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Experimental Studies on Lateral Root Formation in Radish Seedling Roots

II. ANALYSIS OF THE DOSE-RESPONSE TO EXOGENOUS AUXIN

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ABSTRACT

Application of indoleacetic acid (IAA) and other auxins causes cultured radish (*Raphanus sativus* L. 'Scarlet Globe') seedling root segments to produce an increased frequency (FR, no. cm^{-1}) of lateral roots (LR); in the absence of auxin, segments spontaneously form about 6 LR cm^{-1}. A dose-response study has revealed that the increase in FR follows a biphasic Michaelis-Menten relationship with the medium concentration of the undissociated form of IAA ([IAAH]$_m$). The fitted curve for phase I has a maximum response level (R_{max}) of 5.2 LR per centimeter above the spontaneous FR; the [IAAH]$_m$ giving half-maximal response ($C_{1/2}$) is 21 nanomolar. For phase II, the values for R_{max} and $C_{1/2}$ are 56 LR per centimeter and 11 micromolar, respectively. The response is variable in the transition concentration region between the two phases; in that region (but not, or much less commonly, at higher or lower [IAAH]$_m$), LR initiation may resume or continue after the first day. At and above 100 micromolar [IAAH]$_m$, the roots are hyperstimulated and generally fail to respond. The developmental stage of LR formed in medium with very low [IAAH]$_m$ (10 nanomolar) is enhanced compared to LR formed in medium lacking auxin; the stage is diminished at higher auxin levels, in inverse correlation with FR. Trends in the responses to NAA and IBA were similar, but NAA required only 0.03 times the dose of IAA, while IBA required 6 times the dose of IAA. These findings may be of use in a search for possible auxin receptors involved with LR initiation.

LR[1] formation is a major aspect of plant morphogenesis. It is subject to experimental control by hormone application and, in particular, is stimulated by auxins. Building on much earlier work using pea roots (15, 20, 21), we developed the radish seedling root system (5) to study quantitative aspects of LR formation. Root initiation is perhaps the most dramatic and characteristic action resulting from auxin application. The radish system appears suitable for studying the first steps in auxin action, which remain very poorly understood in spite of much work using other systems (*e.g.* auxin-stimulated cell expansion). Thus, new systems may be helpful.

Based on the current understanding of how hormones and other signal molecules act in animal systems, we may postulate that auxin acts by binding to a specific receptor, which leads subsequently to LR initiation via one of several possible transduction mechanisms (8, 13, 24). In straightforward cases, binding between ligand molecules and their receptor molecules follows Michaelis-Menten kinetics that should be reflected in dose-response curves in the absence of complications. We have found that the dose-response curve between FR and [IAAH]$_m$ follows a biphasic Michaelis-Menten relationship, with a variable response occurring in the transition region between the two phases. These observations may help in identifying the ARLR by chemical means.

[1] Abbreviations: LR, lateral root(s); LRP, lateral root primordium(a); FR, frequency of LR formation (no. cm^{-1}); ARLR, auxin receptor involved with LR initiation; IAAH, IAA acid; IAA$^-$, IAA anion; [IAAt], [IAAH] + [IAA$^-$]; IBA, indolebutyric acid; NAA, naphthaleneacetic acid; $_m$, $_s$, $_r$, subscripts denoting medium, symplast, and ARLR compartments respectively; R_{max}, the maximum response expected based on fitting a set of data to a Michaelis-Menten equation; $C_{1/2}$, the concentration expected to elicit half the R_{max} based on fitting data to a Michaelis-Menten equation.

