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(Signed) Chee Kok

PERMANENT ADDRESS:

58, Bidor Road
Tapah Perak
Malaysia

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THE UNIVERSITY OF ALBERTA

STUDIES ON THE RELATION BETWEEN CERTAIN SUCROSE-METABOLIZING ENZYMES AND GROWTH IN EXCISED TOMATO ROOTS

by

© CHEE-KOK CHIN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled 'Studies on the relation between certain sucrose-metabolizing enzymes and growth in excised tomato roots' submitted by Chee-Kok Chin in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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External Examiner

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Date..... Jan 3 1974

ABSTRACT

Enzymes involved in sucrose metabolism, *viz.* sucrose synthetase (UDPglucose:D-fructose 2-glucosyltransferase, EC 2.4.1.13), sucrose phosphate synthetase (UDPglucose:D-fructose-6-phosphate 2-glucosyltransferase, EC 2.4.1.14), sucrose phosphorylase (disaccharide glucosyltransferase, EC 2.4.1.7), α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20), and invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) in excised tomato roots (*Lycopersicon esculentum*, var. Sutton's 'Best of All') were studied. Sucrose synthetase and sucrose phosphate synthetase activities were only detected in roots grown in glucose, not in sucrose medium. The $K_{fructose}$ for sucrose synthetase was calculated as 3.8 mM and the $K_{fructose\text{-}6\text{-}phosphate}$ for sucrose phosphate synthetase as 4 mM. No sucrose phosphorylase activity was detected. α-Glucosidase activity was found in the cytoplasm but not in the cell wall. The partially purified α-glucosidase attacked sucrose at only one third the rate it attacked maltose. Invertase activity was present in the soluble as well as the cell wall fractions. The effects of pH and temperature on the activity of soluble and cell wall invertases were very similar; both enzymes had optimal activity at pH 4.8 and 49°C. The invertase in the cell wall could not be solubilized by carbowax 4,000, Tween 20, Tween 80, Triton X-100, deoxycholate, and borate buffer. It could, however, partially be solubilized with sodium phosphate-citric acid buffer at netural or alkaline pH. The soluble invertase activity was resolved into two species (invertase I and II) by Sephadex G-100 filtration. The molecular weight of invertase I was estimated as 106,000 and that of invertase II as 85,000. The kinetic properties of

these two invertases were very similar. However, the two enzymes differed by their thermostability; invertase I remained stable at temperatures up to 50°C whereas the invertase II was stable only up to 35°C. The half-life at 50°C for invertase I was approximately 12 minutes and that for invertase II was approximately 1 minute.

The distribution of soluble and cell wall invertases along the root axis was studied. While the soluble invertase activity rose rapidly in the growing region and was maintained at a high level in the region that had ceased to grow, the cell wall invertase showed an obvious peak of activity in the growing region.

A close correlation between growth rate and invertase activity was found with roots cultured in different sugars and varying sugar concentrations. When roots were transfe ed from one carbon source to another, changes in invertase activity preceded the change in growth rate. Reducing growth rate by lowering incubation temperature did not reduce invertase activity. These results suggested that sucrose rather than high growth rate was important in maintaining high invertase levels.

Treatment with gibberellic acid or abscisic acid caused a decline in invertase activity and growth. Naphthalene-acetic acid lowered the invertase activity but did not affect growth rate consistently. Kinetin had no obvious effects on roots grown in 1.5% sucrose medium; however, it increased the cell wall invertase activity, but not the soluble activity nor growth rate, of roots in 0.5% sucrose medium.

It is suggested that proper regulation of carbohydrate metabolism is essential to the growth of excised tomato roots and invertases play an important role in this regulation.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ABA	: abscisic acid
ADPglucose	: adenosine-5'-diphosphate glucose
F-6-P	: fructose-6-phosphate
GA	: gibberellic acid
G-1-P	: glucose-1-phosphate
NAA	: naphthalene acetic acid
Pi	: inorganic orthophosphate
Tris	: Tris (hydroxymethyl) aminomethane
UDP	: uridine-5'-diphosphate
UDPglucose	: uridine-5'-diphosphate glucose
α-Glucosidase	: α-D-glucoside glucohydrolase (EC 3.2.1.20)
Invertase	: β-D-fructofuranoside fructohydrolase (EC 3.2.1.26)
Sucrose phosphate synthetase	: UDPglucose:D-fructose-6-phosphate 2-glucosyltransferase (EC 2.4.1.14)
Sucrose phosphorylase	: (disaccharide)glucosyltransferase EC 2.4.1.7)
Sucrose synthetase	: UDPglucose:D-fructose 2-glucosyltransferase (EC 2.4.1.13)

INTRODUCTION

The technique of plant tissue culture, which uses defined media and controlled environments in the culture of excised plant parts, has been developed since the thirties (White, 1931; Gautheret, 1937). With this type of technique many plant cultures can be propagated vegetatively and clones can be established. Materials obtained by clonal culture are useful in studies which do not require genetic variations. As the composition of the medium and the environment in which the cultures are grown can be controlled, the cultures are useful in studying the nutritional requirements, morphogenesis, nutrient absorption, senescence, and other physiological activities of plants.

The continuous culture of roots was first established by White (1934) with tomato roots of variety 'Bonny Best'. In his pioneer work of searching for suitable carbohydrates to support the growth of excised tomato roots, White found that sucrose was markedly superior to any other sugar tested. With another tomato variety, 'Sutton's Best of All', Dormer and Street (1949); Street and Lowe (1950); and Ferguson, Street, and David (1958) obtained similar results.

Many workers, intrigued by the apparently unique role of sucrose in the culture of excised tomato roots, have tried to pin point the role of this sugar in growth. On comparing different sugars and

phosphorylated sugars in respiration studies on excised tomato roots, Morgan and Street (1959) found a large number of carbohydrates to be inactive, and galactose, maltose, raffinose, xylose, glucose-1-phosphate and glucose-6-phosphate to support only a low level of respiration. The poor growth-supporting ability of these sugars might be attributed to their ineffectiveness as respiratory substrates. In contrast, glucose and fructose were found to support a similar level of oxygen uptake to sucrose, showing that the inefficiency of glucose and fructose to maintain growth was not due to their inability to support respiration.

Morgan and Street (1959) observed, however, that the high rate of oxygen uptake established in the presence of sucrose persisted for a longer time after the removal of exogenous sugar than was the case with either glucose or fructose. They, therefore, suggested that despite the equally high oxygen uptake in the presence of these three substrates sucrose incubation supported a larger uptake and consequently larger build-up of endogenous sugar. Weston (1967) later studied the absorption of sugars by feeding the roots with radioactive sucrose and glucose. On the basis of weight of sugar absorbed, at concentrations of one per cent or below, no significant difference in the uptake of sucrose was found. Yet the growth of roots in 1% sucrose was markedly higher than that in 1% glucose, showing that the poor growth in the glucose medium was not caused by a low absorption rate.

Dormer and Street (1948) observed that sucrose-grown roots were larger and had a greater number of cells than the glucose-grown roots. Also, the sucrose-grown roots usually had well developed secondary xylem, whereas in glucose-grown roots the secondary xylem was usually

absent. This led Dormer and Street to speculate that sucrose was more readily utilized than glucose. However, Thomas *et al.* (1963) determined the distribution of radioactivity in respired carbon dioxide, alcohol soluble hemicellulose and cellulose of roots fed with radioactive sucrose and glucose and found no significant difference.

Thus, although there have been many attempts to account for the unique ability of sucrose to support the growth of excised tomato roots, no satisfactory answer has yet been found. Nevertheless, these studies help to rule out some of the speculations. Thus there is evidence to show that the ineffectiveness of glucose is not due to a low absorption rate nor its inability to serve as a substrate for respiration or polysaccharide synthesis. The view has been expressed (Thomas and Weir, 1967) that tomato roots require a critical level of sucrose in their meristems and that this level is established and maintained only by the provision of exogenous sucrose. This presupposes the absorption of the undegraded sucrose molecule, and the inability of roots to synthesize sufficient quantities of sucrose from glucose or any of a wide range of other substrates. The present investigation was designed to directly test these two assumptions, and also to determine the relation between growth and the levels of sucrose-metabolizing enzymes under a number of different growth conditions. It was hoped that these studies would refute or corroborate the views of Thomas and Weir, and also throw light on the nature of the critical requirement for sucrose.

REVIEW OF LITERATURE

There are several enzymes which could be involved in the metabolism of sucrose, *viz.* invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26), α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20), sucrose phosphorylase (disaccharide glucosyltransferase, EC 2.4.1.7), sucrose synthetase (UDPglucose:D-fructose 2-glucosyltransferase, EC 2.4.1.13), and sucrose phosphate synthetase (UDPglucose-D-fructose-6-phosphate 2-glucosyltransferase, EC 2.4.1.14); some studies on these will now be reviewed.

A. INVERTASE

1. Acid and neutral invertases

Invertase has long been known to be an important enzyme in sucrose utilization (Berthelot, 1860). This enzyme attacks not only sucrose but also related glycosides that possess an unsubstituted β-D-fructofuranosyl residue. Its action produces either hydrolysis or a transfer of the fructosyl group.

Based on their pH optimum, invertases from plant tissues can be divided into two types. One has its pH optimum between 4 and 5 and is called acid invertase; the other has optimum activity at pH 7 to 7.5 and is either known as neutral or alkaline invertase. Both types are widespread in plant tissues. Acid invertases have been found in sugar beet roots (Vaughan and MacDonald, 1967), pea roots (Lyne and Ap Rees, 1971). Alkaline invertases have been found in sugar cane (Sacher, Hatch, and Glasziou, 1963), carrot roots (Ricardo and Ap Rees,

1970), and sycamore cells (Copping and Street, 1972).

2. Localization of invertases

Ricardo and Ap Rees (1970), in their studies on the invertase of carrot, proposed that the acid invertase was located in the cell wall and the tonoplast, and active in the rapid hydrolysis of exogenous and endogenous sucrose. The neutral invertase was considered to be in the cytoplasm to function in the release of hexose from sucrose in cells with a lower sugar demand. In sugar cane the actively growing tissue was characterized by high activity of the acid invertase, but, during maturation of the tissue, the acid enzyme was replaced by a neutral enzyme (Sacher *et al.*, 1963). Sacher *et al.* proposed that the acid invertase was located in two separate locations, the storage compartment and the outer space. They suggested that the outer space enzyme was confined to the cell wall or cell surface, and that its function was to control the flow of sucrose from the conducting tissue to the growing cells. In this tissue sucrose hydrolysis was a prerequisite for its absorption. They also proposed that the neutral invertase had a similar function for directing the movement of sucrose. So far no studies have been reported substantiating the suggestions put forward by Sacher *et al.* and Richardo and Ap Rees about the intracellular locations of invertases. It is, however, known that in many plant tissues invertase is present in the cell wall and the cytoplasm (Copping and Street, 1972; Straus, 1962; Hawker, 1969). In addition, invertases of yeast and *Neurospora* were also found in the cytoplasm as well as the cell wall (Gascon *et al.*, 1968; Chang and Trevithick, 1972).

3. Solubilization of invertase

Hawker (1969) reported that the insoluble invertase in grapes could be solubilized by treatment with borate, polyethylene glycol, and non-ionic detergents, and therefore suggested that insoluble invertase from grapes was an artefact of extraction caused by the formation of a tannin-protein complex and/or a protein-tannin-cell wall complex. The insoluble invertase of carrot roots or corn coleoptiles, however, was not solubilized by these treatments (Hawker, 1969) indicating that the formation of the suggested complex did not account for the occurrence of acid invertase in the cell wall fraction of these tissues. Ricardo and Ap Rees (1970) reported that the wall bound acid invertase of carrot roots could be partially solubilized by high pH. They concluded that the pH affected the binding of the acid invertase to the cell wall or to a component of the homogenate that sedimented with the cell wall. Recently, partial solubilization of invertase from the cell wall of tomato fruit (Nakagawa *et al.*, 1972) and cultured sycamore cells (Copping and Street, 1972) with extraction media of high pH was also reported. Neville (1972) studied the effects of 5% Tween, 5% carbowax 4000, 5% Triton-X 100, 5% dimethyl sulfoxide, 2.5% dimethyl formamide, 1% β-mercaptoethanol, 1% bovine serum albumin, 5% polyvinylpyrrolidone and 20% glycerol on solubilization of wall bound invertase of Larder barley rootlets and found none of the reagents effective in freeing the insoluble enzyme. Chang and Trevithick (1972) tried to solubilize the wall bound invertase of *Neurospora* with Triton-X 100, β-mercaptoethanol, EDTA, 1 M potassium chloride, cellulose and buffers of pH from 3 to 10 and found that these treatments failed to release significant amounts of enzyme from the wall. However, with snail gut

juice or β-1-3-glucanase prepared from *Bacillus circulaus* WL-12, more than 90% of the wall enzyme was released. They, therefore, concluded that the invertase was bound to the wall by covalent bonds and was released only when these bonds were disrupted. Although the binding of invertase to the cell wall is a very widespread phenomenon, the studies on the solubilization of the enzyme from the wall reveal that the effectiveness of a particular treatment varies with the tissue and there is no single treatment that could release the enzyme from all the tissues studied.

With histochemical staining Vaughan and MacDonald (1967) observed that during the ageing of carrot disks invertase developed first in the cell wall and subsequently in the cytoplasm. Using fluorescein-labelled antibodies Tkacz and Lampen (1973) found that most of the newly formed invertase in yeast was at the surface of the wall surrounding the developing bud. These direct observations thus confirm that invertase does occur naturally in the wall and is not an artefact of extraction.

4. Relationship between cytoplasmic and cell wall invertase

In comparing the soluble and the cell wall bound invertases of tomato fruits, Nakagawa *et al.* (1972) found no significant differences in kinetics between the two enzymes with regard to the effects of pH, substrate concentration and several organic and inorganic inhibitors. However, the two forms of enzymes showed considerable difference in temperature stability and temperature dependency. The soluble enzyme was totally inactivated by incubation at 55°C for five minutes, while the cell wall enzyme retained 65% of its activity after the treatment. There was a marked break in the Arrhenius plot at 19°C with the cell wall invertase but a linear line was obtained at a

temperature of 10°C to 30°C with the soluble invertase. Neville (1972) reported that the soluble and cell wall invertases of barley roots had similar pH optima and Michaelis constants. However, the two forms of enzymes differed in heat stability, the cell wall enzyme was slightly more stable at 50°C than the soluble enzyme. The soluble and the external invertases of yeast were also found to have very similar K_m values, V_{max} values, pH activity curves and serological cross-reacting properties (Gascon *et al.*, 1968). Moreover, both enzymes were absent in the three sucrose-negative mutants. They differed, however, in having different pH-stability curves and different mobilities in polyacrylamide gel electrophoresis.

