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Growth-limiting Proteins in Relation to Auxin-induced Elongation in Lupin Hypocotyls

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ABSTRACT

The role of protein synthesis in auxin-induced cell elongation in lupin hypocotyl segments was studied using cycloheximide. Cycloheximide inhibited protein synthesis by 9 minutes. Experiments adding cycloheximide at various times before and after indolyl-3-acetic acid are reported. Estimates of the relative amounts of growth-limiting protein(s), and a first order rate constant for the apparent turnover of the growth-limiting protein(s) were made. It was shown that there is a sizeable growth promotion by auxin after protein synthesis has essentially ceased. It is concluded that the initial phases of auxin action do not require protein synthesis but that its action depends on the existing pool of growth-limiting proteins which is rapidly depleted, and protein synthesis is then required for continued elongation.

It has been shown that inhibitors of protein synthesis have a rapid effect on IAA-induced growth (1, 5, 9, 13, 16, 19). This has led to the suggestion that an unstable protein, which has been called growth-limiting protein (5), is necessary for auxin-induced cell expansion. It is not possible, however, to determine from these studies with inhibitors whether auxin acts on the synthesis of GLP[1] or whether the requirement for protein synthesis is a secondary (albeit rapid) effect of auxin action.

Other evidence indicates that auxin acts on pre-existing macromolecules and that protein synthesis is not necessary for the initial action of auxin. This is the rapid change of growth rate by: (*a*) the methyl ester of indolyl-3-acetic acid (21, 22); (*b*) high temperature at high auxin concentration (14); and (*c*) the initial decline in growth rate before the auxin-induced stimulation (22). Although we have tried, without success, to obtain these results with lupin hypocotyl segments, they do illustrate the point for coleoptiles at least.

In this paper the quantitative study of the effect of cycloheximide leads to a similar conclusion that protein synthesis is not required for the initial action of auxin on cell elongation in lupin hypocotyl.

MATERIALS AND METHODS

Seedlings of *Lupinus angustifolius* cv. Bitter were grown for 4 days at 22 C under continuous illumination of 14.06 wm^{-2}. The precise conditions are detailed by Penny (17).

The measurements of elongation were made every minute in the apparatus described by Penny (17). Briefly, a segment was held in a chamber which was held on the stage of a microscope. The desired solution flowed through the chamber, and the flow rate of solution was maintained by a Watson Marlow model MHRE flow inducer. Tris-maleate buffer, pH 6.1, 20 mM, was used throughout; the concentration of IAA (when used) was 30 μM and the concentration of CH, when used, was 10 μg/ml. The solutions were aerated and the light energy at the surface of the segments was 3 wm^{-2} supplied by an incandescent lamp. Temperatures, except where otherwise stated, were maintained at 28 C. There was a tendency for the temperature to increase over the course of a long experiment, but the total difference in temperature during the experiment was never more than 0.5 C.

For measurement of protein synthesis, groups of 120 segments were pretreated in buffer for 2 hr and then blotted and placed in 10 ml of buffer containing either L-leucine-1-^{14}C (34.2 mc/mmole) or L-leucine-U-^{14}C (344 mc/mmole). After 16 min, CH was added to make a final concentration of 10 μg/ml. At varying times groups of 10 segments were removed, rinsed for 30 sec in 40 ml of buffer, and then extracted as outlined by Cleland (5). Total protein in the extract was determined by the Lowry method (10) on a 0.5-ml aliquot and duplicate aliquots of 0.1 ml were counted using 10 ml of Triton X-100—toluene (1:2) scintillation fluid (15). There was no effect of CH on uptake of leucine as determined by counting aliquots of the first ethanol extract.

RESULTS

Effect of Cycloheximide on Protein Synthesis. Figure 1 illustrates the time course of inhibition of protein synthesis. Cycloheximide inhibits the incorporation of leucine into protein within 3 to 9 min. These kinetics of inhibition show that it takes about twice as long to inhibit protein synthesis as in *Avena* coleoptile segments and carrot discs (2, 5). This may be because the lupin hypocotyl segment is a solid cylinder of cells. Protein synthesis would be inhibited first in the outer cells and later in the inner cells. However, it is probable that it is the outer cells that are limiting growth (D. Penny, K. F. Miller, P. Penny, unpublished results) and that protein synthesis in these cells is inhibited well within 9 min.

Effect of Cycloheximide Pretreatment on IAA-induced Growth. Individual segments were pretreated for 0, 5, 10, 15, 20, or 30 min in CH before the solution was changed to IAA plus CH. The treatment for each time was carried out on at least five segments. Figure 2 illustrates the short term kinetics of growth of three segments pretreated for different times.