MATERIALS AND METHODS

Radish (*Raphanus sativus* L. 'Scarlet Globe') seeds were germinated in the dark at 24°C. After 3 d, root segments were excised between 0.5 and 3 cm behind the tip. Four segments were inoculated into 25 ml of medium contained in 125 ml Erlenmeyer flasks. Three or more replicate flasks were used for each treatment. The culture medium consisted of Murashige-Skoog salts (14), 88 mM sucrose, and 10 mM Mes and/or 10 mM succinic acid. Mes was used to buffer pH in the range 5.5 to 6.5, while succinic acid was used for the range 4 to 5.5; both were added to the medium when the pH was varied across the two ranges. Autoclaving caused pH$_m$ to change by as much as 0.1 unit. The pH$_m$ did not change significantly during subsequent incubation with root segments. The values of pH$_m$ presented are averages of the medium in the flasks (typically 3) used for each treatment, as determined at harvest. Variation in pH$_m$ among replicate flasks at harvest was typically less than ±0.01 unit. Segments were harvested after 2 d of culture (except as noted otherwise). The number of LRP occurring in the central 1 cm (the FR), as well as their distribution among the six developmental stages (5), was determined for each segment. Briefly, the developmental stages range from 1, a local plate-like darkening at the pericycle, to 6, a fully emerged lateral. A mean developmental stage was calculated for each segment. Custom and commercial software was used to aid in the gathering and analysis of data. The Student-Newman-Keuls test (19) was used to test the significance of any differences among a group of treatment means. [IAAH]$_m$ was calculated based on [IAAt]$_m$, pH$_m$, and the pK for IAA, 4.7 (18), using the Henderson-Hasselbalch relationship. The pK values for IBA and NAA were taken to be 4.8 (1) and 4.2 (18), respectively.

When carrying out nonlinear least squares curve fitting of treatment mean FR data, we first subtracted 6 (the average spontaneous FR) from the means. A hyperbolic curve of the Michaelis-Menten type, descriptive of the relationship between observed FR and [IAAH]$_m$, would thus have this form:

$$FR = \frac{R_{max} \times [IAAH]_m}{C_{1/2} + [IAAH]_m} + 6$$

RESULTS

Spontaneous LRP Formation. In medium containing no auxin, LRP were produced with a spontaneous frequency of about 6 cm^{-1}. There was little or no effect of pH_m over the range of 4 to 6.4 (Fig. 1). In the absence of exogenous auxin (but not in its presence), there was a tendency for the LRP to occur in higher frequency at the acropetal ends of the segments and in lower frequency at the basipetal ends (Fig. 2).

Dose-Response to IAA. We previously found that variation in pH_m had a major effect on the response to a given medium concentration of IAA and that the inclusion of pH buffers in the medium was necessary in order to obtain reproducible results (4). Our earlier studies indicated that variation in treatment mean FR was best correlated with variation in $[IAAH]_m$ (2, 7).

Treatment mean FR values are plotted against calculated treatment $[IAAH]_m$ in Figure 3. The pH_m for each treatment mean is indicated on the graph. The FR begins to rise over the spontaneous FR level as $[IAAH]_m$ rises above 10^{-8} M. As $[IAAH]_m$ rises above 10^{-7} M the FR appears to plateau, but the response is variable. The FR begins to rise steeply as $[IAAH]_m$ is increased above 10^{-6} M. As $[IAAH]_m$ nears 10^{-4} M, LRP are less well formed and more difficult to count, while most segments are killed at $[IAAH]_m$ levels above 10^{-4} M.

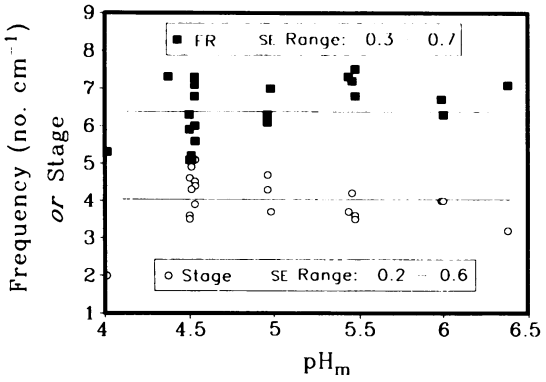

FIG. 1. Spontaneous treatment mean FR values and developmental stages. Data are presented for 21 treatments in which $[IAA]_m = 0$.

