Regulation of Growth and Invertase Activity by Kinetin and Gibberellic Acid in Developing *Avena* Internodes

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Abstract

Evidence presented here indicates that there is a complex interaction between kinetin, gibberellin, and sucrose in the regulation of turnover of invertase *in vivo*. The synthesis of invertase is maintained in the presence of GA_3 and sucrose over relatively long periods of time. Kinetin, on the other hand, inhibits the full development of invertase activity seen in the sucrose and gibberellin control treatments. Moreover, the peak in invertase activity occurs earlier with kinetin treatment. During invertase turnover, once the peak is reached, kinetin enhances the rate of decay of enzyme activity relative to synthesis. The regulatory significance of invertase in intercalary growth and possible modes of kinetin action in this process are discussed.

Introduction

In a previous study, evidence was presented that protein synthesis is necessary for the induction of invertase by gibberellic acid (GA_3) in Avena stem segments (Kaufman *et al.* 1968). It was also found that the activity rises, then falls after 12 h in water treated tissue, indicating turnover of the enzyme. This substantiates comparable findings of Bacon *et al.* (1965) for invertase induction in water-treated beet root storage tissue. More recently, it was concluded that gibberellin must be involved in regulating the turnover of invertase in Avena stem segments (Kaufman *et al.* 1971). That native gibberellin is implicated in this response is strongly supported by recent work of Bradshaw and Edelman (1969) with Jerusalem artichoke tuber discs, where they found that gibberellin-like substances are produced in significant amounts preceding invertase induction. It has recently been reported that during turnover of invertase in the Avena system (Kaufman *et al.* 1971), both GA_3 and sucrose play a major role in inducing the enzyme *in vivo*. This synthesis is maintained in the presence of gibberellin and sucrose over relatively long periods of time. This compares well with lens epicotyls, where Seitz and Lang (1968) reported that growth and invertase activity were stimulated by GA_3 applications. Ethylene at physiological concentrations has been shown to inhibit GA_3-promotion of both invertase activity and growth in lettuce hypocotyls (Scott and Leopold 1962). The promotion of growth by GA_3 in developing Avena internodes is also markedly suppressed by the application of kinetin at physiological concentrations (Jones and Kaufman 1971). The GA_3-promoted growth is a substrate-requiring process (Adams, P. A., Ph. D. Diss. Univ. Mich., 1969). Also, the addition of sucrose to the incubation medium partially relieves kinetin of its inhibitory action. In light of these findings, it was of interest to ask the question, does kinetin, in regulating GA_3-promoted growth in Avena, act through a modification of the turnover of an enzyme such as invertase? Specifically, might it (1) inhibit the induction of invertase activity or (2) possibly promote the decay of the enzyme activity? These questions constitute the primary focal points for this paper.

Methods

Preparation and incubation of segments. One cm stem segments containing intercalary meristems (IM) were isolated from the bases of next-to-last internodes of 45 day old Avena shoots grown in the greenhouse (Kaufman 1965). The segments were surface-sterilized with a 10 per cent solution of 'Chlorox' (sodium hypochlorite) for two minutes, then thoroughly washed with 8–10 changes of distilled water. This treatment effectively prevented any microbial contamination in the incubation medium. Ten IM segments were supported upright in raised perforated plexiglas disks on filter paper in a 5 cm petri dish. The bases of the segments were immersed in 2 ml of treatment solution. Freshly prepared gibberellic acid (GA_3) was used at 1 μM, and sucrose where

used, at 0.1 *M*. These concentrations of GA_3 and sucrose, respectively, give maximum growth responses (Kaufman *et al.* 1971). Kinetin (6-furfurylaminopurine) was used at 10^{-4} *M*, a concentration which allows a half-maximal growth response to GA_3 (1 μ*M*) in these segments (Jones and Kaufman 1971). Segments were incubated in the dark at 23°C. Growth was measured with a mm ruler to the nearest 0.5 mm and the standard error of the mean was determined on a sample size of 20 segments. All experiments were repeated at least three times.

