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Timing of the Response of Coleoptiles to the Application and Withdrawal of Various Auxins

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Summary. A study was made of the time courses of growth promotion and the reversal of growth promotion upon the addition and withdrawal of various auxins. Growth promotion by 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) occurs more slowly and is less vigorous than growth promotion by the same concentration of indoleacetic acid (IAA).

The time required for the reversal of the stimulation of elongation by auxin is many times greater for 2,4-D-stimulated growth than for IAA- or NAA-stimulated growth (80 min vs. about 10 min). This difference appears to be due to the sluggish exit of 2,4-D since (1) experiments with labeled auxins show that 2,4-D moves out of the tissue more slowly than IAA, and (2) it is possible to shorten the time required for a decline in elongation rate after the removal of 2,4-D to 13 min by adding an auxin antagonist (p-chlorophenoxyisobutyric acid).

The rapid reversal of the hormonal stimulation of growth is discussed in relation to possible mechanisms of action of auxin.

A. Introduction

Recently it has become increasingly clear that continuous RNA and protein synthesis is necessary during long-term growth responses to auxin (Key and Shannon, 1964; Nooden and Thimann, 1963, 1965, 1966). A number of authors have shown correlations between the degree of inhibition of RNA or protein synthesis and the degree of inhibition of auxin-induced elongation (Key and Ingle, 1964; Nooden and Thimann, 1963). These observations, in conjunction with evidence that auxin treatment enhances RNA and protein synthesis in some tissues (Key and Shannon, 1964; Masuda, 1963), have led to the suggestion that the primary action of auxin may be a stimulation of the synthesis of a growth-regulatory protein, either at the transcriptional or translational level (Bonner, 1965; Key, 1964; Nooden and Thimann, 1963).

However, alternative explanations of the growth-inhibitory effects of antibiotics have been offered by various authors. It has been pointed out, for example, that the primary action of auxin may be dependent

upon the presence of a particular protein not directly involved in the growth process (NOODEN and THIMANN, 1966) or that the cells of higher plants may possess a feed-back regulatory mechanism that shuts down elongation processes in the absence of active protein synthesis (EVANS and RAY, 1969).

If auxin were to promote elongation by somehow inducing the synthesis of a growth-specific protein, then the protein in question could be one of two types, an enzymatic protein playing directly or indirectly some catalytic role in cell wall loosening, or a structural protein needed in the mechanism of the growth process.

These two alternatives lead to very different predictions in the timing of the decline in growth rate upon the removal of auxin. If the growth-regulating protein were enzymatic, one would expect the timing of the decline in growth rate to be a function of the stability of the enzyme. If, for example, auxin were to induce cell wall loosening by promoting the synthesis of an enzyme catalyzing the introduction of cell wall material from an essentially inexhaustible supply of carbohydrate or the hydrolytic breakdown of cell wall polymers, then, after the removal of auxin, one would expect cell wall loosening and rapid elongation to continue as long as the growth-specific enzyme remained functional. That is, the timing of the decline in growth rate after the removal of auxin should be a function of the half-life of the growth-specific enzyme.

If, however, auxin were to stimulate the synthesis of a protein which causes cell wall loosening by being incorporated into the cell wall as a structural material then the timing of the decline in growth rate after the removal of auxin should be a function of the pool size or steady-state level of the protein. The half-life of such a structural protein would be an important factor in the timing of the decline in growth rate only if the protein were unstable and were used slowly enough as to have an appreciable pool size.

This study was made in order to compare the short-term growth-promoting properties of various auxins and to examine the time course of changes in elongation rate upon the application and withdrawal of these auxins. The data are interpreted in light of the above-mentioned alternative roles for auxin in the promotion of elongation.

