The Influence of Carbon Dioxide On Growth and Metabolism of Etiolated *Avena sativa* L. Coleoptiles

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To my beloved wife and son.

ABSTRACT

The influence of carbon dioxide on growth and protein synthesis of etiolated *Avena* coleoptiles was investigated. Evidence is presented that 0.03% carbon dioxide stimulated both these processes; and that carbon dioxide stimulated growth depends on carbon dioxide stimulated protein synthesis, In addition the evidence indicates that carbon dioxide stimulated growth is mediated by metabolism, and that carbon dioxide stimulates growth through a dark fixation process. Growth studies also demonstrated that IAA and carbon dioxide stimulated growth in a synergistic manner.

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1. INTRODUCTION

The growth and development of plants depends ultimately on the growth and development of plant cells. The process of plant growth and morphogenesis is initiated by the enlargement of daughter cells following cell division. It is common for the plant cell to expand predominantly in one dimension resulting in elongation. Cell elongation, a major component of plant growth, involves an extension of the cell wall and it is important to recognize that growth of a plant is a function of cell wall extension.

Cell wall extensibility, which is believed to be a controlling factor in cell elongation, is increased by auxin (Cleland, 1968; Masuda, 1968; Coartney, Morre and Key, 1967), low pH (Nitsch and Nitsch, 1956; Rayle and Cleland, 1970) or carbon dioxide (Reinhold and Glinka, 1966). Thesefactors also induce rapid cell elongation and are thought to do so by increasing the extensibility of the cell wall so that turgor pressure on the cell wall will result in cell expansion. Two general theories have been proposed to account for the increase in extensibility; either it may be controlled by synthesis of new cell wall materials (Ray, 1967) or it may be due to degradation of cell wall materials already present (Heyn, 1970).

Growth of etiolated *Avena* coleoptiles has been studied by different investigators using different growth promoting substances, such as auxin (Evans and Ray, 1969; Nissl and Zenk, 1969), low pH (Rayle and Cleland, 1970) or carbon dioxide. There have been many reports of carbon dioxide stimulated extension growth of non-photosynthetic plant tissue and Yamaki (1954), Nitsch and Nitsch (1956) and Harison (1965a; 1965b) have reported that

carbon dioxide can stimulate the extension growth of etiolated *Avena* coleoptiles. Increased carbon dioxide concentration can also stimulate growth of *Avena* mesocotyl and etiolated coleoptile tissues (Mer and Richards, 1950; Mer, 1957). However, the mechanisms by which auxins, low pH values and carbon dioxide stimulate growth is not yet understood.

Recently, Evans, Ray and Rienhold (1971) using excised etiolated *Avena* coleoptile sections, have shown that carbon dioxide saturated water rapidly produced a pronounced stimulation of coleoptile extension. They showed that after a 1 minute lag period carbon dioxide saturated water produced an increase in the growth rate and that the growth rate was 15 times greater than that found in water alone. Auxin, on the other hand, stimulated growth after a lag of 10 - 12 minutes and resulted in a growth rate 8 times that in water alone. They also showed that a citrate buffer and carbon dioxide saturated water, both at pH 3.8, stimulated elongation after a similar short lag period but that the rate of elongation in the buffer was less than that in carbon dioxide saturated water. Evans et al (1971) also demonstrated that carbon dioxide-induced elongation was not the same as auxin-induced growth since the former phenomenon was insensitive to a variety of metabolic inhibitors which suppressed auxin stimulated growth. Furthermore, they showed that IAA did not further accelerate the elongation rate when this had already been promoted by carbon dioxide. However, carbon dioxide could promote the elongation rate of coleoptiles which had been treated with IAA. They suggested that the carbon dioxide effect was a physical phenomenon, independent of immediate biochemical participation, and proposed that carbon dioxide acted by acidification.

Rayle and Cleland (1970) have demonstrated that the mechanisms of hydrogen ion and carbon dioxide stimulated growth are different. They showed that saturating carbon dioxide concentrations would stimulate growth even when the medium was buffered at a pH which did not stimulate growth. Furthermore, they demonstrated that growth stimulation by low pH values could be inhibited by a short exposure to pH 7.0, after which solutions at pH 3.0 would no longer stimulate growth, although solutions saturated with carbon dioxide could still do so. They also pointed out that the maximal growth rate caused by carbon dioxide was greater than the maximal growth rate produced using hydrogen ions. Rayle, Haughton and Cleland (1970) proposed that the response to hydrogen ions more closely resembled the auxin response than the carbon dioxide response because *in vivo* auxin and low pH responses were similar in regard to the maximum rate of extension and the temperature dependence of elongation.

Splittstoesser (1966), using buffered solution with 0.03% carbon dioxide and carbon dioxide free air, showed that carbon dioxide could stimulate growth in a variety of non-photosynthetic tissues, such as excised maize roots, carrots slices and tomato roots. He found growth of excised tomato roots aerated with air was 50% more than growth of the tissue aerated with carbon dioxide free air. He also found that malate and aspartate were the major labelled products when $^{14}CO_2$ was fed to the tissue. Saltman, Kunitabe, Stitt and Spotter (1956) and Saltman, Lynch, Kunitabe and Stitt (1957) also found that malate and aspartate were the initial compounds labelled when young *Bryophyllum* leaves were exposed to $^{14}CO_2$. After relatively long period in the dark, there was a spread of label to other carboxylic acids and amino acids. They concluded that the first product of dark carbon dioxide fixation would be OAA and that malate and

aspartate were secondary products.

Splittstoesser (1966) proposed that carbon dioxide stimulated growth involves carbon dioxide fixation resulting in four carbon acids which can replace Krebs acids lost to the cycle during the biosynthesis of amino acids and other metabolites. For each turn of the Krebs cycle one molecule of oxaloacetate is regenerated to initiate the succeeding turn of the cycle. However, for several intermediates of this cycle, particularly oxaloacetate, α-ketoglutarate and succinyl CoA, there are other metabolic fates alternate to those of the Krebs cycle. Synthesis of aspartate from oxaloacetate, and glutamate from α-ketoglutarate, would inevitably decrease the rate at which the cycle could operate unless the loss of these acids were offset by a replacement of Krebs acids.

In microorganisms Krebs acids are replenished by carbon dioxide fixation with pyruvate or phosphoenolpyruvate (Wiame, 1957). The importance of phosphopyruvate carboxylase (EC. 4.1.1.31) [reaction 1] in replacing Krebs cycle intermediates was indicated by Ashworth, Kornberg and Ward (1965) who showed that phosphopyruvate carboxylase mutants of *E. Coli* could not grow in the presence of glucose unless the medium was supplemented with four, five, or six carbon acids. Also Lips and Beevers (1966) demonstrated that in corn roots malate produced by fixation of carbon dioxide was utilized much faster under conditions which would be expected to increase the demand for acetyl group acceptors in the mitochondria. The carboxylation of phosphoenolpyruvate to yield OAA by phosphoenolpyruvate carboxylase is essentially irreversible and *in vivo* the oxaloacetate is presumably converted to malate by malic dehydrogenase. Phosphoenolpyruvate carboxylase has a high affinity of CO_2 which allows it to fix carbon dioxide even in

CO_2 tension less than 0.03% (Rauson and Thomas, 1960).

$$PEP + H_2O + CO_2 \longrightarrow OAA + Pi \qquad (1)$$

Bown and Lampman (1971) have shown that the presence of the enzyme phosphopyruvate carboxylase in etiolated coleoptiles of *Avena sativa*. They also found that malate and aspartate were the first detectable products of ^{14}C-bicarbonate fixation. Rauson (1953) also demonstrated that carbon dioxide stimulated the levels of malate, aspartate, glutamate and alanine in *Avena* coleoptiles.

Splittstoesser (1966), using ^{14}C-leucine incorporation as an indicator of rates of protein synthesis in carrot slices and tomato roots, showed that these tissueswhen aerated with air incorporated 33% more ^{14}C-leucine into protein than those aerated with carbon dioxide free air. Bown and Lampman (in press) have also found that protein synthesis and radioactive leucine incorporated were reduced in carbon dioxide free air. Thus, carbon dioxide promoted protein synthesis and growth may be related to the role of carbon dioxide fixation in replacing Krebs acids lost to the cycle during the biosynthesis of amino acids required for protein synthesis.

Many investigarors, while studying the growth of plant tissue, use the classical growth promoting auxin indole-3-acetic acid (IAA). It was found in *Avena* coleoptiles by Went (1935) who also showed that the addition of IAA increased the growth of the coleoptiles. Yamaki (1954) found that the elongation of *Avena* coleoptile cylinders was greater in the presence of both IAA and carbon dioxide than in the presence of carbon dioxide alone. He also found that the effect of carbon dioxide was most pronounced when sucrose and IAA were included in the incubation mixture.

Nooden and Thimann (1963) showed that auxin stimulated the growth

of artichoke tuber disks, oat coleoptiles and pea stem sections by 22% to 39%. They also demonstrated that auxin-induced cell enlargement was accompanied by a 17% to 59% increase in incorporation of ^{14}C-leucine into protein, which they considered to be a growth limiting factor. Inhibition of auxin-induced growth by actinomycin D indicates a dependence of continued growth on the synthesis of RNA and they proposed that the action of auxin in cell enlargement is on a nucleic acid system controlling the synthesis of proteins essential for growth.

Key and Shannon (1964) and Key (1964), using soybean hypocotyl tissue, found that puromycin and 8-azaguanine, inhibitors of protein synthesis, inhibited both cell elongation and incorporation of ^{14}C-leucine into protein. In addition, they found that actinomycin D inhibited incorporation of radioactive nucleotides into RNA, radioactive leucine into protein and growth of the tissue. As a result, they concluded that RNA and protein synthesis are essential for growth. Similarly Nooden and Thimann (1966) using a variety of protein synthesis inhibitors, demonstrated a parallel relationship between inhibition of auxin-induced growth and inhibition of auxin-stimulated protein synthesis and proposed that these two processes are closely related to each other and that protein synthesis is a limiting factor in auxin-induced cell expansion.

Patterson and Trewavas (1967), using subapical sections of etiolated peas and the double labelling technique to demonstrate differential protein synthesis, showed that 2×10^{-5} M IAA could change the type of protein synthesized. They proposed that IAA not only increased the incorporation of amino acids into proteins but also changed the pattern of protein synthesis. The effect of IAA may not be general and the rate of synthesis of many proteins may be unaltered by IAA.

Because many reports show that the growth of plant tissue depends on protein synthesis the term growth limiting protein (GLP) has been coined. Cleland (1970, 1971) using *Avena* coleoptile segments, studied the effect of cycloheximide and puromycin on the stability and pool size of the GLP. Cleland found that inhibition of growth followed inhibition of protein synthesis by 20 - 25 minutes regardless of the growth rate, which indicated that the disappearence of the growth limiting protein was due to its functional instability rather than its consumption in the growth process. He also found that the size of this proposed GLP pool was low in the absence of IAA but on the addition of IAA rapidly expanded and reached a maximum within 20 - 25 minutes. Cleland suggested that auxin-induced growth is determined by the size of this growth limiting protein and proposed that auxin could cause either *de-novo* synthesis of this protein or activate GLP which is synthesized independently of auxin. These results indicate that continued cell elongation depends on continued protein synthesis but which proteins are needed and how they contribute to cell elongation is still far from clear.

Despite all the evidence demonstrating that auxins stimulate RNA and protein synthesis, there is much evidence that these processes are stimulated indirectly and that the primary action of auxin is on some other process. Many possible sites of action for auxin have been proposed and include an influence on transcription, translation allosteric enzymes and membranes.

Key (1966) showed that 5-fluoro-uracil selectively inhibited ribosomal and soluble RNA without affecting D-RNA, which he believed to be an "essential RNA" for auxin-induced growth. It is called D-RNA because of DNA like properties and is very different from soluble and ribosomal RNA.

It was first called AMP-rich RNA, because of a high AMP and low GMP ratio. Its composition, sedimentation (stability) are similar to those of m-RNA in bacterial systems and therfore it is suspected that this RNA functions as messenger RNA in auxin-induced growth of plant tissue. Key and Ingle (1968) also found that actinomycin D would inhibit the auxin-induced growth of tissue treated with 5-fluorouracil and as a result of these experiments, they suggested that the ability of auxin to stimulate growth depended upon the synthesis of AMP-rich RNA which has properties similar to those of messenger RNA (Ingle, Key and Holm, 1965).

This type of data has led to the advancement of the gene activation hypothesis which proposes that the primary action of auxin occurs through a specific stimulation of RNA synthesis on a DNA template. Specific RNA synthesis could result in the synthesis of particular enzymes or proteins essential for auxin-induced growth.

Another hypothesis is that auxins stimulate the protein synthesizing mechanism of the cell and that this effect on the translational process leads to the specific synthesis of essential protein(s) or enzyme(s). Bendana and Galston (1965) followed the metabolism of ^{14}C-IAA in excised green pea stem segments. Separation of extracted RNA in a sucrose density gradient showed that ^{14}C-IAA was concentrated in a 4S peak, which also showed greater stability to ribonuclease than a similar fraction obtained from tissue not treated with IAA. Bendana, Galston, Kauer-Sawhney and Penny (1965) also found that the kinetics of labelled IAA incorporation into RNA resembled the kinetics of growth induced by IAA. They found that ^{14}C from methylene--labelled IAA and carboxyl and methylene--labelled 2,4-dichlorophenoxyacetic acid (2,4-D) were also incorporated into RNA, but with lower efficiency than carboxyl-labelled IAA.

Furthermore a "substantial fraction" of the bound label appeared to be recoverable as IAA and they demonstrated that ^{14}C-label was associated with adenylate and cytidylate. In his review on auxins, Gordon (1954) presented evidence for the existence of a protein-bound auxin fraction which was recoverable following acid or enzymic hydrolysis. As a result of this kind of evidence, Armstrong (1966) proposed that auxin may stimulate the translation of mRNA by supplementing or replacing N-formylmethionine on transfer RNA as a signal for polypeptide chain initiation. Nooden and Thimann (1966) showed a parallel relationship between inhibition of growth and inhibition of protein synthesis by a variety of protein synthesis inhibitors. They suggested that auxin may stabilize an unstable messenger RNA leading to the synthesis of protein(s) or enzyme(s) necessary for cell elongation.

On the other hand, many authors have obtained evidence that the site of action of auxin in stimulating growth cannot be on the transcriptional or translational processes. Nissl and Zenk (1969), using oat coleoptiles, studied the time required for auxin induced cell elongation. They found that using 10^{-5} M IAA at $21^{o}C$ a lag phase of about 10 minutes was obtained before growth was stimulated. By increasing the IAA concentration from 10^{-5} M to 10^{-3} M and temperature from 21^{o} to $29^{o}C$, they eliminated this lag phase completely without affecting the growth rate. They pointed out that the induction of protein synthesis via transcription or translation is a time-consuming process and is preceeded by a characteristic lag phase. Since they obtained stimulated growth with no lag phase, they concluded that the primary site of auxin action was not on the transcriptional or translational processes. They also opposed the proposal put forward by

Bendana et al (1965) who stated that labelled IAA was incorporated into RNA. They demonstrated that when radioactive auxins were added to tissue less than 1% of the radioactivity in the 80% ethanol extract represented metabolized auxin, and that no radioactivity was associated with the macromolecular components. As a result they concluded that auxin is active in causing an elongation response only in the free state.

