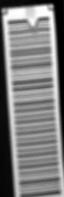# INVOLVEMENT OF PROTEIN SYNTHESIS IN
AUXIN-INDUCED ELONGATION

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
Keith K. Schlender
1966

This is to certify that the

thesis entitled

INVOLVEMENT OF PROTEIN SYNTHESIS
IN AUXIN-INDUCED ELONGATION

presented by

Keith K. Schlender

has been accepted towards fulfillment
of the requirements for

___Ph. D.___ degree in _Biochemis_try

Date _Novembe_

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ABSTRACT

INVOLVEMENT OF PROTEIN SYNTHESIS IN

AUXIN-INDUCED ELONGATION

by Keith K. Schlender

The exact mechanism of auxin-induced cell elongation
is not known. One process which has been implicated in cell
enlargement is protein synthesis. The role of protein syn-
thesis in auxin-induced elongation was investigated by employ-
ing chloramphenicol, cycloheximide, and gougerotin.

In _Avena_ and _Triticum_ coleoptiles, auxin-induced
elongation and protein synthesis were inhibited by the same
concentrations of chloramphenicol. _Avena_ coleoptiles were
inhibited between 5×10^{-4} to 5×10^{-3} $\underline{M}$ concentration. Chloram-
phenicol inhibited both protein synthesis and elongation at
5×10^{-3} $\underline{M}$ in _Triticum_ coleoptiles. In the _Avena_ coleoptile,
preincubation and kinetic experiments supported the view that
protein synthesis was necessary for the initiation as well as
the continuation of auxin-induced elongation.

^{14}C-Leucine and ^{14}C-α-aminoisobutyric acid uptake were
inhibited by chloramphenicol. ^{14}C-α-Aminobutyric acid uptake
was also inhibited by chloramphenicol. However, this analog
of protein amino acids was not a satisfactory tool for inves-
tigating amino acid uptake. ^{14}C-α-Aminobutyric acid was

rapidly metabolized and its radioactivity incorporated into protein at a rate comparable to that of ^{14}C-leucine. Chloramphenicol inhibited the uptake of ^{14}C-indole-3-acetic acid, but the inhibition was small and did not contribute to the inhibition of elongation.

Chloramphenicol uptake and metabolism were not involved in the high concentrations required for growth inhibition in _Avena_. When treated with a 5×10^{-3} _M_ solution of chloramphenicol, the internal concentration exceeded 10^{-3} _M_ within 30 minutes. After 4 hours, the internal concentration, of which 80-90% was unchanged chloramphenicol, equaled the external concentration.

The action of chloramphenicol was not stereospecific in several plant systems. Auxin-induced elongation, ^{14}C-leucine uptake and incorporation into protein, ^{14}C-α-amino-isobutyric acid uptake, buckwheat root elongation, and gibberellic acid-induced synthesis of α-amylase were inhibited by the four stereoisomers of chloramphenicol.

Cycloheximide inhibited auxin-induced elongation in _Avena_ and _Triticum_ coleoptiles. In _Avena_ coleoptiles, there was a parallel between the degree of inhibition of elongation and protein synthesis throughout the concentration range of 10^{-5} to 10^{-7} _M_. Kinetic studies of cycloheximide inhibition of auxin-induced elongation and inhibition of protein synthesis indicated a temporal relationship between the two phenomena. The repression of protein synthesis preceeded inhibition of elongation.

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Gougerotin inhibited auxin-induced elongation in <u>Avena</u> coleoptiles, 50% inhibition being reached at 10^{-6} <u>M</u> concentration. The compound was an effective inhibitor of protein synthesis in the plant coleoptiles.

The relationship between the inhibition of elongation and inhibition of protein synthesis reported in this thesis are consistent with the viewpoint that protein synthesis is an essential requirement for both the initiation and the continuation of auxin-induced elongation.

INVOLVEMENT OF PROTEIN SYNTHESIS IN

AUXIN-INDUCED ELONGATION

By

Keith K. Schlender

A THESIS

Submitted to
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1966

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Abbreviations

Avena . . . . *Avena sativa*

CAMP . . . . Chloramphenicol

CPM . . . . Counts per minute

Cyclo . . . . Cycloheximide

IAA . . . . Indole-3-acetic acid

TCA . . . . Trichloroacetic acid

Triticum . . . . . . *Triticum vulgare*

INTRODUCTION

INTRODUCTION

> "The biochemist will proudly show the
> row of vials containing these mysterious
> hormones mostly in the form of crystal-
> line powders and will be able to give us
> the structural formula of most of the
> substances. The really intriguing prob-
> len, however, is not what these struc-
> tures are, but what they do, how they act
> on the molecular level, and how they pro-
> duce their actions. There is no answer
> to this question."
>
> Szent-Gyogyi (1960)

Over a third of a century has passed since Went (111)
first described auxin as an extractable and measurable
chemical substance. In the ensuing years great strides have
been made in elucidating both the chemical nature and the
physiological role of auxins in the growth and development
of higher plants (57). Progress on the biochemical mechan-
ism of auxin action has not been as rewarding. The basic
mechanism of auxin-induced cell elongation still remains
unknown.

Many early investigations on the changes in protein
content and enzyme activity during the elongation process
were not successful in determining the role of protein syn-
thesis in auxin-induced elongation (19). Further progress
was not possible until recent advancements in biochemistry
revealed the basic pathway of protein biosynthesis and some
of the factors which control it.

2

Selective inhibitors of protein synthesis, which act at specific sites in the biosynthetic pathway, have been of immeasurable value in determining the role of protein synthesis in complex physiological systems. In this study, chloramphenicol, cycloheximide, and gougerotin, compounds which are specific inhibitors of protein synthesis in microbial systems, were employed to assess the involvement of protein synthesis in auxin-induced elongation. Concentration and kinetic relationships of inhibition of auxin-induced elongation and repression of protein synthesis were compared. In addition, since the success of this approach depends upon the specific inhibition of protein synthesis, a detailed investigation of the uptake, metabolism, and specificity of chloramphenicol suppression in plant systems was undertaken.

LITERATURE REVIEW

LITERATURE REVIEW

Mechanism of Auxin Action

A large volume of experimental evidence has accumulated indicating that, the stimulatory effect of auxin on cell enlargement involves a softening of the cell wall and thus, an increase in the cell wall plasticity (57). Auxin softening of the cell wall was first demonstrated by Heyn in 1932 (41, 42). Later Bonner (7) showed that a striking parallel existed between the concentration of auxin required for plastic bending and the stimulation of elongation. It is important to note in this report, that the plasticity was measured after 60 minutes while the growth measurements were taken 18 hours after treatment.

Recently, this phenomenon was investigated by obtaining load-extension curves from a "constant-rate-of-extension" instrument. The latter instrument, Instron Universal Testing Instrument, was originally designed to analyze the effects of chemical modification on the mechanical properties of textile fibers (72). Using this instrument, Olson, Bonner, and Morre (72) studied the mechanical properties of isolated Avena sativa coleoptile cell walls. By the use of isolated cell walls, they eliminated the complications caused by internal tugor stresses. Their results indicated that the difference between the extensibility of IAA-treated and non IAA-treated tissue was not dependent upon the presence of an intact protoplast.

Various chemical and enzymatic treatments helped Olsen, et al. to characterize the portion of the cell wall involved with auxin-induced extensibility. Pronase treatment of the isolated cell wall, which removed 97% of the protein nitrogen, did not effect the extensibility. The latter experiment provided evidence that the extensibility was not a characteristic of the disrupted protoplast, but of the cell wall itself. Hot acid treatment of the cell wall, which removed hemicellulose, did not disrupt the IAA effect on extensibility. Cellulase treatment, which interfered with the cellulose microfibril interaction, had a profound effect on the extensibility. Therefore, the authors concluded that the interaction between the fibrils of cellulose were responsible for the IAA-induced changes in the cell wall properties. They also reasoned that the polymers themselves had been altered, but that the chemical modifications which resulted in the altered mechanical properties were small.

Although the changes in the physical properties of the cell wall are now known, the biochemical mechanisms are not well defined. An early theory (105) suggested that the cell wall rigidity was dependent upon the number of calcium cross linkages between the pectin chains. Auxin treatment was believed to decrease the number of cross linkages by promoting methylation of the carboxyl groups in the pectin molecule. In some expanding tissues auxin does enhance the rate of ^{14}C-methionine incorporation into pectin (74). However, not all tissue which can be induced to elongate by auxin, show a corresponding increase in methylation (20). Furthermore, Cleland

(23), working with <u>Avena</u> coleoptiles, demonstrated with the aid of ^{14}C-methionine and ethionine that auxin-induced elongation occurred under experimental conditions where methyl transfer was completely eliminated.

Further evidence against the involvement of pectin cross linkages was secured by employing radioactive calcium. In preincubation experiments where radioactive calcium was incorporated into the cell walls, there was no confirmation of auxin-induced loss of cell wall calcium (22).

Another method for auxin to affect cell wall properties would be the synthesis of new cell wall material. There have been a number of observations that auxin-induced cell elongation is accompanied by an increase in cell wall material (2). However, there was no detectable increase in cell wall synthesis in <u>Avena</u> coleoptile sections when elongation was inhibited by mannitol, even though isotonic mannitol did not prevent the loosening of the cell wall as measured by the Instron stress-strain analyzer (73). Hence, the increased synthesis which accompanied elongation could be caused by elongation, rather than the cause of elongation. However, other experiments employing ^{14}C-glucose, and calcium to inhibit elongation, indicated that there was some cell wall synthesis in the absence of growth (2). Cell wall synthesis without growth was called a direct auxin effect. The latter effect seen in the presence of calcium was in the synthesis of matrix polysaccharides and not α-cellulose (84). The indirect effect, due to elongation, promoted α-cellulose synthesis (84).

An important contribution to this discussion would be
to study the plasticity of the tissue prevented from elonga-
tion by calcium. If the loosening of the cell wall occurs in
the presence of calcium as it did in isotonic mannitol (73)
there would be a correlation between direct effects on cell
wall synthesis (matrix polysaccharides) and cell wall exten-
sibility.

Recent evidence indicates that more is involved in
cell enlargement than a simple softening of the cell wall
followed by a concomitant passive entry of water as suggested
by Leopold (57). Cleland (24) studied cell wall loosening in
Avena coleoptiles in the presence of actinomycin D. After an
initial lag period actinomycin D inhibited RNA synthesis (24),
protein synthesis (70), and effectively prevented elongation
(24, 70). The addition of auxin three hours after actinomycin
D treatment induced a considerable increase in cell wall exten-
sibility (24). This reaction occurred under the same condi-
tions where RNA synthesis was inhibited by 90% and elongation
was completely blocked. Cleland concluded that RNA synthesis
must be required for some other process such as an adequate
supply of water and osmotic solutes.

In an independent investigation, Morre (63) investigat-
ing the effect of actinomycin D on RNA synthesis, cell elonga-
tion, and tissue deformability in pea (Pisum sativum L.) and
soybean (Glycin max) hypocotyls arrived at a similar conclusion.
His results revealed clearly that the action of actinomycin D
was not simply an inhibition of auxin-induced tissue deform-
ability. Morre postulated that at least two sets of factors

(S_1 and S_2) were involved in cell elongation. He designated arbitrarily the first set (S_1) as those involved in cell wall loosening. In pea stems S_1 was not as sensitive to actinomycin D as S_2 since, actinomycin D greatly reduced the ability of the sections to elongate under conditions where tissue deformability was adequate to permit cell expansion. In soybean tissue both S_1 and S_2 seemed to be depleted by pretreatment with actinomycin D. Morre suggested that both cell elongation and tissue deformability were dependent upon RNA synthesis. However, these two sets of conditions were independent.

Effect of Auxin on Enzyme Activity

Early studies on the mode of action of auxin were centered around the effect of auxin on both _in vivo_ and _in vitro_ enzyme activity. Most of the _in vitro_ studies have been on enzymes and enzyme systems involved in oxidative or respiratory activities. Auxin in concentrations which promote growth are almost entirely without effect upon _in vitro_ enzymes (8, 19). At high concentrations some enzymes are influenced by auxin, either inhibition or stimulation, but it is difficult to show that these changes in activity have any relationship to auxin-induced cell elongation.

The activity of enzymes _in vivo_ are usually increased by auxin treatment (57). However, the increase in enzyme activity usually is much slower than the growth response and most likely is secondary, resulting from the increased elongation rather than the cause of auxin-induced growth.

Protein Synthesis and Auxin-Induced Elongation

The role of protein synthesis in auxin-induced cell elongation is still not completely known. In some tissue there is an increase in protein content during auxin-induced growth. Protein synthesis in artichoke (Helianthus tuberosus) slices was strongly promoted and to a lesser extent protein synthesis was enhanced in potato (Solanum tuberosum) slices during auxin treatment (104). In addition, aged artichoke tuber disks treated with auxin incorporated more ^{14}C-leucine than did the controls (69, 71). Christiansen and Thimann (16) showed that there was considerable synthesis of protein in pea stem segments in the presence of auxin. However, there was also a considerable synthesis of protein in the controls. When growth was inhibited by various metabolic inhibitors there was a corresponding decrease in protein synthesis. The problem of protein synthesis in pea stem tissue has recently been investigated with the aid of ^{14}C-amino acids. The incorporation of ^{14}C-leucine was enhanced but most of the enhancement may have been due to the increased uptake of the radioactive amino acid (71). Indole-3-acetic acid at concentrations which promoted weight increases of fresh tissue enhanced ^{14}C-glycine uptake and incorporation into protein (35). Inhibitory levels of IAA decreased uptake and incorporation. Datta and Sen (29) incubated pea internodes for 15 minutes with ^{14}C-phenylalanine. After incubation, the subcellular fractions were isolated by differential centrifugation. Auxin strongly increased amino acid incorporation into the nuclear protein fraction. None of the other fractions were affected.

In other tissues there is no change or a net decrease in protein content during auxin treatment. There was no increase in protein content during the cell elongation of wheat (<u>Triticum</u> <u>sativum</u>) roots (12). Protein nitrogen decreased in corn (<u>Zea</u> <u>mays</u>) mesocotyl sections during cell elongation (30). The decrease was not altered by auxin. Insoluble nitrogen did not change in <u>Avena</u> mesocotyl tissue during either control or auxin-induced elongation (46). There was a loss of protein content during incubation of excised soybean hypocotyl sections. Although auxin greatly stimulated the fresh weight of the hypocotyl sections, there was only a slight difference in the protein content of the two treatments. Key (54) later indicated that auxin slightly stimulated the incorporation of ^{14}C-leucine into the TCA insoluble fraction.

In 1953, Boroughs and Bonner (9) investigated the effect of auxin on protein synthesis in both corn and oat coleoptiles. Protein levels remained constant in excised sections over a period of 6 hours and were independent of auxin. In addition, auxin did not alter the rate of incorporation of either ^{14}C-glycine or ^{14}C-leucine into the protein fraction. This study was confirmed by Thimann and Nooden (71).

Another approach to the study of the role of protein synthesis in auxin-induced cell elongation was the use of amino acid analogs. Bonner (6) demonstrated that canavanine, an antagonist of arginine, inhibited auxin-induced growth in <u>Avena</u>. The inhibition was reversed by arginine. In the same study hydroxyproline, an antagonist of proline, suppressed

cell elongation and this inhibition was reversed by proline
(21). Ethionine, an analog of methionine, repressed elonga-
tion and the inhibition was overcome by the addition of
methionine (22, 23, 90).

The use of canavanine, ethionine, and hydroxyproline
as evidence for a requirement of protein synthesis has been
criticized by Nooden and Thimann (71). The interpretation
of the results is limited because of the participation of
arginine, methionine, and hydroxyproline in reactions other
than the synthesis of proteins. Indeed, recently, Cleland
(25) in a detailed investigation of the mechanism of hydroxy-
proline inhibition concluded that hydroxyproline may inhibit
elongation by preventing the normal formation of hydroxypro-
line-rich cell wall proteins. It is interesting to note that
4-azaleucine, an analog of leucine which inhibits the growth
of bacteria, did not inhibit auxin-induced growth in Avena
coleoptiles (Unpublished results). However, p-fluorophenyl-
alanine did inhibit effectively auxin-induced growth in Avena
and the inhibition was reversed by phenylalanine (70).

Thus far, the most effective approach to the study of
the involvement of protein synthesis in auxin-induced growth
has been the use of selective inhibitors of protein synthesis.
Although the use of inhibitors of protein to determine the
participation of protein synthesis in a physiological response
is not new (13), Thimann and Nooden were the first to success-
fully use this tool in the study of auxin-induced cell elonga-
tion (71). Several early attempts were unsuccessful due to
the low concentrations of inhibitors employed (49, 92).

Thimann and Nooden (71) reasoned from the published data on protein content and auxin-induced growth that auxin may promote the synthesis or turnover of a protein or proteins. This protein may comprise only a small fraction of the total cell protein and thus auxin-induced synthesis of one or even a series of enzymes may not be detected among the total cell proteins. The inhibitors they used in their original study were chloramphenicol and puromycin, inhibitors of protein synthesis (39, 114) and actinomycin D, an inhibitor of DNA-dependent RNA synthesis (47). Their results reported in this communication (71) and two following papers (69, 70) demonstrated a correlation between the concentrations of these three inhibitors required to inhibit auxin-induced growth and protein synthesis. On the evidence that, A. compounds which were known to selectively inhibit protein synthesis also inhibited auxin-induced cell elongation; and that B. a parallel existed between the degree of growth inhibition and the degree of inhibition of protein synthesis, Nooden and Thimann proposed that the locus of auxin action is on a nucleic acid system controlling the synthesis of some essential enzyme or enzymes required for growth.

Since these studies were published, there have been several research reports dealing with the interrelationship between auxin-induced elongation and protein synthesis in the presence of various inhibitors which are presumed to be specific inhibitors in plant systems. Key (54) found in soybean tissue that puromycin as well as actinomycin D inhibited both elongation and ^{14}C-leucine incorporation into the protein

fraction. Actinomycin D, puromycin, and chloramphenicol inhibited both auxin-induced growth and control growth in sunflower (Helianthus annuus L) hypocotyls (56). The same three inhibitors also inhibited water uptake in potato disks and leaf cells of Rhoeo discolor (62). In these two studies, it was not determined whether the inhibitors being used actually did inhibit protein synthesis in the systems being studied. Penny and Galston (80) reported a detailed study of the kinetics of the inhibition of auxin-induced elongation in green pea stem segments by actinomycin D, ribonuclease, puromycin, chloramphenicol, and p-fluorophenylalanine. Unfortunately, they did not relate the kinetics of inhibition of elongation to the kinetics of inhibition of RNA or protein synthesis.

Chloramphenicol Inhibition of Protein Synthesis

$$O{=}C{-}CHCl_2$$

$$O_2N{-}\bigcirc{-}\underset{OH}{\overset{H}{\underset{|}{\overset{|}{C}}}}{-}\underset{H}{\overset{NH}{\underset{|}{\overset{|}{C}}}}{-}CH_2OH$$
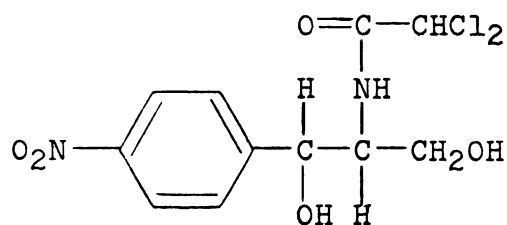
Chloramphenicol

Chloramphenicol was discovered independently in 1947 by two groups. Ehrlich and co-workers (31) isolated the broad spectrum antibiotic from an unidentified Streptomyces found near Carocas, Venezuela while a group at the University of Illinois (14) isolated the same substance from a Streptomyces found near Urbana, Illinois. Rebstock and

co-workers (27, 85) characterized and synthesized the compound in 1949. Chloramphenicol inhibits the growth of a wide variety of bacteria at concentrations between 1-100 µg/ml (10). It inhibits the growth of plants (66) and algae (28, 103), but requires concentrations which are 100-1000 times greater than those required for bacteria.