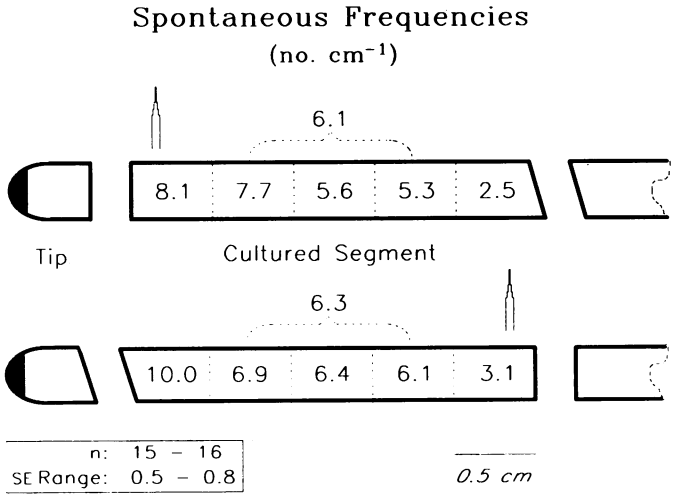

FIG. 2. Spontaneous FR related to distance behind the tip in 2.5 cm cultured segments. Two sets of segments were used. In one set (upper part of diagram), the basal end of the segments was identified by using an angled cut; in the other set (lower part), the acropetal end was marked by using an angled cut. The forceps icons indicate which end of the segments was handled during inoculation. The treatment mean FR (no. cm^{-1}) is indicated for each 0.5 cm region; the treatment mean FR in the central cm is also indicated.

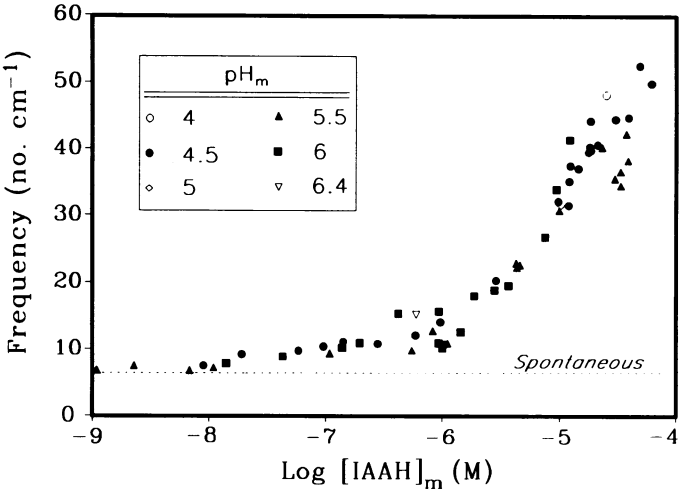

FIG. 3. Treatment mean FR data plotted as a function of $[IAAH]_m$ (calculated using the Henderson-Hasselbalch relationship, using pK = 4.7). Roots were harvested after 2 d in culture. The sample size was 12 in most instances. The spontaneous FR is set at 6 cm^{-1}.

We refer to these four distinct regions of the dose-response curve as phase I, the transition region, phase II, and the region of hyperstimulation. They are described in more detail below.

Phase I and Phase II. A Michaelis-Menten hyperbolic curve was fitted to the data points for treatments in which $[IAAH]_m$ fell in the range between 0 and 300 nM. The result is shown in the upper part of Figure 4. As seen from the data shown on the graph, $C_{1/2}$ was determined to be 21 nM, and the limiting phase I FR to be 5.2 above the spontaneous FR. Likewise, a hyperbolic curve was fitted to data for treatments in which $[IAAH]_m$ fell in the range between 3 and 20 μM (Fig. 4, lower). A limiting FR value of 56 LRP cm^{-1} above the spontaneous is suggested by the curve fitting results; $C_{1/2}$ is 11 μM. In all of our work with higher levels of IAA, treatment means have rarely been over 60 LRP cm^{-1}, although exceptional individual segments have formed up to slightly over 100 LRP cm^{-1}. The FR values for treatments in which $[IAAH]_m$ was above 20 μM, and in which the pH_m was above 5, tended to be well below the FR predicted by the curve. This might be a consequence of the very high $[IAAt]_m$—over 200 μM.