The area under the curve, above endogenous growth rate level, was assumed to be an estimate of the amount of GLP available when auxin was added. The areas were calculated for all treatments and an average area at each pretreatment time

[1] Abbreviations: GLP: growth-limiting protein; CH: cycloheximide.

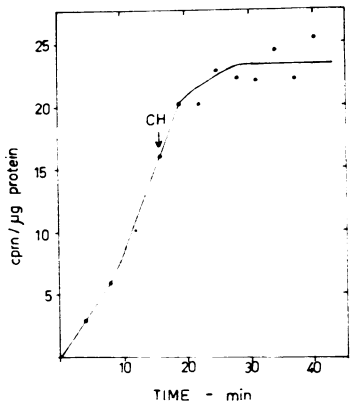

FIG. 1. Effect of cycloheximide on protein synthesis. One hundred and twenty segments were placed in ^{14}C-leucine at time 0, and 10 segments were removed at various times for total protein and radioactive protein determinations. CH was added at arrow at 16 min.

obtained. A plot of the log area against pretreatment time, shown in Figure 3, yields a straight line whose equation obtained by the method of least squares is:

$$Y = 0.023X + 3.022 \quad (1)$$

When this equation is converted to the form $A = A_o e^{-kt}$ (where A is the amount of GLP at any time t and A_o is the initial pool size), it becomes

$$A = 1052e^{-0.052t}$$

The correlation coefficient of this equation with the points is 0.981. The half life and the mean life of the protein were calculated from the values of equation 2 (23) to be about 12 min and 17 min, respectively. This is direct evidence that protein synthesis is not necessary for the initial phase of IAA-induced growth because growth occurs even with a 30 min CH pretreatment.

Effect of Adding CH after Adding IAA. The above experiments on pretreatment in CH permitted estimates to be made of the pool size of GLP at the time of auxin addition. It was of interest to determine the effect of IAA on the GLP for two reasons: (*a*) for comparison with the effect on pool size in *Avena* (5), and (b) to provide data to compare with the computer output of a negative feedback model, thus providing a test for the model (16).

The short term kinetics of the effect of CH added 10, 20, 30, 50, and 110 min after IAA were measured. These may be seen in Figure 4. The curve for each post-treatment time is an average of five individual segments. The effect of CH became apparent about 8 min after addition. It appears from Figure 4 that CH addition after 20 min led to an immediate inhibition. However, due to the variability of the height of the first peak, this apparently immediate inhibition is not significant. It was, therefore, difficult to tell exactly the time at which inhibition took place but was assumed to be the same as the other treatments, *i.e.*, 8 min.

It was assumed that the growth that occurred after CH inhibition of protein synthesis was a measure of the size of the GLP pool. Therefore, the total growth for each CH treatment time was calculated, starting with the growth that occurred in the 9th min after CH addition. The results can be seen in Figure 5. It appears that the pool size of GLP increases and reaches a maximum at 30 min and then diminishes to about 50% above the initial by 110 min after IAA addition. However, as can be seen by the large standard errors the maximum may not be significant. It is interesting that the apparent in-

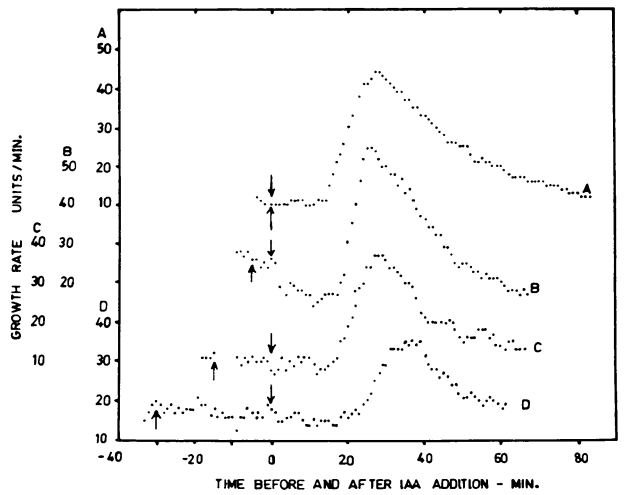

FIG. 2. Effect of cycloheximide on IAA-induced growth. IAA was added at downward pointing arrow at time 0. CH was added at upward pointing arrow at time 0, A; −5, B; −15, C; and −30, D: 1 unit = 0.23 μm. 30 μM IAA. 10 μg/ml CH.