The first report on the mode of action of chloramphenicol was published by Gale and Folkes (39). Their study demonstrated that chloramphenicol preferentially inhibited protein synthesis in intact Staphylococcus aureus and that any changes in RNA and DNA metabolism were of secondary nature. These same observations were soon extended to a number of other bacterial systems (10).

Protein synthesis in several microbial cell-free systems was also sensitive to chloramphenicol (110). Detailed studies of cell-free systems established that the activation of amino acids and the transfer of activated amino acyl soluble-RNA was not altered by chloramphenicol (67). The exact mechanism of inhibition of protein synthesis is not known, but it is clear that chloramphenicol in some manner prevents the transfer of the amino acyl soluble-RNA to the growing peptide chain. Weisberger and co-workers (109, 110) have suggested that chloramphenicol acts by blocking the attachment of messenger-RNA to the ribosomes, but further work will be necessary before the details of the mechanism will be established.

Although, some controversy exists (32, 59), chloramphenicol is reported to inhibit protein synthesis in plant tissue.

Chloramphenicol suppressed the synthesis of phosphatase and amylase in germinating peas (116), a number of enzymes in the chloroplasts of beans (Phaseolus vulgaris) (61), thymidine kinase in the microspores of the lily (Lilium longiflorum) (43), and the gibberellin-induced synthesis of α-amylase in barley (Hordeum vulgare) aleurone layers (106). In addition, there have been numerous reports of chloramphenicol inhibition of ^{14}C-amino acid incorporation into protein (32, 45, 50, 51, 69, 71, 77, 78, 79). However, in the latter studies the uptake of the ^{14}C-amino acids was also repressed and it was difficult to separate the two processes. In this regard, the incorporation of ^{14}C-amino acids in several plant cell-free systems where problems of uptake are eliminated was repressed by chloramphenicol. Inhibition was found in systems from corn (83), tobacco (Nicotiana glutinosa) (100), and wheat (65).

The concentration of chloramphenicol necessary for inhibition in both intact cells and in cell-free systems was much greater than the corresponding concentration needed for an analogous microbial system. Since the mechanism of protein synthesis is similar in bacterial and plant systems, the basis for the difference in sensitivity is not clear. Several possibilities exist: first, the plant cells may not absorb chloramphenicol. Vazquez (108) found in several bacteria that there was parallel between the activity of chloramphenicol and its absorption. Uptake of course would not explain the results obtained from the cell-free systems. In the same paper (108) Vazquez noted a relationship between antibiotic activity and

the binding of chloramphenicol to the ribosome. Ribosomes obtained from peas did not bind ^{14}C-chloramphenicol as effectively as those obtained from E. coli.

A second factor which could be involved in the difference of sensitivity is the metabolism or inactivation of chloramphenicol. Certain strains of bacteria produce an extracellular substance capable of inactivating chloramphenicol and this ability is believed to be widespread among bacteria (11). In studies of a variety of animals including man, about 90% of an administered dose was recovered in the urine within 24 hours (91). Of this recovered fraction, less than 10% was free active chloramphenicol. Most of the chloramphenicol was recovered as the inactive glucuronic acid conjugate. There are no reports of chloramphenicol metabolism in plants.

Cycloheximide Inhibition of Protein Synthesis
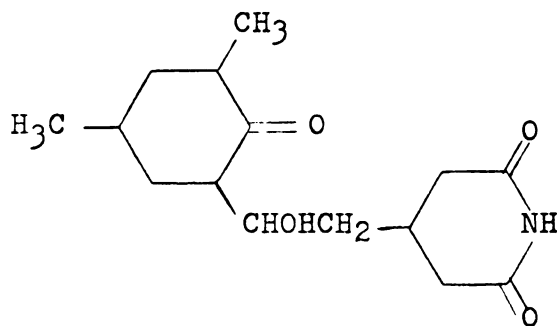
Cycloheximide

Cycloheximide was isolated from a culture of Streptomyces greiseus in 1946 (112). Although this compound did not inhibit the growth of bacteria (112), it did inhibit the growth of fungi (112), algae (75), protozoa (58), animal cells (80), and higher plants (79).

In 1958, Kerridge (53) reported that cycloheximide inhibited both protein and DNA synthesis but not RNA synthesis in yeast. Several recent reports (4, 34, 64) have provided evidence that the effect on DNA synthesis was a secondary characteristic of cycloheximide inhibition.

Evidence that protein synthesis is the primary site of cycloheximide inhibition has come from investigations on cell-free systems. Cycloheximide inhibited ^{14}C-amino acid incorporation into protein by cell-free preparations from yeast (97), mouse tumor cells (4), rat liver (33), and reticulocytes (26). It did not inhibit synthesis in a cell-free system from E. coli (34). The site of inhibition was after the formation of amino acyl soluble-RNA (33, 95) and appeared to be at the ribosomal level (96).

Cycloheximide inhibits protein synthesis in plant tissue. Varner and co-workers (107) found cycloheximide inhibited both ^{14}C-amino acid incorporation and gibberellin-induced synthesis of α-amylase by 90% in barley aleurone layers. Parthier (76, 77) reported cycloheximide inhibited radioactive amino acid incorporation into protein without affecting RNA synthesis in green tobacco leaf disks. The concentration required for inhibition, around 5×10^{-6} M for 50% inhibition, makes cycloheximide the most effective inhibitor of protein synthesis known in plants.

Gougerotin Inhibition of Protein Synthesis
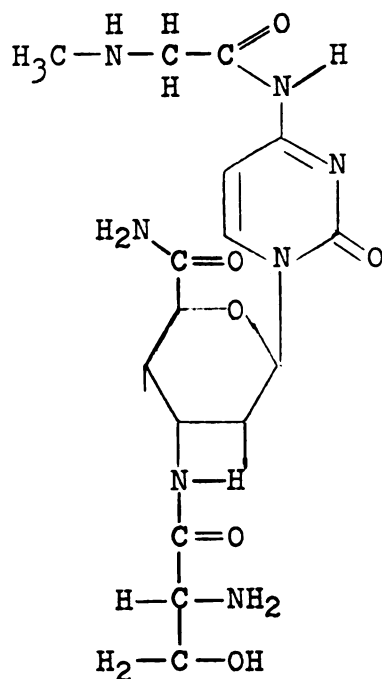
Gougerotin

Gougerotin was isolated from **Streptomyces gougerotii** by Iwasaki in 1962 (48). The antibiotic inhibited protein synthesis in cell-free systems from **E. coli** (17), mouse liver tissue (98), and reticulocytes (15). In a detailed study of the mode of action, Casjens and Morris (15) demonstrated that gougerotin inhibited the transfer of amino acyl soluble-RNA on to the growing peptide chain but did not affect the release of completed protein chains from the ribosomes. They suggested that gougerotin, which could be considered a structural analog of amino acyl soluble-RNA, interacted with the enzyme which catalyzes the formation of the peptide bond. Since there was no peptide bond formed between gougerotin and the growing peptide chain, the polypeptide chain remained attached to the

ribosomes. The presence of gougerotin at the active site of the polymerase prevented further synthesis of the peptide chain.

There are no reports of the action of gougerotin in plants.

MATERIALS AND METHODS

MATERIALS AND METHODS

Plant Material

Avena coleoptiles

Avena sativa (var. Torch) seeds were soaked in the dark
at 26.5° for 2-3 hours in tap water. The seeds were then
spread evenly on moist vermiculite in glass trays and allowed
to germinate under a dim red light (trays were placed 6 feet
below two 60 watt Ruby Red light bulbs) at 26.5° for 24 hours.
The seeds were covered with a thin layer of vermiculite and
placed in the dark at 26.5°. All further operations were made
under a green safe-light (68). About 70 hours after planting
when the coleoptiles were 2-3 cm in length, 4.5 mm sections
were cut 2-3 mm below the tip of the coleoptiles. These sec-
tions were floated for 2 hours on a glass-distilled water
solution containing 1 mg of $MnSO_4 \cdot H_2O$ per liter.

Triticum coleoptiles

Triticum vulgare (var. Thatcher) seeds were soaked in
tap water for 2 hours in the dark at 26.5°. All operations
were performed under the green safe-light. The seeds were
spread on moist vermiculite, covered with a thin layer of
vermiculite and germinated in total darkness at 26.5°. After
70 hours, when the coleoptiles were 2.5-3.5 cm in length, a
4.5 mm section was removed about 3-4 mm below the tip and

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floated on glass-distilled water for 1 hour.

Straight growth assay (68)

A pH 5 assay solution was prepared by placing 1 ml of Tween 80, 1.794 g of dipotassium phosphate, and 1.019 g of citric acid monohydrate in a 1 liter volumetric flask and adjusting to volume with glass-distilled water. The buffer solution was stored at 4° until used. Immediately before use, the buffer solution was made to 2% (w/v) sucrose and the appropriate chemicals added. Under the green safe-light 10 coleoptiles, either wheat or oat, were placed in a 6 inch test tube, 2 ml of the assay solution added, and the tubes placed in a revolving drum and turned at 1 revolution per minute. After the specified time of incubation at 26.5^o in the dark, the sections were removed and measured to the nearest 0.1 mm using a photographic enlarger.

Uptake of ^{14}C-Compounds

Using the _Avena_ assay buffer, coleoptiles were incubated with the appropriate ^{14}C-compound in the dark at 26.5^o on the roller-drum. After the incubation period, the coleoptiles were placed on a wire screen, rinsed with water, and transferred to a 10 ml beaker. The coleoptiles were rinsed for 1 minute in 8 ml of distilled water, then blotted dry on a paper towel. The coleoptiles were then transferred to a scintillation vial and 15 ml of scintillation fluid was added. The scintillation fluid was prepared from 10 g of 2,5-diphenyloxazole, 0.1 g of α-napthylphenyloxazole, and 160 g of naphthalene dissolved in 770 ml of xylene, 770 ml of p-dioxane,

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and 462 ml of absolute ethanol. For the studies of ^{14}C-compounds
which were not incorporated into protein, the coleoptiles and
scintillation fluid were equilibrated for 12 hours at 4° and
then counted directly. When ^{14}C-leucine or ^{14}C-α-aminobutyric
acid was employed, the coleoptiles were sonicated (Branson Sonic
Power Sonifier) directly in the scintillation fluid before
counting. The samples were counted on several different
Packard Tri Carb Scintillation Spectrophotometers. The count-
ing efficiency of the instruments ranged from 50-70%. However,
within any given experiment, the same instrument was used for
all of the samples. The data were expressed as cpm per 10 sec-
tions or μμm per 10 sections.

Fractionation of Proteins

Fifteen coleoptiles were incubated with the buffer used
for the straight growth assay along with the ^{14}C-amino acid.
After incubation the coleoptiles were rinsed and blotted as
previously described. Five coleoptiles were employed for
uptake study and 10 of them were placed in a 5 ml glass vial
with a plastic cap and placed on dry ice until the proteins
were fractionated. For protein isolation, 2 ml of ice cold
water and 1 ml of ice cold Bovine serum albumin (15 mg/ml) was
added to the vial containing the coleoptiles. The tissue was
sonicated until completely disrupted (about 60 seconds). The
contents of the vial were transferred into a 5 inch test tube
and the proteins were precipitated by the addition of 1 ml of
25% (w/v) trichloroacetic acid (TCA). The tubes were placed
in an ice bath for 20 minutes and then centrifuged at 1500xg

for 5 minutes. The precipitate was suspended in 5% TCA, placed in an ice bath for 5 minutes, then centrifuged for 5 minutes at 1500xg. The pellet was dissolved in 0.5 ml of 1 $\underline{N}$ NaOH, and again adjusted to 5% TCA. After 20 minutes in a ice bath the sample was centrifuged for 10 minutes, the supernatant was removed, the pellet rinsed with 5% TCA, the pellet was dissolved in 0.5 ml of 1 $\underline{N}$ NaOH, and transferred to a scintillation vial. To this preparation was added 15 ml of a scintillation gel containing 7 g of 2,5-diphenyloxazole, 150 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene, 50 g naphthalene, and 36 g of thixotropic gel powder dissolved in 200 ml of toluene, 30 ml of absolute ethanol, and 800 ml of p-dioxane (15). Samples were counted on a Packard Tri Carb Scintillation Spectrophotometer. When ^{14}C-leucine was added to unlabeled coleoptiles immediately after sonification and the homogenate treated as previously described, all of the radioactivity was removed. The data was expressed as cpm per 10 sections. Since there was no change in protein content during the assay (9), any change in the cpm reflects a change in the specific activity of the protein.

Throughout this investigation the primary leaf which does not respond to auxin was not removed. To determine the distribution of the ^{14}C-leucine between the responding coleoptile and the primary leaf, sections were incubated for 6 hours with ^{14}C-leucine, the primary leaf and the coleoptile separated, and the distribution of the radioactivity measured. Less than 2% of the total radioactivity taken up was incorporated into the protein of the primary leaf (Table 1).

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TABLE 1

Distribution of ^{14}C-Leucine in the Coleoptile and Primary Leaf of the Avena

	Uptake	TCA Insoluble
Leaf*	822	119
Coleoptile*	8,120	4,234

*Expressed as cpm/10 sections.

Metabolism of Chloramphenicol

Incubation and preparation for chromatography

Avena coleoptiles were incubated with 5×10^{-3} $\underline{M}$ ^{14}C-chloramphenicol (18,420 cpm/ml) in the dark at 26.5°. After 4 hours the internal concentration was equal to the external concentration. The coleoptiles were placed in a 5 ml vial, 3 ml of acetone added, the vial covered, and placed in the dark at 4°. After 5 hours the coleoptiles were removed and their radioactivity determined. About 3% of the initial radioactivity remained in the tissue. Extending the extraction period to 24 hours did not remove any further activity. The acetone was removed in vacuo and the residue taken up in a small volume of acetone for chromatography.

Thin-layer chromatography

Since there were no good procedures developed for thin-layer chromatography of chloramphenicol a number of solvent systems were studied. For these studies, small instant thin-layer sheets, 2x6 2/3 cm, were cut from 20x20

cm Eastman silica gel chromatogram sheets. The small sheets
could be developed in 5-7 minutes and a rapid survey made of
many different solvent systems. After development, the chro-
matograms were sprayed with a 0.05% solution of Rhodamine B
in ethanol. The chloramphenicol was located by viewing the
chromatogram under short-wave ultraviolet light. Two solvent
systems gave good results. In the first system of chloroform:
ethyl acetate:formic acid (5:4:1) the R_f value of chloramphen-
icol was 0.75. In the second system of chloroform:benzene:
ethanol (7:3:1), the R_f value was 0.39.

^{14}C-Chloramphenicol extracts were chromatographed on
4x20 cm thin-layer sheets. One side of the chromatogram was
spotted with unlabeled chloramphenicol and the other side
with the ^{14}C-chloramphenicol extract. The chromatogram was
developed in one of the above solvent systems for 15 cm.
After drying, the chromatogram was cut down the middle to
separate the marker spot from the extract. The location of
the unlabeled chloramphenicol was determined with Rhodamine B.
For location of the radioactive metabolic products of chloram-
phenicol, the other half of the chromatogram was cut into 15
equal segments and each one placed in a scintillation vial
with 5 ml of scintillation fluid and the radioactivity deter-
mined.

Incubation and preparation for bioassay

The 4 isomers of chloramphenicol at $5x10^{-3}$ $\underline{M}$ were incu-
bated with _Avena_ coleoptiles (8 coleoptiles/ml) for 4 hours in
the dark at 26.5°. As determined from the quantitative experi-
ments with ^{14}C-chloramphenicol, there were about 8 μg of

chloramphenicol per coleoptile section. For the extraction, 9 coleoptiles were placed in a 5 ml vial. Three ml of acetone was added, the vial closed, and placed in the dark at 4°. After 20 hours, the coleoptiles were removed, and 1 ml of acetone extract was transferred into each of three test tubes. The 5 inch test tubes contained about 24 µg of extracted chloramphenicol. The acetone was removed _in vacuo_ at room temperature and the residue was used directly for the assay of chloramphenicol activity in _E. coli_.

Bioassay of Chloramphenicol

Growth of cells

The culture medium was prepared by dissolving 10 g of tryptone, 10 g of yeast extract, 5 g of K_2HPO_4, and 10 g of glucose in 1 liter of glass-distilled water. The medium was autoclaved before use. Using sterile technique, 8 ml of culture medium in a 6 inch test tube was inoculated with 0.2 ml of a stock culture of _Escherichia coli_ (Crooks strain). The culture was incubated at 35°. The growth of the culture was followed by determining the optical density at 660 mµ (Coleman Jr. Spectrometer). After about 3-4 hours the cell suspension was in the log phase of growth with an optical density between 0.2-0.3.

Determination of protein synthesis

The rate of protein synthesis in the _E. coli_ cell suspension was determined by following the incorporation of ^{14}C-leucine into the protein fraction. One ml of the above cell

suspension was transferred to a 5 inch test tube. The cells
were incubated for $\frac{1}{2}$ hour at 35° with the appropriate chemical
or plant extract, then ^{14}C-leucine (40,000 cpm) was added and
the incubation continued for 1 hour. The reaction was stopped
by the addition of 2 ml of 10% TCA. The mixture was heated at
80° for 10 minutes, and the insoluble protein was collected on
a glass fiber filter disk using a Millipore filter system.
The disk was washed twice with 5% TCA, once with ethanol:ether
(1:1 v/v), and once with ether. The disk was placed in a scin-
tillation vial and counted in 5 ml of scintillation fluid.

α-Amylase Assay

Barley (Hordeum vulgare, var. Himalaya) seeds were cut
in half on the equatorial axis and the embryo-half discarded.
The tips were removed from the half-seeds and the half-seeds
were soaked in Chlorox (5% sodium hypochlorite) diluted five-
fold with distilled water for 15 minutes. All remaining steps
were performed aseptically. The half-seeds were rinsed in
sterile distilled water and transferred to sterile moist sand
in a Petri dish. After preincubation for 3 days at room tem-
perature in the dark, the seed coat was slit on one side and
the endosperm removed from the seed coat and aleurone layers.
Ten layers were incubated in a 25 ml Erlenmeyer flask with 2
ml of 10^{-5} M gibberellic acid in a 0.001 M acetate buffer
(pH 4.8) and 0.01 M $CaCl_2$ along with the appropriate chemical
treatment. After 24 hours incubation in the dark at 21°, the
medium was poured off and the layers rinsed with 3 ml of the
acetate buffer. The layers were ground in a mortar with sand

and 5 ml 0.2 $\underline{M}$ NaCl. After centrifugation at 1000xg the medium
and extract were assayed separately.

α-Amylase activity was measured as described by Shuster
and Gifford (94). A starch solution containing 67 mg of solu-
ble starch in 100 ml of 0.06 $\underline{M}$ KH_2PO_4 was prepared. One ml of
this solution was added to enzyme and water to give a final
volume of 2.0 ml. After 5 minutes of incubation at 25°, the
reaction was stopped by the addition of 1.0 ml of an iodine-
HCL solution prepared from 60 mg of KI and 6 mg of I_2 in 100
ml of 0.05 $\underline{N}$ HCl. Then 5 ml of water was added, and the optical
density (OD) of the resulting solution was measured at 620 mμ.
The activity of the enzyme was expressed as mg of starch hydro-
lyzed per 10 layers per minute.

Metabolism of α-Aminobutyric Acid

Incubation and fractionation

Coleoptiles, 160, were incubated with ^{14}C-α-aminobutyric
acid for 2 hours in the dark at 26.5°. Ten coleoptiles were
picked at random to determine the total uptake. After counting,
the incorporation of ^{14}C-α-aminobutyric acid into the protein
fraction was estimated by extraction with 70% ethanol (113).
The coleoptiles were extracted with hot 70% ethanol for three
hours. Extracting with 70% ethanol in a Soxhlet extractor did
not remove any further radioactivity.

