The relation between cell wall protein and nucleic acid synthesis in tall pea plants treated with a growth retardant AMO-1618.

Yu Tu
University of Windsor

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THE RELATION BETWEEN CELL WALL PROTEIN AND NUCLEIC ACID SYNTHESIS IN TALL PEA PLANTS TREATED WITH A GROWTH RETARDANT AMO-1618

BY

YU TU

A THESIS
Submitted to the Faculty of Graduate Studies through the Department of Biology in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Windsor

WINDSOR, ONTARIO, CANADA
1970

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ABSTRACT

Ways of modifying plant growth continue to interest plant physiologists. Recently the growth retardant AMO-1618 (2-isopropyl-4-(trimethyl ammonium chloride)-5-methylphenyl piperidine-1-carboxylate) has been shown to slow cell division and cell elongation in stem and regulate plant height without formative effects. The present study was initiated to gain additional information on the mode of action of AMO-1618 on tall pea plants with regard to changes in dry weight, cell wall, protein and nucleic acid synthesis during internode expansion. Data obtained suggests that AMO-1618 retards plant growth by altering the nucleic acid synthesis which in turn affects the protein synthesis, leading to the altered cell wall synthesis.
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INTRODUCTION

The use of chemicals to retard the growth of plants is a recent development in the field of plant growth regulation. A number of chemical compounds which have the specific effect of retarding stem elongation have been described by Wirwille and Mitchell (1950), Tolbert (1959) and Cathey (1964). Although these compounds are chemically unrelated to each other, they have been found to give the same general growth response with various plant species. Hence, they have been commonly designated as "Plant growth retardants". These compounds include 2-chloroethyl trimethyl ammonium chloride (CCC), 2,4-dichlorobenzyl tributyl phosphonium chloride (Phosfon-D), alkyl trimethyl ammonium bromide (AMAB), 2-isopropyl-4-(trimethyl ammonium chloride)-5-methylphenyl piperidine-1-carboxylate (AMO-1618) and N-dimethylamino succinamic acid (Alar).

The most striking symptoms resulting from treatment with these compounds are a marked decrease in stem and petiole elongation and an increase in the green color of

The following abbreviations have been used throughout this thesis: GA, gibberellic acid; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; lr-RNA, light ribosomal RNA; hr-RNA, heavy ribosomal RNA; MAK, methylated albumin on kieselguhr; m-RNA, messenger RNA; and s-RNA, soluble RNA.
of leaves. Some effects on flowering and leaf expansion have also been observed. The marked specificity of action of retardants on stem elongation not only suggests immediate practical applications, but interesting physiological questions. Since their effects on plant growth and development are in many respects opposite and antagonistic to those of gibberellins, they are also designated as "anti-gibberellins" (Tolbert, 1961; Lockhart, 1962). Most of the existing literature on these compounds deals with their effects on morphological changes and their relationship with gibberellins. Very little is known about their effects on metabolic processes in plants except for several reports on enzyme activity.

The present study was undertaken to gain additional information on the metabolic effects of a growth retardant AMO-1618, with particular attention to its relationship to synthesis of cell wall, protein and nucleic acid in addition to growth.
LITERATURE REVIEW

A group of newly synthesized quaternary ammonium carbamate compounds discovered by Wirwille and Mitchell (1950) was observed to have marked regulatory activity on growth phenomena of several species of plants. The addition of small amounts of these compounds to growing plants caused a reduction in internode length which resulted in a plant with a dwarfed appearance.

The synthesis and biological activity of a number of quaternary ammonium compounds were described by Krewson et al. (1959). The compound most extensively used in their growth studies on plants was AMO-1618. With further studies on the effect of several analogs of this compound, on the growth of Black Valentine beans, they suggested that the two nitrogen atoms, one a quaternary nitrogen and the other a carbamate nitrogen, were essential for biological activity. They also found that the substitution of either a methyl, or isopropyl or tertiary butyl groups on the 2 and 5 positions of the aromatic ring was important in determining the activity of this compound.
In addition to this chemical specificity there is a high degree of species specificity. Marth, Preston and Mitchell (1954) studied the response of 44 species of plants to AMO-1618 and classified a large group as non-responsive, another group as moderately responsive and 7 species as highly responsive. Included in this last group were Black Valentine bean, chrysanthemum, lettuce, peas and sunflower. There is no obvious correlation between taxonomic classification and plant response to the compound. For example, Cathey (1958) found that closely related strains of chrysanthemums responded quite differently to applications of AMO-1618. From other studies by Halevy and Cathey (1959), it appears that individual species responded quite differently to growth retardants, e.g., a particular plant might be non-responsive to one but quite responsive to another slightly different quaternary ammonium carbamate.

Although the most pronounced effect of AMO-1618 on plant growth is the reduction in internode length, other effects have also been observed. Marth and Mitchell (1959) applied AMO-1618 to Black Valentine beans (1) as a spray, (2) as a soil treatment, and (3) as a lanolin paste mixture. Treated plants had an intensified green leaf color, retarded flower development and increased longevity or delayed maturity. Marth et al. (1954) indicated that the effect of AMO-1618 carried over by the seeds to the progeny was evident in the two succeeding generations. But the

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intensity of these effects decreased with each successive generation and no effect could be observed after three or four generations.

One interesting effect of AMO-1618 is its ability to increase resistance to chemical and physical changes in the plant environment. Lona (1962) reported that AMO-1618 caused considerable frost hardiness of the stems and petioles of herbaceous plants. Young soy bean (Marth and Frank, 1961) and wheat plants (Miyamoto, 1962) retarded with AMO-1618 showed a marked increase in resistance to high salt content and pH change in the soil.