The Transition Region. On the basis of either the phase I or II curves, FR values were often higher than expected among segments cultured in medium with [IAAH] in the range 0.1 to 3 μM. This was especially so in our earlier studies, when segments were harvested after 4 (rather than 2) d in culture. Among segments harvested after 4 d in medium with $[IAAH]_m$ in the range 0.1 to 3 μM, many segments had a broad and usually bimodal distribution in their LRP developmental stages (Fig. 5). The bimodality is due primarily to 'late-formers' rather than 'laggards' in development, as shown by time course studies (3; Fig. 6). It was usually found that initiation of LRP ceased within 1 d (or 2 d at high $[IAAH]_m$) after inoculation. At high FR (*e.g.* see the 14 μM $[IAAH]_m$ data in Fig. 6), small, closely spaced stage 1 LRP are often not well enough resolved during the first 24 h for accurate counting, and this may account for the observed increase in LRP during the second day. However, in the time course for 1.2 μM $[IAAH]_m$ shown in Figure 6, there was clearly a re-

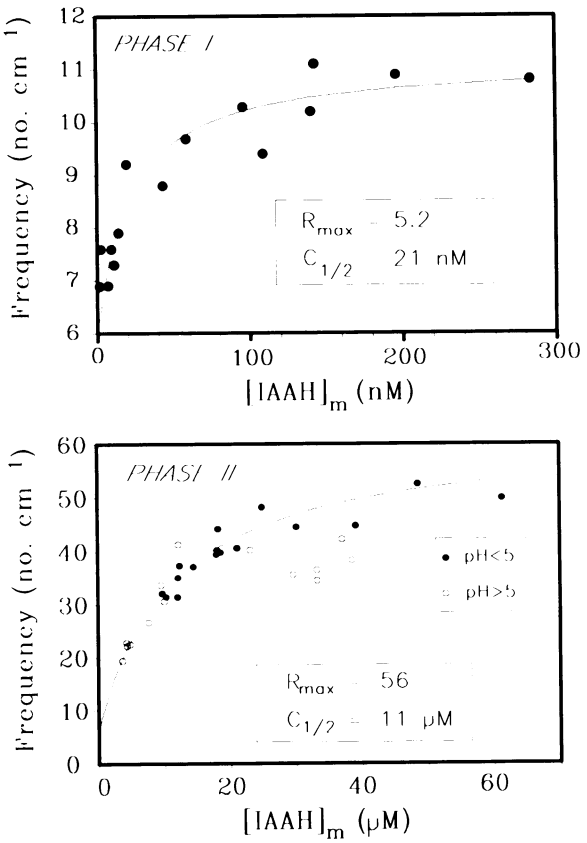

FIG. 4. Treatment mean FR data plotted as a function of $[IAAH]_m$, along with Michaelis-Menten curves fitted to two ranges of the data shown in Figure 3. In the upper graph (phase I), the curve is a result of fitting between 0 and 300 nM $[IAAH]_m$. In the lower graph (phase II), the curve results from fitting to data between 3 and 20 μM $[IAAH]_m$. R_{max} is the maximum response (above the baseline spontaneous FR of 6 LRP cm^{-1}) expected, based on curve fitting; $C_{1/2}$ is the concentration ($[IAAH]_m$) expected to elicit one-half the maximum response, based on curve fitting.

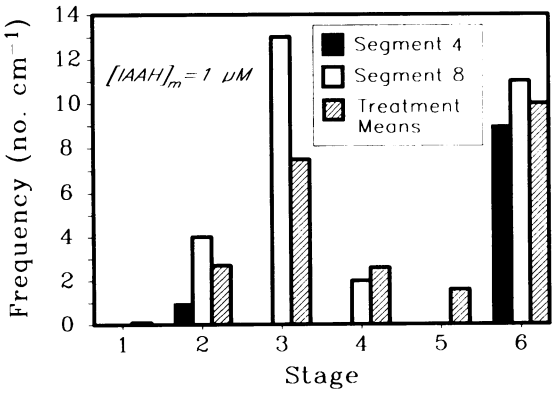

FIG. 5. Bimodal distribution of LRP among the six developmental stages in segments cultured 4 d in medium with 1 μM IAAH. In this treatment, segment number 4 had only one late-former or laggard at stage 2; segment number 8 had many. As the treatment means indicate, most segments had many. ($[IAAt]_m$ was 5.9 μM, and pH_m was 5.4. Overall treatment mean values were: FR, 24.4 ± 3.8; stage, 4.7 ± 0.2; n = 12.)