Enzyme extraction and assay. For the preparation of the crude invertase fraction, 10 IM segments were removed at various times and frozen in a deep freeze until the segments in a set of experiments were accumulated. Freezing has been found not to affect invertase activity upon subsequent extraction (Kaufman *et al.* 1971). The 10 segments were ground in a 70 ml porcelain mortar by hand with 4 ml cold 5 m*M* phosphate-citrate buffer (pH 5.0) and about 1.0 g sand. This and all subsequent steps were carried out at 0–4°C. The resulting mixture was centrifuged at 1700 × *g* for 5 min with an International clinical centrifuge. Invertase activity was assayed in the supernatant fractions. In order to remove endogenous substrate and possible inhibitors, the supernatant was dialyzed against distilled water with 3 changes during a 60 min period at 4°C.

The soluble enzyme reaction mixture consisted of 0.5 ml of enzyme, 30 μmol of phosphate-citrate buffer (pH 5.0) and 60 μmol sucrose in a total volume of 1.0 ml. The enzyme reaction mixture was incubated at 30°C in a water bath. The enzyme reaction was started with the addition of enzyme and was stopped after 20 min of reaction by the addition of 1 ml of alkaline reducing sugar reagent (Nelson 1944). The reducing sugar content was then determined by the standard method of Somogyi (1952) with arsenomolybdate. The reducing sugar was read with a Klett-Summerson colorimeter with a green (no. 54) filter. Protein was determined according to the method of Lowry *et al.* (1951). The invertase specific activity is expressed as μmol glucose per mg protein and hour at 30°C. The enzyme experiments were repeated at at least three times.

Gibberellic acid was obtained from Imperial Chemical Industries, Ltd., England; Kinetin from Nutritional Biochemicals, Inc.; and 'Victory' oat seed from the Swedish Seed Assoc., Svalöf, Sweden.

Results

Time-course changes in invertase activity in the absence of exogenously supplied sucrose. The first part of this study was concerned with the question, does kinetin play a role in the regulation of invertase activity? To investigate this possibility, the time-course changes in invertase activity in the presence and absence of kinetin (10^{-4} *M*) were determined. Figure 1 illustrates the effects

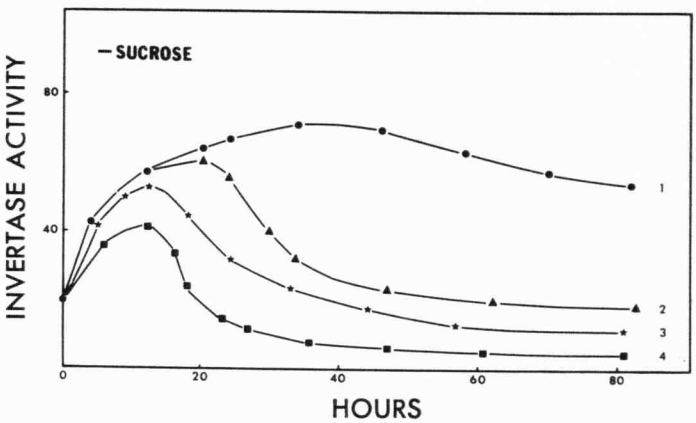

Figure 1. *Time-course changes in invertase activity of Avena intercalary meristem segments treated with GA3* (curve 1), *kinetin* (curve 4), *kinetin plus GA3* (curve 2), *all in the absence of sucrose* (H_2O control, curve 3). Ordinate: glucose $\mu mol \cdot h^{-1} \cdot mg^{-1}$ protein.

of kinetin on invertase activity in segments incubated in the dark at 23°C for 82 h in the absence of exogenously supplied sucrose. The two control treatments, GA_3 and H_2O, (curves 1 and 3, respectively) show the same pattern as found earlier (Kaufman *et al.* 1971). Treatment with kinetin (curve 4) indicates that there is a very early suppression of invertase activity; at 5 h of incubation, it is 25 per cent of the H_2O control. Invertase activity at 12 h reaches a maximum peak of 40.9 μmol glucose per mg protein and hour. This is approximately a 20 per cent reduction in the peak activity achieved in the water control (50.8 μmol glucose per mg protein and hour). After the peak, there is a rapid loss in invertase activity, with a half-time loss of 4.8 h. In contrast, the H_2O control, which also reaches maximum invertase activity at 12 h, has a half-time loss of 9.6 h.