B. Materials and Methods

a) Plant Material. Corn (*Zea mays* L., ev. "Golden Bantam") obtained from Vaughan's Seed Co., Chicago, Ill. was soaked for 30 min in about 2.6% hypochlorite (Chlorox, diluted 1 to 1) and submerged in cold, running tap water overnight. The seeds were then planted embryo upward on 4 layers of wet paper toweling

in a covered plastic box $33 \times 26 \times 10$ cm and moved to an environmental growth chamber at 26° where they were positioned about 40 cm under a 25-watt ruby red lamp. After 24 hours the light was turned off and the seedlings were allowed to grow in complete darkness for 48 hours. Seedlings 2.5 to 3.0 cm long were selected for harvesting. Segments 8 mm long were cut from each coleoptile beginning 3 mm from the tip, using a double-bladed cutting device. The leaf was removed from each segment.

b) Method of Measurement. The high-resolution growth recording device used in these experiments is described in detail in an earlier paper (EVANS and RAY, 1968). Briefly, 13 hollow coleoptile segments are strung onto a thread with a supporting platform at the bottom. A piece of red pyrex glass capillary tubing weighing 92 mg is also strung onto the thread so that it rests upon the uppermost coleoptile segment and serves as a weight. The thread assembly is then inserted into a tubular glass chamber 14 ml in volume, and the chamber is clamped into a vertical position. The glass chamber is filled with a particular growth medium and an arc lamp is used to cast a sharp shadow of the glass weight onto a narrow vertical slit in a piece of cardboard placed about 1 m from the chamber. Immediately behind the slit, a long piece of photographic paper is drawn horizontally by a kymograph drum turning at a rate of 3.08 mm min^{-1}. This arrangement allows continuous shadowgraphic recording of the elongation of the entire column of coleoptile segments by recording the vertical displacement of the shadow of the glass weight as the latter is pushed upward along the thread by the growing segments below. All curves shown in this report are direct tracings of original shadowgraphic records with time scales indicated. Since the magnification differs slightly for each record, a marker representing 1 mm increase in length of the column of segments is shown at the end of each curve.

During each experiment the growth medium surrounding the coleoptile segments was continuously gassed with oxygen. Solutions could be drained and replenished within 15 sec through the appropriate outlet and connecting funnel. All solutions contained 10^{-3} M potassium phosphate buffer at pH 6.5. In changing solutions from hormone to any other medium, the chamber was rinsed thoroughly with buffer before adding the new solution. All experiments were done under dim red light in an environmental growth chamber at 26°.

c) Measurement of Efflux of Radioactive Auxins. In order to examine the time course of auxin exit from the coleoptile segments, the segments were mounted as described above and allowed to equilibrate for 35 min with a solution of the radioactive auxin to be studied. The chamber was then drained, rinsed with buffer, and refilled with buffer. One-ml aliquots of buffer were then withdrawn at specified intervals and placed in 25-ml plastic counting vials. For determination of activity remaining in the tissue, the coleoptile segments were then placed into a counting vial containing 1 ml of methanol. 5 ml of Bray's solution [4 g 2,5-diphenyloxazole (PPO) and 0.2 g 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), both from Packard Instrument Co., Downers Grove, Ill., USA; 60 g naphthalene; 100 ml methanol; diluted to 1000 ml with p-dioxane] was added to each vial and the vials were counted in a liquid scintillation counter (Beckman CPM-100) at room temperature at 85% efficiency.

d) Chemicals and Radioactive Compounds. Parachlorophenoxyisobutyric acid (PCIB) was purchased from Calbiochem, Los Angeles, Calif. 3-indoleacetic acid (IAA) was obtained from Fisher Scientific Co., Chicago, Ill., 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) from Nutritional Biochemicals Corp., Cleveland, Ohio. IAA-1-^{14}C (specific activity, 31 mc mM^{-1}) and 2,4-D-1-^{14}C (specific activity, 31 mc mM^{-1}) were obtained from Nuclear Chicago.

