface tension using artificial membranes, and found that ethylene had very little effect on the surface

tension. This indicated that such physical actions on membrane structure could not be the mechanism for the biological activity of ethylene.

The third possible mechanism for ethylene action involves a possible alteration of enzyme patterns through alteration of RNA directed protein synthesis. Ethylene is known to bring about alterations in the amounts or effectiveness of numerous enzymes. Peroxidase enzyme has been shown to be induced by ethylene. Stahmann, Clare and Woodbury (1966) demonstrated that the production of ethylene in sweet potato tissues in response to black rot fungus infection resulted in tremendous increases in peroxidase levels. They were able to show increases in peroxidase levels by supplying ethylene in the absence of fungus. Gahagan, Holm and Abeles (1968) further showed that CO_2 could prevent the ethylene enhancement of peroxidase and that inhibitors of RNA and protein synthesis could prevent the increase in peroxidase levels.

Chalutz, DeVay and Maxie (1969) reported that exposure of carrot roots to ethylene leads to enhancement of isocoumarin biosynthesis and that this change was prevented by elevated CO_2 levels. Riov, Monselise and Kahan (1969) demonstrated that ethylene induced increases in phenylalanine ammonia-lyase in citrus peels, and that the increase could be prevented by CO_2 or by cycloheximide. This enzyme is often considered a limiting step in the biosynthesis of phenolics in plants. Several other enzymes are known to be induced by ethylene, including phosphatases (Olson and Spencer, 1968) and a number of enzymes involved in the fruit ripening process (Rhodes, Galliard, Wooltorton and Hulme, 1968). The enzymes were involved in organic acid metabolism, specifically with respect to a malate decarboxylating system that was

induced by ethylene during aging of apple peel discs.

Ethylene effects on cellulase enzymes have been reported. The stimulation of abscission in bean leaves by ethylene is known to be associated with an increase in some cellulase enzymes in the region of separation (Abeles, 1969; Ridge and Osborne, 1969). Lewis and Varner (1970) reported that appearance of at least one of these cellulases was through de novo synthesis. The rapidity of ethylene actions on abscission development reported by Dela Fuente and Leopold (1969) was not consistent with enzyme synthesis kinetics (Abeles and Leather, 1971). When Abeles re-examined the distribution of the enzymes he found that the rapid response to ethylene correlated with a rapid release of cellulase from a bound form into a soluble form in the intercellular spaces of the petiole. Jones (1969) had reported ethylene-stimulated release of α-amylase from a bound form into the liquid medium from barley aleurone layers. From these results it was evident that enzymatic changes induced by ethylene might not necessarily involve an alteration in enzyme biosynthesis.

Numerous ethylene effects have been effectively inhibited by nucleic acid or protein synthesis inhibitors, implying that ethylene regulation of growth may result from regulated enzyme synthesis through RNA-directed protein synthesis. Holm and Abeles (1967) reported a small increase in $^{32}PO_4$ incorporation into RNA of bean abscission zones in the presence of ethylene. With the inhibition of abscission by actinomycin D and certain other inhibitors, it was suggested that ethylene acted through a synthesis of some new RNA, possibly mRNA. Holm, O'Brien, Key and Cherry (1970) further demonstrated

that in soybean hypocotyls ethylene exposure lead to a marked increase in DNA-directed RNA synthesis and brought about the formation of RNA with an altered base composition. Shimokawa and Kasai (1968) applied radioactive ethylene to morning glory seeds and recovered some of the label in a light RNA fraction which was thought to be a tRNA.

It could be deduced from these numerous lines of evidence that ethylene might regulate growth functions through RNA and RNA-directed protein synthesis. This however, might be too simple an explanation for rapid effects of ethylene. Three types of evidence suggest that ethylene effects might not occur directly through changes in protein synthesis:

1. As previously mentioned, some rapid ethylene actions seem to involve alterations of enzyme secretion rather than synthesis (Abeles and Leather, 1971; Jones, 1969).
2. Timing measurements of ethylene inhibitions of growth show that altered growth rates set in within a 5 minute period after ethylene exposure, and this is probably too rapid for an effect via protein synthesis (Warner and Leopold, 1971).
3. At least some ethylene responses are not inhibited by cycloheximide or actinomycin D (Burg and Burg, 1967a).

It can be concluded then that ethylene has a dynamic part in the regulation of growth and of many diverse developmental phenomena. The mechanism of action of ethylene is no clearer than that of auxins or the other major groups of growth regulators. There is strong evidence that points to nucleic acid involvement in the ethylene responses, but it must be remembered that some rapid responses attributed to ethylene seem to be independent of nucleic acid and protein synthesis.

D. The Role of Carbon Dioxide in Growth Regulation and its Relationship to Hormonal Effects.

The ability of carbon dioxide to stimulate growth in various non-photosynthetic tissues has been carefully examined over the past two decades. Recently, it has been reported that two types of growth response to carbon dioxide can be distinguished (Bown, Dymock and Aung, 1974). Carbon dioxide-saturated solutions have been shown to produce immediate increases in growth that are insensitive to cycloheximide and the elevated rate is greater than that obtained by auxins or low pH values (Rayle and Cleland, 1970a; Evans and Ray, 1969). These growth characteristics are indicative of a mechanism that is independent of protein synthesis and which is not mediated by time-consuming metabolic processes. Although the growth response to CO_2-saturated solutions is dependent on a pH below 6, it appears that the response to high levels of CO_2 is not due to acidification by dissolved CO_2 alone (Rayle and Cleland, 1970a; Bown et al, 1974).

In contrast, low concentrations of carbon dioxide are known to stimulate the growth of various non-photosynthetic tissues. In both root tissues (Leonard and Pinckard, 1946; Stolwijk and Thimann, 1957) and etiolated coleoptiles (Harrison, 1965) carbon dioxide levels of 1% or less have been shown to produce increased growth. In addition, Bown, et al (1974) and Splittstoesser (1966) have demonstrated that reduced growth rates have been observed in carbon dioxide-free air. It has also been reported that the growth response of Avena coleoptiles to atmospheric levels of carbon dioxide (300µl/l) occurs after a lag period of 12 to 15 minutes, is not due to acidification of the

medium, and is inhibited by cycloheximide (Bown, et al, 1974). These characteristics clearly separate the growth response to atmospheric levels of carbon dioxide from the response elicited in the same tissue to carbon dioxide-saturated solutions that were mentioned previously.

It has been suggested also that growth stimulation by low levels of carbon dioxide is a result of dark carbon dioxide fixation. The fixation process results in the production of four-carbon acids which are required to replace Kreb's cycle acids lost to the Kreb's cycle during the biosynthesis of amino acids and proteins (Bown and Aung, 1974; Splittstoesser, 1966). This hypothesis is supported by observations that some organic acids, most notably malate, could significantly stimulate the growth of Avena coleoptile sections (Thimann, 1940; Thimann and Bonner, 1948); that malate and carbon dioxide are interchangeable in their ability to stimulate coleoptile growth; and that neither factor is able to stimulate growth in the presence of the other (Bown et al, 1974). It has also been shown that the major dark carbon dioxide fixation enzyme, phospho-enolpyruvate carboxylase (EC 4·1·1·31) has been detected in Avena coleoptiles and that malate and aspartate are the first detectable products of ^{14}C-bicarbonate fixation (Bown and Lampman, 1971).

The influence of IAA on the growth response of low levels of carbon dioxide in etiolated Avena coleoptiles has been reported by a number of authors (Bown et al, 1974; Cockshull and Heath, 1964; Yamaki, 1954). Thimann (1940) reported that IAA added to coleoptile sections in the presence of malate resulted in a marked rise in respiration and that concentrations active in this reaction closely

paralleled those active in accelerating growth. Thimann (1949) further reported that pretreatment with malate made possible a direct demonstration that auxin influenced the respiration in the coleoptile. This was taken to mean that malate was required for auxin to exert its full effect, and that when this effect was exerted on growth it was by way of a respiratory system.

Yamaki (1954) made simultaneous measurements of coleoptile growth, oxygen consumption and RQ values. He demonstrated that carbon dioxide stimulation of growth was more pronounced in the presence of IAA and that RQ values dropped significantly before IAA stimulated growth. He suggested that this rapid change in RQ value involved an increase in dark carbon dioxide fixation, and not a change in the respiratory substrate. More recently, it has been shown by Bown et al (1974) that IAA and carbon dioxide stimulate the growth of Avena coleoptiles in a synergistic manner. They also demonstrated a similar lag period for growth responses to these two factors as well as for malate.

1-4 Hypothesis for Ethylene Inhibition of Coleoptile Growth

The inhibition of growth by ethylene has been well documented in a variety of tissues (Goeschl, Pratt and Bonner, 1967; Radin and Loomis, 1969) and has been well summarized in a review article by Abeles (1972). Carbon dioxide-mediated reversal of developmental responses to ethylene have been reported by Abeles and Gahagan (1968) and Chadwick and Burg (1967). Burg and Burg (1967a) reported that growth data obtained using pea seedlings suggested that atmospheric levels of carbon dioxide could compete with ethylene for a common site within the cell. Might it then

be possible that ethylene could influence the growth response to low levels of carbon dioxide in Avena coleoptile tissues?

Bown and Aung (1974) reported preliminary data which indicated that ethylene inhibited coleoptile elongation with a lag period of 10 - 15 minutes. In view of this data, growth stimulation by CO_2 and the well known antagonism between CO_2 and ethylene in their influence on growth (Burg and Burg, 1967a,b; Kang, Yocum, Burg and Ray, 1967), it was decided to investigate further the ethylene inhibition of coleoptile growth. Since it appeared probable that CO_2 stimulated growth through dark fixation, then ethylene inhibition of coleoptile growth could result from an inhibition of dark CO_2 fixation. This hypothesis is outlined in Figure 1.

Ethylene inhibition of growth could result from competition between CO_2 and C_2H_4at the active sites of the enzyme PEP carboxylase (EC 1·1·1·37). The antagonism between CO_2 and C_2H_4 observed by Burg and Burg (1967a,b) lends support to this hypothesis for a common site of action for CO_2 and C_2H_4.

Two tests of the hypothesis were used:

1. The first test involves the investigation of growth in the presence of malate. If ethylene blocks the fixation step as proposed, then the simultaneous application of malate with ethylene should result in malate being able to counteract the inhibition of growth by ethylene, since malate, one of the initial fixation products would be present regardless of any effect ethylene had on fixation. The ability of malate to replace CO_2 in the stimulation of growth of Avena coleoptiles has been documented (Bown et al, 1974). Malate

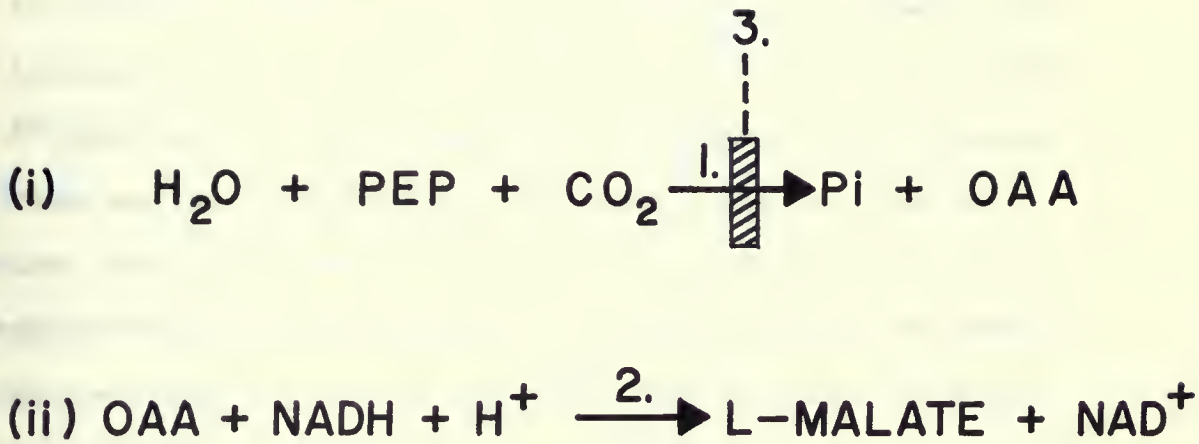
Hypothesis for the Inhibition of Coleoptile Growth by Ethylene

Malate supplies 4-carbon skeletons for essential amino acid biosynthesis and ultimately for the growth phenomenon. Two possible tests of the hypothesis that C_2H_4 inhibits growth by acting at the fixation site are:

Test 1 Investigate C_2H_4 inhibition of growth in the presence of malate.

Test 2 Investigate the influence of C_2H_4 on dark carbon dioxide fixation.

FIGURE 1



1. PEP CARBOXYLASE.

2. MALATE DEHYDROGENASE.

3. POSTULATED INHIBITION BY ETHYLENE OF CARBON DIOXIDE FIXATION STEP.

FIGURE 2

should then protect the tissue from ethylene inhibition.

2. The second test of the hypothesis involves the investigation of the influence of C_2H_4 on dark carbon dioxide fixation. If ethylene inhibited the fixation process, then coleoptiles supplied with ^{14}C-bicarbonate and treated with C_2H_4 would show lower levels of total ^{14}C-bicarbonate fixation compared to non-ethylene treated tissues.

There are two reasons that make this approach to the study of ethylene inhibition of growth attractive: (i) inhibition of fixation by ethylene could be very rapid; (ii) it would account for the observed antagonism between CO_2 and C_2H_4 (Burg and Burg, 1967a,b). The events that occur prior to stimulation of growth by CO_2 and IAA, or the inhibition of growth by ethylene within 10 minutes are of prime importance in considering the mechanisms by which these growth regulators act. The short lag periods are consistent with arguments against the more time-consuming synthesis of RNA and protein as means by which these growth regulators act. Since we are looking for immediate or somewhat rapid effects that occur before the growth rate changes, and since the antagonism between CO_2 and ethylene observed by Burg and Burg (1967b) lends support to the hypothesis as outlined in Figure 1, then the two proposed tests of the hypothesis would present a reasonable approach to the investigation.

The purpose of this investigation then is to attempt to answer relevant questions regarding the growth regulatory mechanisms involved in Avena coleoptile tissue with respect to the involvement of CO_2, IAA and C_2H_4.

The investigation of the hypothesis that ethylene inhibition of

growth is mediated by an ethylene inhibition of dark CO_2 fixation has therefore been examined by studying the influence of ethylene on growth rates, rate of incorporation and fixation of ^{14}C-bicarbonate and on malate metabolism, both in the absence and presence of IAA. These investigations and their subsequent results are therefore reported here in an attempt to provide further insights into the growth promoting mechanisms of IAA, CO_2 and malate, and into the mechanisms by which ethylene can inhibit growth. Close relationships exist between these endogenous plant growth regulators, and clarification of their independent and related roles would provide further insights into the problems involved in characterizing the regulatory mechanisms involved in plant growth and development.

2. METHODS

2 - 1 Materials

A. Biological Materials

Seeds of *Avena sativa* L. var 'Victory' were purchased from Ward's Biological Supply House, Rochester, New York, U.S.A. Vermiculite (Industrial Number 4 grade - fine) was purchased from Ball-Superior Limited, Mississauga, Ontario.

B. Chemicals

With the exception of those chemicals listed below, all commercial chemicals were purchased either from BDH (Canada) limited, Toronto, Ontario, or from Fisher Scientific Company, New Jersey, U.S.A. and were analytical grade. Indole-3-acetic acid (IAA) was purchased from Sigma Chemical Company, St. Louis, Mo., U.S.A.; ^{14}C-sodium bicarbonate (57 mCi/m Mole), L-[U-^{14}C]- malic acid (58.3 mCi/m Mole) and NCS tissue solubilizer were purchased from Amersham/Searle Corporation; ethanolamine was purchased from Packard Instrument Company, Montreal, Quebec; all photographic materials were purchased from Eastman Kodak (Canada) Limited, Toronto, Ontario, and gas mixtures were purchased from Linde Speciality Gases, Oakville, Ontario.

C. Reagents

a) Potassium phosphate buffer, 10 mM

i) 10 mM KH_2PO_4 solution

1.36 g of potassium dihydrogen phosphate was dissolved in and made up to one litre with distilled water

ii) 10 mM K_2HPO_4 solution

1.742 g of dipotassium hydrogen phosphate was dissolved in and made up to one litre with distilled water.

Solution (i) and solution (ii) were mixed together with constant stirring until the pH of the solution was 7.0.

b) IAA stock solution

0.175 g of indole-3-acetic acid was dissolved in 10 ml of 80% (v/v) ethanol in phosphate buffer (10 mM, pH 7.0) to give a final concentration of 10 mM IAA. The stock IAA solution was stored in a light tight flask at $4^{o}C$ and was prepared fresh weekly in order to maintain a stable IAA solution.

c) Malate stock solution

0.670 g of L-malic acid was dissolved in phosphate buffer. The pH of the solution was corrected to 7.0 with 1 N KOH and the final volume made up to 500 ml with phosphate buffer (10 mM, pH 7.0). The final concentration of the malate stock solution was 10 mM.

d) IAA working solution

1.0 ml of IAA stock solution was diluted 500 times with phosphate buffer (10 mM, pH 7.0) to give a final concentration of 20µM IAA.

e) Malate working solution

50 ml of malate stock solution was diluted to 500 ml with phosphate buffer (10 mM, pH 7.0) to give a final concentration of 1.0 mM malate.

f) IAA and malate working solution

1.0 ml of IAA stock solution and 50 ml of malate stock solution were diluted to 500 ml with phosphate buffer (10 mM, pH 7.0) to give a final concentration of 20µM IAA and 1.0 mM malate.

g) Radioactive solutions

i) ^{14}C-bicarbonate solution

The 5 ml stock radioactive ^{14}C-bicarbonate solution (57 mCi/m Mole was added to a 250 ml volumetric flask containing phosphate buffer (10 mM, pH 7.0). The vial was repeatedly rinsed with buffer and the contents added to the flask. The ^{14}C-bicarbonate was made up to volume with phosphate buffer. The working solution was then divided equally into four 250 ml flasks, stoppered tightly and placed in the freezer until ready for use. Before use the flask was thawed and triplicate 100 µl samples of the working ^{14}C-bicarbonate solution prepared for radioactive counting. Initial activity of each flask was determined to be 8.0 million dpm per ml of solution. The pH of the final solution was 7.0.

ii) L-[U-^{14}C]-malic acid solution

The contents of the vial of the stock L-[U-^{14}C]-malic acid (58.3 mCi/m Mole) were added to a 100 ml volumetric flask containing phosphate buffer (10 mM, pH 7.0). The stock vial was repeatedly rinsed with buffer and the volume made up to 100 ml. The working radioactive malate solution was divided equally into two 250 ml flasks and kept tightly sealed in the cold (4^{o}C). Prior to use the working solution was warmed to room temperature and triplicate 100 µl samples were prepared for radioactive counting. The initial activity of the stock radioactive malate was determined to be 1.1 million dpm per ml of solution. The pH of the final solution was 7.0.