The proteins from 150 coleoptiles were precipitated by
the TCA procedure previously described with the exception that
carrier protein was not added. After TCA precipitation, the
TCA soluble fraction was taken to dryness _in vacuo_ and the

amino acids dissolved in 4 ml of 0.1 N HCl. The TCA was extracted from the acid solution with ether and the aqueous phase taken to dryness in vacuo. To remove the excess HCl the residue was taken up in 4 ml of water and the water removed in vacuo. The residue was taken up in 10% solution of 2-propanol for chromatography.

The TCA insoluble material was hydrolyzed in 6 N HCl in a sealed glass tube at 107° for 60 hours. After hydrolysis the solution was filtered and the HCl solution removed in vacuo. The amino acids were taken up several times in 4 ml of water and taken to dryness to remove the excess HCl. The residue was taken up in 10% solution of 2-propanol for chromatography.

Chromatography of the amino acids

The amino acids were paper chromatographed in two dimensions as previously described (5). The chromatogram was developed in the first direction with phenol saturated with water and in the second direction with butanol:propionic acid: water. The amino acids were located by exposure of the dried chromatogram to Kodak No-Screen X-ray film for 2 weeks. The radioactive spots were then counted with a thin-window (Du Pont Mylar film) gas flow counter using a Nuclear Chicago scaler. For preparative chromatography, chromatograms were run in one direction with butanol:propionic acid:water and the compounds located by direct scan.

Chemicals

The isomers of chloramphenicol were kindly supplied by

Dr. M. Rebstock, Park Davis and Company. Dr. H. Petering of
the Upjohn Company supplied the cycloheximide and the gouger-
otin was a gift of Dr. A. Mayake, Chemical Industries Ltd.,
Osaka, Japan. All of the radioisotopes were obtained from
New England Nuclear Corporation. The specific activity of the
compounds were: leucine, 250 $\mu c/\mu m$; α-aminobutyric acid, 4.1
$\mu c/\mu m$; α-aminoisobutyric acid, 4.0 $\mu c/\mu m$; indole-3-acetic acid,
13.5 $\mu c/\mu m$; and chloramphenicol, 3.08 $\mu c/\mu m$. All other chemi-
cals and reagents were obtained from commercial sources.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

Effect of Auxin on Elongation and Protein Synthesis

The kinetics of elongation induced by auxin in Avena

coleoptiles is shown in Figure 1. The auxin used in this

experiment and throughout this study was a 10^{-5} M solution of

indole-3-acetic acid. The time course followed the well-known

bilinear curve for Avena coleoptile elongation (93). In the

first 8 hours a linear rate of elongation was observed in both

the control and the IAA treated sections. The rate of elonga-

tion of the auxin treated coleoptiles was about 4 times greater

than was the control elongation. Elongation from 8-24 hours

continued at a reduced linear rate. The ratio of IAA to con-

trol growth during the second phase was about 2.

Throughout this study 2% sucrose and 0.01% Tween 80 were

included in the buffer system. Thus, it was important to deter-

mine how these additives affected the rate of elongation.

Nitsch and Nitsch (68) reported that over a 24 hour period

sucrose increased elongation and Tween 80, which was used to

facilitate dissolution of the chemical treatments, had little

effect on elongation. The effect of the deletion of either

sucrose or Tween 80 on the kinetics of elongation is illus-

trated in Figure 2. The experiment without Tween 80 did not

affect auxin-induced elongation. On the other hand, after a

two hour lag period, the deletion of sucrose markedly reduced

Figure 1

Kinetics of Auxin-Induced Elongation in the <u>Avena</u> Coleoptile
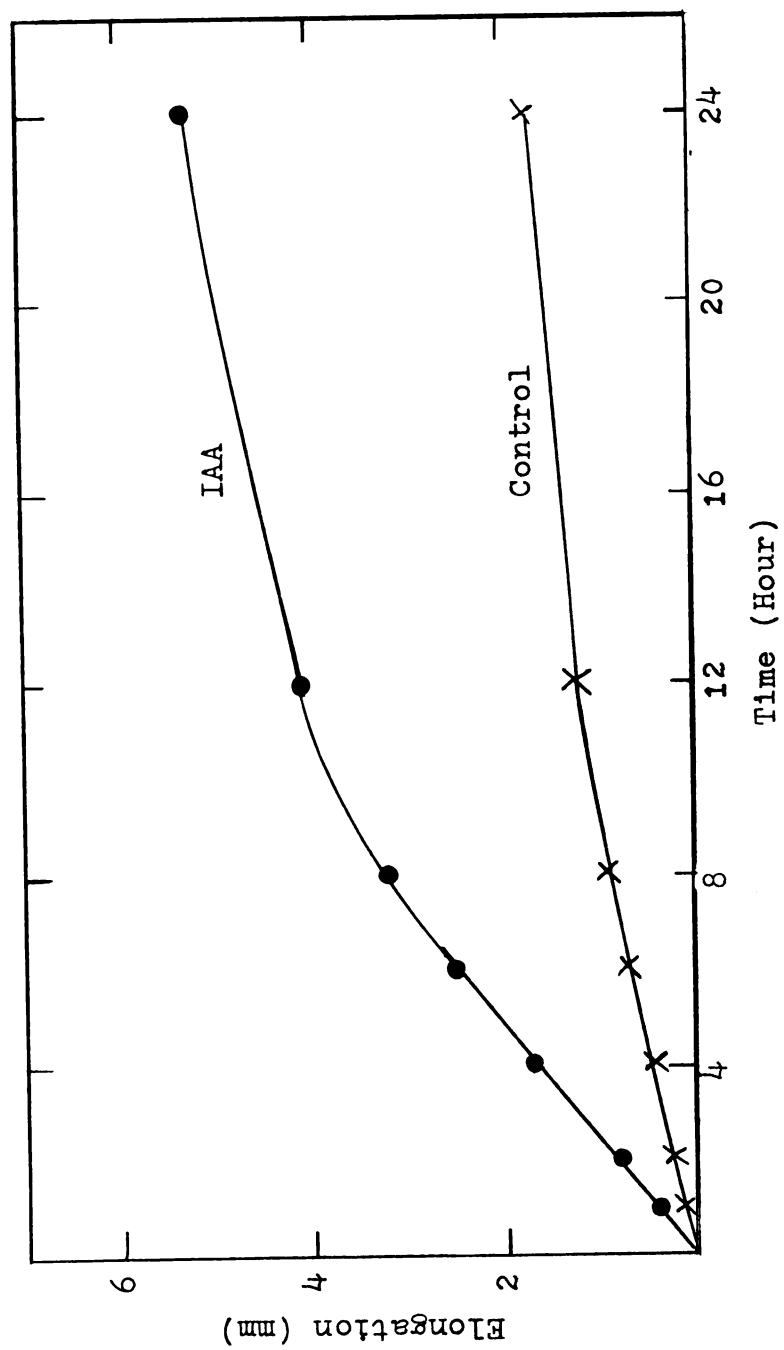
Figure 2

Effect of Sucrose and Tween 80 on the Kinetics of Auxin-
Induced Elongation in the <u>Avena</u> Coleoptile
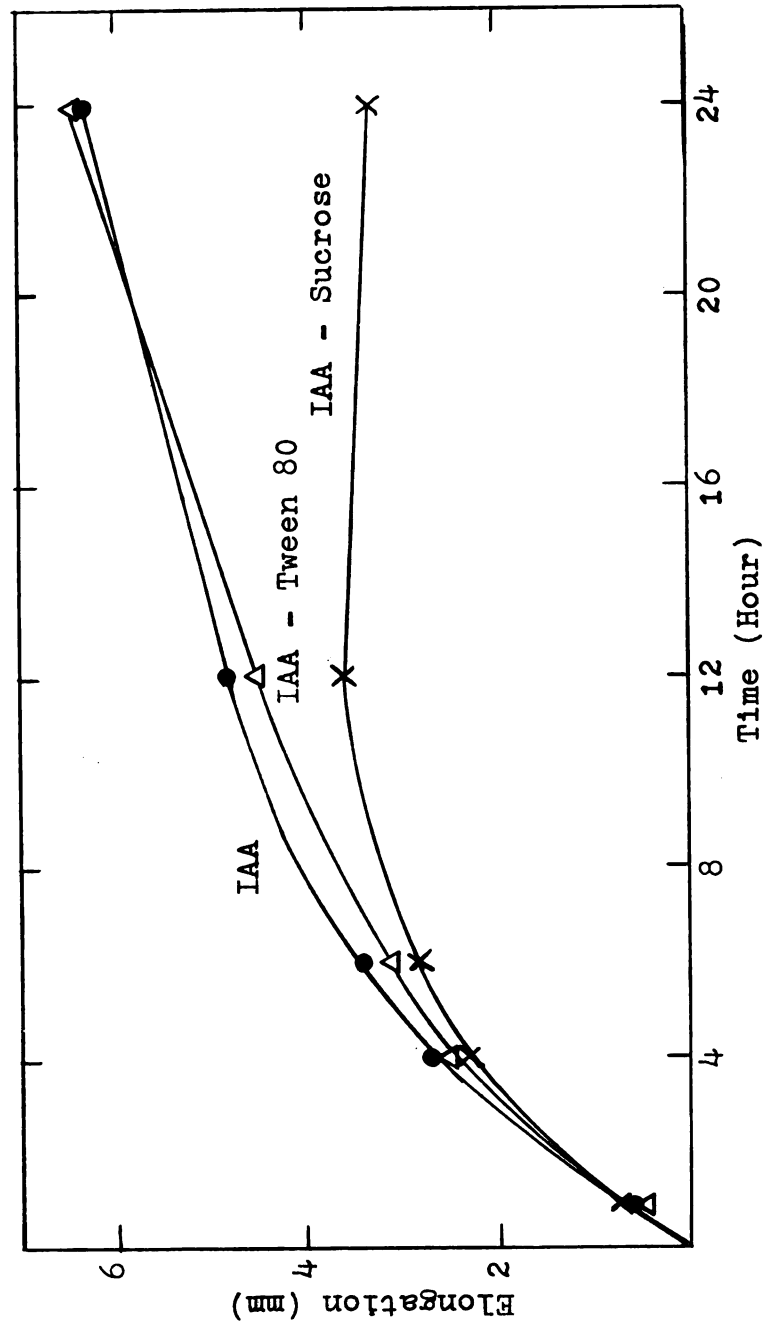
the rate of elongation and all growth was eliminated after 12 hours. Little change was noted in the first 6 hours in the ratio of auxin-induced elongation to control elongation when sugar was excluded from the medium (data not shown). Thus, it appears that sucrose does not directly induce elongation, but rather is a source of energy for auxin-induced elongation.

The effect of auxin on the incorporation of ^{14}C-leucine into protein was investigated. The lack of stimulation of protein synthesis during auxin-induced growth has already been reported (9, 71). However, in these reports, the incubation period was 5 and 6 hours while the growth response was much more rapid, there being a marked stimulation during the first hour (Figure 1). The effect of auxin on the elongation, uptake, and incorporation of ^{14}C-leucine into protein is presented in Table 2. Although the growth rate in the presence of auxin after 1 hour is more than double the control, there was little stimulation of protein synthesis as measured by ^{14}C-leucine incorporation into the TCA insoluble protein fraction. Two hours after incubation the growth rate was 4 times greater than the control, but little effect was noted on protein synthesis.

Since protein synthesis is required for auxin-induced elongation as has been postulated, an interpretation of the above results is not directly obvious. Incorporation of ^{14}C-leucine into the protein fraction of the controls was rapid, but this incorporation was not enhanced by the addition of IAA under experimental conditions where elongation was increased 2-4 fold. This does not eliminate the possibility

TABLE 2

Effect of Auxin on the Elongation, Uptake, and Incorporation of ^{14}C-Leucine into the Protein of the <u>Avena</u> Coleoptile

	Time			
	1 Hour		2 Hours	
	<u>IAA</u>	<u>Control</u>	<u>IAA</u>	<u>Control</u>
Elongation[*]	0.5	0.2	1.2	0.3
Uptake[**]	2,976	3,055	5,711	5,146
TCA Insoluble[**]	1,394	1,248	2,928	2,657

[*]Expressed in mm.
[**]Expressed in cpm/10 sections.

that auxin may induce the synthesis of some new protein(s){
essential for elongation, but only reflects the overall rate
of protein synthesis. Auxin could induce the synthesis of a
few specific enzymes and if the amount were small compared
to the total protein synthesis, it would not be observed by
this method. A second alternative could be a re-direction
of protein synthesis. Thus, the lack of stimulation of
^{14}C-leucine incorporation into protein does not in itself
exclude an essential role for protein synthesis in auxin-
induced elongation.

<div align="center">

Chloramphenicol Inhibition of

Auxin-Induced Elongation and Protein Synthesis

</div>

The parallel between the concentration of chloramphen-
icol required to inhibit auxin-induced elongation and protein
synthesis in Avena coleoptiles, previously reported by Nooden
and Thimann (20, 25), was confirmed. The concentration range
for inhibition was between $5x10^{-4}$ and $5x10^{-3}$ M (Figure 3).
Investigation of wheat coleoptiles again revealed that a par-
allel existed between suppression of protein synthesis and
elongation. Triticum coleoptiles required even higher concen-
trations than did the Avena. These results are shown in
Table 3. Incubation with a $5x10^{-4}$ M solution of chloramphen-
icol and auxin for 22 hours increased elongation 25% over that
observed for the auxin control. Incubation with a 10^{-3} M
solution of chloramphenicol had little effect while a concen-
tration of $5x10^{-3}$ M almost completely eliminated all growth
over a 22 hour period. Perhaps, the marked stimulation of
elongation by the $5x10^{-4}$ M solution was due to the bactericidal

Figure 3

Effect of Chloramphenicol on Auxin-Induced Growth in the _Avena_
Coleoptile: Concentration Range
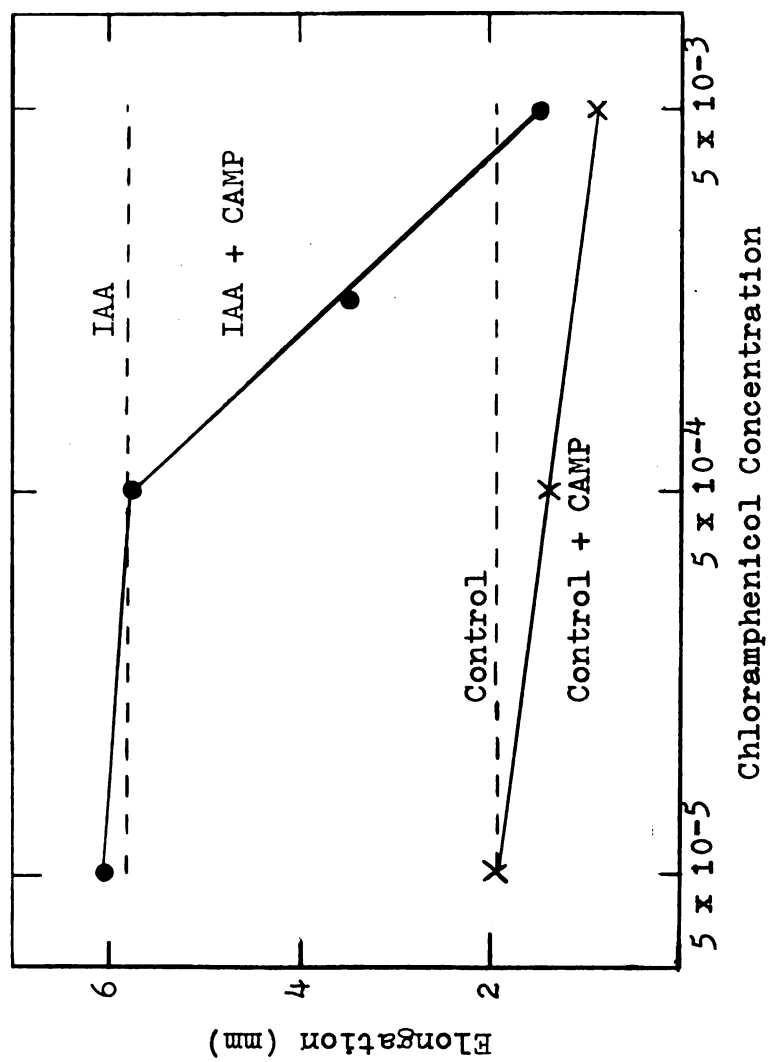
TABLE 3

Elongation, Uptake, and Incorporation of ^{14}C-Leucine into the Protein of Triticum Coleoptiles Treated with Chloramphenicol

		Concentration			
	Time (Hr)	0	5×10^{-4} $\underline{M}$	1×10^{-3} $\underline{M}$	5×10^{-3} $\underline{M}$
Elongation*	22	7.4 (0)	9.2 (-25)	7.8 (-6)	0.6 (92)
Elongation*	4	2.6 (0)	2.5 (4)	2.3 (12)	0.9 (65)
Uptake**	4	5,380 (0)	5,292 (2)	4,652 (14)	2,299 (57)
TCA Insoluble**	4	2,969 (0)	2,774 (7)	2,360 (21)	918 (69)

*Expressed as mm and % inhibition.
**Expressed as cpm/10 sections and % inhibition.

action of chloramphenicol rather than a direct effect on the plant tissue.

Chloramphenicol inhibition of elongation, ^{14}C-leucine uptake, and incorporation into the protein of Triticum after 4 hours treatment is given in Table 3. At 5×10^{-4} M, chloramphenicol was almost without effect on all 3 parameters. The stimulation of elongation by 5×10^{-4} M chloramphenicol after 22 hours was not observed after 4 hours where bacterial contamination was not a problem. There was only a slight inhibition at a concentration of 10^{-3} M while a 5×10^{-3} M solution of chloramphenicol inhibited elongation by 65% and protein synthesis by 69%. As in the Avena coleoptiles, chloramphenicol inhibited the uptake of ^{14}C-leucine making a direct comparison of the repression of protein synthesis and elongation difficult.

