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Importance of Time after Excision and of pH on the Kinetics of Response of Wheat Coleoptile Segments to Added Indoleacetic Acid[1]

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ABSTRACT

Segments of coleoptiles of 3-day-old wheat (*Triticum* × *Aestivum* L. cv. Kharkov M.C. 22) grown at 24 C were strung on a glass rod and the kinetics of their elongation in 0.01 M K-phosphate buffer was examined photometrically. Measured rates of elongation in response to treatments were corrected by subtraction of endogenous rates. The customary practice of testing the effects of growth regulators added between the two endogenous surges of growth, that is, up to 3 hours after segments were excised from coleoptiles, gave erroneous kinetic data. Rates of response were then limited by the passive penetration of added auxin and the second endogenous surge interfered with late responses. It was necessary to wait for a phase of more rapid but more steady elongation after the second endogenous surge was over, about 4 hours for wheat at 25 C, to attain the active uptake required for nearly synchronous response through the segment. The more active uptake in this steady phase was confirmed with β-[2-^{14}C]indoleacetic acid and it was greater at pH 5 than at pH 7. The degree of dissociation of indoleacetic acid added at pH 7 was an impediment to penetration that could be compensated for by removal of intercellular air. The pH did not influence the endogenous rate of elongation. The dependence of the rate of elongation on the concentration of indoleacetic acid added at pH 5 was bell-shaped with maximum rate at 10 μM indoleacetic acid, in confirmation of previous measurements made over long intervals of time. The relation between the response and suboptimal concentrations was not sigmoid but was indicative of greater binding affinity than previously reported.

Vertically oriented excised coleoptile segments do not elongate endogenously (spontaneously) at a constant rate (6, 13). We reported previously that in coleoptile segments of Kharkov wheat at 25 C an initial endogenous acceleration or surge of elongation starts about 15 min after excision, extends through another 15 min, and declines to a low rate which is terminated in 75 min by a 30- to 120-min long second surge that finally slows to a more steady rate (20). We now describe experiments on the most suitable time for the addition of IAA, from which we conclude that most published responses of coleoptile segments to IAA may have been subject to restricted penetration limited chiefly by the absorbing activity of the cells and by the charge on the IAA.

The initial endogenous surge in the 1st hr after excision was described as a tactile effect (12) or as a wounding or residual IAA effect (16). Most investigators float their decapitated coleoptile segments for 0.5 to 2.5 hr (usually 1.5 hr) on buffer until this effect appears to be over, then add IAA. The response to IAA was reported apparently without correction for the highly variable endogenous rate and its second surge. Some investigators felt justified in ignoring endogenous rates because pulse treatments with 1 μM IAA showed stimulation followed by a return to the slow preaddition rates (7, 11, 13, 25).

The larger, second endogenous surge of elongation started at 1.75, 2.5, or 4 hr after excision of coleoptile segments of wheat (20), oat (6, 10, 13, 17) or corn (13), respectively, at about 25 C, and this time was inversely dependent on the temperature. This spontaneous growth response was identified with the physiological regeneration of the tip or the resumed synthesis of IAA (13, 30). It may be considered the start of another phase of elongation which was more rapid than the initial phase, and because this surge subsided to a steady rate that was prolonged over many hours, we refer to the stabilized rate as the steady phase of elongation. No previous continuous records of the effects of IAA added clearly in this steady phase have been made, yet there is evidence that it is the time at which metabolism has returned closest to normalcy (24, 29).

The dependence of the rate of coleoptile extension growth on the concentration of IAA is also unsettled in the literature. In early experiments based on measurements made more than 12 hr after application of IAA, a bell-shaped curve always resulted from the inhibitory effect of 10 μM to 1 mM IAA (3, 5, 14, 19, 22). At suboptimal concentrations, the dependence was hyperbolic. The hyperbolic form was extended to all concentrations in later data for which initial rates were derived from measurements at 2-hr (2) or 5-hr (31) intervals. Recent continuously recorded kinetic data, however, led to statements that the dose response curve is actually sigmoid (5, 22). In each of the two independent investigations, this conclusion hinged on a single datum for the lowest concentration of IAA tested 0.1 nM (22), 10 nM (5). A more detailed experiment (5, Fig. 6) established the hyperbolic curve. No inhibition by high concentrations of IAA was observed in the rapid measurements. Clarification of these kinetic data is clearly required for a given material and method.