It has been found that the binding of invertase to supporting materials affects the kinetics and physical properties of the enzyme. Filippusson and Hornby (1970) devised a chemical method of attaching invertase to a polystyrene chain. The bound invertase was found to have a similar pH activity curve as the unbound form, but the V_{max} of the bound enzyme was found to decrease nine fold and the K_m to increase two fold. They concluded that the attachment of the enzymes to the polystyrene might insolate the enzyme from the bulk of the substrate solution and thus alter its accessibility. Alternatively, the hydrophobic polystyrene might not favor equal partition of the substrate between substrate solution and surface of the polymer. If the concentration of substrate is lower in the environment of the bound enzyme than in the bulk of the substrate solution then an increase in the observed K_m would result. Invertase was also found capable of covalently coupling to porous glass particles (Mason and Weetall, 1972). The kinetic values for the bound enzyme were similar to the

soluble enzyme with the exception of the pH optimum; the bound enzyme showed optimal activity at a higher pH.

In studying the origin of the cell wall invertase of *Neurospora*, Trevithick and Metzenberg (1964) observed that intact *Neurospora* secreted only a small amount of invertase into the medium. However, if the cell wall of the fungus was digested with snail-gut juice large amounts of invertase were secreted. They suggested that the low secretion of invertase by intact cells was the result of the retention of the enzyme by the cell wall. Bigger *et al.* (1972) recently studied the invertase secretion of the slime mutant of *Neurospora* by electron microscopy. They observed that the slime mutant was like an artificially prepared protoplast in that it lacked a cell wall. The mutant secreted over 95% of the invertase into the medium whereas cells of the wild type retained almost all their invertase. These results indicate that the external invertase arose by secretion followed by binding to the cell wall. Although cell wall invertases are common in higher plant tissues, no information on their origin is available.

5. Purification of invertase

Metzenberg (1964) found that the invertase from *Neurospora* could be separated into heavy (H) and light (L) forms by gel filtration. The H form could be converted into subunits which resembled the natural L form by heat, high salt concentration and high or low pH. No evidence was obtained for more than one type of subunit. The conditions used for the dissociation of the H form caused a small amount of inactivation. However, there was no change in activity upon reaggregation of the subunits to the H form enzyme. Thus it appeared that the subunit has the same activity whether present in monomeric or polymeric form.

Both forms of invertase were found in the cytoplasm and in the cell wall. However, the H form predominated in the cytoplasm.

Heavy and light forms of invertase were also found in yeast (Gascon *et al.*, 1968). Unlike *Neurospora*, in which both H and L forms were found in the cytoplasm as well as the cell wall, yeast H and L invertase corresponded to the external and internal enzymes. The purified L invertase had a molecular weight of 135,000 and was free of carbohydrate. The molecular weight of the H form was 270,000 and only approximately half of this was protein, the other half being carbohydrate. There were other differences between these two forms. The H invertase contained cysteine and L invertase did not. Further, the H form was more stable to heat and acid pH. Since three sucrose-negative mutants lacked both H and L forms of invertase, Gascon concluded that the biosynthesis of the two forms was related.

Sasaki *et al.* (1971) examined the invertase from low-temperature-treated potato tuber with ammonium sulphate fractionation and DEAE-cellulose column chromatography. They found that the invertase could be resolved into five fractions. Fraction 3 and 4 were not detected in significant amounts and fraction 5 was retained on the top of the column. These three fractions were not studied closely. Fraction 1 and 2 had optimum activity at pH 3 to 4.5. At 50°C fraction 1 was more stable than fraction 2. The Michaelis constant for fraction 1 was 23 mM and for fraction 2 was 12 mM.

In studying the invertase of barley roots with electrofocusing, Neville (1972) found that the soluble enzyme had only one peak of activity, isoelectric at pH 9.8. The enzyme from shoots, however, had two peaks; one isoelectric at about pH 4.2 called the acid enzyme and

the other at about 9.8, called the basic enzyme. The root enzyme had optimum activity at pH 4.1 and was stable to long exposure to a range of pH from 4 to 10. The molecular weight of the enzyme was estimated to be 92,000 ± 3,000 and the Michaelis constant calculated to be 9 mM. The basic shoot invertase had properties similar to the root enzyme except that its Michaelis constant was 3 mM. The acid shoot enzyme also had a molecular weight of around 92,000 but it differed from the root enzyme and the basic enzyme in having a pH optimum at 5, being heat sensitive, labile at pH 7 and having a Michaelis constant of 5 mM.

The studies on the purification and characterization of invertases thus show that multiple forms of invertase are rather common.

6. Natural inhibitors of invertase

The presence of natural inhibitors of invertase was first reported by Schwimmer *et al.* (1961). Based on the kinetic data obtained they suggested that potato tuber possessed an inhibitor of invertase. This was later confirmed by Pressey (1967) who successfully isolated the inhibitor and showed it to be a low molecular weight protein (about 17,000). The mechanism of inhibition was not known. However, it was known that the inhibition was non-competitive, probably due to the formation of an undissociable complex. The effect of potato inhibitor on various plant invertases ranged from negligible to total inhibition. The inhibitor did not affect yeast or *Neurospora* invertases. Invertase inhibitors have also been found in red beet, sugar beet, and sweet potato roots (Pressey, 1968). Recently, an invertase inhibitor was also detected in maize endosperm (Jaynes and Nelson 1971). This inhibitor was probably a protein and it inhibited only one of the two forms of invertase found in maize.

7. Correlation between invertase activity and growth

In studying the distribution of invertase along the axis of *Vicia faba* roots, Robinson and Brown (1952) reported a sharp peak of invertase activity at the region of rapid cell enlargement. This correlation between invertase activity and elongation has also been observed in other roots (Hellebust and Forward, 1962; Sutcliffe and Sexton, 1969). In these experiments no attempt was made to detect soluble and insoluble enzymes. Recently, Lyne and Ap Rees (1971) demonstrated that roots of *Pisum sativum* possessed acid and neutral invertases. They found that during the rapid expansion of cells there was no change in neutral invertase, but the acid invertase increased sharply. A correlation between invertase activity and growth was also found in sugar cane (Hatch and Glasziou, 1963). Developing sugar cane internodes contained an acid invertase (Glasziou 1962), which was located in both the cytoplasm and the cell wall. The level of total soluble acid invertase correlated with the growth rate, whereas the enzyme located in the cell wall was relatively stable (Hatch and Glasziou, 1963). The soluble acid invertase disappeared when internode growth ceased and at the same time the level of a neutral invertase increased. While the soluble acid invertase was found to correlate with growth, the neutral invertase was found to correlate with the levels of hexoses in the tissue.

Acid and neutral invertases were found in both the cytoplasm and cell walls of cultured sycamore cells (Copping and Street, 1972). Their activities were low in stationary phase cells, but increased following sub-culture and reached peaks of activity towards the end of the period of most active cell growth and division. The activities

declined gradually as the cells re entered the stationary phase.

8. Effect of plant growth regulators on invertase activity and growth

Many investigators have studied the correlation of growth and invertase activity by using plant growth regulators to modify the growth rate of the plant tissues. Kaufman, Ghosheh and Ikuma (1968) showed that gibberellic acid (GA) promoted growth and induced invertase activity within six hours in *Avena* stem segments. The increase in invertase activity closely paralleled the growth promotion caused by the hormone. This growth promotion and increase in invertase activity was inhibited by the protein synthesis inhibitor, cycloheximide. Actinomycin D, an inhibitor of m-RNA synthesis, had little or no effect on the GA-promoted growth except at high concentrations (40 and 80 µg/ml) where i depressed both growth and invertase activity. Kaufman *et al.* concluded that the GA-promoted growth and invertase activity involved synthesis of new protein. The full development of activity and growth was inhibited by kinetin and there was a correlation between the decay of invertase activity caused by kinetin and the cessation of growth (Jones and Kaufman, 1971). Jones and Kaufman suggested that part of the effect of kinetin on the growth of *Avena* internodes was to enhance the decay of invertase activity. With lentil epicotyls, Seitz and Lang (1968) also found that GA enhanced growth and increased invertase activity. When GA was added in the mid-course of growth, the GA-dependent increase in invertase activity preceded the increase in growth rate. The osmoticum, polyethyleneglycol, inhibited the increase of both growth and invertase activity. This led them to conclude that the GA-induced growth of lentil epicotyls involved an increase in the synthesis of invertase.

They further observed that the enhancement of both growth and invertase activity was inhibited by cycloheximide, Actinomycin D and 5-fluorodeoxyuridine. Since cycloheximide caused the complete inhibition of growth and a decrease in invertase activity with no appreciable lag phase, they concluded that the increase in invertase activity resulted from *de novo* synthesis of protein. Actinomycin D, showed an effect only after a lag period of approximately two to three hours. This was interpreted as a measure of the stability of invertase m-RNA. The promotion of growth and invertase activity by GA were also found with dwarf pea internodes (Antje, 1971) and rice coleoptile sections (Sarvjit and Kaufman, 1972).

9. Effect of sugars on invertase activity

In addition to plant growth regulators, sugars were also found to affect the levels of invertase. Jones and Kaufman (1971); and Kaufman *et al.* (1973) reported that the growth and invertase activity of developing *Avena* internodes were promoted by sucrose treatment. Promotion of invertase activity and growth by sucrose was also found with rice coleoptile sections (Sarvjit and Kaufman, 1972). Antje (1970) reported that the invertase activity in potato varied according to the sucrose levels in the tissues. The part of the potato with the highest sucrose content always had the highest invertase activity. In addition, Pressey and Shaw (1966) reported that the accumulation of sucrose in tubers stored at cold temperature induced the production of invertase.

Davies (1956) reported a five to ten-fold increase in invertase activity in yeast treated with 0.005 to 0.02 M sucrose, glucose or fructose. However, with higher sugar concentrations (0.05M), glucose

and fructose were strongly inhibitory to invertase development. Dodyk and Rothstein (1964) found that several intermediates of glucose metabolism, *viz.* acetaldehyde, ethanol, acetate, succinate, fumarate, and malate were also inhibitory to invertase formation. They observed that with increasing glucose concentration, the rate of glucose uptake and pyruvate formation were increased. The pyruvate could be metabolized either by the oxidation pathway with the production of acetyl-CoA or by a decarboxylation pathway with the formation of acetaldehyde, ethanol and acetate. When the glucose concentration of the medium was low the products of the decarboxylation were minimal but as the glucose concentration increased these products accumulated. As the addition of these two-carbon intermediates in the presence of non-suppressive concentrations of glucose inhibited invertase development, Dodyk and Rothstein suggested that the inhibitory effect of glucose was due to the accumulation of one or more intermediates of the decarboxylation pathway. This suggestion was supported by their finding that the inhibitory effect of glucose occurred at a lower glucose concentration under anaerobic conditions, under which the decarboxylation pathway prevailed.

Repression of invertase synthesis by glucose has also been described in higher plants. In sugar cane, invertase synthesis was inhibited by glucose, fructose and mannose (Glasziou, Waldron and Most, 1967). Glucose prevented invertase synthesis under conditions where Actinomycin D had little or no effect. When tissues were transferred to water following a glucose treatment, Actinomycin D completely repressed the increase in activity and this led Glasziou *et al.* to propose that m-RNA required for invertase synthesis was

destroyed during glucose treatment. The half-life for loss of the invertase activity of sugar cane was calculated to be two hours and this half-life was found not to be affected by the protein synthesis inhibitor chloramphenicol (Glasziou, 1969). The results indicate that the glucose effect was on the rate of synthesis rather than on the destruction of invertase (Glasziou *et al.*, 1966).

The regulation of invertase synthesis by sucrose and glucose is not universal. Ricardo *et al.* (1972) studied the effects of sugars on invertase production by carrot cells and found that the production of acid invertase in both tissue culture and disks of storage tissue was neither repressed by hexose nor enhanced by sucrose. Hexoses also did not repress the invertase formation in cultured sycamore cells. In fact, Copping and Street (1972) found that glucose-grown cells had higher invertase levels than cells grown in sucrose or a mixture of sucrose and glucose.

B. α-Glucosidase

In addition to invertase, α-glucosidase can also hydrolyze sucrose, but differs from invertase by attacking sucrose from the glucosyl end. This enzyme is not specific for sucrose; it attacks a great number of glycosides that possess an α-D-glucopyranoside end (Harvoson and Ellias, 1958; Phillips, 1959). The specificity of this enzyme is confined to the glucose moiety and to the anomeric character of the linkage and not to the particular aglycon moiety.

α-Glucosidase has been found in different strains of yeast (Halvorson and Ellias, 1958; Phillips, 1959; Chiba *et al.*, 1962), molds (Pazur and Takahido, 1960) and other plant tissues (Nigam and Giri, 1959;

Takashashi *et al.*, 1968; Jogensen and Jogensen, 1963), and in some cases the enzyme has been purified to yield a homogenous protein in ultracentrifugal analysis (Halvorson and Ellias, 1958; Phillips, 1969; Takashashi *et al.*, 1968). Crystalline form α-glucosidase obtained by Sugawara *et al.* (1959) was estimated to have a molecular weight of 85,000 ± 30,000. The pH optimum of yeast α-glucosidase is pH 6.6 to 7 (Chiba *et al.*, 1962) and that of buckwheat is pH 5.0 (Takashashi *et al.*, 1968).

C. Sucrose phosphorylase

Sucrose phosphorylase catalyzes the transfer of the glucosyl moiety of sucrose to inorganic phosphate forming fructose and glucose-1-phosphate (G-1-P).

$$\text{D-sucrose} + H_3PO_4 \rightleftarrows \text{D-G-1-P} + \text{fructose}$$

The reaction is reversible, *i.e.* G-1-P can act as a glucosyl donor and fructose as acceptor, and the enzyme catalyzes the exchange of ^{14}C-fructose into sucrose (Fitting and Doudoroff, 1952) and of ^{32}P into G-1-P (Doudoroff *et al.*, 1947). Besides the transfer reaction, sucrose phosphorylase also catalyzes the hydrolysis of sucrose and G-1-P although the hydrolytic reaction proceeds much more slowly than the phosphorylic reaction (Mieyal *et al.*, 1972).

