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Invertase Activity and Cell Growth in Lentil Epicotyls[1]

Konrad Seitz[2] and Anton Lang
MSU/AEC Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48823

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Abstract. The activity of invertase and its relation to growth were studied in the epicotyls of lentil seedlings incubated in the presence and absence of gibberellic acid (GA_3).

Invertase activity per epicotyl increases relatively more rapidly than does length, reaches a maximum during most active elongation, and declines upon cessation of growth.

GA_3 enhances both growth and increase in invertase activity, without altering the kinetics of the 2 processes. If GA_3 is added during incubation invertase activity increases more rapidly than does elongation rate.

Incubation of the seedlings in solutions of polyethyleneglycol inhibits the increase of both growth and invertase activity, the latter actually undergoing a decline, but causes no great change in the relative effect of GA_3. In presence of polyethyleneglycol GA_3 has however a relatively greater effect on invertase activity than on growth.

Sugars in the incubation medium have no significant effect on growth and invertase activity in the epicotyl, except inhibition at relatively high concentrations.

Cycloheximide, actinomycin D, and 5-fluorodeoxyuridine (FUDR) inhibit both growth and the increase in invertase activity. Added during incubation cycloheximide causes complete inhibition of growth and a decrease in invertase activity with no appreciable lag phase. With actinomycin D and FUDR the inhibition occurs after lag periods of 2 to 3 and of at least 10 hours, respectively. Thus the increase in enzyme activity is very probably based on *de-novo* synthesis, and the enzyme is in a state of turnover during growth.

The enzyme is present in soluble form in the cytoplasm, not firmly bound to any cell structures.

Expansion growth of plant cells and the action of plant growth hormones in this process depend upon synthesis of RNA and protein, and in some cases also on DNA synthesis (*e.g.* 2, 11, 13, 14). It seems possible that among the proteins which are synthesized during cell growth are enzymes which in turn play a regulatory function in growth, *e.g.* by determining the availability of substrates. One such enzyme could be invertase. Invertase can be envisaged to control cell growth by regulating the hydrolysis of sucrose and also its transport to cells as well as between different compartments of the cell. It is known to have its maximal activity in the most rapidly elongating part of growing roots (9, 15) and stems (6), and the activity has been found to increase when the growth of the tissue was promoted by applied gibberellin (8, 10, 16).

In an effort to learn more about the possible function of invertase in the growth of plant cells and especially in cell expansion the relationship between the activity of this enzyme and growth was studied both in presence and absence of exogenous gibberellic acid (GA_3). The experimental system consisted of epicotyls of etiolated lentil seedlings. It was chosen because the lentil epicotyl, *i.e.* the internode between the points of attachment of the cotyledons and of the first scale leaf, is morphologically a clearly defined organ, is known to elongate predominantly by cell expansion, and is responsive to gibberellin (13).

Materials and Methods

According to the methods of Nitsan and Lang (13), lentil seeds (*Lens culinaris* Medik. = *L. esculenta* Moench, cv. Persian; Washington State University, Pullman, Washington) were soaked for 8 hours in distilled water and then germinated for 40 hours in the dark on moist filter paper. After this time the seed coats were removed and the radicle cut back. Seedlings in this condition are referred to as seedlings in the initial state. The seedlings were then incubated for the experimental treatments under shaking in the dark in 0.1 Hoagland's nutrient solution containing 1000 units of penicillin GK per ml. For incubation with sugars the seedlings were surface sterilized and chloramphenicol (20 μg/ml) was added as an additional bacteriostatic agent. The antibiotics had, at the concentrations used, no effect on growth or on invertase activity. Usually there were 20 seedlings per 20 ml of solution. All handling and transfer operations were carried out in daylight.

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[2] Present address: Botanisches Institut der Universität, D-63 Giessen, Germany.