Considerably more published data are concerned with the latent period or lag time between the addition of the auxin and the response of the coleoptile segments (17, 25). Most evidence does not support the view that the lag is the time required for the synthesis of new protein (12, 22, 28), but very short response times are in question (9, 10, 28). Experiments on [^{14}C]-IAA uptake (22) showed that the entry of IAA is limited to the cut ends of the coleoptile segments and that the lag involved a delay in the longitudinal distribution of auxin. Subsequent work in other laboratories sought further significance in the lag which we feel is unjustified.

Most investigators of rapid kinetics of coleoptile responses

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chose to experiment in the range of pH 6 to 7. A few have used pH 4.5 to 5.5. The promotion of elongation of *Avena* coleoptiles by acidic buffers was initially (4) believed to be a result of the activity of nondissociated endogenous or added IAA (pK_a = 4.75) (8, 23). Acidic buffers promoted the elongation of coleoptile segments maximally at pH 3 (27) or 3.9 (15), and the uptake of [^{14}C]IAA also was shown to be maximal at pH 3.3 (22). The true optimum pH for elongation as well as uptake (above) may be pH 5, as the former applied to the acid buffer effect on elongation after the epidermis was peeled from the segments to facilitate penetration (26). Our data show that the bulk protons at a continuous pH of 5 only facilitate the response to IAA by promoting uptake of IAA.

MATERIALS AND METHODS

Seeds of *Triticum* × *Aestivum* L. cv. Kharkov M.C. 22, a winter wheat, were germinated and grown in the dark at 24 C for 64 to 70 hr. Coleoptiles 20 to 25 cm long were excised near their bases, drawn from the primary leaf, and floated on 0.01 M K-phosphate buffer at the experimental pH. Timing started upon the excision of the fifth coleoptile. Ten 1-cm segments were cut, strung onto a glass rod in buffer, and placed in an oxygenated, 50-ml reservoir thermostated at 25 C in a photometric recording assembly (20). All data presented below are averages from three to nine separate experiments.

The lag period between the time of addition of IAA and the stimulated rate of elongation was measured as the intercept time (22). The maximum rate of response to the added IAA, or to the endogenous IAA in the second endogenous surge of elongation, was corrected by subtraction of the preresponse rate because variability prevented the correction of the rate of response of an experimental column by the rate of a control column. The validity of this routine correction was supported by tests on the time of addition of IAA. The time and rate of the second endogenous surge represented a limit or baseline for the intercept time and for growth responses in the initial phase because early added IAA of lowest concentration did not produce an earlier response or affect the magnitude of the second surge (Table I). The responses to 10 μM IAA added at about 35 min in the initial phase are given in Table I. If, however, IAA was added later in the initial phase so that the response occurred near the time of the second endogenous surge, the resulting rate was the sum of the initial response and the second surge and exceeded the response to IAA added later in the steady phase.

The rate of uptake of β-[2-^{14}C]IAA (New England Nuclear) was determined by incubating rods of 10 coleoptile segments in 10 ml of five different concentrations of aerated labeled IAA for four to eight periods from 1 to 30 min at 25 C. Upon removal from the tracer solution, each rod was rinsed by a series of four dips in fresh, distilled H_2O and the segments were removed with forceps (spaced to prevent pinching), flushed through by a 10 ml/min jet of tap water from a 26-gauge hypodermic needle, blotted, and plunged into 2 ml hot 80% ethanol. One ml was mixed with 10 ml Bray's solution and counted in an Ansitron liquid scintillation counter (Picker Nuclear). Rates were computed from the slopes of the linear plots of cpm *versus* time (22) and converted through dpm to μμmol IAA/min · 1-cm segment. Rates of uptake of IAA from a given concentration were read from plots of rate *versus* concentration. Average rates from quadruplicate tests at various concentrations had a coefficient of variation of 25%.

