PHYSIOLOGICAL STUDIES OF ELONGATION OF GRAND RAPIDS LETTUCE SEEDLINGS

TERRY WAYNE MERRELL

To the Graduate Council:

I am submitting herewith a Thesis written by Terry Wayne Merrell entitled "Physiological Studies of Elongation of Grand Rapids Lettuce Seedlings." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biology.

Benj. L. Stone
Major Professor

We have read this thesis and recommend its acceptance:

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PHYSIOLOGICAL STUDIES OF ELONGATION OF GRAND RAPIDS LETTUCE SEEDLINGS

An Abstract

Presented to

the Graduate Council of

Austin Peay State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in Biology

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by

Terry Wayne Merrell

August 1970

ABSTRACT

Seeds of Lactuca sativa L. var. Grand Rapids were used for studying the effects of various plant growth regulators upon hypocotyl elongation. This study was undertaken to observe: (1) the interaction between various combinations of ethionine, methionine, and gibberellic acid upon elongation of lettuce seedling hypocotyl; (2) the effects of blue, red, far-red, and white light upon elongation of lettuce seedling hypocotyl; and (3) the effects of various inhibitors of RNA and protein synthesis upon elongation of lettuce seedling hypocotyl.

Seedlings were allowed to elongate in combination with various treatments for a period of 48 hours, after which the roots and hypocotyls were measured.

Results indicate that ethionine severely inhibits elongation of lettuce seedling hypocotyl, while addition of methionine to ethionine-treated seedlings results in an almost complete reversal of ethionine-induced inhibition of elongation. Gibberellic acid causes a definite enhancement of hypocotyl elongation. However, addition of ethionine to gibberellic-acid treated seedlings results in an inhibition very similar to seedlings treated with ethionine alone. When methionine is added to seedlings treated with ethionine and GA_3, elongation is enhanced to a level similar to seedlings treated with GA_3 alone. Addition of adenosine triphosphate to ethionine-treated seedlings does not significantly reverse the inhibitory effect of ethionine. It is concluded that ethionine inhibits protein synthesis other than by decreasing synthesis

of adenosine triphosphate, possibly by inhibiting methylation of transfer ribonucleic acid.

The ethionine inhibition and GA_3 stimulation of lettuce seedling hypocotyl elongation is prevalent in red, blue, and far-red light as well as darkness. In all qualities of light tested, methionine is capable of reversing the ethionine inhibition and restoring the GA_3 stimulation of hypocotyl elongation which had been nullified by ethionine treatment.

Seedlings treated with far-red light showed hypocotyl elongation similar to that of seedlings grown in darkness. Likewise, seedlings grown in red light elongated to a level similar to those treated with blue light. The effect of darkness and far-red light upon hypocotyl elongation was distinctly different from seedlings treated with blue and red light.

The studies with actinomycin-D showed that hypocotyl elongation is most severely inhibited at a concentration of 75 micrograms per milliliter. The threshold concentration of inhibition is 25 micrograms per milliliter.

The degree of inhibition exerted by 5-fluorouracil is only slight as compared to that of cycloheximide and 6-methylpurine. This may suggest that synthesis of all RNAs is not essential for normal growth to occur. The severe inhibition of hypocotyl elongation by cycloheximide and 6-methylpurine are consistent with the idea that protein synthesis and some RNA synthesis is necessary for cell elongation to occur.

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ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation for the advice and guidance offered by Dr. Benjamin P. Stone, Department of Biology, Austin Peay State University. Appreciation is also expressed to Dr. Haskell Phillips, Department of Biology, and Mr. Floyd Brown, Department of Biology, both of Austin Peay State University, for their critical reading of the manuscript.

Gratitude is expressed to my wife, Fran, whose encouragement and patience during this study are greatly appreciated.

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CHAPTER I

INTRODUCTION

Lettuce seeds represent a unique system for studying the effect of various plant growth substances upon germination and subsequent growth. Recent interest in the physiology of lettuce seedling elongation has been mainly concerned with the action of inhibitors and promoters on hypocotyl elongation. Gibberellic acid greatly stimulates the elongation of the lettuce seedling hypocotyl (Frankland and Wareing, 1960). While the germination of the lettuce seeds has probably received more attention, their subsequent elongation is of utmost importance for studies with plant growth regulators.

This study was undertaken to observe: (1) the interaction between various combinations of ethionine, methionine, and gibberellic acid upon elongation of Grand Rapids lettuce seedlings; (2) the effects of red, far-red, blue, and white light upon lettuce seedling hypocotyl elongation; and (3) the effects of various inhibitors of ribonucleic acid (RNA) and protein synthesis upon lettuce seedling hypocotyl elongation.

CHAPTER II

REVIEW OF LITERATURE

Ethionine, the ethyl analogue of methionine, has been widely used as a competitive inhibitor in studies of methionine metabolism. Boll (1960) reported that ethionine inhibition of the growth of excised tomato roots was relieved by methionine. Schrank (1956) reported that elongation of Avena coleoptiles was inhibited by 50 percent with ethionine, but methionine was shown to completely reverse the inhibition. He suggested that the ethionine might interfere with the utilization of methionine for protein syntheses which are required for elongation of the Avena coleoptile.

Cleland (1960) investigated two possibilities of ethionine action in inhibiting elongation of Avena coleoptiles: (a) blocking protein synthesis and (b) inhibiting transfer of methyl groups from methionine to the hot-water-soluble fraction of the cell wall. He separated total elongation of the coleoptiles into reversible and irreversible components and found that ethionine caused an immediate inhibition of reversible elongation. However, a period of 3-6 hours had to elapse before irreversible elongation was inhibited. Cleland thus suggested that the inhibition of methylation was the cause of inhibition of reversible elongation, while protein synthesis inhibition was responsible for inhibition of irreversible elongation. Although he did not specifically consider a mechanism by which protein synthesis was inhibited by ethionine, Cleland concluded that ethionine acts by affecting some facet of methionine metabolism.

While studying the effects of ethionine upon rat liver, Shull (1962) observed that adenosine triphosphate (ATP) concentration in the liver was greatly decreased within a few hours after injection of ethionine. Villa-Trevino et al. (1963) attempted to explain a possible relationship between the decreased level of ATP and the inhibition of protein synthesis induced by ethionine. Their data suggested that the primary effect of ethionine is on the cellular ATP level and that inhibition of protein synthesis is secondary.

Methionine reacts with ATP to form S-adenosylmethionine via the enzyme methionine adenosyltransferase (Shapiro and Schlenk, 1960). Shapiro and Schlenk (1960) also reported that ethionine is a substrate for this enzyme and reacts with ATP to form S-adenosylethionine. Farber (1963) reported that S-adenosylethionine is not as readily utilized as S-adenosylmethionine. It was further suggested by Smith and Salmon (1965) that an imbalance between the rates of formation and of breakdown of S-adenosylethionine may occur in the ethionine-treated rat and may lead to an accumulation of this intermediate in the liver. Two modes of action concerning the excess S-adenosylethionine have been postulated. According to Stekol et al. (1960), the excess S-adenosylethionine inhibits mitochondrial oxidation and oxidative phosphorylation with a resulting decrease in the rate of synthesis of ATP from ADP. According to a second hypothesis (Stekol, 1963), ATP reacts with ethionine to form S-adenosylethionine at a faster rate than the cell can synthesize ATP from available precursors. This results in the conversion of ATP to a much less reactive form and thereby effectively traps increasing amounts of adenine as an adenosine derivative. Shull et al. (1966) reported that the injection of ethionine into rat liver resulted in a decrease of

ATP concentration to a level of 20 percent of the control. They further showed that mitochondrial respiration and oxidative phosphorylation are intact when measured in isolated mitochondria or in other systems *in vitro* obtained from ethionine-treated animals. Shull *et al.* (1966) thus concluded that the primary effect of ethionine is to trap adenosine as S-adenosylethionine by reacting with ATP. This adenine trapping effect had previously been shown in yeast (Schmidt *et al.*, 1956).

Norris (1964) also supports the concept that ethionine exerts its inhibitory effect by interference with ATP metabolism. He presented data which showed that 0.01 molar (M) ethionine inhibited elongation of *Avena* coleoptile segments by 70 percent, but the application of ATP at concentrations of 0.25 millimole (mM) and 0.5 mM stimulated the growth of the coleoptile sections and partially reversed the ethionine-induced inhibition of their elongation.