Elongation and the activity of invertase were measured only in the epicotyls. The invertase activity was measured in the following way. Forty or 60 epicotyls were ground in a mortar with sand and 10 ml of phosphate buffer (0.02 M; pH 5.3). The extract was then centrifuged for 8 minutes at 1200 × *g* to clear it from sand, unbroken cells and wall debris. The activity of the cell-wall fraction can be neglected, as will be shown below. To an aliquot of the supernatant an equal volume of 0.1 M sucrose, dissolved in buffer, was added. The sugar concentration was determined polarimetrically at the beginning and the end of a 2-hour period. Under these conditions the enzyme activity was well within the linear range with regard to both enzyme concentration and duration of assay. The only products of sucrose hydrolysis were 2 substances which cochromatographed on paper (ethyl acetate-pyridine-water, 8:2:1) with fructose and glucose, and there was no hydrolytic action on maltose. There is no doubt that the lentil-epicotyl enzyme is a typical plant invertase (beta-fructofuranosidase). The sucrose concentration used, while resulting in maximal rate of hydrolysis and thus optimal for the activity test, was too low to observe formation of trisaccharides.

Protein was measured in the extract by the Lowry method, using bovine serum albumin as a standard. The temperature for germination, incubation and enzyme assay was 22°. All experiments were run in duplicate and repeated at least 3 times.

Results

Time Course of Growth and Invertase Activity. Length, invertase activity, and protein content in lentil epicotyls at the start of the experimental treatment are given in table I. For a quantitative comparison of the changes in invertase activity and length during growth the values of these parameters are given as percent of this initial state.

Upon incubation of the seedlings in basal medium the epicotyls grow to about 6 times their original length, growth being completed after approximately 48 hours (fig 1, top). The increase in invertase activity per epicotyl is greater, to about 10 times the initial value, whilst invertase per mg protein increases parallel to length during the first 34 hours of growth, to a maximum of about 4 times the initial value. The most rapid increase in invertase activity coincides closely with the period of most rapid elongation, actually preceding it slightly. As soon as growth ceases, the activity of the enzyme declines, dropping, after 96 hours of incubation, to less than half the maximal level.

Effect of Gibberellic Acid. Exogenous GA_3 added at the start of incubation results in a great increase in the rate of elongation and in invertase (fig 1, bottom). Length increases to 12, invertase per epicotyl to 18 and invertase per mg protein to 7 times the initial value. The kinetics of growth and invertase increase remain, however, essentially the same as in basal medium.

Measured as percent of the untreated controls GA_3 increases growth and invertase activity to the same extent. The optimal concentration for either effect is also the same, namely 30 mM. However, the maximum of the GA_3 effect on invertase and on elongation is reached after different times of incubation (table II). The effect on invertase is greatest after 24 hours, being about 200 % for invertase per mg protein, and even more for invertase per epicotyl since the protein content in the GA_3-treated epicotyls is also increased by about 10 %. There is also an increase of invertase per mg fresh weight of the epicotyls but it is less pronounced. On the other hand the effect of GA_3 on elongation is greatest after longer times of incubation, reaching also about 200 %, but only at 72 hours.

Table II. *Effect of GA on Growth and Invertase Activity After Different Times of Incubation*

Values calculated from the data of figure 1, and expressed as percent of control. GA_3 concentration was 30 mM.

Hours of incubation	9	24	34	48	72
Length	119	170	171	188	193
Invertase/mg protein	151	196	154	145	95

When GA_3 is added to the samples after 24 hours of incubation in basal medium, that is, during the time of approximately constant and maximal rate of elongation (compare fig 1, top), there is no lag for the increase in invertase activity but there is a noticeable lag for the effect on growth (fig 2).

If added during the extraction procedures GA_3 has no effect on the activity of the enzyme.

Osmotic Inhibition of Growth. Invertase activity being evidently closely correlated with growth it seemed interesting to determine whether the activity of the enzyme could be enhanced independently from growth. An attempt to answer this question was made by inhibiting elongation osmotically by means of polyethyleneglycol (MW 1000).