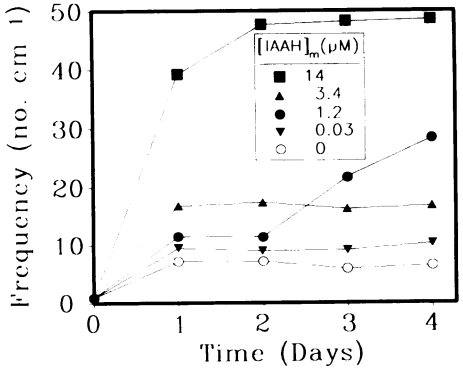

FIG. 6. Time course of LRP appearance in segments cultured through 4 d in media containing various levels of IAAH. A sample of 40 segments harvested at inoculation time (time 0) had mean FR 0.9 ± 0.3 LRP cm^{-1}; 33 had none, while the remainder had 2 to 7 stage 1 LRP. For the other points, n (*i.e.* sample size) ranged from 10 to 20; SE values were typically about 7% of their associated means (except d 3 and 4 at 1.2 μM $[IAAH]_m$, where they were 13%). The pH_m was 5.5 for each time course except the one using 0.03 μM $[IAAH]_m$, in which case it was 4.5.

sumption in LRP initiation after 2 d. At 4 d, the FR of only those LRP at stages 5 and 6 was 10.6 cm^{-1}, nearly equal to the total FR at d 1 and 2, which was 11.5 cm^{-1} in each case. The remaining 17.8 LRP cm^{-1} at d 4 (those at stages 1 to 4) were therefore mostly late-formers.

Data from the earlier experiments, in which segments were cultured for 4 d, are shown in Figure 7. All treatment means are shown in the upper graph, plotted against $[IAAH]_m$. The data are very similar to the 2 d data for $[IAAH]_m$ below 0.1 μM. Between 0.1 and 3 μM, there is considerable variation in FR, which is not correlated with $[IAAH]_m$. A Michaelis-Menten curve was fitted to data for which $[IAAH]_m$ fell between 3 and 20 μM. The resulting phase II curve for the 4 d data is shown in the upper graph; the 2 d phase II curve is shown for comparison. The values of $C_{1/2}$ for the two curves are very similar (14 and 11 μM). R_{max} is higher for the 4 d data, suggesting a degree of resumed or continued initiation of LRP at the higher levels of $[IAAH]_m$.

In the lower portion of Figure 7, we show treatment mean data, broken into two classes (stage range 1–4, and stage range 5–6), for the transition region and phase I. The treatment mean FR values of LRP at stages 5 to 6 clustered around the 2 d phase I curve (shown on the graph). There was considerable variation among the means for LRP at stages 1 to 4 in the transition region. There was no clear correlation between the latter means and any of the treatment variables (pH_m, $[IAAH]_m$, $[IAA^-]_m$, or $[IAAt]_m$), and we do not yet know what might foster resumed or continued LRP initiation. Further study may be warranted as the behavior in this region might provide clues to the nature of the mechanism that responds to auxin.

Two root segments are pictured in Figure 8. The upper photograph shows a portion of a segment cultured in medium with 1 μM $[IAAH]_m$ for 4 d, illustrative of segments with late-formers. The lower photograph shows a portion of a segment cultured 4 d in medium with 3.8 μM $[IAAH]_m$ which, having no late formers, is representative of segments cultured in medium just above the transition region.

Hyperstimulation Region. Signs of stress become apparent at high auxin levels. Symptoms of hyperstimulation were poorly developed, merged, or indistinct LRP, a continuous uneven darkening of the pericycle, or the absence of any visible response. Segments in which LRP could not be clearly counted, or within