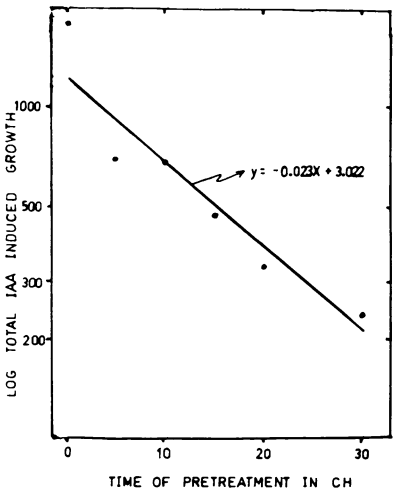

FIG. 3. Effect of cycloheximide pretreatment on total IAA-induced growth. The equation is the line of best fit obtained by the method of least squares. Each point is the average of the auxin-induced growth above endogenous level for at least five segments.

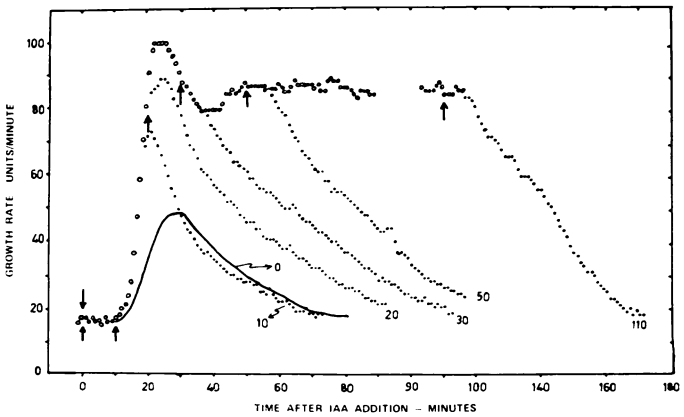

FIG. 4. Effect of adding cycloheximide at various times after adding IAA. IAA (30 μM) was added at downward pointing arrow. CH (10 μg/ml) added at upward pointing arrows at 0, 10, 20, 30, 50, 110 min after IAA. Numbers indicate kinetics of growth of segments treated with CH at that time. 1 unit = 0.23 μm.

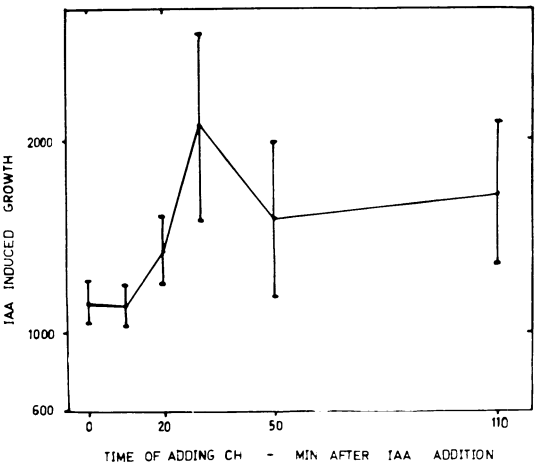

FIG. 5. Effect of adding CH 0, 10, 20, 30, 50, and 110 min after adding IAA on subsequent IAA-induced growth. Each bar is two standard errors.

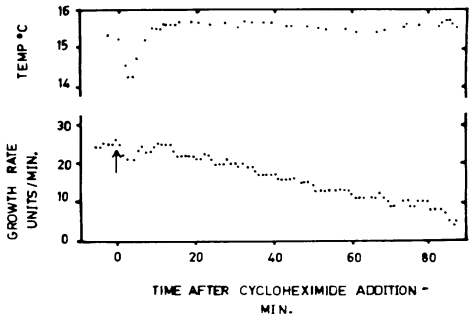

FIG. 6. Effect of temperature on cycloheximide (10 μg/ml) inhibition of IAA-induced steady-state growth rate. Upper curve, temperature; lower curve, growth rate. 30 μM IAA. 1 unit = 0.23 μm.

crease in pool size is much less than the increase in growth rate.

Effect of Temperature on CH Inhibition of IAA-induced Steady-State Growth. These results are reported because they bring some evidence to bear on a possible explanation of the short half-life of the protein outlined above. Figure 6 illustrates the effect of CH on IAA-induced steady-state growth rate at about 15 C. The upper line is the actual temperature, and the lower line is the growth rate averaged for four experiments. The time elapsed before complete inhibition of IAA-induced growth is slightly longer than at 28 C (71 min at 15 C and 65 min at 28 C [see Fig. 4]). This provides an estimate of the relative rates of depletion of the GLP pool at the different temperatures.

DISCUSSION

The major conclusions of this work are that protein synthesis is not necessary for the initial IAA-induced growth, but at least one protein is necessary for growth and its synthesis is required within a few minutes of the initial action of auxin. The evidence for this is:

1. IAA-induced growth occurs even when the segments have been pretreated for 30 min in CH. The amount of IAA-induced growth decreases exponentially with increasing time of pretreatment in CH.