One should keep in mind that other possibilities for the mechanism of ethionine-induced inhibition still exist. Dunn *et al.* (1966) found that the methylated purine and pyrimidine bases which occur as components in nucleic acids are localized primarily in transfer RNA (t-RNA). Peterkofsky (1964), working with *Escherichia coli* and yeast, investigated the role of methylated bases in connection with the amino acid transfer function of t-RNA. His data showed that the *E. coli* enzyme of the amino acid leucine (leucyl-T-RNA synthetase) successfully attached the leucine to t-RNA regardless of whether or not it contained the methylated bases. However, the yeast enzyme was non-functional in the absence of methylated bases. Peterkofsky further concluded that methylation has a profound effect on the codon recognition properties of t-RNA. Moore and Smith (1969) presented evidence that ethionine exerts

its inhibitory effect by preventing methylation of t-RNA. Their results show that S-adenosylethionine (2.0×10^{-4}M) inhibited incorporation of methyl groups into t-RNA by 66 percent in bacteria, while in rat liver, total inhibition of methylation occurred. This work seems to be in agreement with the earlier suggestion of Cleland (1960), who stated that ethionine inhibition of growth was due, at least in part, to a prevention of cell wall methylation.

Although both methods of ethionine inhibition could presently be accepted as valid evidence, further work is necessary to determine which one plays the major role in the inhibition of plant growth.

The germination of many seeds is affected by light. Borthwick et al. (1952) made observations which led them to postulate the existence of what is now known as the red, far-red reversible pigment system. Many aspects of growth and development are affected by this red, far-red interaction. Some of these, as reported by Hendricks and Borthwick (1967), are flower initiation and development, germination of seeds and spores, and enlargement of structures such as stems and leaves. Downs (1956) reported that flowering of Xanthium pennsylvanicum was enhanced by red radiation and inhibited by far-red radiation.

The pigment responsible for this reversible system, now called phytochrome, is a blue or bluish-green protein that exists in two forms (Borthwick and Hendricks, 1960). These two forms are interconvertible by light, with 660 nanometers (nm.) and 730 nm. being the absorption maxima of each form. Form P_{730} is the active form of phytochrome, changing in darkness to the inactive form P_{660}.

The mechanism by which phytochrome action takes place has not been narrowed to a single mode of action. Hendricks and Borthwick (1967) observed a number of plant responses which indicate that phytochrome acts on cell permeability as an early step in its control of plant growth and development. Mohr (1966) proposed that some of the actions of phytochrome are due to gene activation, which enhances new RNA and protein synthesis and subsequently results in differentiation and development. However, the effects for which Mohr's mechanism has been proposed occur several hours after the first light exposure, and it is well known that many effects attributable to phytochrome are detectable within an hour or so.

The responses of plants to light given at high intensities for several hours have frequently shown 2 wave length regions of maximum effectiveness. One is in blue from 420-480 nm. and one in far-red from 700 to about 750 nm. Mohr and Wehrung (1960) illustrated this high energy light reaction by demonstrating that hypocotyl lengthening of Grand Rapids lettuce seedlings was slightly influenced by red radiation, while the action of white, blue, and far-red light strongly inhibited hypocotyl lengthening in the seedlings. They concluded that a "blue, far-red reaction system" was evidence of a second light reaction other than phytochrome.

Turner and Vince (1968), working with lettuce seedling hypocotyls, reported that both blue and red light modified the response to far-red in a similar manner, and a prior irradiation with either was shown to reduce the effect of subsequent far-red treatment. The differences in the response to blue and far-red under high-energy conditions indicated that two separate reactions were involved. The authors concluded that a

distinct blue light absorbing pigment was present and that this pigment mediated a reaction inhibitory to elongation.

Esashi and Odi (1966) suggested that the active function of the dual effect of blue and far-red light used was not related with the phytochrome reaction. This blue, far-red reaction system can be physiologically demonstrated only by high energy irradiation. Thus far it has been referred to as the "high energy reaction" (Mohr, 1962).

Mohr (1962) strongly supports the theory of phytochrome and high energy reaction as being two entirely different reaction systems. His investigations of the movements of the plumular hook of Grand Rapids lettuce seedlings strengthened this concept. While no appreciable plumular hook is formed on dark-grown seedlings, it can be induced by red light. The formation of the hook by red light can further be nullified by immediate far-red irradiation, thus showing the effect of phytochrome. The hook which had been closed by red light was shown to open only when blue or far-red light was applied at high intensity. Mohr thus presented evidence that the "high energy reaction" was present.

Conversely, as one might expect, there are those who are not in agreement with the theory of Mohr. Regardless of the validity of Mohr's proposal, his work was a major contribution toward a better understanding of phytochrome and its related processes.

The development of chlorophyll is light-dependent and requires a lag period during which the conversion of protochlorophyllide to chlorophyll occurs, followed by a period of rapid chlorophyll synthesis (Gassman and Bogorad, 1967). It has been proposed that light induces the synthesis of enzymes necessary for chlorophyll production (Marcus, 1960).

After working with various inhibitors of protein and RNA synthesis, Gassman and Bogorad (1967) concluded that nucleic acid and protein synthesis are essential for normal chlorophyll production. They further suggested that light regulates chlorophyll synthesis by controlling the availability of gamma-aminolevulinic acid, possibly by causing the formation of an enzyme which regulates the synthesis of aminolevulinic acid.

Red light stimulates chlorophyll synthesis, while far-red light reverses the effect of red light (Price and Klein, 1961). It was suggested by Augustinussen and Madsen (1965) that the effect of red on chlorophyll synthesis is probably due to an effect on protochlorophyllide resynthesis which follows the phototransformation of the pigment to chlorophyll.

The application of gibberellic acid (GA_3) results in a significant increase of plant growth. Frankland and Wareing (1960) found substantial stimulation of the growth of lettuce seedling hypocotyls with increasing concentrations of GA_3 up to 100 parts per million (ppm.). While there is no doubt that growth of plants is enhanced by GA_3, the mechanism by which it exerts its affect is not clearcut.

Recent studies on the incorporation in vivo of labeled amino acids into proteins of aleurone layers of barley suggest de novo synthesis of α-amylase in response to added GA_3 (Varner, 1964; Varner and Ram Chandra, 1964). It was shown by Varner and Ram Chandra (1964) that actinomycin-D, an inhibitor of deoxyribonucleic acid (DNA)-dependent RNA synthesis, inhibits the GA_3-dependent formation of α-amylase in isolated aleurone layers. The authors suggested that GA_3 is involved in the formation of a specific messenger RNA (m-RNA) for

α-amylase. Further work by Chrispeels and Varner (1967) showed that addition of the protein synthesis inhibitor cycloheximide rapidly and completely inhibited amylase synthesis, regardless of when GA_3 was added. If added before or at the same time as GA_3, actinomycin-D also prevented amylase formation. However, when actinomycin-D was added after 4 hours of GA_3 treatment, a reduced effect on amylase synthesis was observed. After 8 hours of GA_3 treatment actinomycin-D addition was without effect. Even so, RNA synthesis was inhibited 65 percent during the 4-hour interval which followed the addition of 100 micrograms per milliliter (µgm./ml.) of actinomycin-D at 8 hours.

Johri and Varner (1968) showed that nuclei isolated from dwarf pea and incubated in the presence of GA_3 (10^{-8}M) incorporated up to 80 percent more ^{3}H-nucleotide into RNA than control nuclei. The later GA_3 was added during the isolation, the smaller the response. The failure of purified nuclei to respond to GA_3 was apparently caused by the loss during isolation of some essential hormone-sensitive factor. The mechanism for the GA_3 stimulation of RNA synthesis in nuclei is not known, although Johri and Varner suggested that it could be due to an increase in template sites on DNA or due to an increase in RNA polymerase activity. Similarly, Jarvis et al. (1968) attributed the breaking of dormancy in seeds of Hazel (Corylus avellana) by GA_3 to an increase in RNA polymerase activity and increased DNA template availability.

