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# Additional Evidence for Separable Responses to Auxin in Soybean Hypocotyl[1]

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## ABSTRACT

**Additional evidence for two separable responses to auxin is presented. The average of 24 control experiments indicated lag times of 12.4 and 35.4 min, and maximum rates of 0.57 and 0.54 mm·hr$^{-1}$, for the first and second response, respectively. The auxin analog 4-azido-2-chlorophenoxyacetic acid increased the lag time of the second response (but not the first), resulting in the temporal separation of the two responses. Plots of elongation rates against time, taken from the literature, allowed the characterization of the two responses in monocotyls and dicotyls. Study of published rate-time elongation curves showed that the maximum rate of the first response is frequently greater than the maximum rate of the second response; however, the maximum rate of the second response has not yet been shown to exceed the maximum rate of the first response.**

The short lag time between auxin application and increased elongation rate was first described by Yamaki (27) and Köhler (9). The possible implications of this work received considerable attention when Evans and Ray (3), using a unique growth apparatus that continuously measured and recorded growth, continued the earlier work of Ray and Ruesink (22). The rapid response of stem, hypocotyl, and coleoptile cells to auxin has since been cited frequently as evidence that auxin-induced cell elongation is not mediated by gene activation (21, and references therein), as first suggested by Skoog and co-workers (23, 24), and later proposed for elongating cells by Nooden and Thimann (11-13) and Key and co-workers (6-8).

The cytokinin, isopentenyladenine, inhibited auxin-induced elongation in long term (6-8 hr) experiments (26, and references therein). This naturally occurring hormone, however, did not inhibit the rapid response of elongating soybean hypocotyl cells to auxin. The study of the auxin-cytokinin interaction in elongating cells has produced evidence that there are separable responses to auxin (25). This possibility has been previously considered (19), and there is some biochemical evidence from work by Morré and Cherry and co-workers (5) that there may be two responses. The first response, very similar to the low pH-induced response in these cells, was not inhibited by cytokinin; however, the second response, which began approximately 35 min after auxin application, was inhibited (25). While gene activation by auxin has certainly not been proven (6, 8, 21), it was the important conclusion from this work (25) that neither is it disproven by experiments which describe the fast response to auxin.

The experiments described herein further characterize the separable responses to auxin, and present additional evidence that two elongation reactions to auxin do indeed occur in soybean hypocotyl cells.

## MATERIALS AND METHODS

Soybean seedlings (*Glycine max* L. Merr. var. Wayne) were germinated in the dark and the elongating segment of the hypocotyl was excised as described (26), except that all procedures were performed under green light (460-590 nm) at 30 C.

Hypocotyl extension was measured continuously with a linear transducer. The apparatus was modified after that reported by Green and Cummins (4). To facilitate clamping of the segment in the growth chamber, a 2-cm section, which included the 1-cm elongating section directly below the hypocotyl hook plus a centimeter of tissue basal to it, was excised from a 3-day-old etiolated soybean seedling. The basal centimeter was used to attach the segment to the growth chamber. For each growth measurement, five segments were cut into 20 ml of 5 mM $KH_2PO_4$, pH 6, in 30 mM sucrose, and were incubated for 1 hr at 30 C. Where indicated, cytokinin (49 $\mu$M isopentenyl adenine) was also present. Segments were rinsed and blotted, and the straightest one was clamped into the chamber in 100 ml of the buffered sucrose solution. The solution was continuously gassed with air while growth was monitored. After 15 min, this solution was thoroughly aspirated from the chamber and replaced with 100 ml of buffered sucrose containing 45 $\mu$M auxin (2,4-D). The photoaffinity labeled auxin, 4-azido-2-chlorophenoxyacetic acid (10), was used (Fig. 2) at its optimum concentration of 91 $\mu$M. Changing solutions took a maximum of 1 min. Growth rates were calculated from the growth curve for each 2.5- or 5-min interval.

The postulated overlapping reactions were constructed under published summation curves (Fig. 3). These constructions were dependent upon the nature of the summation response and known characteristics of the first response (only the first response occurs in the presence of cytokinin, or in response to low pH; hence, it can be studied in the absence of the second response; see ref. 25).