Sucrose phosphorylase was first found in *Pseudomonas saccharophila*, which grew more actively when supplied with sucrose than with glucose (Doudoroff *et al.*, 1943). The synthesis of sucrose phosphorylase by *P. saccharophila* is induced by sucrose (Mieyal *et al.*, 1972). Doudoroff postulated that sucrose phosphorylase was essential for the absorption of sugar and considered that the inability of *P.*

saccharophila to utilize glucose was a consequence of the specificity of this enzyme. Sucrose phosphorylase was also considered to play a role in the absorption of sucrose by excised tomato roots (Street and Lowe, 1950). They found a similarity between the symptoms of phosphorus and carbohydrate deficiency and were able to demonstrate a phosphate activation of sucrose absorption by phosphorus-deficient roots. Phloridzin, a compound known to inhibit certain phosphorylations (Hartt, 1943), inhibited the growth of excised tomato roots. The phloridzin-inhibition of growth was reversible, proportional to the concentration of phloridzin used and partially counteracted by high sucrose concentrations. Moreover, no similar phloridzin inhibition was observed with attached seedling roots. This led Street and Lowe to suggest that sucrose phosphorylase was involved in sucrose absorption. However, they did not directly demonstrate the presence of this enzyme in excised tomato roots. Sucrose phosphorylase has been detected mainly in bacteria, *e.g. P. saccharophila* (Doudoroff *et al.*, 1943) and *P. putrefaciens* (Doudoroff *et al.*, 1949). Reports of sucrose phosphorylase in higher plants are scarce and attempts to detect it by Hassid and Doudoroff (1950) and Gibbs (1959) were unsuccessful. Shukla and Prabhu (1960) and Pandya and Ramakrishnan (1956) have published brief descriptions of the presence of the enzyme in preparations from sugar cane juice and leaves, respectively. Hatch, Sacher and Glaszlou (1963), however, were not able to detect the enzyme in a number of sugar cane tissue extracts.

D. Sucrose synthetase and sucrose phosphate synthetase

Two mechanisms for the biosynthesis of sucrose in plants have been

demonstrated:

(1) UDPglucose + D-fructose $\rightleftarrows$ UDP + sucrose

(2a) UDPglucose + D-fructose-6-phosphate $\rightleftarrows$ UDP + sucrose-6-phosphate

(2b) Sucrose-6-phosphate + H_2O $\rightarrow$ Sucrose + P_i

Reaction 1 is catalyzed by sucrose synthetase, which was first detected in wheat germ (Cardini *et al.*, 1955). The reaction is freely reversible and the equilibrium constant in the direction of sucrose synthesis was estimated to be between 1.6 and 8 (Cardini *et al.*, 1955). Although the equilibrium is in favor of sucrose synthesis this enzyme is considered to be active in the degradation of sucrose (Avigad, 1964; Milner and Avigad, 1964). The complete breakdown of sucrose is possible if the UDPglucose is used up in other metabolic processes. Sucrose synthetase has been demonstrated in a variety of plant tissues, *e.g. Phaseolus* seedlings (Grimes *et al.*, 1970), sugar beet (Avigad and Milner, 1966), potato tuber (Lavintman and Cardini, 1968), and artichoke tuber (Avigad, 1964). In *Phaseolus* seedlings activity was high in non-photosynthetic tissues, but significantly lower in photosynthetic tissues (Delmer and Albersheim, 1970). Based on this distribution pattern, Delmer and Albersheim concluded that sucrose synthetase was unlikely to play a role in the biosynthesis of sucrose; rather, they suggested that this enzyme served predominantly to catalyze the conversion of translocated sucrose to UDPglucose and fructose in non-photosynthetic tissues. Sucrose synthetase is not specific for UDP-glucose, but is capable of utilizing other nucleotides, especially ADPglucose (Grimes *et al.*, 1970) which is a precursor of starch. Slabnik, Frydman and Cardini (1968) suggested that since ADP

competed with UDP in reaction 1, sucrose synthetase might serve as a link between sucrose and the formation of starch. This was corroborated by the fact that a number of phenolic glycosides, which inhibit the synthesis of starch, also inhibited the formation of sucrose from UDPglucose or ADPglucose. In this context sucrose synthetase has been found in numerous starch-synthesizing tissues, *e.g.* sweet corn (Fekete and Cardini, 1964), rice (Murata *et al.*, 1964), and potato tuber (Pressey, 1969) and has been implicated in sucrose-starch transformation in these tissues.

Reaction 2a is catalyzed by sucrose phosphate synthetase (Leloir and Cardini, 1955). The equilibrium constant of this reaction in the direction of sucrose-6-phosphate formation was calculated to be 3250 at 38°C and pH 7.5 (Hassid, 1960). When this reaction is coupled with reaction 2b, catalyzed by phosphatase, the biosynthesis of sucrose can be considered essentially irreversible. Sucrose phosphate synthetase has been demonstrated in many plant tissues, *e.g.* wheat germ (Leloir and Cardini, 1955), tobacco leaves (Bird *et al.*, 1965), spinach leaves (Mendicino, 1960), sugar beet leaves (Rorem *et al.*, 1960), seeds of maize, broad bean, and castor bean (Hawker, 1971). Although reaction 2a is reversible, the high equilibrium constant in the direction of synthesis suggests that this enzyme is operating for sucrose-6-phosphate synthesis *in vivo*. Infiltration of ^{14}C-glucose into *Canna* leaf disks resulted in the fructofuranosyl moiety of sucrose becoming highly labelled before any label appeared in the free fructose pool, indicating that free fructose was not an intermediate in sucrose synthesis *in vivo* (Putman and Hassid, 1954). Therefore, reactions 2a and 2b and not reaction 1 are considered responsible for sucrose synthesis.

MATERIALS AND METHODS

MATERIALS

1. Chemicals

Uridine-5'-diphosphate (UDP) and uridine-5'-diphosphate-glucose (UDPglucose) were obtained from Sigma Chemical Company, Missouri, USA. Fructose-6-phosphate (F-6-P), bovine albumin, kinetin and naphthalene acetic acid (NAA) were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, USA. Raffinose, gibberellic acid (GA), nicotinic acid, 2-thiobarbituric acid and thiamine hydrochloride were purchased from Eastman Organic Chemicals, Rochester, New York, USA. 1,4-*bis*[2-(5-phenyloxazolyl)] benzene and 2,5-diphenyloxazole were obtained from Nuclear Chicago, Des Plaines, Illinois, USA. Ferric chloride solution was supplied by Johnson Mathey Chemicals Ltd., London, England. Sephadex was obtained from Pharmacia Canada Ltd., Montreal, Canada. Sucrose-U-^{14}C and fructosyl-^{14}C-sucrose were purchased from Amersham/Searle, Des Plaines, Illinois, USA. Cycloheximide was obtained from Calbiochem, California, USA. Abscisic acid (ABA) was a gift from Shell Development Co., California, USA. All other chemicals were purchased from Fisher Scientific Co., Edmonton. All chemicals used were of the highest analytical grade available.

2. Biological material

A clone of tomato root was established from a single seed of *Lycopersicon esculentum* variety Sutton's Best of All. The seed was surface sterilized by immersion in 1% bromine water for 5 minutes and washed 5 times in sterile distilled water. It was then transferred to

a sterile petri dish containing filter paper moistened with distilled water and incubated at 28°C until the radicle was 25 to 30 mm long. A 10 mm radicle tip was then cut and transferred to a 150 ml Erlenmeyer flask containing 50 ml of modified White's medium (White, 1943; Sheat *et al.*, 1959) and incubated at 28°C. After incubation for seven days, sectors of the main axis bearing 4 to 5 lateral roots were excised and transferred to fresh medium. At the end of seven days 10 mm tips were excised from the laterals of these sectors and transferred to fresh medium. In this way it was possible to build up a large clone and maintain the tissue continuously in culture. All manipulations were carried out in a sterile transfer room. Tip cultures only were used in experiments described herein.

3. (a) Medium composition

The medium used in this study was basically that of White (1943) but was modified by: (1) the addition of molybdenum and replacement of $FeCl_3$ by Fe-EDTA (Sheat *et al.*, 1959), and (2) the omission of glycine (Weston, 1970). The composition of the medium is listed in Table 1. The pH of the medium was adjusted to 4.8 with 0.1 N NaOH.

(b) Medium sterilization

Sucrose medium was sterilized by autoclaving at 15 lb/sq. in. for 5 minutes. Glucose medium prepared by autoclaving the medium complete is detrimental to the growth of excised tomato roots (Ferguson *et al.*, 1958). Glucose, fructose, and raffinose media were therefore prepared by autoclaving aqueous sugar solutions and salt-vitamin solutions separately and then mixing them together aseptically. To avoid the possibility that plant growth regulators may be decomposed by autoclaving, media supplemented with plant growth regulators were prepared by

TABLE 1

Standard Culture Medium

Constituents	mg/litre
$Ca(NO_3)_2$	200.00
$MgSO_4$	360.00
KNO_3	80.00
KCl	65.00
Na_2SO_4	200.00
NaH_2PO_4	16.50
KI	0.75
$MnCl_2$	4.50
$ZnSO_4$	1.50
H_3BO_3	1.50
$HMoO_4$	0.0017
$CuSO_4$	0.013
Aneurin HCl	0.10
Pyridoxin	0.10
Nicotinic acid	0.50
Fe-EDTA*	10.00 ml/litre
Sucrose	15.00 g/litre

*Fe-EDTA was prepared by mixing 0.08% $FeCl_3$ solution with 0.62% Na-EDTA solution in equal volume.

adding filter-sterilized solutions of plant growth regulators to autoclaved medium.

METHODS

A. Extraction and partial purification of enzymes

1. Invertase

Step 1 -- Seven-day-old roots were harvested, incubated in distilled water for two hours, and washed 3 times with distilled water. The roots were homogenized with 0.05 M sodium phosphate-citric acid buffer, pH 7.0 with a Ten Broeck glass homogenizer. The homogenate was centrifuged at 12,000 x *g* for 10 minutes and the supernatant used as the soluble enzyme. The pellet was washed with the same buffer and centrifuged at 12,000 x *g* for 10 minutes and the washing process repeated twice. The final sediment was resuspended in 0.05 M sodium phosphate-citric acid buffer, pH 7, with a glass homogenizer and used as the cell wall enzyme.

In studying the enzyme distribution along the main axis, roots were sectioned with specially constructed metal rig, similar to that described by Hellebust and Forward (1962) and the invertases extracted as described above.

The soluble and cell wall fractions obtained were tested for invertase, α-glucosidase and sucrose phosphorylase activity. Enzyme preparations from step 1 were used in the studies in Sections III, IV, V, and VI.

Step 2 -- Solid ammonium sulphate was added slowly to the crude soluble extract to bring it to 20% saturation. The suspension was allowed to stand for 2 hours after which the precipitated protein was

removed by centrifugation for 10 minutes at 12,000 x *g* and discarded. The supernatant solution was then brought to 80% saturation with ammonium sulphate and again allowed to stand for 2 hours. After centrifugation for 10 minutes, at 12,000 x *g*, the precipitated protein was retained and the supernatant fluid discarded. The precipitated protein was dissolved in 0.1 M sodium phosphate-citric acid buffer, pH 7.0, and the ammonium sulphate removed by dialysis against the same buffer overnight.

Step 3 -- Sephadex G-100 was allowed to swell for 4 days in cold 0.05 M sodium phosphate-citric acid buffer, pH 7.0, with occasional stirring. The fines on the top were removed by suction and the gel packed in a column (2.5 x 50 cm) as described in Pharmacia bulletin. The column was then equilibrated with 0.05 M sodium phosphate-citric acid buffer, pH 7.0.

An aliquot (0.5 ml) of the enzyme preparation from Step 2, containing approximately 50 mg of protein, was applied with a micropipette to the top of the column by layering under a small volume of buffer already present. The column was eluted with 0.05 M sodium phosphate-citric acid buffer, pH 7.0. Elution rate was adjusted to give a flow rate of 20 ml per hour. The effluent was collected in fractions of 2.5 ml with a fraction collector (Instrumentation Specialities Co., Inc., Model 326). The protein content in each tube was determined by spectrophotometric method (Warburg and Christian, 1941). The invertase, α-glucosidase and sucrose phosphorylase activities in each fraction were assayed. The fractions containing each enzyme were then pooled and the protein levels of the pooled fractions determined by Folin phenol method (Lowry *et al.*, 1951).

All the above operations were carried out at 4°C.

2. Sucrose synthetase and sucrose phosphate synthetase

Step 1 -- Seven-day-old roots were incubated in distilled water for 2 hours and then washed three times with distilled water. The roots were homogenized with 0.05 M Tris-HCl buffer, pH 7.3, containing 0.01 M cysteine, in a glass homogenizer. The homogenate was centrifuged for 10 minutes at 12,000 x *g* and the supernatant used as the crude preparation after dialysis overnight against four litres of 0.05 M Tris-HCl buffer containing 0.001 M cysteine, pH 7.3.

Step 2 -- The dialyzed extract from Step 1 was brought to 60% saturation with ammonium sulphate, stirred slowly for 2 hours and the precipitate removed by centrifugation at 12,000 x *g* for 10 minutes. The pellet was redissolved in a volume of 0.05 M Tris-HCl buffer containing 0.001 M cysteine equal to half of the volume of the initial extract. The resuspended pellet was dialyzed against 2 changes of the buffer used in the extraction and then tested for sucrose synthetase and sucrose phosphate synthetase activities.

Step 3 -- An equal volume of 2% protamine sulphate solution (adjusted to pH 7.3 with NaOH) was added slowly with constant stirring to the enzyme preparation from Step 2, and suspension centrifuged at 12,000 x *g* for 10 minutes. The sucrose phosphate synthetase was precipitated whereas sucrose synthetase remained in the supernatant.

Step 4 -- The supernatant for Step 3 was brought to 70% saturation with solid ammonium sulphate. The suspension was centrifuged at 12,000 x *g*, the precipitate redissolved in 0.05 M Tris-HCl buffer, pH 7.3, containing 0.01 M cysteine, and dialyzed against the same buffer overnight. A precipitate formed during dialysis; this was removed by centrifugation at 12,000 x *g* for 10 minutes and discarded.

The enzyme preparation obtained was the partially purified sucrose synthetase.

The precipitate obtained from Step 3 was redissolved in Tris-HCl buffer, 0.05 M, pH 7.3, containing 0.001 M cysteine and brought to 60% saturation with solid ammonium sulphate. The precipitate was collected by centrifugation at 12,000 x *g* for 10 minutes and redissolved in Tris-HCl buffer, 0.05 M, pH 7.3, containing 0.001 M cysteine. The preparation was dialyzed with the same buffer overnight and the dialysate used as the partially purified sucrose phosphate synthetase.

All the above operations were carried out at 4°C.

B. Enzyme assay

1. Invertase

The invertase assay system contained 290 µmoles sucrose, 90 µmoles sodium phosphate-citric acid buffer, pH 4.8, and enzyme preparation (0.1 mg protein) in a final volume of 2 ml. The reaction mixture was incubated at 28°C. At the end of the reaction an aliquot of the reaction mixture was rapidly transferred to 1 ml of copper reagent (see below). Preliminary study had shown that this reagent effectively stopped the reaction. Controls containing no substrate or enzyme were included in every experiment and the results were corrected against control values.