Table I. Effects of Time of Addition of IAA on Lag Period and Rate of Elongation

Averages of three runs ± standard deviation at pH 7

IAA Added Time (min)	IAA Added Concn (μM)	Stage after Excision	Intercept Time (min)	Elongation Rate ($\mu \cdot min^{-1} \cdot cm^{-1}$) Pre-response	Maximum	Corrected
-	0	Second Surge	75[1] ± 10	0.7 ± 0.3	6.3 ± 1.7	5.6 ± 1.5
		Initial Phase				
35	10	Early	20 ± 2	1.6 ± 0.2	13.2 ± 0.9	11.6 ± 1.3
90	10	Late	19 ± 3	0.9 ± 0.5	18.6 ± 0.9	17.7 ± 1.3
240	10	Steady Phase	15 ± 2	3.7 ± 2.5	15.3 ± 5.7	12.2 ± 2.8

[1] Period between time when auxin otherwise added (20 minutes from start of recording or 35 minutes from mid-excision) and time of intercept between slope in initial phase and maximum slope of second surge.

RESULTS

Rates of Endogenous Elongation. During the initial phase after the first surge, the endogenous rate slowed by 50% (Table II). The average maximum rate of the second surge, not corrected by subtraction of the final rate in the initial phase, amounted to an acceleration of about 6-fold at pH 5 or about 9-fold at pH 7 (Table II). There was no significant effect of pH on the endogenous rate at any stage, including the rate in the steady phase about 4 hr from excision when the rate had subsided 20 to 50% below the maximum rate of the second surge.

Effects of Phase of Elongation and pH on Rate of Uptake of IAA. [^{14}C]IAA was taken up by coleoptile segments, under the same experimental conditions as those used for the determination of elongation, by linear (zero order) kinetics over at least 30 min in contrast with the lag and acceleration in the response of segment elongation to added IAA. This confirmed the data of Nissl and Zenk (22) for *Avena* coleoptile segments, in both kinetic order and magnitude. The rates of uptake (Table III) in the steady state phase were significantly higher than those in the initial phase, and the rates in either phase at pH 5 were significantly higher than those at pH 7.

Effects of Segment Length and Preinfiltration on Lag Period and Elongation Rate. Data for pH 7 on lag periods are plotted inversely in Figure 1A to give a rate expression for comparison with rates of elongation in Figure 1B. It is assumed that 1/intercept time includes the rate of penetration of IAA. Its values in the steady phase of elongation were significantly higher than those in the initial phase, unlike the rates of elongation. The lag period averaged 28% shorter in the steady phase than in the initial phase, but it was significantly extended by the use of coleoptile segments more than 1 cm long which also significantly decreased the elongation rate in the steady phase (Fig. 1B). The particularly low rates for the five 2-cm coleoptile segments, with only 10 cut ends, were not depressed by the use of the 2nd cm of the original coleoptile because when the five 2-cm segments were further cut in half, the IAA-stimulated growth of the resulting 10 1-cm segments was no less than that of 10 first 1-cm segments. The rate of elongation of 1-cm segments in the steady phase was also limited by the number of cut ends available for

Table II. Average Rates of Elongation of Wheat Coleoptile Segments Without Added Auxin at Different Stages after their Excision in Acidic and Neutral Buffers

Averages of numerous determinations ± standard deviations, for 0.01 M potassium phosphate buffer

Stage after Excision	pH 5	pH 7
Initial Phase		
Early	1.9 ± 0.8	1.4 ± 0.5
Late	1.0 ± 0.3	0.7 ± 0.3
Second Surge	6.1 ± 0.9	6.3 ± 1.7
Second Phase	4.9 ± 1.6	3.2 ± 1.5