Ray (1969) and Evans and Ray (1969) studied the gene activation hypothesis by examing the effect of inhibitors of RNA and protein synthesis upon the timing of the growth response to auxin. They expected that if elongation results from auxin-induced transcription or translation the lag phase before auxin induced elongation would be lengthened by inhibitors of these processes. They found, however, that doses of actionomycin D, cycloheximide or puromycin caused no substantial increase in the lag period before auxin stimulated growth. In fact cycloheximide actually shortened the lag period. If auxin stimulates growth via gene activation the lag period should be extended by these inhibitors. This was not the case, however, and Ray proposed that auxin probably does not act by promoting the synthesis of specific mRNA molecules. Evans and Ray (1969), after studying the growth response to auxin, proposed that the site of auxin action could be on the activities of preformed enzymes. Ray, however, agrees that for continued long term growth protein synthesis is necessary.

Pope and Black (1972), using etiolated wheat coleoptiles, studied the influence of IAA and cycloheximide on growth and protein synthesis. They found that cycloheximide (1µg/ml) inhibited growth and ^{14}C-leucine incorporation by 13% and 72% within 1 hour and also found that 10 µg/ml of cycloheximide inhibited ^{3}H-leucine incorporation into protein by at least

90% within 10 minutes. Despite this virtual elimination of protein synthesis, they demonstrated that IAA-induced growth could still occur in the presence of cycloheximide (10 μg/ml) if the tissue were pretreated with the inhibitor up to 2 hours before the addition of IAA. They proposed that IAA induces growth by increasing the activity of a growth limiting factor.

These results, in their entirety, make it highly possible that the primary site of auxin action is not upon transcription or translation. The activity of a few enzymes has been shown to be increased by growth-promoting concentrations of auxin, but whether these changes are due to increases in enzyme synthesis, decreases in enzyme breakdown or auxin stimulated activation of preformed enzymes has not been fully established. For example, Cleland (1967), suggested that the site of auxin action could be on an already synthesized allosteric enzyme.

Pope and Black (1972) showed that for 2 hours after the addition of cycloheximide and almost complete inhibition of protein synthesis, wheat coleoptiles could still exhibit increased growth in the presence of auxin. They proposed that growth depends on the availability of protein and that auxins act directly on pre-existing protein which apparently has a life of about 2 hours. Sarkissian (1968) studied the effect of IAA on citrate synthase extracted from corn scutella. He found that 1.25×10^{-10} M IAA increased the enzyme activity by 35% and that there was a sigmoidal relationship between substrate concentration and reaction rate. This suggests that citrate synthase is an allosterie enzyme and that IAA is a positive effector. Trewavas (1968), using etiolated subapical section of *Pisum sativum*, studied the effect of IAA on RNA and protein synthesis. He found that IAA stimulated RNA synthesis, that the incorporation of

radioactive RNA precusors into RNA was first detected in the nuclear fraction and that IAA could alter the GMP-AMP ratio of the newly synthesized RNA. He used this indirect evidence to suggest that IAA activates a DNA dependent RNA polymerase.

A direct effect of auxin on cell membranes was recently put forward by a number of investigators as a possible site of auxin action. Rayle, Ouitrakul and Hertel (1969), using corn coleoptiles and various auxins, studied the influence of one auxin on the basipetal transport of another auxin. They found that IAA enhanced the transport of 2,4-D, the transport of IAA and α-naphthalene acetic acid (α-NAA) were also stimulated by auxins within 20 minutes. Assuming that the controlling factor with regard to transport was the rate of movement of auxins through the plasma membrane, they suggested that the evidence was consistent with an auxin influence directly on the membrane. Etherton (1970), using etiolated oat coleoptiles, made a preliminary study on the effect of IAA on membrane potentials. The author found that by increasing IAA concentration from 10^{-9} to 10^{-7} M the membrane potentials of coleoptile cells became more negative and also that solutions with IAA concentration from 10^{-8} to 10^{-6} M made the cytoplasmic and vacuolar potentials more negative than solutions with 10^{-9} M or lower IAA concentrations. Etherton speculated that auxin-induced cell elongation might be directly correlated with the effect of auxin on membrane potentials.

The above discussion leads to the conclusion that long term auxin stimulated growth depends on continued protein synthesis. However, despite the work of many people over many years, the mechanism by which auxin stimulates growth and protein synthesis is still far from clear.

On the other hand, the carbon dioxide stimulated growth of etiolated plant tissues has not been so extensively investigated and it is possible that an understanding of this phenomena will yield insights into the growth process. Questions that have not been vigorously investigated include the site of action of carbon dioxide in stimulating growth, whether or not carbon dioxide stimulated protein synthesis is necessary for carbon dioxide stimulated growth; the relationship between carbon dioxide and IAA in stimulating growth and whether or not carbon dioxide and IAA stimulate the synthesis of the same kinds of protein.

This investigation was undertaken to obtain evidence pertaining to these questions and to explore the possibility that carbon dioxide stimulated growth and protein synthesis are mediated by dark carbon dioxide fixation.

2. MATERIALS

2.1 Biological Materials

Seeds of *Avena sativa* (var. Victory) were purchased from Wards Biological Supply House, Chicago, Illinois, U. S. A.

2.2 Chemicals

Except for those listed below, all common chemicals were purchased either from BDH (Canada) Ltd. or Fisher Scientific Co., N. J., and were of analytical grade.

(a)	Phenol Reagent (Folin & Ciocalteu) :	BDH (Canada) Ltd., Toronto.
(b)	Cyanogum 41 (Gelling Agent) :	Fisher Scientific Co., N. J.
(c)	Cyanogum 41 Catalyst (b-dimethylaminopropionitrile) DMAPN :	Fisher Scientific Co., N. J.
(d)	NCS - Solubilizer (0.6 N Solution in toluene) :	Amersham / Searle Corporation, U. S. A.
(e)	Hydroxide of Hyamine 10 - x:	Packard Instrument Co. Inc., Illinois, U. S. A.
(f)	POPOP : 1,4-bis 2 - (4 - Methyl- 5 - Phenyloxazolyl) - Benzene (Scintillation grade)	Packard Instrument Co. Inc., Illinois, U. S. A.
(g)	PPO 2,5 - Diphenyloxazole (Scintillation grade)	Packard Instrument Co. Inc., Illinois, U. S. A.

(h)	Indole-3-Acetic Acid: (1AA)	Sigma Chemical Company, St. Louis, Mo, U. S. A.
(i)	Cycloheximide :	Sigma Chemical Company, St. Louis, Mo, U. S. A.
(j)	Brilliant Blue R (Coomassie Brilliant Blue) :	Sigma Chemical Company, St. Louis, Mo, U. S. A.
(k)	L-Leucine :	General Biochemicals, Chagrin Falls, Ohio, U. S. A.
(l)	Photoflo 200 Solution :	Eastman Kodak Company, Rochester, N. Y., U. S. A.
(m)	Indicarb 10-20 Mesh : (CO_2 absorber)	Fisher Scientific Co., N. J.
(n)	L-Leucine-^{14}C (U) : Specific Activity = 331 m Ci/m mol	Amersham / Searle Corporation, U. S. A.
(o)	Sodium Bicarbonate - ^{14}C : Specific Activity = 59 m Ci/m mol	Amersham / Searle Corporation, U. S. A.

2.3 Reagents

1. Phosphate Buffer, pH 7.5

(a) 0.1 M KH_2PO_4 solution

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

(b) 0.1 M $NaHPO_4$ solution

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

(i) 0.1 M Phosphate Buffer

Solution (a) and solution (b) were mixed together with constant stirring until the pH of the solution was 7.5.

(ii) 0.025 M Phosphate Buffer

Solution (a) and solution (b) were diluted 4 times and then were mixed with constant stirring until the pH of the solution was 7.5.

(iii) 0.001 M Phosphate Buffer

Solution (a) and solution (b) were diluted 100 times and then were mixed with constant stirring until the pH of the solution was 7.5.

2. (i) IAA Stock Solution

0.438 g of indole-3-acetic acid was dissolved in a small volume of ethanol and diluted to 250 ml with phosphate buffer (0.025 M, pH 7.5) to give the final concentration of 10^{-2} M.

(ii) IAA working standard

1 ml of the above IAA solution was diluted 500 times with phosphate buffer (0.025 M, pH 7.5) to give a final concentration of 2×10^{-5} M.

3. 50% (w/v)$(NH_4)_2SO_4$ solution

50 g of ammonium sulphate crystal was dissolved in and made up to 100 ml with distilled water.

4. 2% (w/v)$NaHCO_3$ in 0.1 N NaOH solution

2.0 g of sodium bicarbonate was dissolved in and made up to 100 ml with 0.1 N sodium hydroxide solution.

5. 0.5% (w/v)$CuSO_4$ solution

0.5 g of copper sulphate crystals were dissolved in and made up to 100 ml with distilled water.

6. 0.1% (w/v) Leucine in 25% $(NH_4)_2SO_4$ solution

0.1 g of L-leucine was dissolved in and made up to 100 ml with 25% ammonium sulphate solution, which was prepared by dissolving 25.0 g ammonium sulphate up to 100 ml with distilled water.

7. 0.3% (w/v) Leucine in phosphate buffer

0.3 g of L-leucine was dissolved in and made up to 100 ml with phosphate buffer (0.025 M, pH 7.5).

8. Tris/HCl Buffer

(a) 1 M Tris solution

121.1 g of Tris (hydroxymethylaminomethane) was dissolved and diluted to 1 litre with distilled water.

(b) 1 M HCl

Standard 1 N hydrochloric acid.

(i) Tris/HCl buffer pH 7.5

Solution (a) was added to solution (b) with constant stirring until the pH of the solution was 7.5.

(ii) Tris/HCl buffer pH 9.5

Solution (a) was added to solution (b) with constant stirring until the pH of the solution was 9.5

9. Tris/Glycine Buffer (pH 8.3)

Tris = 4.0 g

Glycine =14.4 g

Tris and glycine were dissolved in and made up to 1 litre with distilled water.

This stock solution was diluted 10 times just prior to use as the coil chamber buffer for electrophoresis.

10. 0.15% (w/v) $(NH_4)_2S_2O_8$ solution

0.15 g of ammonium persulphate crystals was dissolved in and made up to 100 ml with distilled water. This solution was prepared daily.

11. 12.5% (w/v) TCA solution

12.5 g of trichloroacetic acid was dissolved in and made up to 100 ml with distilled water.

12. 10.0% (w/v) TCA solution

10.0 g of trichloroacetic acid was dissolved in and made up to 100 ml with distilled water.

13. 1% (w/v) Aqueous Coomassie Blue (Stock)

1.0 g of coomassie brilliant blue was dissolved in and made up to 100 ml with distilled water.

For the staining solution, this stock solution was diluted 20 times with 12.5% (w/v) trichloroacetic acid solution and was prepared daily.

14. 0.005% Bromophenol Blue Indicator (pH 2.8-4)

1% stock aqueous bromophenol blue indicator solution was diluted 200 times with distilled water.

15. (i) CH_3 COOK Stock Solution

0.2454 g of potassium acetate crystals were dissolved in and made up to 250 ml with distilled water togive a final concentration of 10^{-2} M.

(ii) CH_3 COOK working standard

2.5 ml of the above stock solution was diluted to 50 ml with phosphate buffer (0.025 M, pH 7.5) to give a final concentration of 5×10^{-4} M.

16. (i) Malate Stock Solution

0.335 g of L-malic acid crystal was dissolved in and made up to 250 ml with distilled water to give a final concentration of 10^{-2} M.

(ii) Malate Working Standard

2.5 ml of the above stock solution was diluted to 50 ml with phosphate buffer (0.025 M, pH 7.5) to give a final concentration of 5×10^{-4}M.

17. 2% (v/v) Photoflo Solution

2 ml of photoflo solution was diluted to 100 ml with distilled water.

18. Concentrated Ammonium Hydroxide Solution

(25% NH_3)

19. (i) Scintillation Fluid I

5.0 g of 2,5-diphenylaxazole and 300 mg of 1,4-bis 2-(4-Methyl-5-Phenyl-oxazolyl) - benzene were dissolved in and made up to 1 litre with toluene.

(ii) Scintillation Fluid II

667 ml of scintillation fluid I was added to 333 ml of absolute ethanol.

3. METHODS

3.1 Growing and Harvesting of Coleoptiles

Seeds of *Avena sativa* (var. Victory) were husked and soaked for two hours in deionized 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 1 cm apart, with the coleoptile uppermost and were incubated at 25°C under weak red light for about 72 hours.

Coleoptiles between 2 and 3 cm were selected and harvested under weak green light. The apical 3 mm tips of the coleoptiles were removed and the adjacent 2 cm sections used. The inner leaf from each coleoptile was removed. Coleoptiles were put into preweighed light tight vials, containing 5 ml of phosphate buffer (0.025 M, pH 7.5) with concentrations of various test substances. The vials plus the contents were then weighed again to establish the weight of the tissue.

3.2 Incubation of the Tissue

Weighed batches of tissue were transferred into light tight incubation tubes, containing 10 ml of phosphate buffer (0.025 M, pH 7.5), with concentrations of various test substances. Two basic types of tissue incubation were used:

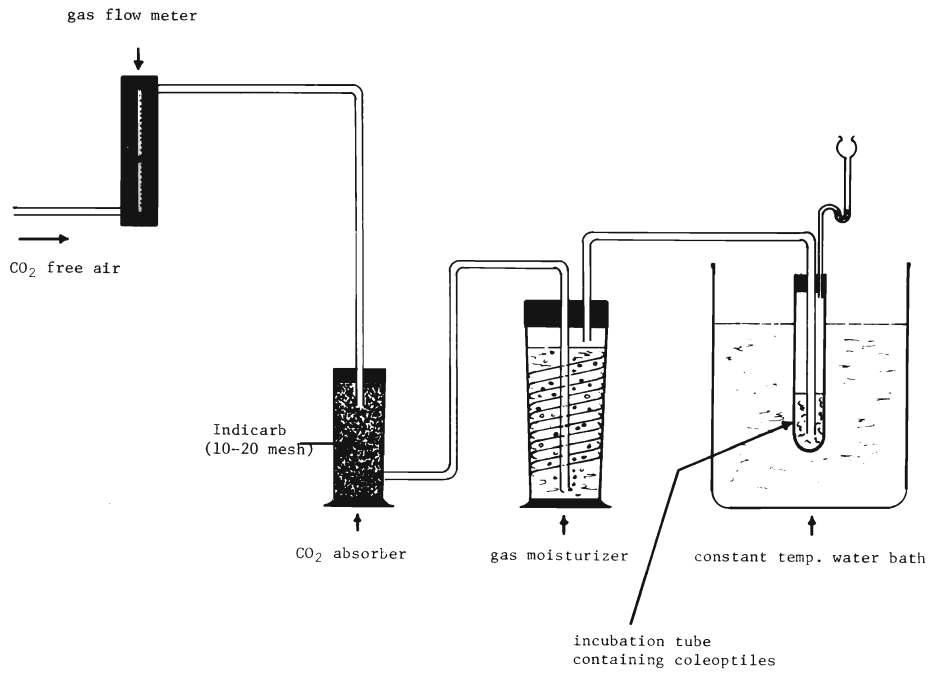
(i) A system in which the tissue was aerated with a gas stream. This was used for growth measurements and ^{14}C-leucine incorporation experiments

(ii) A closed system was used in experiments involving ^{14}C-bicarbonate incorporation.