C. Results

1. Growth Responses to Application and Withdrawal of Auxins

a) Curve *A* in Fig. 1 shows the response of corn coleoptile segments to 5×10^{-5} M IAA and the timing of the decline in growth rate when the growth medium is changed from IAA to buffer alone. The timing of the

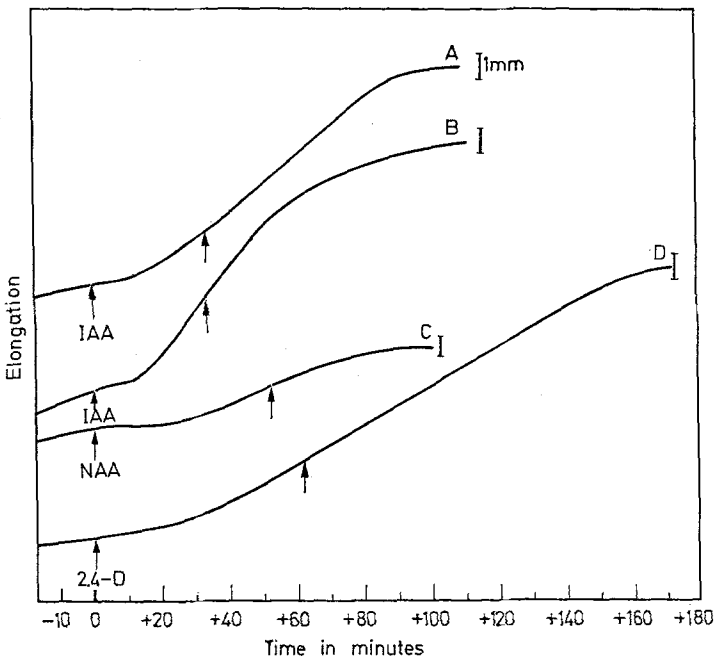

Fig. 1. Timing of growth responses to IAA, NAA, and 2,4-D. Hormone was added at the first arrow in each curve and replaced with buffer at the second arrow. The hormone concentration was 5×10^{-5} M in curve *A*, 10^{-6} M in curves *B* and *D*, and 2×10^{-6} M in curve *C*

response of coleoptile segments has been discussed in an earlier paper (EVANS and RAY, 1969). The response shown in curve *A* is typical for corn in that it possesses a latent period of about 12 min followed by a rather rapid increase in growth rate to a new steady value. The decline in the rate of elongation after the removal of this concentration of IAA, however, occurs quite slowly (curve *A*, second arrow). After the withdrawal of 5×10^{-5} M IAA, the segments continue to elongate at the same auxin-promoted rate (0.6 mm hr^{-1} segment) for about 50 min before showing any sign of a decline. After 50 min the elongation rate drops rapidly to a rate typical of segments that have not been treated

with IAA (0.08 mm hr^{-1} segment). These results conflict both with the claim of BONNER and FOSTER (1955) that withdrawal of auxin is followed immediately by a decline in growth rate and with the report by CLELAND (1965) that a decline in growth rate requires several hours after the withdrawal of exogenous auxin.

This long delay in the decline in the rate of auxin-stimulated growth may be due to one of the following factors: 1. Treatment of the tissue with auxin might lead to the production of some growth intermediate that does not leave the tissue by diffusion. Thus, rapid growth could proceed in the absence of auxin until this intermediate were used up or decayed in some other manner. 2. IAA might move very slowly out of or be destroyed slowly in the tissue so that enough free IAA remains to continue to promote elongation for at least 50 min. 3. Since the total volume of the growth measurement chamber is 14 ml, perhaps enough IAA moves into the buffer to build up a growth-promoting concentration of IAA in the so-called IAA-free medium.

Curve *B* in Fig. 1 indicates that alternatives 2 and 3 are the most likely ones. The experiment is similar to that shown in Curve *A* except that a lower concentration of IAA (10^{-6} M) was used. Notice that, although this lower concentration of IAA is enough to promote elongation to the same extent as the 5×10^{-5} M IAA used in the experiment of curve *A*, the rate of elongation begins to decline only 7 min after the removal of IAA and continues to decline gradually during the next 35 min until the rate of elongation falls to a low value characteristic of unpromoted segments. These data are in close agreement with similar results obtained by RAY and RUESINK (1962) using oat coleoptiles. This implies that most of the long delay in the decline in growth rate after the removal of a high concentration of auxin cannot be explained by the stockpiling of some auxin-induced growth intermediate.

b) Curve *C* in Fig. 1 illustrates the timing of the growth response of corn coleoptile segments to the application and withdrawal of NAA. This experiment was done with a higher concentration of hormone (2×10^{-6} M) in order to get an easily measurable growth response. Notice that the response of corn coleoptile segments to NAA appears to be considerably more sluggish than the response to IAA. Although the growth response begins about 12 min after the application of either hormone, the subsequent increase in growth rate is much more gradual in NAA. The final steady rate of growth is reached about 35 min after the application of NAA, as compared to 20 min after the beginning of IAA treatment. Furthermore, the final steady rate of elongation supported by 10^{-6} M IAA is about 2 times greater (0.72 mm hr^{-1} segment for IAA vs. 0.35 mm hr^{-1} segment for NAA) than that sustained by a higher concentration (2×10^{-6} M) of NAA.