Increasing concentrations of polyethyleneglycol

Table I. *Values for Length and Invertase Activity at the Initial State*

Length	:	2.9 mm
Protein	:	0.08 mg/epicotyl
Invertase	:	0.12 μmole sucrose hydrolyzed/epicotyl hr^{-1}
Invertase/mg protein	:	1.5 μmole sucrose hydrolyzed mg $protein^{-1}$ hr^{-1}

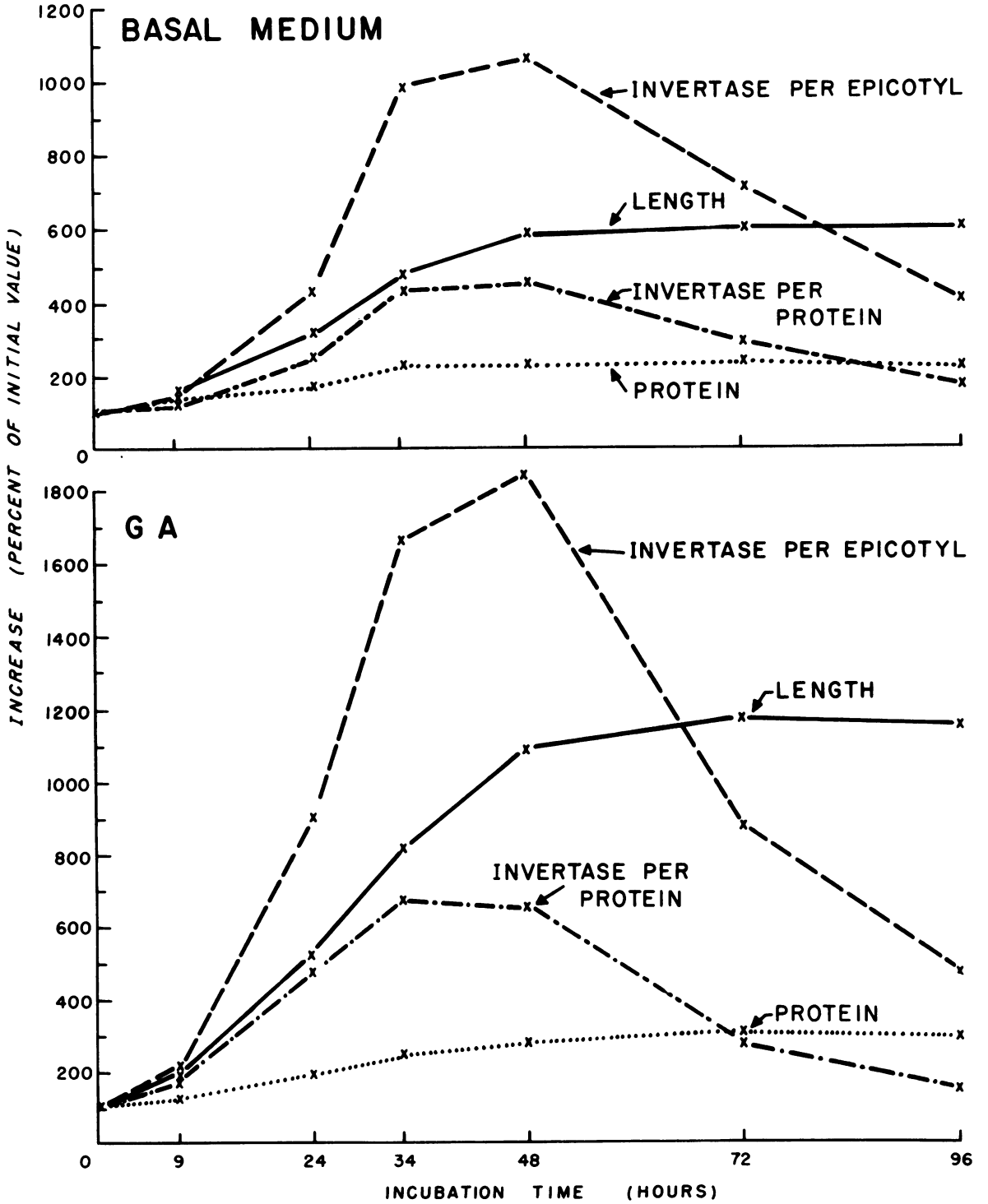

FIG. 1. Kinetics of elongation and invertase activity during growth. Top, seedlings incubated in basal medium; bottom, seedlings incubated in presence of GA_3 (30 mM).