(i) Gas stream system (Figure 1)

Tissue was incubated in a water bath at 25°C with aeration by air (0.03% CO_2) or CO_2 free air at a rate of 0.4 cuft/h/tube. In studies

Figure 1: Gas Stream System



involving the incorporation of ^{14}C-leucine (U) incubation proceeded for 30 minutes before the addition of radioactive leucine. Incorporation was terminated at various times as indicated in different experiments. For growth experiments, tissue was incubated for 2 hours.

(ii) Closed system

Tissue was incubated in a closed container containing 15 ml of phosphate buffer (0.025 M, pH 7.5) with various concentrations of test substances. ^{14}C-bicarbonate was added after 30 minutes incubation and the incubation was continued for various periods of time.

After the incubation with radioactive compounds the tissue was immediately washed with deionized water (about 300 to 400 ml) and was frozen in a freezer for 3 to 4 hours. In the growth experiment, the tissue was immediately washed and the length of each coleoptile section was measured.

3.3 Measurement of Growth

Six batches of tissue were used for every growth experiment. Three batches were exposed to one of IAA, malate, acetate or cycloheximide and 3 batches were not exposed to these substances. From each group of 3 batches, one was used to determine the zero hour lengths and the other 2 batches were used to determine the influence of air or CO_2 free air on growth during a 2 hour incubation. All batches of tissue were then washed with deionized water and the length of the sections measured, using dissecting microscope, after placing the coleoptile in groove of a plastic block. A ruler, which was graduated into one hundredths of an inch, was fastened along the side of the groove. The coleoptile section was placed with one end touching the sealed end of the groove while the other end was used to measure the length. All these procedures were carried out under weak green light at room temperature. After measurement coleoptile lengths were converted from inches into

millimeters.

3.4 Extraction of Soluble Proteins

The frozen tissue was thawed and then ground in a Potter-Elvehjen tissue homogenizer for 5 minutes at 5000 rpm with 5 ml of phosphate buffer (0.001 M, pH 7.5) at 4°C. It was then centrifuged at 13,000 g for 15 minutes in a B-20 International Centrifuge at 4°C. The supernatant was collected and the extraction procedure repeated 2 more times, first with 0.025 M phosphate buffer (pH 7.5) and then with 0.1 M phosphate buffer (pH 7.5). The three supernatant fluids were then combined.

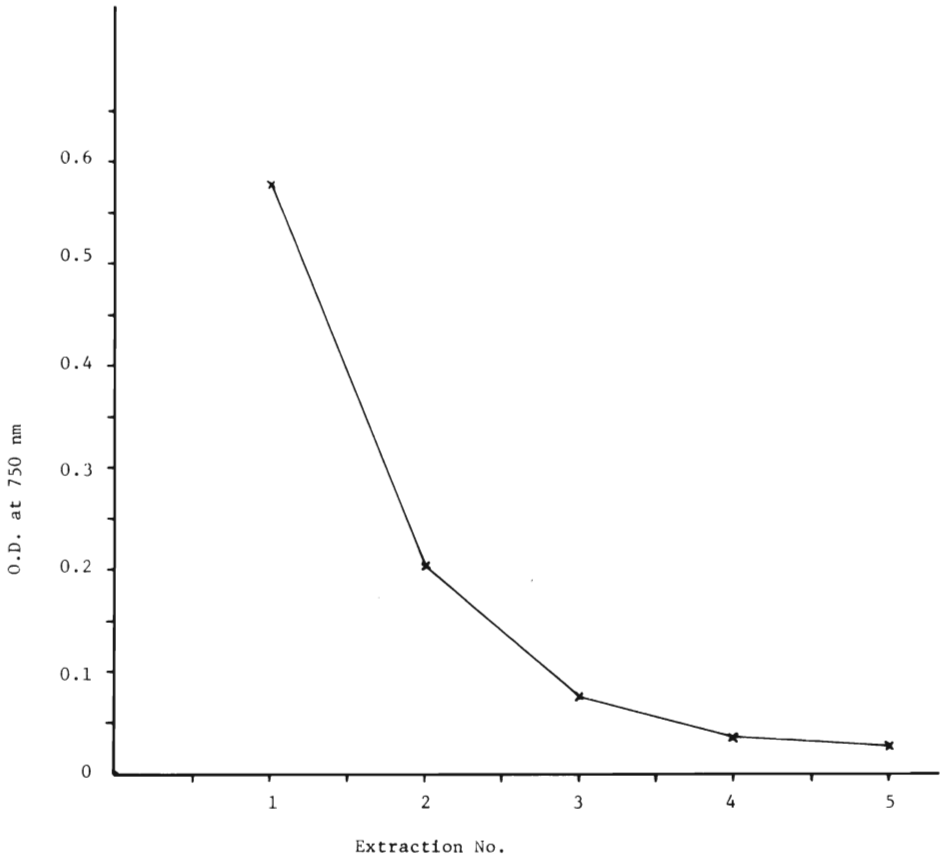
The thoroughness of the extraction procedure in removing Folin positive substances from the insoluble debris was tested by five successive extractions. Figure 2 indicates the amount of Folin positive materials extracted at each step.

3.5 Preparation of Cell Wall Fraction

Five ml of phosphate buffer (0.025 M, pH 7.5) containing non-radioactive leucine (0.3% w/v) was added to the residue of the above extraction procedure (3.4) and was shaken vigorously with a mechanical vibrator for one minute. It was then centrifuged in a B-20 International Centrifuge at 18,000 g for 20 minutes and the supernatant was discarded. The pellets were used for the determination of radioactive material in the cell wall fraction.

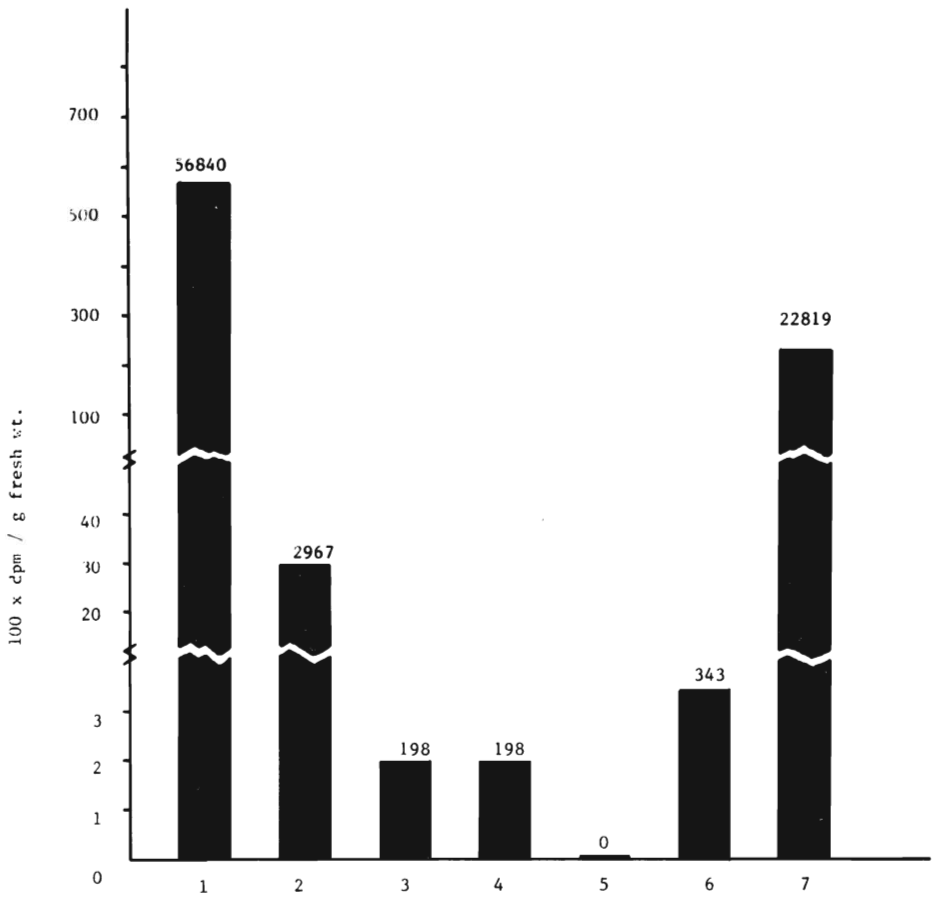
The thoroughness of the extraction procedure in removing extractable radioactivity from the cell wall fraction is indicated in Figure 3.

Figure 2: Efficiency of soluble protein extraction technique.



Note: Approximately 0.5 g of coleoptile tissue was extracted with 5 ml of phosphate buffer (0.001 M, pH 7.5) for 5 minutes and then centrifuged. The supernatent was collected and the pellet was extracted four more times with different concentration of phosphate buffers at pH 7.5. First with 0.025 M, and then three extractions with 0.1 M phosphate buffer. Soluble protein from each extraction was determined. Similar curves were obtained from 4 different batches of tissue.

Figure 3: Distribution of radioactivity at different steps during the preparation of the cell wall fraction of *Avena* coleoptiles.



Note: Approximately 0.5 g of coleoptile tissue was extracted five times with different concentration of phosphate buffers. The experimental conditions were the same as in Figure 2. Radioactivity in each supernatent fluid and the cell wall fraction was determined. Similar histograms were obtained from 4 different batches of tissue.

1 - 5 = extraction with different concentration of phosphate buffer
6 = washing with a phosphate buffer containing leucine
7 = cell wall fraction

3.6 Determination of Soluble Proteins

An equal volume of ammonium sulphate (50% w/v) was added to the combined extracts (3.4) and was left overnight at $4^{o}C$. It was then centrifuged at 25,000 g for 30 minutes in a B-20 Internation Centrifuge and the supernatant was discarded. The pellets were washed with 5 ml of a solution containing non-radioactive leucine (0.1% w/v) in 25% (w/v) ammonium sulphate and was then centrifuged at 25,000 g for 30 minutes. The supernatant was discarded and the pellets were redissolved in phosphate buffer (0.025 M, pH 7.5). Aliquots of 0.2 ml of this solution were used for the protein determination by the standard Folin and Ciocalteau method (Lowery et al, 1950) and bovine serum albumin was used as the reference standard.

3.7 Determination of Radioactivity

Radioactivity was determined using a Packard 3310 liquid scintillation counter. Settings for the instrument were 9.5% gain with 50-1000 window for the red channel and 9.5% gain with 50-1000 window for the green channel. The blue channel which had setting of 2.0% gain and a 300-1000 window was used to determine counting efficiency by the automatic external standard method. The samples were counted for 10 minutes or until 20,000 counts had accumulated. The efficiency of the counting process varied for different scintillation systems.

(i) Soluble Proteins

A 0.2 ml aliquot of the sample solution was added to a scintillation vial, containing 10 ml of scintillation fluid II and shaken to form a one phase liquid. The efficiency of counting ^{14}C in this system fluctuated between 60 and 63%. Duplicate determinations were done for each sample.

(ii) Cell Wall Fraction

The insoluble residue (3.5), after washing with phosphate buffer containing non-radioactive leucine (0.3% w/v), was resuspended in 5 ml of distilled water. The suspension was transferred into a scintillation vial and treated at 50°C until dry. To the vial was added 2 ml of hydroxide of hyamine and the vial was heated at 50°C for 24 hours. Ten ml of scintillation fluid I was added and radioactivity measured. The efficiency of counting ^{14}C in this system varied between 50 and 54%.

(iii) Polyacrylamide gel slices

To a 2 mm section of an acrylamide gel slice in a scintillation vial was added 1.5 ml of MCS solvent and 0.15 ml of concentrated ammonium hydroxide solution (Ward, Wilson & Gilliam, 1970). The vials were heated at 50°C for 24 hours and then 10 ml of scintillation fluid II was added. The efficiency of counting ^{14}C in this sytem varied between 59 and 62%.

3.8 Determination of Oxygen Consumption

Oxygen consumption was measured by the standard Warburg manomentric technique using a Gilson Differential Respirometer. Batches of coleoptile sections containing approximately 0.5 g of tissue were transferred into 2.5 ml of phosphate buffer (0.025 M, pH 7.5) with or without cycloheximide (6 µg/ml). Measurements were taken at 25°C for 3 hours.

3.9 Analytical Polyacrylamide Gel Electrophoresis

Electrophoresis was carried out using a Quickfit electrophoresis unit with a capacity of 8 tubes, each of which was 6.5 cm long and 0.5 cm in diameter. The discontinuous buffer system was used according to the method of Ornstein and Davis (1964) with following modifications.

(i) Preparation of gel

Six per cent (w/v) separating gels were prepared as follows:

Cyanogum 41	= 1.2 g
Tris/HCl buffer (pH 9.5)	= 10 ml
Cyanogum 41 catalyst	= 0.1 ml

10 ml of 0.15 % (w/v) freshly prepared ammonium persulphate solution was added just before the gelling solution was added to the glass tubes, which were presoaked in 2% (w/v) photoflo solution. The tubes were filled up to the 4.5 cm marker level and the gel surface was carefully covered with distilled water, without disturbing the flat gel surface. After 2 hours of polymerization the distilled water was poured off and the surface of the gel was washed with 1.5% Cyanogum 41 in Tris/HCl buffer (pH 7.5) solution.

Three per cent (w/v) stacking gel was prepared as follows:

Cyanogum 41	= 0.6 g
Tris/HCl buffer (pH 7.5)	= 10 ml
Cyanogum 41 catalyst	= 0.1 ml

10 ml of 0.15% (w/v) freshly prepared ammonium persulphate solution was added just before the gelling solution was added on top of the separating gel up to the 5.5 cm marker level of the tube. The gel surface was then carefully covered with distilled water. After 2 hours of polymerization the distilled water was poured off and the surface of the gel was washed with distilled water.

The gel tubes were transferred to the coil chamber unit. The tubes were inserted carefully through rubber grommets and adjusted till the tubes were all the same height.