Inhibitors of RNA and protein synthesis used in this study were actinomycin-D, 5-fluorouracil, cycloheximide, and 6-methylpurine. Probably the most used inhibitor of RNA synthesis is actinomycin-D. Goldberg and Reich (1964) postulated that the antibiotic forms a complex specifically with the amino group of deoxyguanosine in DNA, thus

preventing DNA-dependent RNA synthesis. Key (1964) observed that when sections of soybean hypocotyl were incubated in solutions containing 10 μgm./ml. actinomycin-D, cell elongation proceeded at about 90 percent of the normal rate for 2 hours. However, after 4 hours, hypocotyl elongation was inhibited 75-85 percent. RNA synthesis was inhibited 60 percent during the initial 2 hours and reached 90-95 percent within 4 hours. Key stated that a part of the delay in growth inhibition by actinomycin-D was due to the time required for the antibiotic to penetrate the tissue. He also postulated that part of the delay in inhibition by actinomycin-D was possibly a result of the time required to deplete the supply of unstable template RNA. Penny and Galston (1966), working with green pea stems, reached the same conclusions. After addition of actinomycin-D, a lag phase of 2 hours was required before significant growth inhibition could be detected. Penny and Galston (1966) concluded that the cell can store enough RNA for its short-term growth needs. Although not inhibiting protein synthesis directly, actinomycin-D leads to an inhibition of protein synthesis which increases with an increase in time (Lin and Key, 1968).

The timing of inhibition of cell elongation by actinomycin-D in excised tissues varies from species to species. Courtney et al. (1967) suggested that the degree of inhibition of cell elongation correlates positively with inhibition of RNA synthesis. While growing at a constant rate, cell elongation of soybean hypocotyl was inhibited by actinomycin-D after a lag of 1-2 hours, with the degree of inhibition depending upon the concentration of actinomycin-D (Key, 1964).

Key and Ingle (1964) showed that actinomycin-D, up to concentrations of 0.2 μgm./ ml., inhibited RNA synthesis and cell elongation

similarly. It is thus clear, as suggested by Key (1964), that actinomycin-D inhibits RNA synthesis prior to its inhibition of cell elongation.

The observation that growth can occur while some RNA synthesis is inhibited has been further emphasized by the use of 5-fluorouracil. A possible mechanism of this pyrimidine analogue has been suggested by Horawitz et al. (1960). They suggest that it is incorporated into m-RNA as a substitute for a pyrimidine base (probably uracil), and the resulting modified messenger RNAs serve as templates in producing proteins having abnormal amino acid sequences. Nooden (1968) found that 5-fluorouracil did not inhibit cell enlargement in the presence of auxin, while severe inhibition of RNA synthesis occurred. Along the same line, Key and Ingle (1964) showed that the growth of soybean hypocotyl was not affected by 5-fluorouracil, while total RNA synthesis was inhibited by 50 percent. Inhibition of ribosomal RNA (r-RNA) was 90 percent, transfer RNA (t-RNA) 75 percent, and messenger RNA (m-RNA) inhibition amounted to only 10 percent. Due to the almost complete inhibition of r-RNA and little or no inhibition of m-RNA, Key and Ingle concluded that the synthesis of new ribosomes, and possibly t-RNA as well, is not required during continued cell elongation of excised and intact plant tissues. Key and Ingle (1964) further concluded that synthesis of m-RNA is essential for growth.

Cycloheximide (actidone) is an effective inhibitor of protein synthesis. It was shown by Siegel and Sisler (1965) that this inhibitor prevents the transfer of amino acids from t-RNA to protein, thus causing a direct inhibition of peptide formation. They concluded that amino acid

activation and synthesis of aminoacyl-t-RNA are not affected by cycloheximide. While working with soybean hypocotyl, Key (1966) showed that cycloheximide markedly inhibited accumulation of r-RNA, but that synthesis of r-RNA, per se, was not inhibited. The accumulation of newly synthesized m-RNA was only slightly affected by cycloheximide. These results showed that the inhibition of cell elongation by cycloheximide correlates with the inhibition of protein synthesis, but not with the effect on RNA metabolism.

Another frequently used inhibitor of RNA synthesis is 6-methylpurine. This purine analogue was shown by Key (1966) to effectively inhibit growth, RNA synthesis, and protein synthesis of soybean hypocotyl.

CHAPTER III

MATERIALS AND METHODS

Seedling Growth

Seeds used for germination and subsequent seedling growth were of *Lactuca sativa* variety Grand Rapids, obtained from Joseph Harris Seed Company, Rochester, New York.

Seeds were allowed to germinate in 9 centimeter (cm.) petrie dishes containing Type D filter paper (Matheson Scientific Inc.) moistened with 4 milliliters (ml.) of distilled water. After 18 hours in continuous light, germinated seedlings with radicles approximately 1 millimeter (mm.) in length were removed and transferred into petri dishes containing 4 ml. of the various experimental treatments. Unless stated otherwise, seedlings were allowed to elongate in continuous light at a temperature of 25 ± 2° C for a period of 48 hours. At the end of the 48-hour period the hypocotyls and roots were measured to observe the effects of the various substances upon elongation. There were 20 seedlings per treatment. Replicates were run for each treatment.

The various chemicals used were obtained from the following sources: D-L ethionine from Sigma Chemical Company, St. Louis, Missouri; L-methionine from Aceto Chemical Company, Flushing, New York; gibberellic acid (GA_3) from Eastman Organic Chemicals, Rochester, New York; 5-fluorouracil from Hoffman-LaRoche Incorporated, Nutley, New Jersey; 6-methylpurine, cycloheximide, and adenosine triphosphate (ATP) all from Nutritional Biochemical Corporation, Cleveland, Ohio; and actinomycin-D from Merck, Sharp, and Dohme Research Laboratory, Rahway, New Jersey.

Incorporation of Blue, Red, and Far-Red Light

The effects of blue, red and far-red light upon hypocotyl elongation were studied by attaching a plastic filter to the top of a light-tight box. Far-red light was obtained by using Carolina Biological Supply (CBS) plastic filter No. 750, red by CBS filter No. 650, and blue by CBS filter No. 450. The filters were 31 cm. above the level of the seedlings. The light source was an incandescent 150-watt bulb attached by a clamp on a ring stand 73 cm. above the level of the seedlings. After 48 hours of growth the roots and hypocotyls were measured. The temperature at the level of the filters was 25 ± 2° C.

Chlorophyll Extraction

Approximately 100 seedlings per treatment were extracted for estimation of chlorophyll. The seedlings were removed from the petri dishes after the normal 48-hour growth period and were boiled briefly in two 4 ml. portions of 80 percent ethanol. The extracts were adjusted to 5 ml. with 80 percent ethanol and their absorbancies read at 660 nanometers (nm.). This method is similar to that of Aspinall et al. (1967).

CHAPTER IV

RESULTS

The Interaction Between Various Combinations of Ethionine, Methionine, and Gibberellic Acid Upon Hypocotyl Elongation of Grand Rapids Lettuce Seedlings

The results of various combinations of ethionine, methionine, and gibberellic acid upon lettuce seedling hypocotyl and root elongation are given in tables I and II.

The data indicate that at all concentrations used, ethionine greatly inhibits the elongation of both hypocotyl and root, while addition of methionine to ethionine-treated seedlings results in almost complete reversal of ethionine-induced inhibition. The reversal of this ethionine-induced inhibition by methionine agrees with the data of Boll (1960) and Schrank (1956), who also reported reversal of ethionine-induced inhibition after addition of methionine.

When seedlings are allowed to elongate in the presence of GA_3, an enhancement of root and hypocotyl elongation to a level greater than the control is observed, as indicated by tables I and II. Addition of ethionine along with GA_3 indicates an inhibition of elongation very similar to seedlings treated with ethionine alone. This would suggest that GA_3 has little if any effect on ethionine-induced inhibition. However, addition of methionine to seedlings treated with GA_3 and ethionine indicates a very definite reversal of the inhibition exerted by ethionine. Here again, the ability of methionine to reverse the ethionine-induced inhibition is shown.

TABLE I

EFFECTS OF VARIOUS COMBINATIONS OF ETHIONINE, METHIONINE, AND GIBBERELLIC ACID UPON ELONGATION OF LETTUCE SEEDLING HYPOCOTYL

Treatment	Concentration			
	.01M	.005M	.0025M	.00125M
	Percent of Control			
Ethionine	45	38	31	47
Methionine	89	97	90	79
Ethionine + Methionine	96	85	89	89
GA_3	274	209	295	139
GA_3 + Ethionine	48	41	40	46
GA_3 + Ethionine + Methionine	187	220	248	216

Hypocotyls were measured after the seedlings had elongated for 48 hours in combination with the various treatments. A total of 20 seedlings were contained in each treatment, with replicates being run for each treatment. Results represent mean hypocotyl lengths compared with a water control. The concentration of gibberellic acid (GA_3) was 10 parts per million (ppm.). Data are average of duplicate experiments.