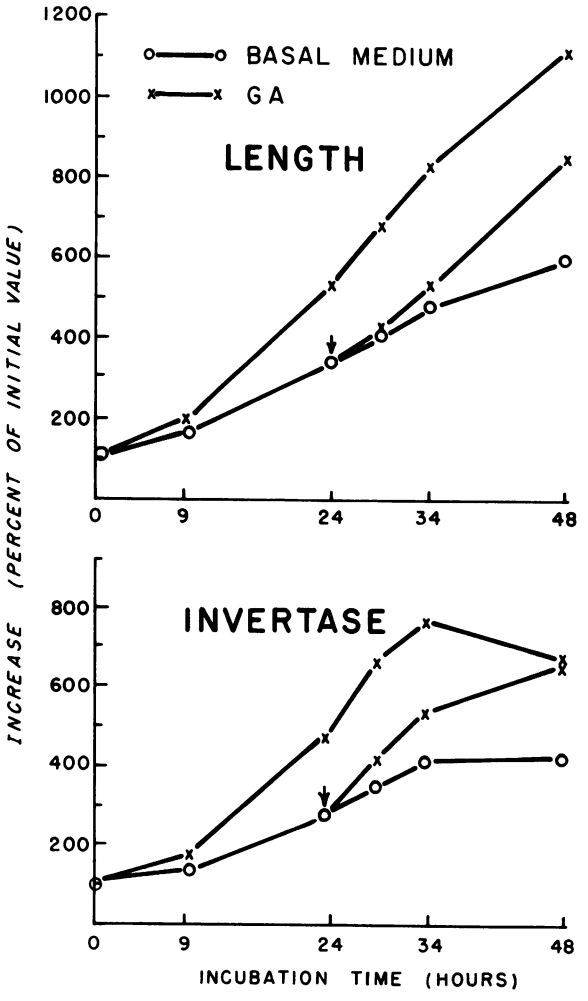

FIG. 2. Effect of GA_3 (30 mM), added in the course of incubation, on growth (top) and invertase activity, measured per mg protein (bottom). Upper curves = control, GA_3 added at start of incubation; middle curves (starting at arrow) = GA_3 added after 24 hours incubation; bottom curves = control, basal medium.

resulted in an increasing inhibition of both growth and invertase activity approximately parallel to one another when measured after 24 hours of incubation. With a concentration of 0.22 M, growth in absence of GA_3 was completely inhibited and invertase activity underwent actually a decrease (table III). But the effect of GA_3, measured as percent of the controls, was nearly the same as in the absence of polyethyleneglycol, being slightly lower for growth and higher for invertase. Thus, it was not possible to separate invertase activity from growth by this approach.

If, after 24 hours incubation in polyethyleneglycol in the absence of GA_3, the tissue was transferred to basal medium growth and invertase activity both recovered during another 24 hours of incubation. This recovery was relatively less when the tissue had been exposed to higher polyethyleneglycol concentrations, but the recovery in growth and in enzyme activity were similar. In presence of GA_3, in contrast, recovery in invertase activity was considerably greater than that in growth. Thus, GA_3 in the presence of polyethyleneglycol has a greater effect on invertase than on length (table III).

Effect of Sugars. Fructose, glucose and sucrose in the medium had no influence on growth or invertase activity during 24 and 48 hours of incubation. At higher concentrations (0.1 M and above) they resulted in an inhibition which was not significantly different from the inhibition caused by the same concentrations of polyethyleneglycol or mannitol.

Effect of Inhibitors. When added at the start of incubation and at a concentration of 10 μg/ml cycloheximide inhibited any increase in length and in invertase activity during 24 hours of incubation, the invertase activity showing in fact a slight decline. A cycloheximide concentration of 1 μg/ml was sufficient to block the promotion caused by GA_3 (table IV).

Actinomycin D (50 μg/ml) also inhibited elongation and invertase increase (table IV).

FUDR (10 mM) added at 0 hours resulted, after 24 hours of incubation, only in a relatively small

Table III. *Effect of Polyethyleneglycol on Growth and Invertase Activity in Basal Medium and GA_3*
Values in percent of the initial state.

Time	Conditions	Length		Invertase per mg protein	
		Basal medium	30 mM GA_3	Basal medium	30 mM GA_3
		%	%	%	%
After 24 hr incubation	Control	426	691	418	676
	Polyethyleneglycol, 0.22 M	104	129	46	85
Recovery after an additional 24 hr of incubation	Control	695	1455	646	884
	Polyethyleneglycol 0.22 M present during the first 24 hr of incubation	279	613	92	477

Table IV. *Effect of Inhibitors on Growth and Invertase Activity in Basal Medium and GA_3*

Values in percent of the initial state, after 24 hours of incubation.

