

Control of Invertase Synthesis in Sugar Cane. Loci of Auxin and Glucose Effects

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Summary. In tissue slices from rapidly expanding internodes of sugar cane the vacuolar invertase level is a function of the balance between synthesis and destruction. The enzyme is destroyed in the tissue at an approximately constant rate with a half time of 2 hours. Invertase synthesis is regulated by both auxin and glucose. From studies with inhibitors of protein and RNA synthesis we conclude that auxin alters the rate of synthesis and glucose increases the rate of destruction of messenger RNA required for the production of invertase.

In *Saccharum officinarum* and most cultivated hybrid varieties of sugar cane, sucrose is the major carbohydrate translocated and stored. Other storage polysaccharides are virtually absent. In stem tissue sucrose is inverted prior to entry into metabolic processes, thus enabling the complex of invertases present to function as key control points for regulating the carbohydrate supply (16). Changes in levels of at least 3 invertases may be correlated with movement and utilization of sugar (6, 8, 9). Levels of the vacuolar invertase of rapidly expanding parenchyma cells of immature internodal tissue were shown to be regulated by both auxin and an end product feedback repression system dependent on the glucose concentration (4, 5, 16). The present communication purports to show which of the partial processes of protein synthesis are affected by these controlling substances.

Methods and Materials

Preparation, Incubation and Extraction of Tissue. Tissue was taken from the basal region of rapidly elongating internodes of field grown sugar cane, variety Pindar. The rind was removed, the tissue cut into slices approximately 1 mm thick, washed, randomized and weighed into 2 g lots. Treatments were carried out in 5 ml solution contained in 50 ml conical flasks, the solutions being changed at 2- to 4-hour intervals. The flasks were shaken at 120 strokes per minute in a water bath at 30°. After treatment the tissue was washed briefly, ground with the aid of a little sand with a pestle and mortar, the juice squeezed out through muslin, and 1.0 ml aliquots dialyzed immediately against distilled water at 1°.

Freedom from Microorganisms. No invertase activity can be detected in the media bathing the tissues. No differences were measured in the amount of invertase in extracts before and after centrifugation at $12,000 \times g$ for 20 minutes, and no activity was found in the precipitate. This result was obtained using our standard techniques in replicated experiments over 8- and 24-hour intervals with and without glucose in the bathing medium. Consequently we believe that our results on invertase

changes are in no way affected by contaminating microorganisms.

Enzyme Assays. Invertase was measured by the method described previously (5). The unit of invertase activity is that amount of enzyme which hydrolyzed 1 μ mole of sucrose in 1 hour under the assay conditions.

Materials. Actinomycin D was obtained from Merck Sharp and Dohme, chloramphenicol from Parke Davis and Company, puromycin from Nutritional Biochemicals Corporation.

Results

Absence of High Molecular Weight Inhibitors. A possible mode of action for the glucose effect is that low or high molecular weight inhibitors may be produced which lower the activity but not the amount of enzyme present. Previously, no evidence for the presence of such inhibitors was obtained (4, 5). An alternative method to detect high molecular weight inhibitors in tissue extracts has been used in which the invertase activity was measured at saturating concentrations of sucrose with the same amount of enzyme but different final volumes of assay solution. If no inhibitor is present, the total amount of product formed should be independent of the volume of the assay solution. If an inhibitor is present it will be diluted as the volume of the assay solution is increased, and the enzyme activity should increase accordingly. In confirmation of previous results no such increase was observed.

Effects of Inhibitors of Protein and RNA Synthesis. Chloramphenicol is a potent inhibitor of protein synthesis and apparently acts by interfering with the correct functioning of the messenger RNA (*m*-RNA) and ribosome combination (19). Its effectiveness in preventing invertase synthesis in cane tissue was reported in a preliminary communication (4). The time course for levels of invertase in tissue treated with chloramphenicol is shown in figure 1. Chloramphenicol effects on tissue entering the rapid phase of invertase synthesis after 6- to 8-hours incubation in water is shown in figure 2.

Puromycin inhibits protein synthesis in many organisms and tissues, by attaching to the carboxyl end

group of the nascent peptide chain following which incomplete peptides are released from the ribosomes (18). The effects of this compound on invertase synthesis are shown in figure 3. Inhibition was incomplete with the concentrations used, but was found to be complete when the concentration was raised to 2.2×10^{-3} M.