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## RESULTS AND DISCUSSION

The two responses to auxin are presented in Figure 1 as an average plot of 24 control experiments. The descending slope (broken line) of the first response was handdrawn to approximate the first response as seen in cytokinin-treated segments (25). The second response was then obtained by subtracting the first response from the control (summation) curve. Data concerning the two responses were determined directly from the recorder output, and/or from the two responses as constructed in Figure 1. The lag times for the first and second responses were 12.4 min and 35.4 min, respectively, with similar maximum elongation rates (5.7 and 5.4 mm·hr$^{-1}$, respectively).

Additional evidence for separable responses came in experiments with the auxin analog, 4-azido-2-chlorophenoxyacetic acid. This photoaffinity labeled compound, in conjunction with experiments directed toward identifying auxin receptor molecules, was used in experiments where the auxin response was continuously monitored. The plot of the average of seven experiments (Fig. 2A) showed a usual biphasic response (although with depressed maximum elongation rates for both responses, agreeing with previous results, ref. 10). The immediately relevant result of experiments with this analog is that in three of the seven experiments the lag time for the postulated second response was increased, completely separating it from the first response (Fig. 2B). The differential effect on the two responses (the lag time for the first response was never affected by the analog), and the actual temporal separation of the two responses, again support the hypothesis that there are separable responses to auxin by elongating cells.

The literature was surveyed for experiments which plotted the data of auxin-induced elongation as rate against time. Data for oat and corn coleoptile, lupine hypocotyl, and pea stem are reproduced, with the postulated overlapping responses added, in Figure 3. For the most part, these graphs indicate that there are two overlapping responses to auxin; especially note the experiments with lupin and pea (Fig. 3, J and S). Using these modified graphs, it is determined that the average lag time for the first response was 10.4 min for monocotyls and 15.2 min for dicotyls. The average lag time for the second response was 28.4 min for monocotyls and 30.6 min for dicotyls. These data compare with 12.4 min (first response) and 35.4 min (second response) for soybean (Fig. 1). In monocotyls the first response is terminated 74.3 min after auxin application (67.7 min in dicotyls), as compared to 78 min in soybean (Fig. 1).

## CONCLUSIONS

Penny *et al.* (19), in an extensive study of the kinetics of auxin-induced elongation, were the first to attempt to explain the multiphasic response to auxin. While they suggested the possibility of two separate effects of auxin, their computer-simulated model was directed at proving their hypothesis that there was negative feedback with underdamping (19). Subsequently, a very elegant demonstration of the usefulness of experiment-

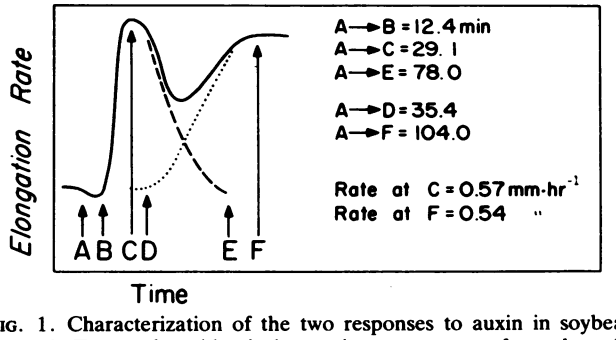


FIG. 1. Characterization of the two responses to auxin in soybean hypocotyl. Twenty-four identical experiments were performed on 21 different days between February 1973 and May 1975. The average curve is normalized about the point of auxin addition at A. The separate responses are constructed as described under "Materials and Methods." Auxin was added at A.

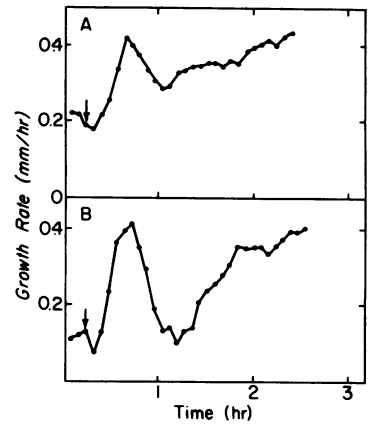


FIG. 2. Separation of the two responses using the auxin analog 4-azido-2-chlorophenoxyacetic acid. After a 60-min preincubation in buffered sucrose, the elongating segment was transferred to an identical solution in the growth apparatus. The auxin analog was added at the arrow. A: average of seven experiments, normalized about the point of auxin addition; B: an individual experiment.