When kinetin is added to the GA_3 incubation medium (curve 2), three differences from the GA_3 control curve (curve 1) are observed: (1) there is a lower peak enzyme activity of 60 μmol glucose per mg protein and hour for kinetin plus GA_3, compared to 71.2 μmol glucose per mg protein and hour for the GA_3 control; (2) this peak for kinetin plus GA_3 treatment is reached 14 h earlier (at 20 h of incubation) than the peak for GA_3 treatment; and (3) the invertase activity then rapidly decays in kinetin plus GA_3 treatment, with a half-time loss of 10 h, with the enzyme activity falling below the initial levels by 60 h. In the GA_3 control, a high level of enzyme activity is maintained over a much longer period of time, with a half-time loss of activity greater than 50 h. Thus, kinetin treatment induces an early and more rapid decay of this enzyme activity.

Time-course changes in growth in the absence of exogenously supplied sucrose. Figure 3 A shows the growth response of Avena stem segments to kinetin and/or GA_3 in the absence of exogenously supplied sucrose. Seg-

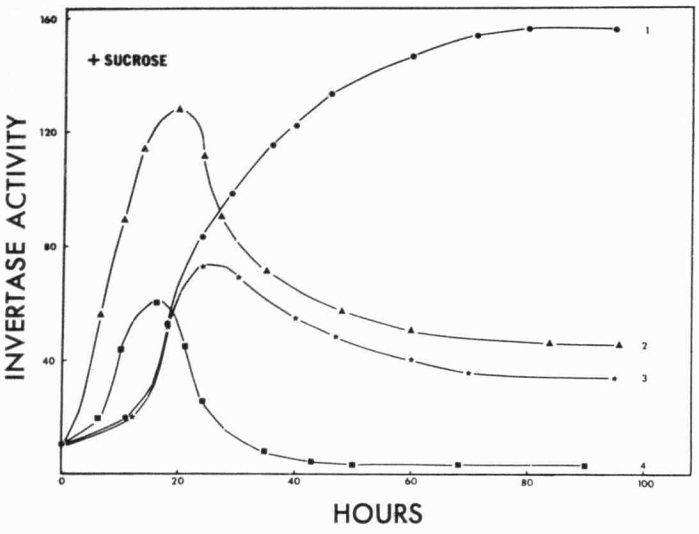

Figure 2. *Time-course changes in invertase activity of Avena intercalary meristem segments treated with GA3* (curve 1), *kinetin* (curve 4), *and kinetin plus GA3* (curve 3), *all in the presence of sucrose* (control curve 2). Ordinate: glucose $\mu mol \cdot h^{-1} \cdot mg^{-1}$ protein.

ments in the H_2O control (curve 3) elongate for approximately 35 h, achieving a mean final growth of 0.14 ± 0.02 cm. Segments treated with kinetin (curve 4) elongate for about 25 h, attaining a mean final growth of 0.18 ± 0.01 cm. It is evident that kinetin- and H_2O-treated segments show very similar growth kinetics. Comparison of Figure 1 with Figure 3 A shows that the induction of invertase activity precedes the period of rapid growth increase in both treatments. While kinetin treatment induces less invertase activity with a slower rate than the H_2O treatment, the final growth attained with kinetin treatment is slightly higher. Further comparison discloses an equally important correlation between the rate of loss of enzyme activity and the time of cessation of cell elongation. Enzyme activity declines, reaching initial levels by 20 and 38 h for kinetin and H_2O-treated tissue, respectively, while growth ceases at 25 and 35 h for these same treatments.

Referring again to Figure 3 A, segments in the GA_3 control (curve 1) elongate for approximately 48 h, achieving a mean final growth of 1.28 ± 0.04 cm. When kinetin is added to the GA_3 incubation medium (curve 2), the segments elongate for approximately 30 h, reaching a mean length of 0.53 ± 0.02 cm. When these two curves are compared with their respective curves in Figure 1, they indicate that (1) the early inhibition of GA_3-promoted growth by kinetin is not preceded by a suppression in the rate of invertase activity induction and (2) kinetin treatment reaches peak enzyme activity at 20 h (14 h earlier than the GA_3 control), which rapidly decays during the following 10 h. The kinetin growth ceases shortly after 30 h of incubation. This comparison indicates a fairly close correlation between the time when growth ceases and the time when invertase activity decreases.