Table III. Effects of Phase of Elongation and pH on Rate of Uptake of ^{14}C-IAA

7.5 μM IAA in 0.01 M potassium phosphate buffer at 25°C

pH	Phase	Rate ($\mu\mu$ mole $\cdot min^{-1} \cdot segment^{-1}$)
5	Initial	0.33
	Steady	1.10
7	Initial	0.10
	Steady	0.43

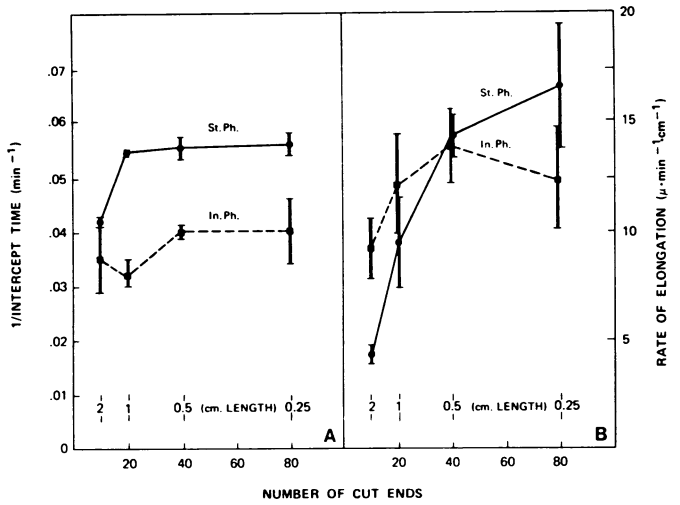

FIG. 1. Responses of 10-cm long columns of coleoptile segments in the initial phase (In. Ph.) and steady phase (St. Ph.) to 1 μM IAA at pH 7 and 25 C as a function of the number of cut ends (or length of segments). Averages of three measurements each ± standard deviation. A: Penetration rate as the inverse of the lag period; B: elongation rate.

entry of IAA, and the hyperbolic trend indicated a maximal rate higher than 17 $\mu m \cdot min^{-1} \cdot cm^{-1}$ that would be theoretically obtainable with more than 80 transverse cuts in the 10-cm column of coleoptile tissue. The less marked effects obtained in the initial phase were not readily discernible from the experimental variance, but similar trends were indicated (Fig. 1, A and B). The rate of elongation was less affected by the cut surface area (*i.e.* the number of cut ends) in the initial phase than in the steady phase.

When 1-cm segments were infiltrated with buffer solution of pH 7 through successive aspirations of air before they were strung on the glass rod, the lag periods in response to 1 μm IAA were decreased by about 25% in both initial and steady phases (Table IV). Preinfiltration had no effect on the rate of elongation of the 1-cm segments in the initial phase in response to 1 μM IAA possibly because the latter response was saturated under these conditions (Fig. 4B), but the treatment doubled the rate in the steady phase (Table IV). As the same rate of elongation (about 20 $\mu m \cdot min^{-1} \cdot cm^{-1}$) was achieved by infiltrated 5-mm segments in their steady phase (Table IV), the elongation rate was independent of the number of cut ends and may represent the saturation value for Figure 1B. The same rate was maximal at pH 5 (below) and it was not enhanced by preinfiltration.

Effects of IAA Concentration and pH on Lag Period. The intercept time decreased with increase in concentration of IAA (Fig. 2). It was longest for segments in the initial phase, when the lowest concentration of IAA tested seemed to postpone the second endogenous surge of elongation. The latter was also postponed by the lower pH (Fig. 2A). The intercept time in the initial phase fell rapidly with increasing IAA concentration and tapered off to a minimum of 15 min at 1 mM IAA at both pH 5 and 7. The intercept time of segments in the initial phase in response to 0.1 μM IAA was considerably shorter at pH 5 (39 min) than at pH 7 (65 min). A plot of the inverse lag *versus* the logarithm of the concentration at pH 5 (Fig. 3) was linear and intersected the abscissa at approximately 1 nM IAA.