(ii) Electrophoresis

A 0.2 ml aliquot of protein solution (3.6) containing 40% (w/v) sucrose was layered on top of the stacking gel and was carefully covered with coil chamber buffer. The lower compartment was filled to a depth of 5 cm with coil chamber buffer, 10 times diluted Tris/Glycine buffer (pH 8.3). The upper chamber was also filled with the same buffer until the top of the electrophoresis tubes were immersed. About 1 ml of 0.005% (w/v) bromophenol blue indicator was added to the upper compartment buffer. Electrophoresis was started by adjusting the applied voltage to give a current of 4 - 5 mA per tube. It was terminated when bromophenol blue dye was approximately 4 mm from the bottom of the tube, usually after 45 - 50 minutes.

(iii) Staining the gel

The gels were removed from the tube by rotating a hypodermic syringe needle around the bottom end of the gels and the loosened gel was then pushed out by loosening the top end with a needle through which was passed a fine stream of water. The gels were immediately soaked in 12.5% (w/v) trichloroacetic acid solution for an hour with occassional shaking. The gels were then transferred into a solution containing 0.05% coomassie blue in 12.5% (w/v) trichloroacetic acid for 24 hours. Destaining was accomplished by soaking the stained gel in 10% (w/v) TCA solution for another 24 hours. (Chramback, Reisfled, Wyckoff and Zaccari, 1967).

(iv) Scanning the protein distribution along the gels

Scanning of the gels was done using a Gilford Spectrophotometer 2440 at 600nm, distilled water being used as a blank. Full scale deflection of the recording pen was calibrated to be equivalent to 2.0 optical density

units, and the slit width of the incident light was 0.1 mm. The chart speed was 2 inches per minute whilst the speed of the scanned gel was 0.5 cm per minute.

(v) Determination of Radioactivity along the gel

After electrophoresis the gel was removed from the tube and without fixing or staining placed snugly in a groove of a plastic block. The gel was sliced into 2 mm sections using another block which had inserted into it razor blades arranged parallel and 2 mm apart. When slicing the gel the razor blades fitted into spaces in the first block which are perpendicular to the gel and 2 mm apart. Radioactive assays of gel slices were performed as in section 3.7 (iii).

4. RESULTS

The results of this investigation are described under the following categories:

1. Growth studies
2. Protein and cell wall metabolism
3. Changes in the pattern of protein synthesis
4. The influence of IAA on the incorporation of radioactive bicarbonate

4.1 Growth Studies

In all growth study experiments, batches of 30 coleoptiles were used for each physiological condition and the experiment was done twice. The influence on growth by various substances was studied by determining the significance of differences in mean lengths between batches of coleoptiles incubated under different conditions. The null hypothesis was used in conjunction with the "t" test to determine significance levels (p values) of these differences.

By examining Table 1, it can be seen that in the absence of IAA 0.03% carbon dioxide stimulated growth by 88%, whereas in the absence of carbon dioxide IAA stimulated growth by 108%. In the presence of both factors maximum growth was obtained and growth was stimulated by 388%. These figures indicate that carbon dioxide and IAA stimulate growth in a synergistic manner and the absence of either factor drastically reduces growth. Table 2 indicates the significance of the differences in the growth values shown in Table 1.

Table 3 shows the influence of malate and carbon dioxide on the growth of the coleoptiles. It can be seen that malate and carbon dioxide

Table 1: Influence of IAA and carbon dioxide on growth of *Avena* coleoptiles.

Treatment	Initial Length (mm)	Final Length (mm)	Growth Rate (mm/h/coleoptile)
IAA + CO_2	21.06±0.06	22.28±0.23	0.610
IAA + no CO_2	21.06±0.06	21.62±0.17	0.260
no IAA + CO_2	20.71±0.05	21.18±0.07	0.235
no IAA + no CO_2	20.71±0.05	20.96±0.06	0.125

Note: Batches of 30 coleoptile sections, approximately 20 mm. long, were placed in phosphate buffer (0.025 M, pH 7.5) in the presence or absence of IAA (2×10^{-5} M) for approximately 1 h. before treatment with or without carbon dioxide (0.03%) for 2 h. Measurements were taken at 0 h. and after 2 h. incubation. Figures represent the average of two experiments. Results are expressed as the mean ± the standard deviation.

Table 2: Analysis of the growth promoting influence of IAA and carbon dioxide by the "t" test.

Treatment	+ IAA	- IAA
Effect of CO_2	$p < 0.001$	$p < 0.001$

Treatment	+ CO_2	- CO_2
Effect of IAA	$p < 0.001$	$p < 0.001$

Note: These figures are derived from the data in Table 1.

Table 3: Influence of malate and carbon dioxide on growth of *Avena* coleoptiles.

Treatment	Initial Length (mm)	Final Length (mm)	Growth Rate (mm/h/coleoptile)
Malate + CO_2	20.78±0.203	21.21±0.203	0.215
Malate + no CO_2	20.78±0.203	21.18±0.057	0.200
no Malate + CO_2	20.73±0.173	21.15±0.228	0.210
no Malate + no CO_2	20.73±0.173	20.99±0.156	0.130

Note: Batches of 30 coleoptile sections, approximately 20 mm. long, were placed in phosphate buffer (0.025 M, pH 7.5) in the presence or absence of malate (5×10^{-4} M) for about 1 h. before treatment with or without carbon dioxide (0.03%) for 2 h. Measurements were taken at 0 h. and after 2 h. incubation. Figures represent the average of two experiments. Results are expressed as the mean ± the standard deviation.

Table 4: Analysis of the growth promoting influence of malate and carbon dioxide by the "t" test.

Treatment	+ Malate	- Malate
Effect of CO_2	N.S.	$p < 0.001$

Treatment	+ CO_2	- CO_2
Effect of Malate	N.S.	$p < 0.001$

Note: These figures are derived from the data in Table 3. N.S. = Not Significant

stimulated growth to the same extent, i.e. approximately 60%. However, neither malate nor carbon dioxide in the presence of the other factor increased significantly the growth of the coleoptiles. The relationship between malate and carbon dioxide in stimulating growth is thus different from the synergistic relationship between IAA and carbon dioxide (Table 1). It appears from the data that malate and carbon dioxide are interchangeable in their growth stimulating capacity. Table 4 shows that in the absence of the other factor both malate and carbon dioxide significantly stimulated growth; whereas in the presence of the other factor malate and carbon dioxide did not significantly stimulate growth.

It is shown in Table 5 that whereas carbon dioxide stimulated growth by 125%, acetate stimulated growth by 100%. However, in the presence of both factors growth was stimulated 135%. Table 6 demonstrates that even though the influence of acetate and carbon dioxide were highly significant ($p < 0.001$), the significance level of the influence of carbon dioxide in the presence of acetate was found to be $p < 0.1$ and acetate did not significantly stimulate growth in the presence of carbon dioxide. Thus whereas carbon dioxide did not stimulate growth in the presence of malate it did stimulate growth in the presence of acetate.

Tables 7 and 8 indicate the influence of cycloheximide (CHI) on carbon dioxide stimulated growth. CHI inhibited not only the carbon dioxide stimulated growth but also the basal level of growth found in the absence of carbon dioxide. Carbon dioxide did not stimulate growth in the presence of CHI but it did in the absence of CHI. It stimulated growth by 116%.

Table 5: Influence of acetate and carbon dioxide on growth of *Avena* coleoptiles.

Treatment	Initial Length (mm)	Final Length (mm)	Growth Rate (mm/h/coleoptile)
Acetate + CO_2	20.70±0.152	21.17±0.224	.235
Acetate + no CO_2	20.70±0.152	21.10±0.143	.200
no Acetate + CO_2	20.64±0.094	21.09±0.172	.225
no Acetate + no CO_2	20.64±0.094	20.84±0.064	.100

Note: Batches of 30 coleoptile sections, approximately 20 mm. long, were placed in phosphate buffer (0.025 M, pH 7.5) in the presence or absence of acetate (5×10^{-4} M) for about 1 h. before treatment with or without carbon dioxide (0.03%) for 2 h. Measurements were taken at 0 h. and after 2 h. incubation. Figures represent the average of two experiments. Results are expressed as the mean ± the standard deviation.

Table 6: Analysis of the growth promoting influence of acetate and carbon dioxide by the "t" test.

Treatment	+ Acetate	- Acetate
Effect of CO_2	$p < 0.1$	$p < 0.001$

Treatment	+ CO_2	- CO_2
Effect of Acetate	N.S.	$p < 0.001$

Note: These figures are derived from the data in Table 5.
N.S. = Not Significant

Table 7: Influence of cycloheximide on carbon dioxide stimulated growth of *Avena* coleoptiles.

Treatment	Initial Length (mm)	Final Length (mm)	Growth Rate (mm/h/coleoptile)
CHI + CO_2	20.63±0.238	20.70±0.236	0.035
CHI + no CO_2	20.63±0.238	20.67±0.234	0.020
no CHI + CO_2	20.74±0.064	21.15±0.127	0.205
no CHI + no CO_2	20.74±0.064	20.93±0.114	0.095

Note: Batches of 30 coleoptile sections, approximately 20 mm. long, were placed in phosphate buffer (0.025 M, pH 7.5) in the presence or absence of cycloheximide (6 µg/ml) for about 1 h. before treatment with or without carbon dioxide (0.03%) for 2 h. Measurements were taken at 0 h. and after 2 h. incubation. Figures represent the average of two experiments. Results are expressed as the mean ± the standard deviation.

CHI = cycloheximide

Table 8: Analysis by the "t" test of the effect of cycloheximide on carbon dioxide stimulated growth.

Treatment	+ CHI	- CHI
Effect of CO_2	N.S.	$p < 0.001$

Treatment	+ CO_2	- CO_2
Effect of CHI	$p < 0.001$	$p < 0.001$

Note: These figures are derived from the data in Table 7. N.S. = Not Significant

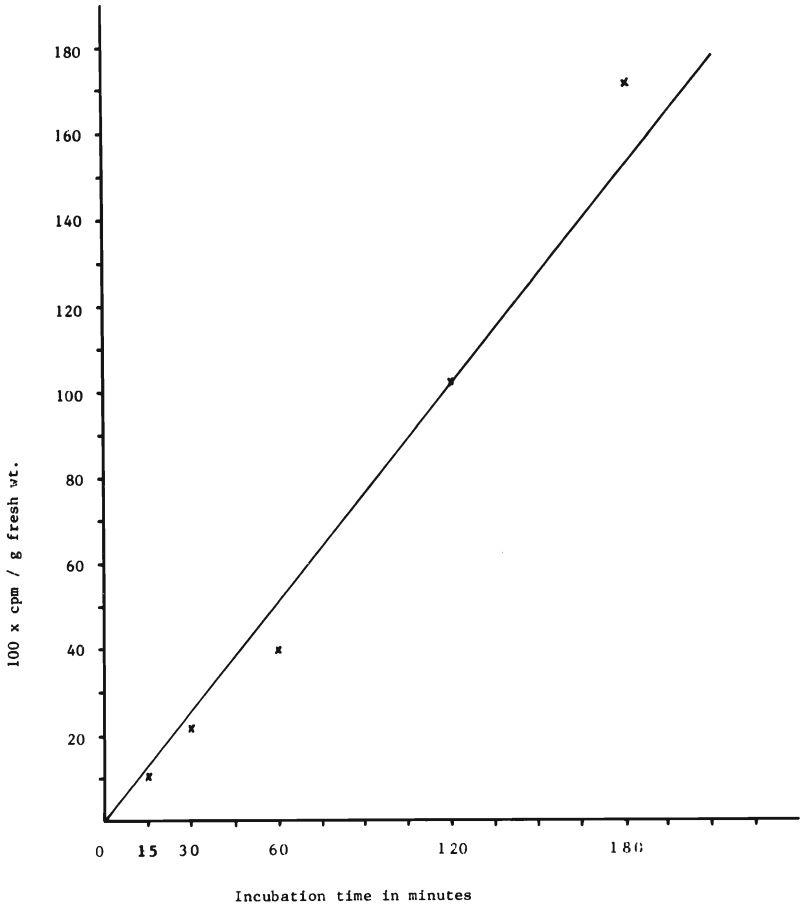
4.2 Protein and Cell Wall Metabolism

In this type of experiment, apart from the cell wall fraction, all samples were analysed by duplicate determination. Moreover every experiment was repeated two or four times and the average figure from these experiments is shown. Data is expressed as the mean value per gram of fresh coleoptile tissue plus or minus the standard deviation.

It can be seen in Figure 4, that in the presence of IAA and carbon dioxide the rate of ^{14}C-leucine incorporation into the soluble protein fraction was linear for up to 3 hours of incubation. The same type of linear incorporation was also found in the absence of both these factors (Figure not shown). Figure 5 demonstrates that in the presence of IAA and carbon dioxide, the protein level rose linearly for nearly 2 hours and then continued to rise more slowly. A similar relationship was also obtained in the absence of both factors (Figure not shown). Figures 4 and 5 together indicate that under the experimental conditions used, protein synthesis is occurring; and that after 2 hours different levels of incorporation of radioactive leucine into the soluble fraction would indicate relative rates of protein synthesis.

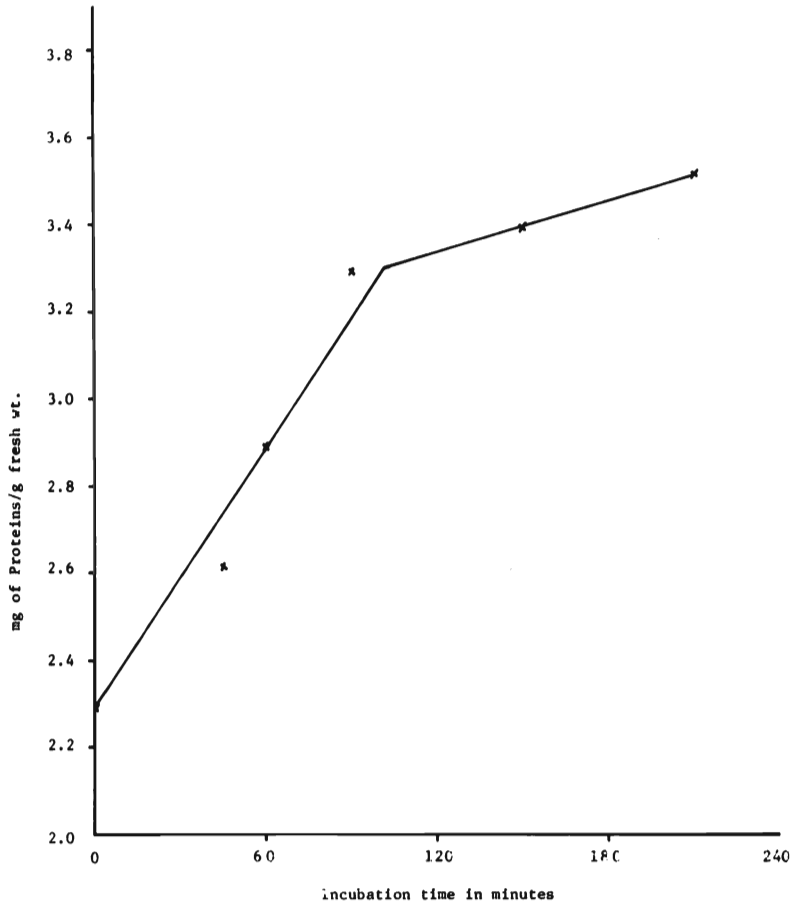
Figure 6 shows a kinetic study of radioactive leucine incorporation into the cell wall fraction of *Avena* coleoptiles, in the presence of both IAA and carbon dioxide. It can be seen that a linear relationship was found for up to 2 hours incubation. This linear relationship indicates that after 2 hours different levels of incorporation of radioactivity from labelled leucine into the cell wall fraction would reflect different rates of synthesis of a cell wall component.