2. When CH is applied after IAA, a decrease in growth rate occurs at roughly the same time that protein synthesis is inhibited.

It has recently been claimed that CH is not a specific protein synthesis inhibitor in nongreen tissue but that, in addition, it uncouples respiration and inhibits the uptake of ions and organic molecules (7). In lupin hypocotyls which are green, light-grown tissues CH does inhibit protein synthesis (Fig. 1) but has no effect on respiration, chloride efflux, chloride and leucine uptake, or ^{32}P-phosphate incorporation into organic phosphates (unpublished results). It must be pointed out that there is no direct evidence for uncoupling of respiration even in nongreen tissues. Chrispeels (2) has shown for one of the tissues used by Ellis and MacDonald (7) (carrot discs) that CH has inhibited protein synthesis within 2 min, whereas in beet tissue increased O_2 uptake does not occur until after 30 min. The critical test which would give direct evidence has not been done by Ellis and MacDonald (7); that is to determine whether uncoupling does, in fact, occur by determining the levels of organic phosphates.

From the CH pretreatment experiments, an estimate could be made of the half life of the protein. If more than one protein is involved, then this would be the half-life of the protein having the shortest half-life. The value which was obtained—12 min—is an unusually short half life for a protein from a higher organism (6, 9), although it appears to be similar to that which is presumably a similar protein in *Avena* (5, 8). This half life may be a measure of one or more of three phenomena:

1. The rate of hydrolysis of a protein. Cases which have been reported for plants have half lives of about several hours to many days (6, 9). The literature is, therefore, against the possibility of hydrolysis, but this, of course, does not rule it out.

2. Conformational change of a protein to an inactive form. A well known example is phytochrome (10) but this also occurs during heat denaturation of enzymes. A conformational change of a protein is often, but not always, indicated by a very high Q_{10} for the reaction (10). In the case of the GLP, the inactivation of the protein should be slower at 15 C than 28 C and, therefore, IAA-induced growth should take much longer to fall to endogenous level at 15 C. This did not happen. However, this possibility can not be ruled out because such changes do not always involve high Q_{10} values or the conditions for measurement could have masked it if it did occur.

3. Rate of transport from a compartment. A hypothetical case would be the transport of protein from the cytoplasm to the wall when auxin can only interact with the protein in the cytoplasm. The apparent turnover of the GLP measured in this paper would be an estimate of the rate of transport from one compartment to another. This is similar to the case reported by Chrispeels (2) for carrot discs. He has shown that the proline of the hydroxyproline rich protein can only be hydroxylated after the synthesis of the protein and before it enters a membrane-bound compartment.

In all of the estimates of pool size made in this paper, as also made by Cleland (5), the endogenous growth was subtracted from the total growth to give the IAA-induced growth. By making this calculation, the assumption is made that the processes leading to endogenous growth are in some way different from those involved in auxin-induced growth. Although this is not proved, it is consistent with observations that CH (18), actinomycin D (19), turgor pressure (3), anaerobic conditions (unpublished), anti-auxins (12), and cyanide (4) have different effects on endogenous and IAA-induced growth.

It was assumed that the amount of growth occurring after CH inhibited IAA-induced steady state growth was a measure of the size of the pool of GLP available at the time protein synthesis was inhibited. Using this assumption, estimates of pool size were made. It is now possible to compare these experimental results with those predicted by the theoretical

model (D. Penny, unpublished) and thus act as a test for the model.

The experimental results indicate that after about 10 min in auxin, the size of the pool of GLP increases to a maximum at about 30 min, and then decreases to a steady state pool size approximately 50% larger than that at the time of auxin application. The computer output of the model, however, predicts that the pool size should diminish after auxin addition, reaching a minimum at about 30 min and then rising slightly to a new steady state size approximately 50% below the initial value.

In this respect, then, the model fails although it did predict fairly well the effect of CH pretreatment on IAA-induced growth. A simple modification to the model which could lead to a more successful prediction would be to assume that another factor (*e.g.*, polysaccharide (16)) is interacting with the GLP to cause the IAA-induced increase in growth.

The only other quantitative work on the effect of CH on GLP is that of Cleland (5). Both his and the present work enabled estimates of the pool size of GLP and both have shown that it has a short half life. This work has in addition enabled estimates of that half life to be made. The initial pool size of GLP is apparently much larger in lupin than *Avena* and this may account for the different growth rate curves exhibited by these two plants in response to auxin. In lupin the size of the first peak is dependent on the initial pool size, but the appearance of the second requires continual synthesis.

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