In spite of these differences in the kinetics of growth promotion by IAA and NAA, the time course of the reduction in the rate of elongation after removal of either hormone is quite similar. This can be seen by comparing curves *B* and *C* in Fig. 1 beginning at the second arrows. The decline in the rate of elongation begins about 10 min after the withdrawal of either NAA or IAA.

c) Curve *D* in Fig. 1 shows the timing of growth responses to the application and withdrawal of a third auxin, 2,4-D. Over the concentration range studied, (10^{-6} M to 5×10^{-5} M), responses to 2,4-D are similar to responses to NAA in that they occur gradually and reach a maximum only after about 35 min. 2,4-D also resembles NAA in being less effective than IAA as a growth promotor (0.36 mm hr^{-1} segment for 2,4-D vs. 0.72 mm hr^{-1} segment for IAA), at least during the first hour and over a concentration range near 10^{-6} M.

One striking feature of rapid elongation promoted by 2,4-D is the sluggishness with which it declines upon the removal of 2,4-D. After replacing 10^{-6} M 2,4-D with buffer alone, the segments continue to elongate at the same rapid rate for about 80 min before beginning to decline in growth rate (Fig. 1, curve *D*). This is about 10 times as long as the time required for a decline in the rate of IAA- or NAA-promoted growth. These data imply that either 2,4-D moves out of the tissue much more slowly than IAA or that the nature of the steady rate of elongation induced by 2,4-D differs significantly from that induced by IAA. In order to test the former alternative the time course of the exit of labeled IAA and 2,4-D from corn coleoptile segments was measured.

2. Exit of Labeled Auxins from Tissue Segments

Fig. 2 illustrates the time course of the movement of 10^{-6} M IAA-1-^{14}C and 2,4-D-1-^{14}C from corn coleoptile segments mounted within the chamber in the normal fashion (see Materials and Methods). About half of the free IAA comes out of the coleoptile segments during the first 10 min after the withdrawal of IAA. Practically all of the free IAA has moved out of the tissue after 30 min. No further exit occurs during the subsequent 90 min interval.

This is not the case with 2,4-D. Fig. 2 shows that 2,4-D is still moving out of the tissue at a substantial rate 120 min after the replacement of 2,4-D solution with buffer. The total amount of 2,4-D taken up by the coleoptile segments during the 35 min period of immersion in the hormone solution was about 3.6 times the amount of IAA taken up during the same length of time (13,850 cpm for 2,4-D vs. 3,819 cpm for IAA).

The rapid uptake and prolonged exit of 2,4-D relative to IAA found here is in close agreement with data of HERTEL and FLORY (1968) who also used corn coleoptile segments. The efficient uptake of 2,4-D relative

to IAA explains the large difference in the total amount of radioactivity leaving the tissue after the withdrawal of 2,4-D as compared to IAA (Fig. 2).

The data of Fig. 2 suggest that the differences in the timing of the decline in growth rate upon withdrawal of 2,4-D and IAA can be attributed to differences in the rate of exit of these two auxins. IAA moves