TABLE II

EFFECTS OF VARIOUS COMBINATIONS OF ETHIONINE, METHIONINE, AND GIBBERELLIC ACID UPON ELONGATION OF LETTUCE SEEDLING ROOT

Treatment	Concentration			
	.01M	.005M	.0025M	.00125M
	Percent of Control			
Ethionine	41	18	31	36
Methionine	101	110	88	98
Ethionine + Methionine	104	123	96	95
GA_3	136	144	164	121
GA_3 + Ethionine	33	22	36	41
GA_3 + Ethionine + Methionine	83	76	81	86

Roots were measured after the seedlings had elongated for 48 hours in combination with the various treatments. A total of 20 seedlings were contained in each treatment. Results represent mean root lengths compared with a water control. Replicates were run for each treatment. Data are average of duplicate experiments. Concentration of GA_3 was 10 ppm.

Reversal of ethionine-induced inhibition by addition of ATP was reported by Norris (1964) and Villa-Trevino et al. (1963). An experiment was performed to observe the results of ATP addition to ethionine-treated lettuce seedling hypocotyls. The results of such an experiment are shown in figure 1. ATP at varying concentrations was added to ethionine and water-treated seedlings. Although a slight stimulation of mean hypocotyl length is indicated, especially at concentrations of 0.1 mM and 0.01 mM, the enhancement cannot be accepted as significant because the controls (water and ethionine) show an overlapping of standard deviations with the seedlings treated with ATP. Thus, the data in figure 1 indicate that ATP does not reverse the ethionine-induced inhibition of elongation in lettuce seedling hypocotyls. These results do not agree with those obtained by Norris (1964) and Villa-Trevino et al. (1963).

A study was made of the elongation of lettuce seedling hypocotyl after addition of ethionine at various time intervals. Results of this work are shown in table III. The data indicate that if ethionine is added at any time between 0 and 8 hours, there is only a slight change in inhibition (44 to 51 percent control). Apparently the critical time at which the ethionine must be present in the tissue for maximum inhibition is between 0 and 12 hours. After the addition at 12 hours, ethionine is less inhibitory to hypocotyl elongation. Addition at 36 hours shows an inhibition of only 21 percent. The data indicate that ethionine-induced inhibition of lettuce seedling hypocotyls is greatest when added between 0 and 12 hours. Thus ethionine apparently is inhibiting biochemical processes that occur in the early stages of hypocotyl elongation.

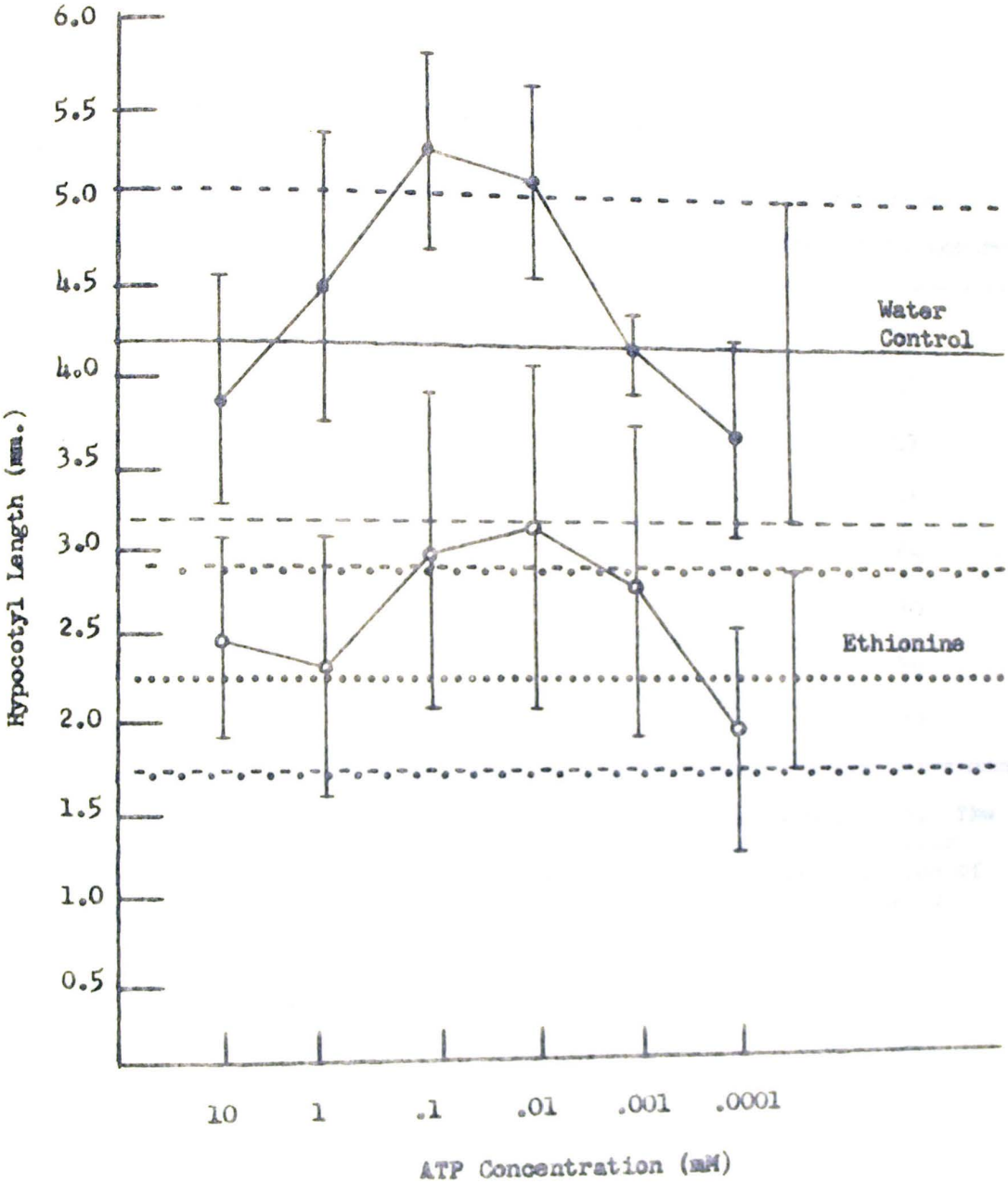

Figure 1. Influence of ATP on elongation of lettuce seedling hypocotyl in ethionine (0.0025M) and in water.

Standard deviations and mean hypocotyl lengths are shown. There were 20 seedlings for each concentration. Replicates were run for each concentration. Data are average of duplicate experiments.

TABLE III

EFFECT OF ETHIONINE AT VARIOUS TIME INTERVALS UPON ELONGATION OF LETTUCE SEEDLING HYPOCOTYL

Time Added	Percent of Control
0-48 Hrs.	44
3-48 Hrs.	46
6-48 Hrs.	49
8-48 Hrs.	51
12-48 Hrs.	65
16-48 Hrs.	70
24-48 Hrs.	74
36-48 Hrs.	79

Hypocotyls were measured after a 48-hour growth period. The mean hypocotyl length of each treatment was compared with a water control. Replicates were run for each treatment. Concentration of ethionine was .0025M. Data are average of duplicate experiments.

Effects of Red, Blue, Far-red, and White Light Upon Lettuce Seedling Hypocotyl Elongation

The lettuce seedling hypocotyl shows sharply defined regions of response to blue and far-red light, while red light has little direct effect (Vince and Turner, 1968). This blue/far-red response of the lettuce seedling was studied in detail by Mohr and Wehrung (1960) and by Vince and Turner (1968).

Vince and Turner (1968) showed that red light increased hypocotyl elongation when used alone over a long period of time. Far-red light was inhibitory for hypocotyls up to 36 hours old, after which the inhibitory effect was relieved. Blue light was inhibitory throughout the entire growth period.