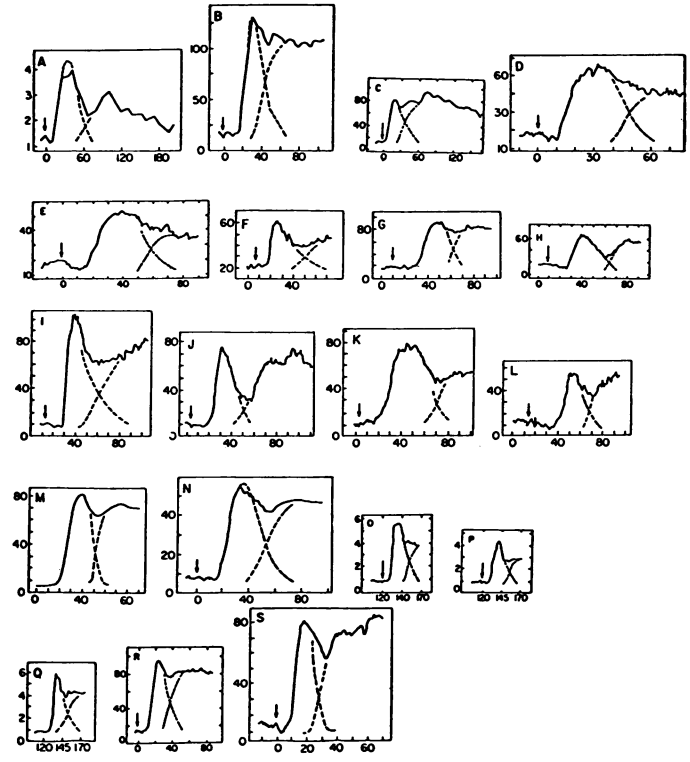

FIG. 3. Construction of the postulated two overlapping auxin responses for previously published elongation curves (rate *versus* time). In all cases the abscissa is time in minutes. The ordinate is units per min, except for B, C, O to Q where it is $\mu$m per min. Auxin (or auxin analog; see H, I) was added at the arrow. Solid line: published curve; dashed lines: postulated two responses constructed as described under "Materials and Methods." A: ref. 9, Fig. 5, *Avena* coleoptile; B: ref. 2, Fig. 2, *Zea* coleoptile; C: ref. 20, Fig. 2, *Avena* coleoptile; D–E: ref. 14, Figs. 4–5, lupine hypocotyl; F: ref. 17, Fig. 3, lupine hypocotyl; G, H, K–M: ref. 18, Figs. 1C, 1D, 3A, 3B, 4, lupine hypocotyl; I, J: ref. 18, Fig., 2 B and C, pea epicotyl; N: ref. 16, Fig. 1, lupine hypocotyl; O–Q: ref. 1, Fig. 1, A to C, *Pisum* stem; R: ref. 15, Fig. 4, lupine stem; S: ref. 19, Fig. 2A, lupine hypocotyl.

computer simulation interaction provided two possible reasons for the latent period after auxin addition; however, the existence of separate responses to auxin was neither proven nor disproven (16). Evidence presented here, and in a previous paper (25), indicates that there are two responses since (*a*) cytokinin differentially affects the two responses, (*b*) 4-azido-2-chlorophenoxyacetic acid differentially affects the lag times of the two responses, (*c*) the acid response approximates the first, but not the second, response, (*d*) rate-time plots from the literature appear to be summations of two overlapping responses, (*e*) 4-azido-2-chlorophenoxyacetic acid occasionally elicited a second response which was temporally separated from the first response. It seems apparent, therefore, that it is necessary that the effects of auxins and auxin inhibitors on both responses be assessed when evaluating experimental data concerning the mode of action of auxin.

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