Figure 4: Kinetic study of the incorporation of radioactive leucine into *Avena* coleoptile protein, maintained in the presence of IAA and carbon dioxide.



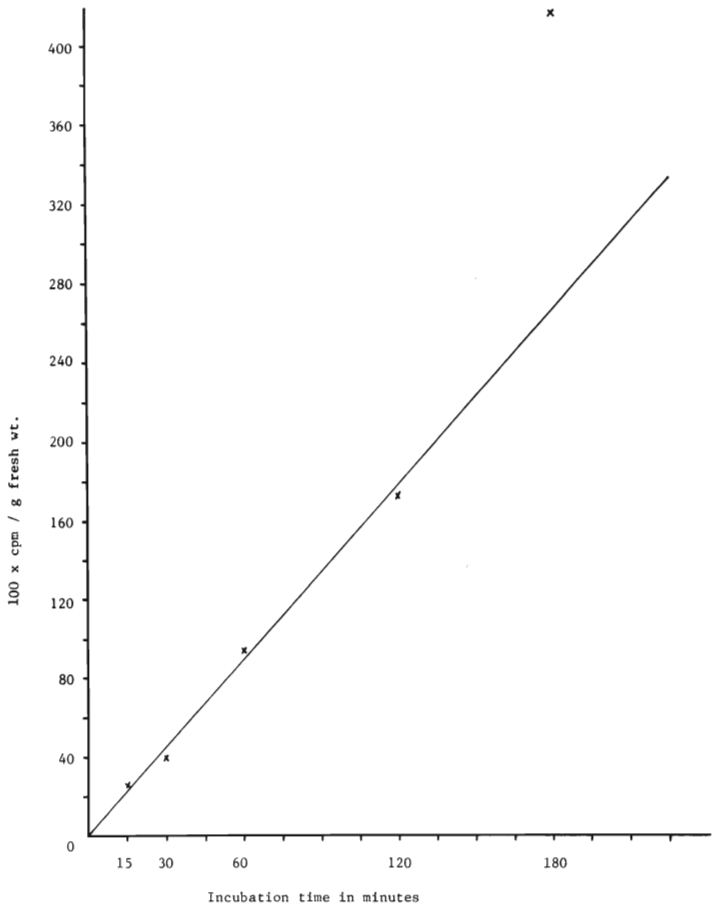
Note: Approximately 0.5 g of coleoptile tissue was treated with carbon dioxide (0.03%) and IAA (2×10^{-5} M) in phosphate buffer (0.025 M, pH 7.5). 8×10^5 dpm of ^{14}C-leucine (U) (specific activity = 331 m Ci/m mol) was added after 30 minutes incubation and the incubation was terminated at the times specified. The experiment was done twice and similar results were obtained.

Figure 5: Kinetic study of changes in protein level of *Avena* coleoptiles maintained in the presence of IAA and carbon dioxide.



Note: Approximately 0.5 g of coleoptiles was treated with carbon dioxide (0.03%) and IAA (2×10^{-5} M) in phosphate buffer (0.025 M, pH 7.5). The incubation was terminated at the times indicated and total soluble protein was determined. The experiment was done twice and similar results were obtained.

Figure 6: Kinetic study of the incorporation of radioactive leucine into the cell wall fraction of *Avena* coleoptiles.



Note: Approximately 0.5 g of coleoptiles was treated with carbon dioxide (0.03%) and IAA (2×10^{-5} M) in phosphate buffer (0.025 M, pH 7.5). 8×10^5 dpm of ^{14}C-leucine (U) [specific activity = 331 m Ci/m mol] was added to the tissue after 30 minutes incubation and the incubation was terminated at the times indicated. Radioactivity in the cell wall fraction was determined. The experiment was done twice and similar results were obtained.

In Table 9 the stimulation of protein levels and rate of ^{14}C-leucine incorporation by IAA and carbon dioxide are shown. Carbon dioxide stimulated the protein level by 23% and ^{14}C incorporation by 17% whilst IAA stimulated the protein level by 37% and ^{14}C-leucine incorporation by 28%. Maximum levels were obtained only when both factors were present and for ^{14}C incorporation into soluble protein the effect of these factors seemed to be weakly synergistic. Table 10 indicates the influence of IAA and carbon dioxide on the incorporation of radioactivity from labelled leucine into soluble protein and the cell wall fraction. IAA and carbon dioxide stimulated the incorporation of label into the cell wall in a synergistic manner and radioactivity was increased by 50% in the soluble protein component and approximately 130% in the cell wall fraction. This trend is also indicated by the percentage distribution of r a dioactivity between the soluble protein and the cell wall fraction. In the presence of IAA and carbon dioxide the ratio is changed with increasing radioactivity in the cell wall fraction. The data in Table 9 and 10 show an influence of carbon dioxide and IAA on the incorporation of label into the soluble proteins and cell wall fractions that is similar to the influence of these agents on growth (Table 1). In all cases IAA and carbon dioxide exhibited a synergistic relationship; and maximum rates were obtained only when both factors were present.

The word "tips" refers to the 3 mm apical sections of *Avena* coleoptile. It can be seen in Table 11 that the maximum rate of ^{14}C incorporation and maximum level of protein were obtained with tips and carbon dioxide present.

Table 12 indicates that malate and carbon dioxide could stimulate the protein level and ^{14}C-leucine incorporation, more or less, to the same

Table 9: Influence of IAA and carbon dioxide on protein metabolism of *Avena* coleoptiles.

Treatment	Per g of fresh wt.	
	mg Proteins	dpm
IAA + CO_2	4.038 ± 0.328	86,318 ± 1011
IAA + no CO_2	3.605 ± 0.439	69,293 ± 888
no IAA + CO_2	3.225 ± 0.373	63,536 ± 821
no IAA + no CO_2	2.660 ± 0.104	54,312 ± 783

Note: Approximately 0.5 g of coleoptile tissues was treated with or without CO_2 (0.03%) in the presence or absence of IAA (2×10^{-5} M) in phosphate buffer (0.025 M, pH 7.5). 8×10^6 dpm of ^{14}C-leucine (U) [specific activity = 331 m Ci/m mol] was added after 30 minutes incubation and the incubation was continued for another 90 minutes. Figures represent the average of duplicate determination from four experiments. Results are expressed as the mean ± the standard deviation.

Table 10: Influence of IAA and carbon dioxide on the incorporation of radioactive leucine into soluble protein and cell wall fraction of *Avena* coleoptiles.

Treatment	dpm/g fresh wt.		% Distribution	
	Soluble Proteins	Cell Wall Fraction	S. Proteins	C.W. Fraction
IAA + CO_2	76,843±936	112,650±203.9	40.55	59.45
IAA + no CO_2	64,375±832	85,746±197.1	42.88	57.12
no IAA + CO_2	59,472±813	62,527±169.4	48.75	51.25
no IAA + no CO_2	50,243±774	48,239±162.3	51.02	48.98

Note: Approximately 0.5 g of tissues was treated with or without carbon dioxide (0.03%) in the presence or absence of IAA (2×10^{-5} M) in phosphate buffer (0.025 M, pH 7.5). 8×10^6 dpm of ^{14}C-leucine (U) (specific activity = 331 m Ci/m mol) was added to the tissue after 30 minutes incubation and the incubation was continued for another 90 minutes. Figures represent the average of duplicate determination from two experiments. Results are expressed as the mean ± the standard deviation.

Table 11: Influence of tips and carbon dioxide on protein metabolism of *Avena* coleoptiles.

Treatment	Per g of fresh wt.	
	mg Proteins	dpm
Tips + CO_2	3.884 ± 0.148	57,762 ± 870
Tips + no CO_2	3.376 ± 0.075	47,443 ± 841
no Tips + CO_2	3.245 ± 0.053	41,088 ± 745
no Tips + no CO_2	2.976 ± 0.120	28,783 ± 718

Note: Approximately 0.5 g of coleoptiles, with or without 3 mm tips, was treated with or without carbon dioxide (0.03%) in phosphate buffer (0.025 M, pH 7.5). 5×10^6 dpm of ^{14}C-leucine (U) [specific activity = 331 m Ci/m mol] was added to the tissue after 30 minutes incubation and the incubation was continued for another 90 minutes. Figures represent the average of duplicate determination from two experiments. Results are expressed as the mean ± the standard deviation.

Table 12: Influence of malate and carbon dioxide on the levels of proteins and the incorporation of radioactive leucine into soluble protein and the cell wall fraction of *Avena* coleoptiles.

Treatment	Per g of fresh wt.		
	mg	dpm	
	Soluble Proteins	Soluble Proteins	Cell Wall Fraction
Malate + CO_2	3.792±0.09	47,520±648	50,284±128
Malate + no CO_2	3.590±0.08	41,874±636	43,685±111
no Malate + CO_2	3.526±0.07	38,756±604	41,580±110
no Malate + no CO_2	3.033±0.11	25,888±552	24,674±104

Note: Approximately 0.5 g of tissue was treated with or without carbon dioxide (0.03%) in the presence or absence of malate (5×10^{-4} M) in phosphate buffer (0.025 M, pH 7.5). 2×10^6 dpm of ^{14}C-leucine (U) [specific activity = 331 m Ci/m mol] was added to the tissue after 30 minutes incubation and the incubation was continued for another 90 minutes. Figures represent the average of duplicate determination from two experiments. The results are expressed as the mean ± the standard deviation.

extent, i.e. about 20% for the protein level and 50% for the ^{14}C incorporation. However, maximum levels were obtained when both factors were included in the incubation medium, and this pattern is slightly different from the influence of these factors on growth where in the presence of one factor the other did not stimulate growth. (Table 3). Table 13 demonstrates the influence of malate and carbon dioxide on the distribution of radioactivity between the soluble protein and cell wall fractions. Either factor increases to the same extent the percentage of radioactivity in the cell wall fraction, but the presence of both factors has an effect quantitatively similar to either factor alone. This pattern is different from the pattern of distribution obtaining using IAA and carbon dioxide (Table 10).

The influence of acetate and carbon dioxide on protein and cell wall metabolism is shown in Table 14. It can be seen that acetate and carbon dioxide together give the largest level of protein and incorporation of radioactivity into soluble protein and cell wall fractions. Either factor alone stimulated these processes to the same extent. The influence of acetate and carbon dioxide on the total protein level and incorporated radioactivity appear to be additive, but not synergistic. The percentage distribution of radioactivity between protein and cell wall fractions was not changed by the presence of both factors (Table 15.)

Tables 16 and 17 show the influence of cycloheximide and carbon dioxide on protein and cell wall metabolism. It is quite clear that CHI completely inhibited the carbon dioxide stimulated level of:

(i) soluble protein

(ii) ^{14}C incorporation into soluble protein

Table 13: Influence of malate and carbon dioxide on the percentage distribution of radioactivity from ^{14}C leucine incorporated into the soluble protein and the cell wall fraction of *Avena* coleoptiles.

Treatment	Per cent Distribution	
	Soluble Protein	Cell Wall Fraction
Malate + CO_2	48.59	51.41
Malate + no CO_2	48.98	51.02
no Malate + CO_2	48.24	51.76
no Malate + no CO_2	51.20	48.80

Note: These figures were derived from the data in Table 12.

Table 14: Influence of acetate and carbon dioxide on the levels of protein and the incorporation of radioactive leucine into soluble protein and the cell wall fraction of *Avena* coleoptiles.

Treatment	Per g of fresh wt.		
	mg	dpm	
	Soluble Proteins	Soluble Proteins	Cell Wall Fraction
Acetate + CO_2	2.875±0.11	28,656±694	28,710±106
Acetate + no CO_2	2.508±0.05	24,469±662	24,972±104
no Acetate + CO_2	2.489±0.06	24,183±648	25,012±109
no Acetate + no CO_2	2.219±0.05	20,044±618	18,718± 97

Note: Approximately 0.5 g of coleoptiles was treated with or without carbon dioxide (0.03%) in the presence or absence of acetate (5×10^{-4} M) in phosphate buffer (0.025 M, pH7.5). 2×10^6 dpm of ^{14}C-leucine (U) [specific activity = 331 m Ci/m mol] was added to the tissue after 30 minutes incubation and the incubation was continued for another 90 minutes. Figures represent the average of duplicate determination from two experiments. The results are expressed as the mean ± the standard deviation.

Table 15: Influence of acetate and carbon dioxide on the percentage distribution of radioactivity from ^{14}C-leucine incorporated into the soluble protein and the cell wall fraction of *Avena* coleoptiles.

	Per Cent Distribution	
	Soluble Protein	Cell Wall Fraction
Acetate + CO_2	49.95	50.05
Acetate + no CO_2	49.49	50.51
no Acetate + CO_2	49.16	50.84
no Acetate + no CO_2	51.71	48.29

Note: These figures derived from the data in Table 14.

Table 16: Influence of cycloheximide and carbon dioxide on the levels of protein and the incorporation of radioactive leucine into soluble protein and the cell wall fraction of *Avena* coleoptiles.

Treatment	Per g of fresh wt.		
	mg	dpm	
	Soluble Proteins	Soluble Proteins	Cell Wall Fraction
CHI + CO_2	2.192±0.05	2,924±190	6,369±46
CHI + no CO_2	2.171±0.07	2,834±188	6,027±44
no CHI + CO_2	2.669±0.12	20,827±486	22,819±84
no CHI + no CO_2	2,280±0.09	15,922±396	14,790±68

Note: Approximately 0.5 g of coleoptile tissues was treated with or without carbon dioxide (0.03%) in the presence or absence of cycloheximide (6 µg/ml) in phosphate buffer (0.025 M, pH 7.5). 2 x 10^6 dpm of ^{14}C-leucine (U) [specific activity = 331 m Ci/m mol] was added after 30 minutes incubation and the incubation was continued for another 90 minutes. Figures represent the average of duplicate determination from two experiments. The results are expressed as the mean ± the standard deviation.

Table 17: Influence of cycloheximide and carbon dioxide on the percentage distribution of radioactivity from ^{14}C-leucine incorporated into the soluble protein and the cell wall fraction of *Avena* coleoptiles.

Treatment	Per Cent Distribution	
	Soluble Protein	Cell Wall Fraction
CHI + CO_2	31.46	68.54
CHI + no CO_2	31.98	68.02
no CHI + CO_2	47.72	52.28
no CHI + no CO_2	51.82	48.18

Note: These figures derived from the data in Table 16.