Compound and conc	Length		Invertase per mg protein	
	Basal medium	30 mM GA	Basal medium	30 mM GA
	%	%	%	%
Control (no addenda)	328	500	365	594
Cycloheximide 1 μg/ml	173	194	183	177
Cycloheximide 10 μg/ml	103	106	88	88
Actinomycin D 50 μg/ml	167	184	180	203
FUDR (10 mM)	251	338	283	385
FUDR + 1 mM thymidine	332	532	359	572

inhibition, both growth and invertase activity being reduced to about 70 % of the control. Thymidine fully reversed the effect of FUDR (table IV).

Preincubation in FUDR strongly reduced the effect of GA_3 subsequently added. This cannot be achieved by preincubation in an equally inhibitory concentration of cycloheximide; with this inhibitor, the action of subsequently added GA_3, measured as percent of control, is unchanged (table V).

The effect of inhibitors added after 24 hours of incubation, when elongation is proceeding at a near-constant rate, is shown in figure 3. The data refer to GA-treated samples only but the effects in basal medium were the same.

Cycloheximide added after 24 hours caused a complete stop in elongation within a very short time. Invertase activity also stopped increasing and then decreased markedly, with a half-life of approximately 14 hours (fig 3, top).

Actinomycin D became effective only after a considerably longer lag period of 2 to 3 hours, and there was no change in the pattern of the decrease in enzyme activity (fig 3, center).

FUDR added at 24 hours caused, after a lag period of at least 10 hours, only a small inhibition of growth and invertase synthesis (fig 3, bottom).

Localization of the Enzyme Inside the Cell. For an understanding of the function of invertase in plant cell growth it is important to know where in the cell the enzyme is localized, and especially whether it is bound to some organelle or other cell structure. However, the centrifuged extract (8 min, 1200 × *g*) had about the same activity per ml as the crude, unfiltered and uncentrifuged extract containing cytoplasm as well as wall debris and unbroken cells. There was no difference in this regard between younger (24 hr incubation) and older (72 hr incubation) epicotyls, and the entire activity stayed in the supernatant even after centrifugation for 2 hours at 165,000 × *g*. When 0.3 % glutaraldehyde, which is known to stabilize some of the cell membranes and to increase the sedimentation rate of some organelles, was added to the medium either 3 hours prior to, or during, or after extraction, there was also no significant effect on the tendency of the enzyme to remain in the supernatant. Thus, the invertase of lentil epicotyl cells appears not to be firmly bound to the cell wall or to any of the cell organelles, but to exist in a free or easily soluble condition in the "ground cytoplasm."

Table V. *Effect of Preincubation in Equally Inhibitory Concentrations of Cycloheximide and FUDR on the Response to GA_3*

Values show the effect of GA_3, expressed as percent of control. GA_3 (30 mM) was added after 24 hours of a total incubation period of 48 hours.

Inhibitor	Time of addition	Length	Invertase per mg protein
		%	%
Control (no inhibitors)		138	145
Cycloheximide (0.15 μg/ml)	Simultaneously with GA	132	139
	24 Hr before GA	147	141
FUDR (10 mM)	Simultaneously with GA	142	136
	24 Hr before GA	114	113