Time-course changes in invertase activity in the presence of exogenously supplied sucrose. Figure 2 indicates the time-course changes in invertase activity for segments treated with kinetin and/or GA_3 in the presence of exogenously supplied sucrose. The results in Figure 2 can be summarized as follows: (1) in the sucrose control (curve 3), the invertase activity rises slowly during the first 10 h of incubation. This is then followed by a period of rapid induction of enzyme activity, rising to a maximum activity of 72.8 μmol glucose per mg protein and hour at 24 h, then declining slowly with a half-time loss in activity of 32 h; (2) the enzyme activity for treatment with kinetin plus sucrose (curve 4) rises very early (*i.e.*, a 2.5 fold increase over the sucrose treatment at 5 h) to a peak activity of 59.5 μmol glucose per mg protein and hour at 16 h, then decays rapidly with a half-time loss of 6 h. Initial levels of enzyme activity are reached by 34 h; (3) in the presence of GA_3 (curve 1), enzyme activity rises parallel to the sucrose control curve for the first 18 h of incubation. The activity continues to rise rapidly, reaching maximal activity of 156 μmol glucose per mg protein and hour at 80 h. The enzyme activity remains stable with very little decay over the next 15 h; (4) it is evident from curve 2 that the addition of kinetin to the medium, containing GA_3 plus sucrose, markedly diminishes the early lag period in the induction of invertase activity found in both sucrose alone and sucrose plus GA_3 (curves 3 and 1, respectively). Enzyme activity reaches a peak of 128 μmol glucose per mg protein and hour at 20 h of incubation. This is an 18 per cent reduction in the maximum activity attained in the GA_3 plus sucrose-treated tissue, which occurs much later, at 80 h of incubation. The activity then decays rapidly with a half-time loss of 16 h. Unlike the kinetin plus sucrose-treated tissue (curve 4), the enzyme activity does not fall below the sucrose control level. In fact, for the last 60 h of incubation, the kinetin-GA_3-sucrose curve follows very closely the slower decay characteristic of the sucrose control curve. Most striking is the observation that the kinetin-GA_3-sucrose treatment (curve 2) very closely approximates the composite of the kinetin plus sucrose and the sucrose control curves, following first the early kinetics of induction and decay in enzyme activity for the kinetin plus sucrose-treated tissue (curve 4) and secondly, the later, slower enzyme activity decay characteristics of sucrose-treated tissue (curve 3).

In order to test a possible leakage of the enzyme into the incubation medium, enzyme activity was assayed in the incubation medium carefully at the various times of tissue incubation for each treatment. The results indicated that *no* detectable invertase activity could be found in the medium for any of the treatments.

Time-course changes in growth in the presence of exogenous sucrose. Figure 3 B shows the growth response of Avena stem segments to kinetin and/or GA_3 in the presence of sucrose. Segments in the sucrose control (curve 3) elongate for approximately 30 h and achieve a mean growth of 0.34 ± 0.01 cm at 48 h. When kinetin is added to the incubation medium at time zero (curve 4), there is a very marked lag in growth induction for the first 6 h of incubation. The mean amount of growth finally attained is only 0.12 ± 0.01 cm, representing a 65 per cent reduction in sucrose-promoted growth. Comparison with Figure 3 A shows that this final growth is even lower than that of segments in the H_2O control (curve 3).

Comparison of Figure 3 B with Figure 2 indicates that (1) the early inhibition of sucrose-promoted growth by kinetin is *not* accompanied or preceded by lower levels of invertase activity. By 6 h there is a 75 per cent reduction in sucrose-promoted growth, whereas invertase activity increases by 2-fold; (2) cessation of growth (by 30 h in kinetin-treated tissue) is paralleled by decay of enzyme activity which reaches initial levels by 34 h; (3) sucrose alone maintains a relatively high level of invertase activity for a considerable length of time after all growth has ceased.