Actinomycin D is an inhibitor of RNA synthesis (7,10). It has variable effects on invertase synthesis, depending on the state of the tissue when treatment commences. For tissue from cane harvested early in the morning and placed immediately in actinomycin D solutions, invertase may fall to a low, constant level with a half time to attain this level of 2 to 3 hours. The rapid rise in invertase level which occurred in the water controls after about 6 hours was prevented by actinomycin D (fig 4a). If actinomycin D was added after the rapid rise had commenced, the effect varied from virtually none (fig 4d) to a substantial rate of loss of enzyme activity (fig 4c). Under the conditions in which actinomycin D had little effect in preventing the continued synthesis of the enzyme glucose had a marked inhibitory effect (fig 4b).

If tissue is held overnight in glucose and glycine, invertase synthesis is stopped but is resumed at a very rapid rate after the tissue is washed and incubated in water (16). In a similar experiment transfer to actinomycin D completely prevented the synthesis of invertase (fig 4b).

Rate of Loss of Invertase. The acid invertase of expanding internodes is situated in 2 cell compartments (15), the outer space (which includes the cell wall) and the storage space (which includes the vacuole). The enzyme from both compartments is extracted when tissue is ground. Fluctuations in total invertase over periods of 24 hours are almost entirely attributable to changes in the level of the storage space invertase (16,17). The rate of loss of the storage space invertase may be measured if its synthesis can be completely suppressed, and the

amount of the outer space invertase taken into account. In a previous measurement, the amount of the outer space enzyme was not considered in estimating the rate of loss of the storage compartment enzyme (5).

When chloramphenicol or glucose is used at concentrations which appear to fully inhibit invertase synthesis, the total enzyme level falls to a low constant value (outer space invertase), and the rate of loss of the labile fraction follows a logarithmic decay curve with an approximately constant velocity. Results from 4 experiments are plotted in figure 5. The half time for loss of the enzyme is estimated at about 2 hours. The maximum rate of loss of storage space invertase when actinomycin D is used as an inhibitor also gave a half time of about 2 hours (fig 4a). With fluoride as the inhibitor the base level was attained between 6 and 8 hours, that is 3 to 4 half lives. All results were with field grown cane and extended over an 18 month interval.

Relationship of Invertase Changes to Endogenous Sugar Levels. Invertase changes in tissue placed in water are quite variable, in some cases an immediate and continuing increase occurring, and in others an initial drop followed by a later rise. We suggested that these differences may be explicable in terms of the size of the cytoplasmic hexose pool responsible for repressing invertase synthesis. This pool would, in turn, be a function of the rate of leakage of hexoses from the vacuole and their rate of utilization in metabolism during their passage to the bathing medium (5). The suggestion is consistent with the results plotted in figure 6, which indicate that the balance of synthesis and destruction of invertase turns in favour of increasing invertase levels when the endogenous level of stored reducing sugar falls below about 0.05 M.

An anomalous situation occurred for tissue which had been incubated overnight in glucose solutions, then washed and transferred to water. In such cases invertase synthesis was extremely rapid (fig 4b), even though the endogenous reducing sugar level was in