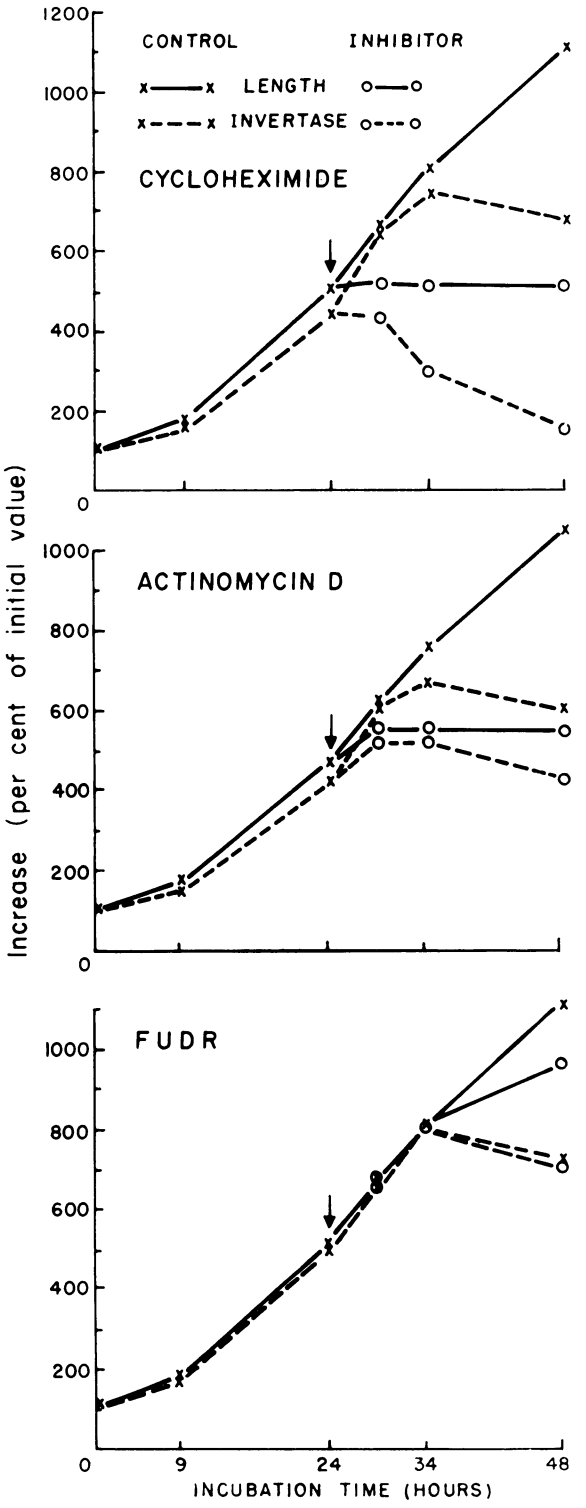

FIG. 3. Effect of inhibitors added during growth. Top, cycloheximide, 10 μg/ml; middle, actinomycin D, 100 μg/ml; bottom, FUDR, 10 mM, all added after 24 hours incubation (arrow!). Values in percent of the initial state for length and for invertase per mg protein. Seedlings were incubated in presence of GA_3 (30 mM).

Discussion

Kinetics of Invertase Activity and Growth. As can be seen from the time course studies (fig 1) invertase activity in lentil epicotyls increases maximally during most active elongation, and drops rapidly once growth is completed. This agrees very well with measurements of invertase activity in growing roots (9, 14) and in sugarcane stems (6).

A careful analysis of the data in figure 1 shows that first there is a maximum in the rate of invertase increase; this is followed closely by a peak in growth rate (both rates measured as slope of the curves in fig 1). But the maximum in invertase activity is reached a considerable time after the maximum in growth rate. This discrepancy might be caused by invertase synthesis proceeding so vigorously that there is an overshoot when growth is beginning to level off.

The increase in invertase activity is not a simple function of an overall increase in protein as the activity of the enzyme, on a protein basis, increased 4-fold in the absence of GA and 7-fold in its presence.

The inhibitor studies indicate that the increase is dependent on protein, RNA and DNA synthesis (table IV). This, and the very rapid effect of cycloheximide (fig 3, top), which is known to be a powerful inhibitor of protein synthesis, suggest that the increase in invertase activity is due to *de novo* synthesis.

Actinomycin D, considered to be an inhibitor of DNA-dependent RNA synthesis, causes less inhibition of invertase synthesis than does cycloheximide and, if added during the course of enzyme synthesis, becomes effective only after a distinct lag period of 2 to 3 hours (table IV and fig 3, center). This may be interpreted as indicating that sufficient amounts of a relatively stable messenger RNA are present in the cells to maintain a certain level of protein synthesis. However, one should keep in mind that such interpretations are based on generalizations of findings made in microorganisms, and that it is not known whether and to what extent these results are valid with respect to higher organisms.