When GA_3 is added to the sucrose incubation medium at 0 h, both growth and invertase activity (Figures 3 B and 2, respectively) rise in parallel fashion during the first 10 h of incubation. This period is followed by a very rapid induction of growth and invertase activity. Growth subsides after 60 h with peak enzyme activity stabilizing at 80 h. Therefore, with GA_3 treatment, cessation of growth is not accompanied by a loss in enzyme activity. Kinetin significantly alters the GA_3 plus sucrose growth kinetics, as can be seen in Figure 3 B. Kinetin maintains a marked lag in GA_3-promoted growth during the first 6 h of incubation, but this does not express itself in a reduced amount of induction in enzyme activity (Figure 2). However, while invertase activity rises rapidly to an early maximum, this activity decays very rapidly with a half-loss in activity by 36 h. This loss in enzyme activity is accompanied by a slower rate of growth.

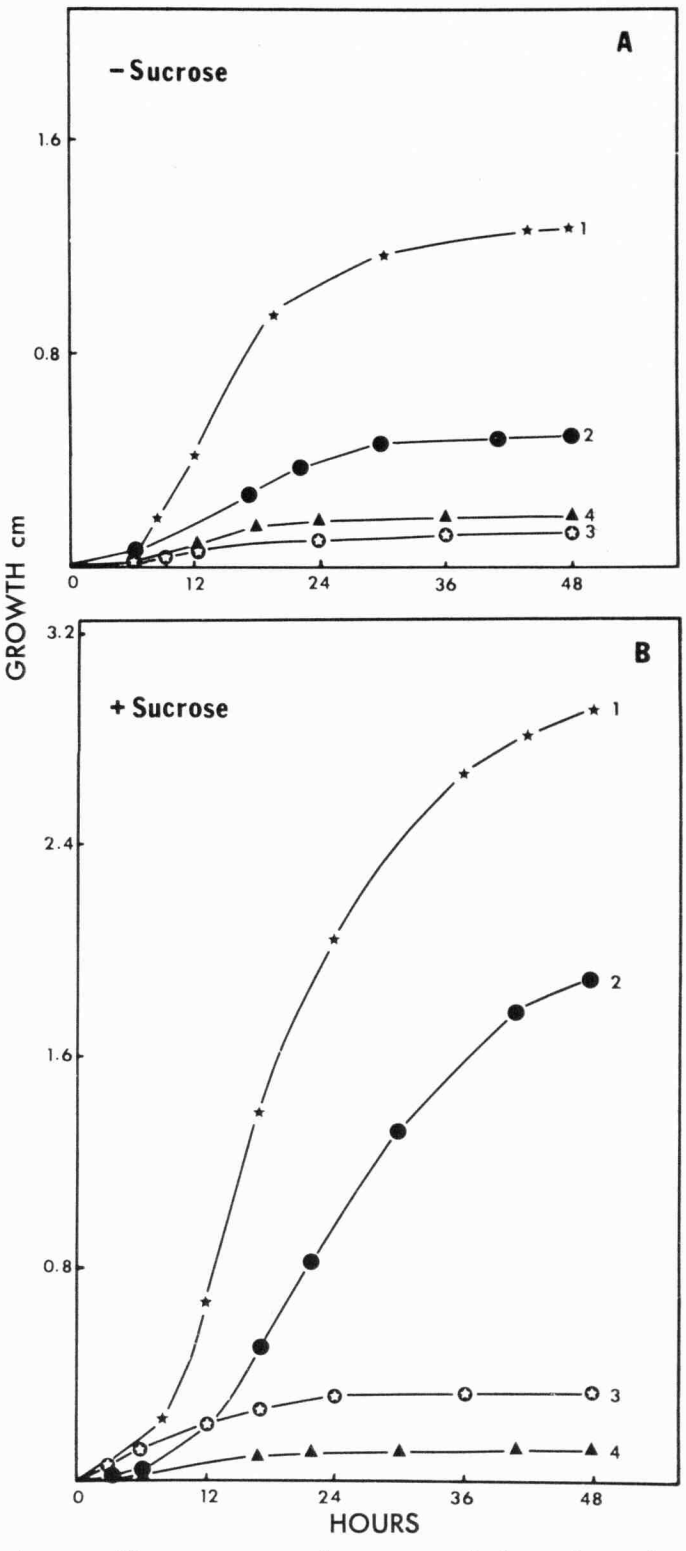

Figure 3. *Time-course growth responses of Avena intercalary meristem segments in the absence* (A), *in the presence* (B) *of sucrose.* Treatments included GA_3 (curve 1), kinetin plus GA_3 (curve 2), kinetin (curve 4), and H_2O or sucrose (curve 3 in *A* and *B*, respectively).