Sachs et. al. (1959, 1960) observed that cell divisions in the subapical meristematic region of AMO-1618 treated chrysanthemums disappeared almost completely. They pointed out the relationship between this reduction in cell division and subsequent internode shortening. Scherf (1952) observed that parenchymatous cortical cells in the first internode of AMO-1618 treated bean plants were 69% shorter than cells in the controls. Similarly, Wheaton (1960) showed that the pith cells in the mature third internode of Alaska Pea treated with AMO-1618 were significantly shorter than those in normal plants. He suggested that application of AMO-1618 resulted in inhibition of cell elongation rather than in reduction in the number of cells.
Attempts have been made to elucidate changes in the metabolic processes of the plant as a result of treatment with AMO-1618. It was shown by Halevy (1962,63, and 64), Monselise and Halevy (1962), Gaspar and Lacoppe (1968) and Pillay (1970) that AMO-1618 markedly increased peroxidase and catalase activities in cucumber, citrus, barley and soybean seedlings. Wheaton (1960) reported that the activity of extractable pectin methylesterase of stem sections of Alaska Pea seedlings treated with AMO-1618 was higher than in untreated seedlings. He further indicated that the short internode condition could not be correlated with any changes in choline metabolism as measured by incorporation of radioactivity from C\textsuperscript{14}-methyl-labeled methionine into free or phospholipid choline.

The action of gibberellins and the growth retardants in altering the growth of plants is mutually antagonistic. This antagonism was found for stem growth of bean (Downs and Cathey, 1960) and (Lockhart, 1961, 1962), cucumber (Halevy, 1962), chrysanthemum (Cathey, 1958), potato (Kawahard, 1962) and wheat (Tolbert, 1960, 1961); for cell division in chrysanthemum (Sachs et al. 1960) and for catalase and peroxidase activity in cucumber seedlings (Halevy, 1962). Based on their biological activities and on kinetic studies, Lockhart (1961, 1962) and Tolbert, (1961) regarded growth retardants as antigibberellins.
Lockhart (1962) concluded that they exerted their influence by reducing the activity of gibberellins in the growth system. Kende, Ninneman and Lang (1963), using a strain of Fusarium moniliforme, found that AMO-1618 suppressed the biosynthesis of gibberellins without affecting growth. Similarly, Baldev et al. (1965) have also shown that AMO-1618 inhibits the accumulation of gibberellin-like substances in developing pea seeds. West and Upper (1965) in their studies of gibberellin biosynthesis found that AMO-1618 inhibits the formation of (-)-kaurene and (-)kaurene-19-ol but not transgeranylgeraniol from mevalonate in homogenates of endosperm and nucellus from Echinocystis macrocarpa seeds. Enzymic cyclization of transgeranylgeranyl pyrophosphate was inhibited by concentrations of AMO-1618. These results suggest that the action of growth retardants on plants is related to inhibition of gibberellin synthesis. However, other results, such as those of Cabler (1964) on chrysanthemum; Cleland (1965) on Avena and Paleg et al. (1965) on α-amylase in barley show little or no mutual antagonism between the retardants and gibberellin.
MATERIALS AND METHODS

Seeds of tall peas *Pisum sativum* L.var. "Tall Telephone" were sown in vermiculite in plastic pans. Plants were grown under controlled environmental conditions in a growth chamber with a light intensity of 1100 foot candles (mixed with fluorescent and incandescent lamps) and a temperature of 25°±2°C. When the fifth internodes of the seedlings emerged, plants were selected for uniformity and sprayed with either 10⁻³M AMO-1618 or 10⁻³M gibberellic acid (GA) or water for control. Duplicate samples of third and fifth internodes were harvested at various times after treatment to determine changes in dry weight, cell wall material, protein and nucleic acids. The plant parts were dried at 100°C for at least two days and stored in a desiccator before making the dry weight determinations.

Determination of Cell Wall Material

The method used for separating cell wall material was based on the work of McComb(1966). Samples of 8 internodes were harvested, weighed and deep-frozed until required. Each sample was thawed and blended for 20 minutes in 40 ml of water, using a Sorvall Homogenizer. The blend was centrifuged for 15 minutes at 1200 x g and the supernatant discarded. The solid residue was taken up in water and maintained at 100°C for three hours. Solids were recovered by centrifugation and the extraction was repeated two more times. The solid fraction was then suspended in a mixture.
of ethanol and benzene (1:2 by volume) and kept at 70°C for 6 hours. Solids were recovered by filtration through a Buchner filter, and the white papery residue was transferred on the filter pad to an oven at 70°C to remove traces of solvent. After drying in a desiccator, the wall extract was removed and weighed. The weight of extract was divided by the number of internodes to give an estimate of the amount of cell wall per internode. When a sample of internode was harvested for wall analysis, a comparable sample was taken for fresh and dry weight determinations. The fresh weights of the two samples were comparable but not necessarily identical. The ratio of dry to fresh weight was determined from the second sample and this value was used to calculate the dry weight of the sample from which wall material was extracted. (See Appendix A).

Determination of Protein and Soluble Nitrogen Content

Protein and soluble nitrogen were extracted by the procedures described by Thimann and Laloraya (1960). A 1 g sample of internodes was ground with 10 ml of cold 10% trichloroacetic acid (TCA) at 4°C to precipitate the protein and centrifuged at 2000 x g for 30 minutes. The supernatant was decanted and the precipitate washed with 5 ml of 10% TCA and again centrifuged. The combined supernatants and washings were analyzed for soluble nitrogen. To the protein precipitate 5 ml of 5% TCA was added and
it was then incubated for 30 minutes at 80°C to remove the nucleic acids. The precipitate now obtained after centrifugation was analyzed for protein and the supernatant discarded. The soluble and protein nitrogen were estimated by the standard microkjeldahl methods. The material was transferred to a kjeldahl flask for digestion with sulphuric acid, using selenium catalyst. Later the digest was placed on a micro steam distilling unit. After adding 5 ml of 6% NaOH, NH₃ was distilled and trapped in 4% boric acid and titrated with 0.0714N sulfuric acid. (See Appendix B)