Measurement of reducing sugars -- Reducing sugars were measured by the method of Somogyi (1952). The reagents for the test were prepared as follows:

Copper reagent:

24 g anhydrous sodium bicarbonate, 10 g sodium potassium tartrate, 4 g $CuSO_4 \cdot 5H_2O$, 16 g sodium bicarbonate and 180 g anhydrous sodium

sulphate were dissolved and made up to 1 litre with glass distilled water. The solution was stored at 25°C to prevent crystallization. A small degree of self reduction occurred during the first three days after storage. The reagent was filtered before use during this period.

Arsenomolybdate reagent:

To 25 g of ammonium molybdate in 450 ml water was added 2 ml concentrated sulphuric acid and 3 g sodium arsenate dissolved in 25 ml water. The solution was made up to 500 ml, incubated at 37°C for 24 hours and stored in a brown bottle.

In the determination of reducing sugars 1 ml of sample was delivered into a test tube containing 1 ml of copper reagent. The mixture was heated in a boiling water bath for 15 minutes, cooled and 1 ml of arsenomolybdate reagent added. After thorough mixing the solution was made to 10 ml with distilled water and the intensity of colour read on a 'Spectronic 20' colorimeter at 525 nm. A blank in which distilled water instead of reaction mixture was used was set up each time. The quantity of reducing sugars was determined by reference to a standard curve prepared under the test conditions with standard glucose solutions. Three replicates of each estimation were carried out each time.

2. α-Glucosidase

The assay system for α-glucosidase contained 290 µmoles maltose or 290 µmoles trehalose, 90 µmoles sodium phosphate-citric acid buffer, pH 4.8, and enzyme preparation (0.1 mg protein) in a final volume of 2 ml. The reaction mixture was incubated at 28°C. At the end of the reaction, an aliquot of the reaction mixture was rapidly transferred to 1 ml of Somogyi's copper reagent. The reducing sugars formed were

determined by the method of Somogyi. No-enzyme and no-substrate controls were set up in each experiment. Maltose and trehalose were much less susceptible to chemical hydrolysis than sucrose; however, results were corrected for controls as before.

3. Sucrose phosphorylase

The assay system for sucrose phosphorylase contained 290 µmoles sucrose, 90 µmoles sodium phosphate-citric acid buffer, pH 6, or 90 µmoles phosphate buffer, pH 6, and enzyme preparation (0.1 mg protein) in a total volume of 2 ml. The reaction mixture was incubated at 28°C. The reaction was stopped by Somogyi's copper solution and the reducing sugars determined as before. Controls in which citric acid-sodium citrate buffer, pH 6.0, was used instead of buffers containing phosphates were set up. Phosphorylase activity was determined as the difference in reducing sugar produced in the presence and absence of phosphate.

4. Sucrose synthetase

Three assay systems were used to test the sucrose synthetase activity.

Method 1 -- This was the method of Grimes *et al.* (1970). In this system the reaction mixture contained 25 µl 0.02 M UDPglucose, 25 µl Tris-phosphate buffer, 0.01 M, containing 0.001 M EDTA, pH 7.3, 25 µl 1 M sucrose solution containing 0.8 µCi ^{14}C-fructose (specific activity 65.8 mCi/mM) and enzyme preparation (0.05 mg protein). The purpose of adding unlabelled sucrose was to reduce invertase hydrolysis of the ^{14}C-sucrose synthesized in the reaction. The reaction mixture was incubated at 37°C.

The ^{14}C-sucrose formed was separated by microzone electrophoresis. At the end of the enzyme reaction sample was applied with a sample applicator (Beckman) to the centre of a strip of electrophoresis membrane (5.7 x 14 cm, Beckman). Markers of sucrose, glucose, and fructose were applied to the margins of the membranes. Electrophoresis runs of half an hour were carried out in sodium tetraborate, 0.05 M, pH 9.6, using a Shandon Universal Electrophoresis Apparatus (Model No. 2549). A constant current of 2.5 mA per strip of membrane was used. After the run the outside lanes bearing the markers were cut and stained by first dipping into a mixture of 1 volume of a saturated aqueous solution of silver nitrate in 200 volumes ethanol and then into 0.5% NaOH in ethanol. The sugars developed as brown spots. The location of the sample lane corresponding to the sucrose spot on reference lanes was cut out and placed in a scintillation vial with 15 ml of fluor containing 0.5% 2,5-diphenyloxazole and 0.03% 1,4-*bis* [2-(5-phenyloxazolyl)] benzene in toluene (w/v). Radioactivity was determined in a Nuclear Chicago liquid scintillation counter (Model Unilux II).

Method 2 -- The reaction system contained 0.5 µmole of UDPglucose, 0.5 µmole of fructose, 5 µmoles of Tris-HCl buffer, pH 7.3, and enzyme preparation (0.05 mg protein) in a final volume of 0.1 ml (Lavintman and Cardini, 1968). The reaction mixture was incubated at 37°C. The reaction was stopped by adding NaOH to a final concentration of 0.25 M and heating at 100°C in a water bath for 15 minutes to destroy unreacted fructose (Cardini *et al.*, 1955). The sucrose formed was estimated by the thiobarbituric acid method (Pencheron, 1962). The thiobarbituric acid system contained 1 ml sample, 1 ml concentrated

HCl, and 1 ml thiobarbituric acid, 0.02 M. The mixture was heated in a boiling water bath for 6 minutes. The mixture was then made up to 10 ml with glass distilled water and read on a colorimeter (Spectronic 20) at 432.5 nm. Controls in which no substrates or no enzymes were set up in each experiment. The quantity of sucrose was referred to a standard curve prepared under the test conditions with sucrose solutions.

Method 3 -- The third method was to determine sucrose cleavage by sucrose synthetase (Pressey, 1969). The reaction mixture contained 250 µmoles sucrose, 10 µmoles UDP, 10 µmoles NaF, 50 µmoles Tris-phosphate buffer, pH 7.3, and enzyme preparation (0.1 mg protein) in a total volume of 1 ml. Controls without UDP or without enzyme were run at the same time. The reaction mixture was incubated at 37°C and the reaction terminated by transferring the sample to copper reagent (Somogyi, 1952). The fructose liberated was determined by Somogyi's method (Somogyi, 1952). The results were corrected for controls.

5. Sucrose phosphate synthetase

Two methods were used to assay sucrose phosphate synthetase activity. These two methods were the same as Method 1 and 2 used for the assay of sucrose synthetase with the exception that fructose-6-phosphate was used instead of fructose.

C. Measurement of growth

Growth was measured by the increase in the fresh weight or the increase in the length of the main axis. Fresh weight was determined after removal of surface water by repeated blotting.

D. Sucrose absorption experiments

In the absorption experiments, 25 7-day-old roots were incubated for four or six hours either in sucrose medium with 10 µCi fructosyl-^{14}C-sucrose added or in glucose or fructose medium with 25 µCi sucrose-U-^{14}C (specificity activity 32 mCi/mmole) added. After the incubation the roots were washed three times with the same medium but without the radioactive sugars. Each wash lasted five minutes. The roots were then extracted with 50 ml boiling 80% ethanol for 20 minutes. The volume of the extract was reduced to 2 ml in a stream of air. Sugars in the extract were separated with descending paper chromatography using Whatman No. 1 paper. Sucrose, glucose, and fructose markers were applied to the margins of the chromatograms. The developing solvent was pyridine:ethanol:water, 8:2:1 (v:v:v) and the chromatogram was developed for 24 hours. The lanes bearing the markers were cut and the sugar spots detected using the following reagents (Trevelyan *et al.*, 1950):

(a) 1 volume of a saturated water solution of silver nitrate in 200 volumes acetone.

(b) 0.5% NaOH in ethanol.

Strips were dipped in reagent (a) briefly, dried, and then dipped in reagent (b). When the strips were dried, the sucrose locations showed up as light brown and the glucose and fructose as deep brown spots. The locations of the sample strips corresponding to the sugar spots on the reference strips were cut out and placed in scintillation vials with 15 ml of scintillation fluor which consisted of 0.5% 2,5-diphenyloxazole and 0.03% 1,4-*bis*[2-(5-phenyloxazolyl)] benzene in toluene (w/v). Radioactivity was determined with a liquid

scintillation counter (Nuclear Chicago Corp., Model Unilux II).

Labelling patterns of sucrose were determined by hydrolyzing the sucrose with yeast invertase (Sigma). The system consisted of 0.1 ml sample, yeast enzyme (0.1 mg protein), and 0.2 ml 0.1 M sodium phosphate-citric acid buffer, pH 5. The mixture was incubated at 37°C for 4 hours. The products were then separated by paper chromatography and the radioactivity of glucose and fructose determined as before.

E. Cell counts

Cell counts were made by the chromic acid maceration technique of Brown and Rickless (1949). Sections of roots were suspended in 5% chromic acid for 24 hours and the final suspension repeatedly forced as a fine jet through a Pasteur pipette. Cells were counted with a haemacytometer.

F. Protein determination

Protein determinations were made by the Folin phenol reagent method of Lowry *et al.* (1951). Crystalline bovine albumin was used as a standard. This method was used in all determinations except in the experiment described in Figure 22 (see page 25) a spectrophotometric method (Warburg and Christian, 1941) was used for convenience.

Experiments I, II B, VI B, VI C, VI D, VI E were repeated once and all the other experiments were repeated at least twice. Only results with good reproducibility were reported and in each experiment only one set of results was presented.

RESULTS

SECTION I

Absorption of sucrose by excised tomato roots

Excised tomato roots were fed with fructosyl-^{14}C-sucrose for 4 and 6 hours. If sucrose was absorbed as such then the asymetrically labelled sucrose should remain unaltered. On the other hand, should hydrolysis be a prerequisite of absorption, then randomization of labelling would result. The results of this experiment are shown in Table 2. Approximately 30% of the radioactivity was found in glucose and fructose, indicating that some fructosyl-^{14}C-sucrose had been hydrolyzed. Further, the appearance of radioactivity in glucose showed that there was conversion of ^{14}C-fructose to ^{14}C-glucose. The bulk of the radioactivity, however, was recovered in sucrose and when this sucrose was hydrolyzed it was found that more than 90% of the radioactivity was retained in the fructosyl moiety. The remarkably small alteration of the labelling pattern of sucrose strongly indicated that sucrose was taken up by excised tomato roots without prior hydrolysis. This was tested further in the following way. Roots were incubated in 0.5% glucose medium or 0.5% fructose medium, both containing 25 µCi ^{14}C-U-sucrose (specificity 32 mCi/mmole). If inversion was essential to sucrose absorption, the sucrose in the roots must be the result of resynthesis. The presence of a large quantity of non-radioactive glucose or fructose in the incubation medium should, therefore, alter the labelling of the resynthesized sucrose pattern. Table 3 shows that this was not the case. The labelling pattern in sucrose

TABLE 2

Distribution of Radioactivity in Excised Tomato Roots Fed with Fructosyl-^{14}C-Sucrose

Time (hr)	^{14}C-glucose (cpm)	^{14}C-fructose (cpm)	^{14}C-sucrose (cpm)	G/F ratio of sucrose*
4	2,080	3,320	19,070	0.04
6	4,210	3,400	14,580	0.05

Roots were incubated for 4 or 6 hours in medium containing fructosyl-^{14}C-sucrose, washed 3 times with non-radioactive medium and extracted with boiling 80% ethanol. Sugars were separated by chromatography. Radioactivity was determined by scintillation counting either directly, or after treatment with invertase.

* G/F ratio of sucrose: ratio of ^{14}C-glucose to ^{14}C-fructose in extracted ^{14}C-sucrose.

again strongly suggests that the recovered sucrose arose from direct absorption rather than from hydrolysis and resynthesis. Table 3 shows several other interesting phenomena, *e.g.*, the fructose-sucrose mixture allowed virtually twice the sucrose absorption than the glucose-sucrose mixture. These interesting observations were, however, taken no further. Thus, the feeding experiments show convincingly that excised tomato roots absorb sucrose without prior hydrolysis.

TABLE 3

The Effect of Non-radioactive Hexose on the Distribution of Radioactivity in Sugars in Excised Tomato Roots Fed with ^{14}C-U-Sucrose

Sugars in the medium	^{14}C-Sugars in the Extract (cpm)			G/F Ratio of sucrose*
	Glucose	Fructose	Sucrose	
^{14}C-U-sucrose + glucose	1,560	6,400	28,242	0.92
^{14}C-U-sucrose + fructose	8,582	922	63,720	1.12

The roots were incubated for 4 hours in 0.5% glucose or 0.5% fructose medium containing 25 µCi ^{14}C-U-sucrose, washed with non-radioactive medium three times and extracted with boiling 80% ethanol. Sugars were separated by chromatography. Radioactivity was determined by scintillation counting either directly, or after treatment with invertase.

* G/F ratio of sucrose: ratio of ^{14}C-glucose to ^{14}C-fructose in extracted ^{14}C-sucrose.

SECTION II

The effect of sugar on the occurrence of sucrose synthetase and sucrose phosphate synthetase in excised tomato roots

A. Sucrose synthetase and sucrose phosphate synthetase activity in sucrose-grown roots

Sucrose synthetase and sucrose phosphate synthetase were extracted from 7-day-old roots as described earlier. The crude extract and the partially purified fractions were tested using three assay systems for sucrose synthetase and two assay systems for sucrose phosphate synthetase, as described in the Methods section. Preliminary studies showed that the extraction and assay methods were effective in this laboratory with wheat germ sucrose synthetase and sucrose phosphate synthetase. No sucrose synthetase in excised tomato roots or sucrose phosphate synthetase activity could be found despite repeated attempts.

B. Sucrose synthetase and sucrose phosphate synthetase activity in glucose-grown roots

With glucose-grown roots both sucrose synthetase (Table 4) and sucrose phosphate synthetase (Table 5) activities were detected. The demonstration of the presence of the two sucrose synthesizing enzymes suggests that the poor growth rate of roots in glucose and fructose is not due to the roots lacking the ability to synthesize sucrose.

To study these two enzymes more closely, they were partially purified with ammonium sulphate and protamine sulphate fractionations.

The purification of sucrose synthetase (Table 4) resulted in approximately a 66-fold increase of specific activity. The total enzyme units after the first ammonium sulphate fractionation exceeded that of the crude extract. This was possibly due to the removal of inhibitory substance(s). This preparation was not completely free of sucrose phosphate synthetase activity but the sucrose phosphate synthetase activity was only approximately 10% the rate of sucrose synthetase.