Grand Rapid lettuce seedlings were allowed to elongate under the influence of red, blue, and far-red light in various treatments for 48 hours. Seedlings allowed to elongate in darkness were used as controls. Results of this experiment are presented in figure 2. In all treatments except GA_3, the mean hypocotyl lengths of seedlings under the influence of red light show an increase over those treated with far-red light. This compares favorably with the data of Vince and Turner, who observed that enhancement of hypocotyl elongation was greater in seedlings treated with red light than those with far-red. The data also indicate that in 3 of the treatments (water control, methionine, methionine + ethionine), the mean hypocotyl length is greater in the presence of blue light than in the presence of red light. However, this enhancement is not significant because there is an overlapping of the standard deviations of the mean hypocotyl length. Figure 2 also indicates that in all

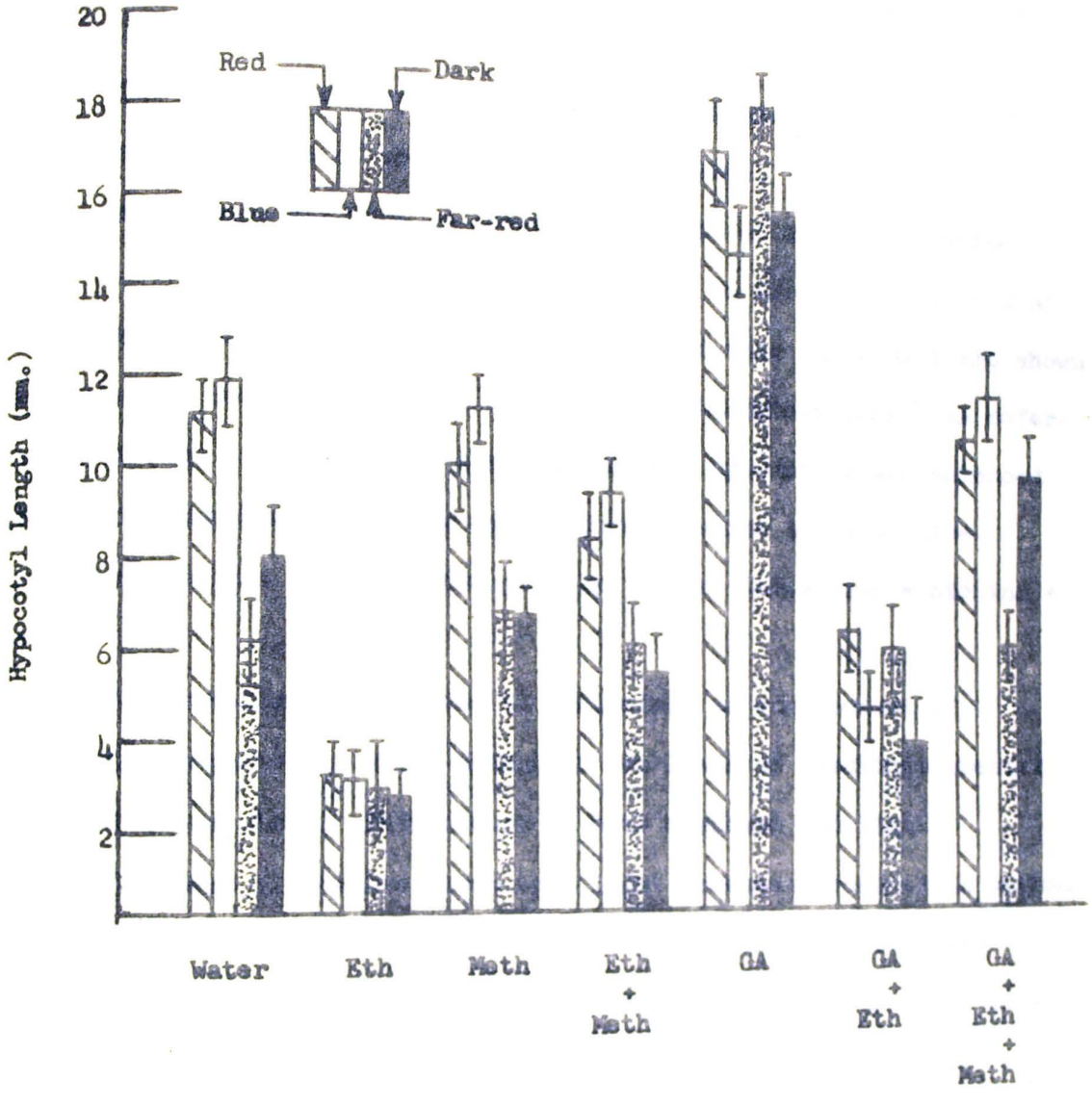

Figure 2. Effects of darkness, blue, red, and far-red light upon elongation of lettuce seedling hypocotyl.

Mean hypocotyl lengths and standard deviations are shown. Seedlings elongated under influence of light treatments for 48 hours. There were 20 seedlings for each treatment, with replicates being run for each treatment. Mean hypocotyl lengths are averages of duplicate experiments. Concentration of ethionine (Eth) and methionine (Meth) was 0.0025M. GA concentration was 10 ppm. Seedlings allowed to elongate in darkness and water served as reference controls.

except 2 treatments (GA_3 and GA_3 + ethionine + methionine), far-red light has an effect upon elongation very similar to seedlings grown in darkness.

The interaction between methionine, ethionine, and GA_3 was prevalent, regardless of the quality of light used.

An attempt was made to study the effects of continuous white light on lettuce seedling hypocotyl elongation in various treatments of ethionine, methionine, and GA_3. The results of this experiment are shown in figure 3. Seedlings allowed to elongate in darkness served as reference controls. The data show that GA_3-treated hypocotyls are enhanced greatly in comparison with the water control, while the ethionine-treated hypocotyls are inhibited greatly, as are those with ethionine + GA_3.

When methionine is added to ethionine, an enhancement of hypocotyl elongation is shown almost to the level of seedlings treated with methionine alone. This occurs in both darkness and light.

Lockhart (1956) has shown that light treatment results in marked inhibition of growth in Alaska pea seedlings. This inhibition was completely reversed by treating the seedlings with GA_3. His data show that GA_3-treated plants in light and darkness grow to almost identical heights, whereas the untreated dark-grown plants show a greater variation in growth rate. Lockhart concluded that some factor in the plant, which may be replaced by gibberellin, controls the relative rate of stem elongation in light and darkness. The enhancement of lettuce seedling hypocotyl elongation by addition of GA_3, as shown in figure 3, does not agree with data obtained by Lockhart. The dark-grown seedlings treated with GA_3 are enhanced to a level significantly higher than the light-