To establish further the relationship between protein synthesis and auxin-induced growth, kinetic studies of the inhibition of elongation and protein synthesis in the Avena coleoptile were conducted. As illustrated in Figure 4, there was a 2 hour lag period before a solution of 10^{-3} M chloramphenicol inhibited auxin-induced elongation. The elongation from 4-24 hours continued at a linear, but at an appreciably reduced rate. Inhibition was obtained within 1 hour when a concentration of 5×10^{-3} M chloramphenicol was used. There-after, the growth rate declined steadily until after 6 hours when the inhibition was complete. Pretreatment of the tissue with a solution of 5×10^{-3} M chloramphenicol before addition of auxin indicated the same lag period. Pretreatment for 45

Figure 4

Kinetics of Auxin-Induced Elongation in <u>Avena</u> Coleoptiles
Treated with Chloramphenicol ($5x10^{-3}$ $\underline{M}$)

Figure 5

Effect of Chloramphenicol on the Kinetics of Auxin-Induced
Elongation in <u>Avena</u> Coleoptiles Pretreated with Auxin
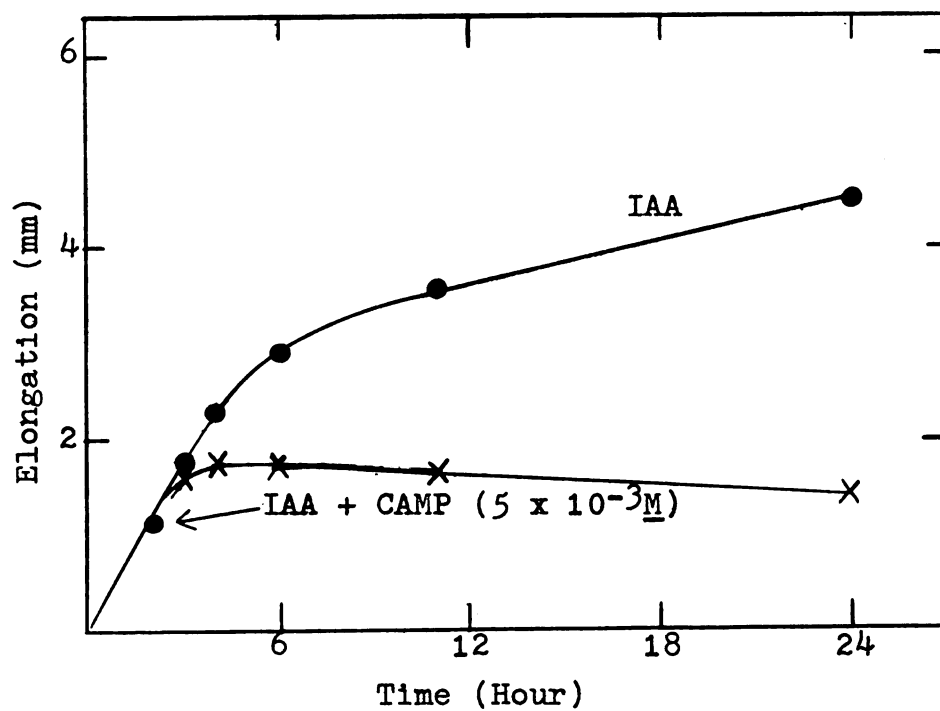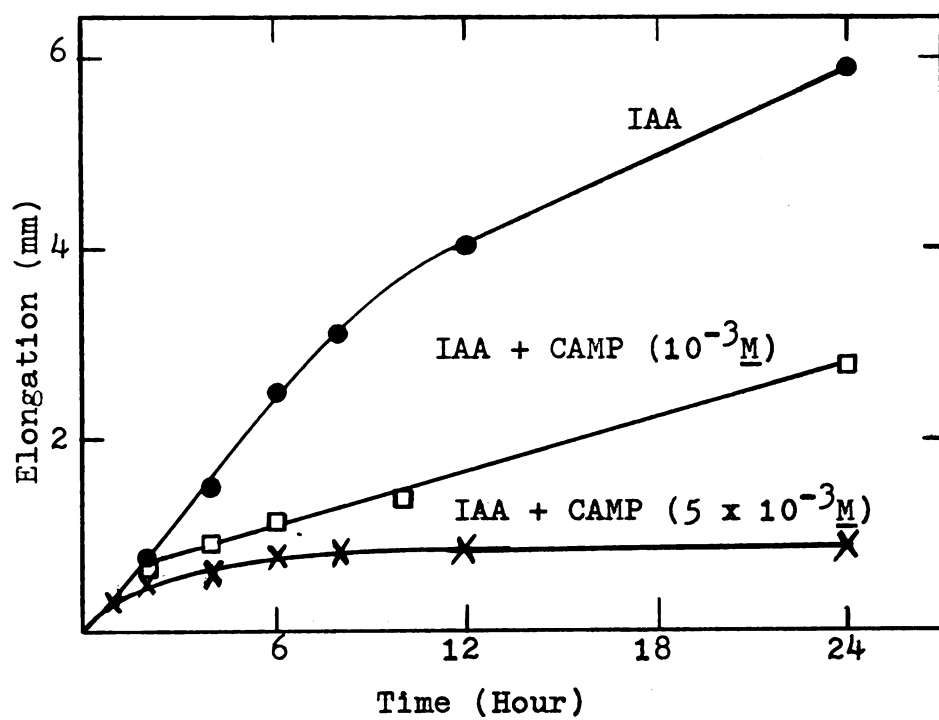
minutes or longer essentially eliminated all auxin-induced

elongation (Table 4). As will be seen later in the section

on uptake of ^{14}C-chloramphenicol, the lag period may have

been due to the rate of diffusion of chloramphenicol into

the cell. In the converse experiment where auxin was

supplied to the tissue before chloramphenicol was added,

elongation only occurred in the first hour after addition of

the inhibitor (Figure 5).

TABLE 4

Auxin-Induced Elongation in <u>Avena</u> Coleoptiles Pretreated with
Chloramphenicol (5x10^{-3} <u>M</u>)

	Elongation		
	Time after addition of IAA (Hr)		
Pretreatment (Hr)	0-2	2-4	4-20
0	0.7	0.1	0.4
1/4	0.7	0.1	0.3
1/3	0.3	0.1	0.1
1 1/2	0.2	0.1	0.1
3	0.1	0.2	0.5
6	0.2	0	0.4
No chloramphenicol	1.0	1.1	2.8

The pretreatment experiments supported the hypothesis

that protein synthesis was required for the initiation as

well as the continuation of auxin-induced elongation. This

conclusion is in conflict with the one reached by Cleland (21).

Cleland using hydroxyproline as an inhibitor suggested that

protein synthesis was required for continuation, but not for

initiation of auxin-induced growth in the <u>Avena</u>. The failure

to extend the lag period after pretreatment with IAA was

evidence that any newly synthesized protein was rapidly being
utilized by the cell.

The effect of a 10^{-3} $\underline{M}$ solution of chloramphenicol on
the uptake and incorporation of ^{14}C-leucine into the protein
of <u>Avena</u> as a function of time is presented in Table 5. These
results indicate no clear cut temporal relationship between
inhibition of elongation and protein synthesis. Inhibition
of protein synthesis varied from 16% to 29% after 1 to 6 hours,
respectively. In agreement with the elongation studies, pro-
tein synthesis was not completely eliminated, but its rate of
synthesis was reduced. The relative inhibition of ^{14}C-leucine
uptake and protein synthesis is confusing. In certain experi-
ments, uptake was inhibited to a greater extent than was pro-
tein synthesis. The pattern did not become clear until higher
concentrations of chloramphenicol were employed.

When a solution of 5×10^{-3} $\underline{M}$ chloramphenicol was added,
the inhibition of ^{14}C-leucine incorporation into protein
closely paralleled the inhibition of elongation (Figure 7).
The inhibition of protein synthesis slightly preceded inhibi-
tion of growth and from 2-6 hours both continued at a dimin-
ished rate.

The inhibition of ^{14}C-leucine uptake paralleled the
inhibition of elongation (Figure 6). Thus, one was confronted
with the difficult problem of assessing whether there was true
inhibition of ^{14}C-leucine incorporation into protein or if it
was an apparent inhibition due to a decreased level of ^{14}C-
leucine in the tissue. To compare the relative inhibition of
the two processes concurrently assayed, the data of Figure 6

TABLE 5

Uptake and Incorporation of ^{14}C-Leucine into the Protein of Avena Coleoptiles
Treated with Chloramphenicol (10^{-3} $\underline{M}$)

Time (Hr)	Uptake**			T.C.A. Insoluble**		
	IAA	IAA + CAMP	% I	IAA	IAA + CAMP	% I
1	2,632	2,087	21	1,044	878	16
2	4,456	4,141	7	2,156	1,788	17
4	10,795	8,953	17	4,940	3,903	21
6	16,556	11,322	32	8,446	6,005	29

**Expressed as cpm/10 sections.

Figure 6

Uptake of ^{14}C-Leucine into _Avena_ Coleoptiles Treated with Chloramphenicol ($5x10^{-3}$ $\underline{M}$)

Figure 7

Incorporation of ^{14}C-Leucine into the Protein of _Avena_ Coleoptiles Treated with Chloramphenicol ($5x10^{-3}$ $\underline{M}$)
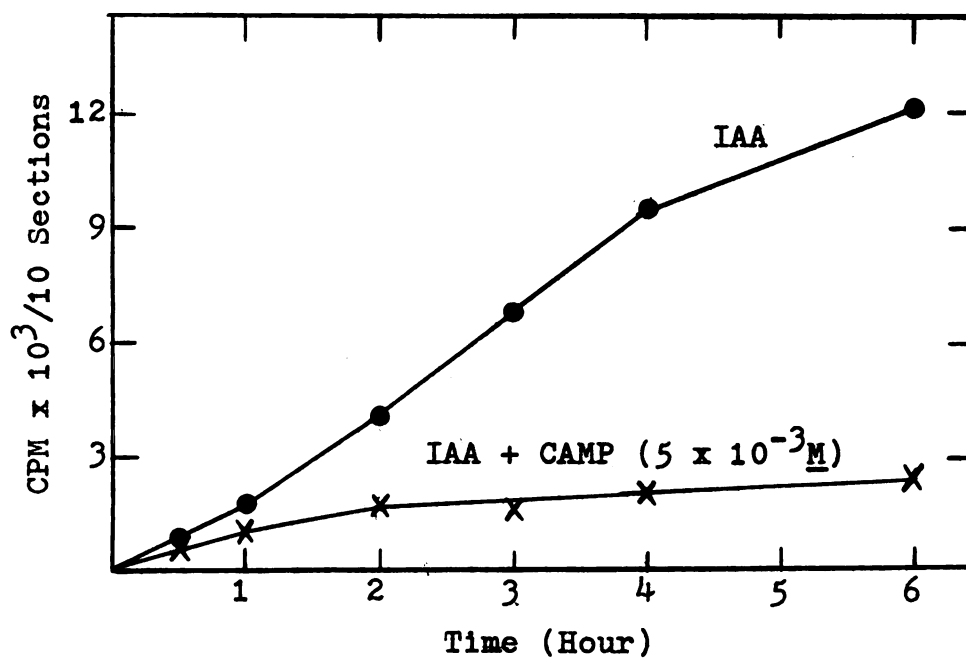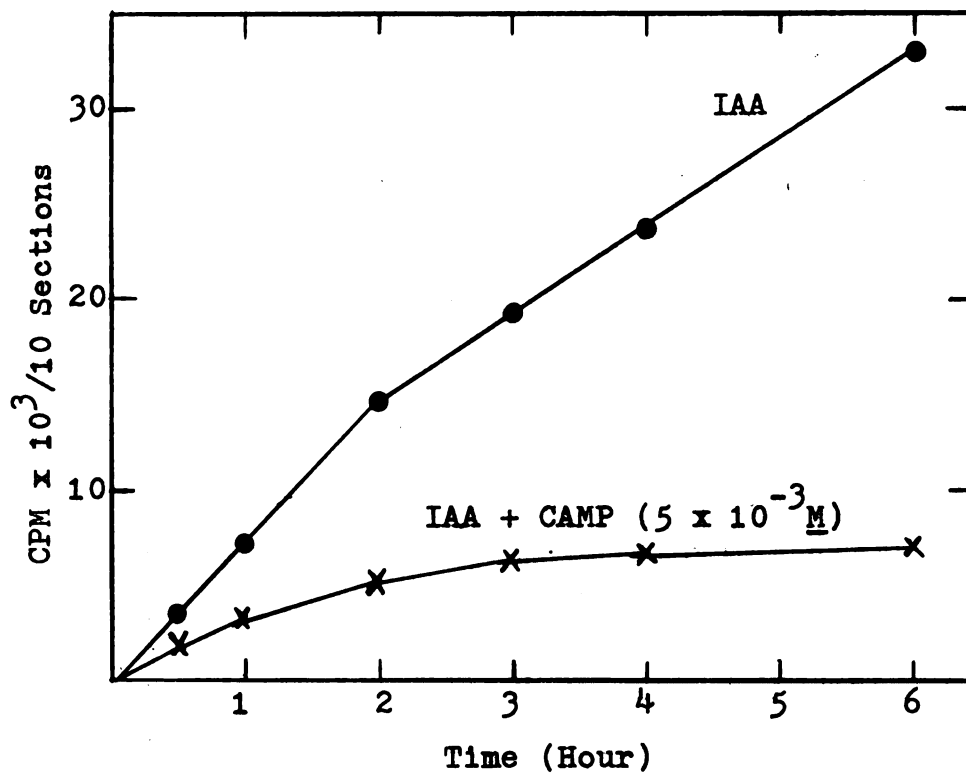
and 7 are assembled in Table 6. These experiments indicate the importance of kinetic information before one attempts to interpret this system. The inhibition of both uptake and incorporation were the same after $\frac{1}{2}$ hour. After 1 and 2 hours of incubation, uptake was inhibited to a greater extent than incorporation and from these experiments one could suggest that chloramphenicol effectively promotes protein synthesis. Thus, in the presence of chloramphenicol a greater per cent of the ^{14}C-leucine, which was taken up into the tissue, was incorporated into the TCA insoluble protein fraction. When the incubation period was for 3 or 4 hours, the incorporation was inhibited more than was uptake. Thus if one examines these two instances, chloramphenicol appears to be an effective inhibitor of protein synthesis. When the incubation was continued for 6 hours, both uptake and incorporation were inhibited to the same extent.

^{14}C-α-Aminoisobutyric acid, an amino acid which is not normally involved in protein synthesis, was employed to obtain a more direct inspection of the inhibition of amino acid uptake. Chloramphenicol (5×10^{-3} $\underline{M}$) inhibited uptake within 30 minutes and completely eliminated all uptake after 1 hour (Figure 8). In the control experiment, uptake was linear over a 4 hour period. In the chloramphenicol treated tissue the internal concentration never exceeded the external concentration, while in the IAA controls this level was exceeded during the first hour. The amino acid continued to concentrate against a gradient for the 4 hour duration of the experiment. No radioactivity was incorporated into the protein fraction (Table 7) and all except minor traces of the 70% ethanol soluble radioactivity

TABLE 6

Uptake and Incorporation of ^{14}C-Leucine into the Protein of Avena Coleoptiles Treated with Chloramphenicol (5×10^{-3} M)

Time (Hr)	Uptake*			T.C.A. Insoluble Protein*		
	IAA	IAA + CAMP	% I	IAA	IAA + CAMP	% I
$\frac{1}{2}$	3,241	1,965	39	820	510	38
1	7,209	3,253	55	1,754	1,028	41
2	14,981	5,036	66	4,100	1,828	55
3	19,486	6,357	67	6,824	1,602	77
4	23,608	6,770	71	9,547	2,146	78
6	33,136	7,024	78	12,241	2,450	78

*Expressed as cpm/10 sections.

Figure 8

Uptake of ^{14}C-α-Aminoisobutyric Acid into <u>Avena</u> Coleoptiles
Treated with Chloramphenicol (5×10^{-3} <u>M</u>)
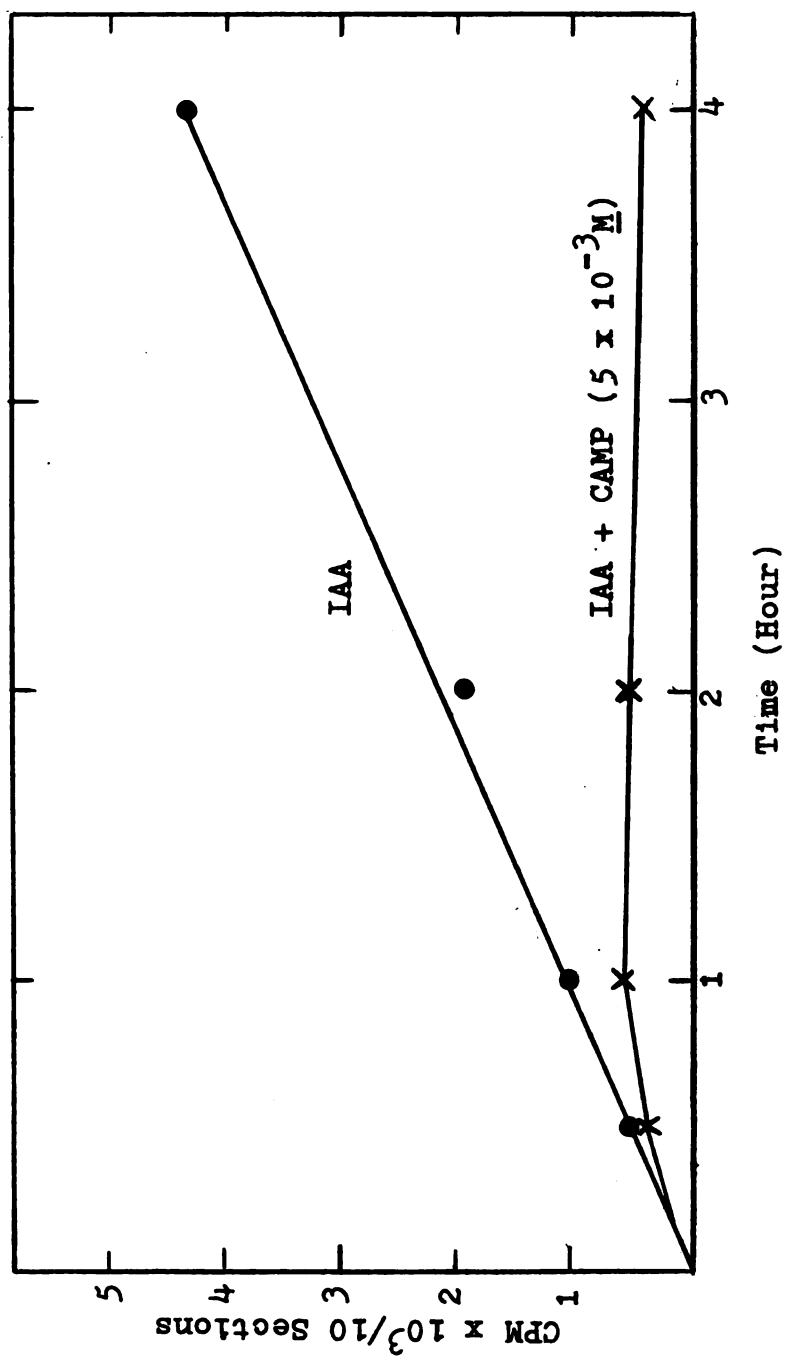
chromatographed as a single spot in the region of α-aminoiso-butyric acid. Thus, there was a true accumulation of ^{14}C-α-aminoisobutyric acid in the tissue.

TABLE 7

Incorporation of ^{14}C-α-Aminoisobutyric Acid into the Protein of the Avena[*]

Uptake	70% Ethanol Insoluble
3,978[**]	3

[*]Incubation was for 4 hours.
[**]Expressed as cpm/10 sections.

How does one explain the kinetics of chloramphenicol inhibition of ^{14}C-leucine uptake and incorporation into protein? Inhibition of solute uptake by chloramphenicol has been interpreted as a dependency of uptake on protein synthesis. It was postulated that the synthesis of a protein, which has a rapid turnover, is required for uptake (51, 101).

Recently several reports contained evidence that the inhibition of protein synthesis by chloramphenicol was not completely non-specific toward the types of protein being synthesized. A number of membrane bound mitochondrial enzymes were particularly sensitive to chloramphenicol (18, 44). In green tobacco leaf disks, chloroplast protein synthesis was more sensitive than ribosomal protein synthesis (78). Sypherd et al. (102) demonstrated in E. coli that inducible enzymes were more sensitive to chloramphenicol than constitutive enzymes, the latter being inhibited to the same degree as total protein synthesis. Thus, it is probable that even if there is

a direct relationship between protein synthesis and certain physiological responses, when there is selective inhibition of protein synthesis by chloramphenicol; it is not true that there is a direct parallel between the inhibition of the response in question and protein synthesis.

Although little is known concerning the enzymes or proteins responsible for amino acid uptake, one inducible protein is involved in the galactoside permease system of E. coli (52). Perhaps protein synthesis required for amino acid uptake was initially more sensitive to chloramphenicol than was gross protein synthesis. Hence, in the first 2 hours, ^{14}C-leucine uptake was inhibited to a greater extent than was total protein synthesis. The direct inhibition of total protein synthesis after longer periods of incubation became more pronounced. As the assay was continued on toward 6 hours, both uptake and incorporation were reduced to an extremely low rate and approached the same level of inhibition.

Although interpretation of the data is complex, under the conditions of this assay, chloramphenicol does appear to inhibit protein synthesis directly and not just as a result of the inhibition of uptake. The inhibition of ^{14}C-α-aminoisobutyric acid uptake was consistent with an obligatory relationship between amino acid uptake and protein synthesis.

Since the primary objective of these experiments was to determine the role of protein synthesis in auxin-induced elongation, an experiment was designed to determine chloramphenicol inhibition of protein synthesis in the absence of

amino acid uptake. <u>Avena</u> coleoptiles were incubated on glass-distilled water containing ^{14}C-leucine. After 2 hours incubation the coleoptiles were removed and rinsed. One group of coleoptiles was used for protein precipitation and the rest transferred to either auxin or auxin and a solution of 5×10^{-3} $\underline{M}$ chloramphenicol. The incubation was continued for 2 additional hours before the protein was precipitated. Therefore, both the coleoptiles in the presence and absence of chloramphenicol had the same amount of ^{14}C-leucine at the start of the experiment. In the second incubation period there was only slightly more radioactivity lost to the medium in the presence of chloramphenicol while protein synthesis was inhibited 44% (Table 8). This was direct evidence that chloramphenicol inhibited protein synthesis under the same experimental conditions where elongation was inhibited.