In response to low concentrations of IAA, lag periods were shorter in the steady phase than in the initial phase and approached a maximal value of approximatley 0.5 hr at both pH 5 and 7. At each pH, the intercept time for 0.1 μM IAA was about 25 min, in contrast with the divergent values obtained in the initial phase. At high concentrations of IAA, the same short intercept time of 15 min was obtained in the steady phase as in the initial phase and at both pH 5 and 7 (Fig. 2). This reveals a common, pH-independent ceiling on the rate of penetration.

Table IV. Effects of Pre-infiltration with Buffer on Lag Periods and Rates of Elongation in Response to 1 μM IAA
Average ± standard deviation of three runs each at pH 7

Segment Length (mm)	Condition	Initial Phase		Steady Phase	
		Intercept Time (min)	Response Rate ($\mu \cdot min^{-1} \cdot cm^{-1}$)	Intercept Time (min)	Response Rate ($\mu \cdot min^{-1} \cdot cm^{-1}$)
5	Control	25 ± 1	13.9 ± 1.7	18 ± 1	14.3 ± 1
	Pre-infiltrated	-	-	15 ± 2	19.4 ± 8
10	Control	31 ± 3	12.1 ± 2.3	18 ± 1	9.4 ± 2.1
	Pre-infiltrated	23 ± 1	12.2 ± 1.9	14 ± 0	20.6 ± 1.3

Effects of IAA Concentration and pH on Rate of Elongation. Sensitivity to added IAA was enhanced in the steady phase over that in the initial phase. Segments in the initial phase required at least 0.1 μM IAA to exceed the rate of their second endogenous surge (the baseline in Fig. 4, A and B), but in the steady phase, significant response was obtained to 20 nM added IAA at pH 5 (Fig. 4C). Since a change of buffer solution alone elicited a measurable response in about two out of five tests, similar marginal activities caused by changes from buffer to low concentrations of IAA were not considered as hormonal responses. In general, the coleoptile extension appeared to be more sensitive to IAA, with respect to both threshold and maximum rate of response, at pH 5 than at pH 7. The maximum rate of elongation was matched at pH 7 when the segments were preinfiltrated (see above).

A bell-shaped curve of rate *versus* concentration was obtained at pH 5 in the steady phase as a result of inhibitory action of IAA above 10 μM (Fig. 4C). There was no evidence at low concentrations to support a sigmoid shape of the rate *versus* concentration curve. When the data were plotted without correction of individual maximum rates by subtraction of the preresponse endogenous rates, the same kinetic form was obtained in a pronounced A-shape.

DISCUSSION

We collected data on the lag periods (intercept times) and elongation rates of wheat coleoptile segments in response to added IAA as functions of the length and infiltration of the segments and the IAA concentration and pH of the solution, in both initial and steady phases of elongation following excision from coleoptiles. The information can be interpreted in terms of the kinetics of penetration of the segments by IAA, which was particularly rate-limiting during the initial phase, and the kinetics of cellular activity including active uptake of IAA which predominated in the steady phase. Poole and Thimann (24) observed two phases of [^{14}C]IAA uptake by segments of *Avena* coleoptiles when the auxin was added immediately after excision. The initial one was physical uptake independent of O_2. The second phase occurred after 2 hr, was O_2 dependent, involved greatly enhanced uptake when the addition of IAA was delayed until 4 hr from excision, and resulted in true accumulation of IAA. We confirmed these phases of uptake of [^{14}C]IAA. Rowan *et al.* (29) observed an increase in respiration of wheat coleoptile segments only after 2 hr from the initial addition of IAA (20 μM) at 25 C.