9) Radioactive standards

i) ^{14}C-glucose solution

One ml stock solution (^{14}C-uniformly labelled glucose in water sealed in) 250 ml aqueous ethanol (ethanol 96% v/v) 750 ml water (CO$_2$ free, i.e. by boiling). Store solution in dark under N$_2$ in the cold. Use ^{14}C-glucose solution to be diluted with ethanol (96% v/v) and water (CO$_2$ free) in the ratio 1:3:9 (v/v/v). Store solution in the dark under N$_2$ in the cold. Use ^{14}C-glucose solution to be diluted with ethanol (96% v/v) and water (CO$_2$ free) in the ratio 1:3:9 (v/v/v). Store solution in the dark under N$_2$ in the cold.

ii) ^{14}C-{^{14}C}-sucrose solution

One ml stock solution (^{14}C-uniformly labelled sucrose in water sealed in) 250 ml aqueous ethanol (ethanol 96% v/v) 750 ml water (CO$_2$ free, i.e. by boiling). Store solution in dark under N$_2$ in the cold. Use ^{14}C-{^{14}C}-sucrose solution to be diluted with ethanol (96% v/v) and water (CO$_2$ free) in the ratio 1:3:9 (v/v/v). Store solution in the dark under N$_2$ in the cold. Use ^{14}C-{^{14}C}-sucrose solution to be diluted with ethanol (96% v/v) and water (CO$_2$ free) in the ratio 1:3:9 (v/v/v). Store solution in the dark under N$_2$ in the cold.

h) Scintillation solution I

This solution was prepared by dissolving, with rapid stirring, 5.0 grams PPO (2,5-diphenyloxazole) and 0.3 grams POPOP (1,4-di-2-5-phenyl-oxazolyl)-benzene in 1 litre of scintillation grade toluene. This solution was routinely used in all radioactive counting procedures.

2 - 2 METHODS

A. Growing and Harvesting of Coleoptiles

Seeds of Avena sativa L. var 'Victory' were husked and soaked for a minimum period of four hours, with stirring, in de-ionized water. The seeds were placed on tissue covering a mixture of vermiculite and water (1:1.5, V:V). The seeds were regularly spaced, about 10 mm apart, with the embryo uppermost and were incubated in moist atmospheric conditions at 25°C under weak red light for approximately 72 hours.

Coleoptiles between 25 and 30 mm were selected and harvested under weak green light. A cutting device was employed that removed the apical 3 mm tip from the coleoptiles and the basal portion nearest the mesocotyl, yielding uniform 20 mm coleoptile sections. The inner leaf was withdrawn from each coleoptile from the basal end. This procedure left the first 10 mm from the cut apex undamaged.

For growth studies the intact apical 10 mm sections were cut off the 20 mm sections and placed in a beaker of phosphate buffer (10 mM, pH 7.0) until sufficient numbers of coleoptile sections were obtained. For all other experiments, the entire 20 mm defoliated sections were placed in light tight, pre-weighed vials containing 5.0 ml phosphate buffer (10 mM, pH 7.0) in sufficient numbers for each type of experiment. The vials were capped, weighed and the tissue fresh weights per vial recorded.

B. Growth Studies

a) Growth Measurement Apparatus

Elongation of ten 10 mm coleoptile sections was measured, using the

high resolution continuous recording technique of Evans and Ray (1969). The growth chamber (Figure 2) and the growth recording apparatus were mounted on an optical bench for stability.

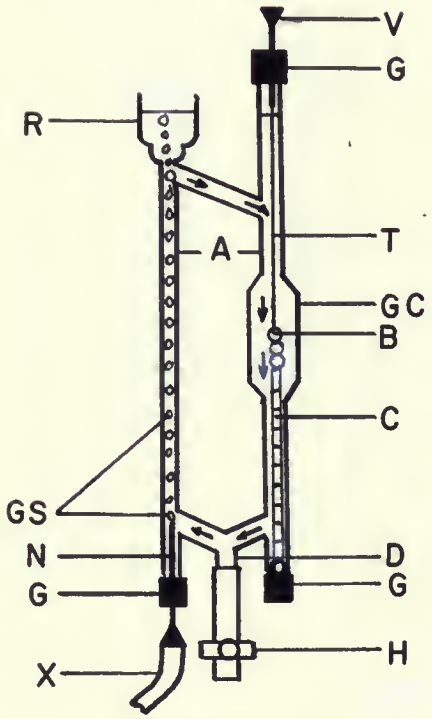
During growth recording, a narrow divergent cone of light passed through the growth chamber from the light source (Figure 3). A baffle (E) with a vertical slit (U) 2 mm wide was placed approximately one metre from the stand supporting the growth chamber. Behind the baffle was located a multispeed kymograph and drum (K) with a strip of Kodak Polycontrast F photographic paper (F) attached. The kymograph moved the photographic paper slowly past the slit. The speed of travel of the paper was set between 5 to 8 mm per minute. Whenever possible the speed of the drum was measured at the beginning and at the end of each recording.

The divergent light emitted from the source of illumination was limited by the iris diaphragm so that a narrow cone of light passed through the growth chamber (GC) and fell upon the beads (B), but not directly on the coleoptile sections. The growth of the column of coleoptile sections resulted in the beads being pushed upwards, interrupting the cone of light and casting a sharp shadow of the beads on the photographic paper behind the slit as shown in Figure 3. The light exposed that portion of the photographic paper that lay above the edge of the shadow. As the segments grew, this boundary moved upward and traced a record of the elongation which was visualized after the development of the photographic paper. Events were marked on the paper by covering the slit for the time necessary to effect either solution or aeration changes. These changes were made within 15 - 30 seconds.

Growth measurement chamber

This diagram illustrates the growth chamber used in the growth experiments. The chamber was made of glass and was designed as shown, with the ten 10 mm coleoptiles inserted. The various parts are: R - reservoir for filling the chamber that also serves as a gas exit vent; A - glass tubing; GC - square glass growth chamber; D - drain; H - drain clamp; G - self-sealing rubber syrings plugs; X - gas line; N - syringe needle for introducing aeration; GS - gas stream; B - plastic bead; T - nylon thread; C - coleoptile section; V - needle vent to prevent any excess gas build-up in glass tubing. The arrows indicate the flow of the buffered medium throughout the chamber.

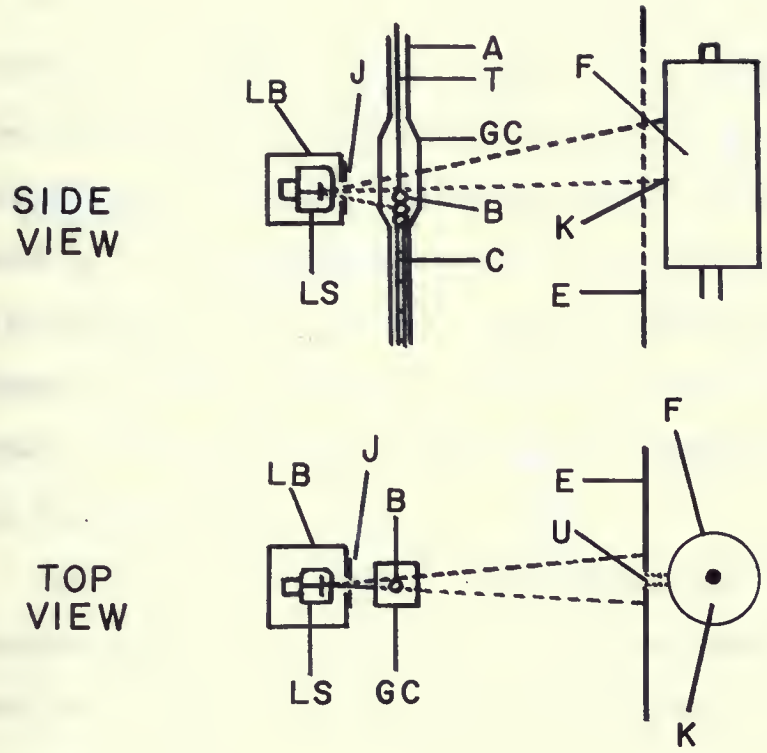
FIGURE 2



Schematic diagram of the shadowgraphic growth recording apparatus (not to scale).

The various parts of the apparatus were mounted on an optical bench. The parts of the growth chamber are indicated as for figure 1. Other parts of interest are: LS - light source, LB - light box; J - iris diaphragm; K - kymograph drum; F - photographic paper; E - baffle; U - 2 mm wide slit in the baffle; both a side view and top view are shown. The dashed lines represent the light beam.

FIGURE 3



b) Growth Measurement Technique

Ten 10 mm hollow coleoptile sections were threaded onto a piece of fine nylon thread about 40 cm in length that had been passed through a self-sealing rubber stopper. Care was taken to ensure that each coleoptile section was strung on the thread with its apical region uppermost. Three opaque plastic beads, weighing a total of 0.14 grams were threaded on top of the column of coleoptile sections and the complete unit was inserted into the growth chamber as shown in Figure 2. Phosphate buffer (10 mM, pH 7.0) was added to the chamber and aeration begun to provide circulation of the buffered medium.

The tissue was allowed to equilibrate in this manner for a minimum period of 45 - 60 minutes. This preconditioning was necessary as both Evans and Ray (1969) and Rayle and Cleland (1970a) reported a period of rapid elongation that results from tactile stimulation during the initial handling of the tissue. This rapid period of elongation, which is known as the "tickle response", can last up to thirty minutes.

After the pre-incubation period, the light source was turned on, giving an intensity of 10 candle power at the slit in the baffle. The kymograph speed was set and measured by a series of timed exposures of the film to the light source.

The initial endogenous growth of the column of coleoptile sections was recorded over a ten minute period. Solution or aeration changes were made within 15 - 30 seconds. The growth was again recorded using one of the following methods: either continuously for a period

of 30 - 40 minutes, or for a ten minute period 30 minutes after the change in the initial growth conditions was made. Subsequent repetitions of the second method of growth recording allowed for repeated solution changes and subsequent growth records to be made on one batch of tissue on the one photographic paper. It was noted that solution and aeration changes themselves did not result in any growth stimulation.

The growth magnification factor was determined at the end of each experiment by moving the growth chamber and consequently the beads up or down in a series of 1 mm steps using a vertically calibrated screw-apparatus on which the chamber was mounted. This caused a vertical displacement of the shadow boundary by an amount dependent on the magnification factor. This calibration allowed for the calculation of absolute elongation rates. The magnification factor was 15 or 20 times.

All procedures during measurement were carried out under photographic safe-light conditions using a 15 watt CGE appliance light bulb in a light tight box equipped with a Kodak OC Series light amber safe-light filter.

c) Development of the Photographic Record:

After each run, the strip of photographic paper was developed for 1.5 to 2 minutes in Kodak Dektol Developer, drained, placed in Kodak Universal Stop Bath for 10 to 15 seconds to stop development, drained, placed in Kodak Fix for 5 minutes and finally rinsed in a flowing water bath at room temperature.

The photographic record was dried and the growth curve traced,

as accurately as possible on lined metric tracing paper. The calibrations for time and magnification factors were duly noted as well as conditions under which growth measurements were carried out.

d) Treatment of Data

The growth rates, response times and duration of responses were calculated from the tracings. The promotion and inhibition times for growth responses were calculated using the method of Cleland (1971) as shown in the representative growth curve in Figure 4. All growth curves presented are representative curves that have the same vertical scale representing elongation as the original photographic record. The horizontal scale, however, has been reduced to a standard of 2 mm per minute of actual growth recording. Placing all the graphs on the same time scale standardizes comparisons much more readily than some of the original tracings and results in considerable reduction of the width of the original recordings.

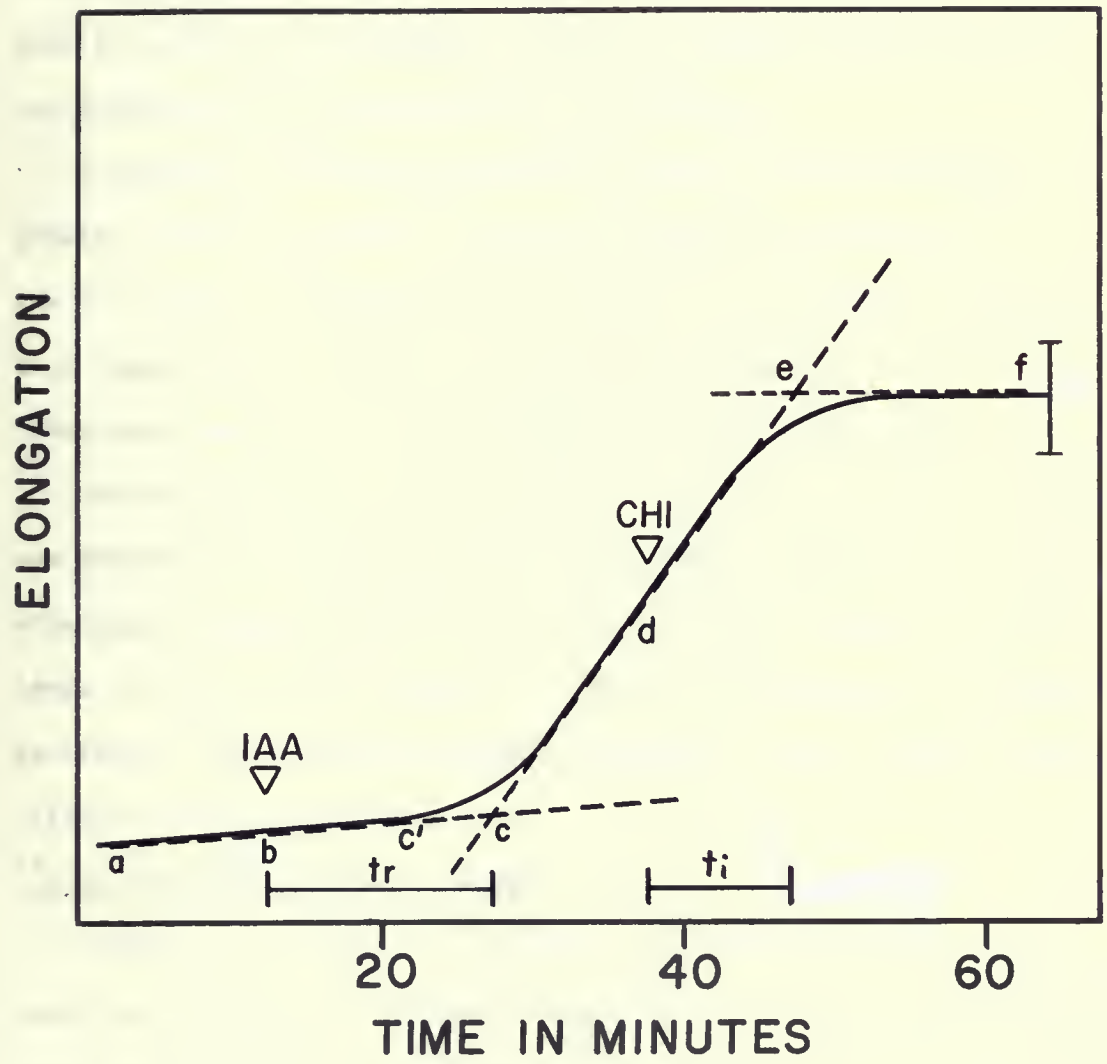
The method resulted in a continuous recording of the magnified cumulative growth of ten 10 mm coleoptile sections against time and all growth data are expressed as mm per hour per 100 mm column of coleoptile sections and the mean ± the standard error of at least five different growth experiments.

Day to day variability was evident between different batches of coleoptile tissue, especially in the measured initial growth rates. Each series of experiments was repeated a minimum of five times. The order of experiments within a series was rotated from day to day so that any possible bias in growth rates that might occur due to their order of performance after initial harvesting could be minimized.

Typical growth record showing the method for calculating response times and growth rates

The following are measured for each growth record: the growth rates before auxin addition (abc), after auxin stimulation of growth (cde), and after cycloheximide inhibition of growth (ef); the response time t_r; the inhibition time t_i. The lag period prior to the initiation of a response is designated by bc^1. The times of addition of IAA and cycloheximide are denoted by the arrows. The magnification factor is denoted by the vertical bar at the end of the growth record and represents 1 mm of elongation of the entire colume of ten 10 mm coleoptile sections. t_r = b-c ; t_i = d-e.

FIGURE 4



The differences observed in mean initial growth rates between batches of tissue were analyzed graphically (Appendix A). This method shows the mean initial growth rates per batch of tissue with their 95% confidence intervals about the mean. The 95% confidence interval represents two standard errors about the mean value. If overlapping occurs in the confidence interval for any of the batches then the differences observed in the mean initial growth rates are not significant at the 95% level of confidence.

Differences between mean initial growth rates and mean final growth rates were analyzed using the Student's t distribution at the 95% level of confidence to determine any significant differences that resulted from changes in experimental conditions. All calculations were performed on a programmable Wang 2200 calculator.

During preliminary growth studies, the pH of the buffered medium was monitored during the course of the growth experiments. It was subsequently noted that there was no significant change in the pH level of the buffered medium as a result of the growth of the tissue sections, during subsequent solution changes, or as a result of the different aeration mixtures used.