TABLE 8

Chloramphenicol (5×10^{-3} $\underline{M}$) Inhibition of the Incorporation of ^{14}C-Leucine into the Protein of <u>Avena</u> Coleoptiles Pretreated with ^{14}C-Leucine*

	Initial	2 Hours	
		IAA	IAA + CAMP
Total**	5,964	5,603	5,140
TCA Insoluble	1,633	2,151	1,806
△ CPM	--	518	344
% Inhibition	--	--	44

*Pretreated for 2 hours with ^{14}C-leucine.
**Expressed as cpm/10 sections.

Effect of Chloramphenicol on the Uptake
of ^{14}C-Indole-3-Acetic Acid

Indole-3-acetic acid uptake by _Avena_ coleoptiles is a metabolic process (81). Since chloramphenicol inhibited the uptake of amino acids, its effect was determined on the uptake of IAA. If chloramphenicol repressed the uptake of IAA, a portion of the observed growth inhibition could have been due to the inhibition of IAA uptake.

Several factors affecting the time course of ^{14}C-IAA (10^{-5} $\underline{M}$) uptake over a 24 hour period are illustrated in Figure 9. The rate of uptake with the complete buffer system was approximately linear in the first 6 hours and then reached a plateau. Between 1 and 2 hours the external concentration equaled the internal concentration. The results were surprising when sucrose or Tween 80 was removed from the buffer system. Without sucrose the rate of uptake continued in a linear manner for the first 12 hours. When Tween 80 was not included, the rate of uptake was considerably reduced. The results were in contrast to the effect of these two factors on elongation (Figure 2). It would be of interest to study the action of sucrose and Tween 80 on the metabolism of IAA in this system.

The effect of chloramphenicol on IAA uptake is depicted in Figure 10. The repression of uptake was not as great as the inhibition of elongation. After 4 hours there was a slight suppression of uptake and severe inhibition of elongation (Figure 4). It was concluded that chloramphenicol did suppress to some extent the uptake of IAA, but this inhibition contributed very little to the repression of elongation during the first few hours of the assay.

Figure 9

Uptake of ^{14}C-Indole-3-Acetic Acid into the <u>Avena</u> Coleoptile

Figure 10

Chloramphenicol Inhibition of the Uptake of ^{14}C-Indole-3-Acetic Acid into the <u>Avena</u> Coleoptile
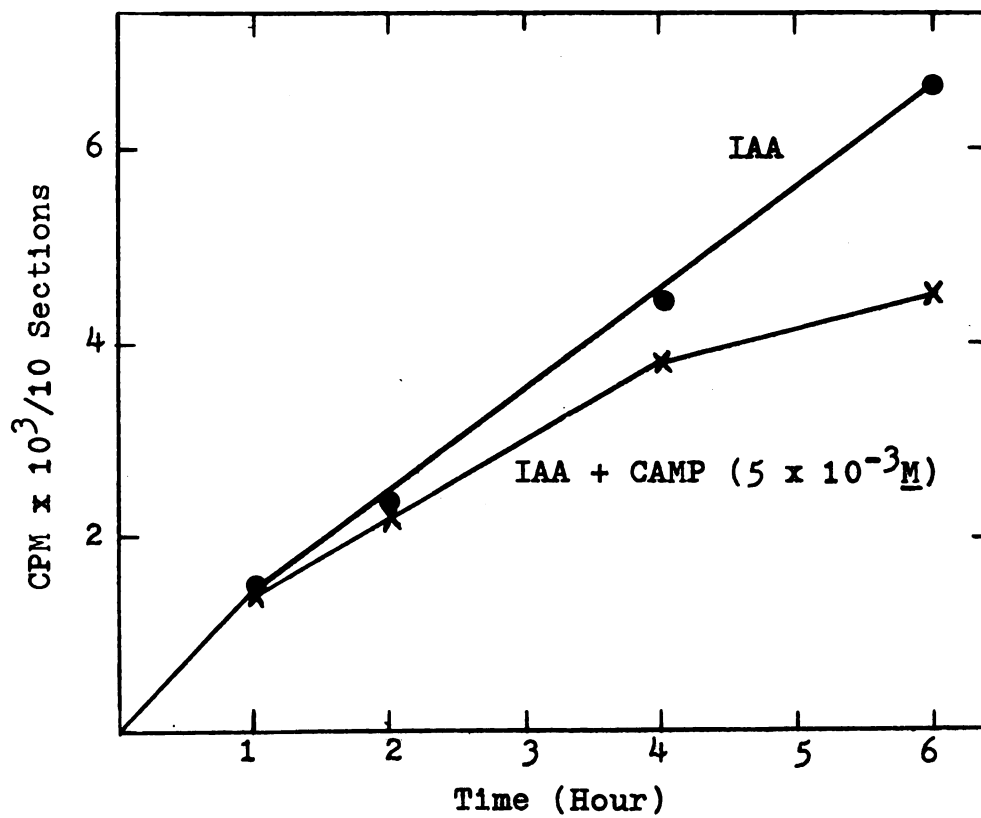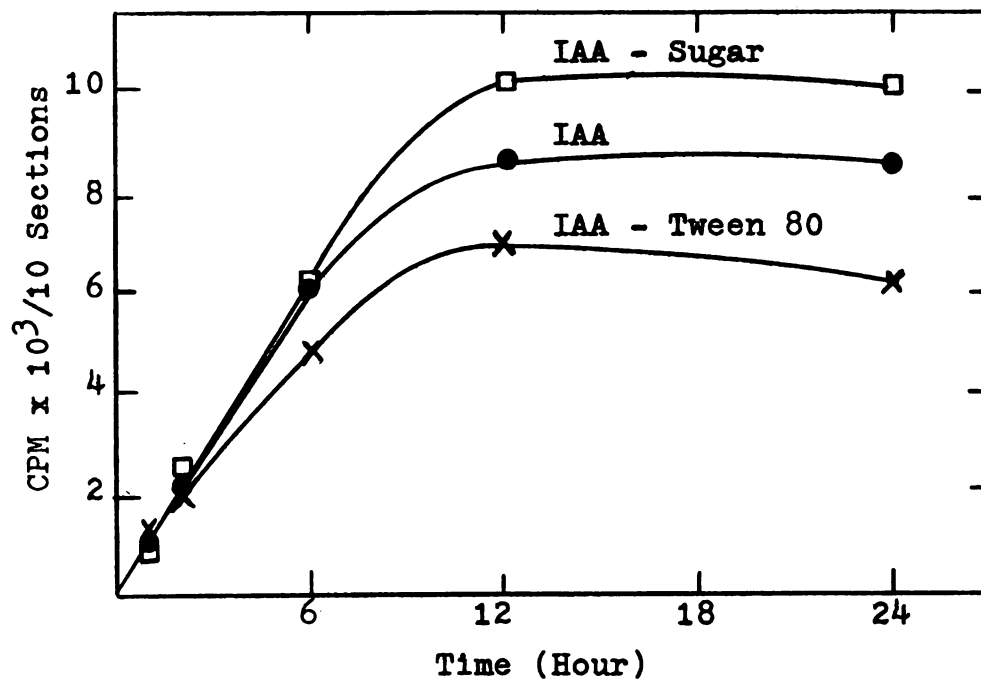
Uptake and Metabolism of Chloramphenicol

The concentration of chloramphenicol required to repress elongation and protein synthesis in Avena and Triticum was 100-1000 times greater than the concentration needed to stop these processes in most bacteria (10). Since the basic mechanism of protein synthesis is similar in both plants and bacteria the basis for the large difference in sensitivity is not clear. Vazquez (108) investigating the problem of resistance in certain bacteria noticed that antibiotic activity of chloramphenicol closely paralleled its absorption. On the basis of results reported on the uptake of chloramphenicol in Nitella (82), Nooden and Thimann (69) suggested that slow penetration was responsible for the low sensitivity in Avena coleoptiles.

A rapid inactivation of chloramphenicol could also contribute to the high concentrations required for inhibition. The ability of bacteria (11) and animals (91) to inactivate chloramphenicol has been established. Experiments were designed to study both the uptake and metabolism of ^{14}C-chloramphenicol in Avena coleoptiles.

The uptake of ^{14}C-chloramphenicol at 5×10^{-3} and 10^{-3} $\underline{M}$ concentration is presented in Figure 11. The entry of chloramphenicol into the tissue was probably by simple diffusion. The internal concentration approached the external concentration in both treatments within the first 4 hours. With a longer period there was no accumulation of chloramphenicol against a gradient. The slight additional uptake of a 10^{-3} $\underline{M}$ solution of chloramphenicol between 4 and 6 hours

Figure 11

Uptake of ^{14}C-Chloramphenicol into the **Avena** Coleoptile
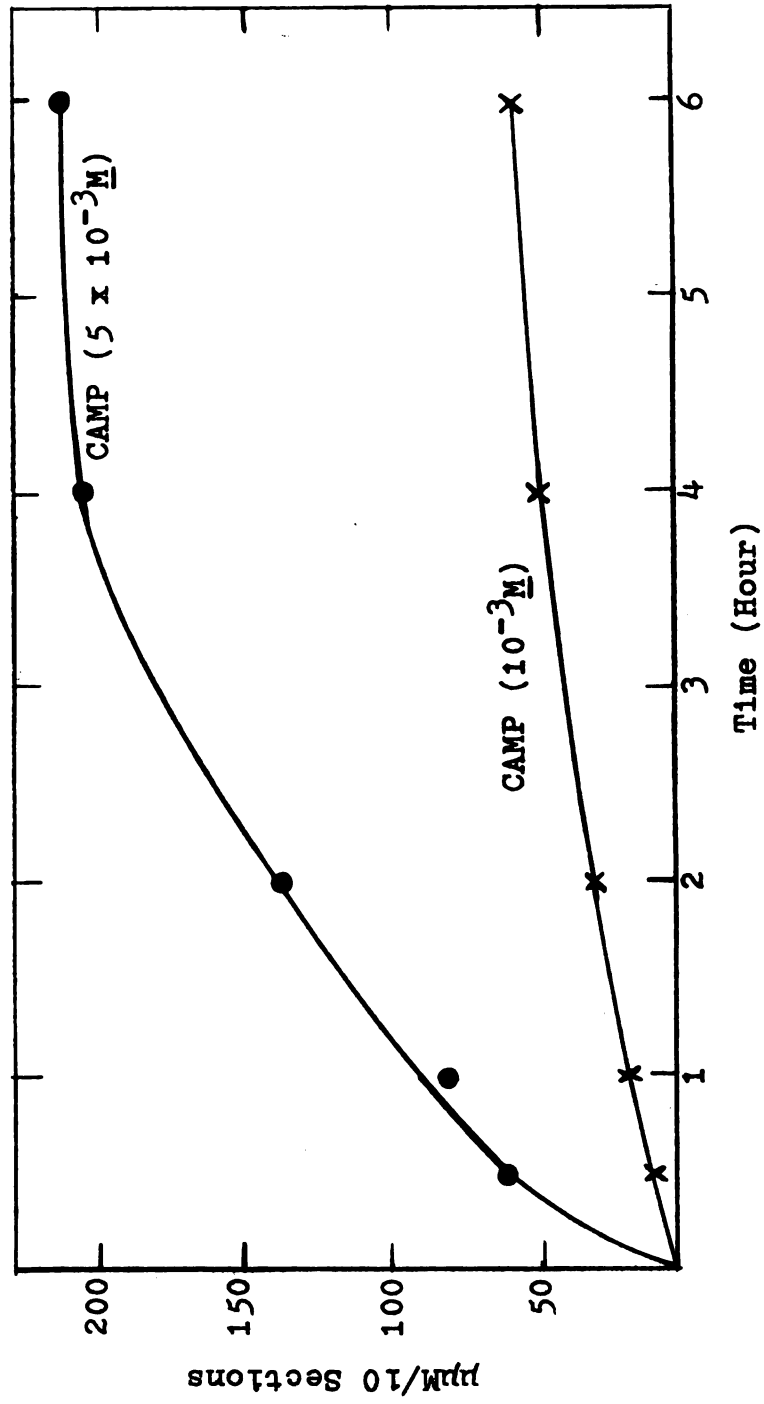
was a reflection of its continued elongation. Although the penetration may explain the lag period before chloramphenicol inhibition was observed, it was not a factor involved in the high concentration required for inhibition. When a 5×10^{-3} $\underline{M}$ solution of chloramphenicol was employed, the internal concentration exceeded 10^{-3} $\underline{M}$ within 30 minutes, a level much greater than that required for inhibition in bacteria (10).

The metabolism of ^{14}C-chloramphenicol was studied by thin-layer chromatography. Four hours after incubation with a 5×10^{-3} $\underline{M}$ solution of ^{14}C-chloramphenicol, the $\underline{Avena}$ coleoptiles were extracted with acetone and the constituents in the extract were separated by thin-layer chromatography. In the first solvent system of chloroform:benzene:ethanol (7:3:1), 81% of the total radioactivity coincided with the R_f of an authentic sample of chloramphenicol (Figure 12). In the second solvent system of chloroform:ethyl acetate:formic acid (5:4:1), approximately 90% of the applied counts agreed with chloramphenicol (Figure 13). The extract still retained its biological activity in $\underline{E}$. $\underline{coli}$ and was, thus, characterized as unchanged chloramphenicol.

In conclusion, the high concentration of chloramphenicol demanded for inhibition was not due to either a lack of uptake or a rapid inactivation by $\underline{Avena}$ coleoptiles. Since in cell-free systems the ribosomes appeared to be the locus of action (67), it would be of interest to study the binding of ^{14}C-chloramphenicol to the ribosomes obtained from $\underline{Avena}$ coleoptiles.

Figure 12

Thin-Layer Chromatography of ^{14}C-Chloramphenicol Extract:

Solvent System of Chloroform:Benzene:Ethanol (7:3:1)

CPM x 10²

Rf →
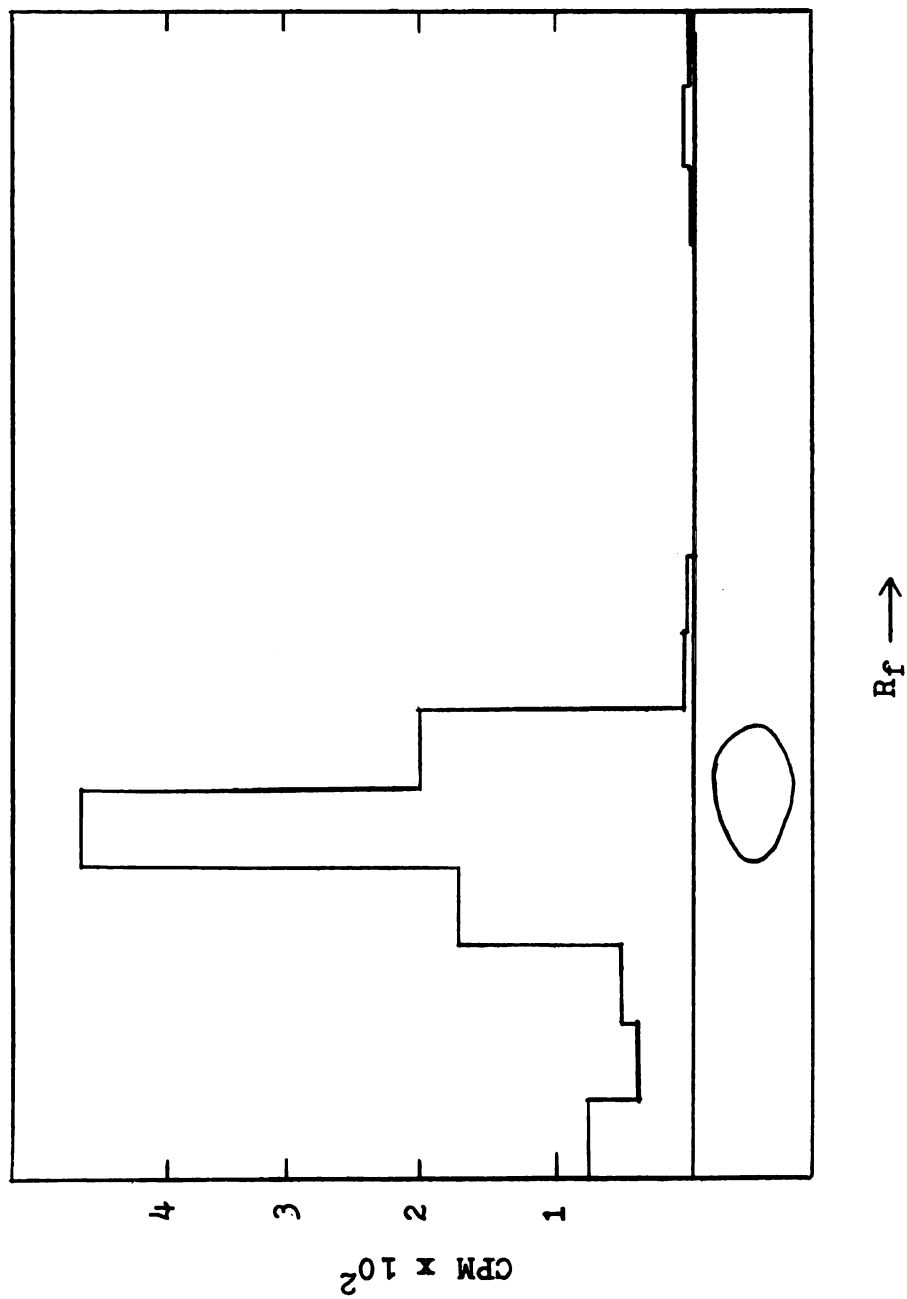
Figure 13

Thin-Layer Chromatography of ^{14}C-Chloramphenicol Extract:

Solvent System of Chloroform:Ethyl Acetate:Formic Acid (5:4:1)

CPM x 10²

$R_f \rightarrow$
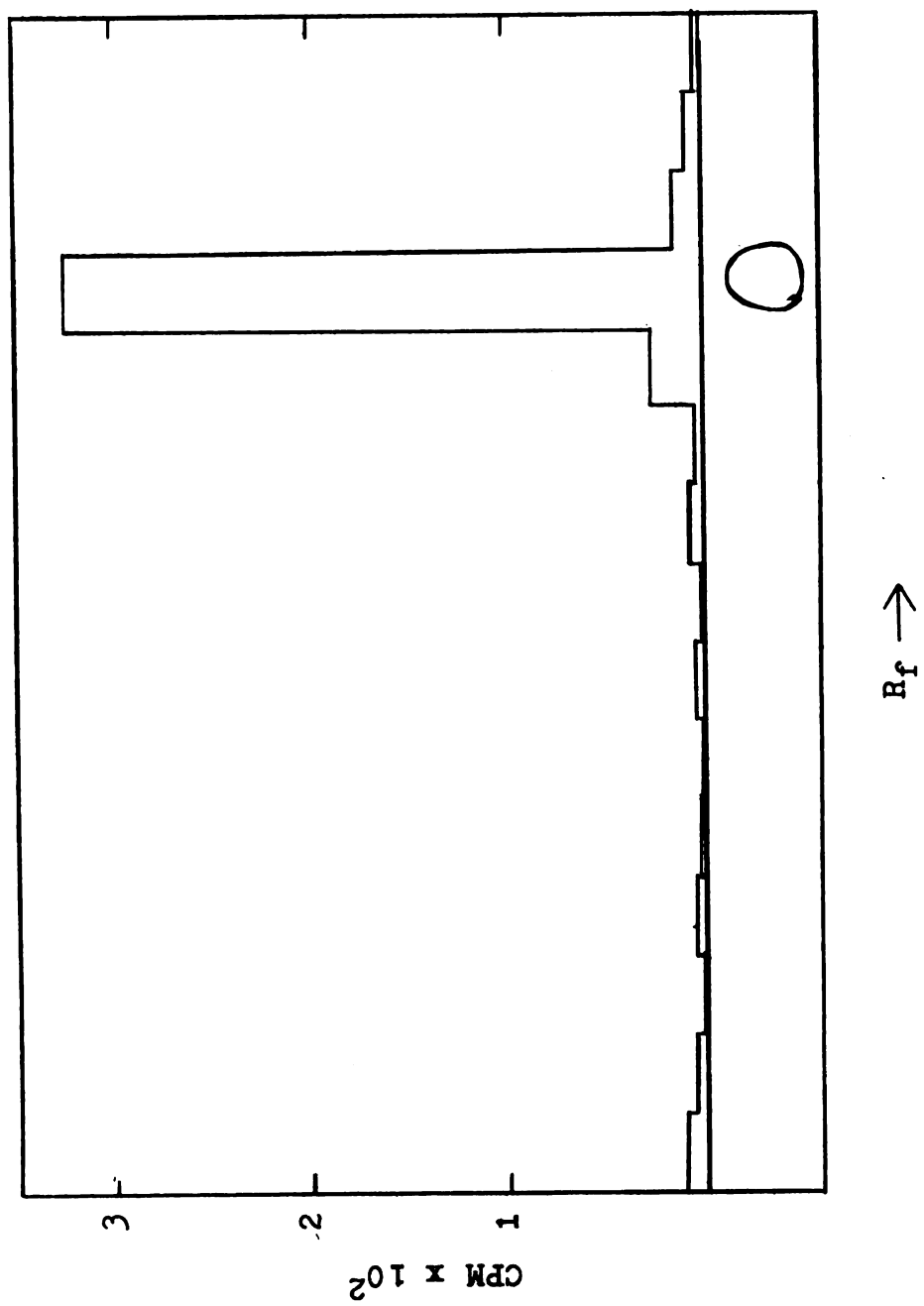
Stereospecificity of Chloramphenicol

Because of the unusually high concentration of chloramphenicol required for inhibition, the stereospecificity of chloramphenicol action in plant tissue was investigated. Of the four possible stereoisomers only the naturally occurring antibiotic D-threo-chloramphenicol showed any significant activity in intact bacteria (10). In a cell-free system obtained from E. coli the L-erythro and L-threo isomers were inactive. However, Jyung, Wittwer, and Bukovac (51) observed that the L-threo isomer repressed protein synthesis in isolated cells from tobacco.