**Determination of Total Nucleic Acid**

Internode samples with fresh weight 0.5 g were homogenized in 2 ml cold methanol in a Sorvall homogenizer. The homogenate was transferred to a centrifuge tube and the homogenizer was washed twice with 2 ml of methanol. The combined homogenate and two washings were centrifuged at 1100 x g and the supernatant was discarded. The pellet was washed twice with 4 ml volumes of cold methanol and the supernatant was discarded. The methanol-insoluble material was extracted twice with 4 ml of 0.2 M HClO₄ to remove acid-soluble phosphates. The acid precipitate was next washed with 4 ml of cold ethanol to remove the acid and other soluble materials. This step was followed by removing lipids with ethanol: ether (2:1) at 50°C for 30
minutes. The extract was then incubated with 5 ml of 5% HClO₄ at 70°C for 40 minutes and then centrifuged. The supernatant was collected. The insoluble pellet was washed once with 1 ml 5% HClO₄, by centrifuging; this supernatant was added to the first. The pH of the supernatant was adjusted approximately to 7 and its volume to 10 ml with H₂O and the soluble KClO₄ was collected in the cold by centrifugation. The supernatant was read at 260 and at 290 μm. The difference between 260 minus 290 μm reading was multiplied by 57 to obtain μg nucleic acid per ml. DNA content was estimated by the diphenylamine test for deoxyribose. 1 ml of the neutralized acid extract was incubated with 2 ml of diphenylamine reagent containing acetaldehyde. Also, tubes which contained 25 μg DNA as a standard and a blank containing 5% KClO₄ were prepared. RNA was calculated by the subtraction of DNA values from total nucleic acid. The above estimation of the nucleic acid content of internodes is based on the method described by Cherry(1962). (See Appendix C).

The Incorporation of Radioactivity from H³-L-Leucine and H³-L-Proline into Protein

Samples of internodes equivalent to 1 gram fresh weight were incubated for various time intervals(2, 6, 10 hours) at 32°C in 8 ml solution of 1% sucrose, 10⁻⁴ M ammonium citrate buffer pH 6.7 and 10⁻³ M AMO-1618,
containing 125 μCi of H³-leucine with a specific activity of 15.8 Ci/mM or H³-proline with a specific activity of 6.7 Ci/mM. (Labelled amino acids were obtained from Amersham-Searle Co., Toronto). At the termination of the incubation period, the internodes were thoroughly washed with unlabelled leucine or proline and subsequently homogenized in 10 ml of 0.5 M mannitol-0.02M tris (pH 9.0), 0.005M MgCl₂ and 0.005M KCl in a Sorvall homogenizer. The homogenates were centrifuged for 10 minutes at 2000 x g. One ml of 15% TCA and 0.5 ml of egg albumin were added to the supernatant and allowed to stand for 20 minutes and centrifuged again at 10000 x g for 15 minutes. The pellet thus formed was washed 3 times with 5% TCA and then dissolved with 0.1N NaOH. Radioactivity was measured with a liquid scintillation counter, using toluene containing 0.3% PPO and 0.01% POPOP. (The method is similar to that used by Kuraishi and Yamaki(1967)).

Isotope labeling, Extraction and Fractionation of Nucleic Acids

Two grams of the fifth internode collected from AMO-1618 treated or control pea plants were incubated for 3 hours in the dark in 25 ml of a medium containing 1% sucrose, 10⁻⁴ M ammonium citrate buffer pH 6.0, 25 μg/ml chloroamphenicol and 0.3 mCi^{32}P. (Sp. activity 7.7 Ci/ml. ^{32}P was obtained from Atomic Energy of Canada Ltd.). Following incubation, the internodes were extracted by a phenol-tris buffer method described by Cherry et.al. (1965) (see Appendix D) and fractionated on MAK
(methylated albumin keiselguhr) column according to the method of Mandell and Hershey (1960) (see Appendix E). Nucleic acids were eluted by means of a linear gradient of NaCl from 0.3M to 1.5M in sodium phosphate buffer in consecutive 5 ml fractions and their U.V. absorbancy recorded at 260 mu. The radioactivity of $^{32}$P incorporated into nucleic acid was determined in a liquid scintillation counter.
RESULTS

Effect of AMO-1618 on fresh and dry weight distribution in tall pea plants

Changes in fresh and dry weights of various organs of young pea seedlings collected at different harvest dates are summarized in Table 1. Following application of AMO-1618 by spraying, the seedlings collected at different times were dissected into roots, shoots (all internodes and leaves), cotyledon, and stem (all internodes) for determinations. It is apparent from the data, that the fresh and dry weights of cotyledon in the treated plants were highest during different time intervals. Because the cotyledon is a storage tissue, which provides nutrients for embryo growth, these data might indicate that the mobilization of organic nutrients was partially inhibited by the application of AMO-1618. There was little change in the fresh and dry weights of root during the experimental period. However, a marked decrease in the rate of incorporation of dry weight into shoots of AMO-1618 treated plants was observed as compared to roots. The most striking effect caused by AMO-1618, was the change in dry weight accumulation by the stem, which was revealed in all harvests. The effect is even more obvious, when data are considered for the fifth internodes, which were expanding during the experimental period (Table 2). The amount of water present in each internode, which is taken as an index of cell volume, was obtained by subtracting dry
TABLE 1

Effect of AMO-1618 on fresh and dry weights of various organs of tall pea plants, harvested at different times after treatment. All weights are in milligrams (mg).