(iii) ^{14}C incorporation into the cell wall fraction

The basal levels (i.e. without carbon dioxide) of these three parameters were also inhibited by CHI. It can be seen in Table 16 that CHI inhibition of the incorporation of radioactivity into the soluble protein fraction was much higher than the inhibition of incorporation into the cell wall fraction. The percentage distribution of radioactivity between the soluble protein and cell wall fraction also indicate this phenomenon (Table 17).

4.3 Changes in the Pattern of Protein Synthesis

In this section the influence of various treatments on protein metabolism was studied by determining the distribution along polyacrylamide gels of total protein and incorporated radioactivity. The data is recorded in three ways:

(i) distribution of protein bands along the gel by scanning.

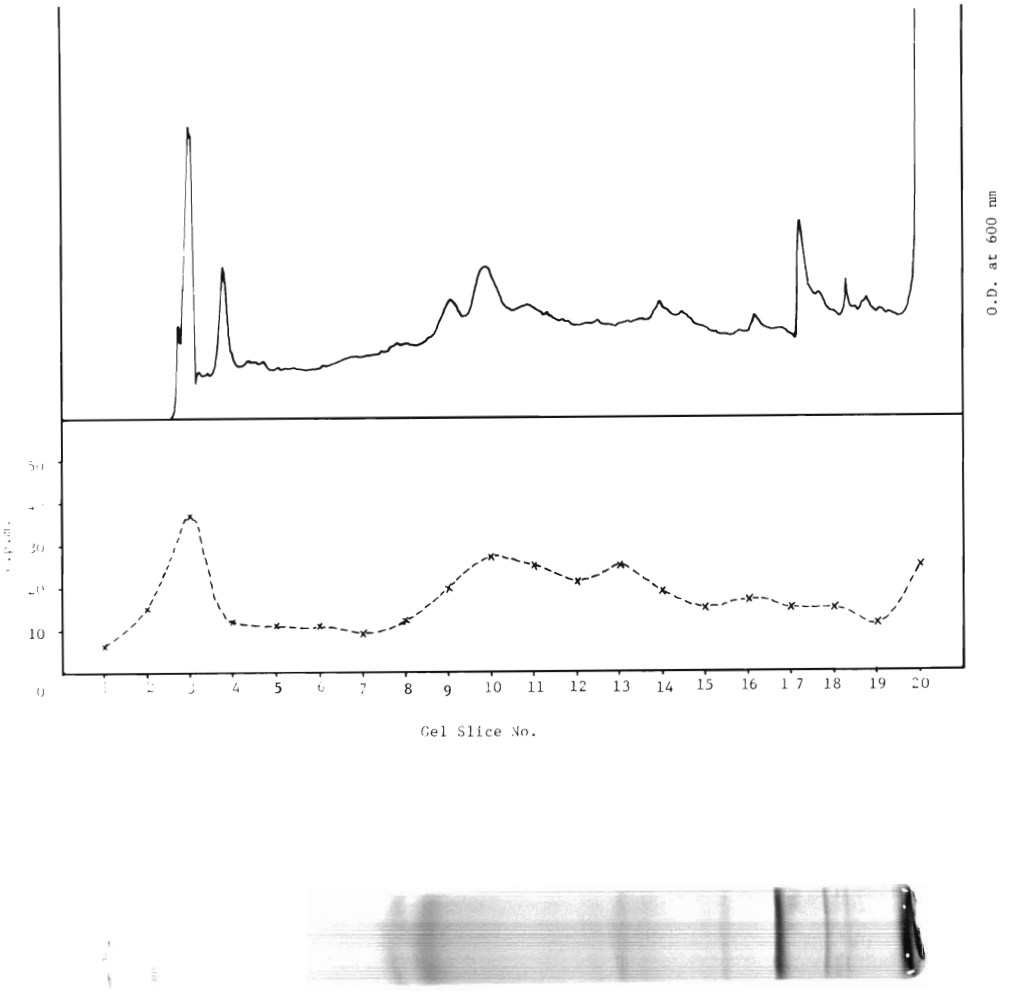
(ii) distribution of incorporated radioactivity by radioactive assay.

(iii) photographic recording.

Figure 7 shows the results obtained after protein extraction from tissue maintained in the presence of radioactive leucine and the absence of both IAA and carbon dioxide. The continuous line represents the distribution of protein along the gel and the dotted line represents the amount of radioactivity incorporated into these proteins. The bottom picture is a photograph of the stained gel.

In Figures 8, 9 and 10 data is presented to illustrate changes in this pattern of protein metabolism (Figure 7) brought about by respectively

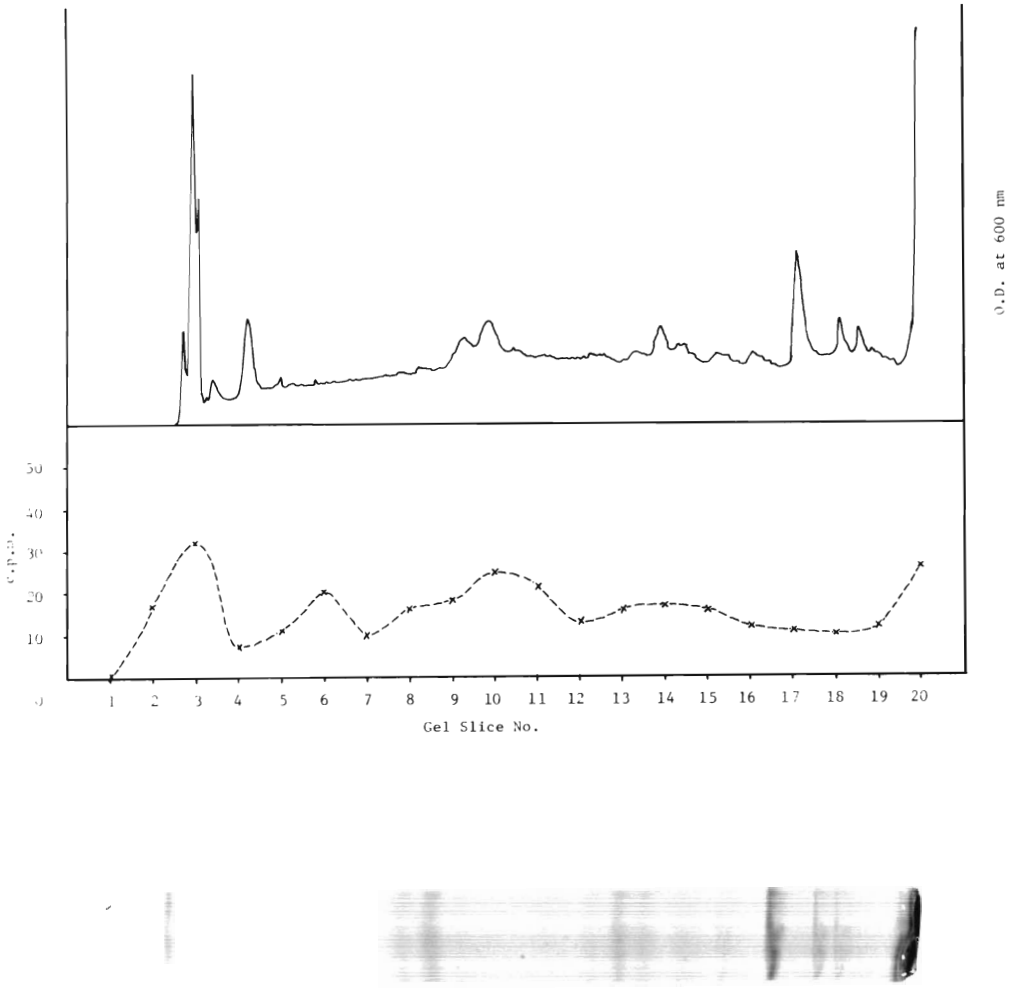
Figure 7: Distribution of protein and radioactivity after gel electrophoresis of soluble proteins extracted from *Avena* coleoptiles maintained in the absence of both IAA and carbon dioxide.



Note: Coleoptile tissue was treated with carbon dioxide free air in the absence of IAA in phosphate buffer (0.025 M, pH 7.5) before soluble proteins were extracted. Two aliquots of protein solution containing approximately 0.2 mg of protein were subjected to analytical polyacrylamide gel electrophoresis. One was fixed with TCA (12.5%) and stained in coomassie blue (0.05%) before scanning for the distribution of protein. The other gel was sliced into 2 mm sections and each section assayed for radioactivity. The experiment was done twice and similar results were obtained.

———— scanned protein
---------- radioactivity

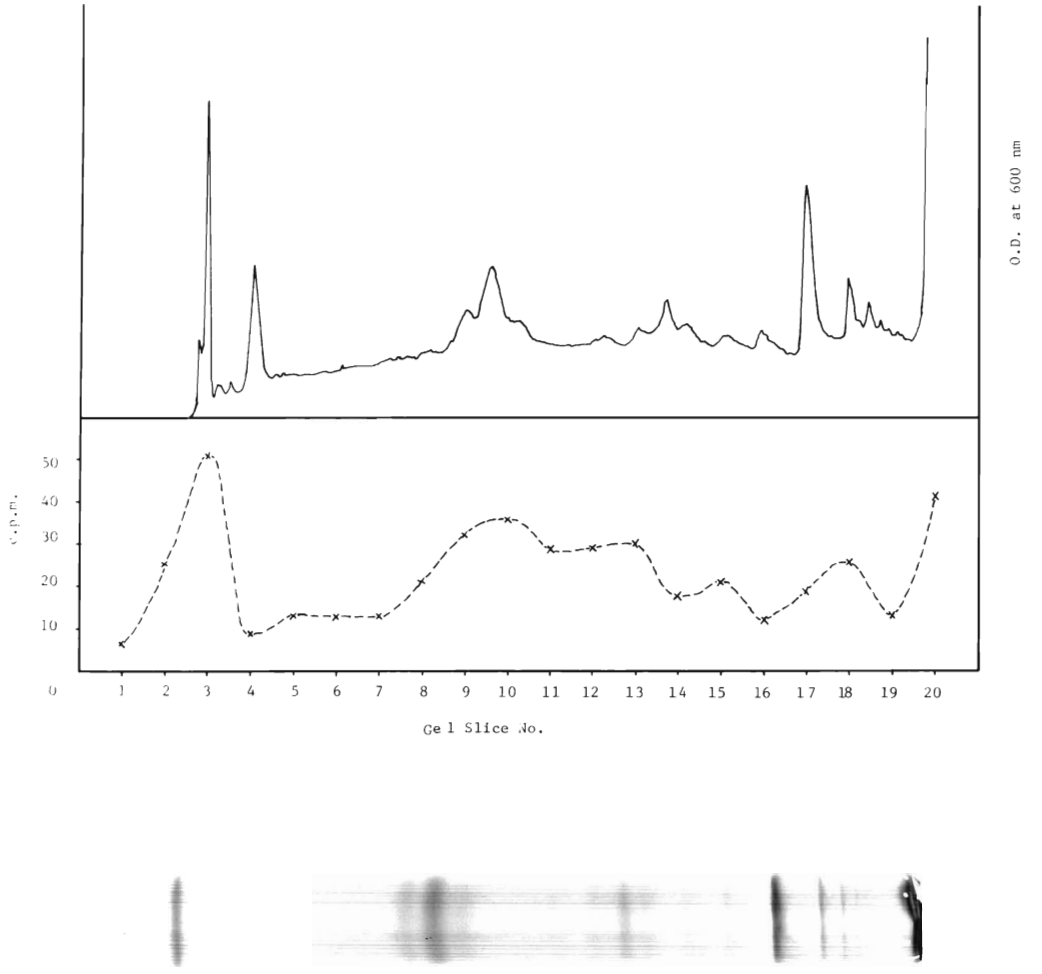
Figure 8: Distribution of protein and radioacitivity after gel electrophoresis of soluble proteins extracted from *Avena* coleoptiles maintained in the presence of carbon dioxide only.



Note: Coleoptile tissue was treated with carbon dioxide (0.03%) in phosphate buffer (0.025 M, pH 7.5) before soluble proteins were extracted. The procedures for electrophoresis, scanning the distribution of protein and determination of radioactivity along the gel were the same as in Figure 7.

———— scanned protein
---------- radioactivity

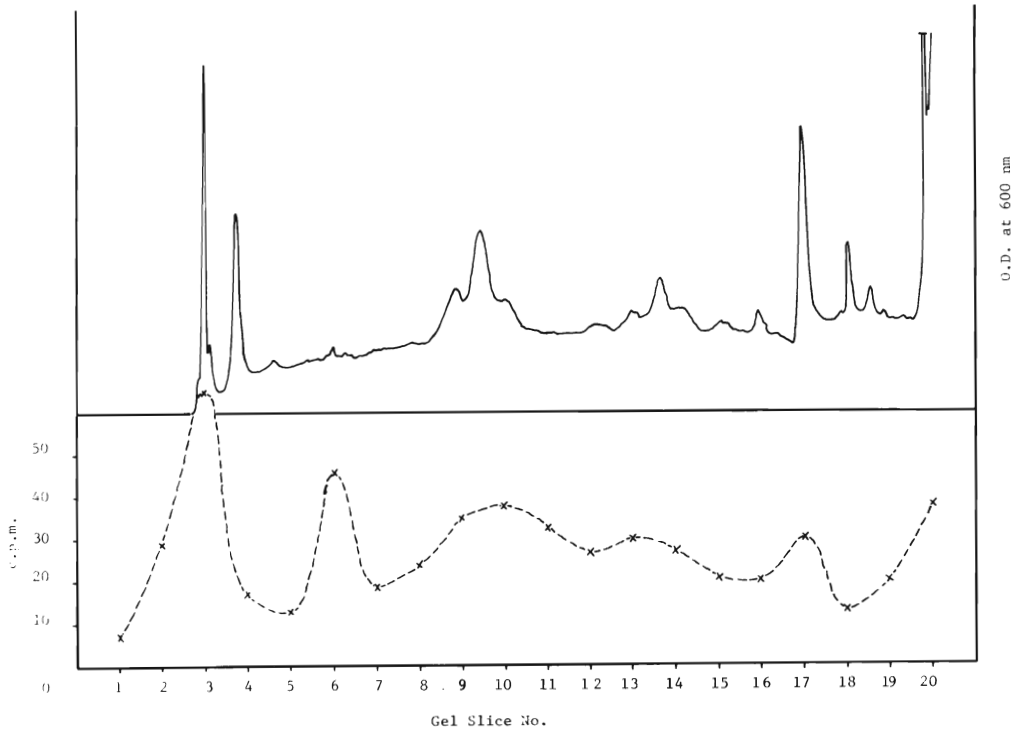
Figure 9: Distribution of protein and radioactivity after gel electrophoresis of soluble proteins extracted from *Avena* coleoptiles maintained in the presence of IAA only.



Note: Coleoptile tissue was treated with IAA (2×10^{-5} M) and carbon dioxide free air in phosphate buffer (0.025 M, pH 7.5) before soluble proteins were extracted. The procedures for electrophoresis, scanning the distribution of protein and determination of radioactivity along the gel were the same as in Figure 7.