The inhibition of auxin-induced elongation in Avena by the four isomers of chloramphenicol is given in Table 9. All four isomers were effective inhibitors of auxin-induced elongation to about the same degree from $5x10^{-4}$ to $5x10^{-3}$ $\underline{M}$ concentration. Furthermore, all of the isomers effectively inhibited incorporation of ^{14}C-leucine into protein (Table 10). As shown for D-threo-chloramphenicol activity the 3 non-antibiotic structures markedly inhibited the uptake of ^{14}C-leucine. In addition, all four isomers were very strong inhibitors of ^{14}C-α-aminoisobutyric acid uptake (Figure 14).

To establish whether this inhibition was a general phenomenon in plants or unique to the Avena, several other plant systems were investigated. Since the L-threo isomer was reported to repress root growth in higher plants (88), all four isomers were tested for activity in the buckwheat (Fagopyrum esculentum) assay (89). D-Threo, L-threo, and L-erythro-chloramphenicol were very effective inhibitors of

TABLE 9

Stereospecificity of Chloramphenicol Inhibition of Auxin-Induced Elongation in the _Avena_ Coleoptile: Concentration Range*

Concentration	D-Threo**	L-Threo	D-Erythro	L-Erthro
5x10^{-4} M	8	2	2	-1
1x10^{-3} M	49	57	46	40
5x10^{-3} M	74	78	70	68

*Incubation was for 24 hours.
**Expressed as % Inhibition.

TABLE 10

Stereospecificity of Chloramphenicol (5x10^{-3} M) Inhibition of Elongation, ^{14}C-Leucine Uptake, and Incorporation into the Protein of _Avena_*

	Control	D-Threo	L-Threo	D-Erythro	L-Erythro
Elongation**	2.5 (0)	1.1 (56)	1.3 (48)	1.6 (36)	1.3 (48)
Uptake***	9,890 (0)	5,294 (46)	3,963 (60)	4,822 (51)	6,744 (32)
T.C.A. Insoluble***	4,417 (0)	1,326 (70)	1,392 (68)	1,954 (56)	1,638 (59)

*Incubation was for 4 hours.
**Expressed in mm and % inhibition.
***Expressed in cpm/10 sections and % inhibition.

Figure 14

Stereospecificity of Chloramphenicol (5×10^{-3} $\underline{M}$) Inhibition of

the Uptake of ^{14}C-α-Aminoisobutyric Acid into the **Avena**

Coleoptile
1. Control
2. L-Erythro-chloramphenicol
3. D-Erythro-chloramphenicol
4. L-Threo-chloramphenicol
5. D-Threo-chloramphenicol
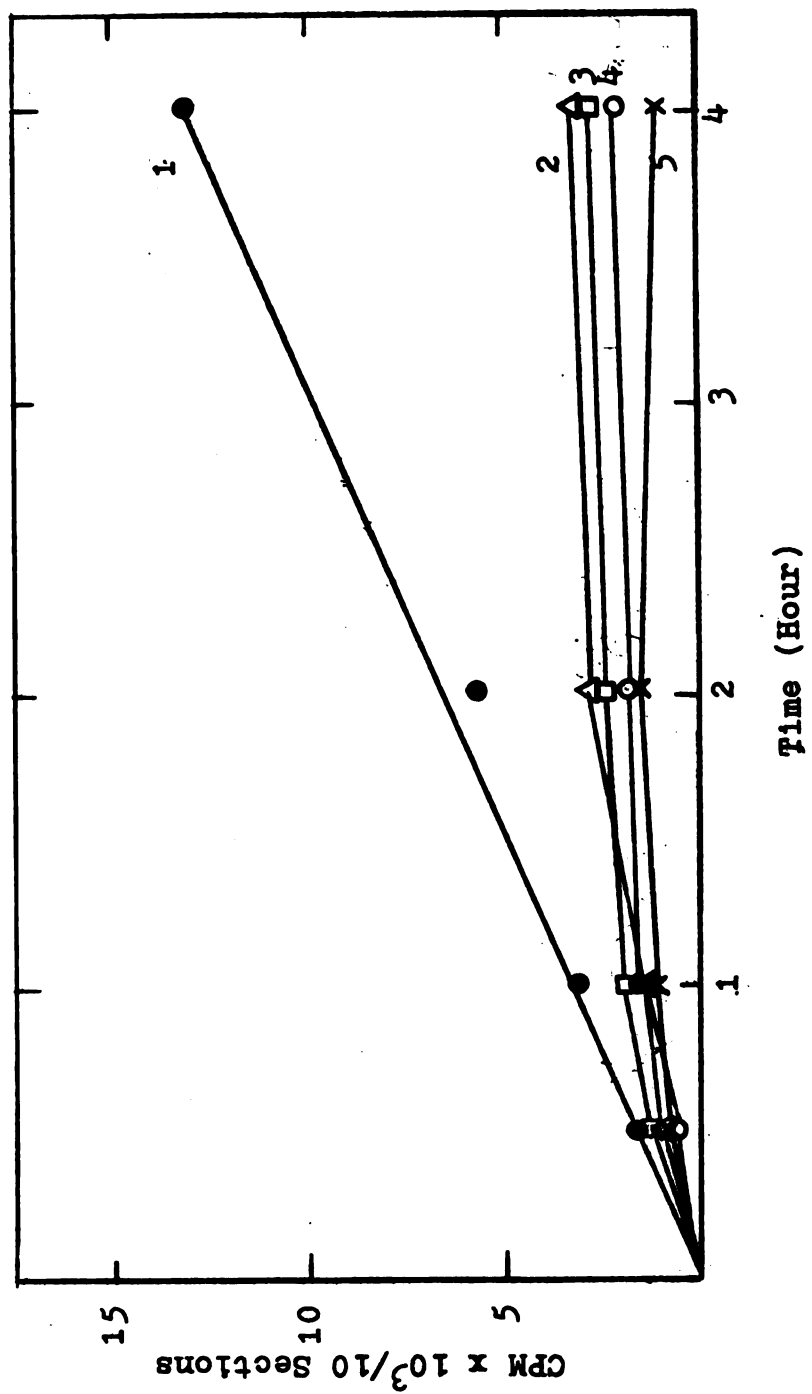
root growth (Table 11). D-Erythro-chloramphenicol also inhib-
ited root gorwth, but to a lesser extent. On a concentration
basis, root growth was more sensitive to chloramphenicol than
was coleoptile growth.

TABLE 11

Stereospecificity of Chloramphenicol Inhibition of Buckwheat
Root Growth

Concentration	D-Threo	L-Threo	D-Erythro	L-Erythro
10^{-6} M	9*	2	-4	6
10^{-5} M	30	15	5	7
10^{-4} M	46	22	13	15
10^{-3} M	57	53	18	49

*Expressed as % inhibition.

The activity of the isomers on auxin-induced elonga-
tion in wheat coleoptiles is shown in Table 12. At 5×10^{-3} M
concentration all of the isomers strongly inhibited elonga-
tion. These data are further evidence that the stimulation of
growth by a 5×10^{-4} M solution of chloramphenicol during the
22 hour assay was due to its interference with bacterial
growth. L-Threo, D-erythro, and L-erythro-chloramphenicol,
which do not inhibit bacterial growth (10), slightly inhibited
elongation. In Table 13, the effect of the stereoisomers on
elongation, uptake and incorporation of ^{14}C-leucine into the
protein fraction of Triticum is presented. The isomers effec-
tively inhibited elongation, uptake, and protein synthesis.

TABLE 12

Stereospecificity of Chloramphenicol Inhibition of Elongation in the _Triticum_ Coleoptile: Concentration Range*

Concentration	D-Threo	L-Threo	D-Erythro	L-Erythro
5×10^{-4} M	-25**	9	8	7
1×10^{-3} M	-6	19	12	10
5×10^{-3} M	92	91	87	93

*Incubation was for 22 hours.
**Expressed as % inhibition.

TABLE 13

Stereospecificity of Chloramphenicol (5×10^{-3} M) Inhibition of Elongation, ^{14}C-Leucine Uptake, and Incorporation into the Protein of _Triticum_*

|	Control	D-Threo	L-Threo	D-Erythro	L-Erythro
Elongation**	2.6 (0)	0.9 (65)	0.8 (69)	1.3 (50)	1.1 (57)
Uptake***	5,380 (0)	2,299 (57)	1,746 (67)	2,891 (46)	2,404 (55)
T.C.A. Insoluble***	2,969 (0)	918 (69)	772 (74)	1,056 (64)	1,306 (56)

*Incubation was for 4 hours.
**Expressed in mm and % inhibition.
***Expressed in cpm/10 sections and % inhibition.

On the basis of ^{14}C-leucine incorporation into the TCA
insoluble protein fraction, it appeared that chloramphenicol
repression of protein synthesis was not stereospecific in
plants. To obtain a more direct assay of protein synthesis
in a plant system, the ability of the isomers to inhibit
gibberellic acid-induced synthesis of α-amylase in barley
aleurone layers was investigated. Varner (106) previously
reported that D-threo-chloramphenicol inhibited gibberellic
acid-induced α-amylase synthesis. The chloramphenicol isomers
at 5×10^{-3} M concentration were incubated for 24 hours with
barley aleurone layers and a 10^{-5} M solution of gibberellic
acid. After incubation, α-amylase activity in the medium and
in the tissue was examined separately. Total activity of the
combined medium and extract ranged from 73% inhibition with
the least active D-erythro to 83% inhibition for the most
active L-threo isomer (Table 14). The release of the enzyme
was also inhibited. The chloramphenicol isomers at the high-
est concentration found in any of the assays did not repress
the activity of α-amylase (Table 15).

The possibility existed that plant tissue had the
capacity to racemize L-threo, D-erythro, and L-erythro isomers
into D-threo-chloramphenicol and only the latter isomer was
active per se. To test this hypothesis a bioassay for D-threo-
chloramphenicol activity was utilized. A cell suspension of
E. coli was preincubated with the appropriate chemical or plant
extract. After the preincubation period, ^{14}C-leucine was added
and the inhibition of its incorporation into hot TCA insoluble
protein was measured. The sensitivity of the E. coli to
chloramphenicol is presented in Table 16.

TABLE 14

Stereospecificity of Chloramphenicol (5×10^{-3} $\underline{M}$) Inhibition of α-Amylase Synthesis

	Control	D-Threo	L-Threo	D-Erythro	L-Erythro
Medium*	12,725	1,875	1,669	2,400	2,425
Extract*	8,462	2,675	1,838	3,400	2,850
Total*	21,187	4,550	3,507	5,800	5,275
% Inhibition	0	79	83	73	75

*Expressed in μg of starch hydrolyzed per minute per aleurone layers.

TABLE 15

Effect of the Isomers of Chloramphenicol on the Activity of α-Amylase*

	Control	D-Threo	L-Threo	D-Erythro	L-Erythro
Activity**	8,462	8,250	8,375	8,462	8,212

*Isomers at 35 μg/ml.
**Expressed in μg of starch hydrolyzed per minute per 10 aleurone layers.

TABLE 16

Effect of Chloramphenicol on Protein Synthesis in E. coli[*]

	Concentration μg/ml			
	0	10	15	25
TCA Insoluble (CPM)	132	9	4	2

[*]Pretreated 1/2 hour with inhibitor. Incubated 1 hour with ^{14}C-leucine.

At a concentration as low as 10 μg/ml, protein synthesis was inhibited by over 90%. D-Threo-chloramphenicol, 25 μg/ml, completely inhibited protein synthesis while the non-antibiotic isomers had little effect (Table 17).

Avena coleoptiles were incubated 4 hours with the chloramphenicol isomers and then extracted with acetone. An E. coli cell suspension was preincubated with a plant extract equivalent to 24 μg/ml of the chloramphenicol isomer. The results given in Table 18 demonstrated conclusively that the plant tissue did not racemize the "inactive" chloramphenicol isomers into the "active" D-threo-chloramphenicol.

In conclusion, chloramphenicol inhibition of plant systems was not stereospecific. This lack of specificity appears to be a general phenomenon since auxin-induced elongation, root growth, ^{14}C-α-aminoisobutyric acid uptake, ^{14}C-leucine uptake and incorporation into protein, and α-amylase synthesis were all inhibited. Although there were some minor differences in the degree of inhibition of the various physiological responses, all four isomers inhibited in the same order of magnitude and over the same concentration range. The

TABLE 17

Stereospecificity of Chloramphenicol (25 µg/ml) Inhibition of Protein Synthesis in E. coli*

	0	D-Threo	L-Threo	D-Erythro	L-Erythro
TCA Insoluble (CPM)	186	-2	163	191	154

*Pretreated 1/2 hour with inhibitor. Incubated 1 hour with ^{14}C-leucine.

TABLE 18

Effect of Chloramphenicol Extracts on Protein Synthesis in E. coli

	25 µg D-Threo	Control	D-Threo	Tissue Extracts L-Threo	D-Erythro	L-Erythro
TCA Insoluble (CPM)	-3	326	8	292	350	273

*Pretreated 1/2 hour with extract. Incubated 1 hour with ^{14}C-leucine.

activity was not due to a racemization to the D-threo isomer.

Effect of Cycloheximide on Auxin-Induced
Elongation and Protein Synthesis

Further evidence on the involvement of protein synthesis in auxin-induced cell enlargement was obtained by investigating the effect of cycloheximide on elongation and protein synthesis. The concentration range of cycloheximide inhibition of auxin-induced and control elongation in Avena and Triticum is given in Figure 15 and 16. A marked inhibition was noted in both auxin-induced and control elongation. The most striking property of cycloheximide activity was its extremely low concentration required for inhibition. In both Avena and Triticum, 50% inhibition was obtained with a concentration of about $2x10^{-6}$ M. Cycloheximide was more active than was any inhibitor previously reported.

Elongation, ^{14}C-leucine uptake, and ^{14}C-leucine incorporation into protein were all inhibited over the same concentration range in the Avena (Table 19). In all cases studied, the suppression of ^{14}C-leucine uptake was smaller than the inhibition of protein synthesis, indicating a direct repression of protein synthesis as well as inhibition of amino acid uptake. As shown in Table 20, the uptake of ^{14}C-α-aminoisobutyric acid was also inhibited by a 10^{-5} M concentration of cycloheximide.

Time course studies with cycloheximide at $2x10^{-6}$ M and 10^{-5} M concentration presented in Figure 17, showed 50% and 40% inhibition after two hours incubation. With a 10^{-5} M

Figure 15

Effect of Cycloheximide on Auxin-Induced Elongation in the
Avena Coleoptile: Concentration Range

Figure 16

Effect of Cycloheximide on Auxin-Induced Elongation in the
Triticum Coleoptile: Concentration Range
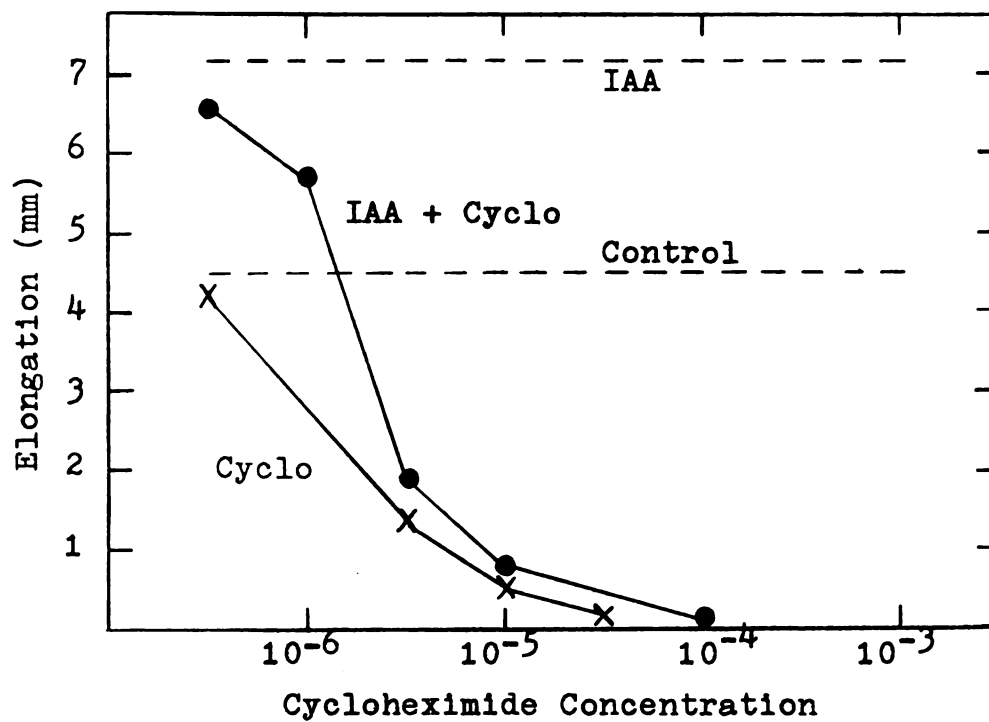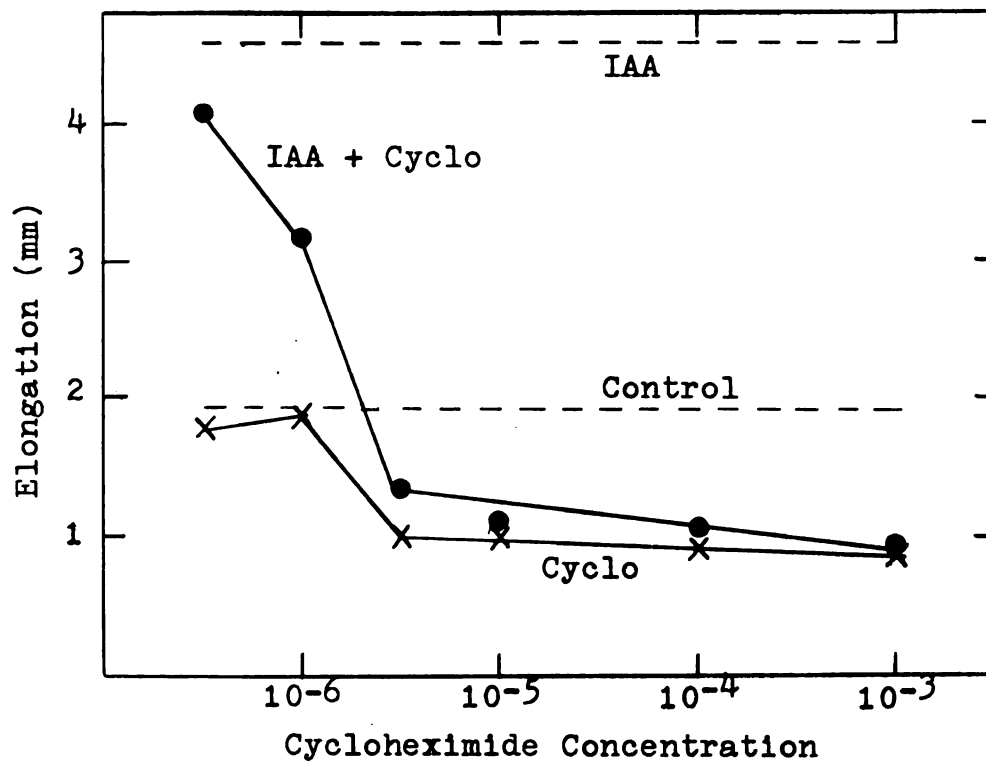
Figure 15