Several observations demonstrated that the rate of penetration of the segments by IAA limited their response. The lag period was shortened and the rate of elongation of segments was increased when there were many severed cross-sections in the 10 cm of coleoptile tissue. At pH 7, the lag period was maximally decreased, but not abolished, by preinfiltration with buffer which also relieved the limitation on the rate of elongation by IAA penetration at that pH. The exponential dependence of the lag period on IAA concentration in the initial phase and the

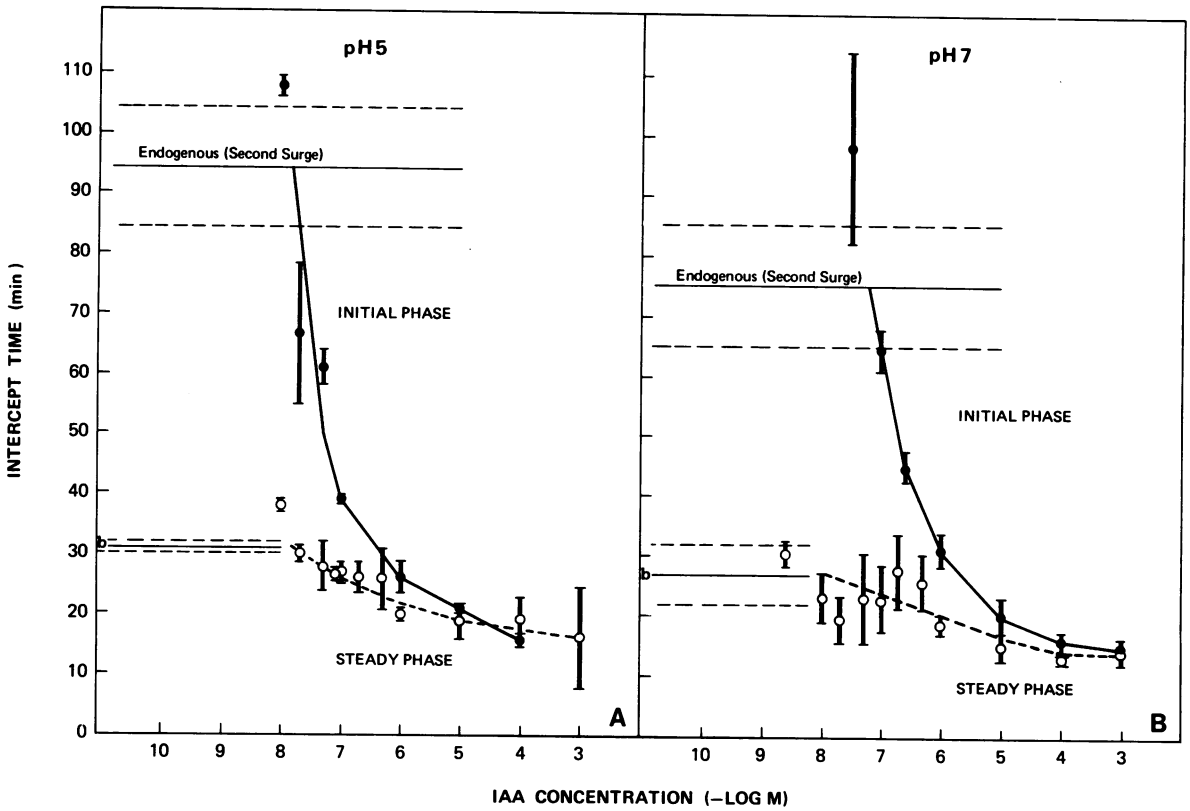

FIG. 2. Influence of IAA concentration on the lag period in the responses of columns of 10 1-cm coleoptile segments in their initial and steady phases of endogenous growth. The period between the time when IAA was added to experimental samples and the time of intercept with the maximum second cycle rate in control columns is represented by the Endogenous line ± the standard deviation (- - -). The average intercept time in response to change of buffer in the steady phase is represented by the line marked b ± its standard deviation (- - -). A: pH 5; B: pH 7.