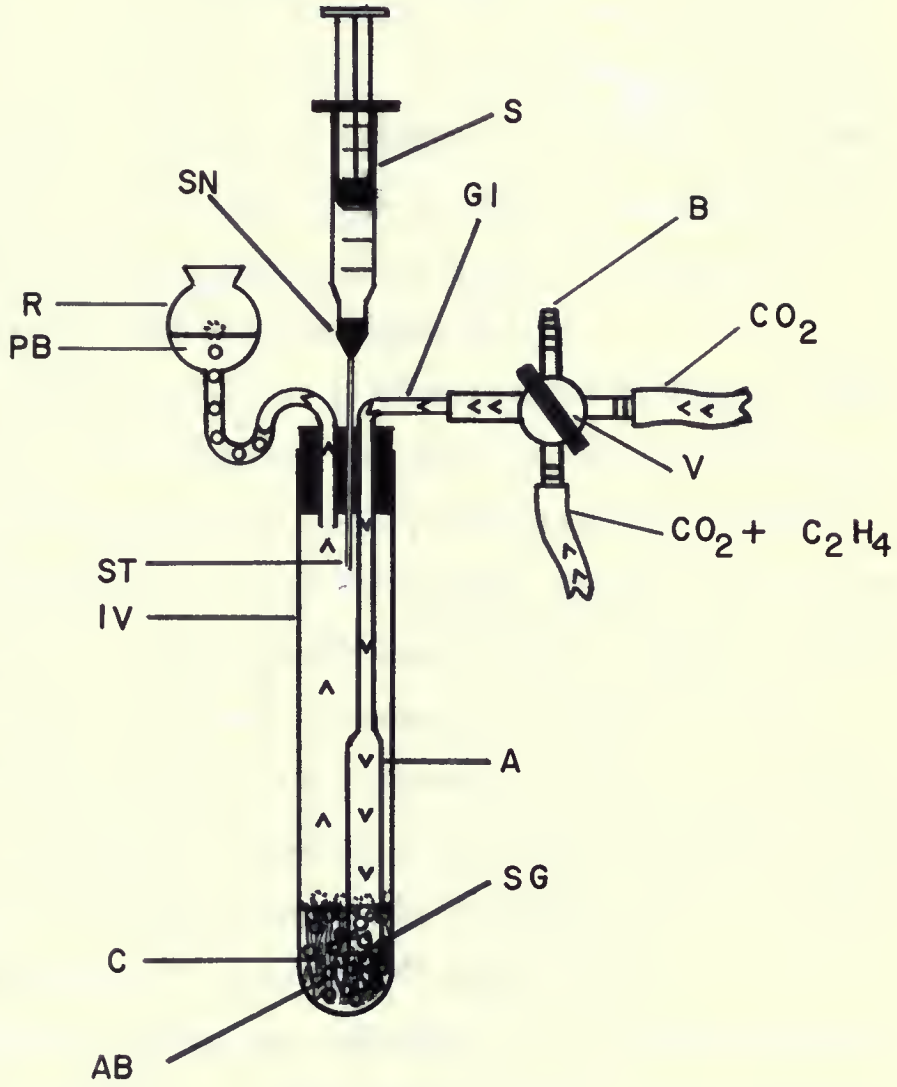
C. ^{14}C-Bicarbonate Fixation Studies

Sixteen 20 mm coleoptile sections were harvested as previously mentioned, weighed and placed in light tight 25 mm diameter incubation tubes containing 9.0 ml phosphate buffer (10 mM, pH 7.0). Each incubation tube was equipped with inlet and outlet tubes for aeration of the buffer and a syringe needle permanently mounted in the tube stopper for the introduction of 1.0 ml of test substance or ^{14}C-bicarbonate solution as shown in Figure 5.

Diagram of the incubation vessel for the study of the influence of various growth regulators on the incorporation and fixation of ^{14}C-bicarbonate by Avena coleoptiles.

The incubation vessel (IV) consisted of a light tight 25 x 150 mm boiling tube with a two-hole rubber stopper RS equipped with inlet (GI) and outlet tubes (R). The inlet tube was expanded to form an aerator (A) that contacted the buffer solution and was equipped with a scintered glass end (SG) to allow for vigorous aeration of the phosphate buffer medium (AB) and the coleoptile sections (C). There was a four way flow valve (V) attached to the inlet tube. This allowed for the rapid changing of gas mixtures by a simple turning of the valve. When flow of one gas mixture was discontinued it was redirected out of the by-pass valve, while the other gas mixture was being simultaneously introduced into the vessel. The outlet tube was fitted with a thistle-tube reservoir which contained phosphate buffer (10 mM, pH 7.0) that acted both as a visual flow regulator (as shown) and as a plug to form a closed system prior to the introduction of the ^{14}C-bicarbonate solution. A syringe needle (SN) was permanently mounted in the rubber stopper (RS). This allowed for the introduction of various test substances to the aerating system as well as the ^{14}C-bicarbonate solution.

FIGURE 5



After a one-hour incubation, with gentle shaking, at 25°C, test substances were introduced by injection through the needle or by changing the aerating gas. After 2 or 30 minutes incubation with the test substance, aeration was stopped, ^{14}C-bicarbonate injected into the system and the closed system incubated for a further six minutes. Incubation was terminated by rinsing the tissue in a suction funnel with 500 ml of distilled water.

Washed tissue was placed in 10 ml of cold 90% acidified ethanol (0.01 N HCl) in a scintillation vial which was agitated gently at 4°C for 18 hours whilst a gas stream removed radioactive carbon dioxide for trapping in 6 N KOH. The soluble fraction was collected and combined with a second soluble fraction that resulted from homogenization of the tissue with a further 5 ml of cold acidified ethanol and centrifugation of the homogenate at 500 g in a clinical centrifuge. Duplicate 1 ml samples of the combined soluble fractions were taken for radioactive counting in a scintillation vial containing 5 ml methanol and 10 ml of Scintillation fluid I.

The insoluble fraction was suspended in 3 ml of Amersham/Searle NCS tissue solubilizer in a scintillation vial, tightly capped and maintained at 48°C for a minimum 48 hours before addition of 5 ml methanol and 10 ml Scintillation fluid I for subsequent radioactive counting.

The ability of the cold acidified ethanol to remove all excess unfixed ^{14}C-bicarbonate was tested by rapid addition of 7.5×10^6 dpm of ^{14}C-bicarbonate to 10 ml acidified ethanol in scintillation vials and shaking gently at 4°C. Duplicate 100µl samples were

removed at timed intervals and immediately placed in scintillation vials containing 1 ml ethanolamine.

Five ml methanol and 10 ml scintillation fluid I were added before radioactive counting. These tests demonstrated that less than 0.02% of the unfixed bicarbonate remained after only one hour in the acidified ethanol.

Tests designed to determine the kinetics of extraction of label from the tissue involved removing acidified ethanol samples at regular intervals over a 48 hour period to determine when the maximum level of radioactivity was attained in the first soluble extract. Figure 6 shows that after 12 - 15 hours extraction the level of radioactivity in the acidified ethanol had reached a plateau and remained constant up to 48 hours. Eighteen hour extraction periods were subsequently used routinely.

After radioactive counting of both the soluble and insoluble fractions it was found that 96 to 98% of the fixed radioactivity was found in the soluble fraction and 2 to 4% in the insoluble fraction.

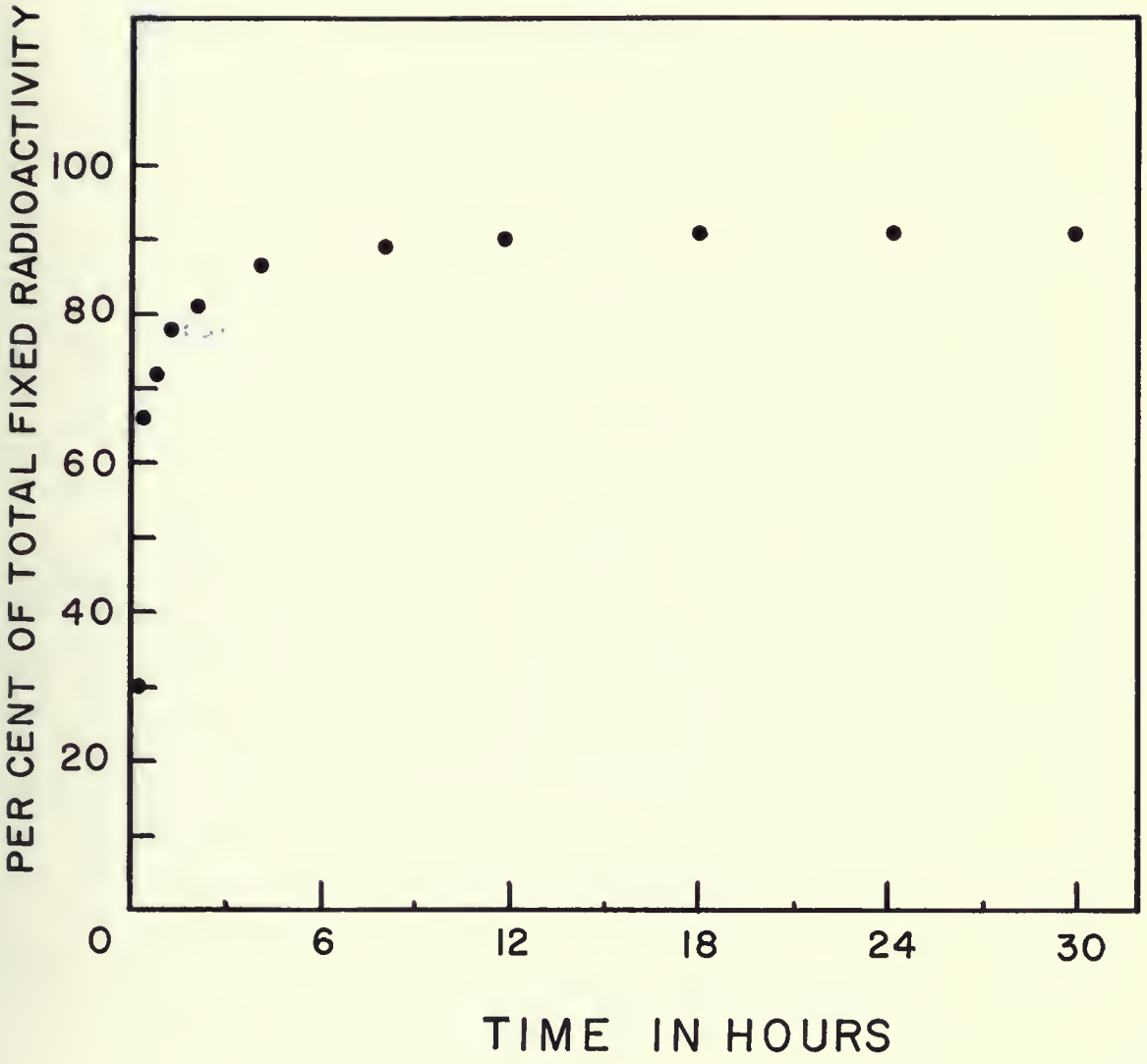
The kinetics of bicarbonate fixation were determined by removing duplicate tissue samples from the ^{14}C-bicarbonate solution at 3 minute intervals and extracting them as above. The kinetics of ^{14}C-bicarbonate fixation (Figure 7) showed linearity for at least 9 minutes. A six minute fixation period was subsequently used routinely.

A further test of the ^{14}C-bicarbonate fixation process involved heating the tissue for 30 minutes at 95^{o}C. This treatment resulted in a 95% inhibition of fixation, indicating the involvement of a

Kinetics of extraction of ^{14}C-label from Avena coleoptile tissue by cold, acidified 90% ethanol (0.01 N HCl).

Batches of coleoptile tissue were prepared for ^{14}C-bicarbonate fixation studies as outlined in Methods. Tests designed to determine the kinetics of extraction of the label from the tissue involved removing duplicate 100 µl samples of the acidified ethanol at regular intervals over a 48 hour period. The samples were prepared for radioactive counting by addition to scintillation vials containing 5 ml. methanol and 10 ml scintillation fluid I. The level of ^{14}C-label for each sample of the first soluble extract was determined and was expressed as a per cent of the total fixed radioactivity. The data was plotted as per cent of total fixed radioactivity for each sampling time in hours. After 12-15 hours of extraction the level of radioactivity in the acidified ethanol had reached a plateau. This remained constant up to 48 hours. Eighteen hour extraction periods were subsequently used routinely.

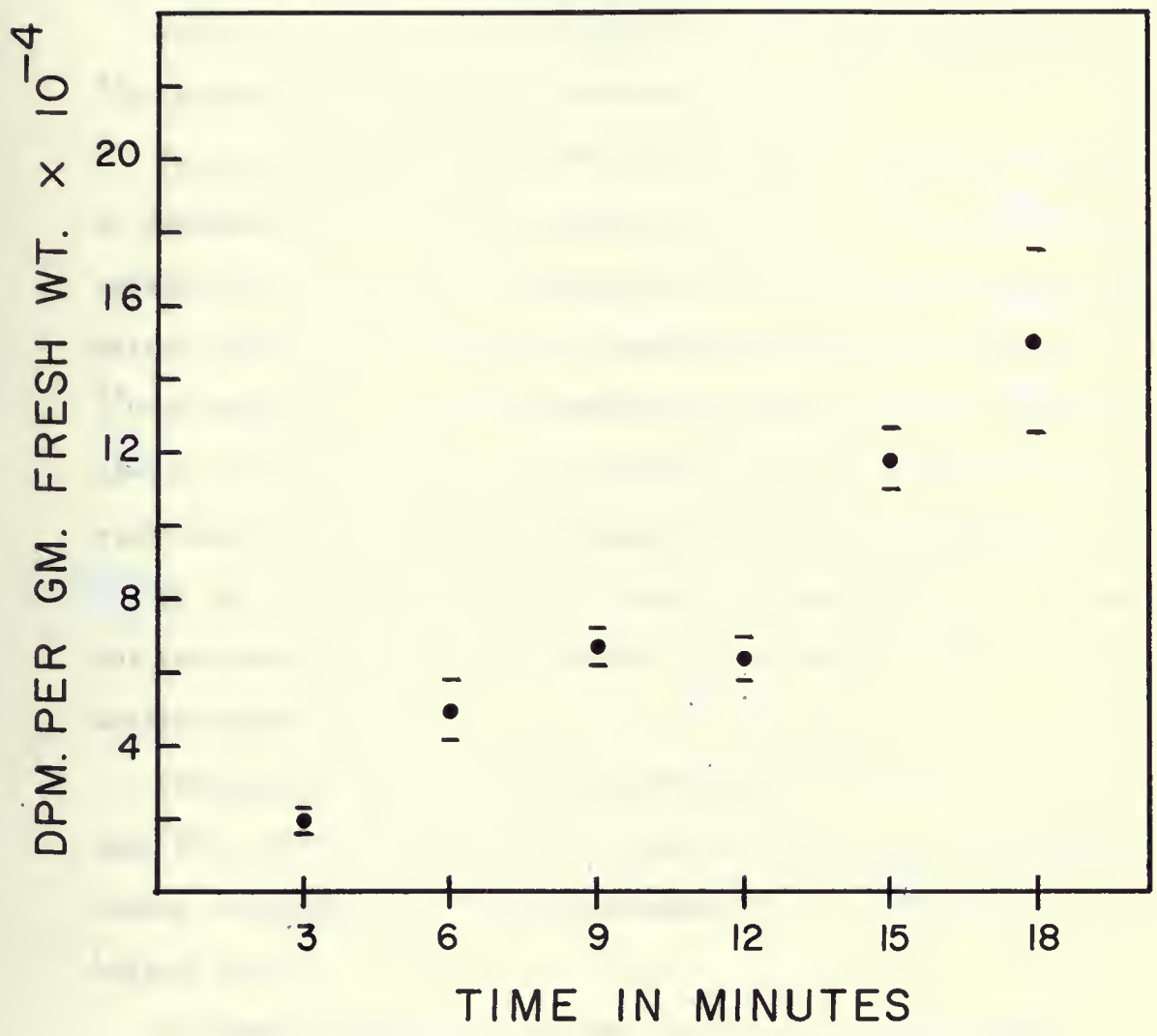
FIGURE 6


PER CENT OF TOTAL FIXED RADIOACTIVITY
100
80
60
40
20
0
6
12
18
24
30
TIME IN HOURS

Kinetics of dark fixation of ^{14}C-bicarbonate in Avena coleoptile tissue.

Batches of 0.25 g of coleoptile tissue in phosphate buffer (10 mM, pH 7.0) were incubated with aeration containing 320 µl/l CO_2 for one hour. Aeration was stopped and 5-7 x 10^6 dpm of ^{14}C-bicarbonate injected into the closed system. Duplicate tissue samples were removed from the ^{14}C-bicarbonate solution at 3 minute intervals and extracted as outlined in Methods. The data was expressed as dpm per gram tissue fresh weight and plotted against the time in minutes of incubation in ^{14}C-bicarbonate. The plotted results represent the mean (•) ± the standard error (=) of five separate experiments.

FIGURE 7



heat labile, metabolic process. All data were expressed as dpm fixed per gram fresh weight of coleoptile tissue.

D. ^{14}C-Bicarbonate Incorporation Studies

Batches of twenty 20 mm coleoptile sections were incubated in ^{14}C-bicarbonate and rinsed as described previously.

Ten sections were removed and extracted as previously described to determine levels of ^{14}C-bicarbonate fixation. The remaining ten coleoptile sections were rinsed again and water injected through the hollow centre of the sections to remove remaining unincorporated ^{14}C-bicarbonate. Pairs of coleoptile sections were then immediately placed in scintillation vials containing 2 ml of ethanolamine, the vials were tightly capped and allowed to sit at room temperature (25^{o}C) for a minimum period of 24 hours. The material was prepared for radioactive counting by addition of 5 ml methanol and 10 ml of scintillation fluid I.

Subsequent removal of unsolubilized cellular debris indicated that 98 to 99% of the incorporated radioactivity was in the ethanolamine or soluble phase and approximately 1% in the debris or insoluble phase.

All data were expressed as dpm incorporated per gram fresh weight of coleoptile tissue.

E. Analysis of ^{14}C-Fixation and Incorporation Data

After radioactive counting with a Model 3310 Packard Tri-Carb Liquid Scintillation Spectrometer the data were expressed as dpm per gram fresh weight of tissue. All experiments involved an investigation of the influence of some test substance on the rate of

^{14}C-fixation and/or incorporation by incubation of 6 tubes of tissue in the presence and 6 in the absence of the test substance. After exposure to the control and experimental conditions for the specified time interval, ^{14}C-bicarbonate was added for a further 6 minutes incubation.

The mean and standard error were calculated for both the control and experimental groups of data, and one way analysis of variance was used as the statistical test to determine the probability of there being a significant difference between these two groups of data. One way analysis of variance allowed for comparison of values obtained for the six vials in either the test or control experiments against their means as well as a comparison between the means and standard errors of the test and control groups. The results of the data analysis were reported at the 0.95 confidence limit.

A sample result of one such fixation experiment and the subsequent treatment of the data and statistical analysis of that data are included in Appendix B. All other incorporation and fixation data show the mean ± standard error for six samples in either the control or experimental groups for any one experiment, and the results of the statistical analysis. All mathematical manipulations were performed on a Wang 2200 programmable calculator.