<table>
<thead>
<tr>
<th>Time elapsing between treatment and harvest (days)</th>
<th>0</th>
<th>3</th>
<th>6</th>
<th>9</th>
<th>12</th>
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<tbody>
<tr>
<td></td>
<td>Fresh</td>
<td>Dry</td>
<td>Fresh</td>
<td>Dry</td>
<td>Fresh</td>
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<tr>
<td>Shoot (Leaves plus internodes)</td>
<td>Water</td>
<td>326</td>
<td>30.1</td>
<td>528</td>
<td>64.3</td>
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<tr>
<td></td>
<td>AMO</td>
<td>-</td>
<td>-</td>
<td>459</td>
<td>51.8</td>
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<tr>
<td>Cotyledon</td>
<td>Water</td>
<td>781</td>
<td>92.0</td>
<td>631</td>
<td>71.3</td>
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<tr>
<td></td>
<td>AMO</td>
<td>-</td>
<td>-</td>
<td>695</td>
<td>83.1</td>
</tr>
<tr>
<td>Root</td>
<td>Water</td>
<td>690</td>
<td>42.8</td>
<td>1012</td>
<td>62.4</td>
</tr>
<tr>
<td></td>
<td>AMO</td>
<td>-</td>
<td>-</td>
<td>1031</td>
<td>64.7</td>
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<tr>
<td>Stem (All internodes)</td>
<td>Water</td>
<td>191</td>
<td>10.2</td>
<td>304</td>
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<tr>
<td></td>
<td>AMO</td>
<td>-</td>
<td>-</td>
<td>245</td>
<td>18.5</td>
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TABLE 2

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<th>12</th>
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<tr>
<td>Internode dry weight (mg)</td>
<td>Water 1.7</td>
<td>4.1</td>
<td>6.3</td>
<td>8.1</td>
<td>8.8</td>
</tr>
<tr>
<td>Weight of water per internode (mg)</td>
<td>AMO 2.7</td>
<td>3.4</td>
<td>4.1</td>
<td>4.5</td>
<td>5.6</td>
</tr>
<tr>
<td>Weight of water per internode (mg)</td>
<td>Water 10</td>
<td>21</td>
<td>44</td>
<td>27</td>
<td>33</td>
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<tr>
<td>Protoplasm weight (mg)</td>
<td>AMO 16</td>
<td>2.9</td>
<td>4.0</td>
<td>2.1</td>
<td>4.9</td>
</tr>
<tr>
<td>Protoplasm weight (mg)</td>
<td>Water 1.4</td>
<td>1.8</td>
<td>4.9</td>
<td>2.3</td>
<td>5.3</td>
</tr>
<tr>
<td>Protoplasm weight (mg)</td>
<td>AMO 21</td>
<td>46</td>
<td>24</td>
<td>50</td>
<td>26</td>
</tr>
<tr>
<td>Internode length (mm)</td>
<td>AMO 17</td>
<td>17</td>
<td>20</td>
<td>24</td>
<td>26</td>
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</table>

Effect of AMO-1618 on dry weight, water weight, protoplasm weight and length in fifth internodes of tall pea plants, harvested at different times after treatment.

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weight from fresh weight and is included in Table 2. In Fig. 1, water content is plotted against dry weight for the fifth internodes during expansion. Water content and the dry weight during the expansion period are strongly correlated. (Correlation coefficient, $r = 0.942$).

**Effect of AMO-1618 upon cell wall synthesis**

Sachs et al. (1960) and Wheaton (1960) presented evidence that the reduction in stem length obtained by AMO-1618 was a result of inhibition of cell division and cell elongation respectively in the subapical meristem. It was therefore, reasonable to suspect, that the inhibition of cell division and elongation may be connected with changes in cell wall synthesis. Accordingly, the effect of AMO-1618 on the rate of cell wall synthesis was investigated. Attention was concentrated on the amount of cell wall deposited in the fifth internode of a plant during its expansion. Because gibberellins are antagonistic to AMO-1618 with respect to internode elongation, some of the plants were also treated with GA for comparison. Samples of 8 internodes were harvested and the cell wall extracted as described previously. Results presented in Fig. 2 show that AMO-1618 caused a pronounced decrease in the rate of cell wall synthesis, while GA caused an increase. The effect was especially noticeable between 6 and 9 days after treatment. The amount of water is plotted against the weight of cell wall in Fig. 3. There is a definite correlation.
FIGURE 1

The relation between the amount of water and the dry weight in fifth internodes of tall pea plants

The data are for the fifth internodes of plants harvested from 0 to 12 days after the commencement of the experiment. (AMO=AMO-1618, CK=Control)
FIGURE 2

The effect of AMO-1618 and GA on the weight of cell wall material in fifth internodes of tall pea plants

Samples of 8 internodes were harvested at different times after treatment and extracted 3 times with water at 100°C for 3 hr and finally with a mixture of ethanol and benzene (1:2, v/v) at 70°C for 6 hr. (AMO=AMO-1618, GA=Gibberellic Acid, CK=Control)
The relation between the amount of water and the weight of cell wall material in fifth internodes of tall pea plants

The data are for the fifth internodes of plants harvested from 0 to 12 days after the commencement of the experiment. (AMO=AMO-1618, CK=Control)
between these parameters (Correlation coefficient, \( r=0.997 \)). The difference between wall weight and total dry weight has been used as an index of protoplasmic dry weight (Table 2). This, like the wall weight, decreased following AMO-1618 treatment. It is necessary to emphasize here that the immature fifth internode was treated with AMO-1618 or GA, prior to internode elongation. In order to determine whether AMO-1618 had any effect on other internodes, the third internode, which was almost fully expanded at the time of treatment, was investigated. Results presented in Table 3 show that there was little change with treatment or with time in the amount of wall material or protein in third internode.

**Effect of AMO-1618 upon protein synthesis**

From Fig. 2 we have concluded that there is a decrease in the rate of wall synthesis following AMO-1618 treatment. Any change in cell wall synthesis might therefore be expected to be accompanied by a change in protein synthesis. The amount of protein in expanding internodes harvested at different dates is presented in Fig. 4. As in the case of cell wall synthesis, the data on protein synthesis clearly indicates that AMO-1618 caused decrease in the rate of protein synthesis in expanding internodes. In contrast, GA caused a striking increase. The ratio between protein and fresh weight presented in Table 4 decreased with time. From Table 3 we recall that there was


**TABLE 3**

Effect of AMO-1618 and GA on cell wall weight and protein content in third internodes of tall pea plants.