FUDR inhibited invertase increase even less, and with a longer lag period, than did actinomycin D (table IV, and fig 3, bottom). This and the more pronounced inhibition when FUDR was added before GA_3 (table V) indicate that its effect is more indirect.

All inhibitors used caused also complete or partial inhibition of growth of the epicotyls. This result is in full agreement with the now well-known fact that growth, even if based on cell enlargement, is dependent on RNA and protein synthesis (*e.g.* 11, 14). The results with FUDR agree with those of Nitsan and Lang (13) and Bopp (2). The degree of inhibition was less than that obtained by the former authors in the same plant material, but

this is probably because of the shorter incubation time used in the present studies. It is worth noting that while cycloheximide, applied 24 hours before GA_3, had no selective effect on GA_3-promoted growth and invertase activity, FUDR did, a concentration of 10 mM reducing the difference with GA_3-free controls from *ca.* 40 to *ca.* 10 % (table V). This is in agreement with the idea that gibberellin-regulated growth is often dependent, in some direct or indirect manner, on DNA synthesis (compare 12).

It was not possible to detect any significant effect of sugars added to the incubation medium, either on invertase activity or on growth. In this connection one should not forget that the seedlings were incubated in presence of the cotyledons which are undoubtedly providing the epicotyl with sugars, thus rendering it independent of exogenous sugar supply. However, these results are quite in contrast to those obtained by Glasziou *et al.* (5) in sugarcane.

Relationship of Invertase and Growth in Lentil Epicotyls. Invertase activity and growth exhibit, in general, very similar kinetics. This is evident both in normal growth and in experiments with inhibitors. Presence of GA_3 results in increases of both growth rate and invertase level, as also reported by several other authors (8, 10, 16), but here as well as in the inhibitor experiments the kinetic pattern of the 2 processes remains unaltered (fig 1). This latter situation is in contrast to the results of Kaufman *et al.* (10) in *Avena* internodes where presence of GA_3 did cause a change in the kinetics of both elongation and invertase activity.

In some cases, however, the close agreement between growth and invertase is disrupted. The increase in invertase per epicotyl during the first 34 hours of incubation is relatively greater than the increase in length (fig 1). Under the influence of an osmotic inhibitor (polyethyleneglycol) GA_3 has a greater effect on invertase activity than on length (table III). Furthermore, when GA_3 is added in midcourse of growth, the gibberellin-dependent increase in invertase precedes that in growth rate (fig 2), which corresponds to the sequence of the maxima in the effects of GA_3 on invertase and on growth during incubation (table II). This result fully agrees with the concept that growth promotion by hormones involves an increase in the synthesis of enzymes (11, 14).

The invertase in lentil epicotyl cells appears not to be associated with the cell walls and cannot be sedimented by centrifugation, even if glutaraldehyde is added before the extraction. The ineffectiveness of glutaraldehyde may be due to the fact that it inactivates invertase before increasing its sedimentation rate. However, on the whole the results suggest that the invertase of the lentil epicotyl cells is present in a free or easily soluble state. Thus, it would not seem to be associated with the tonoplast where it could supply osmotically active sugars into the expanding vacuole, as postulated by Cook (3). The situation is in contrast to that in storage tissues where the enzyme is insolubly bound to the cell walls (1, 4). In sugarcane stems a soluble invertase was found only in immature tissues while in mature parts the enzyme was associated with the cell walls (7). It is however quite unlikely that a similar change, which would simulate a decrease of activity in the soluble fraction, occurs in the lentil epicotyls as there was no difference in invertase activity in the walls of younger and more mature epicotyls.

Control of the Invertase Level. Whenever growth ceases, whether spontaneously after 48 hours of incubation or under the influence of inhibitors, invertase activity decreases. The half-life of the lentil epicotyl enzyme is about 14 hours, considerably longer than the 2 hours reported by Glasziou *et al.* (5) in sugarcane. Thus, the enzyme is not only synthesized but is also degraded during growth, meaning that the invertase of lentil epicotyls is undergoing continuous turnover. This turnover provides the cells with a more effective control system for the actual level of enzyme activity and thus for the level of available sugars than would be provided by only turning on and off the synthesis of the enzyme.

The results indicate that invertase is closely correlated with growth, and suggest that the enzyme is in some way essential for growth, making sugars available for cell expansion.

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