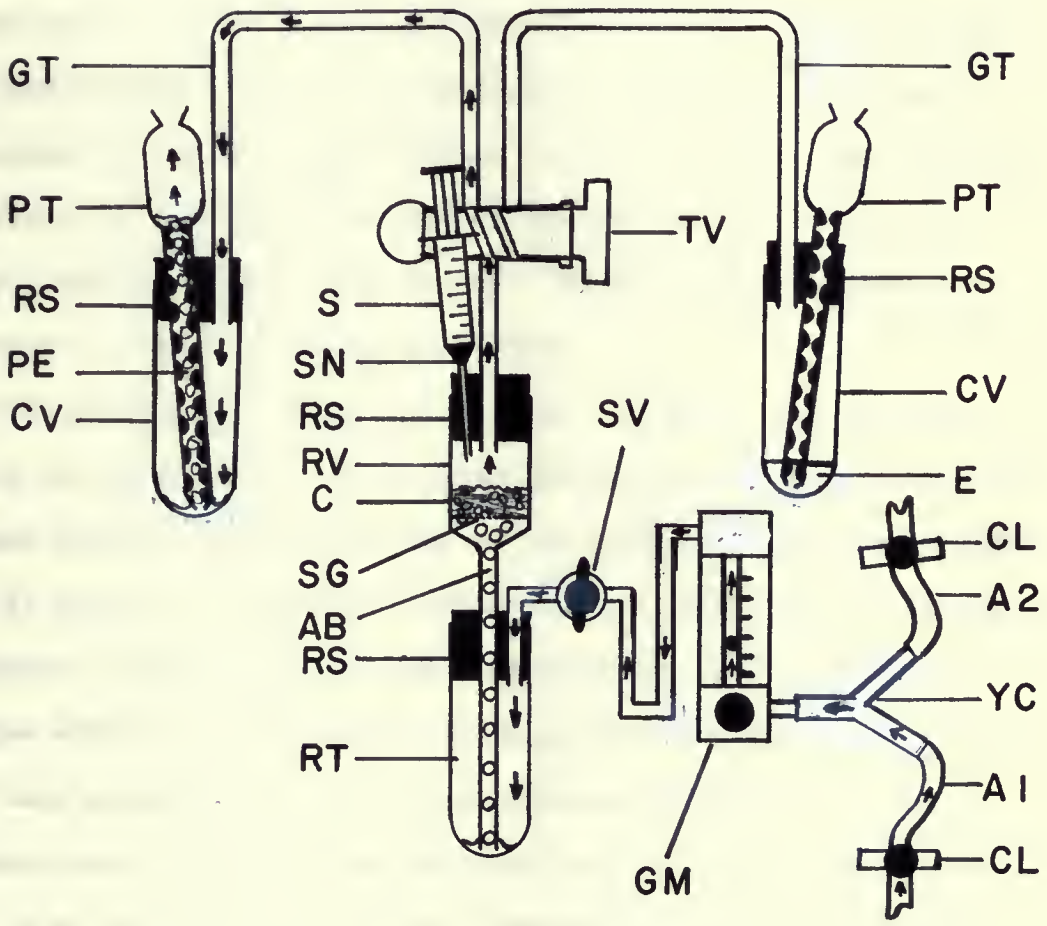
F. Studies on the Decarboxylation of ^{14}C-Malate

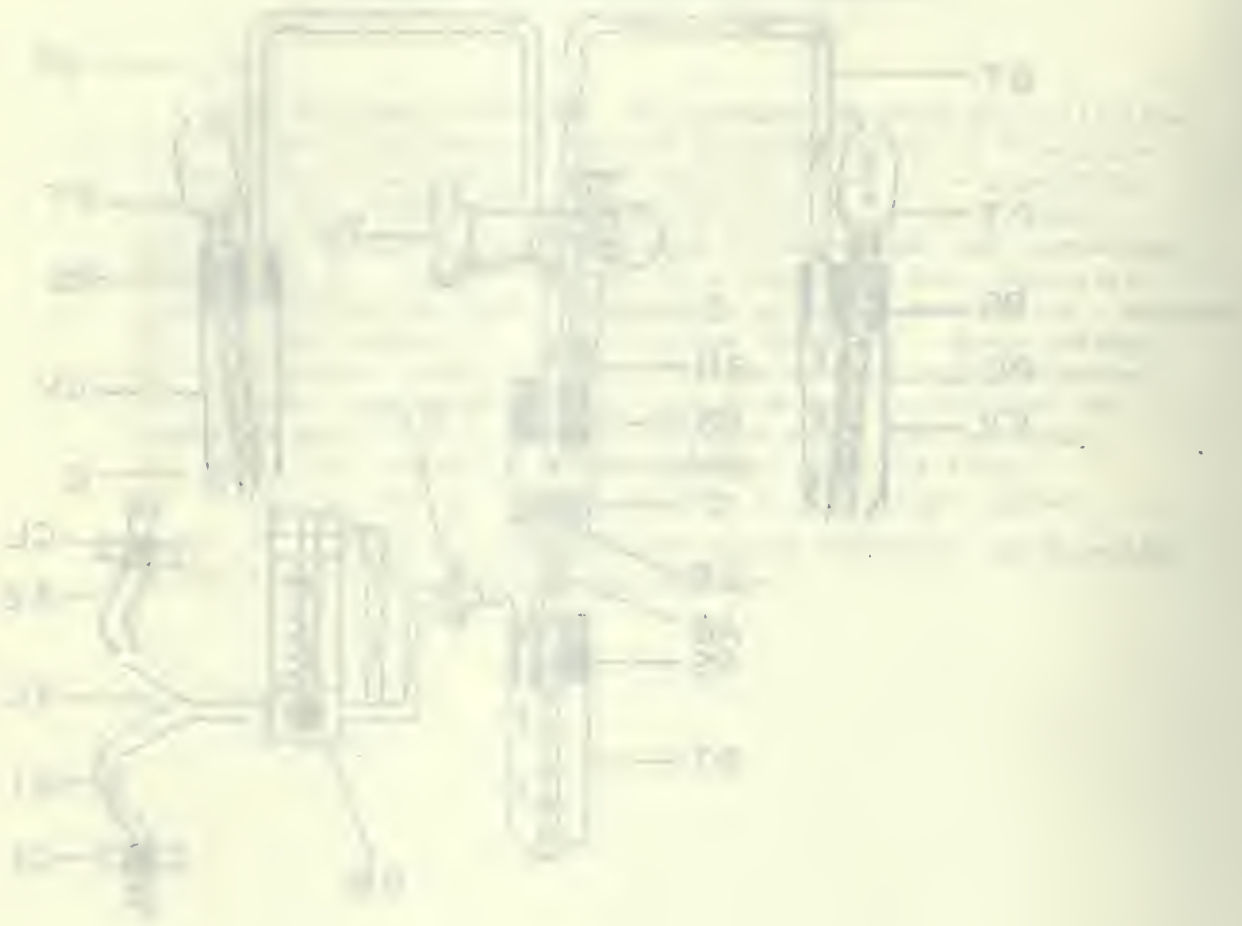
Batches of forty 20 mm coleoptiles were harvested and weighed in light tight vials as previously mentioned. For each experiment the tissue was placed in the upper portion of the light tight reaction vessel (Figure 8). To the bottom tube was added 10 ml of

Diagram of $^{14}CO_2$ collection apparatus

This diagram illustrates the apparatus used in the collection of $^{14}CO_2$ that was released through decarboxylation of ^{14}C-malate by the coleoptile tissue. RV - reaction ressel; C - coleoptile sections; SG - scintered glass platform on which tissue rests and is continually bathed in aerated buffer medium (AB) containing ^{14}C-malate; RS - rubber stopper; RT - reaction tube - initially contains buffer and radioactive malate prior to aeration; S - syringe; SN - syringe needle; TV - two way flow value; GT - glass tubing; CV - collection vessel; PT - percolation tube; E - ethanolamine; SV - single value stopcock; GM - gas flow meter calibrated for measuring glas flow in units of standard cubic feet per hour. This meter is calibrated in 0.2 increment units of SCFH; CL - clamp; A_1 and A_2 - gas lines from different gas mixture tanks; YC - Y junction tubing. The arrows indicated the direction of the flow of the aerating gas mixtures.

FIGURE 8


GT
PT
RS
PE
CV
S
SN
RS
RV
C
SG
AB
RS
RT
TV
SV
GM
GT
PT
RS
CV
E
CL
A2
YC
A1
CL



of phosphate buffer (10 mM, pH 7.0) containing 2.3 to 2.5 X 10^6 dpm of L-[U-^{14}C]-malate (58.3 mCi/m Mole) in the presence or absence of 20μM IAA. Aeration was begun with normal air (300μl/litre carbon dioxide), and the system allowed to equilibrate for a minimum period of one hour. The reaction vessel was transferred to the collection apparatus (Figure 8) and $^{14}CO_2$ collection carried out continuously by passage of the air stream through 2.0 ml of ethanolamine, a highly efficient CO_2 absorber. The flow of the carrier gas was altered every 3 minutes from one side of the $^{14}CO_2$ collection apparatus to the other by way of a two-way flow valve.

Five successive 3 minute collections were made. The test substance was injected into the reaction vessel through the permanently mounted syringe needle in the top of the vessel or by changing the gas flow by way of a two-way valve connecting the different gas mixture cylinders. This addition of the test substance or gas was accomplished within five seconds and ten successive 3 minute collections were made subsequently. After each 3 minute collection 5 ml of methanol were washed down the bubbling tube into the collection vial, then 10 ml of Scintillation fluid I and the entire contents of the collection vial were added to a scintillation vial for radioactive counting.

Each bubbling tube was again rinsed with methanol before attaching the next collection vial. Tests indicated that no significant amount of radioactivity was found in the bubbling tube after this second rinse.

When $^{14}CO_2$ collection was complete, the aeration was stopped, the final volume of the remaining buffer solution was measured, triplicate 100µl samples of the buffer solution were prepared for radioactive counting by adding the samples to scintillation vials containing 5 ml methanol and 10 ml scintillation fluid I.

The coleoptiles were immediately separated into groups of five sections, washed with water and added to scintillation vials containing 2 ml ethanolamine; the vials were tightly capped and then stored at 25°C for 24 hours. The vials and tissue were prepared for radioactive counting by addition of 5 ml methanol and 10 ml scintillation fluid I. Removal of cellular debris after initial counting indicated that 70% of the ^{14}C-labelled compounds were in the soluble phase and 30% remained in the cell wall debris. Incorporation of the label from ^{14}C-malate into the coleoptiles was measured after an average labelling period of 2 hours for each experiment.

Several control tests were carried out in order to assess the capabilities of the apparatus for collection of $^{14}CO_2$. The speed of collection of $^{14}CO_2$ was tested by adding 5.0×10^6 dpm of ^{14}C-bicarbonate to the aerated reaction vessel containing 9.0 ml of acidified 90% ethanol (0.01 N HCl). From 88 to 95% of the added labelled $^{14}CO_2$ was collected in the first one-minute collection and by the end of 15 one-minute collections less than 0.08% of the total amount of ^{14}C label remained in the reaction vessel and 100% of the added label could be accounted for.

A further test of the system was made to determine whether there were significantly high background levels of radioactivity being collected when no tissue was present. This background level of radioactivity could be the result of the bubbling action of the gas flowing through the scintered glass platform of the reaction vessel. This agitation action could have an aerosol effect resulting in the formation of microdroplets of buffer solution containing radioactive malate. These could then pass in the gas stream from the reaction vessel to the collection vessel and become trapped in the ethanolamine. Each of the three reaction vessels was tested in the following manner for background levels of radioactivity.

Each reaction vessel was used in the normal experimental procedure with no tissue present and collections were made at three minute intervals over the normal course of the experiment. This enabled mean background rates of radioactivity to be determined for each vial. The evolution of radioactivity from the experimental system in the absence of plant material was linear after approximately 30 minutes at a flow rate of 0.6 SCFH at 25°C or tended to decline slightly. Therefore the initial three minute collections of $^{14}CO_2$ were begun after a one-hour equilibration period. The background rates of ^{14}C-label accounted for up to 25% of the total collected radioactivity found in the gas stream passing from the reaction vessels.

Observed differences were attributed to varying degrees of porosity of the scintered glass platforms of each reaction vessel. Therefore, each vessel had a different degree of aerosol production

that resulted in different observed background levels of radioactivity for each vessel.

Further tests were carried out to show that in the presence of coleoptile tissue, active metabolic processes were primarily responsible for the production of the $^{14}CO_2$ label that was being collected. Normal experimental procedures were carried out with tissue present. Several metabolic inhibitors were added in different experiments to observe the effects they might have on $^{14}CO_2$ production. 100 mM malonate, a respiratory inhibitor, resulted in approximately 75% reduction in the level of $^{14}CO_2$ being collected within a 10 minute period both in the presence and absence of IAA. 1 mM and 100 mM phenylsuccinate, an inhibitor of dicarboxylic acid (malate) transport resulted in approximately 10% and 35% reductions in the $^{14}CO_2$ that was collected in the presence of IAA.

G. Analysis of Data from ^{14}C-Malate Decarboxylation Studies

The data were recorded graphically as cumulative dpm evolved as $^{14}CO_2$ against time of collection as shown in Figure 13. Calculation of the rates of $^{14}CO_2$ evolved per minute per gram tissue fresh weight were determined from the slopes prior to and after the changes in experimental conditions. The results of the investigation of the influence of C_2H_4 on malate decarboxylation were analysed using one-way analysis of variance to determine the significance of differences between initial and final rates of $^{14}CO_2$ evolution for C_2H_4 treated and non-treated tissues at the 0.95 confidence level.

H. Respirometry Studies

An investigation of the influence of various test substances

on O_2 consumption, CO_2 evolution and the respiratory quotient (RQ value) was undertaken. This was done using the Gilson Differential Respirometer and the standard direct method as outlined by Umbreit, Burris and Stauffer (1964).

Eight batches of twenty 20 mm coleoptile sections were harvested and weighed as previously described. Each batch of tissue was placed in a Warburg Flask containing 3.0 ml of phosphate buffer (10 mM, pH 7.0); four of which contained the test compound, and four which contained none. Two test flasks and two control flasks had 0.2 ml of 6 N KOH added to fluted filter paper in the centre well and the other four flasks had none added.

All preparative procedures were carried out under weak green light. The light tight Warburg flasks were then attached to the respirometer, with the water bath set at 25°C. Measurements were taken at 5 minute intervals for a period of 45 to 60 minutes. Calculations were made to determine the O_2 consumption and CO_2 evolution in µl/hour/gram tissue fresh weight and the RQ values.

3. RESULTS

3-1 Growth data

In the presence of 320 µl/l carbon dioxide, 10.8 µl/l ethylene consistently reduced the growth rate of Avena coleoptile sections. This occurred both in the presence and absence of 20 µM IAA. Figure 9 shows typical growth curves for ethylene inhibition of growth. In the presence of 20 µM IAA, the inhibition times were 12 to 13 minutes, and in the absence of IAA, the inhibition times were 8 to 10 minutes. Table II reveals that the per cent inhibition, but not the absolute inhibition, of growth rate was greatest in the absence of IAA. The growth of control coleoptile sections that are not treated with ethylene are shown to proceed at a relatively linear rate of growth, both in the presence and absence of IAA (figure 9).

In contrast, Table III and Table IV demonstrate that in the absence of carbon dioxide,inhibition of growth rate by ethylene is only slightly greater than the normal slow decline in growth observed with time both in the absence and presence of 20 µM IAA respectively. Representative curves demonstrating the (antagonistic) nature of ethylene inhibition of carbon dioxide stimulated growth in the presence and absence of IAA are shown in figure 10. The application of ethylene with carbon dioxide blocks the usual growth response to the latter both in the absence (Table III) and presence (Table IV) of 20 µM IAA. The graphical analysis of observed differences in initial growth rates (Appendix A) reveals that no significant differences exist between the slightly varied mean initial growth rates for each type of experiment observed either in the presence (Table IV) or absence of 20 µM IAA (Table III).

Ethylene inhibition of coleoptile growth.

Carbon dioxide (320 µl/l) was present throughout, and ethylene (10.8 µl/l) was introduced into the aerating gas stream at the time indicated. Twenty µM IAA was present for the upper recording and absent for the lower recording. The bar indicates 1 mm elongation. The dotted lines represent the growth of coleoptile sections that were not treated with ethylene.

FIGURE 9

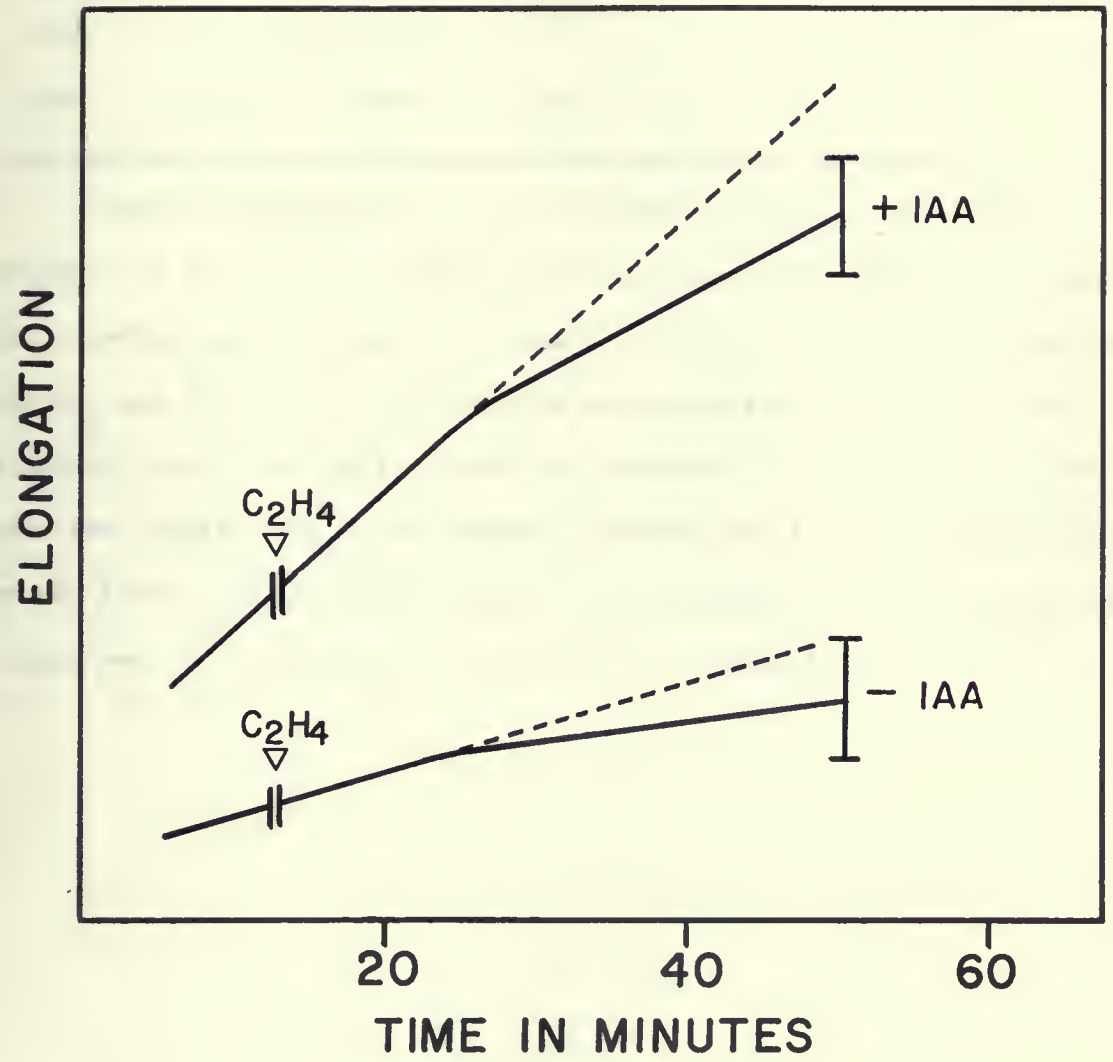


Table II Inhibition of growth by C_2H_4 in the presence and absence of IAA.