<table>
<thead>
<tr>
<th></th>
<th>Time elapsing between treatment and harvest (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td><strong>Cell wall weight (mg)</strong></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>1.3</td>
</tr>
<tr>
<td>AMO</td>
<td>-</td>
</tr>
<tr>
<td>GA</td>
<td>-</td>
</tr>
<tr>
<td><strong>Protein content (µg)</strong></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>61</td>
</tr>
<tr>
<td>AMO</td>
<td>-</td>
</tr>
<tr>
<td>GA</td>
<td>-</td>
</tr>
</tbody>
</table>
FIGURE 4

The effect of AMO-1618 and GA on protein content in fifth internodes of tall pea plants

Protein was estimated in trichloracetic acid extracts, by subjecting the nucleic acid free precipitate to standard micro-kjeldahl procedures. (AMO=AMO-1618, GA=Gibberellic Acid, CK=Control)
TABLE 4

Effect of AMO-1618 and GA on length, ratio of cell wall/fresh weight and protein/fresh weight in fifth internodes of tall pea plants harvested at different times after treatment.

<table>
<thead>
<tr>
<th></th>
<th>Time elapsing between treatment and harvest (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Internode length (mm)</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>6</td>
</tr>
<tr>
<td>AMO</td>
<td>-</td>
</tr>
<tr>
<td>GA</td>
<td>-</td>
</tr>
<tr>
<td>Fresh weight (mg)</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>10</td>
</tr>
<tr>
<td>AMO</td>
<td>-</td>
</tr>
<tr>
<td>GA</td>
<td>-</td>
</tr>
<tr>
<td>Cell wall/Fr.wt. (mg/mg)</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>0.03</td>
</tr>
<tr>
<td>AMO</td>
<td>-</td>
</tr>
<tr>
<td>GA</td>
<td>-</td>
</tr>
<tr>
<td>Protein/Fr.wt. (μg/mg)</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>3.1</td>
</tr>
<tr>
<td>AMO</td>
<td>-</td>
</tr>
<tr>
<td>GA</td>
<td>-</td>
</tr>
</tbody>
</table>
little effect of AMO-1618 and GA on protein in the third internode, which had expanded completely at the time of treatment. In the expanding fifth internode, AMO-1618 and GA elicite, respectively, progressive decreases and increases in soluble nitrogen content (Table 5). The changes elicited by GA are similar to those observed by Rai and Laloraya (1965) for lettuce seedlings.

**Effect of AMO-1618 on the incorporation of $H^3$-leucine and $H^3$-proline into protein**

In addition to the fact that protein in the expanding internode was reduced by AMO-1618 treatment (Fig. 4), it was desirable to determine whether or not AMO-1618 had any effect on *de novo* protein synthesis. This was done by measuring the incorporation of labeled amino acids into protein.

The internodes were incubated for 2, 6 and 10 hours with $H^3$-leucine or $H^3$-proline. Data presented in Table 6 show that tissue incubated with AMO-1618 solution strongly inhibited the incorporation of $H^3$-leucine into water soluble protein. During the shortest incubation period (2 hrs) little decrease in incorporation was found as a result of AMO-1618 treatment. However, with increasing incubation time (6 and 10 hrs) incorporation in treated tissue decreased by more that 50%. This might indicate that AMO-1618 needs a certain lag period to show its effect on the tissue. Decreases in $H^3$-proline incorporation
TABLE 5

Effect of AMO-1618 and GA on soluble nitrogen in fifth internodes of tall pea plants harvested at different times after treatment.

<table>
<thead>
<tr>
<th>Soluble nitrogen (µg)</th>
<th>Time elapsing between treatment and harvest (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Water</td>
<td>27</td>
</tr>
<tr>
<td>AMO</td>
<td>-</td>
</tr>
<tr>
<td>GA</td>
<td>-</td>
</tr>
</tbody>
</table>
### TABLE 6

Effect of AMO-1618 on the incorporation of H₃-Leucine and H₃-Proline into protein of fifth internodes of tall pea plants after various incubation periods.

<table>
<thead>
<tr>
<th></th>
<th>Incubation Time</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 hr</td>
<td>6 hr</td>
<td>10 hr</td>
</tr>
<tr>
<td></td>
<td>cpm</td>
<td>cpm</td>
<td>cpm</td>
<td>cpm</td>
</tr>
<tr>
<td>Leucine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>3024</td>
<td>11657</td>
<td>16197</td>
<td></td>
</tr>
<tr>
<td>AMO</td>
<td>2820</td>
<td>5854</td>
<td>7509</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>3016</td>
<td>8236</td>
<td>10560</td>
<td></td>
</tr>
<tr>
<td>AMO</td>
<td>2724</td>
<td>7908</td>
<td>9342</td>
<td></td>
</tr>
</tbody>
</table>
were not as conspicuous as those in leucine incorporation.