———— scanned proteins
---------- radioactivity

Figure 10: Distribution of protein and radioactivity after gel electrophoresis of soluble proteins extracted from *Avena* coleoptiles maintained in the presence of IAA and carbon dioxide.



Note: Coleoptile tissue was treated with IAA (2×10^{-5} M) and carbon dioxide (0.03%) in phosphate buffer (0.025 M, pH 7.5) before soluble proteins were extracted. The procedures for electrophoresis, scanning the distribution of protein and determination of radioactivity along the gel were the same as in Figure 7.

———— scanned protein
---------- radioactivity

carbon dioxide, IAA and both factors together. It can be seen from a comparison of Figures 7 and 8 that, although the distribution pattern of proteins are similar, a difference in radioactive distribution along the gel is found in gel slices 5, 6 and 7. There is a small peak in Figure 8 whereas Figure 7 shows no such peak. A similar influence of carbon dioxide can be seen in Figure 10 (i.e. IAA plus CO_2 condition) when it is compared with Figure 9 (i.e. IAA plus no CO_2 condition).

On the other hand, a comparison of Figures 7 and 8 (i.e. no IAA) with Figures 9 and 10 (i.e. with IAA) indicates that IAA stimulated the synthesis of a protein fraction which appears in gel slices 16, 17 and 18. Figure 11 shows a photographic recording of gels after the separation of protein extracted from tissue exposed to the above four conditions.

The influence of CHI on protein metabolism is indicated by comparing Figure 12 (i.e. CHI plus no CO_2) with Figure 7 (i.e. no CHI plus no CO_2); or by comparing Figure 13 (CHI plus CO_2) with Figure 8 (no CHI plus CO_2). It is apparent from these comparisons that CHI drastically reduced the incorporation of radioactivity into all protein fractions irrespective of the presence or absence of carbon dioxide. Figure 14 shows the distribution of protein bands after electrophoresis of soluble protein extracted from tissue exposed to the four permutations possible of the two factors, CHI and carbon dioxide. Gels 3 and 4 of Figure 14 are derived from tissue exposed to the presence or absence of carbon dioxide and are similar to gels 3 and 4 of Figure 11 which were derived from different batches of tissue maintained under identical conditions to those of Figure 14.

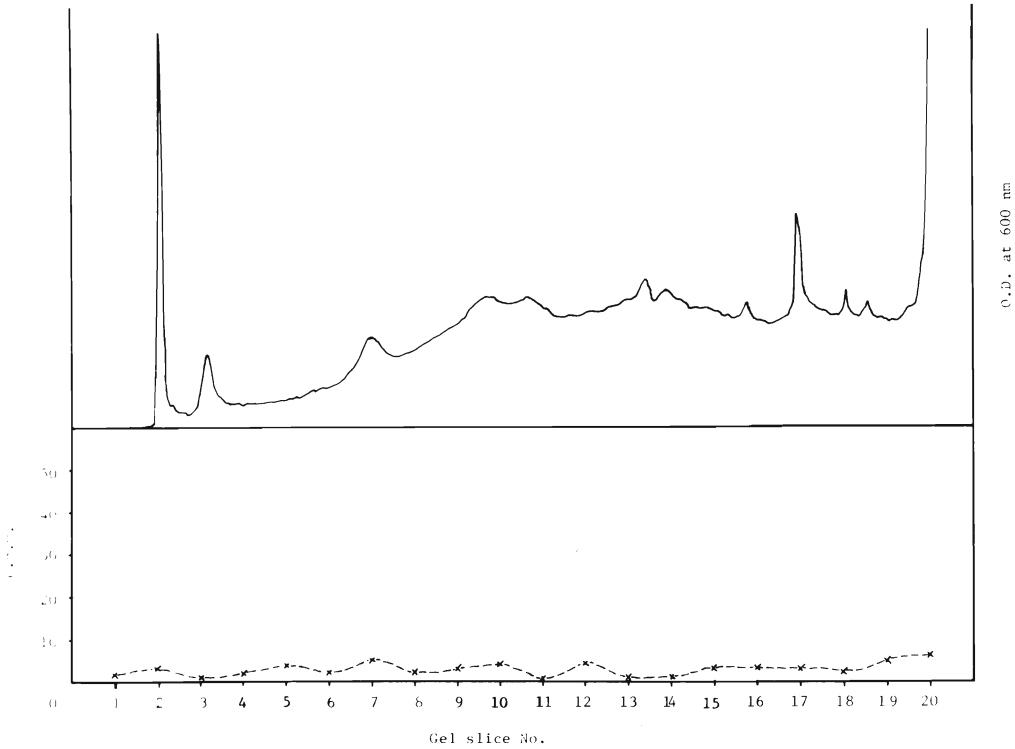
Figure 11: Polyacrylamide gel electrophoresis of soluble proteins extracted from *Avena* coleoptiles maintained under different conditions.

1 2 3 4

Note: Approximately 0.5 g of coleoptile tissue was treated with or without carbon dioxide (0.03%) in the presence or absence of IAA (2×10^{-5} M), in phosphate buffer (0.025 M, pH 7.5), before extracting soluble proteins. Aliquots of protein solution containing approximately 0.2 mg of protein were subjected to analytical polyacrylamide gel electrophoresis. Gels were fixed in TCA (12.5%) and stained in coomassie blue (0.05%).

1 = IAA + CO_2
2 = IAA + no CO_2
3 = no IAA + CO_2
4 = no IAA + no CO_2

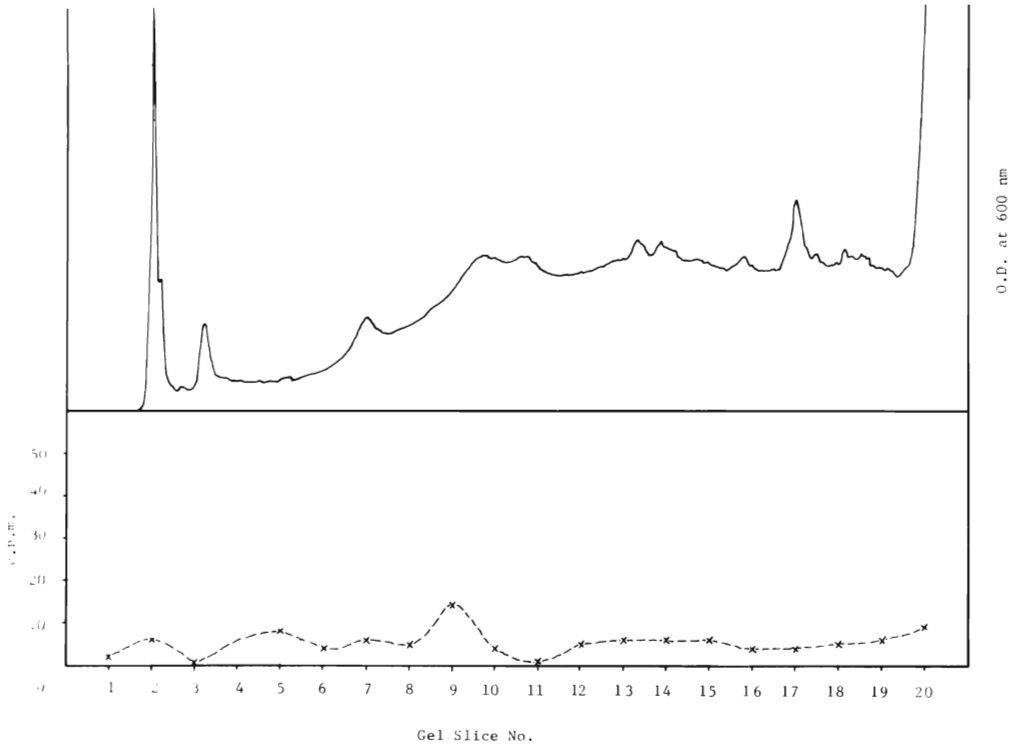
Figure 12: Distribution of protein and radioactivity after gel electrophoresis of soluble proteins extracted from *Avena* coleoptiles maintained in the presence of cycloheximide only.



Note: Coleoptile tissue was treated with cycloheximide (6 µg/ml) and carbon dioxide free air in phosphate buffer (2 x 10^{-5} M, pH 7.5) before soluble proteins were extracted. The procedures for electrophoresis, scanning the distribution of protein and determination of radioactivity along the gel were the same as in Figure 7.

——————— scanned proteins
---------- radioactivity

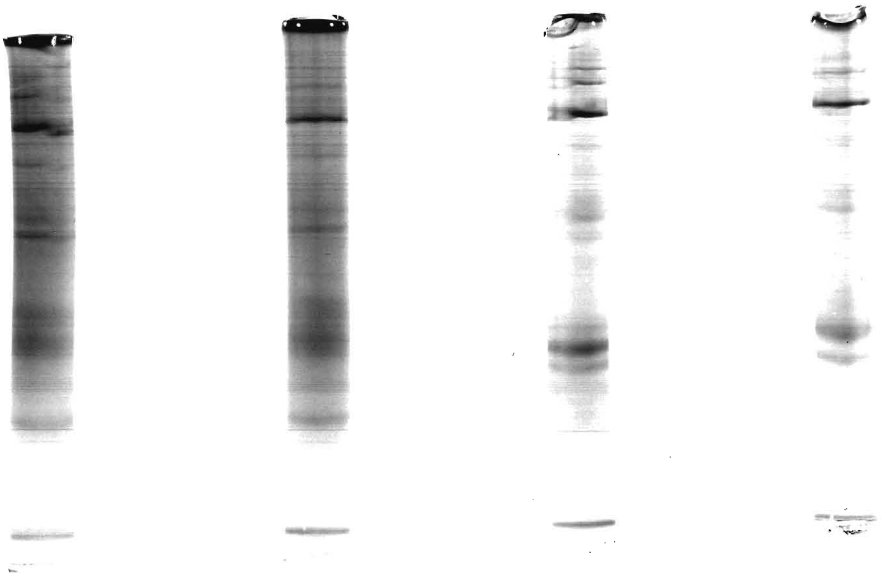
Figure 13: Distribution of protein and radioactivity after gel electrophoresis of soluble proteins extracted from *Avena* coleoptile maintained in the presence of cycloheximide and carbon dioxide.



Note: Coleoptile tissue was treated with cycloheximide (6 µg/ml) and carbon dioxide (0.03%) in phosphate buffer (0.025 M, pH 7.5) before soluble proteins were extracted. The procedures for electrophoresis, scanning the distribution of protein and determination of radioactivity along the gel were the same as in Figure 7.

———————— scanned proteins
---------- radioactivity

Figure 14: Polyacrylamide gel electrophoresis of soluble proteins extracted from *Avena* coleoptiles maintained under different conditions.



1 2 3 4

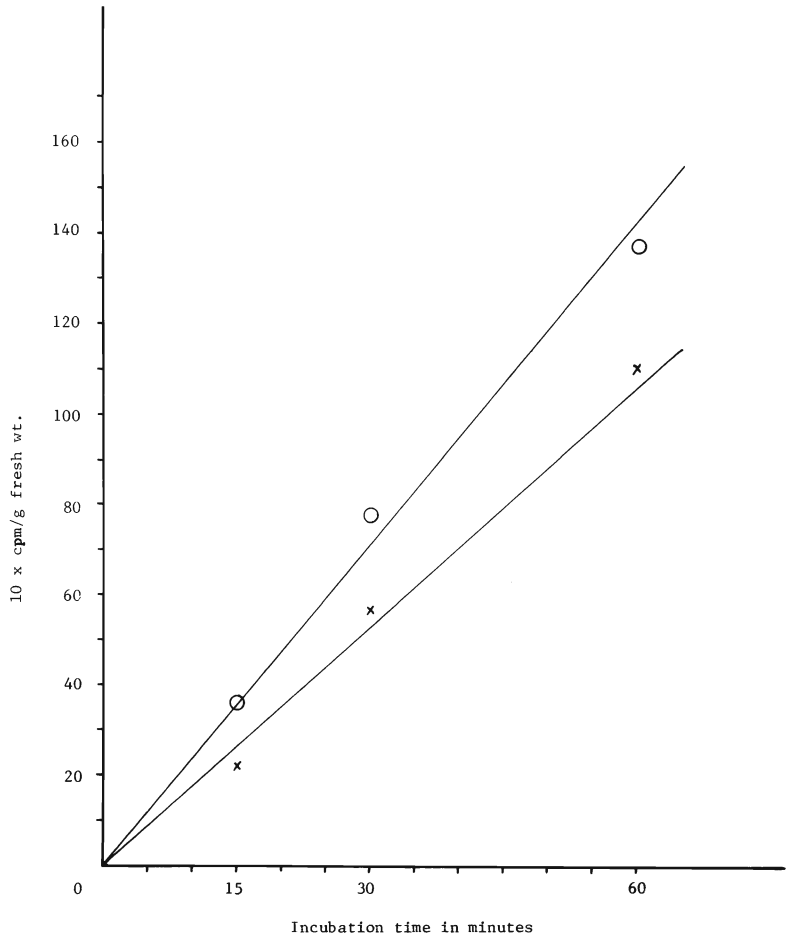
Note: Approximately 0.5 g of coleoptile tissue was treated with or without carbon dioxide (0.03%) in the presence or absence of cycloheximide (6 µg/ml), in phosphate buffer (0.025 M, pH 7.5) before extracting soluble proteins. Aliquots of protein solution containing approximately 0.2 mg of protein were subjected to analytical polyacrylamide gel electrophoresis. Gels were fixed in TCA (12.5%) and stained in coomassie blue (0.05%).

1 = CHI + CO_2
2 = CHI + no CO_2
3 = no CHI + CO_2
4 = no CHI + no CO_2

4.4 The Influence of IAA on the Incorporation of Radioactive Bicarbonate

The influence of IAA on radioactive bicarbonate incorporation into soluble protein is shown in Figure 15 and Table 18. Figure 15 indicates that the kinetics of incorporation were linear up to 1 hour; and that there is a stimulation of incorporation by IAA. To determine the significance level of this apparent stimulation by IAA batches of coleoptile tissue, with or without IAA, were exposed to ^{14}C-bicarbonate for 30 minutes in closed containers. The results in Table 18 show that IAA significantly stimulated the incorporation of radioactive bicarbonate into the soluble protein and cell wall fraction.

Figure 15: Kinetic study of the incorporation of radioactive bicarbonate into *Avena* coleoptile protein.



Note: Approximately 0.5 g of coleoptile tissue were incubated in the presence or absence of IAA (2×10^{-5} M) in phosphate buffer (0.025 M, pH 7.5) in a closed container. 2×10^{6} dpm of ^{14}C-bicarbonate (specific activity = 59 m Ci/m mol) was added to the tissue after 30 minutes incubation and the incubation was terminated at the times specified. The experiment was done twice and similar results were obtained.

0 —— 0 = with IAA
x —— x = without IAA

Table 18: Influence of IAA on the incorporation of radioactive bicarbonate into the soluble protein and the cell wall fraction of *Avena* coleoptiles.

