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Studies on Acidification of Media by *Avena* Stem Segments in the Presence and Absence of Gibberellic Acid[1]

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ABSTRACT

The rate of acidification of media by *Avena* stem segments was studied with a titrimeter. GA_3 increased this rate by an average of 17% if supplied to the segments 90 min prior to measurement. GA_3 inhibited the rate by 15% if supplied 10 min prior to measurement. After 90 min incubation, stimulation of elongation had started; at 10 min, GA_3 had not yet started to stimulate elongation in the segments.

The acidification rates of the nodes (including the sheath-pulvinus), leaf sheath bases, and the internode bases of the stem segments were determined for plus and minus GA_3-treated segments. The internode fraction contributes most to modification of the acidification rate, the node-pulvinus fraction less so, and the nongrowing sheath not at all.

Acidification rates were measured for segments in different stages of elongation (lag, log, and plateau phases of growth). Segments in these growth stages were obtained from intact plants and from segments preincubated in sucrose and sucrose + GA_3. Segments from all sources which are in the log phase of growth have the highest rates, those in the plateau phase the lowest. For lag and log growth phases, segments preincubated in sucrose + GA_3 show the highest rates, those preincubated in sucrose the lowest rates. The opposite occurs for segments in the plateau phase of growth.

Segments stimulated to grow by GA_3 cause the pH of their incubation media to drop to pH 5.15 from an initial pH of 6.5. Nonstimulated segments cause a drop to pH 5.6. Long term growth of the segments is maximal in media buffered to pH 5 in the presence and absence of GA_3.

Our results support the idea that GA_3 stimulates an active acidification process in *Avena* stem segments just after GA_3 starts to stimulate growth in the segments, and that such an acidification process could play an important role in wall-loosening during active growth of the internode.

Evans (6), Cleland and Rayle (5), and Marrè *et al.* (12) have shown that IAA causes a significant increase in proton efflux from *Avena* coleoptile and *Pisum* epicotyl sections. Evans and Schmitt (7) have indicated that such hydrogen ion efflux can also be evoked in oat coleoptile sections by cutting them. These investigators have suggested that IAA-stimulated proton efflux could play a significant role in the initiation of hormone-promoted growth. In contrast to these systems, Fisher and Albersheim (8) report that in sycamore (*Acer pseudoplatanus*) cell suspension cultures, IAA does not stimulate hydrogen ion efflux from the cells.

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Very little information is available on the effects of gibberellins (GAs) on hydrogen ion efflux, or any other acidification process during the relatively short times just before and after GA begins to stimulate growth in *Avena* stem segments. In this paper, we report on acidification induced in the medium by *Avena* stem segments in the presence and absence of gibberellic acid (GA_3).

MATERIALS AND METHODS

Preparation and Incubation of Stem Segments for Growth and Titrimeter Experiments. For the growth experiments 2-cm stem segments, containing next-to-last internodes (p-l^1) were excised from *Avena* shoots according to methods cited in Kaufman (10) and Montague *et al.* (14). These segments include the basal node, the intercalary meristem portion of p-l^1, and the surrounding sheath base. The segments were thoroughly washed with three to four changes of sterile distilled H_2O. They were grown horizontally in Petri dishes (100 × 15 mm) on filter paper saturated with 6 ml of incubation medium. Sucrose (0.1 M) served as the control medium; 0.1 M sucrose plus 30 μM GA_3 was used for the hormone treatment. All cultures were incubated in the dark at 30 C. Growth was measured to the nearest 0.5 mm. All experiments were repeated at least five times.

In the pH experiments, our standard buffers were 50 M citrate for pH 3 and 4 and 50 mM citrate phosphate for pH 5 to 8. Potassium phosphate and acetate buffers were also tested to assess any nutrient effect from the citrate and citrate phosphate buffers. The pH of the medium in each experiment was checked at the end of the experiment with a pH meter and found not to change.

Except for experiments testing the effects of abrasion and cutting, we did not abrade or strip the epidermis. This is often done with *Avena* coleoptile and pea stem segments in order to obtain an acidification response, but is unnecessary with *Avena* stem segments, which have a cuticle $^1/_{10}$ as thick as that in coleoptiles and pea stems.