**Effect of AMO-1618 on nucleic acid synthesis**

The total DNA and RNA content of expanding internodes following AMO-1618 and GA treatment was estimated (Figs. 5 and 6). From these figures we see that the changes in the length that resulted from AMO-1618 and GA treatment are accompanied by changes in the rates of RNA and DNA synthesis. During the early stages of expansion (Fig. 6), the rate of DNA synthesis was relatively low in treated and control plants. AMO-1618 and GA had slight effects on DNA synthesis during this period. However, a marked effect on the rate of RNA synthesis was elicited by AMO-1618 and GA in this stage (Fig. 5). After 48 hours, GA elicited a massive increase in DNA in this expanding internode (Fig. 6). When the experiment was terminated, GA treated plants had approximately twice the amount of DNA as the controls and three times that of AMO-1618 treated plants. In summary, AMO-1618 decreased the rate of both DNA and RNA synthesis, while GA increased it. Furthermore, plants treated with both GA and AMO-1618 showed, that these compounds are mutually antagonistic with respect to effects on nucleic acid synthesis.
FIGURE 5

The effect of AMO-1618 and GA on RNA content in fifth internodes of tall pea plants

RNA was calculated by the subtraction of DNA from total content of nucleic acids. (AMO=AMO-1618, GA=Gibberellic Acid, CK=Control)
FIGURE 6

The effect of AMO-1618 and GA on DNA content in fifth internodes of tall pea plants

DNA content was estimated by the diphenylamine test. (AMO=AMO-1618, GA=Gibberellic Acid, CK=Control)
Effect of AMO-1618 upon the incorporation of $^{32}\text{P}$ into nucleic acids

Since the results obtained for total nucleic acids showed strong treatment effects, this study was extended to include the effect of AMO-1618 on synthesis of specific nucleic acid fractions, using $^{32}\text{P}$ incorporation, followed by MAK column chromatography. The elution profiles of phenol-extracted $^{32}\text{P}$-labeled nucleic acids from internodes of treated and control plants, separated on MAK columns are presented in Figs. 7 and 8. The profiles including at least five major fractions are similar to those obtained from other plant tissues by Cherry (1965) and Ingle and Key (1965). The soluble RNA was separated into two ultraviolet absorbing peaks designated as $S_1$ and $S_2$. The third peak is a DNA-RNA fraction which has been characterized by Cherry (1964) to comprise of 3 components: a rapidly metabolized RNA, a rapidly metabolized DNA and a non-metabolized DNA. Most of the RNA was ribosomal. The fourth and fifth peaks were light ribosomal RNA and heavy ribosomal RNA fractions respectively. However, the so-called mRNA fraction which should elute just after the hrRNA fraction was not detected. Similar findings were also reported by Brook et al. (1967). It is clear from Table 7 that treatment with AMO-1618 resulted in a decreased specific activity for all nucleic acid fractions. Specific activity
FIGURE 7

MAK column chromatography elution profiles of $^{32}$P-labeled, phenol-extracted nucleic acids of fifth internodes of control tall pea plants

Two grams of internodes were incubated in 25 ml aqueous solution containing 0.3 mCi of $^{32}$P for 3 hours. The nucleic acids were extracted with cold phenol and dialyzed for 48 hours. A 5 ml aliquot from the extract was fractionated on an MAK column using a linear elution gradient of buffered NaCl solution from 0.3 M to 1.5 M. Fractions containing 5 ml were collected for determination of U. V. absorbance and radioactivity.
FIGURE 8

MAK column chromatography elution profiles of 32P-labeled, phenol-extracted nucleic acids of fifth internodes of AMO-1618 treated tall pea plants

Two grams of internodes were incubated in 25 ml aqueous solution containing 0.3 mCi of 32P for 3 hours. The nucleic acids were extracted with cold phenol and dialyzed for 48 hours. A 5 ml aliquot from the extract was fractionated on an MAK column using a linear elution gradient of buffered NaCl solution from 0.3 M to 1.5 M. Fractions containing 5 ml were collected for determination of U. V. absorbance and radioactivity.
## TABLE 7

Effect of AMO-1618 on specific activities of various nucleic acid species in fifth internodes of tall pea plants, calculated from elution profiles in Figs. 7 and 8.

<table>
<thead>
<tr>
<th></th>
<th>$s_1$-RNA</th>
<th>$s_2$-RNA</th>
<th>Total s-RNA</th>
<th>DNA-RNA</th>
<th>lr-RNA</th>
<th>hr-RNA</th>
<th>Total r-RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>189</td>
<td>112</td>
<td>301</td>
<td>169</td>
<td>163</td>
<td>212</td>
<td>375</td>
</tr>
<tr>
<td>AMO</td>
<td>81</td>
<td>85</td>
<td>166</td>
<td>92</td>
<td>146</td>
<td>120</td>
<td>266</td>
</tr>
</tbody>
</table>
was calculated by dividing the total radioactivity counts per minute by the optical density. These data demonstrate decreased nucleic acid synthesis in retardant treated tissue and are consistent with report by Key (1964) indicating a necessity for RNA synthesis as a prerequisite for cell elongation.
DISCUSSION

The problems emerging from studies of the mode of action of growth retardants are complicated by the diversity of response to retardant exhibited by different tissues. Literature on the mechanism of action of AMO-1618 is mainly concerned with its suggested competitive relationship with GA. The inadequate and conflicting reports on this subject suggest the possibility that AMO-1618 may have more than one mode of action.

Plant growth retardation by AMO-1618 can be attributed to inhibition of cell elongation or cell division (Sachs et al., 1960; and Wheaton, 1960). Mechanisms involved in regulation of cell elongation and division have been studied from a number of viewpoints. Firstly, cell elongation has been shown to be intimately correlated with primary wall synthesis (Northcote, 1962; Baker, 1965; Ray, 1965 and McComb and Broughton, 1966). Secondly, involvement of DNA, RNA and protein synthesis in hormone induced cell elongation has been demonstrated (Nooden and Thimann, 1963; Key, 1964; Nitsan and Lang, 1966; and Broughton, 1968). Thirdly, DNA synthesis has been reported to be a prerequisite for hormone induced cell division (Skoog, 1956).

From the above, it is reasonable to expect that AMO-1618 inhibition of cell elongation and cell division might be related to changes in cell wall, protein and nucleic acid synthesis.
The following discussion will explore the possible mode of action of AMO-1618 with respect to these parameters.