Table 5 shows the data for the purification of sucrose phosphate synthetase. An approximately 82-fold increase in specific activity was achieved. Both the first ammonium sulphate and the protamine treatment increased the number of enzyme units suggesting that inhibitory substances were again present in the crude extract. This preparation was free of sucrose synthetase activity and used only fructose-6-phosphate as substrate.

C. Effect of fructose concentration on sucrose synthetase activity

The effect of fructose concentration on sucrose synthetase activity was studied with assay method 2. A classical hyperbolic curve (Figure 1) was obtained with fructose concentrations from 0.5 mM to 10 mM. The Lineweaver-Burk plot (Lineweaver and Burk, 1934) shows a straight line (Figure 1, inset). The K_m for fructose was calculated from the plot as 3.8 mM. The K_m was slightly higher than that of wheat germ (Cardini *et al.*, 1955), Mung bean (Grimes *et al.*, 1970) and artichoke tubers (Avigad, 1964). The value of V_{max} was estimated to be 3.8 µmoles/mg protein/30 min.

TABLE 4

Purification of Sucrose Synthetase

Fraction	Total enzyme Units*	Total protein (mg)	Specific activity**	Purification (times)
Crude extract	7.05	168	0.042	
Ammonium sulphate (0 - 60%)	23.13	48.2	0.48	11
Protamine sulphate (1%) + ammonium sulphate (0 - 70%)	15.68	5.6	3.8	66

Enzyme activity was determined by assay method 2.

*One enzyme unit is defined as the amount of enzyme which catalyzes the formation of 1 μmole of sucrose in 30 minutes.

**Specific activity is expressed in units/mg protein.

TABLE 5

Purification of Sucrose Phosphate Synthetase

Fraction	Total enzyme Units*	Total protein (mg)	Specific activity**	Purification (times)
Crude extract	15.12	168	0.09	
Ammonium sulphate (0 - 60%)	34.2	48.2	0.72	8
Protamine sulphate (1%)	61.74	12.6	4.9	54
Ammonium sulphate (0 - 60%)	35.52	4.8	7.4	82

Enzyme activity was determined by assay method 2.

*One enzyme unit is defined as the amount of enzyme which catalyzes the formation of 1 µmole of sucrose-6-phosphate in 30 minutes.

**Specific activity is expressed in units/mg protein.

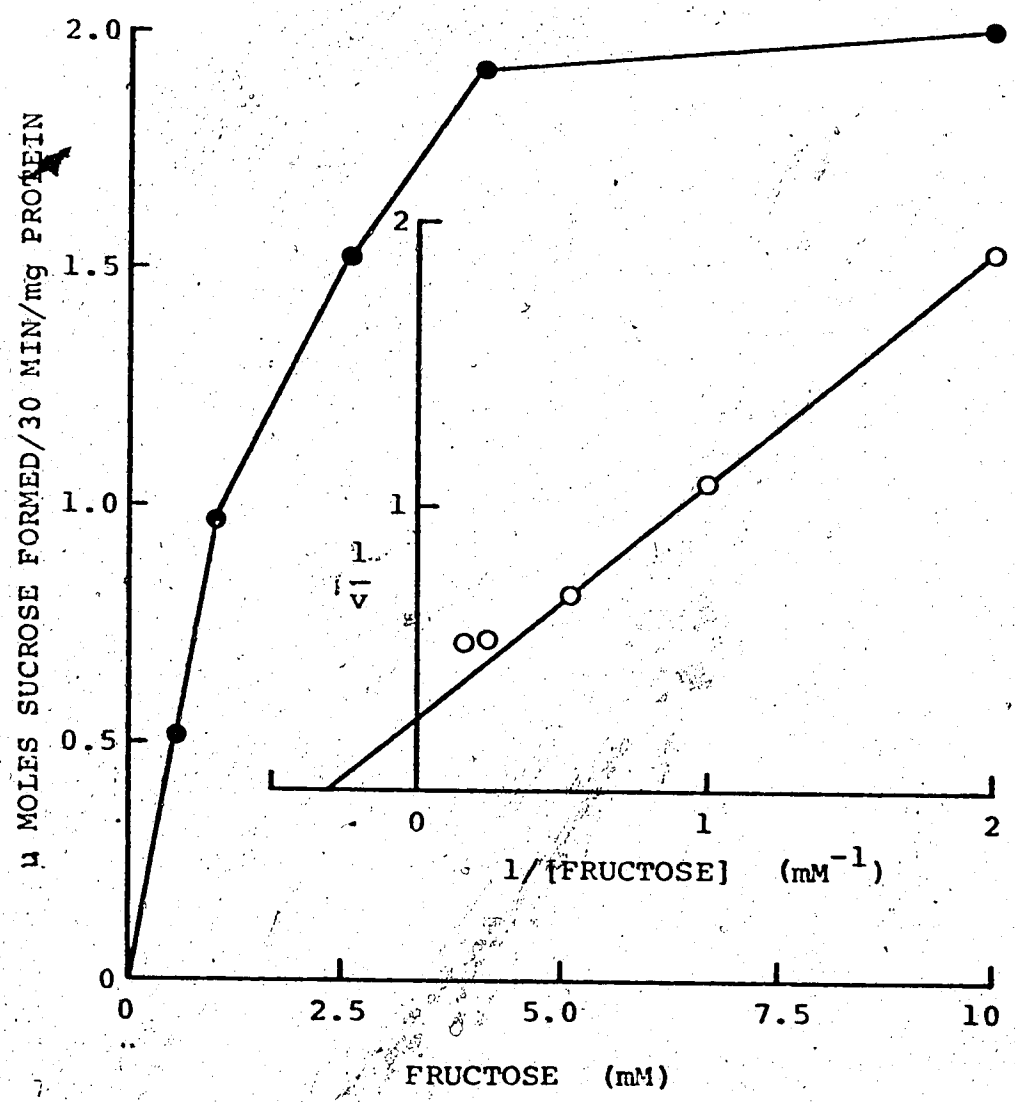
FIGURE 1

The effect of fructose concentration on sucrose synthetase activity

The reaction mixtures contained 0.5 µmole of UDPG, 5 µmoles of Tris-HCl buffer, pH 7.3, 0.05 mg partially purified sucrose synthetase, and varying amounts of fructose in a final volume of 0.1 ml.

The reaction mixtures were incubated at 37°C for 30 minutes. The sucrose formed was estimated by the thiobarbituric acid method (Percheron, 1962).

Inset: Lineweaver-Burk plot of 1/[fructose] versus 1/v.


2.0
1.5
1.0
0.5
0
μ MOLES SUCROSE FORMED/30 MIN/mg PROTEIN
0
2.5
5.0
7.5
10
FRUCTOSE (mM)
2
1
0
1
2
1/v
1/[FRUCTOSE] (mM^{-1})

D. Effect of fructose-6-phosphate concentration on sucrose phosphate synthetase activity

Assay method 2 was used to study the effect of fructose-6-phosphate concentration on sucrose phosphate synthetase activity. A hyperbolic curve was obtained with fructose-6-phosphate concentrations from 2 to 7.5 mM. The Lineweaver-Burk plot gives a straight line (Figure 2, inset). The K_m value was determined from the plot as 4 mM. This value was slightly higher than those of wheat germ (Leloir and Cardini, 1955). The V_{max} was calculated as 10 µmoles sucrose-6-phosphate formed/mg protein/30 min.

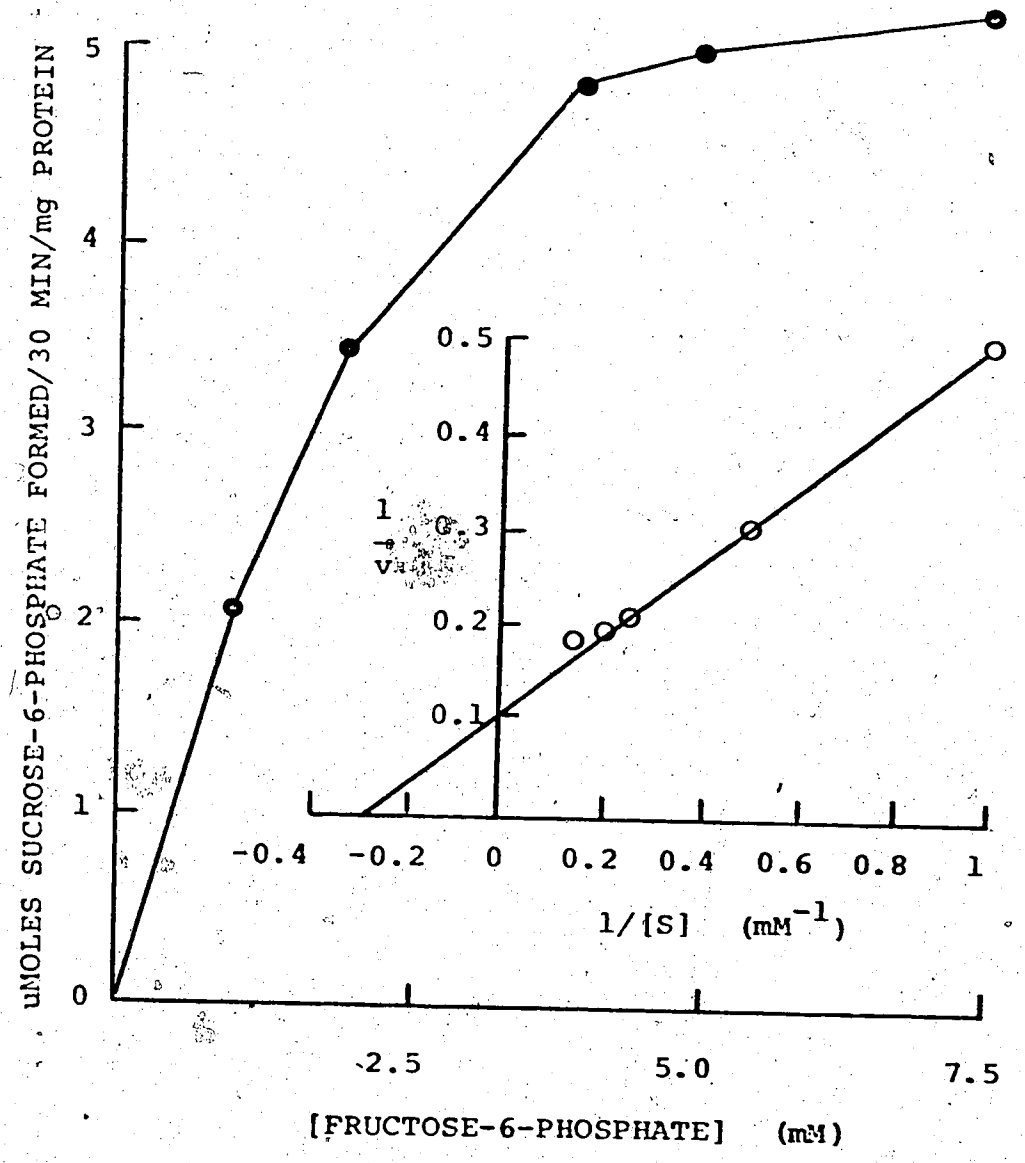
FIGURE 2

The effect of fructose-6-phosphate concentration on on sucrose phosphate synthetase activity

The reaction mixtures contained 0.5 μmole of UDPG, 5 μmoles of Tris-HCl buffer, pH 7.3, 0.05 mg partially purified sucrose phosphate synthetase, and varying amounts of fructose-6-phosphate in a final volume of 0.1 ml.

The reaction mixtures were incubated at 37°C for 30 minutes. The sucrose-6-phosphate formed was estimated by the thiobarbituric acid method (Percheron, 1962).

Inset: Lineweaver-Burk plot of 1/[fructose-6-phosphate] versus 1/v.


uMOLES SUCROSE-6-PHOSPHATE FORMED/30 MIN/mg PROTEIN
0
1
2
3
4
5
2.5
5.0
7.5
[FRUCTOSE-6-PHOSPHATE] (mM)
0.1
0.2
0.3
0.4
0.5
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1
1/[S] (mM^{-1})

SECTION III

Studies on the enzymes involved in sucrose utilization

A. Soluble and cell wall invertase

The most commonly known enzyme to attack sucrose is invertase, and activity was found in both the soluble and cell wall fractions. Excised tomato roots, therefore, are like many other plant tissues in possessing both soluble and cell wall invertases (Glasziou *et al.*, 1963; Straus, 1962; Copping and Street, 1972). To find out whether the presence of invertase activity in both soluble and wall fractions was the result of their incomplete separation, the centrifugation was carried out with varying forces. The results (Table 6) show that the distribution was not affected by the centrifugation force indicating that the soluble invertase activity was not due to cell wall contamination. The presence of cell wall contaminants of the soluble fraction was studied by the microscopic examination of this fraction stained with periodic acid-Schiffs reagent (Hotchkiss, 1948) which stains polysaccharides red. No cell wall material was detected in the soluble fraction.