Determination of Acidification Rates. The rate of acidification of the incubation media was assayed with a Fisher automatic titrimeter model no. 36 equipped with a glass Ag-AgCl combination electrode. The titrimeter was modified to dispense base in amounts of 0.005 ml/increment. A 0.20-ml pipette, fitted with a three-way stopcock for refilling, was used to measure the volume of 20 mM NaOH added to the titrimeter reservoir (a 100-ml beaker). The temperature of the reservoir was maintained at 30 C by a water jacket. The medium (50 ml of 0.1 M sucrose or 0.1 M sucrose plus 30 μM GA_3) was mixed by a glass stirring rod spinning at approximately 250 rpm. After the pH of the medium was adjusted to 6.5, 50 *Avena* stem segments, or fractions thereof, were routinely transferred to the reservoir. The pH was then automatically maintained by the titrimeter. The amount of NaOH added to maintain constant pH was plotted every 15 sec over 20-min periods. The slope of the recorded curve is a

measure of the rate of acidification of the medium caused by the stem segments or their parts.

pH Drop Experiments. The same apparatus was employed for determining to what pH the segments caused the media to drop from an initial pH of 6.5. However, no base (NaOH) was added. pH was recorded on a 25-cm strip chart recorder adjusted to show full scale deflection for a change of 2 pH units. All pH and titrimeter experiments were repeated at least three times.

Gibberellic acid was a gift of Imperial Chemical Industries, Ltd., England. "Victory" oat seed was obtained from Svenska Utsades, A. B., Svalöf, Sweden.

RESULTS

Long Term Growth Responses of *Avena* Stem Segments at Different pHs. Since the cell wall of *Avena* stem segments is plasticized by gibberellin treatment (3), it was of interest to test the idea that gibberellin-promoted growth is favored by low pH, and the possibility that low pH will mimic the GA_3 growth response.

Figure 1, A and B illustrates typical growth responses of *Avena* stem segments at pH values varying between 3 and 8 in the presence and absence of 30 μM GA_3 after incubation for 16 and 47 hr. These curves indicate that a pH effect can be seen at 16 hr, but the differences are very slight from pH 3 to 8. The beginning of a peak at pH 5 for the plus GA_3 treatment is evident, however. By 47 hr, a very pronounced pH profile is evident: the peak growth response is at pH 5 for both minus and plus GA_3 treatments. Both curves are very similar in shape, indicating that the pH response is not peculiar to exogenous GA_3 treatment. The final pH was checked for each treatment at the end of the experiment (66 hr), and it remained unchanged for each of the respective pH values. A 50 mM sodium acetate buffer was used at pH 5 to compare with 50 mM citrate phosphate buffer, pH 5, and a 50 mM K-phosphate, pH 7, to compare with 50 mM citrate phosphate buffer at pH 7, to determine if there was any nutrient effect being derived from the citrate phosphate buffer rather than a pH effect. In either case, the growth was the same, irrespective of the buffer employed.