Effect of Cycloheximide on Auxin-Induced Elongation in the
Avena Coleoptile: Concentration Range

Figure 16

Effect of Cycloheximide on Auxin-Induced Elongation in the
Triticum Coleoptile: Concentration Range
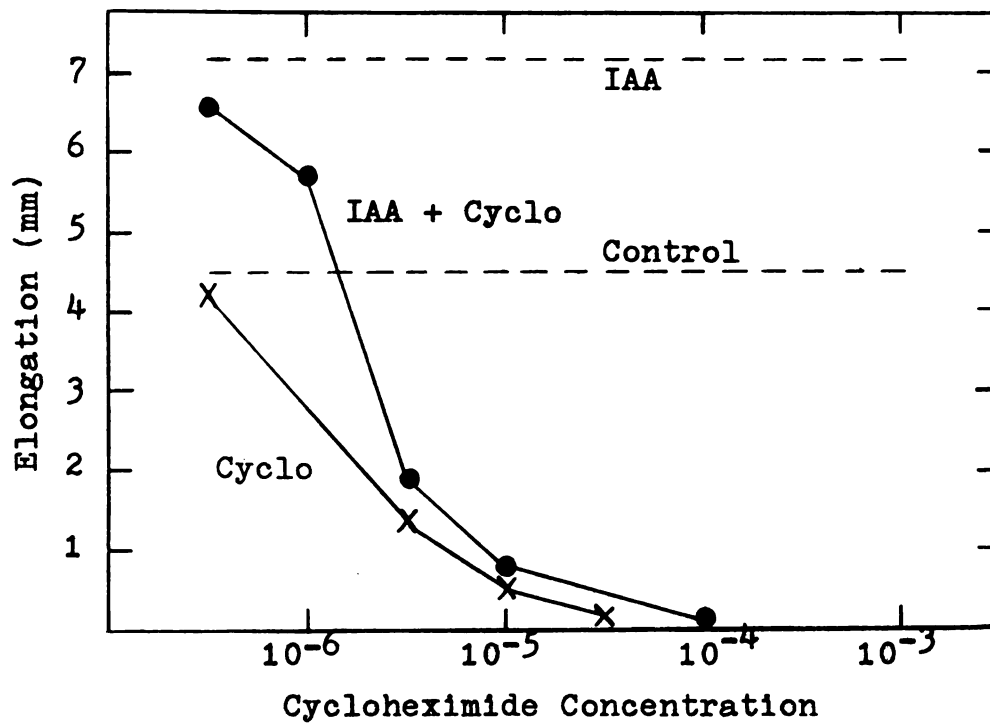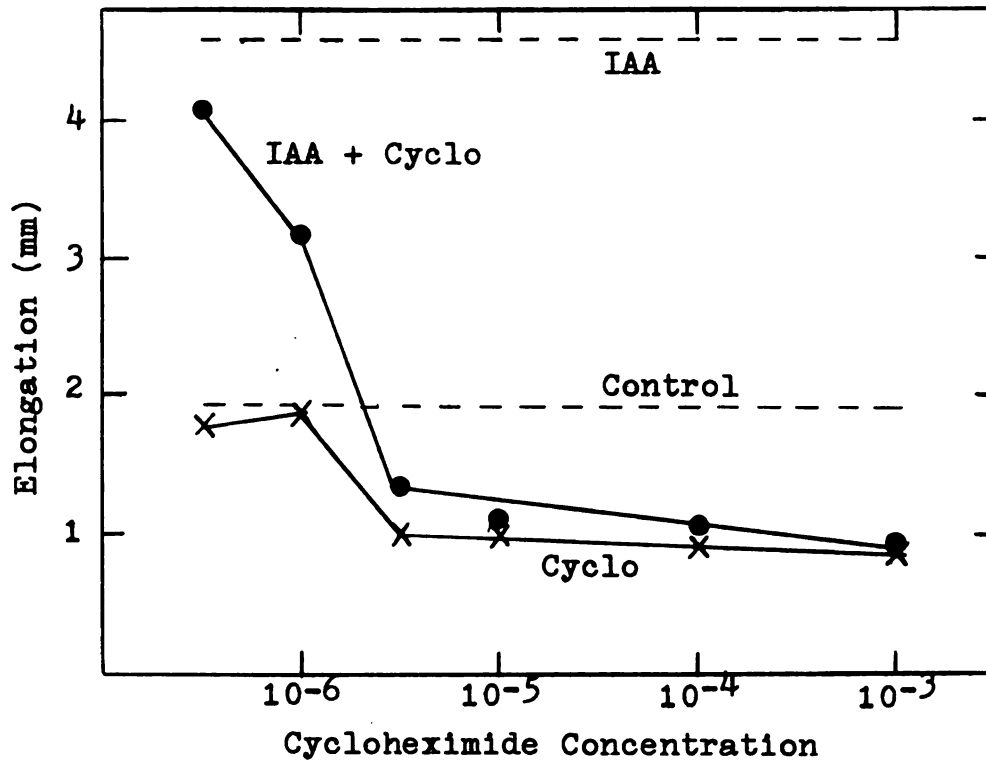
TABLE 19

Effect of Cycloheximide on the Elongation, ^{14}C-Leucine Uptake, and Incorporation into the Protein of Avena*

		Concentration		
	0	10^{-7} M	10^{-6} M	10^{-5} M
Elongation**	2.3 (0)	2.2 (4)	1.4 (39)	0.76 (67)
Uptake***	8,009 (0)	7,303 (9)	4,383 (45)	4,021 (50)
TCA Insoluble***	4,192 (0)	3,674 (12)	987 (76)	654 (85)

*Incubation was for 4 hours.
**Expressed as mm and % inhibition.
***Expressed as cpm/10 sections and % inhibition.

TABLE 20

Effect of Cycloheximide on the Uptake of ^{14}C-α-Aminoisobutyric Acid into the Avena Coleoptile

	Time (Hour)		
Treatment	1	2	4
IAA	2,114*	6,192	10,827
IAA + Cyclo	1,612	2,708	4,564
% Inhibition	24	56	58

*Expressed as cpm/10 sections.

Figure 17

Effect of Cycloheximide on the Kinetics of Auxin-Induced
Elongation in the _Avena_ Coleoptile

Figure 18

Effect of Cycloheximide on the Uptake of ^{14}C-Indole-3-Acetic
Acid
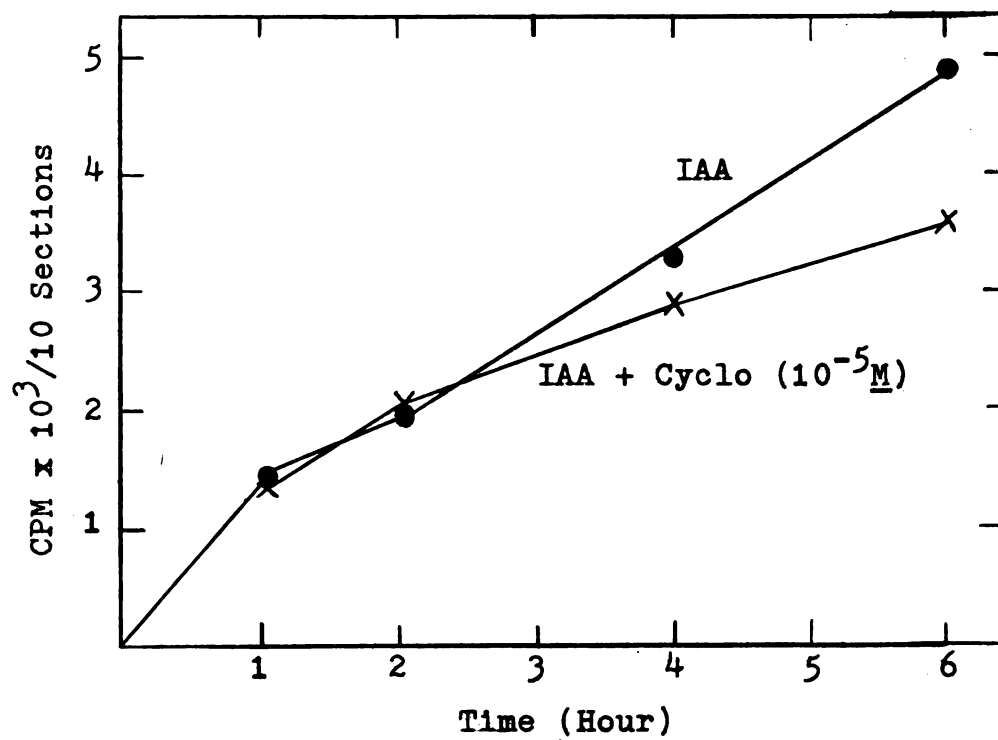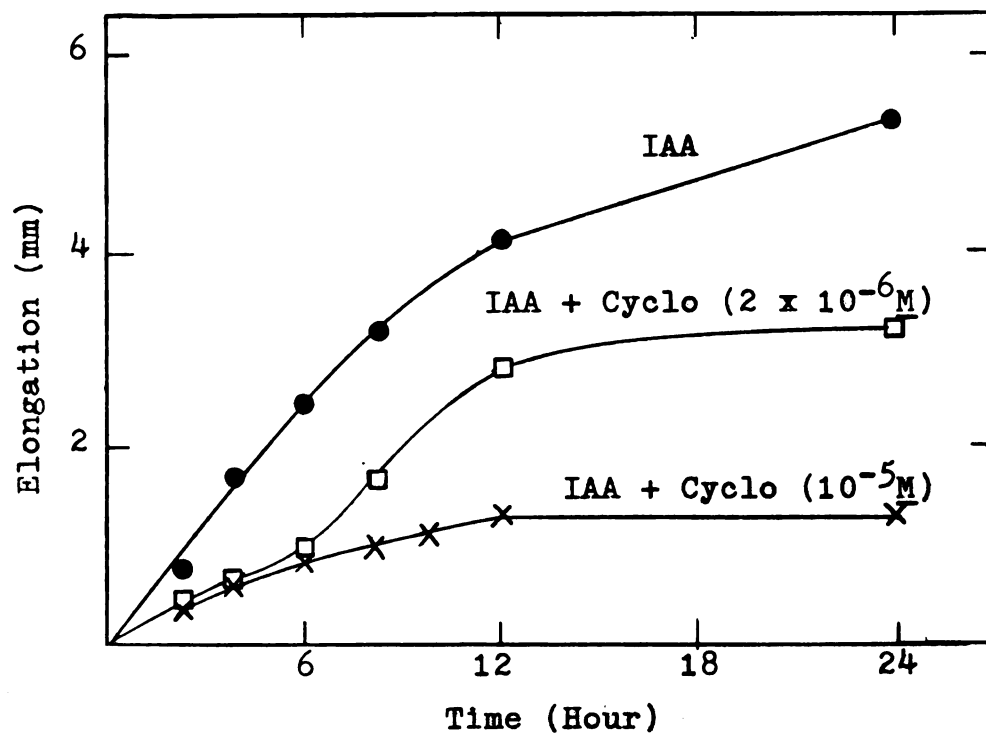
solution of cycloheximide elongation proceeded at a reduced linear rate from 2-12 hours and thereafter elongation was eliminated. The inhibitory action of a $2x10^{-6}$ $\underline{M}$ solution of cycloheximide was lost with time. During the first 6 hours of incubation the elongation was linear and was considerably less than the control. However, the elongation from 6-24 hours closely paralleled the nontreated $\underline{Avena}$.

The strong inhibition of control elongation was an indication that cycloheximide inhibition was not mediated through the repression of IAA uptake. Direct evidence for this observation is illustrated in Figure 18. Only after 4 and 6 hours was there any suppression of uptake and the inhibition was small compared to the repression of elongation.

The time course of ^{14}C-leucine uptake and incorporation into protein with a 10^{-5} $\underline{M}$ solution of cycloheximide was similar to the time course of elongation (Figure 19 and 20). On a per cent basis cycloheximide more effectively inhibited protein synthesis than elongation in the first 2 hours. From 2-6 hours, both processes were inhibited to about the same extent. A short lag appeared between the start of cycloheximide inhibition of protein synthesis and the inhibition of elongation.

The correlation between the concentration of cycloheximide required to inhibit elongation and protein synthesis provides further evidence that protein synthesis is a requirement for auxin-induced elongation. In addition, the lag between the start of inhibition of protein synthesis and the inhibition of elongation is an important observation. These

Figure 19

Effect of Cycloheximide (10^{-5} $\underline{M}$) on the Uptake of ^{14}C-Leucine into the $\underline{Avena}$ Coleoptile

Figure 20

Effect of Cycloheximide (10^{-5} $\underline{M}$) on the Incorporation of ^{14}C-Leucine into the Protein of the $\underline{Avena}$ Coleoptile
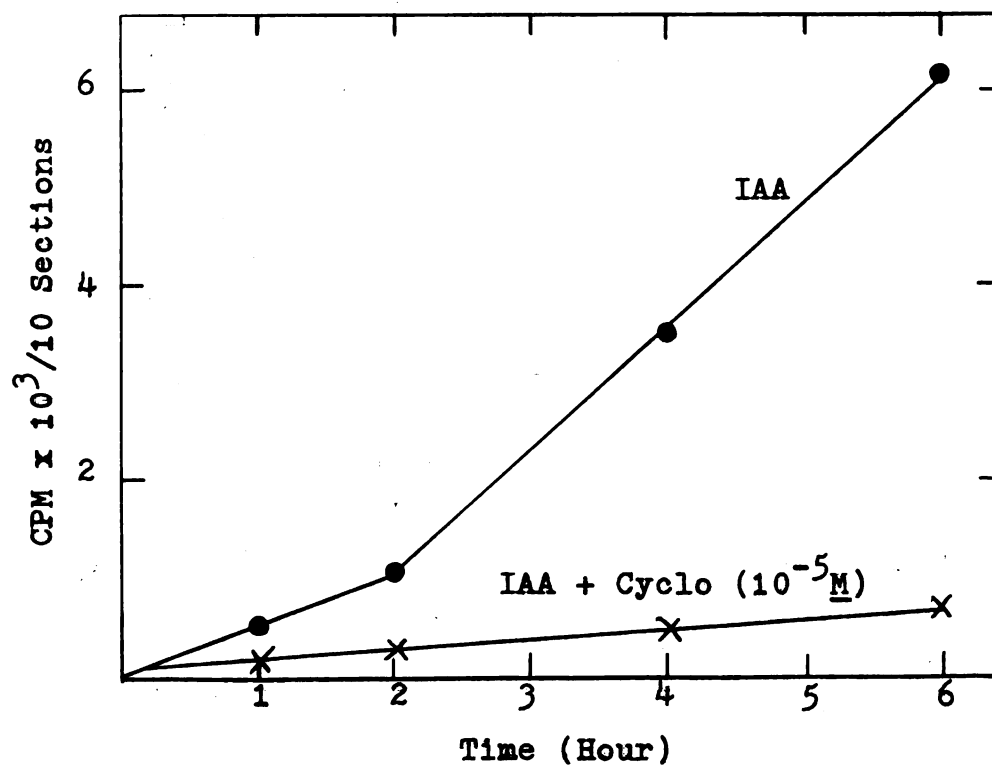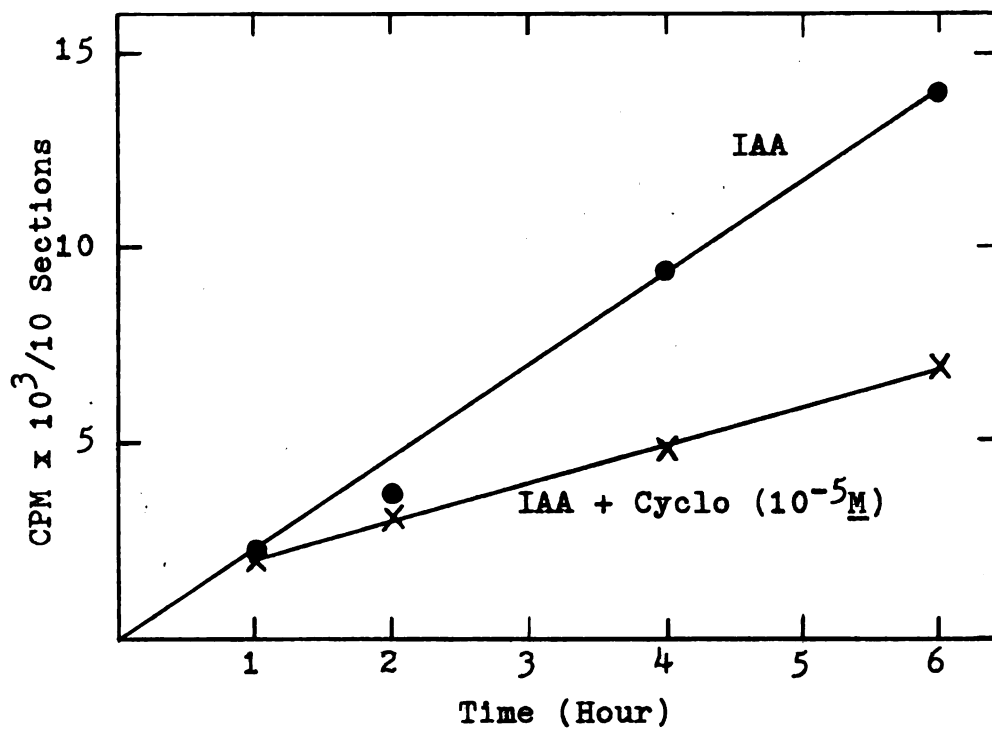
results indicate that the inhibition of protein synthesis was the cause of the inhibition of elongation, and not a reflection of growth inhibition.

Gougerotin Inhibition in Plants

In cooperation with Mr. Allen Burkett, NSF Undergraduate Fellow, the activity of the new antibiotic gougerotin was investigated. Gougerotin is a specific inhibitor of protein synthesis in both bacteria and animal cells (15, 17, 98). No report in the literature has demonstrated the biological activity of this compound in plant systems. Hence, several experiments were devised to determine whether this antibiotic was active in plant systems.

Gougerotin was an effective inhibitor of auxin-induced elongation in the Avena coleoptile (Figure 21). A gougerotin concentration of 10^{-5} M was required for 50% inhibition. The time course of 10^{-4} M inhibition is shown in Figure 22. Within 2 hours, gougerotin repressed elongation by 30%; and elongation continued at a much reduced level through 10 hours. A 10^{-4} M solution of gougerotin inhibited the uptake and incorporation of ^{14}C-leucine into protein by 38% and 50% respectively (Table 21). It also inhibited the uptake of ^{14}C-α-aminoisobutyric acid.