As reported by Sachs et. al. (1960) and Wheaton (1960) AMO-1618 treatment greatly influences the subapical region in the shoot apex which is responsible for its elongation. Such effects are evident in inhibited growth and decrease in dry weight of shoots, which is in agreement with the findings reported in this thesis. Lack of such a region in the root was reflected by the small root responses to AMO-1618 treatment (Table 1). Changes in rates of dry weight accumulation in various organs of the treated plant (Table 1) indicates that there is an apparent decrease for the stem. Considering only the effect of AMO-1618 on internode elongation, one is led to the suggestion that the decrease in internode dry weight is more directly associated with the AMO-1618 action than the dry weight changes in the other organs. Data in Fig. 1 show that there is a close relation between accumulation of dry weight and the weight of water in the internode. Therefore, it might be proposed that expansion of AMO-1618 treated internode is limited by some processes which affect dry weight accumulation. Contrary to this, a growth promoter like GA acts to allow increase in the rate of dry weight accumulation (McComb, 1966). It could also be suggested that the decreased dry weight deposited is a result of decrease in cell volume.

Results presented in Figs. 2 and 3 make it clear that
AMO-1618 brings about a decrease in cell wall synthesis in the expanding internode and that cell wall weight is related to weight of water. This indicates that expansion of the AMO-1618 treated internode was affected by a change in the rate of wall synthesis, or alternatively that the decrease in wall material is a result of a decrease in cell volume. While a decrease in wall synthesis may account for part of the decrease of dry weight in expanding internodes, the remainder may be due to decreased synthesis of proteins and other carbohydrate materials.

AMO-1618 treatment also elicited a marked decrease in the rate of protein synthesis in expanding internodes (Fig. 4). Available evidence exists that protein synthesis (Nooden and Thimann, 1963; and Key, 1964) is a critical factor in auxin induced cell expansion. An experiment on the effect of AMO-1618 treatment on incorporation of $H_3^3$-labeled amino acids into protein of pea internodes revealed a marked inhibition of the rate of incorporation of $H_3^3$-leucine. The relatively slight inhibition of the incorporation rate of $H_3^3$-proline suggests the possibility that AMO-1618 may inhibit the synthesis of protein which has a high ratio of leucine in comparison with proline. Accompanying the decrease in protein nitrogen, there is a decrease in soluble nitrogen.

In the third internode which was fully expanded at the time of treatment, AMO-1618 had little effect on the rate of cell wall and protein synthesis (Table 3). This could be due either to lack of response from mature and elongated cells in
the fully expanded tissue, or AMO-1618 had pronounced effect only when tissue differentiation was taking place. It is interesting to note the relationships between the parameters: internode volume, cell wall and protein. It is evident from the data presented that AMO-1618 produces decreases in all three. It is further clear from Table 4 that the rate of protein synthesis in the treated and untreated fifth internodes does not keep pace with the rate of internode growth, since the amount of protein per unit weight decreased. On the other hand, the amount of cell wall material per unit weight changed little during internode expansion. This indicates that the change in growth rate of internode is more closely related to cell wall synthesis than to protein synthesis. This leads us to suggest that while changes in protein synthesis is an important response of AMO-1618, it is not immediately responsible for such changes in growth rate. However, the fact that there is a decrease in the rate of protein synthesis gives support to the suggestion that AMO-1618 brings about an alteration of protein synthesis leading to changed wall synthesis, which in turn produces the change in growth rate of tall peas. But these kinds of changes in the protein synthesis and its resulting effect upon morphology may ultimately be dictated by the cell genotype. Hence, an investigation of effect of AMO-1618 on the amount of DNA and RNA in the expanding tissue was undertaken.
The data of Figs. 5 and 6 demonstrate that the amounts of DNA and RNA in the AMO-1618 treated internodes were reduced as compared with controls. GA induced increases in DNA and RNA agree with the findings of Broughton (1969). It should be pointed out here that during the early stages of internode expansion AMO-1618 inhibition of RNA synthesis became apparent before inhibition of DNA synthesis (Figs. 5 and 6). Therefore, it is possible that AMO-1618 is acting at two different levels in this tissue. The numerous examples of experimental separation of cell elongation and mitosis (Sachs, 1965), support the possibility that separate controls should also be found in pea stem tissue. Assuming that stimulation of RNA synthesis precedes effects on protein synthesis, then the results suggest that regulation of RNA synthesis is one of the first effects of AMO-1618 in the control of pea internode elongation. Aubel-Sadron (1961) and Brook et. al. (1967) stated that growth retardant could form a complex with nucleic acids and increase the nuclease activity in the retardant treated tissue. In addition to this, the decreased specific activity following AMO-1618 treatment strongly suggests that AMO-1618 has an effect on nucleic acid metabolism.

The above observations are consistent with the DNA-RNA-protein dogma of molecular biology in that AMO-1618 appears to cause a change in nucleic acid metabolism, which in turn alters a wide variety of metabolic processes through altered protein
synthesis, ultimately leading to changed cell wall synthesis and retarded growth.
SUMMARY

Physiological effects of AMO-1618 upon the expanding fifth internode of tall peas were studied. The decreased growth rate of the fifth internode following AMO-1618 treatment was accompanied by decreased rates of protein and cell wall synthesis. However, the change in the growth rate was more related to cell wall synthesis than to protein synthesis since the amount of protein per unit weight decreased, while that of cell wall materials per unit weight remained constant during expansion of fifth internode. The effect of AMO-1618 was also compared with that of a growth promoter, GA. The rate of incorporation of $^3$H-leucine into internodes was markedly reduced by AMO-1618. Further, it was shown that the amounts of DNA and RNA were decreased by AMO-1618 as contrasted with controls. The studies of the incorporation of $^{32}$P into nucleic acid indicated that AMO-1618 decreased the synthesis of nucleic acid by reducing the specific activities of different fractions of nucleic acids. It was concluded that AMO-1618 could cause a change in nucleic acid which presumably alters a wide variety of metabolic processes via altered protein synthesis, leading to a change in cell wall synthesis which is the final step in growth retardation.
APPENDIX A