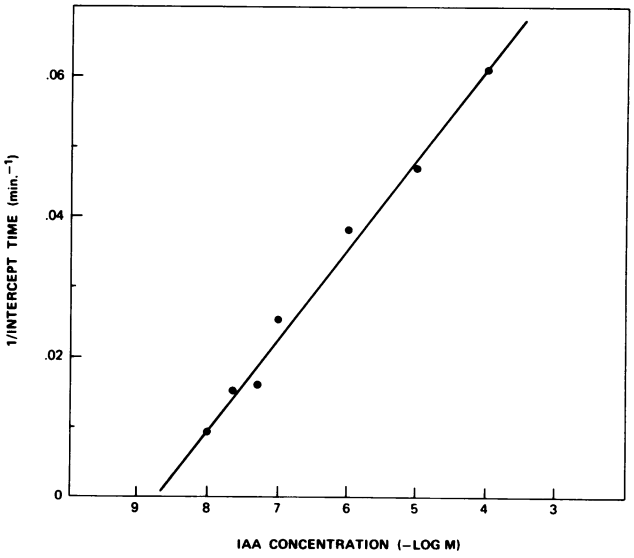

FIG. 3. Relation between the rate of penetration, as the inverse of the intercept time, and the concentration of IAA, for columns of 10 1-cm segments at pH 5 and 25 C.

lowest effective concentration implied that the lag in that phase was primarily determined by the diffusive penetration of the IAA into the segments. As the elongation of long segments at pH 7 caused by 1 μM IAA was faster in the initial phase than in the steady phase, one may suggest that as the IAA entered the ends of long segments in the steady phase, its binding by the end cells impeded its diffusion to the cells in the interior of the segments.

Additional observations showed that kinetic control by the passive penetration of auxin was partially removed by its active uptake in the steady phase. The latter was faster than in the initial phase. The endogenous rate of elongation in the steady phase was also faster than that in the initial phase. The intercept time after the addition of IAA in the steady phase was greatly reduced below that in the initial phase and its dependence on IAA concentration was not clearly exponential. Segments in the steady phase were sensitive to lower concentrations of added IAA, and elongated more rapidly at suboptimal concentrations than did segments in the initial phase.

The response of coleoptile segments, however, must be a statistical function of the diffusive entry of IAA through the cut ends and the number of cells reached and responding. How the elongation of cells follows the concentration gradient of auxin entering the segments from the cut ends determines how soon the maximum rate occurs in response to a given external concentration of IAA. This depends on the time required to respond to absorbed IAA (15 min according to our minimum lag), the maximum rate of elongation of each cell (0.14 $\mu m \cdot min^{-1}$ according to our average cell counts), how long this rate is sustained, and the number of cells responding simultaneously. Thus, the elongation of the segment is a cellular response but it does not kinetically characterize the cell unless all of them are elongating equally and simultaneously. This is approached by the use of metabolically recovered segments in their steady phase of endogenous elongation. At nonoptimal pH and under other inhibitory conditions, the use of preinfiltration or of short segments (5 mm) is advisable. Facilitation of response by the initial removal of the epidermis (26) may have damaging side effects in the initial phase, such as indicated by the response of very short segments here.