Treatment	Growth rate mm/hr/10cm of coleoptile		
	Initial rate CO_2	Final rate $CO_2 + C_2H_4$	Per cent Change
-IAA	1.56 ± 0.18	0.75 ± 0.13	-52 ($P < 0.001$)
+IAA	5.08 ± 0.46	4.07 ± 0.42	-20 ($P < 0.05$)

Tissue was incubated in pH 7.0 buffered medium in the absence or presence of 20 µM IAA. Initial and final rates were obtained by changing the aerating gas from one containing 320 µl/l CO_2 to one containing both the CO_2 and 10.8 µl/l C_2H_4. Results are expressed as the mean ± the standard error. The data obtained was analysed by the t-test to determine the significance of differences between mean initial and mean final growth rates. Results are expressed (in parentheses) after the per cent change as: P = probability level; N.S. = not significant.

Table III Antagonism between CO_2 and C_2H_4 in the regulation of growth in the absence of IAA.

Final Treatment	Growth rate mm/hr/10cm of coleoptile		Per cent Change
	Initial Rate	Final Rate	
	$-CO_2$ $-C_2H_4$		
no change	0.65 ± 0.17	0.60 ± 0.15	-8 (N.S.)[3]
+ C_2H_4	0.48 ± 0.09	0.42 ± 0.05	-12 (N.S.)[3]
+ CO_2	0.67 ± 0.15	1.58 ± 0.18	+138 (P < 0.001)
$+CO_2$ $+C_2H_4$	0.48 ± 0.09	0.48 ± 0.09	0 (N.S.)

Initial growth rates were obtained from tissue incubated in pH 7.0 buffered medium in the absence of CO_2 and C_2H_4. Final rates obtained after the introduction of 320 µl/l CO_2 and 10.8 µl/l C_2H_4 as indicated. Results expressed as the mean ± the standard error. The data obtained was analysed by the t-test to determine the significance of differences between mean initial and mean final growth rates. Results are expressed (in parentheses) after the per cent change as: P = probability level; N.S. = not significant.

[3] Control data also shown in Table VII

Table IV Antagonism between CO_2 and C_2H_4 in the regulation of growth in the presence of IAA.

Final Treatment	Growth rate mm/hr/10 cm of coleoptile Initial Rate $-CO_2$	$-C_2H_4$	Final Rate	Per Cent Change
No change	4.25 ± 0.50		4.23 ± 0.40	- <1% (N.S.) [4]
+ C_2H_4	4.67 ± 0.59		4.29 ± 0.33	- 8% (N.S.) [4]
+CO_2	3.04 ± 0.53		4.32 ± 0.40	+ 42% ($P < 0.05$)
+CO_2 +C_2H_4	4.86 ± 0.46		4.97 ± 0.38	+ 2% (N.S.)

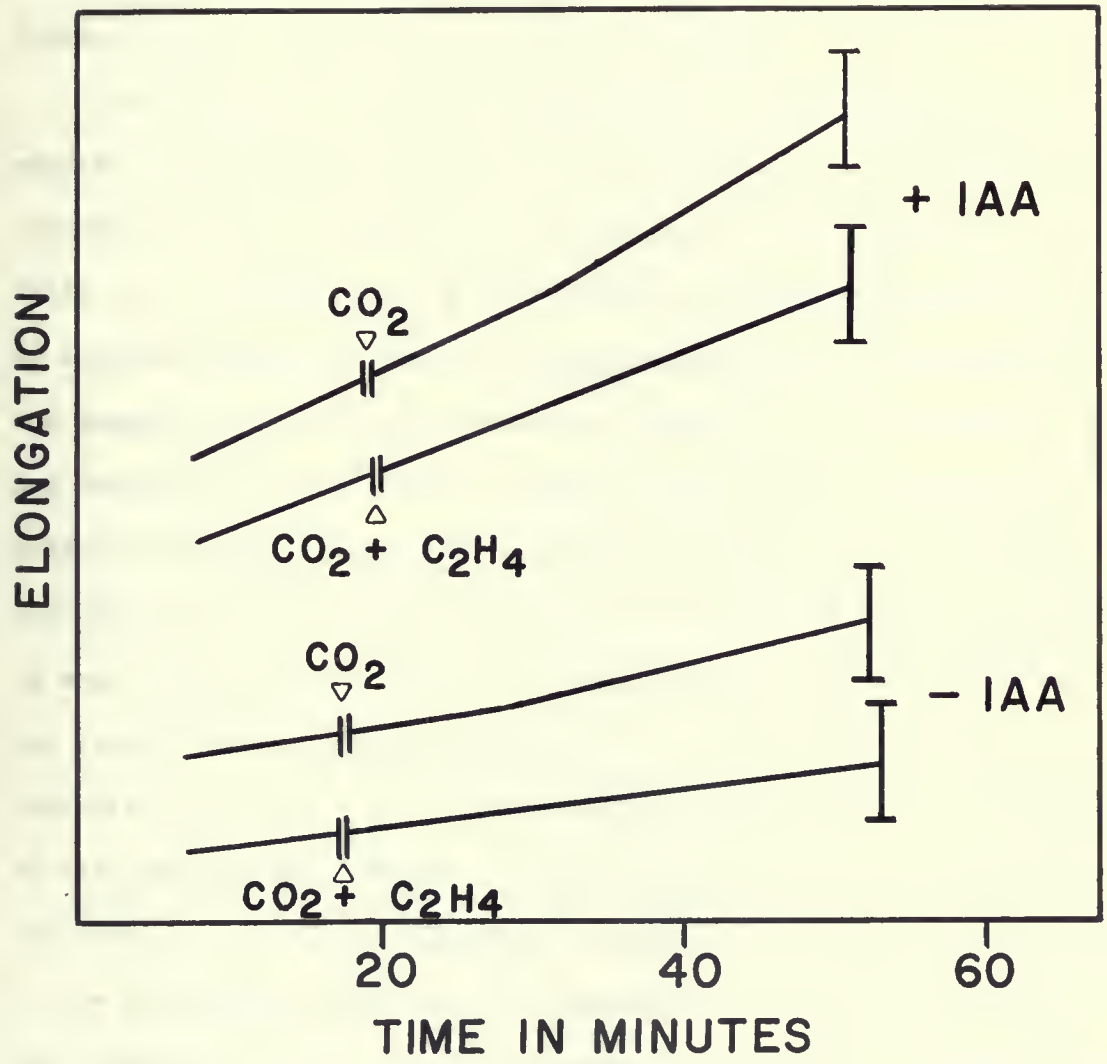
Initial growth rates were obtained from tissue incubated in pH 7.0 buffered medium in the absence of CO_2 and C_2H_4 and the presence of 20 µM IAA. Final rates obtained after the introduction of 320 µl/l CO_2 and 10.8 µl/l C_2H_4 as indicated. Results are expressed as the mean ± the standard error. The data obtained was analysed by the t-test to determine the significance of differences between the mean initial and mean final growth rates. Results are expressed (in parentheses) after the per cent change as: P= probability level; N.S.= not significant.

[4] Control data also shown in Table VIII

Ethylene inhibition of carbon dioxide stimulated growth.

Carbon dioxide was absent from the initial aerating gas stream and 20 μM IAA was present in the upper two curves and absent for the lower two curves. Carbon dioxide (320 μl/l) or carbon dioxide plus ethylene (10.8 μl/l) were introduced as indicated. The bar indicates 1 mm elongation.

FIGURE 10


ELONGATION
+ IAA
CO_2
CO_2 + C_2H_4
CO_2
- IAA
CO_2 + C_2H_4
20
40
60
TIME IN MINUTES

Growth rates obtained in the absence of carbon dioxide demonstrate that 1 mMmalate does not protect the tissue from ethylene inhibition, and that inhibition is actually greater when malate is present (Table V). This is observed both in the presence and absence of 20 μM IAA. The data in Table VI however, indicates that this phenomenon is dependent on the absence of carbon dioxide since there is the same per cent inhibition of growth observed in the presence of 320 μl/l carbon dioxide with or without addition of 1 mM malate.

The inhibition of malate stimulated growth when ethylene is applied simultaneously (Table VII) is analogous to the ethylene inhibition of carbon dioxide stimulated growth as indicated in Table III. Representative growth curves demonstrating the nature of ethylene inhibition of malate stimulated growth in the presence and absence of 20 μM IAA are shown in Figure 11. In the absence and presence of IAA the normal observed stimulations of growth by malate were eliminated by the simultaneous application of ethylene with malate. The graphical analysis of the observed small variations in mean initial growth rates in the absence of IAA (Table VII) were not significantly different (Appendix A). Some significant variability did exist in the mean initial growth rates in the presence of IAA (Table VIII). However, it was observed from Table VIII that the normal 44 per cent stimulation of growth by malate was eliminated by the simultaneous application of ethylene, even though the initial IAA stimulated growth rates in the absence of malate or ethylene were significantly lower than the first two initial IAA stimulated growth rates. This variability was probably due to inherent tissue variability and possible loss of activity of the IAA in the working solutions used in those particular experiments.

The data from Table IX shows the influence of malate and IAA,

Table V Influence of malate on C_2H_4 inhibition of growth in the absence of CO_2 and in the presence and absence of IAA.

Initial Treatment	Growth rate mm/hr/10 cm of coleoptile		Final Treatment	Per cent Change
	Initial Rate	Final Rate		
	-IAA			
-malate $-C_2H_4$	0.48 ± 0.09	0.48 ± 0.09	-malate $+C_2H_4$	0 (N.S.)
+malate $-C_2H_4$	1.74 ± 0.22	1.14 ± 0.25	+malate $+C_2H_4$	-34 ($P < 0.05$)
	+IAA			
-malate $-C_2H_4$	4.67 ± 0.23	4.20 ± 0.30	-malate $+C_2H_4$	-10 (N.S.)
+malate $-C_2H_4$	3.66 ± 0.21	2.78 ± 0.23	+malate $+C_2H_4$	-24 ($P < 0.01$)

Initial growth rates were obtained from tissued incubated in pH 7.0 buffered medium in the presence and absence of 20 µM IAA and 1mM malate as indicated. Final rates obtained after the introduction of 10.8 µl/l C_2H_4 into the aerating gas stream. Results expressed as the mean ± the standard error. The data obtained was analysed by the t-test to determine the significance of differences between the mean initial and mean final growth rates. Results are expressed (in parentheses) after the per cent change as : P = probability level; N.S. = not significant.

Table VI Influence of malate on C_2H_4 inhibition of growth in the presence of CO_2 and in the presence and absence of IAA.

Initial Treatment	Growth rate mm/hr/10 cm of coleoptile Initial rate	Final Rate	Final Treatment	Per cent Change
		-IAA		
-malate $-C_2H_4$	1.56 ± 0.18	0.75 ± 0.13	-malate $+C_2H_4$	-52 ($P < 0.01$)
+malate $-C_2H_4$	1.88 ± 0.11	0.91 ± 0.14	+malate $+C_2H_4$	-52 ($P < 0.001$)
		+IAA		
-malate $-C_2H_4$	5.08 ± 0.45	4.07 ± 0.42	-malate $+C_2H_4$	-20 ($P<0.05$)
+malate $-C_2H_4$	5.14 ± 0.67	4.42 ± 0.62	+malate $+C_2H_4$	-14 (N.S.)

Initial growth rates were obtained from tissue incubated in pH 7.0 buffered medium in the presence and absence of 20 µM IAA and 1 mM malate as indicated. Final rates obtained after the introduction of 10.8 µl/l C_2H_4 to the aerating gas stream which contained 320 µl/l CO_2 throughout. Results expressed as the mean ± the standard error. The data obtained was analysed by the t-test to determine the significance of differences between the mean initial and mean final growth rates. Results are expressed (in parentheses) after the per cent change as: P = probability level; N.S. = not significant.

Table VII Antagonism between malate and C_2H_4 in the regulation of growth in the absence of IAA.

Final Treatment	mm/hr/10 cm of coleoptile		Per cent change
	Initial Rate -malate $-C_2H_4$	Final Rate	
no change	0.65 ± 0.17	0.60 ± 0.15	- 8 (N.S.) [3]
$+C_2H_4$	0.48 ± 0.09	0.42 ± 0.05	-12 (N.S.) [3]
+malate	0.51 ± 0.13	1.63 ± 0.14	+219 ($P < 0.001$)
+malate $+C_2H_4$	0.50 ± 0.10	0.38 ± 0.12	-24 (N.S.)

Initial growth rates were obtained from tissue incubated in pH 7.0 buffered medium in the absence of malate and C_2H_4. Final rates obtained after the introduction of 1mM malate and 10.8 µl/1 C_2H_4 as indicated. Results expressed as the mean ± the standard error. The data obtained was analysed by the t-test to determine the significance of differences between the mean initial and the mean final growth rates. Results are expressed (in parentheses) after the per cent change as: P = probability level; N.S. = not significant.

[3] Control data also shown in Table III

Ethylene inhibition of malate stimulated growth.

Carbon dioxide was absent from the aerating gas stream and 20 μM IAA was present in the upper two recordings and absent from the lower two. Malate (1mM) or malate plus ethylene (10.8 μl/l) were introduced as indicated. The bar indicates 1 mm elongation.

FIGURE 11

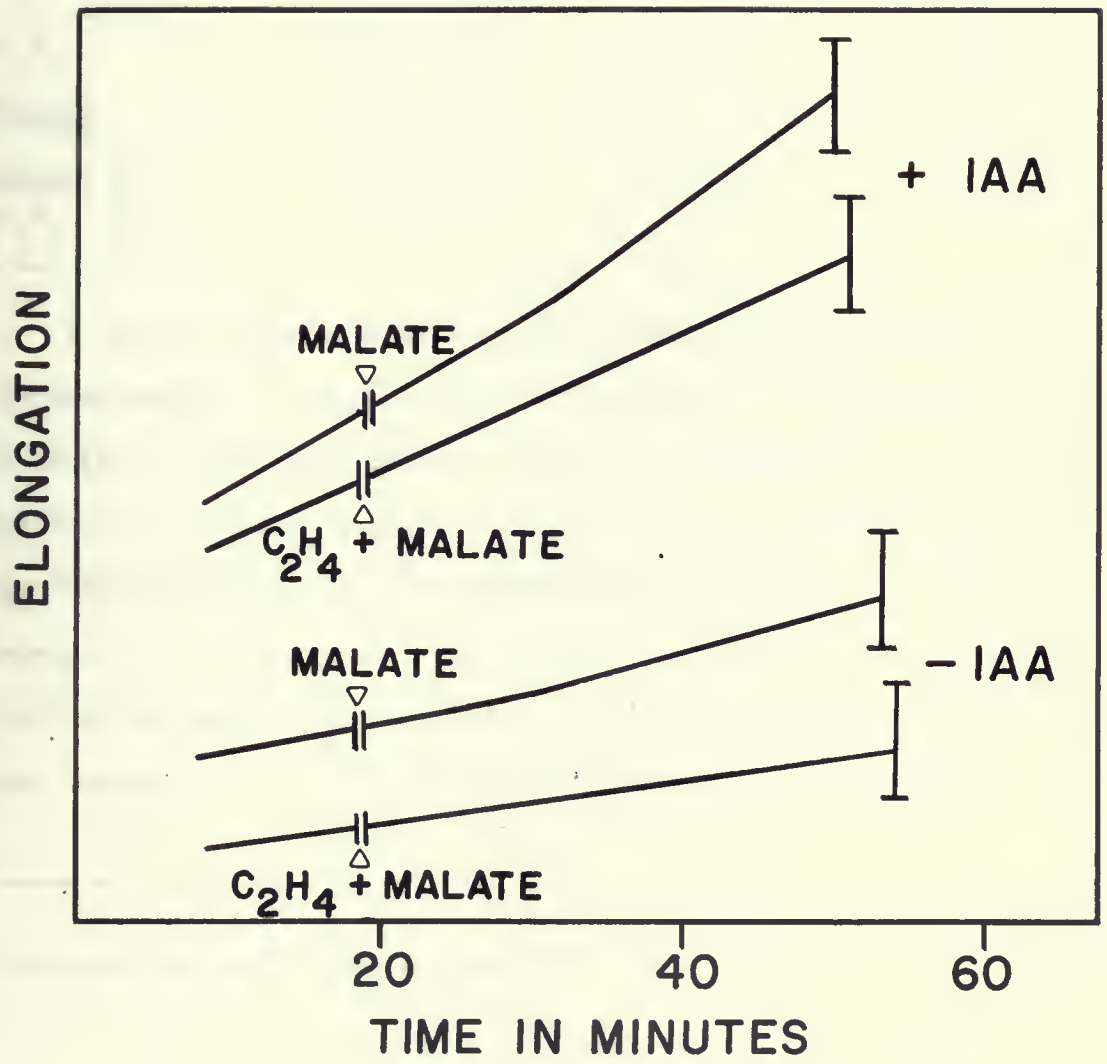

ELONGATION
+ IAA
MALATE
C_2H_4 + MALATE
MALATE
- IAA
C_2H_4 + MALATE
20
40
60
TIME IN MINUTES

Table VIII: Antagonism between malate and C_2H_4 in the regulation of growth in the presence of IAA.