1. Effect of pH on soluble and cell wall invertase activity

The effect of pH on soluble and cell wall invertase activity was studied with sodium phosphate-citrate buffer (Figures 3a and 3b). The effect of pH was very similar and both invertases showed a pH optimum of 4.8. No distinguishable peak of activity at neutral or alkaline pH was observed indicating that both the fractions contained mainly acid invertase. However, both soluble and cell wall invertases appeared to

TABLE 6

The Effect of Increasing Centrifugation Force on the Distribution of Invertase Between the Soluble and Cell Wall Fractions

Centrifugation Force x *g*	Invertase Activity mg hexose/mg protein/2 hrs	
	Soluble	Cell Wall
270	3.65	1.24
1,085	3.51	1.20
12,100	3.49	1.20
48,200	3.49	1.18

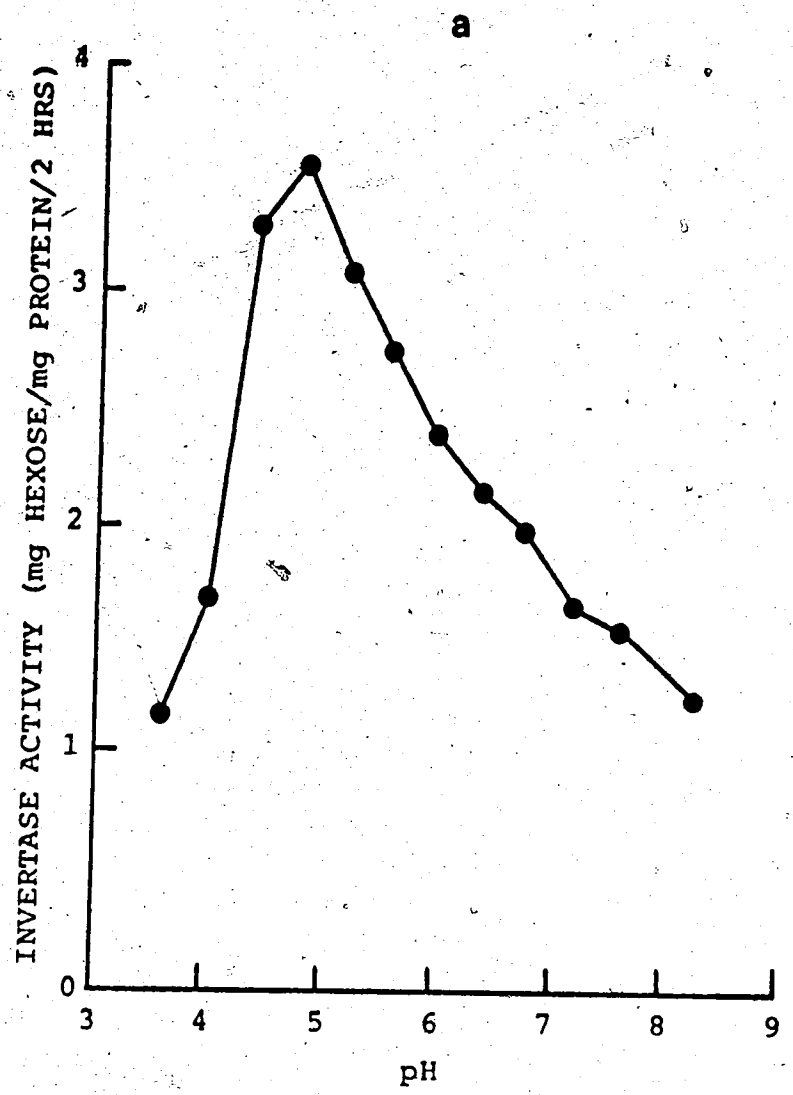
Seven-day-old roots were homogenized with 0.05 M sodium phosphate-citrate buffer, pH 7.0, and centrifuged under varying forces for 10 minutes. The precipitate was washed with the same buffer and recentrifuged. The process was repeated twice and the final pellet was resuspended in 0.05 M sodium phosphate-citrate buffer, pH 7.0.

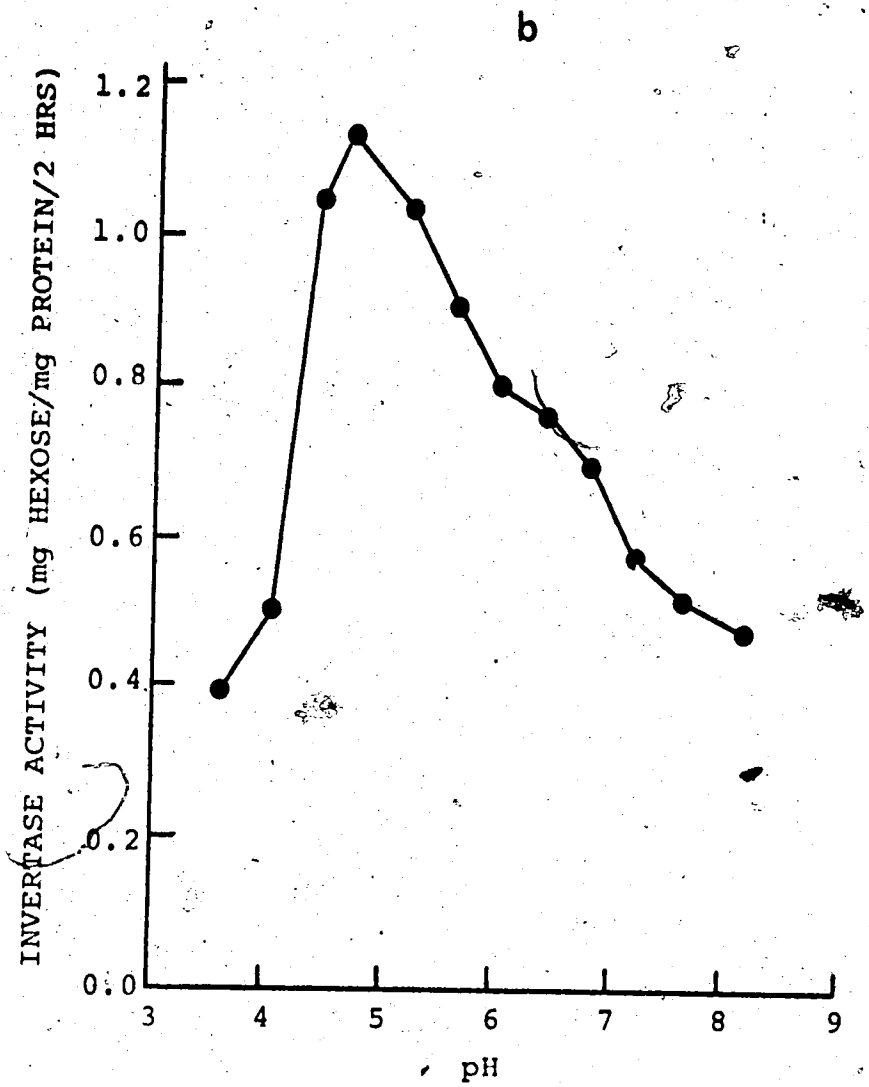
FIGURE 3

Effects of pH on (a) soluble and (b) cell wall invertase activities

The reaction mixtures contained 290 µmoles sucrose, 90 µmoles sodium phosphate-citric acid buffer, and enzyme preparation (0.1 mg protein) in a final volume of 2 ml.

Reaction mixtures were incubated at 28°C for 2 hours.


a
INVERTASE ACTIVITY (mg HEXOSE/mg PROTEIN/2 HRS)
0
1
2
3
4
3
4
5
6
7
8
9
pH


b
INVERTASE ACTIVITY (mg HEXOSE/mg PROTEIN/2 HRS)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
3
4
5
6
7
8
9
pH

have a shoulder in the region of pH 6.0 - 8.0 and this could be caused by a trace of neutral invertase. To determine if this was the case the soluble fraction was subjected to ammonium sulphate fractionation, a method which has been used successfully to separate the acid and neutral invertases of carrot root (Ricardo and Ap Rees, 1970) and cultured sycamore cells (Copping and Street, 1972). Figure 4 shows that no neutral invertase activity could be resolved, indicating that the shoulder on the soluble fraction curve was not caused by a neutral invertase. Since it has not been possible to release the wall enzyme from the wall (Section VII) it was not feasible to subject the cell wall fraction to this treatment.

2. Time course of sucrose hydrolysis

The time course of sucrose hydrolysis by soluble and cell wall preparations was followed for 4 hours. The rate of hydrolysis was found to be linear for 4 hours for both soluble and cell wall fractions (Figure 5). In the following experiments a 2 hour incubation time was used for convenience.

3. Effect of enzyme concentration

The soluble enzyme and cell wall enzyme with concentrations equivalent to from 0.05 to 0.25 mg protein showed a linear relationship with reaction velocity (Figure 6). The results thus show that reaction rates reflected the amounts of enzyme present. Schwimmer *et al.* (1961) using potato extract of varying concentrations showed that the apparent activity per unit weight of tuber increased with decreasing amounts of extract. Based on kinetic considerations they concluded that the deviation from linearity was due to the presence of

FIGURE 4

Separation of soluble fraction by ammonium sulphate precipitation

 : invertase activity assayed at pH 4.8.

 : invertase activity assayed at pH 7.0.

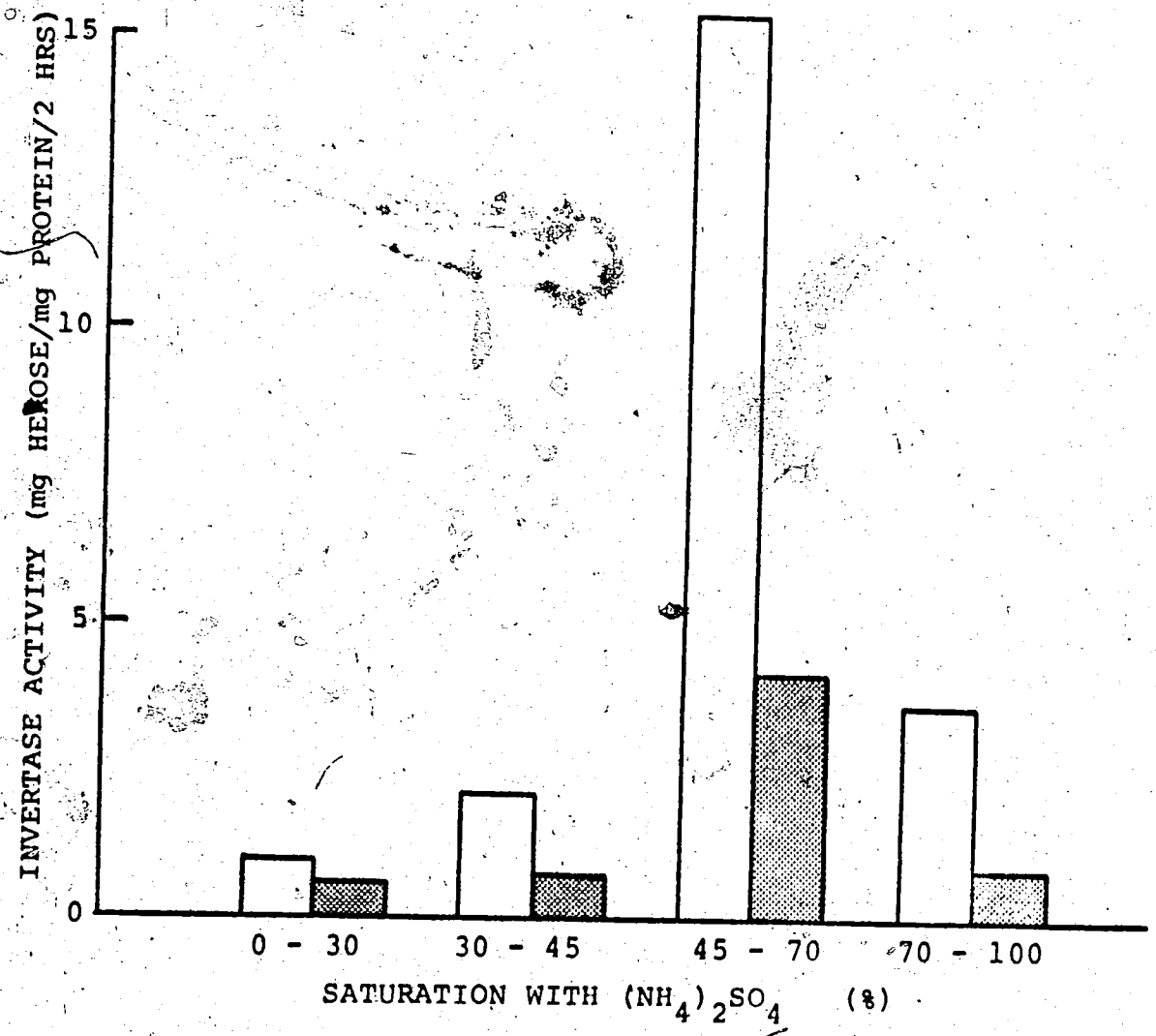

INVERTASE ACTIVITY (mg HEXOSE/mg PROTEIN/2 HRS)
15
10
5
0
0 - 30
30 - 45
45 - 70
70 - 100
SATURATION WITH $(NH_4)_2SO_4$ (%)

FIGURE 5

Time course of hydrolysis of sucrose by soluble and cell wall fractions

The reaction mixtures contained 290 µmoles sucrose, 90 µmoles sodium phosphate-citric acid buffer, pH 4.8, and enzyme preparation (0.1 mg protein) in a final volume of 2 ml.

Reaction mixtures were incubated at 28°C.

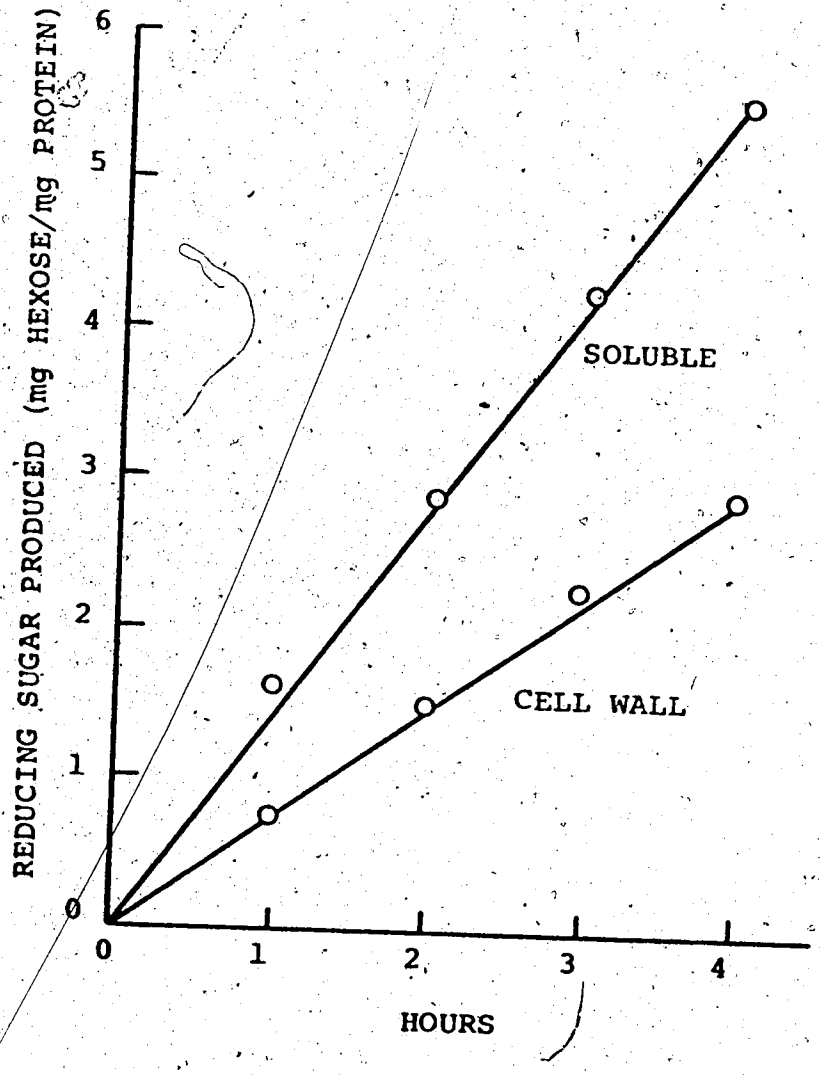
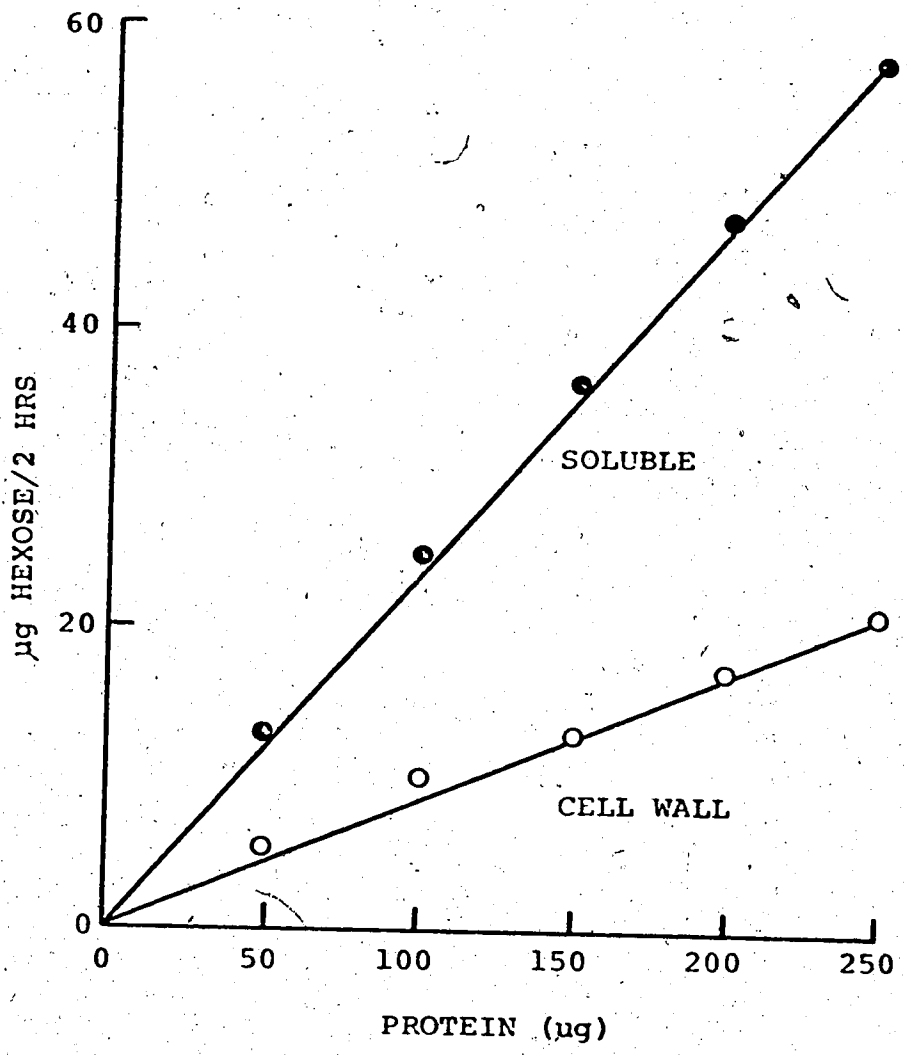