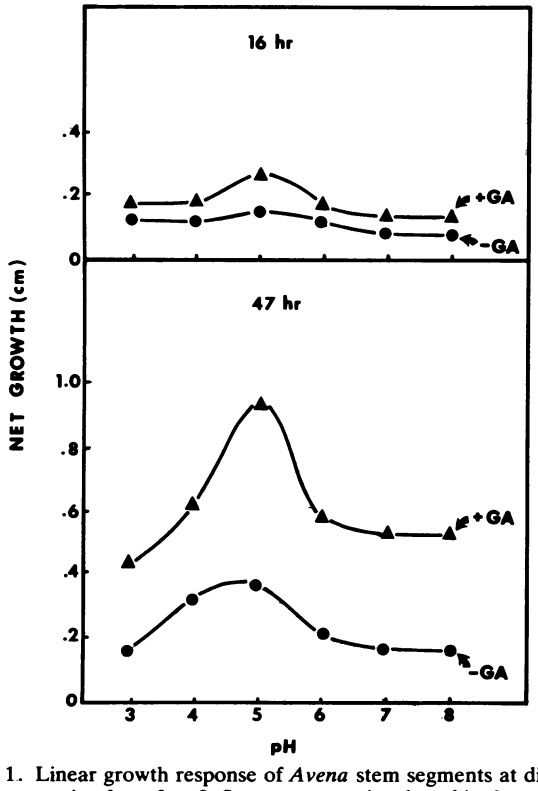

FIG. 1. Linear growth response of *Avena* stem segments at different pH values varying from 3 to 8. Segments were incubated in the presence of 30 μM GA_3 ($+GA_3$) or absence of GA_3 ($-GA_3$) for 47 hr in the dark at 27 C. Buffers employed to obtain pH regimes are indicated in "Materials and Methods." Standard error of the means ($=6/\sqrt{n}$) at pH 5 were as follows: ± 0.01 ($-GA_3$) and ± 0.03 ($+GA_3$) at 16 hr; ± 0.05 ($-GA_3$) and ± 0.08 ($+GA_3$) at 48 hr. Standard errors at pH 8 were as follows: ± 0.01 ($-GA_3$) and ± 0.02 ($+GA_3$) at 16 hr; ± 0.08 ($-GA_3$) and ± 0.08 ($+GA_3$) at 48 hr.

The above experiment clearly shows that maximum growth is attained, over long periods of time, at pH 5 in the segments, both in the presence and absence of GA_3. However, a pH of 3 or 4 does not elicit as much growth in the segments as does GA_3 at any of the pH values tested. It appears that we obtain an acid pH effect on growth of *Avena* stem segments, but it does not occur at very low pH values from 3 to 4, as with *Avena* coleoptile sections, nor is the GA_3 effect mimicked by very low pH values.

Development of a Standard Curve for Acidification of the Medium by *Avena* Stem Segments. The number of segments in the titrimeter reservoir was varied from 12 to 75 and the rate of NaOH addition to maintain a pH of 6.5 was determined. Results are shown in Figure 2, in which it is seen that the rate of acidification varies linearly with the number of segments. For our basic experiments, we chose to use 50 segments.

Figure 3 illustrates a standard curve for acidification of the medium by 50 stem segments over a 20-min period. The segments were obtained from next-to-last internodes of *Avena* plants in the lag phase of growth (1-2 cm in length). The curve shows that the acidification of the medium begins within 30 sec after segments are placed in the titrimeter reservoir. It also indicates that the acidification rate is linear over a 14-min period. The acidification rate, expressed as ml 20 mM NaOH added/10 min, is usually taken at midpoint on the linear part of the curve; in this experiment, it has a rate of 0.181 ± 0.002 ml NaOH added/10 min. In essentially all of our other runs with the titrimeter (over 240), the curves are linear for 14-min experimental periods. Between 14 and 20 min, the rate drops and approaches a value of zero. The segments also stop growing after 14 min in the titrimeter reservoir parallel with the time when acidification ceases.

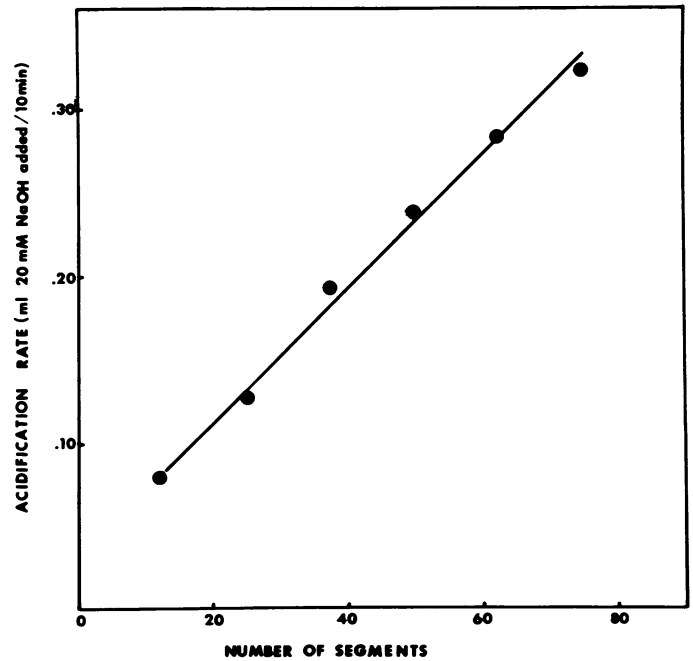

FIG. 2. Acidification rates plotted against number of *Avena* stem segments contained in titrimeter reservoir.