Final Treatment	Growth rate mm/hr/10 cm of coleoptile Initial rate $-CO_2$ $-C_2H_4$	Final Rate	Per cent change
no change	4.25 ± 0.50	4.23 ± 0.04	- <1% (N.S.) [4]
$+C_2H_4$	4.67 ± 0.59	4.29 ± 0.33	- 8% (N.S.) [4]
+malate	2.60 ± 0.33	3.75 ± 0.49	+ 44% (P <0.01)
+malate $+C_2H_4$	1.45 ± 0.19	1.46 ± 0.14	+ 1% (N.S.)

Initial growth rates were obtained from tissue incubated in pH 7.0 buffered medium in the absence of CO_2 and C_2H_4 and the presence of 20 µM IAA. Final rates obtained after the introduction of 320 µl/l CO_2 and 10.8 µl/l C_2H_4 as indicated. Results are expressed as the mean ± the standard error. The data obtained was analysed by the t-test to determine the significance of differences between the mean initial and mean final growth rates. Results are expressed (in parentheses) after the per cent change as: P = probability level; N.S. = not significant.

[4] Control data also shown in Table IV

both separately and together on coleoptile growth. Analysis of the data from Table IX shows that both malate and IAA have significant effects on the stimulation of growth in the absence of the other factor (Table X) and that together they synergistically stimulate growth of the tissue. Figure 12 shows a typical growth recording that demonstrates the synergistic stimulation of growth by IAA and malate. The data from Table IX, that summarizes growth data from several such recordings, indicates that after subtracting the control rate (no IAA or malate) the combination of IAA and malate stimulated growth 25 per cent above the sum of the growth increases due to these factors acting alone.

Table XI summarizes the data on inhibition times for ethylene inhibition of growth to occur for both carbon dioxide and malate stimulated growth in the presence and absence of IAA. Intercept times for ethylene inhibition of both malate and carbon dioxide-stimulated growth were similar both in the presence (12-13 minutes) and absence of IAA (8-10 minutes).

3-2-2 ^{14}C-bicarbonate incorporation data

The influence of ethylene, IAA, and malate on the rate of incorporation of ^{14}C-bicarbonate after a thirty minute exposure of the tissue to each factor prior to addition of radioactivity is shown in Table XII. None of the three factors involved significantly altered the rate of incorporation of ^{14}C-bicarbonate by the tissue. It is evident for Table XII that some variability existed between experiments in the ability of the tissue to incorporate bicarbonate. However, one way analysis of variance for each experiment revealed no significant influence of ethylene, IAA or malate on incorporation of bicarbonate by the tissue.

Table IX Influence of IAA and malate on coleoptile growth

	Growth rate mm/hr/10 cm of coleoptile	
	- IAA	+ IAA
- malate	0.55 ± 0.11	2.60 ± 0.33
+ malate	1.06 ± 0.21	3.75 ± 0.49

Individual columns of tissue were used to obtain the four growth rates above. After obtaining an initial growth rate in the presence of 1 mM malate the rate in the absence of any growth promoting factor was measured. Rates were then obtained in the presence of 20 μM IAA; and in the combined presence of malate and IAA. The data represents mean values from six separate experiments, each of which demonstrated synergism between IAA and malate.

Table X Analysis of growth promotion by malate and IAA

	Effect of	
	malate	IAA
-IAA	P <0.01	
+IAA	P <0.02	
-malate		P <0.001
+malate		P <0.001

Data obtained from the experiments described under Table IX were analysed by the t-test to determine the significance of differences in mean growth rates. P = probability level.

The influence of IAA and malate on coleoptile growth.

Malate (1mM) and IAA (20 μM) were introduced to and removed from the tissue at the times indicated such that growth rates were obtained in the absence of any added growth promoting factor and in the separate and combined presence of IAA and malate. Numbers refer to minutes during which no recording of growth was made. The bar indicates 1 mm elongation.

FIGURE 12

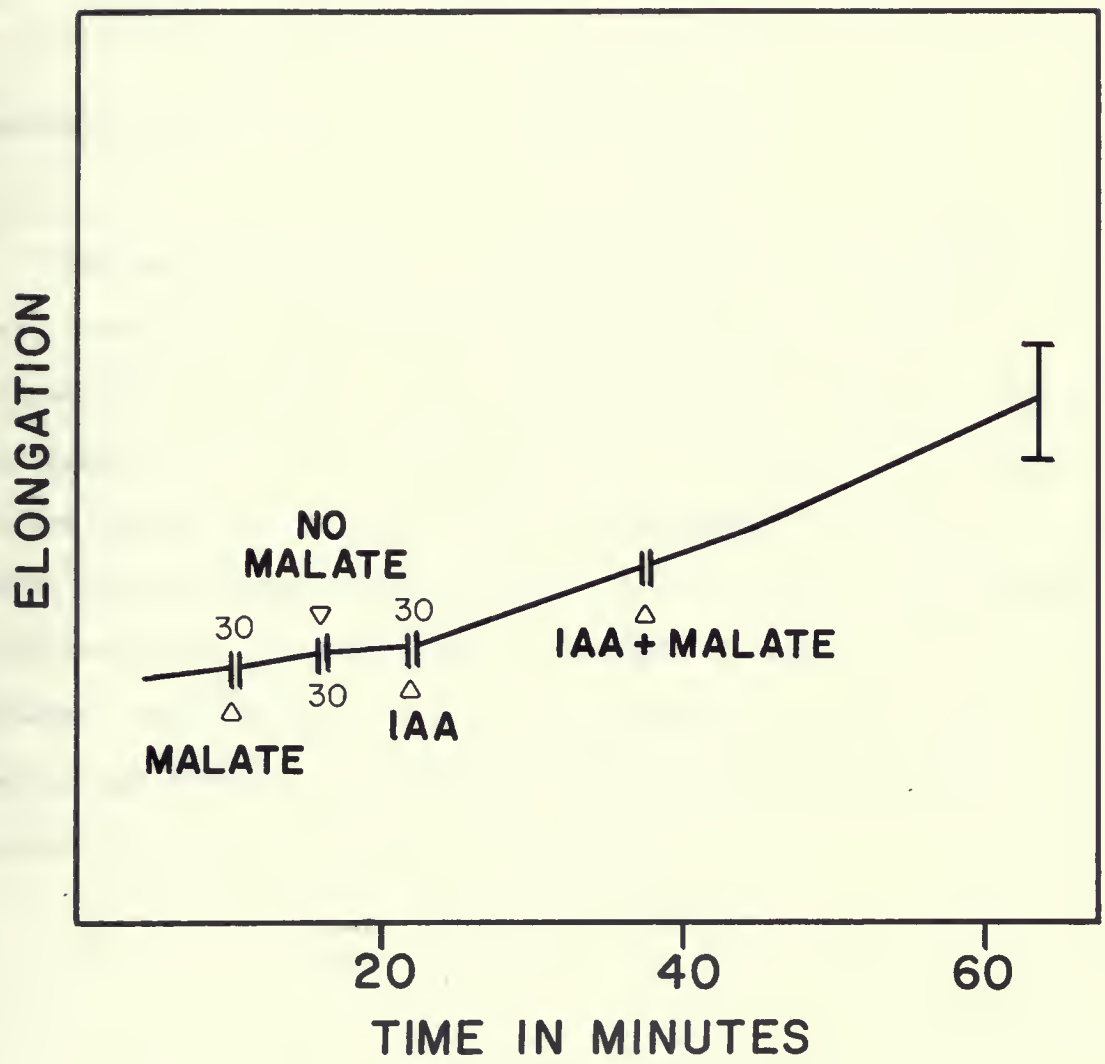

ELONGATION
NO
MALATE
30
30
30
MALATE
IAA
IAA + MALATE
20
40
60
TIME IN MINUTES

Table XI Intercept times for C_2H_4 inhibition of growth to occur

Growth conditions		Intercept time in minutes
CO_2 + C_2H_4	-IAA	8 - 10 minutes
	+IAA	12 - 13 minutes
malate + C_2H_4	-IAA	8 - 10 minutes
	+IAA	12 - 13 minutes

The intercept times for C_2H_4 inhibition of coleoptile growth were determined for the various growth conditions on individual columns of coleoptiles. In each case, the initial growth was recorded in the presence of 320 µl/l CO_2 or 1 mM malate in phosphate buffer (10 mM; pH 7.0) in the absence or presence of 20 µM IAA. After a ten minute recording of growth 10.8 µl/l C_2H_4 was introduced into the system and growth recorded continuously for up to 40 minutes. The intercept times for C_2H_4 inhibition of growth were determined as previously mentioned. The data represents the mean values of at least five growth experiments.

Table XII. The influence of C_2H_4, IAA and malate on the rate of bicarbonate incorporation into Avena coleoptiles.

	Dpm per Gram Fresh Weight	
Treatment	- C_2H_4	+ C_2H_4
Experiment 1	168040 ± 28789	155582 ± 18301
Experiment 2	139682 ± 5805	163399 ± 36597
Treatment	- IAA	+ IAA
Experiment 1	50341 ± 2476	49255 ± 2341
Experiment 2	82845 ± 10333	79000 ± 4620
Treatment	- malate	+ malate
Experiment 1	152252 ± 4355	140437 ± 15913

Each experiment consisted of 12 batches of tissue which were incubated in pH 7.0 buffered medium and aerated with air containing 320 µl/l CO_2. after 1 hour, 6 batches were incubated for 30 minutes in the presence of the test substance (10.8 µl/l C_2H_4, 20 µM IAA, or 1 mM malate). Aeration of all 12 batches was then stopped, 1 ml of ^{14}C-bicarbonate solution of known activity injected (4-7 x 10^6 dpm depending on the experiment), and the coleoptile sections incubated a further 6 minutes in the sealed tubes. Incubation was terminated and the level of incorporation determined by procedures described under the Methods section. Results are expressed as the mean ± the standard error. One way analysis of variance demonstrated that in each experiment the difference between the means was not significant at the 0.95 confidence limit for each of the three treatments.

3-3 ^{14}C-bicarbonate fixation data

Parallel experiments to determine incorporation and dark fixation levels of ^{14}C-bicarbonate revealed that 25 to 35 per cent of the incorporated label was fixed by the tissue within the six minutes exposure of the tissue to ^{14}C-bicarbonate (data not shown).

The influence of ethylene on the rate of dark ^{14}C-bicarbonate fixation during the inhibition time prior to ethylene inhibition was determined in the absence and presence of IAA. The data from Table XIII shows there is considerable variability between experiments in the ability of the tissue to fix bicarbonate. However, one way analysis of variance for each experiment revealed no evidence for a significant influence of ethylene on dark fixation. The results of parallel experiments, on the influence of ethylene on fixation rates after the establishment of ethylene-inhibited growth, again indicated the absence of any influence of ethylene on fixation (Table XIV) either in the absence or presence of IAA.

The influence of 20 μM IAA on the rate of dark carbon dioxide fixation was investigated in a similar manner. The data from Table XV indicates there is no influence of IAA on dark fixation prior to IAA stimulation of growth. The data from Table XVI further demonstrates that there is no influence of IAA on dark carbon fixation after IAA has stimulated the growth of the tissue. Again, the data in Tables XV and XVI demonstrates that between experiments there could be a large variability in the rates of ^{14}C-fixation but within each experiment, however, much less variability existed.

The influence of 1 mM malate on the rate of dark carbon

Table XIII The influence of C_2H_4 on the rate of dark CO_2 fixation prior to C_2H_4 inhibition of growth in the absence and presence of IAA.

Treatment		Dpm per Gram Fresh Weight	
		- C_2H_4	+ C_2H_4
Experiment 1	-IAA	30,687 ± 3,969	38,935 ± 3,349
Experiment 2	-IAA	64,296 ± 6,553	68,015 ± 8,735
Experiment 3	-IAA	79,017 ± 7,270	86,253 ± 8,715
Experiment 4	-IAA	41,915 ± 3,486	36,524 ± 4,688
Experiment 5	+IAA	60,092 ± 8,041	52,413 ± 3,939
Experiment 6	+IAA	34,655 ± 2,923	32,825 ± 4,611
Experiment 7	+IAA	76,006 ± 4,349	81,357 ± 10,104

Each experiment consisted of 12 batches of tissue which were incubated in pH 7.0 buffered medium in the absence or presence of 20 µM IAA, and aerated with air containing 320 µl/l CO_2. After 1 hour, 6 batches were aerated for 2 minutes with air containing 10.8 µl/l C_2H_4 and 320 µl/l CO_2. Aeration of all 12 batches was then stopped, 1 ml of ^{14}C-bicarbonate solution of known activity injected (4-7 x 10^6dpm depending on the experiment); and the coleoptile sections incubated a further 6 minutes in the sealed tubes. Incubation was terminated and the level of fixation determined by the procedures described under the Methods section. Results are expressed as the mean ± the standard error. One way analysis of variance demonstrated that in each experiment the difference between the means was not significant at the 0.95 confidence limit.

Table XIV The influence of C_2H_4 on the rate of dark CO_2 fixation during C_2H_4 inhibition of growth in the absence and presence of IAA.

Treatment	Dpm per Gram Fresh Weight	
	- C_2H_4	+ C_2H_4
Experiment 1 -IAA	40,930 ± 9,252	43,330 ± 5,274
Experiment 2 -IAA	35,333 ± 4,394	41,242 ± 3,752
Experiment 3 -IAA	35,567 ± 3,193	31,943 ± 5,306
Experiment 4 -IAA	45,206 ± 3,731	47,585 ± 6,745
Experiment 5 +IAA	24,924 ± 3,802	22,418 ± 3,992
Experiment 6 +IAA	60,740 ± 5,213	59,209 ± 4,986
Experiment 7 +IAA	58,276 ± 3,203	50,957 ± 7,757
Experiment 8 +IAA	48,112 ± 1,876	53,887 ± 5,470

The experimental procedures were identical to those in Table XIII except that aeration with the C_2H_4 containing gas mixture was for 30 minutes not 2 minutes. Results are expressed as the mean ± the standard error. One way analysis of variance demonstrated that in each experiment the difference between the means was not significant at the 0.95 confidence limit.

Table XV The influence of IAA on the rate of dark carbon dioxide fixation prior to IAA stimulation of growth

	Dpm per Gram Fresh Weight	
	-IAA	+IAA
Experiment 1	52,214 ± 4,744	52,646 ± 5,183
Experiment 2	54,206 ± 6,456	64,261 ± 9,589
Experiment 3	60,798 ± 3,458	59,652 ± 7,399

Each experiment involved 12 batches of tissue which were incubated in pH 7.0 buffered medium, and aerated with air containing 320 µl/l CO_2. After 1 hour 20 µM IAA was introduced to 6 batches, aeration of all 12 batches was stopped 2 minutes later, and 1 ml of ^{14}C-bicarbonate solution of known activity (4 to 7 x 10^6 dpm, depending on the experiment) injected to all tissue samples. After a further 6 minutes, incubation in the sealed tubes was terminated, and the level of fixation determined by the procedures described under Materials and Methods. Results are expressed as the mean ± the standard error. One way analysis of variance demonstrated that in each experiment the difference between the means was not significant at the 0.95 confidence limit.

Table XVI The influence of IAA on the rate of dark carbon dioxide fixation during IAA stimulation of growth

	Dpm per Gram Fresh Weight	
	-IAA	+IAA
Experiment 1	61,237 ± 4,046	66,579 ± 7,238
Experiment 2	39,943 ± 2,757	41,532 ± 4,220
Experiment 3	75,731 ± 6,125	78,320 ± 8,052
Experiment 4	37,665 ± 4,191	33,985 ± 4,632

The experimental procedures were identical to those in Table XV shown in legend except that 20 μM IAA was applied 30 minutes before aeration ceased and ^{14}C-bicarbonate was injected. Results were expressed as the mean ± the standard error. One way analysis of variance demonstrated that in each experiment the difference between the means was not significant at the 0.95 confidence limit.

fixation prior to and after malate stimulation of growth was also investigated. Analysis of the data in Tables XVII and XVIII indicate that 1 mM malate did not influence the rate of ^{14}C-bicarbonate fixation either before or after the onset of malate-stimulated growth. Again there was some variability in the ability of the tissue to fix ^{14}C-bicarbonate between experiments, but within each experiment this variability was much reduced.

3-4 Malate decarboxylation and $^{14}CO_2$ evolution data

The influence of 10.8 µl/l ethylene on the rate of decarboxylation of L-[U-^{14}C]-malate by batches of Avena coleoptiles was investigated both in the presence and absence of 20 µM IAA. Figure 13 shows a representative figure demonstrating the collection of $^{14}CO_2$ that is evolved with time by the tissue in the absence of IAA. Similar rates of $^{14}CO_2$ evolution were evident for both the control tissue and the ethylene-treated tissues. Both showed a similar per cent increase in the rate of $^{14}CO_2$ evolution with time (Table XIX) and no significant influence of ethylene treatment was evident either in the presence of absence of 20 µM IAA.

3-5 Respirometry data

The influence of 1 mM malate on oxygen consumption and CO_2 output was investigated. The data from Table XX indicates that 1 mM malate stimulated oxygen consumption by 12 per cent, but does not indicate any significant influence of malate on carbon dioxide evolution. The 12 per cent increase in oxygen consumption observed was analysed by the t-test. Analysis of the increase in rate of oxygen consumption showed that it was not significantly different from the untreated control.

Table XVII The influence of malate on the rate of dark carbon dioxide fixation prior to malate stimulation of growth

	Dpm per Gram Fresh Weight	
	- Malate	+ Malate
Experiment 2	45,735 ± 3,228	38,727 ± 2,605
Experiment 2	41,008 ± 4,419	41,812 ± 2,027

Each experiment involved 12 batches of tissue which were incubated in pH 7.0 buffered medium, and aerated with carbon dioxide-free air. After 1 hour, 1 mM malate was introduced to 6 batches, aeration of all 12 batches was stopped 2 minutes later, and 1 ml of ^{14}C-bicarbonate solution of known activity (4 to 7 x 10^6 dpm, depending on the experiment) injected to all tissue samples. After a further 6 minutes, incubation in the sealed tubes was terminated, and the level of fixation determined by the procedures described under Materials and Methods. Results are expressed as the mean ± the standard error. One way analysis of variance demonstrated that in each experiment the difference between the means was not significant at the 0.95 confidence level.