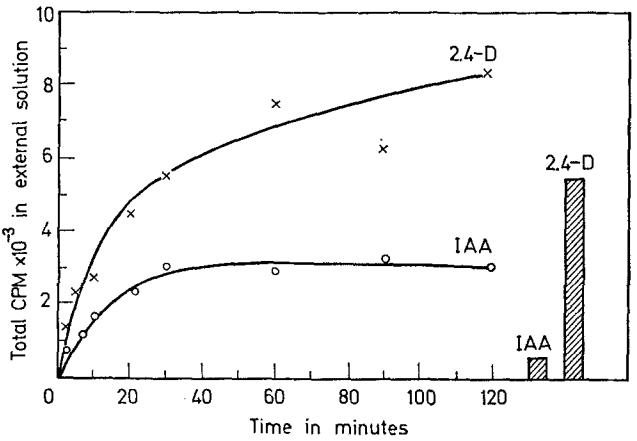

Fig. 2. Comparison of the efflux of labeled IAA and 2,4-D from coleoptile segments. Segments were mounted in the chamber as described in the text and allowed to equilibrate with 10^{-6} M IAA-1-^{14}C or 2,4-D-^{14}C for 35 min. The hormone was then replaced with buffer (zero time) and 1-ml aliquots of buffer were taken periodically for counting. Vertical bars represent activity remaining in the tissue 120 min after the solution change. CPM were corrected for background

out of the tissue segments rapidly and the growth rate sustained by IAA declines rapidly after the removal of IAA. 2,4-D moves out of the tissue slowly and the growth rate sustained by 2,4-D declines slowly after the removal of 2,4-D.

3. Effect of an Auxin Antagonist

If the long delay in the decline in the rate of elongation after the removal of 2,4-D is due simply to the slow exit of 2,4-D it should be possible to shorten the decline time by using an auxin antagonist. The auxin antagonist selected for study was PCIB. However, since PCIB and other auxin antagonists have heretofore been studied only in long-term experiments (FOSTER et al., 1955), and since the properties of auxin-antiauxin interaction are not well characterized, the preliminary experiment shown in Fig. 3, curve A was performed. It is clear that growth promoted by 10^{-6} M IAA can be rapidly and strongly inhibited

by changing the medium to 10^{-6} M IAA + 10^{-4} M PCIB (curve A, second arrow). The third portion of curve A shows that this inhibition is completely reversible by increasing the ratio of IAA to PCIB (5×10^{-4} M IAA + 10^{-4} M PCIB). In these short-term experiments, PCIB appears to act reversibly as a specific antagonist of auxin action.

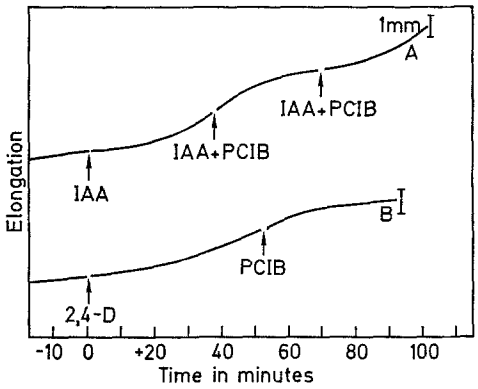

Fig. 3. Effect of PCIB on IAA- and 2,4-D-promoted elongation. Curve *A*: growth medium changed from buffer to 10^{-6} M IAA at the first arrow and to 10^{-6} M IAA + 10^{-4} M PCIB at the second arrow. The third arrow indicates a solution change to 5×10^{-4} M IAA + 10^{-4} M PCIB. Curve *B*: growth medium changed from buffer to 10^{-6} M 2,4-D at the first arrow and from 2,4-D to 10^{-3} M PCIB at the second arrow

Curve *B* in Fig. 3 demonstrates that PCIB can be used to greatly shorten the time required for a reduction in the rate of elongation of coleoptile segments after the withdrawal of 2,4-D. When the 2,4-D growth medium was replaced with PCIB, the rate of elongation began to decline 13 min after changing solutions as compared to 80 min when buffer alone was added (Fig. 1, curve *D*). This supports the suggestion that the slow decline in growth rate upon withdrawal of 2,4-D can be attributed to the slow rate of exit of 2,4-D from corn coleoptile segments.