Previous investigators of rapid responses to IAA used meas-

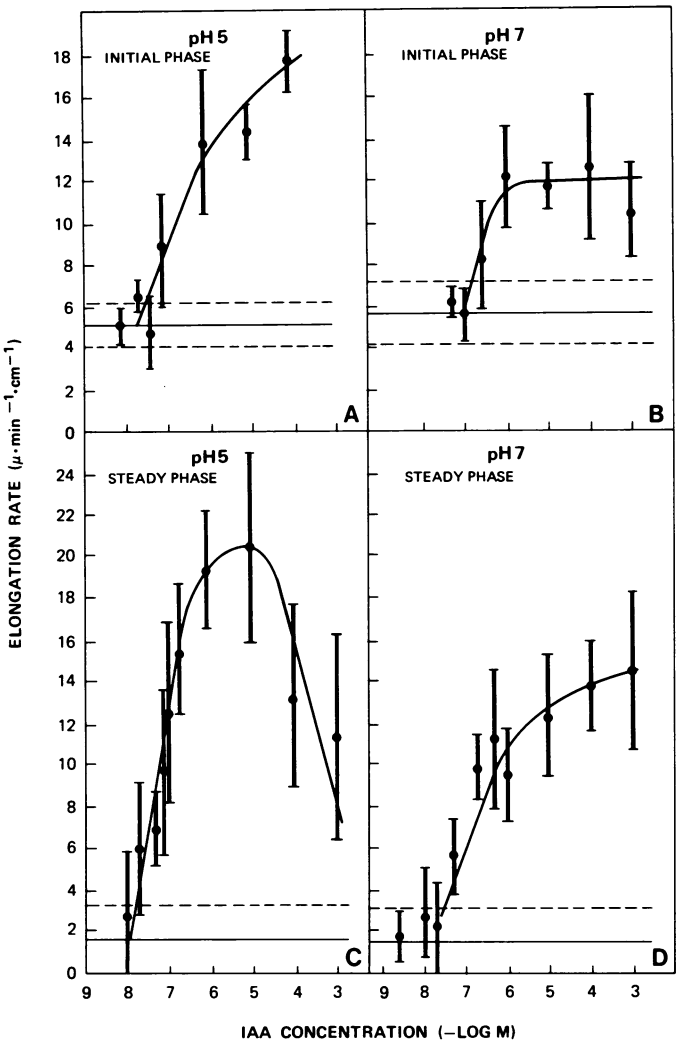

FIG. 4. Effects of IAA concentration on the responding rate of elongation in the initial and steady phases at pH 5 and 7. Points are averages of three to eight measurements, with vertical bars representing ± standard deviations. A and B: Initial phase with solid horizontal baseline representing average corrected maximum rate of the second spontaneous surge ± standard deviation (- - -); C and D: steady phase with solid horizontal baseline representing response to change of buffer alone ± standard deviation (- - -); A and C: pH 5; B and D: pH 7.

urements of penetration-limited responses of tissues to describe erroneously the kinetics of cell response to IAA. Most published kinetic measurements of the responses of coleoptile segments were made with segments in the initial phase. Some investigators have even mistaken the second surge of endogeous elongation as the experimental response. Numerous publications further diminish the power of kinetics by presenting as data only the individual time courses of elongation before and after the addition of the test substance, when in fact the considerable variance among coleoptiles (1, 19) requires repeated rate determinations. The long time experiments of Bonner *et al.* (3, 14) were not limited by the rate of diffusion of IAA into the segments and by the insensitivity of cells observed shortly after the shock of excision. Their bell-shaped dependence of elongation on the concentration of IAA was verified by our rapid measurements under conditions of high cell activity. The acidic pH promoted this in confirmation of Bonner's early work (4).

Our data on elongation in the steady phase at pH 5 (Fig. 4C) are clearly our only valid data for kinetic considerations. A Lineweaver-Burk (18) plot of the data of Figure 4C yields an apparent K_m of 68 nM which might be compared with McRae and Bonner's (21) value fo 355 nM for *Avena* coleoptiles at pH 4.5 and 25 C. Their lower binding affinity for IAA and their lower V_{max} of 7 $\mu m \cdot min^{-1} \cdot cm^{-1}$ are probably consequences of rates based on a measurement after 12 hr of incubation, as *Avena* coleoptiles are models of high sensitivity to IAA, and Nissl and Zenk (22) showed that they responded to less than 1 nM IAA at pH 4.7 and 21 C. In the latter rapid kinetic analysis, however, the dose response curve was maximal and flat from 10 nM to 1 mM IAA.

The high affinity for IAA is indicative of specific binding, and because activity is high at pH 5 near the pH at which IAA is least dissociated (8, 23), a nonpolar site of action or at least a hydrophobic barrier to the entry of IAA is indicated. In general, cell membranes are best permeated by undissociated molecules, and ATPases, one of which may be activated by IAA (15), are lipid-requiring proteins of membranes. We have not examined the growth transients that are known to be caused by changing the buffer to pH 5 from a higher value, but the pH of unchanged buffer did not influence the rate of elongation without added IAA. A pH of 5 did enhance the response to added IAA over that at pH 7, but it did so by expediting the uptake of IAA probably by diminishing its dissociation. Active cell extension in response to added IAA, therefore, appears to include the participation of an IAA complex subject to the pH dependence of IAA uptake.

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