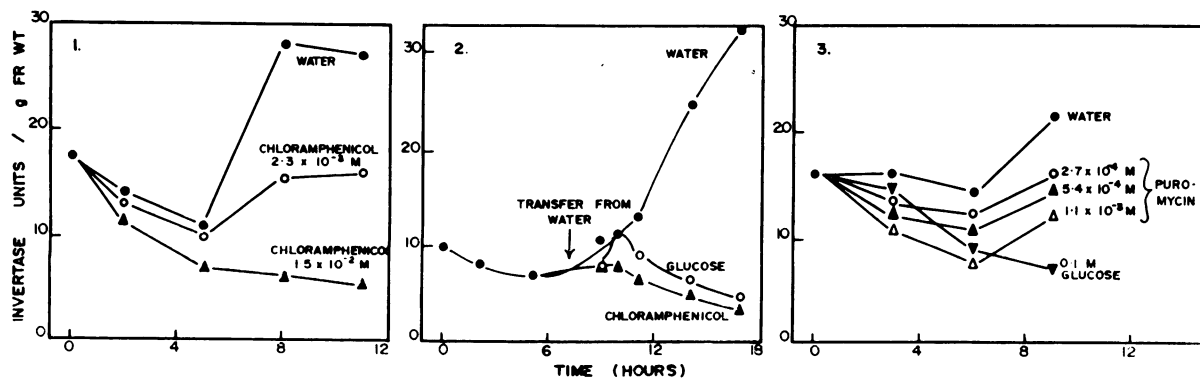


FIG. 1. Time course of changes of invertase level in the presence of chloramphenicol. Tissue was cut, washed for 30 to 60 minutes, then placed in the solutions indicated. At the conclusion of treatment, the tissue was ground, the juice squeezed through muslin, dialyzed at 1° and assayed for invertase activity.

FIG. 2. Effect of 1.7×10^{-2} M chloramphenicol and 0.12 M glucose on tissue entering the rapid phase of invertase synthesis. Experimental detail as in figure 1.

FIG. 3. Time course of changes of invertase level in the presence of puromycin. Experimental detail as in figure 1.

excess of 0.08 M. Previous experiments showed that the rate of destruction of invertase was not altered by the treatment. An explanation for the lack of repression of invertase synthesis by the endogenous sugar pool was sought in terms of rates of sugar leakage which might affect the cytoplasmic hexose pool. Data from 5 experiments for fresh tissue placed in water showed that the rate of sugar loss followed a logarithmic decay curve with an approximately constant half time of from 7 to 8 hours. Contrary to this, sugar leakage from tissue which had been incubated overnight in glucose was very much reduced (fig 7), indicating that substantial changes in the permeability of membranes had occurred thereby altering the normal relationship between sugar level and degree of repression of invertase synthesis.

Escape of Invertase Synthesis from Repression by Glucose. In most batches of tissue, incubation in glucose at concentrations greater than 0.05 M causes invertase to decline to a constant low level where it remains for more than 16 hours. However, in occasional batches, large increases in enzyme level occur despite the presence of glucose in the medium (fig 8). Although we think that escape from glucose control has no significance in vivo, cognizance must be taken of the fact that it can occur in formulating a hypothesis on the mechanism of the glucose effect.

Absence of Endogenous Circadian Rhythm. Slack (17) has reported on diurnal fluctuations in invertase levels in expanding internode tissue of cane growing under field conditions. It seemed possible that the control on invertase exercised by the auxin:

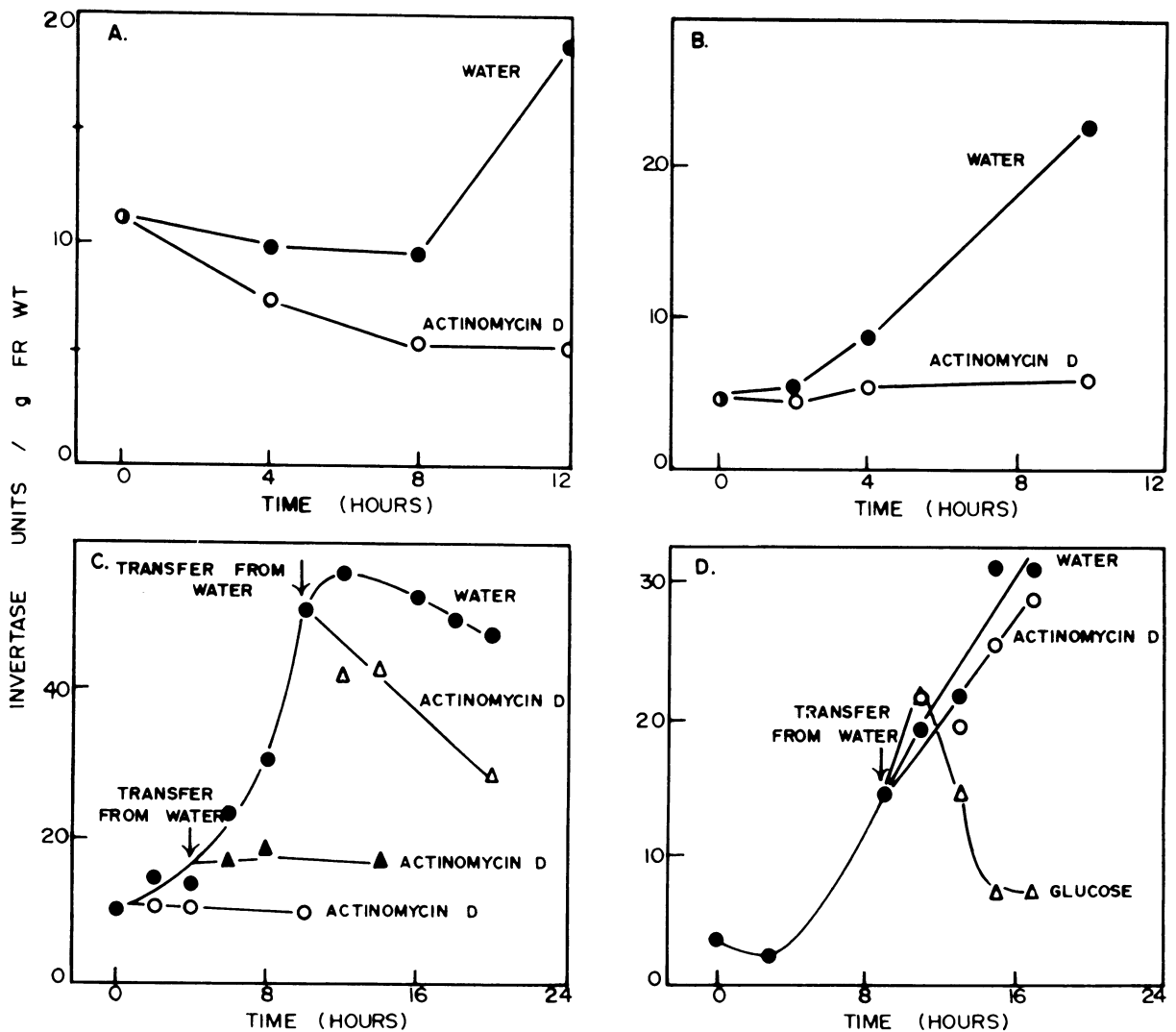


FIG. 4. Effects of 8×10^{-6} M actinomycin D on invertase levels. A) Tissue transferred immediately after cutting; B) transferred after 12 hours pretreatment in 0.11 M glucose and 0.05 M glycine; C) transferred after pretreatment in water for the time interval shown by the arrows; D) transferred to actinomycin D or 0.11 M glucose after 9 hours pretreatment in water. Experimental detail as in figure 1.

and glucose systems, could be superimposed on a third system involving an endogenous circadian rhythm which would occur independently of diurnal environmental variation. No evidence was found for the existence of such a rhythm in tissue slices incubated in water and sampled over a 2-day period (fig 9).

Effects of Auxins. The response obtained to auxins has been much more variable than for glucose repression, and has been partially attributed to varying endogenous auxin level in the tissue due to environmental effects (16). That the picture is more complicated than previously supposed is shown by results depicted in figure 10. Concentrations of α -naphthaleneacetic acid (NAA) of about 10^{-6} M may promote an increase in invertase level compared with

the water control during the first stages of treatment, but later may be inhibitory.

Studies on the uptake of C^{14} -labeled NAA show that concentrations of NAA less than 10^{-4} M may be depleted very rapidly from the medium. Within the tissue, NAA is partially converted to at least 2 other compounds which have been purified but not yet identified. The results obtained in any experiment are not only dependent on the time interval at which harvest is made but also on the ratio of tissue volume to volume of solution and the number of times the solutions are changed during the experiment. With these complications in mind, new experiments were devised to shed light on the auxin effect.

Results presented in figure 11 show that NAA has no significant effect on the rate of loss of invertase in the presence of chloramphenicol. The rate of loss of sugars from NAA-treated tissue was not different from water controls, although substantial effects on invertase levels were recorded (fig 6c). The media bathing NAA-treated tissue were examined for their content of substances reacting with ninhydrin. The chromatographic patterns of these substances were also examined. No differences from water controls were obvious.

In the same experiment in which actinomycin D did not diminish the rate of increase of invertase level compared with water controls and in which 0.11 M glucose gave substantial repression (fig 4d), NAA at 1×10^{-6} M had no significant effect (fig 12).