Acidification Rates in Lag, Log, and Plateau Phases of Development. Segments were obtained from next-to-last internodes in lag (mean length = 2.0 ± 0.1 cm), log (mean length = 4.5 ± 0.2 cm), and plateau (mean length = 10.5 ± 0.3 cm) phases of development. Fifty segments from each stage were placed directly in the titrimeter reservoir immediately after cutting them. Typical acidification rates for each stage are shown in Table I. Segments from lag and log phase internodes have essentially the same acidification rates, whereas those in the plateau phase manifest a significantly lower rate. Thus, relatively high acidification rates are associated with rapidly growing internodes and low rates with internodes which are decelerating in their growth rate.

A comparable experiment was performed with segments initially taken from next-to-last internodes in the lag phase of development (1–2 cm in length). These segments were preincubated in either 0.1 M sucrose or 0.1 M sucrose + 30 μM GA_3 for 6, 24, and 72 hr, corresponding to lag, log, and plateau phases of growth *in vitro*, respectively (2). Data in Table I present typical acidification rates for these respective stages and treatments. The log phase segments show the highest acidification rates and plateau phase segments the lowest. Furthermore, GA_3 stimulates the acidification rate only in the lag and log phases and causes a decrease in the rate during the plateau phase.

Contribution of Different Parts of the *Avena* Stem Segment to the Acidification Response. Segments (Fig. 4) were fractionated into three portions: node-pulvini, internodes, and sheath bases, in order to determine to what extent each of these parts contributed to the acidification process. The sheath base is a nongrowing portion, the node-pulvinus the primary geotropically sensitive part of the segment, and the internode base the portion which shows marked sensitivity in its linear growth response to gibberellins.

Table II shows that 37% of the total acidification is from the node-pulvinus and 23% from the sheath base. Forty per cent of the total acidification comes from the internode base. The sum of the acidification rates for the three fractions is greater than that for the intact segment. This is probably due to a wound respiration effect on acidification rate. These results demonstrate that the node-pulvinus and internode fractions account for most of the acidification response in the segment. Interestingly, the node-pulvinus acidification rate is essentially the same as that of the internode. The node-pulvinus is necessary for a maximum growth response in the internode to GA_3 (14).

Acidification Rates Before and After GA_3-Stimulated Growth. Stem segments were obtained from internodes in the lag phase of growth (1–2 cm in length) and incubated in either 0.1 M sucrose or 0.1 M sucrose + 30 μM GA_3. The preincubation period was either 10 min (before GA_3 stimulates the growth rate) or 90 min (just after GA_3 begins to stimulate the growth rate) (2). For 10-min preincubation segments, or fractions thereof, were used immediately after preincubation. Fractions not used immediately were discarded. For 90-min preincubation, all segments were placed in a refrigerator held at 4 C. One set of 50 segments was fractionated after 5 min in the refrigerator into node-pulvinus, internode, and sheath portions (Fig. 4). The other set of 50 segments was not fractionated and served as intact controls. All material was kept at 4 C until ready for placing in the titrimeter reservoir. Different refrigeration times (0–60 min) had no significant effect on acidification rates.

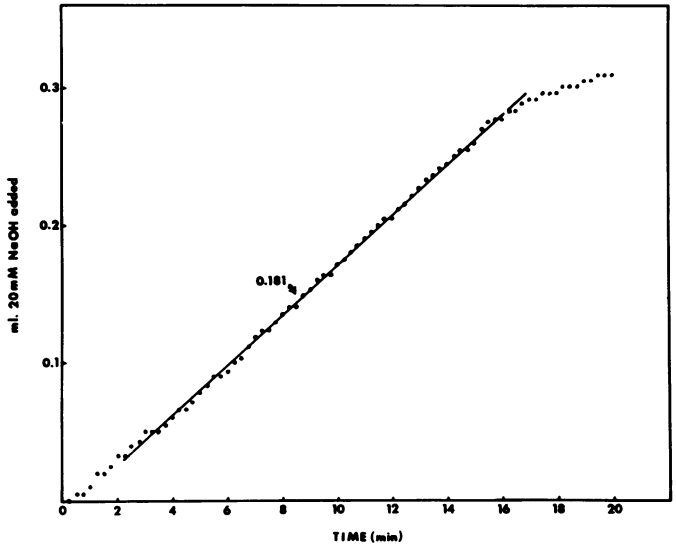

FIG. 3. Standard curve of the time course of addition of 20 mM NaOH to titrimeter reservoir for 50 *Avena* stem segments, run at 30 C.