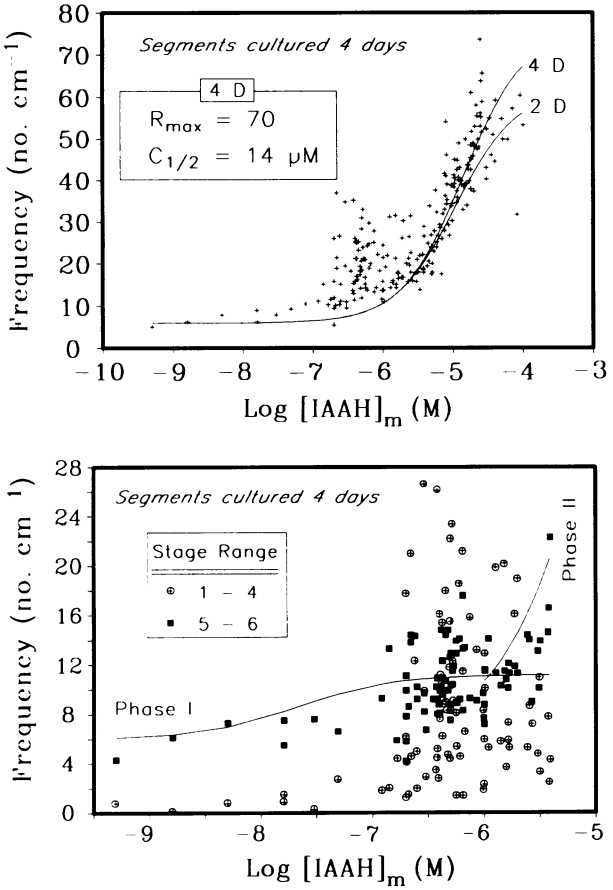

FIG. 7. Data from treatments in which segments were cultured for 4 d. The upper graph shows all the treatment mean FR values plotted against calculated $[IAAH]_m$. On the upper graph, '4 D' refers to the Michaelis-Menten hyperbolic curve which was determined by fitting to the 4 d data in the range 3 to 20 μM $[IAAH]_m$. '2 D' indicates the curve fitted to 2 d data over the same $[IAAH]_m$ range (*cf.* Fig. 4). In the lower graph, means associated with $[IAAH]_m$ between 0 and 4 μM were broken into two classes by stage range, as indicated on the graph. The curve fitted to the 2 d phase I data (*cf.* Fig. 4) is shown, as well as a short length of the 2 d phase II curve at and beyond their point of intersection. Most of the LRP at stages 1 to 4 are presumed to be late-formers.

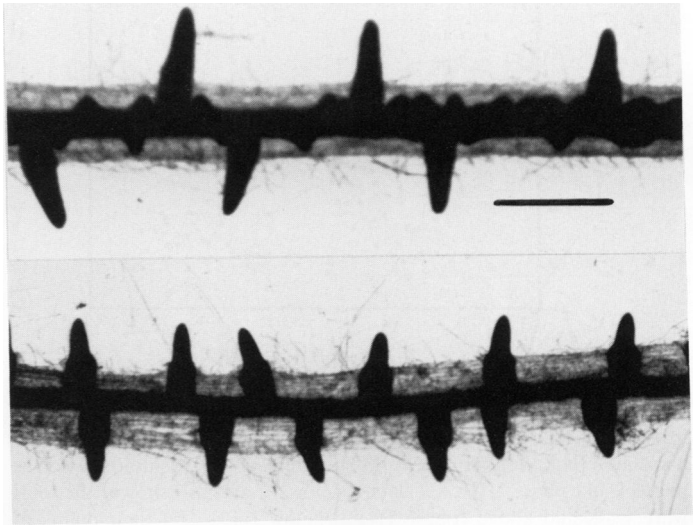

FIG. 8. Photographs showing portions of segments cultured for 4 d in medium containing IAA. The upper segment, which includes apparent late-forming LRP, was cultured in medium containing 1 μM $[IAAH]_m$ (the pH_m was 5.4). The lower segment was cultured in medium containing 3.8 μM $[IAAH]_m$ (the pH_m was also 5.4). The bar represents 1 mm.