Fluoride ions are potent inhibitors of invertase synthesis in this tissue. The inhibitory effect of fluoride is reversed on washing the tissue, but synthesis of invertase which occurs after removal of fluoride and transfer to water may be almost completely prevented by actinomycin D. Results of an experiment in which tissue was held in fluoride for 12 hours, then washed and transferred to water or NAA solutions are shown in figure 13. For the first 6 hours of treatment, maximum promotion of invertase synthesis occurred at a concentration of about 10^{-6} M, but by 9 hours the optimum concentration had shifted to 10^{-7} M.

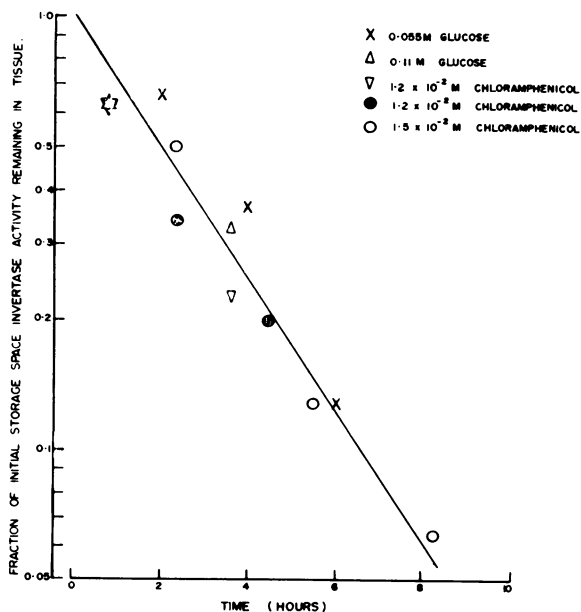


FIG. 5. The loss of invertase from tissue in chloramphenicol or glucose. Data are from 4 different experiments in which logarithmic decay curves for storage space invertase were found. Experimental detail as in figure 1.

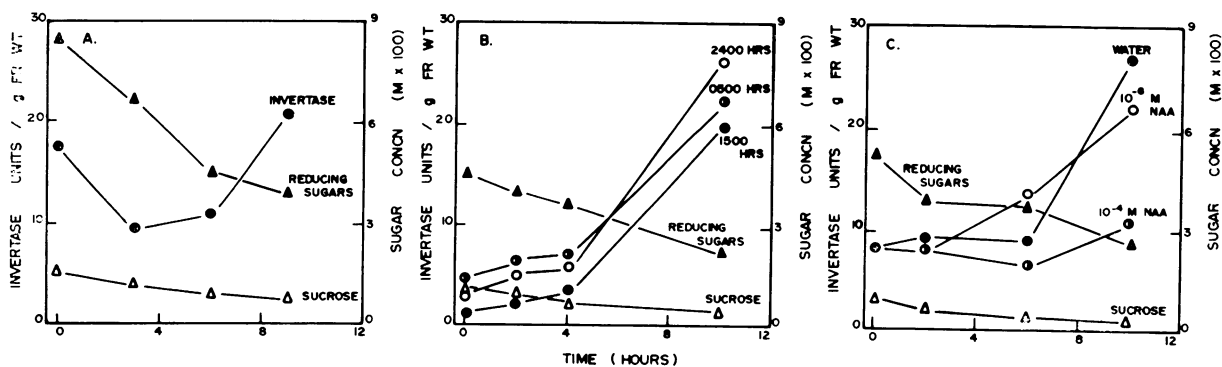


FIG. 6. Relationship between endogenous reducing sugar and change in invertase level. A) Tissue cut at 0800 hours. B) Tissue cut at times shown. Sugar analyses were not significantly different for the 3 harvest intervals. C) Tissue cut at 0800 hours and incubated in NAA or water. NAA made no difference to the level of sucrose or reducing sugar. Experimental detail as in figure 1.

Discussion

In current and previous experiments we have failed to obtain evidence for activation or inactivation of this enzyme by low or high molecular weight substances. Glucose, thought to be the naturally occurring substance responsible for lowering the enzyme level, does not affect the activity of the isolated enzyme when added in physiological concentrations (5). Presently we have reported that inhibitors of protein synthesis such as chloramphenicol, puromycin, actinomycin D and fluoride may cause a rapid drop of invertase level. Both the inhibition due to glucose and to fluoride are readily reversed when the inhibitor is removed from the bathing medium. We conclude that the observed changes in invertase are due to changes in the balance between enzyme synthesis and destruction.