D. Discussion

IAA, NAA, and 2,4-D at low concentrations all elicit easily measurable short-term growth responses. Stimulation of elongation by NAA or 2,4-D occurs much more gradually than stimulation of elongation by IAA. Although the absolute latent period (time after the addition of hormone during which there is no detectable increment in growth rate) is about the same for all three auxins, the response to IAA, once it begins, occurs more rapidly than the response to NAA or 2,4-D. Thus the

time required to reach the final steady rate of elongation after the addition of hormone is about 1.75 times longer for NAA or 2,4-D than for IAA. This is in spite of the fact that NAA and 2,4-D seem to be taken up more efficiently than IAA (Fig. 2; HERTEL and FLORY, 1968; EVANS, unpublished data).

It is also clear that, at the concentrations studied, NAA and 2,4-D are less effective than IAA in promoting growth during short-term experiments. In most experiments NAA- and 2,4-D-induced elongation rates are about half as great as those supported by the same external concentration of IAA, even though, according to uptake studies, the internal concentration of NAA and 2,4-D must be about 4 times greater than the internal concentration of IAA.

A major difference between growth supported by 2,4-D and growth supported by IAA or NAA is in the extended time required to allow a reduction in the rate of elongation after the removal of 2,4-D (about 10 times as long as with IAA or NAA). This difference appears to be due to the sluggish exit of 2,4-D from the coleoptile segments. By treating the coleoptile segments with an auxin antagonist after the removal of 2,4-D, the time required for a decline in the rate of elongation is reduced to about the same value as is required for a decline in the growth rate after the removal of either IAA or NAA.

What does this rapid reversal of the promotion of elongation by auxin imply about the nature of auxin-promoted elongation? If it is true that auxin acts as a growth promotor by inducing the synthesis of a particular growth-specific protein, then after removal of auxin or addition of an auxin antagonist, growth should continue at the same promoted rate until the level of the protein in question begins to diminish. As mentioned earlier, there seem to be only two possible roles for the growth-specific protein, an enzymatic or a structural one. The decline in rate of growth begins about 10 min after the removal of exogenous IAA or NAA and continues during the next 28 min. Therefore, if one assumes that the rate of growth is in direct proportion to the amount of auxin-induced protein present, it is clear that the growth protein itself must begin to decay within 10 min after the removal of auxin and must possess a half-life on the order of 10 min. We feel that a half-life of this magnitude argues against the hypothetical growth specific protein acting as an enzyme. Available indications are that the half-lives of proteins in higher organisms are on the order of days or at least several hours (AFRIDI and HEWITT, 1964; GLASZIOU et al., 1966; HOLLAENDER, 1965; INGLE et al., 1966; SCHIMKE, 1967, 1968; SEITZ and LANG, 1968).

AFRIDI and HEWITT (1964), in a study on the induction of nitrate reductase in cauliflower, refer to the enzyme as being unusually labile

in that quantitative losses are detectable within a few hours after the removal of inducer. If the stability of this higher-plant enzyme is representative, it is highly unlikely that the hypothetical growth-specific protein induced by auxin should possess a half-life as short as 10 min.

The possibility remains, however, that the hypothetical auxin-induced growth protein participates as a structural protein. Suppose the rate of elongation were determined by the amount of structural protein available for cell wall loosening. Upon treatment with auxin one may envision an increase in the rate of synthesis of this protein which would in turn lead to an increase in the rate of elongation. A steady, promoted rate of elongation might be established when the protein is being consumed in the growth process at the same rate as it is being synthesized. Thus, during auxin-promoted elongation, there would exist a specific, steady state pool size of the protein in question. Upon removal of auxin and consequent decline in the rate of formation of the protein, one might expect an essentially immediate reduction in the rate of elongation as the protein was consumed and its pool size diminished.

The timing of the decline in growth rate after the removal of auxin therefore suggests that either the mechanism of action of auxin does not involve promotion of the synthesis of a growth-specific protein at all, or suggests that if auxin does act by inducing the formation of a particular protein, that protein must participate in the growth process as a structural protein, not an enzyme. This is in agreement with the conclusion reached in an earlier paper (Evans and Ray, 1969) dealing with the timing of the promotion of elongation by auxin rather than its reversal.

It should be emphasized that there exist a number of alternatives to the gene-activation hypothesis of auxin action, any of which might depend indirectly on continual protein synthesis in order to remain functional. Serious consideration should be given to possible effects of auxin on intracellular transport processes or membrane permeability properties, for example.

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