Table XVIII The influence of malate on the rate of dark carbon dioxide fixation during malate stimulation of growth

	Dpm per Gram Fresh Weight	
	- Malate	+Malate
Experiment 1	54,361 ± 3,334	60,213 ± 4,964
Experiment 2	44,212 ± 3,778	53,116 ± 7,237
Experiment 3	49,450 ± 2,930	39,643 ± 3,393

The experimental procedures were identical to those in Table XVII as shown in the [illegible] except that 1 mM malate was applied 30 minutes before aeration ceased and ^{14}C-bicarbonate was injected. Results are expressed as the mean ± the standard error. One way analysis of variance demonstrated that in each experiment the difference between the means was not significant at the 0.95 confidence limit.

The influence of ethylene on the rate of malate decarboxylation in the absence of IAA.

Batches of coleoptiles were incubated in pH 7.0 buffered medium, with approximately 2.5 x 10^6 dpm L-[U-^{14}C] malate, in the absence of IAA and aerated with air containing 320 µl/l carbon dioxide. After one hour, the reaction vessel was transferred to the $^{14}CO_2$ collection apparatus and five successive three minute collections made. Then 10.8 µl/l ethylene was added to the aeration stream and ten successive three minute collections made. The level of radioactivity in each vial was determined. The rates of $^{14}CO_2$ evolution were determined and plotted as cumulative dpm $^{14}CO_2$ evolved per gram tissue fresh weight against time of collection in minutes. The control tissue data is represented by ○-; and the ethylene-treated tissue data is represented by ●. Time of addition of ethylene is indicated as shown.

FIGURE 13

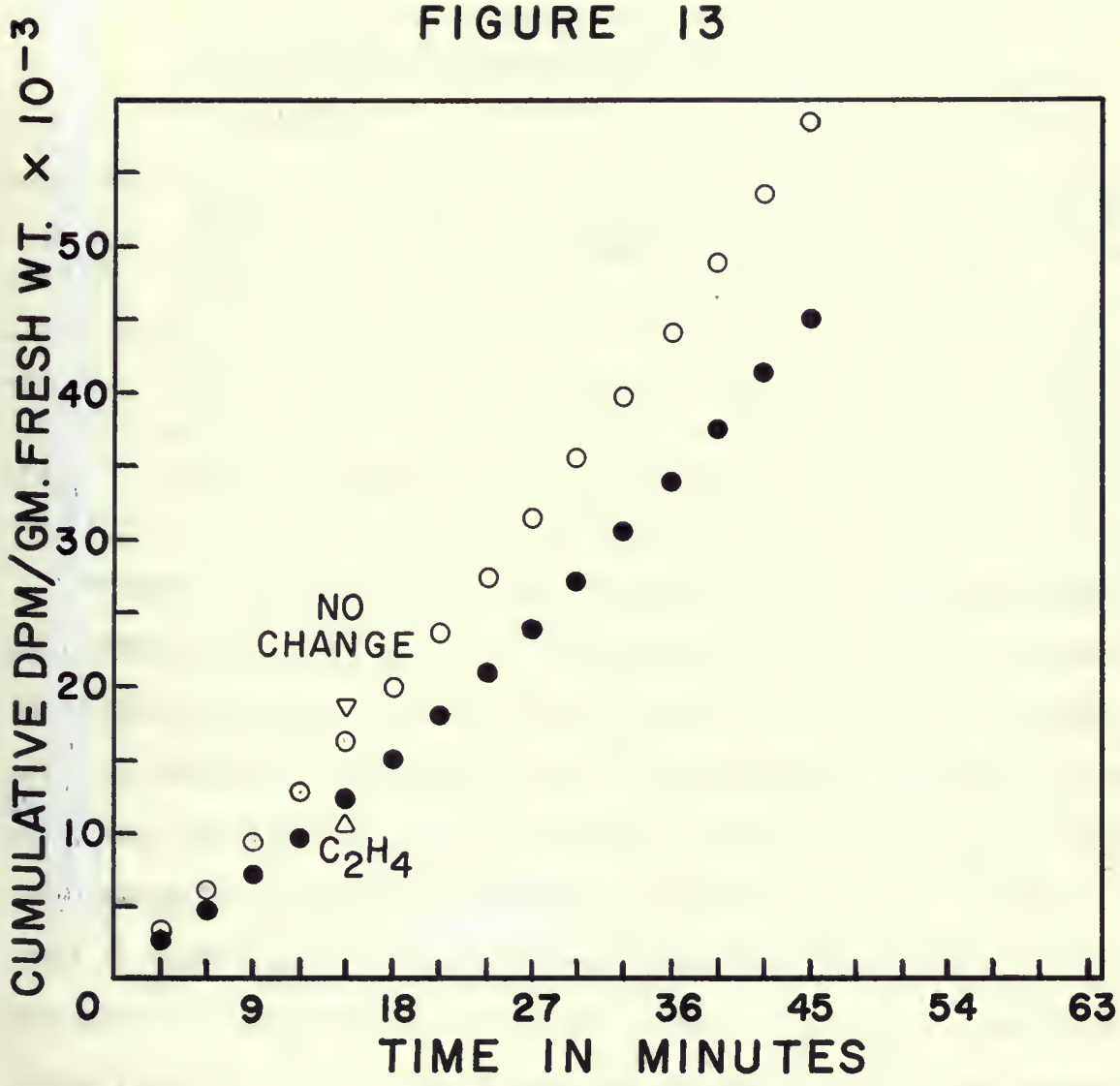

CUMULATIVE DPM/GM. FRESH WT. X 10⁻³
0
10
20
30
40
50
0
9
18
27
36
45
54
63
TIME IN MINUTES
NO CHANGE
C2H4

Table XIX The influence of C_2H_4 on the rate of malate decarboxylation in the presence and absence of IAA.

	Rate of Malate Decarboxylation Dpm $^{14}CO_2$ Evolved/Minute/Gram Fresh Weight					
	Experimental			Control		
	Initial rate $-C_2H_4$	Final rate $+C_2H_4$	Per cent change	Initial rate $-C_2H_4$	Final rate $-C_2H_4$	Per cent change
-IAA	2797 ± 756	3623 ± 895	+29.5	3128 ± 1871	4220 ± 2645	+34.9
+IAA	1102 ± 428	1413 ± 523	+28.2	1649 ± 515	2031 ± 627	+23.2

Batches of 40 coleoptiles were incubated in pH 7.0 buffered medium, with approximately 2.5 x 10^6 dpm L-[U-^{14}C] malate, in the presence or absence of 20 µM IAA and aerated with air containing 320 µl/l CO_2. After one hour, the reaction vessel was transferred to the $^{14}CO_2$ collection apparatus and five successive three minute collections made. Then 10 µl/litre C_2H_4 was added to the aeration stream and ten successive three minute collections made. The level of radioactivity in each vial was determined. The rates of $^{14}CO_2$ evolution were determined and expressed as Dpm $^{14}CO_2$ evolved/minute/gram tissue fresh weight. The data represents the mean ± the standard error of five experiments. One way analysis of variance demonstrated that the differences between means of C_2H_4 treated and non-treated batches of tissue were not significant at the 0.95 confidence level either in the presence or absence of IAA.

Table XX Influence of malate on oxygen consumption and carbon dioxide output.

	µl/hr g fresh weight		RQ
	oxygen consumption	carbon dioxide evolution	
- malate	337 ± 30	174 ± 63	0.46
+ malate	423 ± 36	165 ± 20	0.39

Approximately 0.5 g batches of weighed coleoptile tissue were placed in buffered mediums in light tight Warburg flasks in the presence or absence of 1 mM malate. Rates of oxygen consumption and carbon dioxide output were measured in a Gilson differential respirometer using the standard direct technique. The data represents mean values from four determinations. Analysis of the data for oxygen by the t-test showed there was no significant difference between tissue treated with or without malate with respect to either oxygen consumption or carbon dioxide evolution.

4 - Discussion and Conclusions

The data from Figure 9 and Table II clearly illustrate ethylene inhibition of coleoptile growth in the presence of carbon dioxide both in the presence and absence of IAA. Further to this, data from Tables III and IV and Figure 10 demonstrates that the simultaneous application of carbon dioxide and ethylene in the absence or presence of IAA results in the complete elimination of the normal increased growth rate in response to carbon dioxide. This data is consistent with previous reports of antagonism between carbon dioxide and ethylene in growth responses (Burg and Burg, 1967 b) and is also consistent with the hypothesis that ethylene inhibition of growth is through inhibition of the dark fixation process (Figure 1).

The data from Table XII demonstrates the absence of any detectable influence of ethylene on ^{14}C-bicarbonate incorporation into coleoptile tissue. This would indicate that the rates of ^{14}C-bicarbonate fixation were not influenced by an ethylene mediated control of ^{14}C-bicarbonate availability. Consequently the data from Tables XIII and XIV demonstrates that the rate of dark fixation was not measurably affected by ethylene either within the 10-12 minute period prior to growth inhibition or after inhibition of growth had occurred. This was true both in the absence and presence of IAA. This data then does not support the hypothesis that ethylene inhibition of growth results from an inhibition of dark fixation (Figure 1).

The hypothesis also predicts that products of dark fixation such as malate, which can replace carbon dioxide in growth stimulation (Bown et al, 1974) would protect the tissue from ethylene inhibition.

If this were the case then malate, a product of dark fixation in Avena coleoptile tissue (Bown and Lampman, 1971) could be added to the ethylene-treated tissue and result in increased growth even in the presence of the proposed ethylene inhibition of fixation. The products of fixation would be supplied irregardless of the ethylene block and normal growth stimulation by malate could occur.

The presence of malate, however, resulted in a greater inhibition of growth by ethylene (Table V) and the simultaneous application of ethylene and malate, both in the absence and presence of IAA, resulted in the complete elimination of the normal increased growth rate in the response to malate (Figure 11 and Tables VII and VIII). The data shows a similar antagonism between ethylene and malate, and ethylene and carbon dioxide in the growth of coleoptiles. This data does not support the idea that malate might protect the tissue from ethylene inhibition and consequently does not support the hypothesis. In addition ethylene added in the presence of carbon dioxide inhibited growth to the same extent as it inhibited growth in the presence of carbon dioxide and malate.

Therefore the results of the two tests of the hypothesis as outlined in Figure 1 have not supported it. A third possible test of the hypothesis would be to examine the in vitro influence of ethylene on the fixation enzymes. Enzyme assays of phosphopyruvate carboxylase (EC. 1.1.1.37) and malic enzyme (EC.1.1.1.40) from etiolated coleoptiles indicates that ethylene does not inhibit these carbon dioxide fixing enzymes under conditions of limiting bicarbonate and ethylene saturation of the assay system (Bown and Hill - unpublished data). Under conditions of limiting bicarbonate and ethylene saturation, one would expect an inhibition of enzyme activity if ethylene and carbon dioxide were competing for the active

sites of these enzymes.

In contrast to the hypothesis, the data suggests that ethylene action results from an inhibition of the utilization of the products of fixation, rather than an inhibition of the fixation process itself. Tables III and VII demonstrate that growth stimulation by carbon dioxide and malate was blocked by the simultaneous application of ethylene in the absence of IAA. Similar results, in Tables IV and VIII, were obtained in the presence of IAA. Data from Tables V and VI demonstrate that ethylene inhibition of malate stimulated growth was only observed in the absence of carbon dioxide. These results are consistent with a previous report indicating a common function of carbon dioxide and malate in growth regulation (Bown et al, 1974), and suggest a relationship between malate metabolism and ethylene inhibition of growth.

Growth data, reported by Burg and Burg (1967 b), involving the interaction of carbon dioxide and ethylene has been interpreted as indicating competition between these two agents for a common molecular binding site. This present study however, suggests that the relationship between carbon dioxide and ethylene may not be as direct as this competition hypothesis indicates. It is a difficult to extrapolate growth data into a molecular model with any confidence; and it is not easy to reconcile ethylene and carbon dioxide competition for a common molecular site with data indicating that some aquatic plants exhibit a synergistic growth stimulation in the presence of these two factors (Suge and Kusanagi, 1975). The data suggests then that ethylene action may result from an interaction with some process that is subsequent to carbon dioxide fixation. This interpretation thus changes the emphasis somewhat from that of the usual idea of carbon dioxide inhibition of an ethylene-stimulated process

to one of an ethylene inhibition of a carbon dioxide-stimulated process.

There are many reports that propose that carbon dioxide - stimulated growth involves dark fixation and the generation of four carbon skeletons which are used to maintain levels of Krebs cycle acids which would otherwise be depleted through utilization in amino acid biosynthesis (Ashworth, Kornberg and Ward, 1965; Bown and Aung, 1974; Bown et al., 1974). The first detectable products of dark fixation in Avena coleoptile tissue are malate and aspartate (Bown and Lampman, 1971). Splittstoesser (1966) using various non-autotrophic tissues demonstrated that malate, aspartate and glutamate were the major products of fixation, and that removal of carbon dioxide from the air supply resulted in lower rates of growth and protein synthesis.

Consequently the present data suggests that ethylene action may involve the control of malate transport into the mitochondria, subsequent malate utilization within the mitochondria, or an inhibition of amino acid utilization leading to a decrease in the synthesis of protein essential for growth.

Data reported by Rhodes, Galliard, Wooltorton and Hulme (1968) and Rhodes, Wooltorton, Galliard and Hulme (1968) demonstrated an influence of ethylene on the development of a malate decarboxylating system in discs of pre-climacteric apples. If this phenomenon was occuring in Avena coleoptiles ethylene would be expected to increase the rate of release of labelled carbon dioxide from labelled malate. The data from Table XIX and Figure 13 shows the absence of any influence of ethylene on the rate of malate decarboxylation both in the absence and presence of IAA. The technique employed might not have been sensitive enough to detect changes in the rates of $^{14}CO_2$ evolution

resulting from ethylene induced changes in malate decarboxylation. Alternatively an ethylene influence on malate metabolism may not involve malate decarboxylation. The technique, as outlined in the methods, can be used to show both small and large changes in rates of $^{14}CO_2$ evolution. A 10 per cent decrease in the rate of $^{14}CO_2$ evolution was observed in the presence of 1 mM phenylsuccinate and a 75 per cent reduction of $^{14}CO_2$ evolution was observed in the presence of 100 mM malonate (Methods section).

It can be supposed that the ability of malate to replace carbon dioxide results from malate decarboxylation. However, Table XX shows that 1 mM malate had a slight (but not significant) influence on oxygen consumption and no influence on the rate of carbon dioxide evolution. If malate did replace carbon dioxide through a decarboxylation process and an increased intracellular carbon dioxide level, then an increased evolution of carbon dioxide in response to malate might be expected.

Secondly, the timing of malate stimulation of growth is similar to that for carbon dioxide stimulation of growth (Figures 10 and 11). If malate stimulation of growth resulted from malate decarboxylation then a longer promotion time before malate increased growth would be expected. Thirdly, carbon dioxide free air does not prevent malate stimulation of growth. The addition of carbon dioxide free air has been shown to reduce the growth rate (Bown et al., 1974), presumably through a reduction of intracellular carbon dioxide levels. This condition could be expected to prevent a malate stimulation of growth through malate decarboxylation.

The data from Tables XVII and XVIII shows that malate did not significantly influence the rates of dark fixation either prior to or during malate stimulation of growth, and the data from Table XII

demonstrates that malate had no significant influence on the incorporation of ^{14}C-bicarbonate by Avena coleoptile tissue. Therefore the ability of malate to replace carbon dioxide does not appear to result from an influence on the availability of bicarbonate or on fixation rates.

The most likely explanation for the ability of malate to replace carbon dioxide in growth stimulation is that carbon dioxide fixation generates malate and that carbon dioxide fixation is required in the growth process (Splittstoesser, 1966).

Table II, Table XI and Figure 9 demonstrate that in the presence of IAA ethylene inhibition of growth is less severe and occurs after a longer lag period. These phenomena may well be due to the prior presence of high ethylene levels in response to IAA as reported by Abeles and Rubinstein (1964), but an alternative explanation might be that IAA stimulated growth is less sensitive to ethylene.

The synergistic growth stimulation between carbon dioxide and IAA has been previously reported (Bown et al, 1974). Thimann (1949) reported that malate significantly increased the growth rate of Avena coleoptile sections above the normal rate in the presence of IAA. The data from Tables IX and X, and Figure 12 demonstrate that malate and IAA stimulate coleoptile growth in a similar synergistic manner. This is consistent with these factors influencing a common growth limiting process. IAA, however, did not influence the incorporation of labelled bicarbonate into the coleoptile tissue (Table XII), consequently the data from Tables XV and XVI demonstrate that IAA does not influence the rate of dark fixation. The synergism between malate and IAA (Figure 12, Table IX) indicates that the latter regulates some event which is

subsequent to fixation. Given the present lack of information concerning the mechanism of action of IAA, it is difficult to envisage the relationship between IAA and malate stimulated growth. However, an influence of IAA on malate utilization by the mitochondria or at some subsequent step would be consistent with this data.

The inability of IAA to stimulate the rate of dark fixation (Tables XV and XVI) does not support Yamaki's suggestion of a rapid influence of IAA on this process (Yamaki, 1954). His suggestion, however, resulted from observations demonstrating a reduction in carbon dioxide evolution immediately following IAA application, and is consequently dependent on both carboxylation and decarboxylation processes.

The data suggest that IAA stimulated growth does not involve changes in the rate of fixation, but may involve an influence on malate metabolism and thus is complementary to the data suggesting a close relationship between malate and ethylene inhibition of growth.

The data have shown that there are three types of growth response evident from the study. These are: 1) A control rate that is observed in the absence of any of the growth promoters. This is a low rate of endogenus growth that is insensitive to ethylene inhibition. 2) An auxin-stimulated growth that is not significantly affected by ethylene exposure. 3) Carbon dioxide and malate stimulated growth that is very significantly inhibited by the presence of ethylene, but at some process subsequent to that of dark carbon dioxide fixation. The first two types of growth are insensitive to ethylene exposure and probably involve somewhat different mechanisms from that of CO_2 or malate stimulated growth, which are inhibited by ethylene. However,

the observed synergism between IAA and CO_2 or malate indicates that there is some degree of interaction between the mechanisms for promoting growth that result in the synergistic stimulation of coleoptile growth. Dark carbon dioxide fixation is a necessary step prior to the stimulation of growth observed with atmospheric levels of CO_2. The fixation process generates malate, which in turn has been shown to stimulate growth of coleoptiles in a manner similar to that of carbon dioxide. The mechanism for malate-stimulation of growth, however, remains to be determined.