REDUCING SUGAR PRODUCED (mg HEXOSE/mg PROTEIN)
6
5
4
3
2
1
0
0
1
2
3
4
HOURS
SOLUBLE
CELL WALL

FIGURE 6

Invertase activity as a function of protein concentration

The reaction mixtures contained 290 µmoles sucrose, 90 µmoles sodium phosphate-citric acid buffer, pH 4.8, and varying amounts of invertase preparation in a final volume of 2 ml.

Reaction mixtures were incubated at 28°C for 2 hours.


60
40
20
0
µg HEXOSE/2 HRS
SOLUBLE
CELL WALL
0
50
100
150
200
250
PROTEIN (µg)

an endogenous inhibitor. The linear relationship between enzyme concentration and invertase activity obtained with both soluble and cell wall invertase shows that no similar invertase inhibitor exists in excised tomato roots.

4. Effect of vigorous blending on invertase activity

The presence of an invertase inhibitor in potato tuber stored at cold temperature was reported by Pressey (1966) who found that the inhibitor could be destroyed by vigorous blending. Tomato root extracts were blended at maximum speed with a VirTis '23' blender for a total of 30 minutes and with the operation interrupted every 5 minutes to cool the extract to 4°C. Aliquots were removed every 5 minutes and assayed for invertase activity. Invertase activity in soluble and cell wall extracts so treated are shown in Table 7. No consistent change in invertase activity was obtained. This suggests that no invertase inhibitor similar to that found in potato tuber was present in excised tomato roots.

5. Effect of temperature on invertase activity

The effects of temperature on the soluble and cell wall invertases were almost the same (Figure 7). The optimum temperature of both of these enzymes was 49°C. This experiment is evidence in favor of the theory that the cell wall and soluble enzymes are of the same protein species.

B. α-Glucosidase activity

In addition to invertase α-glucosidase hydrolyses sucrose to glucose and fructose, but differs from invertase by attacking sucrose

TABLE 7

The Effect of Vigorous Blending on Invertase Activity

Duration of Blending (minutes)	Invertase Activity mg hexose/mg protein/ 2 hrs	
	Soluble	Cell Wall
0	3.31	1.34
5	3.23	1.30
10	3.34	1.34
15	3.36	1.31
20	3.38	1.28
25	3.48	1.33
30	3.18	1.24

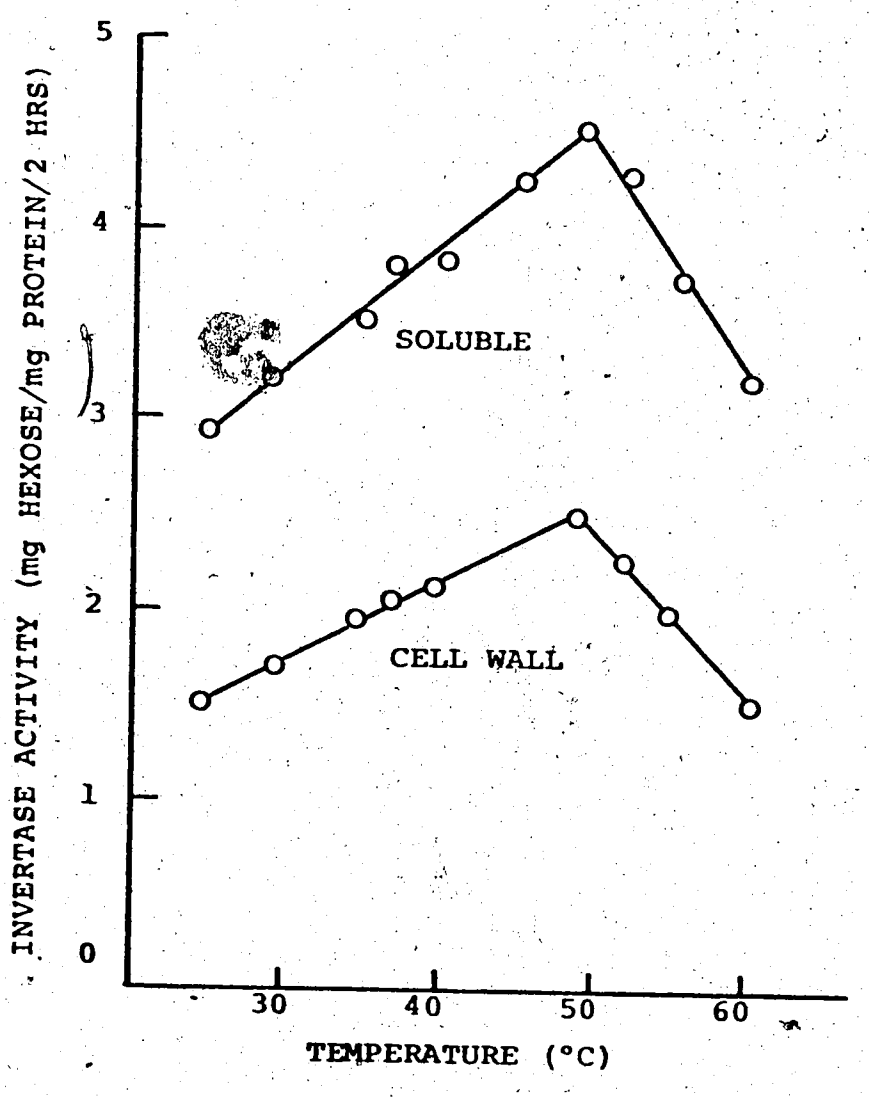
Crude extracts were blended with a VirTis '23' blender at maximum speed.

FIGURE 7

Effect of temperature on the activities of soluble and cell wall invertases

The reaction mixtures contained 290 µmoles sucrose, 90 µmoles sodium phosphate-citric acid buffer, pH 4.8, and enzyme preparation in a final volume of 2 ml.

Reaction mixtures were incubated for 2 hours.


INVERTASE ACTIVITY (mg HEXOSE/mg PROTEIN/2 HRS)
5
4
3
2
1
0
SOLUBLE
CELL WALL
30
40
50
60
TEMPERATURE (°C)

at the α-glucosyl end instead of the β-fructosyl end. In fact, it attacks not only sucrose but a number of sugars that possess a free α-glucosyl end, *e.g.* trehalose, maltose, α-methyl glucoside (Myrback, 1960). Maltose and trehalose do not possess a β-fructosyl moiety and are, therefore, not attacked by invertase. Therefore, invertase will not interfere with the assay of α-glucosidase, if these two substrates are used. The rate of hydrolysis of maltose, trehalose, and sucrose by soluble and cell wall extracts is shown in Table 8. Only the soluble fraction possessed α-glucosidase activity; no α-glucosidase activity was detected in the cell wall fraction. Later studies (Section VII) demonstrate that purified α-glucosidase attacked sucrose at only one third the rate it attacked maltose. Thus, only approximately 3 to 4% of the sucrose hydrolytic activity of the soluble extract was due to α-glucosidase activity. As the α-glucosidase in the soluble extract was low, its interference in the assay of invertase activity was considered minimal. Thus the soluble extract was used in subsequent studies without further purification.

C. Sucrose phosphorylase activity

To detect the presence of sucrose phosphorylase in excised tomato roots, 7-day-old roots were extracted with sodium citrate-citric acid buffer, 0.05 M, pH 7.0, and the fractions prepared as described in the 'Methods'. The preparation was dialyzed overnight against two changes of 4 litres of 0.05 M sodium citrate-citric acid buffer, pH 7.0. The extracts prepared in this way were tested for their ability to hydrolyze sucrose by using sodium phosphate-citric acid, or sodium citrate-citric acid buffers. The results (Table 9) show that soluble

TABLE 8

The Rate of Hydrolysis of Sucrose, Maltose, and Trehalose by Soluble and Cell Wall Fractions

Sugars	Activity µmoles hydrolyzed/mg protein/2 hrs	
	Soluble	Cell Wall
Sucrose	8.57	2.34
Maltose	0.95	0
Trehalose	0.88	0

Reaction mixtures consisted of 290 µmoles of substrate, 90 µmoles sodium phosphate-citric acid buffer, pH 4.8, and 0.1 ml enzyme preparation in a final volume of 2 ml.

Reaction mixtures were incubated at 28°C for 2 hours.

TABLE 9

The Effect of Phosphate on Hydrolysis of Sucrose by Soluble and Cell Wall Fractions

Buffers	Activity mg hexose/mg protein/2 hrs	
	Soluble	Cell Wall
Sodium phosphate-citric acid	2.98	0.97
Sodium citrate-citric acid	2.86	0.95

Soluble and cell wall extracts were prepared with sodium citrate-citric buffer, 0.05 M, pH 7.0. The extracts were dialyzed overnight against two changes of 0.05 M sodium citrate-citric acid buffer, pH 7.0.

Reaction mixture contained 90 µmoles buffer (pH 6), 290 µmoles sucrose and 0.1 mg enzyme preparation in a final volume of 1 ml. Mixture was incubated at 28°C for 2 hours.

and cell wall invertases hydrolyze sucrose at the same rate with or without the presence of phosphate. The results thus indicate that excised tomato roots, like other plant tissues (Hassid and Doudoroff, 1950; Gibbs, 1959; Hatch *et al.*, 1963), do not possess sucrose phosphorylase activity.

SECTION IV

The Relationship Between Invertase Activity and Growth of Excised Tomato Roots

A. Distribution of soluble and cell wall invertase along the root axis

The distribution of soluble and cell wall invertase activity and cell number in successive 1.5 mm segments of the main axis of the roots is shown in Figure 8. The cell number was high in the first segment and dropped markedly in the second segment indicating that the main elongation region was between 0.75 and 2.25 mm from the tip. From the second to the fourth segment the cell number fell slowly and from the fourth segment onwards the cell number remained almost constant, showing that the cells enlarged slowly in the region 2.25 to 5.25 mm from the tip and from here onwards no further enlargement occurred. When expressed on a per segment basis both soluble and cell wall activities were highest in the actively growing zone. Both soluble and cell wall invertases declined as the enlargement process slowed down, however, the latter declined with a faster rate than the former. In the region where there was no apparent cell enlargement, while cell wall activity became very low, the soluble invertase activity remained high. When the invertase activity was expressed on a unit protein basis (Figure 9) the distribution patterns of the soluble and cell wall enzyme were shown to be different. The soluble invertase activity rose rapidly in the growing region but remained relatively uniform thereafter. The cell wall invertase activity also rose rapidly in the growing region but unlike the soluble invertase it fell rapidly in the region where growth slowed down. Thus, although both activities were

FIGURE 8

Distribution of number of cells and invertase activity along root axis, expressed as per 1.5 mm segment

Roots were sectioned into 1.5 mm segments with specially constructed metal rig, similar to that described by Hellebust and Forward (1962). Invertases in these segments were extracted and activities determined.

The reaction mixtures contained 290 µmoles sucrose, 90 µmoles sodium phosphate-citric acid buffer, pH 4.8, and enzyme preparation in a final volume of 2 ml.

Reaction mixtures were incubated at 28°C for 2 hours.