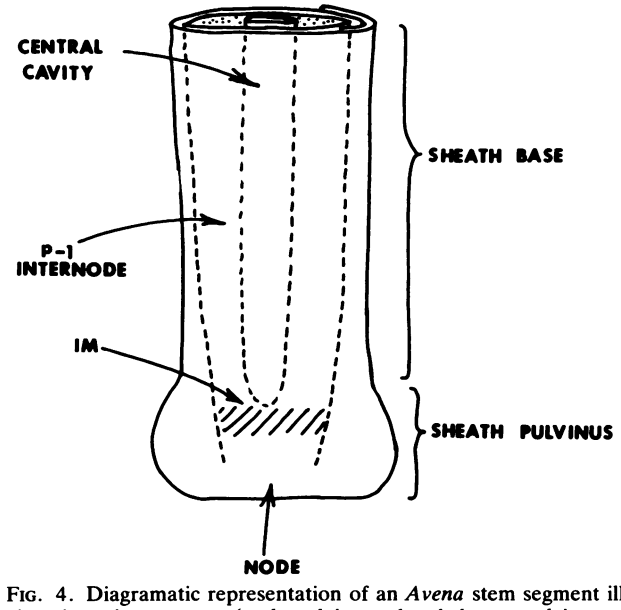

FIG. 4. Diagramatic representation of an *Avena* stem segment illustrating the primary parts (node-pulvinus, sheath base, and internode base) used in titrimeter experiments and general gross structural features of the segment that are referred to in the text.

Table I. Rate of Acidification of the Medium by Avena Stem Segments During Lag, Log, and Plateau Phases of Internodal Extension in Intact Shoots and Preincubated Segments
Data presented are average acidification rates. Experiments were repeated three times.

	Segments From Intact Plants		Preincubated Segments			
			0.1 M Sucrose		0.1 M Sucrose + 30 μM GA_3	
Growth Phase	Mean Length[2]	Acidification Rate[1]	Mean Length[3]	Acidification Rate[1]	Mean Length[3]	Acidification Rate[1]
	cm		cm		cm	
Lag	2.0 ± 0.10	.201± .004	0.2± 0.01	.179± .002	0.7± .04	.221± .010
Log	4.5 ± 0.17	.211± .004	0.4± 0.02	.194± .005	2.5± 0.10	.237± .008
Plateau	10.5 ± 0.31	.131± .003	0.5± 0.03	.157± .008	4.0± 0.22	.107± .012

[1] Expressed as ml of 20 mM NaOH added per 10 min. 50 stem segments were used for each titrimeter run.

[2] Mean length of next-to-last internodes (p-1^i) at respective growth phases. Fifty 1-cm stem segments were excised from the bases of these internodes for the titrimeter runs.

[3] Net internodal extension after 6 hr (lag), 24 hr (log), and 72 hr (plateau) of growth of the segments in vitro (incubated in dark at 30 C).

Table III contains the results of these experiments. For intact segments, 10-min preincubation in sucrose + GA_3 caused a 15% decrease in acidification rate compared with sucrose control segments. In fractionated segments, the internodes contributed 59%, the node-pulvini 31%, and the sheaths 10%, to a total drop for all fractions of 11%. In contrast, 90-min preincubation in sucrose + GA_3 caused a 17% increase in the acidification rate compared with sucrose controls. In fractionated segments, the internode contributed 72%, the node-pulvini 33%, and the sheaths −5%, to a total increase for all fractions of 12%. These results suggest that some time between 10 and 90 min is a crossover point between inhibition and promotion of the acidification rate.

As in Table II, the data in Table III show that fractionation causes an increase in acidification rate when one compares the sums of the acidification rates of the three fractions with the rate for intact segments. Furthermore, the node-pulvini in these 10- and 90-min preincubated segments contribute 33 to 34% to the acidification rate, the internodes 38 to 45%, and the sheaths the least, 21 to 28%. These relative percentages are the same as obtained from intact shoots where no preincubation treatments were employed (Tables II and III).