	Per g of fresh weight					
	mg		dpm			
	Soluble Protein				Cell Wall Fraction	
	+IAA	-IAA	+IAA	-IAA	+IAA	-IAA
1	3.048	2.390	5844±257	3767±240	5488±55	3945±47
2	2.982	2.535	6326±259	3245±238	5768±61	3583±45
3	3.082	2.484	5877±265	4395±243	5474±54	4507±51
4	3.184	2.407	5993±261	3913±241	6692±61	4069±48
5	3.234	2.443	7421±271	4819±250	7562±64	5755±57
6	3.180	2.432	6539±264	4968±251	6182±59	5424±55
Mean ±S.D.	3.118 ±0.10	2.448 ±0.05	6333±263	4180±244	6194±59	4547±51
"t" Test	$p < 0.001$		$p < 0.001$		$p < 0.001$	

Note: Twelve batches of approximately 0.5 g of coleoptiles from the same lawn were harvested and were treated with or without IAA (2×10^{-5} M) in phosphate buffer (0.025 M, pH 7.5) in closed containers. 8×10^{6} dpm of ^{14}C-bicarbonate (specific activity = 59 m Ci/m mol) was added to the samples after 30 minutes incubation and the incubation was continued for another 30 minutes. Total soluble proteins, radioactivity incorporated into soluble proteins and radioactivity in the cell wall fraction were then determined.

5. DISCUSSION

There is substantial evidence that carbon dioxide can stimulate growth (Harrison, 1965a; Rayle and Cleland, 1970; Evans et al., 1971) and protein synthesis (Splittostoesser, 1966; Bown and Lampman, in press) in non-photosynthetic plant tissue. The present investigation also indicates that the level of carbon dioxide normally found in air (0.03% CO_2) stimulated growth (Tables 1, 3, 5 and 7) and protein synthesis (Tables 9, 11, 12, 14 and 16) in etiolated *Avena* coleoptiles. Despite all this work, the question as to whether carbon dioxide stimulated growth depends on carbon dioxide stimulated protein synthesis has not been vigorously investigated. Splittstoesser (1966) showed that in carbon dioxide free air non-photosynthetic plant tissue exhibited lower growth rates and lower rates of radioactive leucine accumulation into soluble proteins. However, higher levels of accumulated label in proteins can be interpreted as either inhibition of breakdown or stimulation of protein synthesis; in addition Splittstoesser did not demonstrate whether or not protein synthesis was required for carbon dioxide stimulated growth.

Figure 4 inconjunction with Table 9 indicates that carbon dioxide stimulated protein synthesis, resulting in both an increase in total protein and an increased incorporation of radioactive leucine. An e x planation of the different levels of radioactivity in the soluble protein based on different rates of breakdown would imply an extremely rapid turnover rate of the soluble protein in the 2 hour period of the experiment. This kind of explanation is not likely in the light of half lives for plant proteins between 3 hours and several days (Glasziou, Waldron and Bull, 1966; Glasziou, 1969

Hock and Beevers, 1966). Evidence that carbon dioxide stimulated protein synthesis is required for carbon dioxide stimulated growth is presented in Tables 7 and 16. A 6 μg/ml concentration of cycloheximide completely inhibited carbon dioxide stimulated growth and protein synthesis. Respiration of *Avena* coleoptiles, on the other hand, was inhibited by only 20% (Data not shown). Because of the complete inhibition of carbon dioxide stimulated growth and protein synthesis by cycloheximide and only partial inhibition of respiration the data support the hypothesis that carbon dioxide stimulated growth depends on carbon dioxide stimulated protein synthesis.

This interpretation which indicates that carbon dioxide stimulated growth is mediated by a biochemical process differs from the interpretation of Evans et al. (1971). Using carbon dioxide saturated water, they showed that the carbon dioxide stimulated growth rate was higher than the auxin stimulated growth and insensitive to a variety of metabolic inhibitors which suppressed auxin action; they also demonstrated an almost instantaneous growth response to carbon dioxide. Evans et al. suggested that carbon dioxide stimulated growth results from a lowering of pH in the carbon dioxide saturated water, and that growth stimulation, is not mediated by metabolism, but results from a physical process. Rayle and Cleland (1970), have shown that the stimulation of growth by carbon dioxide saturated water was not due to a lowering of pH and that the carbon dioxide response was different to response to auxin and low pH values.

In the present study the tissue was maintained in buffered solutions and exposed to 0.03% carbon dioxide which did not change the pH of the buffer (Data not shown). This demonstrates that with this system the carbon dioxide response was not due to an acidifying action of carbon dioxide. Furthermore

it has been shown in Figure 15 and Table 18, that radioactive bicarbonate was incorporated into soluble protein. This evidence plus the data which suggest that carbon dioxide stimulated growth depends on carbon dioixde stimulated protein synthesis indicates that the carbon dioxide response is mediated by a biochemical process.

The role of dark carbon dioxide fixation in carbon dioxide stimulated growth of non-photosynthetic plant tissue was proposed by Splittstoesser in 1966. He suggested that carbon dioxide stimulated growth is related to the biosynthetic role of the Krebs cycle. The continued operation of this cycle depends on the presence of oxaloacetate, the four carbon molecule which accepts the two carbon acetyl unit. If, however, Krebs acids are used for biosynthesis (eg. α-ketoglutarate to glutamate and then to protein), the level of oxaloacetate will fall. This would inevitably decrease the rate at which the cycle operates unless Krebs acids were replaced. One mechanism for doing this is by carbon dioxide fixation which can generate four carbon acids from three carbon intermediates. Bown and Lampman (1971) have shown that phosphopyruvate carboxylase (EC. 4.1.1.31) is present in etiolated *Avena* coleoptiles and aspartate and malate are the first detectable labelled products after feeding ^{14}C-bicarbonate. The importance of phosphopyruvate carboxylase in replacing Krebs cycle intermediates is well established in *E. coli* (Ashworth et al., 1965; Canovas and Kornberg, 1965; Smith 1970). Lips and Beevers (1966), using corn roots, have demonstrated that the major product of bicarbonate fixation is malate, and that this labelled malate is utilized faster in the presence of malonate which inhibits succinic dehydrogenase and therefore the generation of oxaloacetate. This type of evidence indicates that dark carbon dioxide fixation could play an important role in carbon dioxide stimulated growth and protein synthesis.

Thus it appears that the site of action of carbon dioxide in stimulating growth could be through carbon dioxide fixation resulting in the production of four carbon acids which are necessary to maintain the biosynthetic role of the Krebs cycle. If this hypothesis is correct it means that malate should be interchangeable with carbon dioxide in stimulating growth. Tables 3, 4 and 12 show that malate, like carbon dioxide, stimulated growth and protein synthesis. In addition these tables illustrate that malate could replace carbon dioxide in stimulating these processes, and malate or carbon dioxide could not stimulate growth in the presence of the other factor. This data indicates, therefore, that malate and carbon dioxide have the same site of action in stimulating growth. On the other hand, carbon dioxide stimulated growth (Tables 5 and 6) and protein synthesis (Table 14) in the presence of acetate, a two carbon molecule.

A further consequence of this hypothesis is that labelled bicarbonate should be fixed into Krebs acids and related amino acids and eventually turn up in protein. Bown and Lampman (1971) have already shown that the first detectable labelled products of radioactive bicarbonate fixation in etiolated *Avena* coleoptile were malate and aspartate and the present study showed the incorporation of radioactive bicarbonate into soluble protein (Figure 15). Moreover, because IAA stimulates protein synthesis, it should stimulate the incorporation of ^{14}C-bicarbonate into protein and this phenomenon was demonstrated (Table 18).

The role of auxin in stimulating growth and protein synthesis has been well documented (Key, 1964; Courtney et al., 1967). Some data has also accumulated on the relationship between IAA and carbon dioxide in stimulating the growth of non-photosynthetic plant tissues. Yamaki (1954) showed that

atmospheric concentration of carbon dioxide from 0 to 0.5%, in the presence of IAA, promoted growth of *Avena* coleoptiles linearly with respect to increasing carbon dioxide concentrations. Nitsch and Nitsch (1956) also presented evidence which suggested that carbon dioxide could promote the growth response to auxin of *Avena* coleoptiles. However, Cockshull and Heath (1964) have shown that 0 to 20% concentration of carbon dioxide in air would promote extension growth in the absence of externally added auxin, but at the higher concentration of carbon dioxide (20%) the period of growth stimulation was shortened and might be followed by inhibition.

The present study shows that the maximum stimulation of growth and protein synthesis was obtained only when both IAA and carbon dioxide were present (Tables 1, 9 and 10). Although the exact relationship between IAA and carbon dioxide in stimulating growth and protein synthesis is not clearly understood, both factors stimulated growth (Table 1) and incorporation of radioactive leucine into soluble protein and the cell wall fraction (Table 10) synergistically. Since IAA stimulated growth depends on protein synthesis, and the absence of carbon dioxide apparently reduces the rate of protein synthesis, it might be expected that in the absence of carbon dioxide the tissue would exhibit a much reduced response to IAA. Labelled bicarbonate fixation into soluble protein and the cell wall fraction was stimulated in the presence of IAA. This stimulation is consistent with the hypothesis that with a greater rate of protein synthesis due to IAA, the Krebs cycle would have an increased biosynthetic role and therefore an increased demand for acetyl acceptors which are generated by bicarbonate fixation. An alternative hypothesis to explain this stimulated fixation could involve an auxin stimulated increase in the permeability of cell membranes to bicarbonate or some

labelled product of bicarbonate fixation. For instance available evidence indicates (Lips and Beevers, 1966) that dark carbon dioxide fixation, generating malate, occurs outside the mitochondria; and increased utilization of this malate by the Krebs cycle could be brought about by a change in the permeability of the mitochondrial membrane to malate.

Rayle and Cleland (1970), however, have shown that the stimulation of growth by carbon dioxide saturated water and auxin are different and the latter phenomenon more closely resembles that of H^+ ions stimulations. The present study does not indicate whether the stimulation of growth of *Avena* coleoptiles by auxin and carbon dioxide are by similar or different mechanisms. Figures 8 and 9 shows that carbon dioxide and auxin stimulated the incorporation of ^{14}C-leucine into apparently different proteins. The influence of carbon dioxide on the incorporation of radioactivity into soluble protein in the absence of IAA is shown in Figures 7 and 8. The corresponding influence in the presenc e of IAA is shown in Figures 9 and 10. Although apparently different protein are synthesized in response to the presence of IAA and carbon dioxide there is no indication as to whether or not these stimulated protein fractions play an essentail role in the growth process. It is quite possible that both IAA and carbon dioxide could stimulate the synthesis of a small amount of a growth essential protein which would be undetectable by the techniques used in this study.

Patterson and Trewavas (1967) have shown that 2×10^{-5} M IAA changed the pattern of incorporation of ^{14}C-labelled amino acids into protein, and that changes were greatest in the proteins of the nuclear fractions. It has been suggested that rapid cell elongation is limited by the unidentified growth-limiting-protein (GLP) [Key and Shannon, 1964; Nooden, 1968; Cleland,

1971] whose synthesis or activation is stimulated by auxin. Fan and Maclachlan (1967) and Masuda and Yamamoto (1970) have demonstrated that IAA stimulated the activity of certain enzymes in plant tissue. In a similar manner carbon dioxide can stimulate the synthesis of a certain type of protein which may or may not be necessary for cell elongation.

The pattern of stimulated protein synthesis obtained with IAA in the presence of carbon dioxide (Table 9) could also be obtained using undecapitated coleoptiles in the presence of carbon dioxide (Table 11). Thimann and Grochowska (1968) have shown that the tips of *Avena* coleoptiles contain maximum biosynthetic capacity for synthesizing auxin and therefore, it can be suggested that applied IAA in the present investigation influenced metabolism of *Avena* coleoptiles in a manner similar to auxins in the intact tissue.

Growth rates obtained with IAA and carbon dioxide (Table 1) were closely parallel to the rates of labelled leucine incorporation into the cell wall fraction of *Avena* coleoptiles (Table 10). In other words maximum synthesis of cell wall component(s) was obtained when maximum growth of the coleoptiles occurred. IAA not only increased the incorporation of labelled bicarbonate into the soluble protein, it also increased the ^{14}C-bicarbonate incorporation into the cell wall fraction (Table 18). Cycloheximide, which inhibits growth and protein synthesis, inhibited ^{14}C-leucine incorporation drastically both into the soluble protein and cell wall fractions (Table 16). Even though IAA and carbon dioxide stimulated a greater percentage increase in incorporation of radioactivity into the cell wall fraction as opposed to the soluble protein (Table 10), cycloheximide resulted in a larger percentage inhibiton of ^{14}C-leucine incorporation into the soluble protein (Table 17).

Thus although there appears some evidence for a relationship between growth rates and incorporation of radioactive leucine and bicarbonate into the cell wall fraction, incorporation of radioactivity into soluble protein appears to be more sensitive to cycloheximide than incorporation of label into the cell wall fraction.

Cleland (1968) showed that, in *Avena* coleoptiles, the incorporation of labelled proline into the hydroxyproline-rich protein of the cell wall was increased in the presence of IAA and sucrose. However because of a lack of correlation between the amount of hydroxyproline synthesis and the growth rate (Cleland and Karlsnes, 1967) he suggested that hydroxyproline formation is not directly involved in auxin-induced cell wall loosening, but that it might be necessary for the cell wall to retain its capacity for elongation. Cleland also showed that actinomycin D (an inhibitor of RNA synthesis) inhibited auxin-induced growth, but it only slightly inhibited the incorporation of label from proline into the cell wall and he suggested that hydroxy-proline-rich protein could not be a growth limiting protein.

Lamport (1970) has suggested that hydroxyproline-rich protein, which he called "extensin", is involved in auxin-induced cell elongation. He believes that extensibility of the cell wall is governed by a number of hydroxyproline-arabinose cross links within the cell wall and that auxins stimulated the activity of an enzyme that can break these linkages. In addition he has proposed that these bonds are relatively acid labile and that low pH values stimulate extension by cleaving these linkages.

Thus there is evidence that incorporation of amino acids into cell walls is important in plant growth, and a hypothesis exists which implicates cell wall proteins in the growth process. In this study it was found that

IAA and carbon dioxide which stimulate growth also stimulated the incorporation of labelled leucine into the cell wall (Table 10) and IAA also stimulated the incorporation of labelled bicarbonate into the cell wall fraction (Table 18).

From the results of this investigation it can be concluded that both IAA and carbon dioxide elevate the rates of growth and protein synthesis in etiolated *Avena* coleoptiles. In addition the data indicates that IAA and carbon dioxide stimulate growth in a synergistic manner. Evidence is presented that carbon dioxide stimulated growth depends on carbon dioxide stimulated protein synthesis, that stimulation of growth by carbon dioxide is mediated by a biochemical process and that stimulated growth depends on carbon dioxide fixation.

6. BIBLIOGRAPHY

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