Locus of the Glucose Effect. Under optimal conditions the half time for loss of the labile invertase fraction from the tissue is the same whether glucose or an inhibitor of protein synthesis is present in the bathing medium (fig 5), indicating that the glucose effect is on the rate of synthesis rather than destruction of invertase.

Actinomycin D lowers the invertase level of freshly cut tissue, and prevents the rapid rise which occurs after incubation in water for several hours. If the addition of actinomycin D is delayed until rapid invertase synthesis has commenced, then it may have little effect or else cause a sharp drop (fig 4a, c, d). Assuming that actinomycin D prevents *m*-RNA synthesis (7, 10), the results cited are explicable in terms of whether the rate of invertase synthesis is wholly or partially limited by the levels of *m*-RNA for invertase, or by substrates or enzymes required in later steps. Since glucose repression occurred when actinomycin D was ineffective in preventing invertase synthesis, that is when *m*-RNA for invertase is nonlimiting (fig 4d), we conclude that glucose does not affect *m*-RNA synthesis.

When invertase synthesis was stopped by pretreating tissues in glucose and glycine, and the tissues then washed and transferred to water, rapid synthesis of invertase occurred after a short lag period. However, no synthesis occurred if the transfer was made to actinomycin D (fig 4b). Therefore after incubation in glucose, new *m*-RNA must be formed before invertase synthesis can be resumed. If the effect of glucose was to interfere directly with peptide bond formation or release of completed invertase molecules from the ribosomes, resumption of synthesis of the enzyme should have been at least partially independent of new *m*-RNA formation, and hence independent of actinomycin D effects. As this was not the case, we conclude that glucose must act by accelerating the destruction of *m*-RNA required for invertase synthesis.

Escape from Glucose Repression. The simplest hypothesis compatible with the experimental evidence (fig 8) is that permeability changes occur in some batches of tissue such that the sum of the effects of the endogenous and exogenous sugar supply becomes inadequate to maintain a repressing level of glucose in the appropriate cytoplasmic compartment. Evidence that marked permeability changes do occur when tissue has been incubated in glucose is shown in figure 7.

Destruction of Invertase. The same maximum rate of loss is attained under optimum conditions and concentrations of chloramphenicol, actinomycin D and glucose. The estimated half time for invertase loss is 2 hours, this result being obtained from combined data of experiments done more than a year apart and with tissue from cane growing under a variety of conditions (fig 5). A similar observation has been reported for loss of tryptophan pyrrolase from rat liver which is also destroyed at a constant rate and under a variety of conditions (2).

Locus of the Auxin Effect. Both indoleacetic acid and NAA will promote or inhibit the synthesis of

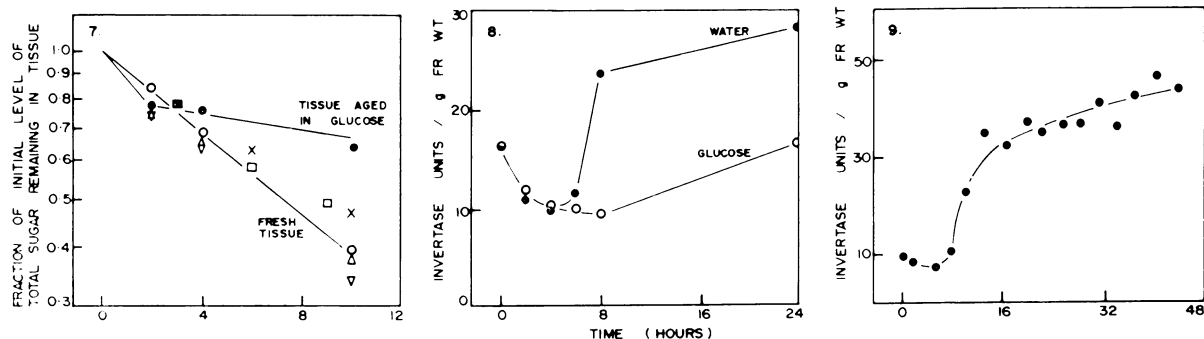


FIG. 7. Leakage rates of endogenous sugars before and after incubation for 16 hours in 0.11 M glucose. Experimental detail as in figure 1. At harvest tissues were washed for 10 minutes, then placed in 3 volumes of ethanol, and the ethanol extract analyzed for sugar content. The initial drop over the first 2 hours for glucose-treated tissue is attributed to incomplete removal of glucose from the outer space.