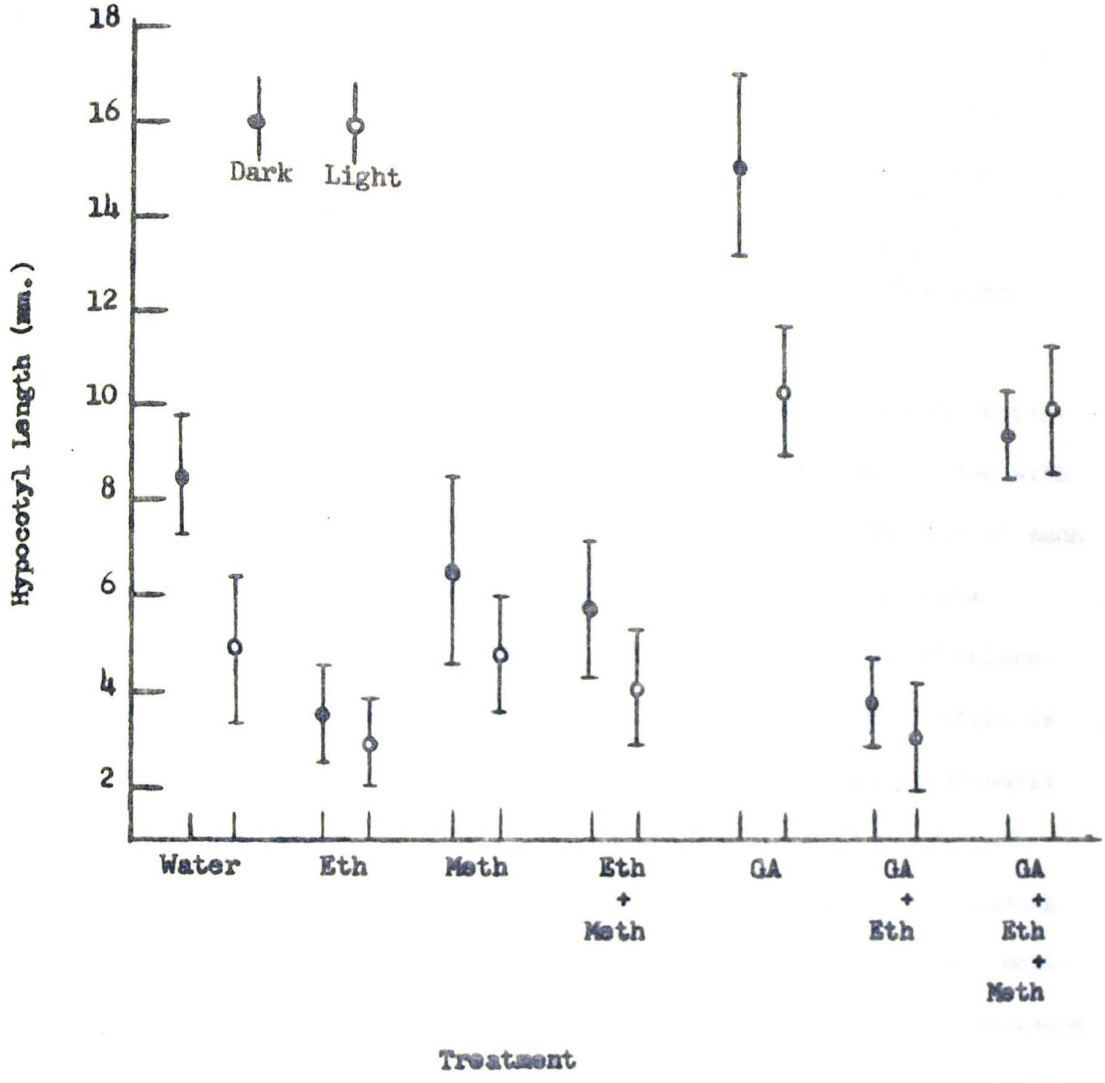

Figure 3. Effect of darkness and continuous light upon lettuce seedling hypocotyl elongation.

Mean hypocotyl lengths and standard deviations are given. Water treatment was used as control. There were 20 seedlings per treatment, with replicates being run for each treatment. Data are average of duplicate experiments. Seedlings elongated in various treatments for 48 hours. Concentration of ethionine (Eth) and methionine (Meth) was 0.0025M. Concentration of gibberellic acid (GA) was 10 ppm.

grown seedlings treated with GA_3. The increase in elongation of dark-grown seedlings treated with GA_3 over dark-grown seedlings in water is very similar to the increase of GA_3-treated seedlings in continuous light over those of the light-treated water control. The data also indicate that the light-grown seedlings treated with GA_3 are not stimulated significantly more than dark-grown seedlings of the water control.

A further study was made of the effects of darkness and continuous light upon chlorophyll synthesis in lettuce seedlings treated with various combinations of ethionine, methionine, and GA_3. Results of such an experiment are shown in figure 4. It is evident from the data presented that the absorbance readings, and thus the amount of chlorophyll synthesis, are indeed higher in all treatments in which light is present. This indicates the necessity of light for normal chlorophyll synthesis to take place.

Seedlings treated with GA_3 have the highest absorbance reading in the presence of light, while ethionine-treated seedlings are lowest. Seedlings treated with GA_3 and ethionine show almost the same absorbance as ethionine alone, while methionine addition increases the reading very near the level of seedlings treated with methionine alone. The methionine-treated seedlings in light are very near the level of those in the water control. As the data indicate, the amount of chlorophyll synthesis is negligable in darkness in all treatments, as compared to those in light. The data further indicate that chlorophyll synthesis in ethionine-treated seedlings is greatly inhibited. The same can be said of seedlings treated with ethionine and GA_3. The treatment of seedlings with GA_3 alone apparently results in an increase in the amount of

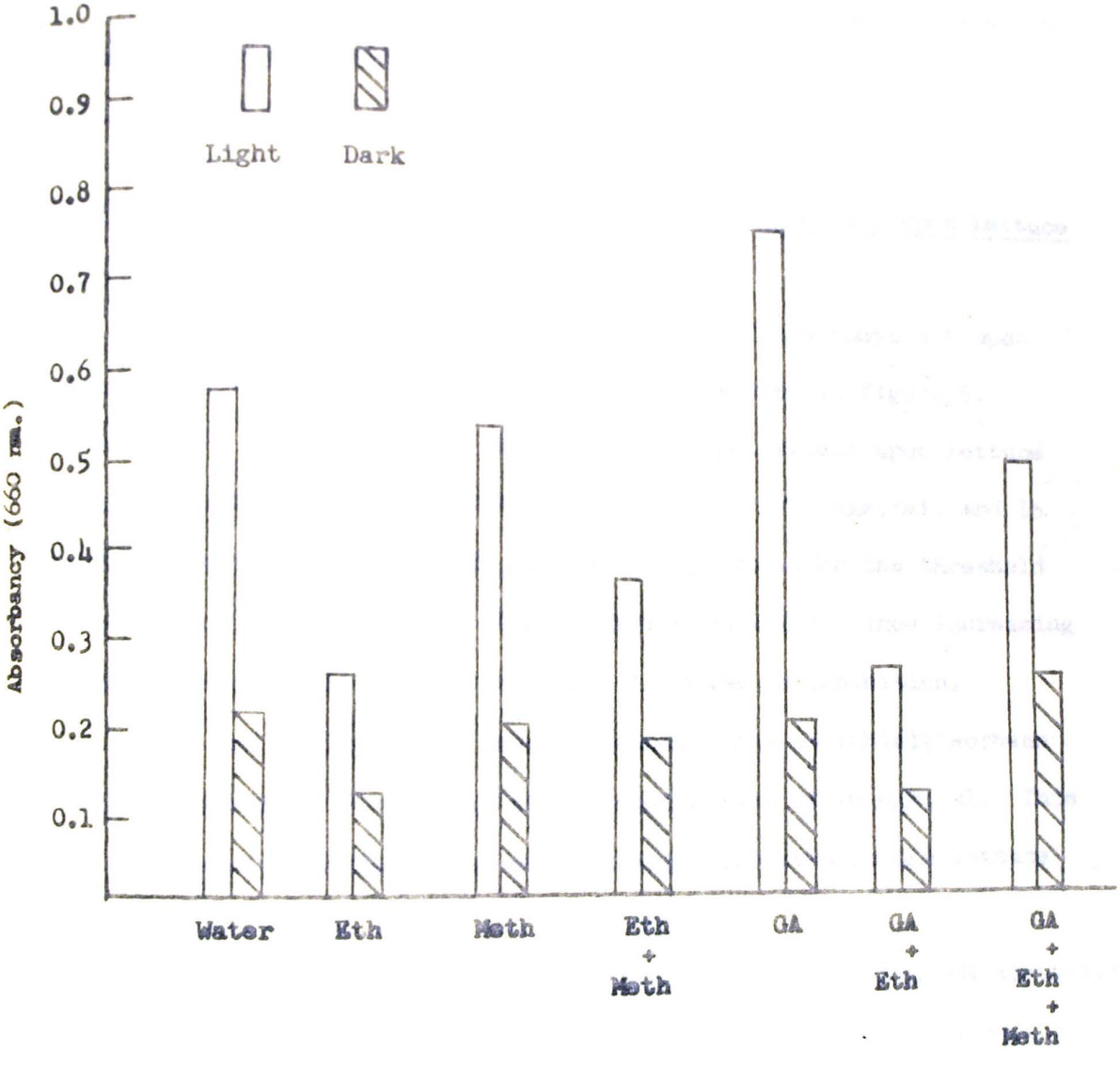

Figure 4. Effects of darkness and continuous light upon chlorophyll synthesis in lettuce seedlings.

Seeds were extracted after a growth period of 48 hours in combination with various treatments. A total of 100 seedlings were contained in each treatment. Replicates were run for each treatment. Data are average of duplicate experiments. Concentration of methionine and ethionine was .0025M. GA concentration was 10 ppm.

chlorophyll being synthesized in light. Addition of methionine to ethionine indicates a partial reversal of ethionine-induced inhibition of chlorophyll synthesis, while the inhibition resulting from seedlings treated with GA_3 and ethionine is almost completely reversed by methionine addition.

Effect of Various Inhibitors of RNA and Protein Synthesis Upon Lettuce Seedling Hypocotyl Elongation

The results of varying concentrations of actinomycin-D upon lettuce seedling hypocotyl elongation are presented in figure 5. Actinomycin-D is indicated to have only a slight effect upon lettuce seedling hypocotyl elongation at concentrations of 5 µgm./ml. and 15 µgm./ml. Actinomycin-D, at 25 µgm./ml., appears to be the threshold concentration for inhibition of hypocotyl elongation, since increasing concentrations result in only a slight increase in inhibition.

Key and Ingle (1964) showed actinomycin-D to inhibit soybean hypocotyl growth by 70 percent at a concentration of 10 µgm./ml. This does not agree with the amount of inhibition observed in the lettuce seedling hypocotyl at such a low concentration.