Close relationships exist between carbon dioxide and malate, IAA, and ethylene in the regulation of Avena coleoptile growth. The exact nature of the mechanism of action of these growth regulators is still uncertain, but it would appear that ethylene and IAA action involves an influence on some process subsequent to dark carbon dioxide fixation.

In concluding the discussion of ethylene inhibition of coleoptile growth and the interrelationships with carbon dioxide, malate and IAA, the following points should be emphasized:

1) The hypothesis that ethylene inhibition of coleoptile growth results from an inhibition of dark carbon dioxide fixation is not supported by the data.
2) IAA stimulation of growth does not involve changes in the rate of dark carbon dioxide fixation.
3) A close relationship exists between growth regulation by IAA and ethylene and the mechanism by which malate stimulates growth.

Future investigations of these relationships might better provide further insights into the regulatory mechanisms involved in plant growth and development.

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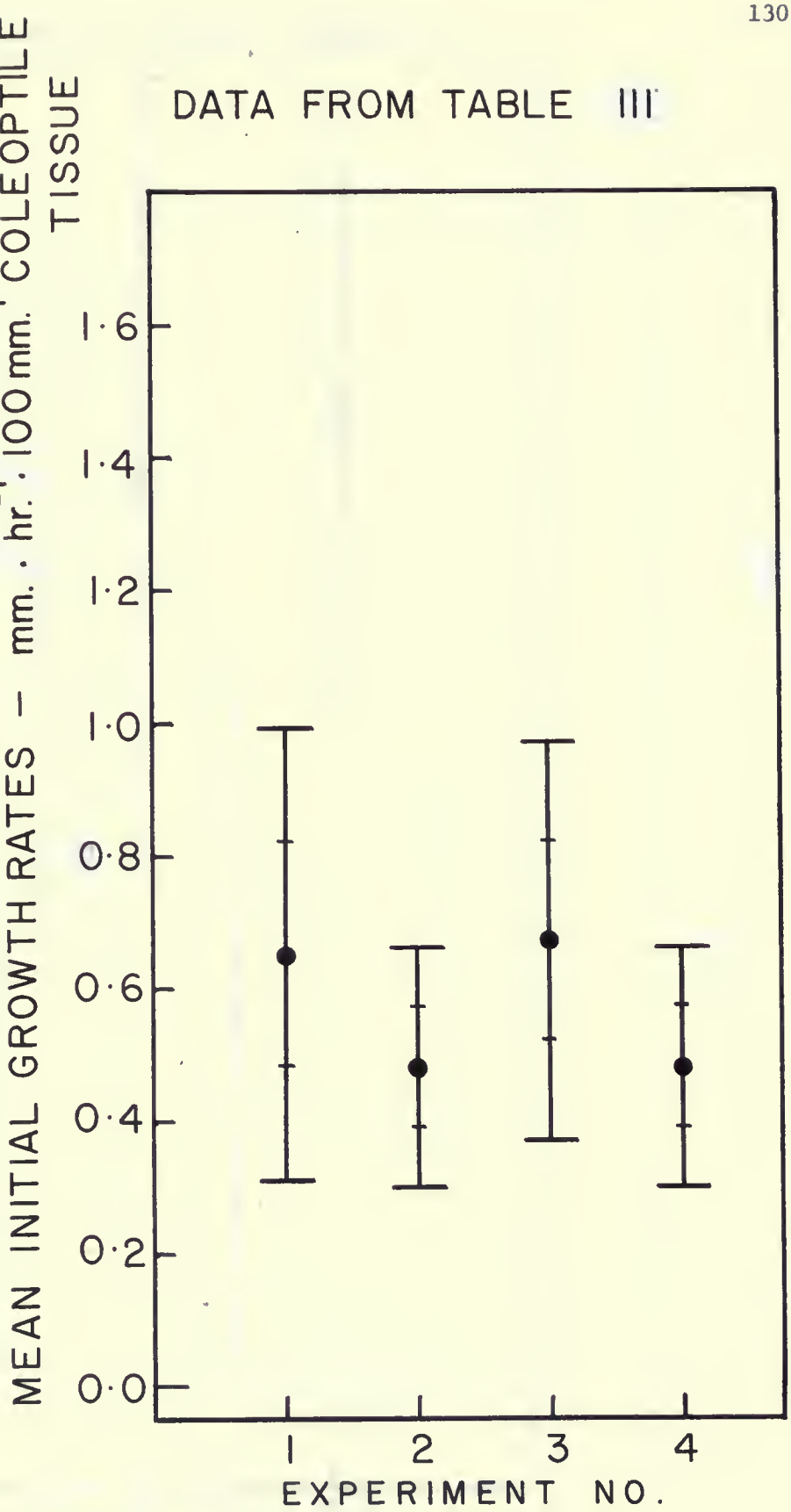
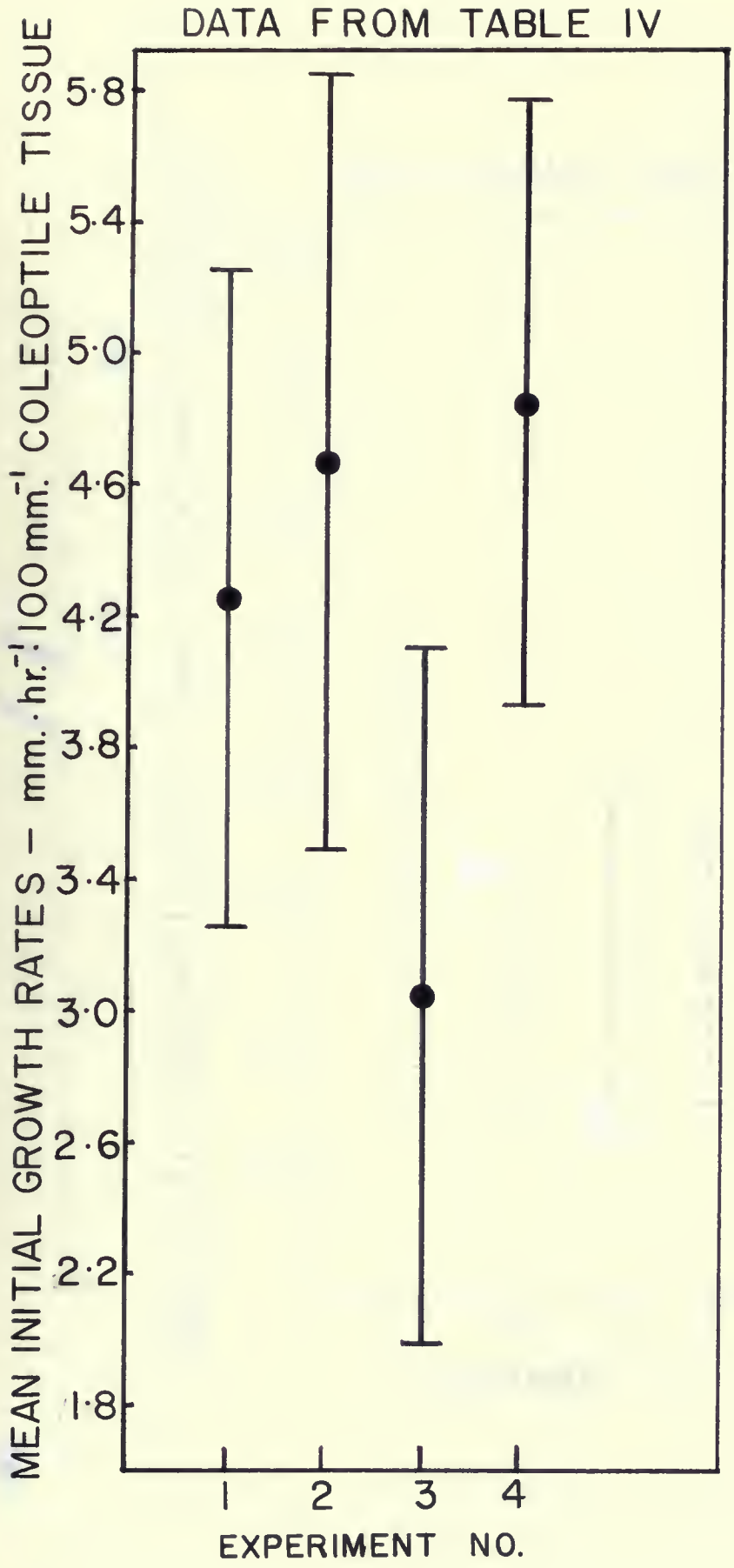
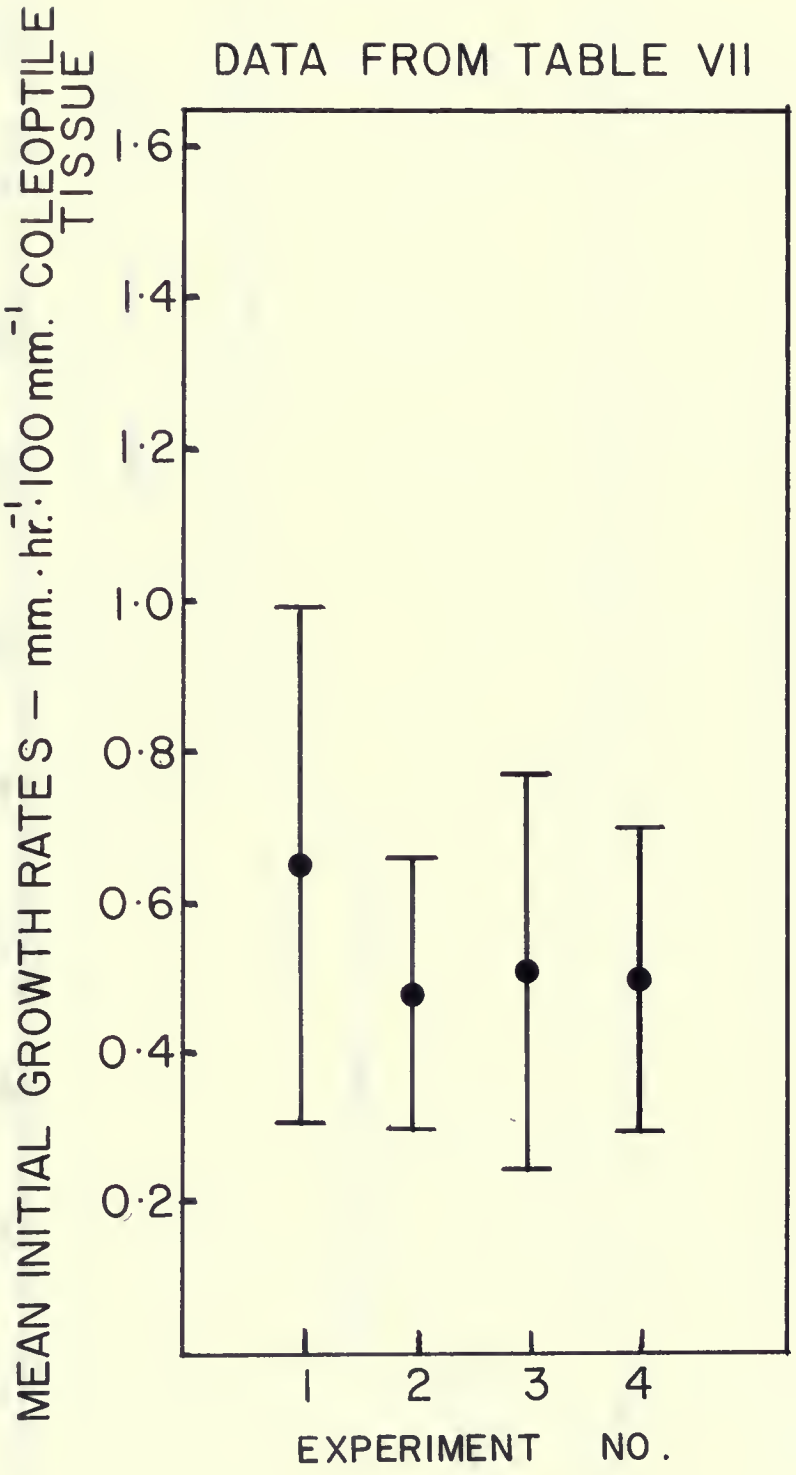
Appendix A

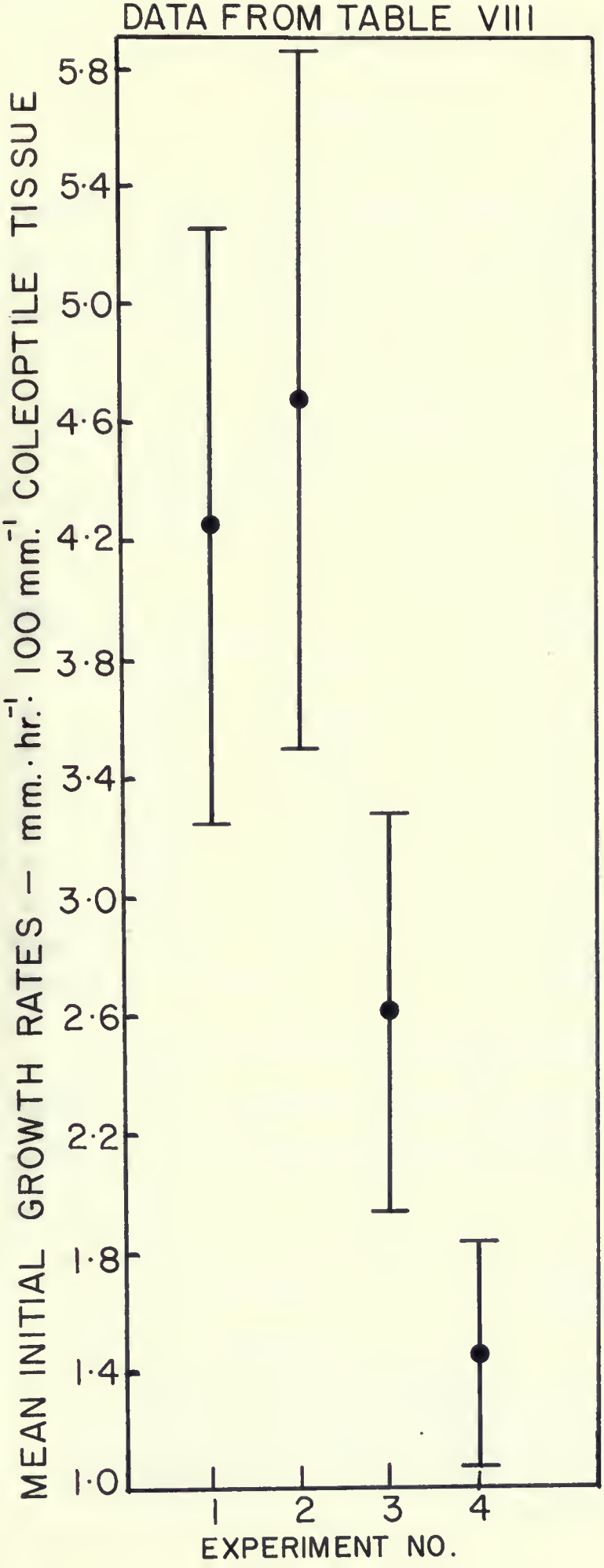
Graphic analysis of mean initial growth rates

This section presents the results of the graphical analysis of the mean initial growth rates in the experiments that examine the antagonistic nature of CO_2 or malate with C_2H_4 on the growth response. The mean initial growth rates (•), both in the absence and presence of IAA are plotted on their respective graphs with their 95% confidence intervals shown as the vertical line between the two short horizontal bars (⊢•⊣). The 95% confidence interval is approximately 2 standard errors about the mean value. If there is no overlapping between two confidence intervals evident, then the two means would be significantly different. If overlapping exists between the confidence intervals for two or more mean initial rates, then there is no significant difference between those mean values with overlapping confidence intervals. Graphic analyses of the mean initial growth rates for Tables III, IV, VII and VIII are presented.


DATA FROM TABLE III
MEAN INITIAL GROWTH RATES – mm. · hr.⁻¹ · 100 mm.⁻¹ COLEOPTILE TISSUE
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·0
1
2
3
4
EXPERIMENT NO.


DATA FROM TABLE IV
MEAN INITIAL GROWTH RATES – mm. · hr.⁻¹ · 100 mm.⁻¹ COLEOPTILE TISSUE
5·8
5·4
5·0
4·6
4·2
3·8
3·4
3·0
2·6
2·2
1·8
1
2
3
4
EXPERIMENT NO.


DATA FROM TABLE VII
MEAN INITIAL GROWTH RATES – mm. · hr.⁻¹ · 100 mm.⁻¹ COLEOPTILE TISSUE
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
1
2
3
4
EXPERIMENT NO.


DATA FROM TABLE VIII
MEAN INITIAL GROWTH RATES — mm. · hr.⁻¹: 100 mm.⁻¹ COLEOPTILE TISSUE
5·8
5·4
5·0
4·6
4·2
3·8
3·4
3·0
2·6
2·2
1·8
1·4
1·0
1
2
3
4
EXPERIMENT NO.

Appendix B

Sample analysis of ^{14}C-bicarbonate fixation data for one experiment on the influence of ethylene on dark fixation after growth inhibition occurs in the absence of IAA.

Total ^{14}C-fixation

Control Conditions $+CO_2/-C_2H_4$			Experimental Conditions $+CO_2/+C_2H_4$		
Vial No.	Dpm ^{14}C/g.fresh weight	log	Vial No.	Dpm ^{14}C/g.fresh weight	log
1	37442	4.5734	1	27315	4.4364
2	55811	4.7467	2	51310	4.7102
3	57346	4.7585	3	65420	4.8157
4	44116	4.6446	4	65142	4.8139
5	38207	4.5821	5	46142	4.6668
6	38317	4.5834	6	29900	4.4757
mean ($\bar{x}$) = 45206			mean ($\bar{x}$) = 47585		
standard error ($S_{\bar{x}}$) = 3731			standard error ($S_{\bar{x}}$) = 6745		

The $\log_{10}$ of the figures for fixation data were computed. These figures were then employed in the data analysis using one way analysis of variance on a Wang 2200 programmable calculator. Results of the analysis are as follows:

Calculated F value = 0.004394

$F_{.95} = 4.96$ (1,10)

	degrees of freedom	
Between groups	Within groups	Total df
1	10	11

Since the calculated F value is $<<4.96$ (1,10) then there is no significant difference between the ethylene treated and the non-treated fixation data. Therefore, ethylene had no influence on dark carbon dioxide fixation after ethylene inhibition of growth had occurred in the absence of IAA.