FIG. 8. Time course for invertase levels in tissue incubated in 0.12 M glucose showing escape from glucose repression. Experimental detail as in figure 1.

FIG. 9. Absence of endogenous circadian rhythm in invertase levels in tissue slices incubated in water. Experimental detail as in figure 1.

invertase (5, 16). Our present work shows that concentrations which promote during the initial stages of an experiment may later inhibit invertase synthesis. We do not know whether the change in invertase level is a response to auxin in the bathing medium only or to the auxin accumulated or metabolized in some tissue compartment. A number of possible loci of action may be excluded, and deductions made as to the mode of operation on the basis of the following considerations.

Since added auxin does not change the rate of loss

of invertase in the presence of an inhibitor of protein synthesis (fig 11), it is unlikely that it directly affects invertase destruction.

When the rate of invertase synthesis was not limited by the level of *m*-RNA (actinomycin D was ineffective), no significant effect of NAA was observed over an 8-hour interval. Under the same conditions glucose repression was expressed (fig 4d, 12). We have concluded that glucose accelerates destruction of *m*-RNA required for invertase synthesis. These results imply that NAA neither accelerates *m*-

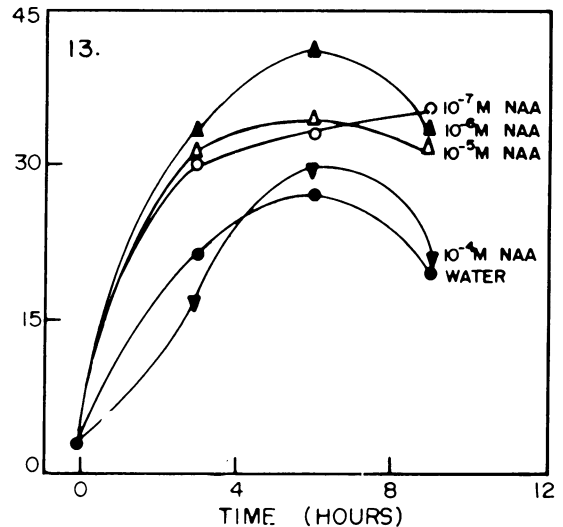
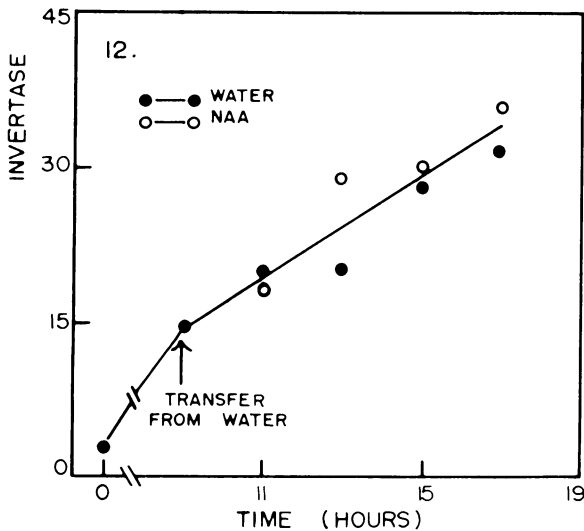
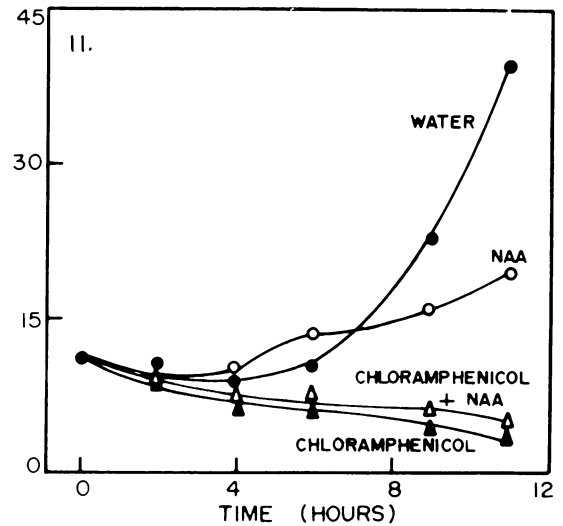
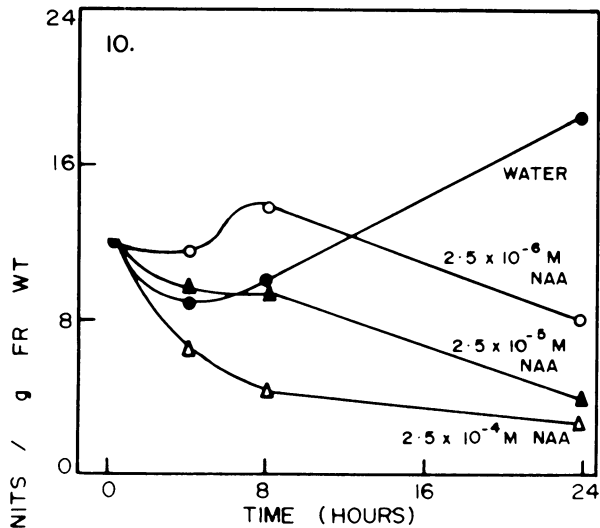