Key and Ingle also showed 5-fluorouracil to inhibit RNA synthesis by 50 percent, while having no effect on growth. However, the data in table IV indicate a definite effect on growth by this pyrimidine analogue. At all concentrations of 5-fluorouracil used, hypocotyl elongation of lettuce seedlings is indicated to be slightly inhibited. Here again, this does not agree with the data obtained by Key and Ingle (1964).

The effects of cycloheximide and 6-methylpurine upon lettuce seedling hypocotyl elongation are also presented in table IV. The

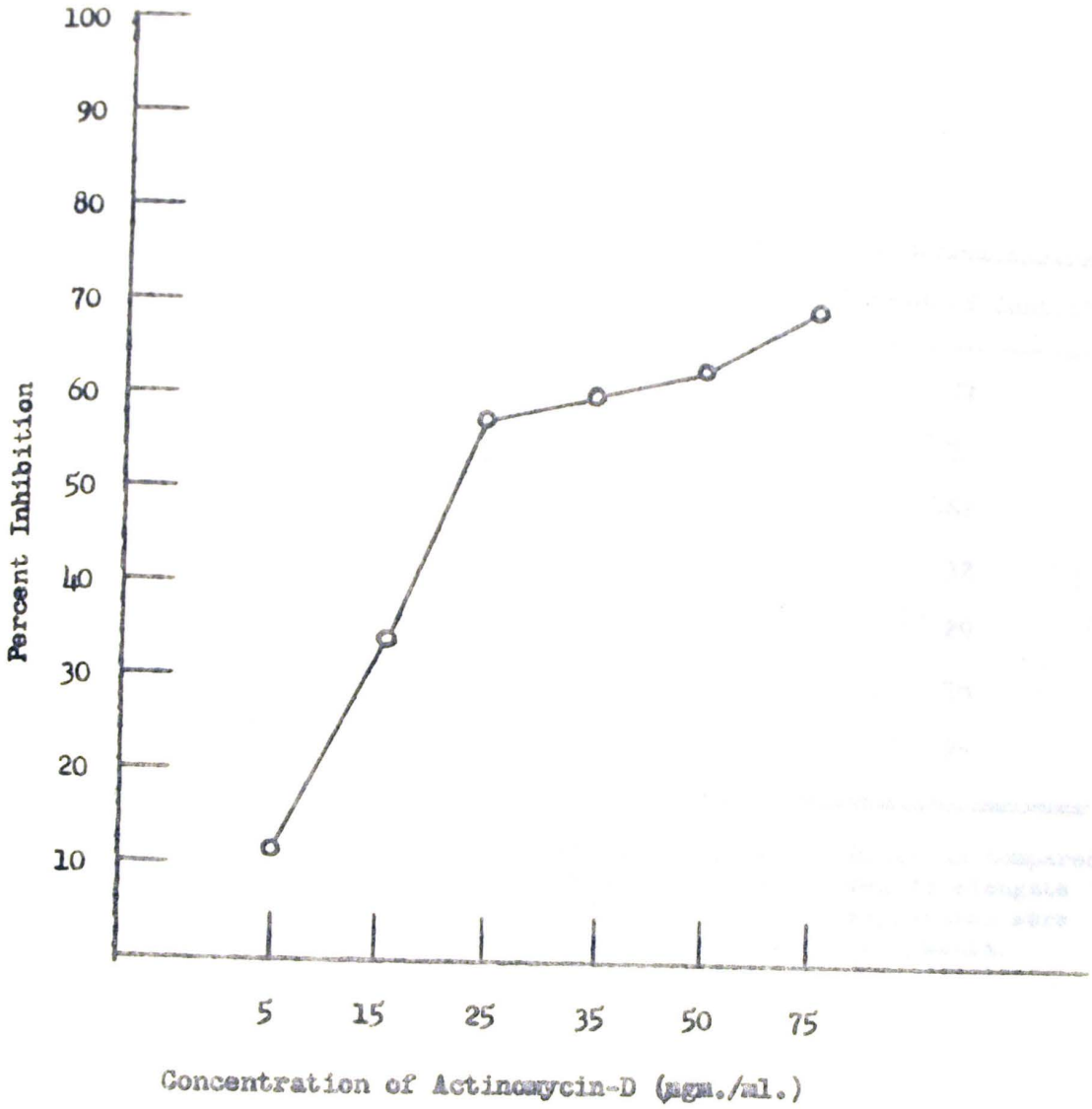

Figure 5. Effect of actinomycin-D upon elongation of lettuce seedling hypocotyl in continuous light.

Each plotted point represents the mean hypocotyl length expressed as percent inhibition as compared with a water control. There were 20 seedlings contained in each treatment, with replicates being run for each concentration. Seedlings were allowed to elongate for 48 hours after transferal to respective concentrations. Data are average of duplicate experiments.

TABLE IV

EFFECTS OF 5-FLUOROURACIL, CYCLOHEXIMIDE, AND 6-METHYLPURINE UPON ELONGATION OF LETTUCE SEEDLING HYPOCOTYL

Treatment	Percent of Control
5-Fluorouracil (50 µgm./ml.)	77
5-Fluorouracil (150 µgm./ml.)	71
5-Fluorouracil (250 µgm./ml.)	61
Cycloheximide (10 µgm./ml.)	32
Cycloheximide (20 µgm./ml.)	29
6-Methylpurine (10 µgm./ml.)	36
6-Methylpurine (20 µgm./ml.)	28

Percentages given represent the mean hypocotyl length as compared with a water control. A total of 20 seedlings were allowed to elongate in combination with a specific treatment for 48 hours. Replicates were run for each treatment. Data are average of duplicate experiments.

effects of these inhibitors upon growth and protein synthesis have been described by Key (1966). The data in table IV indicate that both cycloheximide and 6-methylpurine greatly inhibit elongation of lettuce seedling hypocotyl, regardless of which concentration is used. While 6-methylpurine inhibits RNA synthesis and cycloheximide inhibits protein synthesis (Key, 1966), there is no significant difference in the amount of inhibition being exerted by the inhibitors. The data indicate that protein synthesis may be essential for normal cell elongation, as suggested by Nooden and Thimann (1963).

The inhibition of hypocotyl elongation by 5-fluorouracil is not nearly so severe as that of 6-methylpurine and cycloheximide. This may indicate that 5-fluorouracil does not cause a significant decrease in growth, as suggested by Key and Ingle (1964).

CHAPTER V

DISCUSSION OF RESULTS

Tables I and II show that ethionine severely inhibited elongation of the lettuce seedling hypocotyl and root. The addition of methionine to ethionine-treated seedlings resulted in almost complete reversal of the inhibition. This agrees with data obtained by Boll (1960) and Schrank (1956), who also showed reversal of ethionine-induced inhibition by methionine addition. However, when GA_3 alone was introduced to the seedlings, a definite enhancement of elongation was observed.

The stimulation of RNA synthesis caused by GA_3 has been suggested to be the result of an increase in DNA template and RNA polymerase activity (Johri and Varner, 1968). Although no valid data is presented, the inability of GA_3 to reverse the ethionine-induced inhibition may indicate that protein synthesis is essential for growth, as suggested by Nooden and Thimann (1963).

Another possible mode of action of GA_3 is through methylation of pectic substances in the cell wall and nucleotides of RNA. However, if GA_3 enhances methylation, it does not interact with ethionine in the same manner as does methionine. Although no direct data is presented that normal methylation has to be operative before GA_3 can stimulate elongation, the inability of GA_3 to reverse the ethionine-induced inhibition may suggest that GA_3 is not able to cope with the severity of ethionine inhibition.

The addition of ATP to ethionine-treated lettuce seedlings did not reverse the inhibition of hypocotyl elongation, although other workers (Norris, 1964; Villa-Trevino et al., 1963) have obtained data which suggest that ATP does indeed reverse ethionine-induced inhibition. There have been 2 mechanisms proposed for ethionine-induced inhibition of protein synthesis. One is through a decrease of ATP, as proposed by Norris (1964) and Villa-Trevino et al. (1963), and the other is the inhibition of protein synthesis via inhibition of methylation, as suggested by Moore and Smith (1969). Addition of ATP to lettuce seedlings in this study did not significantly reverse the ethionine-induced inhibition. Since the data indicate that methionine reversed ethionine inhibition and ATP did not, it is possible that some ethionine-methionine interaction is responsible for inhibition of plant growth and subsequent protein synthesis. The fact that Norris used a different system (Avena coleoptiles) could be partially responsible for the difference in the two findings. The inability of ATP to reverse the ethionine-induced inhibition may suggest that ethionine is preventing methylation of t-RNA and subsequent protein synthesis. It is also possible that ethionine may be incorporated into proteins instead of methionine. Regardless of the mode of action by which ethionine acts, the data in table III suggest that ethionine is inhibiting some biochemical process that occurs during the first 8 hours of elongation.