Measurement of the pH Drop in the Medium in the Presence and Absence of GA_3. Since *Avena* stem segments cause a significant acidification of the medium after pretreatment in sucrose or sucrose + GA_3, we wanted to monitor the pH over the time of this drop. Using 50 stem segments for each treatment, we preincubated the segments in either 0.1 M sucrose or 0.1 M sucrose + 30 μM GA_3 in the dark at 30 C for 90 min, then immediately placed the segments in the titrimeter reservoir with base being added to maintain a pH of 6.5. When a steady rate of addition of NaOH was attained, the supply of base was shut off and a trace was run on the pH drop in the medium (Fig. 5). The pH of the medium for the sucrose treatment dropped from 6.5 to 5.6 within 5 min and remained at this pH. For segments preincubated in sucrose + GA_3, the pH dropped from 6.5 to 5.3 in 5 min and stabilized at 5.15 within 10 min. Thus, the acidification response is greater with GA_3 treatment than with sucrose alone.

Table II. Rate of acidification of the medium by Intact Avena Stem Segments Compared with Node-Pulvinus, Internode, and Sheath Fractions of Avena Stem Segments.
Data are averages of three experiments. Errors are standard error of mean $\sigma/\sqrt{n}$

Type of Fraction[1]	Acidification Rate[2]	Total Acidification Rate
		%
Node-Pulvini	0.125± .004	37
Internodes	0.135± .004	40
Sheaths	0.082± .003	23
Total for Fractions	0.344± .011	
Intact Segments	0.217± .005	

[1]All segments were obtained from Avena next-to-last internodes in lag phase of growth (1-2 cm in length). All segments or parts thereof were placed directly into the titrimeter reservoir after cutting. There was no preincubation.

[2]Expressed as ml of 20 mM NaOH added per 10 min. Fifty segments or parts were used for each run.

DISCUSSION

As with IAA in other systems (9, 12, 17) GA_3 causes a decrease in the pH of the medium in which *Avena* stem segments are incubated in comparison with control segments. The fact that the acidification stimulation by GA_3 occurs primarily in the node-pulvini as well as in the internode portions of the segments (Fig. 4 and Table II) may be of significance. The node-pulvini play a major role in determining the amount of GA_3-stimulated growth in the internode (14); deletion of the node-pulvinus greatly reduces linear growth in the internode either in plus or minus GA_3-treated segments. The node-pulvinus is the second richest source of native gibberellin in the *Avena* shoot (11), so that the native gibberellins in this locus could play an active role in stimulating acidification *in situ*. Since the distance between the node-pulvinus and the intercalary meristem at the base of the internode is only of the order of 0.5 mm (Fig. 4), the stimulation of acidification in the node-pulvinus by GA_3 could contribute significantly to any acid-promoted wall-loosening in the internode base. This internode base, the locus of the internodal intercalary meristem, is that part of the internode where GA_3 has its primary accelerating influence on linear growth (1, 2), where GA_3 causes a marked increase in cell wall plasticity (3), and where it causes a significant increase in cell wall synthesis (13).

The acidification rate increase elicited by GA_3 in *Avena* stem segments causes a drop in pH of the medium to 5.2 from an initial pH of 6.5; segments in control media cause the pH to drop to 5.6 from 6.5. Such an effect of GA_3 on acidification of the

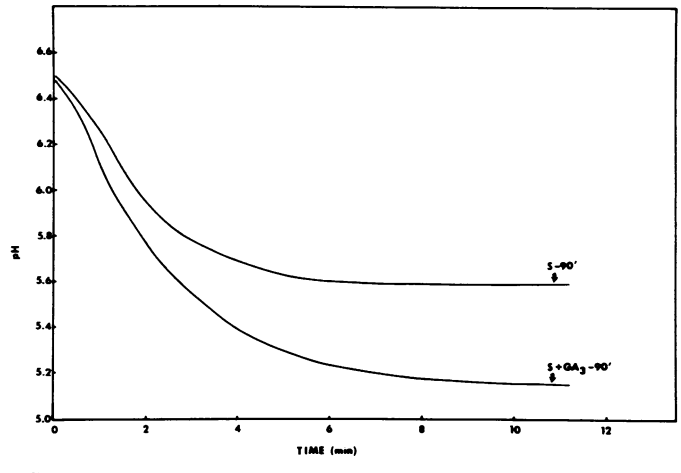

FIG. 5. Plots of change in pH over a 16-min experimental period for segments incubated in 0.1 M sucrose compared to segments incubated in 0.1 M sucrose plus 30 μM GA_3.