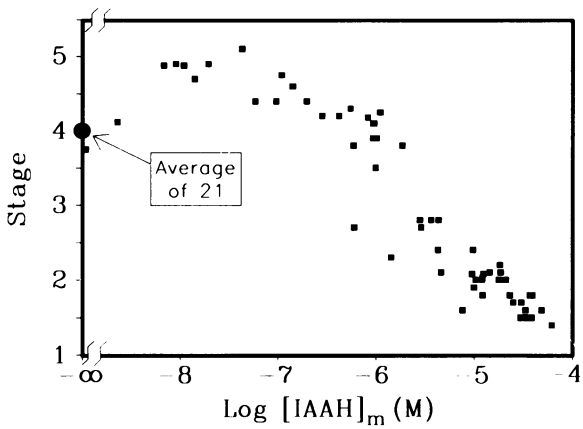

FIG. 9. Treatment mean developmental stage, related to $[IAAH]_m$. The 21 treatment means making up the large point at 0 $[IAAH]_m$ are shown individually as circles in Figure 1.

which only a part of the central cm responded, were not used for determining FR values. Segments harvested from media with $[IAAH]_m$ above 10^{-4} M showed no sign of pericycle activity; they were typically without turgor and presumed dead.

Developmental Stage *versus* $[IAAH]_m$. The developmental stage of LRP varied with the auxin content of the medium. As shown in Figure 9, treatment mean developmental stages at very low $[IAAH]_m$ (10^{-8} M) were higher than in medium with no auxin. Therefore, it can be concluded that LRP development is stimulated by low levels of auxin. However, stages declined at higher auxin levels, in close correlation with increases in FR (*cf.* Fig. 3).

Dose-Response to NAA and IBA. We have gathered dose-response data for the synthetic auxins NAA and IBA. As with IAA, the responses to IBA and NAA were found to be best correlated with medium concentrations of their protonated forms. In Figure 10, treatment mean FR values are plotted against calculated medium concentrations of the undissociated auxins. Also shown are the phase I and phase II curves for IAAH. Trends in the responses to NAA and IBA were similar to those for IAA, but the response to NAAH required only 0.03 times the dose of IAAH, while the response to IBAH required 6 times the dose of IAAH. For NAA, hyperstimulation set in as $[NAAH]_m$ approached 10 μM; between 1 and 10 μM, treatment means of over 50 well-formed LRP cm^{-1} were obtained. On the other hand, hyperstimulation set in with IBA before such high treatment means were realized. The highest IBA treatment mean FR values were 43 and 39, obtained at $[IBAH]_m$ values of 60 and 69 μM, respectively. At higher $[IBAH]_m$, segments generally failed to respond. Late formation of LRP was much less common with IBA than with IAA, while the response to NAA was similar in that regard to IAA.

DISCUSSION

The pericycle of the radish seedling root exhibits a rapid and potentially massive morphogenetic response to exogenous auxin. Cell division commences with a lag time of only 2 h (6). With this study, we have begun a more focused characterization of the response to auxin. Once the biological characterization is well enough advanced, investigations may begin on the first step

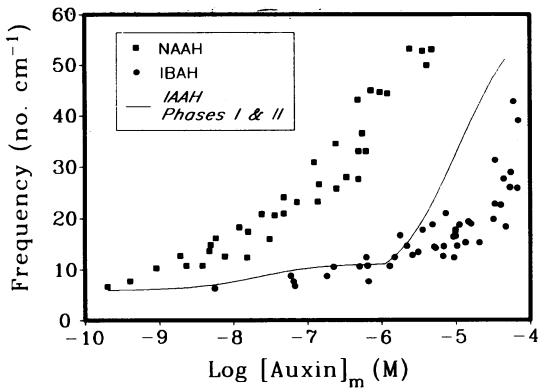

FIG. 10. Treatment mean FR values, from a number of experiments in which the auxins NAA and IBA were used, plotted against their associated $[NAAH]_m$ or $[IBAH]_m$. For comparison, the fitted curves for phase I and phase II IAAH data (*cf.* Fig. 4) are also shown; the lines were truncated above (phase I) and below (phase II) their points of intersection.

in auxin action: (presumably) binding to a specific receptor. It is possible to interpret our data in a manner consistent with current concepts of auxin uptake and responses based upon ligand-receptor interaction. Although other interpretations are certainly conceivable, we will focus the discussion on the above, which seems the simplest in light of our current understanding of the system and its response to auxin.

IAA Uptake Considerations. If we knew, for each medium auxin concentration and its associated FR, the concentration of auxin at the sites where auxin interacts with the ARLR, then we would have a better signature of the ARLR to help in detecting it by radioligand binding. It is not possible at present to determine the auxin concentration at the sites of auxin action in LR initiation because, for one thing, we don't know where the sites are (they presumably reside in the pericycle, but in what local environment?). Nevertheless, the data presented here may already give a good indication of what to begin looking for.