Vince and Turner (1968) showed that far-red light inhibited lettuce seedling hypocotyl elongation up to 36 hours, after which the inhibition was relieved. Blue light was shown by Vince and Turner (1968) to inhibit elongation throughout the entire growth period. In three of the treatments used in this study, blue and red light showed a greater

enhancement of hypocotyl elongation than did far-red light. This is not in agreement with the data of Vince and Turner (1968), who reported blue light to be inhibitory throughout. Although they reported far-red to be inhibitory for 36 hours, it appears that the far-red inhibition, in relation to blue and red light in the 3 treatments shown in figure 2, remains present throughout the entire 48-hour growth period. Vince and Turner (1968) suggested that a measurement made at 48 hours after light treatment began could result in considerable variations. It is possible that an elongation period of more than 48 hours could result in an enhancement of hypocotyls in far-red light to a higher level than seedlings treated with blue light.

It was indicated in figure 2 that hypocotyl elongation of ethionine-treated seedlings was severely inhibited, regardless of which light treatment was used. Addition of methionine to ethionine-treated seedlings resulted in complete reversal of seedlings treated with far-red. The interaction between ethionine, methionine, and gibberellic acid remain operational, regardless of the light treatment used.

Figure 4 showed that the addition of GA_3 in light resulted in an enhancement of chlorophyll synthesis. Gassman and Bogorad (1967) suggested that m-RNA provides information for the synthesis of a protein required for the production of gamma-aminolevulinic acid, which is partially responsible for chlorophyll synthesis. If GA_3 causes an increase of m-RNA, as suggested by Ram Chandra and Varner (1965), then the possibility exists that the increased synthesis of m-RNA is resulting in an enhancement of gamma-aminolevulinic acid, which further causes an increase in chlorophyll synthesis. Regardless of the mechanism involved,

a definite enhancement of chlorophyll synthesis has been shown in seedlings treated with GA_3. This is similar to the effect that GA_3 has upon hypocotyl elongation. The weight of the seedlings in each treatment was not taken into consideration for estimation of chlorophyll. The amount of chlorophyll was based upon 100 seedlings per treatment. If the absorbance had been calculated per milligram (mg.) of fresh weight, it is possible that the amount of chlorophyll synthesis in each treatment might have been different from that as shown in figure 4.

There is a possibility that chlorophyll could require methylation for normal synthesis, possibly at positions 1, 3, or 5 of the porphyrin complex. However, further work is necessary to verify this methylation requirement.

The data in figure 3 showed that dark-grown seedlings in both water and GA_3 were significantly enhanced to a level greater than those in light. The inhibition of elongation in light agrees with data obtained by Lockhart, who observed a similar inhibition of pea stems in light. It is apparent from the data in figure 3 that some factor in the lettuce seedling, which may be replaced by GA_3, is controlling the relative rate of hypocotyl elongation in both light and darkness.

The addition of actinomycin-D at concentrations of 5 µgm./ml. and 15 µgm./ml. had only a slight effect upon hypocotyl elongation. However, an increase in the concentration to 25 µgm./ml. resulted in an inhibition of 58 percent. Increasing concentrations of the antibiotic caused a slight increase in the amount of inhibition. It is concluded that the threshold concentration of inhibition is 25 µgm./ml.

Data acquired by Key and Ingle (1964) showed soybean hypocotyl elongation to be inhibited by 70 percent at a concentration of only 10 µgm./ml. of actinomycin-D. However, the soybean hypocotyls were measured after an incubation period with actinomycin-D of only 8 hours. Had Key and Ingle taken the measurements after a longer period of incubation with actinomycin-D, it is most probable that the amount of inhibition would have been less. However, the ability of actinomycin-D to penetrate the different kinds of tissue cannot be overlooked. Since actinomycin-D inhibits DNA-dependent RNA synthesis (Goldberg and Reich, 1964), it is possible that at certain concentrations of the antibiotic, enough RNA synthesis is occurring to allow growth to proceed at a near normal rate.

The slight inhibition of lettuce seedling hypocotyl elongation by 5-fluorouracil does not agree with data of Key and Ingle (1964), which shows no inhibition of soybean hypocotyl elongation, even though RNA synthesis was inhibited 50 percent. Here again, as with actinomycin-D, Key and Ingle measured soybean seedlings after only 8 hours of incubation with 5-fluorouracil. They showed synthesis of ribosomal RNA (r-RNA) to be inhibited by 90 percent, t-RNA by 75 percent, and m-RNA inhibition was only 10 percent. They further concluded that m-RNA synthesis was essential for growth.

Cycloheximide and 6-methylpurine show severe inhibition of lettuce seedling hypocotyl elongation. Key (1966) also showed data to this effect. Although no direct data is presented, it is suggested that cycloheximide inhibits elongation of lettuce seedling hypocotyl by inhibiting protein synthesis. This necessity of protein synthesis for normal cell elongation has been suggested by Key (1966). Similarly, Key (1966) suggested that RNA synthesis is also essential for cell elongation.

Thus, the inhibition of hypocotyl elongation by 6-methylpurine agrees with the idea that some RNA synthesis is essential for normal growth.

The inhibition of hypocotyl elongation by 5-fluorouracil was slight as compared to that of cycloheximide and 6-methylpurine. Key and Ingle (1964) showed that 5-fluorouracil inhibits the synthesis of r-RNA by 90 percent in soybean hypocotyl. They observed no inhibition of growth. Due to only a slight inhibition of elongation in the lettuce seedling by 5-fluorouracil, it is possible that normal cell elongation may not require synthesis of r-RNA, while synthesis of m-RNA or t-RNA may be essential for normal cell elongation. Key and Ingle (1964) have also made this suggestion.

CHAPTER VI

SUMMARY

This study was undertaken to observe: (1) the interaction between various combinations of ethionine, methionine, and gibberellic acid upon elongation of lettuce seedling hypocotyl; (2) the effects of blue, red, far-red, and white light upon elongation of lettuce seedling hypocotyl; and (3) the effects of various inhibitors of RNA and protein synthesis upon elongation of lettuce seedling hypocotyl.

Results indicate that ethionine severely inhibits elongation of both lettuce seedling hypocotyls and roots while addition of methionine to ethionine-treated seedlings results in an almost complete reversal of ethionine-induced inhibition of elongation. Gibberellic acid causes a definite enhancement of hypocotyl and root elongation. However, addition of ethionine to gibberellic acid-treated seedlings results in an inhibition very similar to seedlings treated with ethionine alone. Addition of methionine to seedlings treated with ethionine and gibberellic acid results in an enhancement similar to seedlings treated with gibberellic acid alone. Addition of adenosine triphosphate to ethionine-treated seedlings does not significantly reverse the inhibitory effect of ethionine. It is concluded that ethionine inhibits protein synthesis other than by decreasing synthesis of adenosine triphosphate, possibly by inhibiting methylation of transfer ribonucleic acid.

The ethionine inhibition and GA_3 stimulation of lettuce seedling hypocotyl elongation is prevalent in red, blue, and far-red light as well as darkness. In all qualities of light tested, methionine is

capable of reversing the ethionine inhibition and restoring the GA_3 stimulation of hypocotyl elongation which had been nullified by ethionine treatment.

Seedlings treated with far-red light showed hypocotyl elongation similar to that of seedlings grown in darkness. Likewise, seedlings grown in red light elongated to a level similar to those treated with blue light. The effect of darkness and far-red light upon hypocotyl elongation was distinctly different from seedlings treated with blue and red light.

The studies with actinomycin-D showed that hypocotyl elongation is most severely inhibited at a concentration of 75 micrograms per milliliter. The threshold concentration of inhibition is 25 micrograms per milliliter.

The degree of inhibition exerted by 5-fluorouracil is only slight as compared to that of cycloheximide and 6-methylpurine. This may suggest that synthesis of all RNAs is not essential for normal growth to occur. The severe inhibition of hypocotyl elongation by cycloheximide and 6-methylpurine are consistent with the idea that protein synthesis and some RNA synthesis is essential for cell elongation to occur.

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