Table III. Rate of Acidification of the Medium by Intact Avena Stem Segments Compared with Node-Pulvinus, Internode, and Sheath Portions of Segments before (10 min. Pretreatment) and After (90 min. Pretreatment) GA_3 Stimulation of Growth.
Data are average acidification rates. Most experiments were repeated three times.

Type of Fraction[1]	10 min Pretreatment of Segments in Dark at 30 C: 0.1 M Sucrose — Acidification Rate[2]	Total Acidification Rate	0.1 M Sucrose + 30 μM GA_3 — Acidification Rate[2]	Total Acidification Rate	Percent Promotion	Weighted % Promotion[3]	% Contribution[4]
		%		%	Δ%		
Node-Pulvini	0.123 .004	33	0.112 .003	34	-10	- 3.5	31
Internodes	0.153 .008	41	0.127 .006	38	-17	- 6.7	59
Sheaths	0.096 .003	26	0.092 .001	28	- 4	- 1.1	10
Total for Fractions	0.373 .015		0.331 .010		-11	-11.3	
Intact Segments	0.230 .011		0.195 .008		-15		
	90 min Pretreatment of Segments in Dark at 30 C						
Node-Pulvini	0.122 .003	33	0.137 .002	34	12	4.0	33
Internodes	0.153 .004	42	0.183 .011	45	20	8.7	72
Sheaths	0.090 .010	25	0.087 .006	21	- 3	- 0.7	- 5
Total for Fractions	0.365 .017		0.407 .019		12	12.0	
Intact Segments	0.195 .007		0.229 .009		17		

[1]All Segments were obtained from Avena second-to-last internodes in lag phase of growth (1-2 cm in length). Fractions were prepared subsequent to treatment and used immediately (10 min pretreatment) or refrigerated (90 min pretreatment).

[2]Expressed as ml of 20 mM NaOH added per 10 min. 50 segments or parts thereof were used for each titrimeter run.

[3](% promotion x average of % of total acidification rate)/100 = weighted % promotion.

[4](weighted % promotion/sum of weighted % promotion) · 100 = % contribution.

medium is nicely correlated with GA_3-stimulated growth at different pH values. Maximum growth occurs at pH 5. The same is true of non-GA_3-treated segments (Fig. 1). In addition, GA_3-promoted growth over a wide pH range of 3 to 8 is greater than growth at pH 3 in the absence of GA_3. pH 3 has been shown to stimulate growth in *Avena* coleoptiles and in pea epicotyl segments, usually showing similar kinetics as for IAA-stimulated growth (4, 17); this pH of 3 causes the pH in the vicinity of the cell wall to drop to pH 5 (5). In contrast, in *Avena* stem segments, pH 3 strongly suppresses growth both in the presence and absence of GA_3 (Fig. 1). The maximal growth response at pH 5 in these segments is one which appeals to us in that many cell wall hydrolases show maximal enzyme activity at around pH 5 (15, 16, 18, 19). Thus, increase in acidification rates elicited by GA_3, resulting in attainment of pH values approximating 5 (compared with pH 5.6 in control segments) could be of major significance in GA_3-stimulated growth, especially since GA_3 starts to increase cell wall plasticity in the internode base within 60 min (3). These results, furthermore, are consistent with several models for wall-loosening (*e.g.* stimulated cell wall endohydrolase activity, increased rate of xyloglucan creep, increased rate of turnover of cell wall polymers).

The drop in rate of acidification caused by GA_3 after a 10-min pretreatment in GA_3, before growth is stimulated by the hormone (at least 30 min is required for GA_3 to cause an acceleration in growth rate-2), is the earliest of any response we have obtained with GA_3 in the *Avena* internode system. This response may be caused by hormone-stimulated diversion of substrate from respiratory processes to cell wall synthesis machinery. Whatever the cause, the rate decrease points to an initial trigger of GA_3 action in stimulating growth of *Avena* internodes that is not due to acidification-mediated wall-loosening. Perhaps this rate decrease accounts for the 40- to 50-min lag before GA_3 begins to stimulate growth in *Avena* stem segments.

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