Procedure for determination of cell wall weight

Plant tissue (8 internodes)

↓

Blend in 40 ml of water for 20 minutes and centrifuge for 15 minutes at 1200xg

↓

Supernatant discarded

Residue

↓

Extract 3 times with water at 100°C for 3 hours

↓

Supernatant discarded

Residue

↓

Suspend in ethanol: benzene(1:2) at 70°C for 6 hours

↓

Supernatant discarded

Residue (cell wall material)

↓

Dry and weigh
APPENDIX B

Procedure for determination of protein and soluble nitrogen

Plant tissue (1 gram)

Grind with 10 ml of 10% TCA at 4°C and centrifuge at 2000xg for 30 minutes

Supernatant (soluble nitrogen) → Wash residue with 5 ml of 10% TCA

Supernatant (soluble nitrogen) → Incubate with 5 ml of 5% TCA for 30 minutes at 80°C

Supernatant discarded → Residue (protein)

Estimate by standard microkjeldahl method
APPENDIX C

Procedure for determination of nucleic acid

Plant Tissue (500 mg)

- Homogenize in 2 ml cold methanol
- Wash insoluble pellet twice
- 2 ml cold methanol

 ethanol-soluble material (discard)

- Extract twice with 4 ml cold 0.2M HClO₄

 acid-soluble material (discard)

- Wash with 4 ml cold ethanol

 ethanol-soluble material (discard)

- Extract with 5 ml ethanol: ether (2.1), 50°C, 30 min

 lipid material (discard)

- Contains protein, nucleic acids, starch, cellulose, etc.

- Extract with 5 ml 5% HClO₄

 hydrolyzed nucleic acid (Discard)

- U.V. absorption

 total nucleic acids

- minus DNA

 RNA

- determine deoxyribose by diphenylamine test

 DNA

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The method for extraction of nucleic acid is given by Cherry (1965). Homogenize the 2 gm of P$^{32}$-labeled tissue in a solution containing 40 ml 0.01 M Tris, pH 7.6, 0.06 M KCl, 0.01 M MgCl$_2$; 1 ml bentonite (40 mg/ml); 4.6 ml 11% Dupanol and 60 ml phenol with a Sorvall homogenizer. Homogenize for 1 minute at full speed, then for another minute at low speed and finally for another minute at full speed. Centrifuge the homogenate at 20,000 xg for 10 minutes. Carefully remove the aqueous layer which contains the nucleic acid with a large syringe and needle. The precipitated protein remains at the interphase of the phenol and water. Add 1 ml of bentonite and 40 ml of phenol to this aqueous layer and shake occasionally for about 5 minutes. (Keep ice cold). Centrifuge this mixture at 20,000 xg for 10 minutes and again remove the aqueous layer. Repeat the phenol treatment with 20 ml of phenol at 0°C for 5 minutes. Again centrifuge at 20,000 xg for 10 minutes and remove the aqueous layer. At this stage of extraction there should be very little protein in the aqueous solution. Now add potassium acetate to the aqueous solution to make 0.2 M (approximately 20 mg per ml of solution) and 2 volumes of cold 95% ethanol. Collect the RNA precipitate by centrifuging at 30,000 xg for 20 minutes. Decant the supernatant and dissolve the nucleic acid in 5-10 ml 0.05 M sodium phosphate buffer, pH 6.7. Place the nucleic acid in dialysis tubing and dialyze for 48 hours against 0.05 M sodium phosphate buffer, pH 6.7 in the cold with the buffer being changed three times.
APPENDIX E

The methylated albumin keiselguhr (MAK) column used in this experiment is prepared according to the method of Mandell and Hershey (1960) in the following manner:

a) Methylation of Protein: Suspend 5 gm bovine serum albumin (fraction v) in 500 ml absolute methanol. Add 4.2 ml conc. HCl, tightly seal the bottle and incubate at 37°C for 5 days in the dark. At the termination of the incubation period, collect the precipitate by centrifugation and wash with methyl alcohol twice. The removal of HCl should be done as rapidly as possible to prevent hydrolysis of the methyl ester. Following the methanol washing, wash the precipitate with anhydrous ether. Evaporate most of the ether in the air and store the powdered material in vacuo over KOH. A 1% solution of the methylated albumin is made in H₂O for the column. (The 1% MA should have a pH not lower than 4.0).

b) Preparation of Column: The MAK column used for analysis of nucleic acids is composed of 3 layers in a 2 cm glass tube and is prepared as follows: Boil and cool suspensions of kieselguhr in two beakers: (1) 10 gm in 50 ml of 0.1 M NaCl - 0.05 M sodium phosphate, pH 6.7; (2) 1 gm in 10 ml of 0.1 M NaCl - 0.05 M sodium phosphate, pH 6.7. Add 2.5 ml of 1% MA to the first beaker and stir. The first layer of the column is made by suspending 1 gm of standard grade paper powder in 20 ml of 0.1 M NaCl - 0.05 M sodium phosphate, pH 6.7 and adding to a 2 x 40 cm
glass column equipped with a porous glass disk. Let the liquid drip from the column until the liquid level is nearly down to the pad of powder. Form the second layer of the column by gently adding the contents of beaker 1 to the column with a pipette. Wash down the excess MAK with 0.1 saline. The final layer of the column is made by adding the content of beaker 2 and washing any excess kieselguhr down with 0.1 M saline.


Lona, F. (1960). *L'Ateneo Parmense.* 33; suppl. 6:1


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VITA AUCTORIS

Born:

September 24, 1943. Chungking, China.
Son of Mr. and Mrs. Heng Tu

Elementary Education:

Taipei, Taiwan, China

Secondary Education:


University Education:

Taiwan Prov. Chung Hsing University, Taichung, Taiwan, 1961-1965.
B. Sc. in Plant Pathology.


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