These preliminary studies indicate that gougerotin does repress elongation and protein synthesis in plant tissue. The concentration required for inhibition was about 10 times greater than those reported for a cell-free system from E. coli (17), but comparable to those reported for animal

Figure 21

Effect of Gougerotin on Auxin-Induced Elongation in the <u>Avena</u>
Coleoptile: Concentration Range

Figure 22

Effect of 10^{-4} <u>M</u> Gougerotin on the Kinetics of Auxin-Induced
Elongation in the <u>Avena</u> Coleoptile
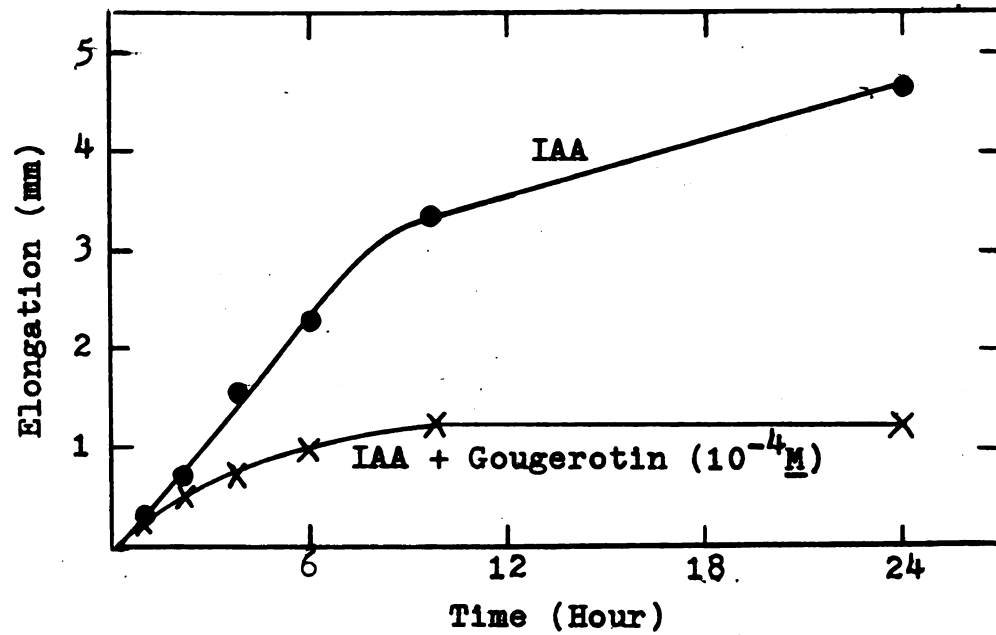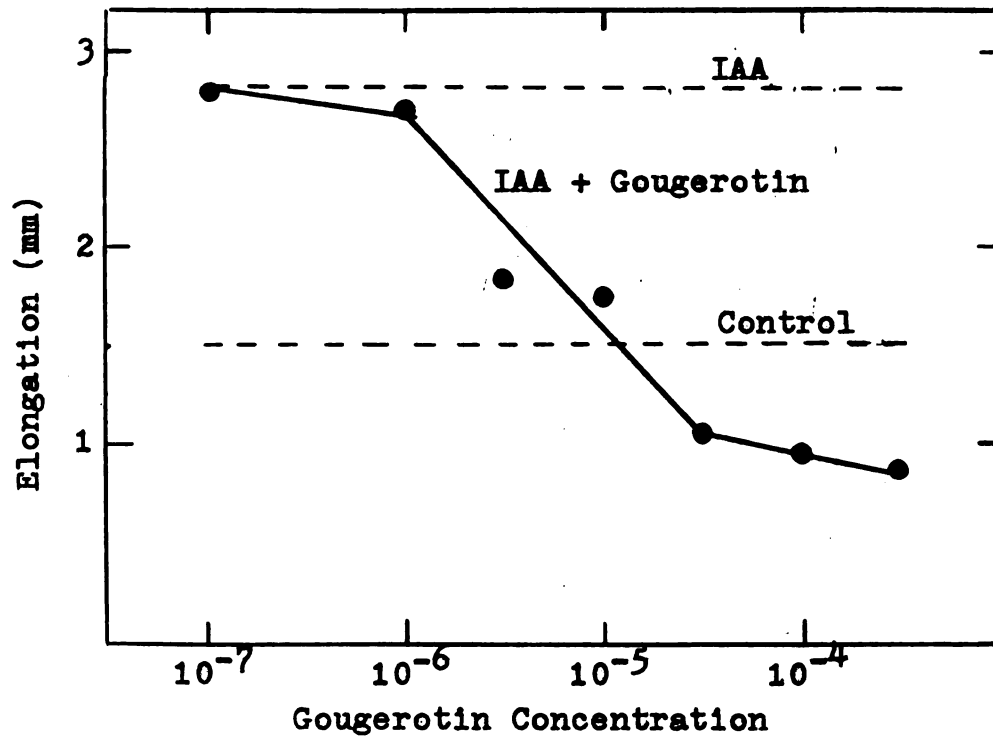
systems (15, 98). Since the mode of action of gougerotin is
known in detail (15), this antibiotic should prove to be a
valuable tool for additional study of auxin-induced growth
and other plant responses requiring protein synthesis.

TABLE 21

Gougerotin (10^{-4} $\underline{M}$) Inhibition of Amino Acid Uptake and
Protein Synthesis in the Avena Coleoptile*

	Elongation	^{14}C-α-Aminoisobutyric Acid Uptake
% Inhibition	42	33
	^{14}C-Leucine Uptake	^{14}C-Leucine TCA Insoluble
% Inhibition	38	50

*Incubation was for 4 hours.

Uptake and Metabolism of ^{14}C-α-Aminobutyric Acid

While working with chloramphenicol, an attempt was
made to separate inhibition of amino acid uptake from protein
synthesis. In this study as reported in another section,
^{14}C-α-aminoisobutyric acid was used. However, before this amino
acid was employed, several studies were made using ^{14}C-α-
aminobutyric acid. In these investigations it was assumed,
as others had assumed, (60) that ^{14}C-α-aminobutyric acid was
not incorporated into protein. When the uptake of ^{14}C-α-
aminobutyric acid was followed in the presence of chloram-
phenicol, uptake was strongly inhibited within 1 hour and

thereafter it was taken up at a much reduced level (Figure 23).

To test the possibility that some of the radioactivity might be incorporated into the protein fraction, coleoptiles were incubated for 1 and 2 hours with ^{14}C-α-aminobutyric acid, then total and 70% ethanol insoluble radioactivity were determined. The radioactivity from ^{14}C-α-aminobutyric acid was readily incorporated into the 70% ethanol insoluble fraction (Table 22). To confirm this observation, coleoptiles were incubated with ^{14}C-α-aminobutyric acid either in the presence or absence of chloramphenicol (5×10^{-3} $\underline{M}$). After 4 hours incubation total uptake and incorporation into TCA insoluble protein were determined. By this procedure 25% of the radioactivity taken up in the absence of chloramphenicol was incorporated into the protein fraction (Table 23). In the chloramphenicol treated coleoptiles, uptake was greatly reduced, but 14% of the radioactivity was transferred to the protein fraction.

A number of plants were surveyed for their ability to incorporate ^{14}C-α-aminobutyric acid into protein. For this study 4.5 mm sections were removed from either the coleoptile or hypocotyl of 4 day old etiolated seedlings. After incubation for 4 hours in ^{14}C-α-aminobutyric acid, the total uptake and incorporation into the 70% insoluble fraction was determined (Table 24). Although there was a considerable variation, all of the plants tested showed significant incorporation into the protein fraction. The amount ranged from 28% of the total for oat coleoptiles to 6.4% for barley coleoptiles.

Figure 23

Effect of Chloramphenicol on the Uptake of ^{14}C-α-Aminobutyric

Acid into the _Avena_ Coleoptile
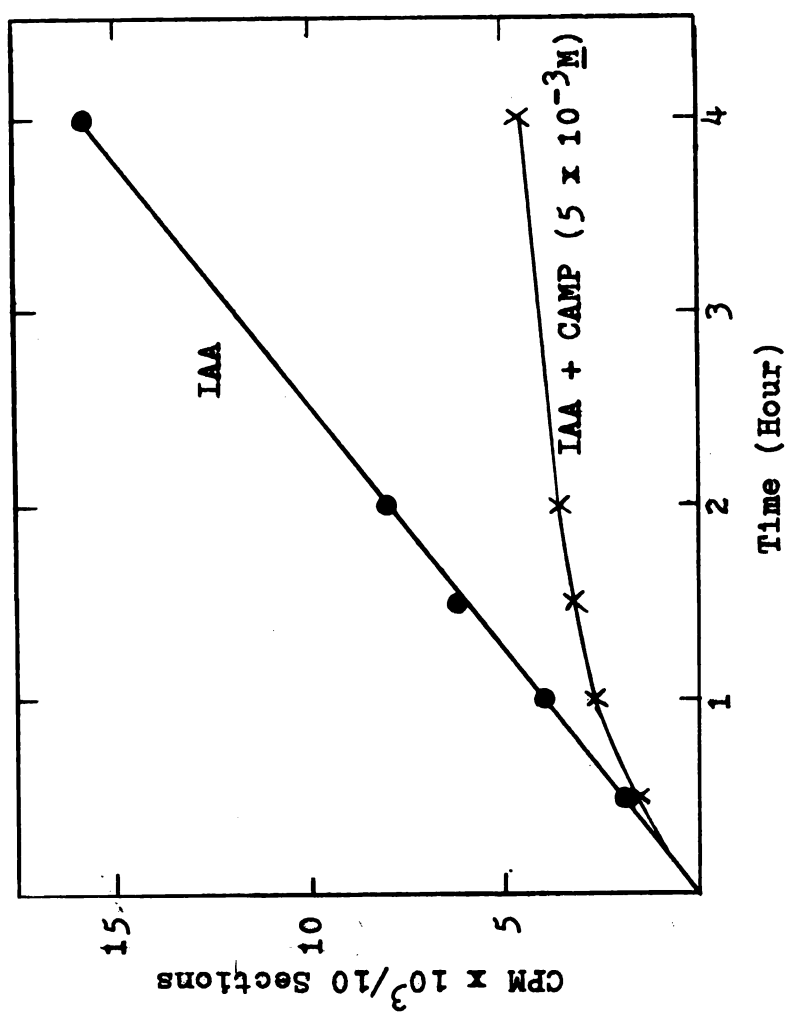
TABLE 22

Uptake and Incorporation of ^{14}C-α-Aminobutyric Acid into the 70% Ethanol
Insoluble Fraction of the _Avena_ Coleoptile

Time	Uptake	70% Ethanol Insoluble
1	8,046*	1,297
2	16,385	4,185

*Expressed as cpm/10 sections.

TABLE 23

Uptake and Incorporation of ^{14}C-γ-Aminobutyric Acid into the TCA Insoluble
Fraction of the _Avena_ Coleoptile

Total Uptake		TCA Insoluble	
Control	CAMP**	Control	CAMP
20,634***	6,002	5,326	819

*Incubation was for 4 hours.
**Chloramphenicol at 5x10^{-3} M.
***Expressed as cpm/10 sections.

TABLE 24

Uptake and Incorporation of ^{14}C-α-Aminobutyric Acid into the Protein of Several Plants*

	Barley	Corn	Cucumber	Lentile
Uptake	13,846**	8,809	10,214	2,849
70% Ethanol Insoluble	882	1,500	2,638	458

	Oat	Pea	Wheat
Uptake	9,350	7,581	10,133
70% Ethanol Insoluble	2,606	1,486	1,505

*Incubation was for 4 hours.
**Expressed as cpm/10 sections.

The incorporation of radioactivity into the protein fraction could have been due to a direct incorporation of ^{14}C-α-aminobutyric acid as reported previously for other nonprotein amino acids (38, 87, 115). Another alternative would be the rapid metabolism of ^{14}C-α-aminobutyric acid into some other amino acid and its subsequent incorporation into protein. Therefore, Avena coleoptiles were incubated for 2 hours with ^{14}C-α-aminobutyric acid and the amino acid fraction (TCA soluble, ether insoluble) and the protein fraction (after hydrolysis) separated by paper chromatography. There were 3 radioactive spots in the amino acid fraction. The major spot, 53% of the total radioactivity, cochromatographed with ^{14}C-α-aminobutyric acid. The two other spots were not rigorously identified. However, the upper spot (21%) cochromatographed with leucine and isoleucine. The middle spot (26%) cochromatographed with valine and methionine.

Chromatography of the protein hydrolyzate indicated only 2 radioactive spots. There was no radioactivity in the region of α-aminobutyric acid giving conclusive proof that α-aminobutyric acid was not incorporated into protein per se. The 2 radioactive spots chromatographed with leucine-isoleucine (13%) and valine-methionine (87%). Oxidation with H_2O_2 before chromatography did not convert the latter amino acids to oxidized methionine. On the basis of comparative biochemistry, the upper spot was tentatively identified as isoleucine. α-Aminobutyric acid was an effective precursor of isoleucine in E. coli (1), Neurospora crassa (40), and a

plant tissue culture (D. K. Dougall, Unpublished data),

Presumedly, α-aminobutyric acid is transaminated to α-keto-butyric acid which is a normal precursor of isoleucine.

The rigorous identification of these amino acids will require further work. However, it is significant that; A. ^{14}C-α-aminobutyric acid is rapidly metabolized, B. its metabolites are incorporated into protein, and C. it cannot be used to separate factors which affect uptake of amino acids from factors which affect protein synthesis. These results emphasize that in all individual cases where amino acid analogs are used, their possible incorporation into protein should be examined.

SUMMARY

SUMMARY

Kinetic analysis of auxin-induced elongation in <u>Avena</u> coleoptiles revealed a rapid rate of elongation from 1-8 hours followed by a reduced rate from 8-24 hours. In the first linear phase, the rate of auxin-induced elongation was 4 times the rate of control elongation. The ratio of IAA to control elongation during the second phase was 2.

After an initial lag period, the deletion of sucrose from the assay medium reduced the rate of elongation. Tween 80 did not affect the kinetics of elongation. Sucrose appeared to serve as a source of energy rather than directly affecting elongation.

Under experimental conditions where elongation was stimulated by auxin 2-4 fold, the incorporation of ^{14}C-leucine into the protein fraction was not enhanced.

Chloramphenicol inhibited auxin-induced elongation, ^{14}C-leucine uptake, and protein synthesis in the <u>Avena</u> coleoptile. The concentration range for these parameters was $5x10^{-4}$ to $5x10^{-3}$ $\underline{M}$. Higher concentrations were required for inhibition in <u>Triticum</u> coleoptiles. Both elongation and protein were markedly inhibited by a solution of $5x10^{-3}$ $\underline{M}$ chloramphenicol. At lower concentrations (10^{-3} and $5x10^{-4}$ $\underline{M}$) elongation was stimulated. The stimulation appeared to be due to the bactericidal action of the lower concentrations of chloramphenicol.

$\underline{\text{Avena}}$ coleoptile elongation was inhibited within the first hour when they were treated with a 5×10^{-3} $\underline{\text{M}}$ solution of chloramphenicol. When treated with a 10^{-3} $\underline{\text{M}}$ solution, there was a 2 hour lag period before inhibition. Repression of protein synthesis by chloramphenicol (5×10^{-3} $\underline{\text{M}}$) followed a time course similar to inhibition of elongation.

A direct measure of protein synthesis was difficult to obtain because of the simultaneous inhibition of ^{14}C-leucine uptake. ^{14}C-α-Aminoisobutyric acid uptake was also inhibited by chloramphenicol. The latter amino acid was not incorporated into protein and was not metabolized. In the absence of chloramphenicol it was accumulated against a gradient. Chloramphenicol prevented any accumulation of ^{14}C-α-aminoisobutyric acid. Chloramphenicol also repressed the uptake of IAA, but the inhibition was slight and it was not a principal contributor to the inhibition of elongation.

Pretreatment of $\underline{\text{Avena}}$ coleoptiles with ^{14}C-leucine provided direct evidence that protein synthesis as well as amino acid uptake was being inhibited under experimental conditions where elongation was inhibited.

The uptake of ^{14}C-chloramphenicol into $\underline{\text{Avena}}$ coleoptiles was by diffusion. The internal concentration approached that of the external concentration within 4 hours, but the external concentration was not exceeded with continued incubation. The entry of chloramphenicol into the tissue accounted for the lag period before inhibition was observed. However, penetration was not a factor in the low sensitivity of the $\underline{\text{Avena}}$ coleoptiles to chloramphenicol.

As determined by thin-layer chromatography and biolog-
ical assay, chloramphenicol was not rapidly metabolized by
the Avena tissue to an inactive form. After 4 hours incuba-
tion of Avena coleoptiles in a solution of ^{14}C-chloramphen-
icol, 80-90% of the extracted radioactivity cochromatographed
with authentic chloramphenicol. In addition, the extract
still maintained its biological activity in E. coli.

Chloramphenicol inhibition was not stereospecific in
the plant systems investigated. L-Threo, D-erythro, and
L-erythro-chloramphenicol were effective inhibitors of auxin-
induced elongation in Avena and Triticum coleoptiles, ^{14}C-
leucine uptake and incorporation into the protein of Avena
and Triticum coleoptiles, ^{14}C-α-aminoisobutyric acid uptake
into Avena coleoptiles, buckwheat root elongation, and gib-
berellic acid-induced synthesis of α-amylase in barley
aleurone layers. Although there was some variation in the
assays, all three isomers had activity similar to the anti-
biotic, D-threo-chloramphenicol.

The non-specific activity of chloramphenicol in plant
tissue was not a result of the non-antibiotic isomers being
racemized to D-threo-chloramphenicol.

Cycloheximide inhibited auxin-induced growth in Avena
and Triticum coleoptiles. With a solution of 2×10^{-6} M,
elongation was inhibited by 50%. Solutions of 10^{-5}, 10^{-6} and
10^{-7} M were equally effective in inhibiting auxin-induced
elongation and protein synthesis in Avena coleoptiles. ^{14}C-
Leucine and ^{14}C-α-aminoisobutyric acid were inhibited to a
lesser degree.

In kinetic studies, auxin-induced elongation and protein synthesis were repressed in the first hour and both continued at a much reduced rate throughout the 6 hour incubation. Cycloheximide inhibition of protein synthesis appeared to proceed suppression of elongation.

Gougerotin, a specific inhibitor of protein synthesis in bacteria and animal cells, inhibited auxin-induced elongation and protein synthesis in <u>Avena</u> coleoptiles. A 10^{-5} <u>M</u> solution inhibited elongation by 50%. This was comparable to the concentration required for animal systems. Gougerotin should be a valuable tool for additional study of the role of protein synthesis in auxin-induced elongation.

^{14}C-α-aminobutyric acid was rapidly taken up into the <u>Avena</u> coleoptile. The radioactivity was incorporated into the protein fraction as readily as ^{14}C-leucine. Six other plants including cucumber, wheat, pea, lentile, barley, and corn all incorporated radioactivity from ^{14}C-α-aminobutyric acid into their protein fraction.

^{14}C-Labeled protein obtained from <u>Avena</u> coleoptiles incubated with ^{14}C-α-aminobutyric acid was hydrolyzed and the resulting amino acids separated by paper chromatography. ^{14}C-α-aminobutyric acid was not incorporated into protein, but 2 of its metabolites were incorporated. Hence, ^{14}C-α-aminobutyric acid cannot be used to separate factors which affect amino acid uptake from factors which affect protein synthesis.

In conclusion, the relationship between the repression of auxin-induced elongation and the inhibition of protein synthesis by chloramphenicol, cycloheximide, and gougerotin

support the hypothesis that protein synthesis plays an essential role in auxin-induced elongation. Complete proof of this hypothesis must await the isolation and characterization of the enzymatic activity associated with the newly synthesized protein(s).

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