Discussion

At the beginning of this study it was suggested that kinetin might specifically act in regulating GA_3-promoted growth by inhibiting the development of invertase activity. Results presented here indicate that the inhibition of the development of invertase activity most likely would not in itself account for the early and marked inhibition of the GA_3-growth process by kinetin. With kinetin treatment, the invertase activity rises early, as in the H_2O control tissue, although the maximum activity reached is lower than that of the H_2O control tissue. Of particular interest, however, is the sucrose series (Figure 2). In both the kinetin plus sucrose and the kine-

tin plus GA_3 plus sucrose-treated segments, invertase activity develops very rapidly, reaching maximum activity at 16 and 20 h, respectively, and then both decay rapidly. This is the period when active cell division is taking place within the intercalary meristem (Kaufman *et al.* 1965). It was previously reported that kinetin reverses the gibberellin-inhibition of cell division activity in the intercalary meristem (Jones and Kaufman 1971). Thus, the very early rise in invertase activity (9 h earlier than the gibberellin plus sucrose control treatment) would be of importance to the meristematic region by providing the necessary energy from hexoses in the growing region, in addition to supplying carbon for the many biosynthetic reactions which occur there.

Our experiments indicate that the early rate of induction of invertase activity is almost constant, with hormone treatment having little effect on this rate. However, it is apparent that hormone treatment can alter the temporal appearance of activity (occurring earlier with kinetin treatment) and the magnitude of activity (very high with GA_3 treatment, lower with kinetin treatment, as compared to control treatments). Cytokinin is known to cause enhanced synthesis of RNA in isolated nuclei. This effect is inhibited by actinomycin D but is reversed by high concentrations of the cytokinin (Roychoudhury *et al.* 1965), suggesting that cytokinin or a cytokinin-mediator complex might bind to DNA and thus compete with repressors for operator sites or might bring about the derepression by another mechanism. Relating this result to the functional aspect of developmental induction of enzymes might provide an explanation for the early induction of invertase by kinetin. An equally plausible explanation might be that kinetin somehow affects the transfer of sucrose from an inaccessible compartment to a more accessible compartment where it can induce the synthesis of invertase.

The possibility was also suggested earlier in this paper that kinetin may specifically act in regulating GA_3-promoted growth by increasing the rate of decay of the enzyme activity. It has previously been suggested that the primary role of the GA_3-induced invertase is to provide substrate for the GA_3-promoted growth process (Kaufman *et al.* 1968). This would sustain the high growth rates seen in segments treated with GA_3 alone and the even higher growth rates attained when sucrose is included in the incubation medium. Data presented here show a correlation between the decay of invertase activity and the cessation of growth. Evidence is also provided that during the GA_3-accelerated growth, the previous addition of kinetin to the incubation medium at 0 h causes the very rapid decay in invertase activity, when hexoses are needed most for the GA_3-growth process. Therefore, one cannot escape the conclusion that kinetin has a marked effect on GA_3-promoted growth, in part, by enhancing the decay of invertase activity.

The actual method by which kinetin influences the decay process is not known. It may involve an enhancement in the levels of specific proteolytic enzymes, similar to the role of cytokinins in squash cotyledons (Penner and Ashton 1967), or it may involve specific invertase inhibitors (Pressey 1966). It should be emphasized that a loss of detectable enzyme activity may merely reflect a reversible inactivation or inhibition, rather than an irreversible degradation of enzyme. However, because of the long duration of the loss of invertase activity, the resultant effect is the same whether the process is reversible or irreversible. Kinetin did not block the induction of enzyme activity. Further work with inhibitors of protein synthesis is required in order to provide evidence that the observed rise in activity reflects an increase in the amounts of protein moiety of the enzyme. Such a requirement of protein synthesis for induction of invertase activity by GA_3 has been established in this system (Kaufman *et al.* 1968). Thus, it is evident that kinetin may not only regulate the synthesis of enzymatic proteins, but just as importantly, it may also act in controlling the decay of enzyme activity.

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