Earlier, when our harvesting was done after 4 d of culture, we considered (2) the possibility that there was only a single phase in the FR/$[IAAH]_m$ relationship (corresponding to what we now call phase II). We thought the high response (higher than expected of a Michaelis-Menten hyperbola) at lower $[IAAH]_m$ (what we now call phase I and the transition region) might be a result of active uptake of $[IAA^-]$, a phenomenon thought to be operative in other systems (9, 11, 18). We believe that the 2-d data are a clearer reflection of the LR initiating events; considering the close correlations seen in Figure 4, we now conclude that there is a biphasic Michaelis-Menten relationship between FR and $[IAAH]_m$.

It has long been recognized that uptake of exogenously supplied IAA into cells, tissues, or membrane vesicles is often strongly influenced by medium pH in a manner consistent with passive diffusional uptake of the lipophilic IAAH (18). The promotion of LR formation in the radish by exogenous auxin appears to be solely a response to $[IAAH]_m$. By varying pH_m and $[IAAt]_m$, it is possible to prepare media with a wide range of $[IAAH]_m$ and $[IAA^-]_m$. In two media with very different $[IAA^-]_m$, but with the same $[IAAH]_m$, the response in the medium with the higher $[IAA^-]_m$ will be the same as (or, at very high $[IAA^-]_m$, even lower than) in the medium with the lower $[IAA^-]_m$ (*cf.* Fig. 3). We proposed that the endodermis in the radish seedling root is an effective barrier to free diffusion of $[IAA^-]$ between the external medium and responsive sites, presumably residing in the pericycle (4). It seems plausible to assume that IAAH would equilibrate in concentration between all aqueous membrane-bound compartments, moving between them by diffusion through their membranes. The concentration of $[IAA^-]$ in each aqueous compartment would depend on the local pH. For example, if $[IAAH]_m$ and $[IAAH]_s$ were 11 μM (the $C_{1/2}$ in phase II), $[IAA^-]_s$ would be expected to be 2 mM if the cytosolic pH were 7 (17).

It appears that IAA in the medium is quite stable during a culture period, based on the response of roots to aged or reused medium (data not shown). We have no data on metabolism of IAA in the roots. However, if IAA is metabolized in the roots, it would most likely be quickly replaced by IAA from the vast reservoir in the medium. The ratio of medium volume to total root volume is over 1000 (20 mg of root in 25 ml of medium); the distance between the medium interface at the root surface and the pericycle is only about 170 μm (5).

Dose-Response Curves and the ARLR. There has been much discussion in the literature of dose-response curves for plant hormones (12, 16, 22, 23). The response to a plant hormone has often been found to increase over several decades of log hormone concentration, rather than only over the 2 decades expected for a response based on a simple case of ligand-receptor binding. Even at the level of gene transcription (auxin-specific stimulation of mRNA accumulation), it has been found that the response may increase over several decades (10). Often, extended dose-response curves may be described by a multiphasic isotherm; the transition from one phase to another has been suggested to be related to a conformational change in the receptor (16).

Our 2-d FR/IAAH dose-response data rather clearly describe a biphasic isotherm. Perhaps the ARLR undergoes a conformation change in the transition region, which occurs in that part of the dose-response curve where phase I has reached its maximum, and where there is an interesting variable and perhaps adaptive response. If the quantitative relationship between auxin-receptor binding and the observed response (FR) were a simple one, then $C_{1/2}$ values should coincide with receptor K_d values (13).

Plant tissues can be expected to contain a variety of proteins with specific auxin-binding capability: perhaps more than one type of 'true' hormone receptor; auxin uptake and efflux carriers, with roles in auxin transport (9, 11, 18); possibly plasma membrane ATPases (18); enzymes involved with auxin metabolism; as well as proteins unlikely to be involved with any auxin function (13, 18). Thus, it will be essential to have a distinctive receptor signature to look for when carrying out binding studies with radio-labeled auxin. The findings presented here, along with additional biological characterization of the response to auxin, may help point the way to an ARLR and associated transduction mechanisms.

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