FIG. 10. Effect of α -naphthaleneacetic acid (NAA) on invertase levels in tissue slices over a 24-hour period. Experimental detail as in figure 1.

FIG. 11. Effect of α -naphthaleneacetic acid (NAA) on invertase levels in tissue slices in the presence of chloramphenicol. NAA was used at a concentration of 2.5×10^{-5} M and chloramphenicol at 1.2×10^{-2} M. Solutions were changed after 3, 6, and 9 hours incubation. Other experimental details as for figure 1.

FIG. 12. Effect of 1×10^{-6} M α -naphthaleneacetic acid (NAA) on invertase levels in tissue slices re-incubated for 9 hours in water. For this tissue there was no difference from the water control when transfer was made to 8×10^{-6} M actinomycin D (see fig 4d). Experimental detail as in figure 1.

FIG. 13. Effect of α -naphthaleneacetic acid (NAA) on invertase levels in tissue preincubated in 0.01 M potassium fluoride and 0.05 M glycine for 12 hours. The tissue was washed in 5 volumes of water for 10 minutes, then a second time, the water removed immediately and NAA or water added, and these solutions changed at 3-hourly intervals. Other experimental details as in figure 1.

RNA breakdown, nor alters the levels of substrates or enzymes which limit the rate of peptide bond formation.

Auxin effects on tissue permeability are well known (3). However we have been unable to detect any substantial auxin effect on losses of endogenous sugars or ninhydrin-reacting substances from this tissue.

The results enumerated lead us to speculate that the auxin effect should be on the rate of *m*-RNA synthesis. In reticulocytes, it has been shown that 10^{-2} M fluoride prevented protein synthesis and caused the polysomes to disrupt. The reticulocytes contained no ribonuclease activity, and on removal of fluoride the polysomes reformed and protein synthesis was resumed at the same rate as previously (1, 13). Fluoride inhibition of invertase synthesis in cane tissue is also reversible, even after 12 hours of treatment. However, this tissue contains considerable ribonuclease. Tissue was incubated in 1×10^{-2} M fluoride and 5×10^{-2} M glycine for 12 hours with the expectation of depleting endogenous sugar levels to concentrations which are subinhibitory for invertase synthesis, and of disrupting polysomes and losing the *m*-RNA for invertase through endogenous ribonuclease action. The glycine was added as a carbon and amino nitrogen source to prevent substrate limitation of invertase synthesis (16). Following removal of fluoride we expected to have a system in which invertase synthesis would be limited by the rate of production of new *m*-RNA, and in which an auxin effect on this phase of protein synthesis would be demonstrable. The results of the experiment showed a substantial promotive effect of NAA with an optimum at 10^{-6} M within 3 hours of removal of fluoride (fig 13). We conclude therefore that the auxin effect on invertase synthesis in this tissue is on the rate of *m*-RNA production, or possibly its combination with ribosomes.

It seems unlikely that the auxin effect on invertase could provide a satisfactory explanation for the manifestations of auxin action on other tissues. We suggest the more general interpretation, that in some manifestations of auxin action, the cause is a changed rate of *m*-RNA production with consequent short-term effects on enzymes which have a high turnover rate. Other workers have reached similar conclusions (11, 12, 14).

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