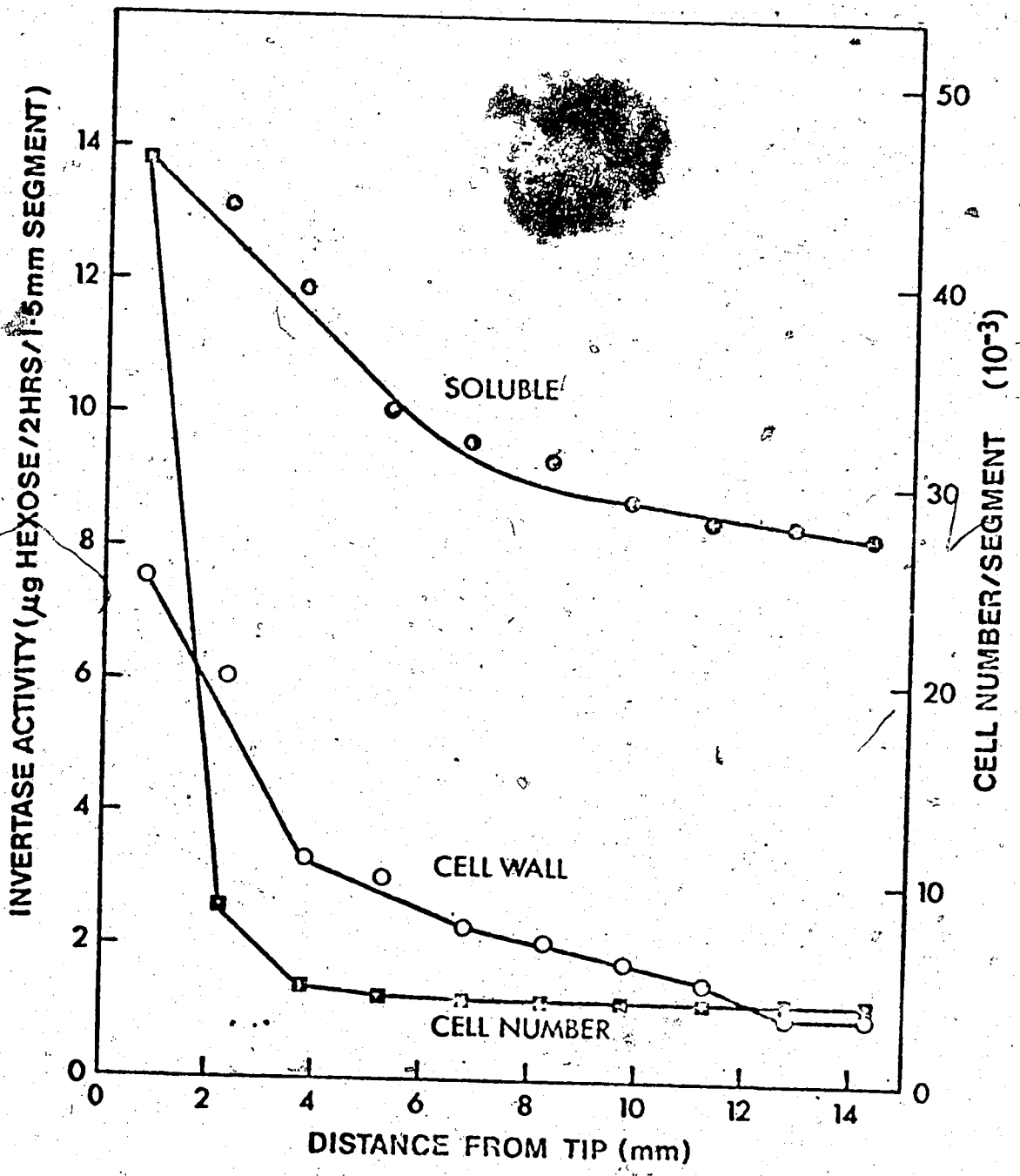

INVERTASE ACTIVITY (µg HEXOSE/2HRS/1·5mm SEGMENT)
CELL NUMBER/SEGMENT (10⁻³)
DISTANCE FROM TIP (mm)
SOLUBLE
CELL WALL
CELL NUMBER
0
2
4
6
8
10
12
14
0
10
20
30
40
50

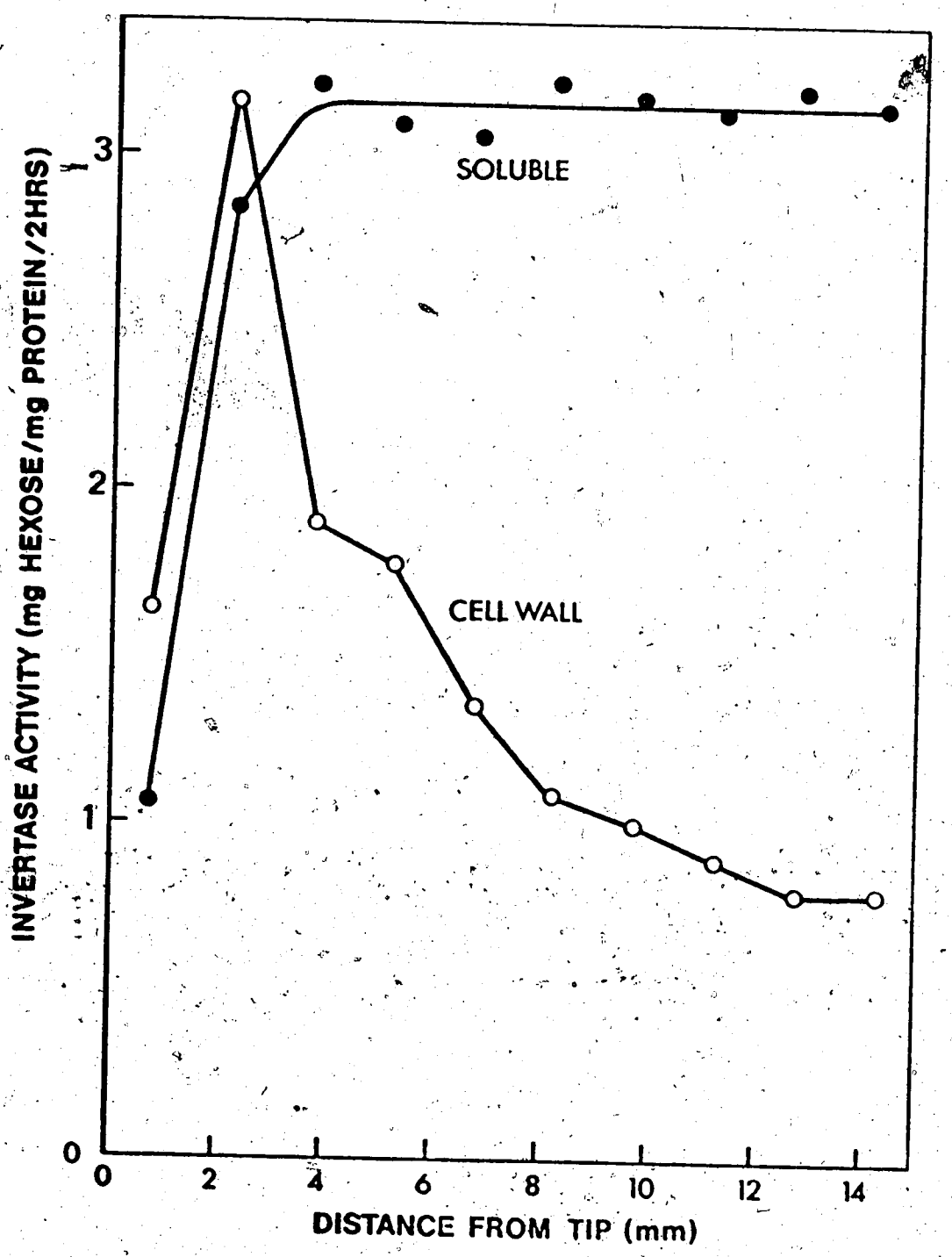
FIGURE 9

Distribution of soluble and cell wall invertases along root axis, expressed as per unit protein

Roots were sectioned into 1.5 mm segments with specially constructed metal rig, similar to that described by Hellebust and Forward (1962). Invertases in these segments were extracted and activities determined.

The reaction mixtures contained 290 µmoles sucrose, 90 µmoles sodium phosphate-citric acid buffer, pH 4.8, and enzyme preparation in a final volume of 2 ml.

Reaction mixtures were incubated at 28°C for 2 hours.


INVERTASE ACTIVITY (mg HEXOSE/mg PROTEIN/2HRS)
0
1
2
3
SOLUBLE
CELL WALL
0
2
4
6
8
10
12
14
DISTANCE FROM TIP (mm)

high in the growth zone, only the cell wall invertase showed a peak of activity.

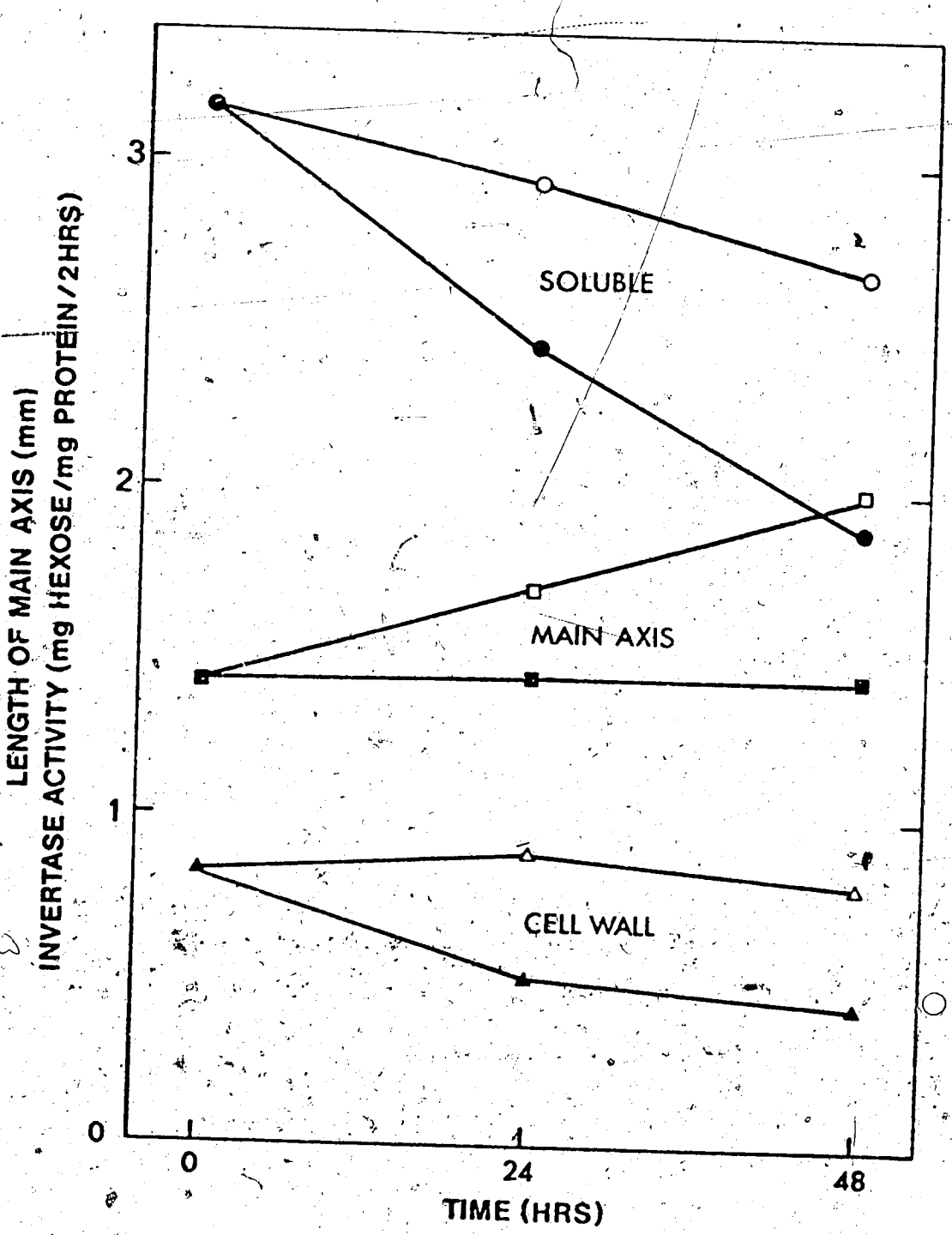
B. The effect of cycloheximide on invertase levels and growth

The rapid rate of decline of the specific activity of cell wall invertase along the main axis in Figure 9 was reduced from the third segment onwards, thus producing a tail to the peak. The sharpness of a peak depends upon the rate of synthesis and breakdown of the enzyme as well as on the rate of growth of the tissue. To study the rate of breakdown of invertase, the protein synthesis inhibitor, cycloheximide (Siegel and Sisler, 1964) was used. Roots were grown in standard medium for 5 days and then transferred to standard medium plus 2 mg/l cycloheximide. The results of this experiment (Figure 10) showed that growth of the roots was almost immediately stopped by cycloheximide. The half-lives of the soluble and cell wall enzymes were found to be approximately 48 hours. This is longer than the turnover rate of both sugar cane invertase, which has a half-life of only 2 hours (Glasziou *et al.*, 1967) and *Lentil* epicotyl, with a half-life of 14 hours (Seitz and Lang, 1968). Figure 10 shows that the growth rate of control 5-day-old excised tomato roots from the fifth to the seventh day was approximately 30 mm per day. This growth rate is 3 times as fast as the growth rate of the pea roots used by Sutcliffe and Sexton (1969). The slower rate of breakdown of the enzyme together with the faster growth rate of the tissue were probably the factors causing the tail of the peak.

FIGURE 10

Effect of cycloheximide (2 mg/l) on growth of the main axis and invertase activity during a 28-hour period

At zero time 5-day-old roots were transferred either to fresh medium (open figures) or fresh medium containing cycloheximide (closed figures).


LENGTH OF MAIN AXIS (mm)
INVERTASE ACTIVITY (mg HEXOSE/mg PROTEIN/2HRS)
3
2
1
0
SOLUBLE
MAIN AXIS
CELL WALL
0
24
48
TIME (HRS)

SECTION V

of Different Carbon Sources on Growth and Invertase Activity

A. The effect of glucose and fructose on activity of extracted tomato root invertases

Growth of excised tomato roots in sucrose medium is associated with the accumulation of glucose and fructose in the medium (Dormer and Street, 1949). To determine whether the accumulation of hexose and growth are related the levels of hexose in the medium and the growth of the roots were followed (Figure 11). Probably due to injury caused by excision the growth of excised tomato roots showed an initial lag phase during the first three days after transfer. From the third to the seventh day the roots grew rapidly, but after that the growth rate declined. In contrast to growth, reducing sugars in the medium increased after the transfer. By the seventh day the reducing sugars in the medium reached a concentration of approximately 0.05%. This concentration of reducing sugars might conceivably exert product inhibition of invertase activity and be responsible for the reduction in growth rate after the seventh day. Due to these results the effects of glucose and fructose on soluble and cell wall invertases were studied. Glucose and fructose up to a concentration of 0.05% did not inhibit soluble or cell wall invertase activity (Table 10) indicating that the decline of growth rate at the latter stage of the growth period was not due to inhibition of invertase by glucose or fructose.

FIGURE 11

The levels of reducing sugar appearing in the medium in relation to growth of excised tomato roots, (a) reducing sugars, (b) growth of roots.

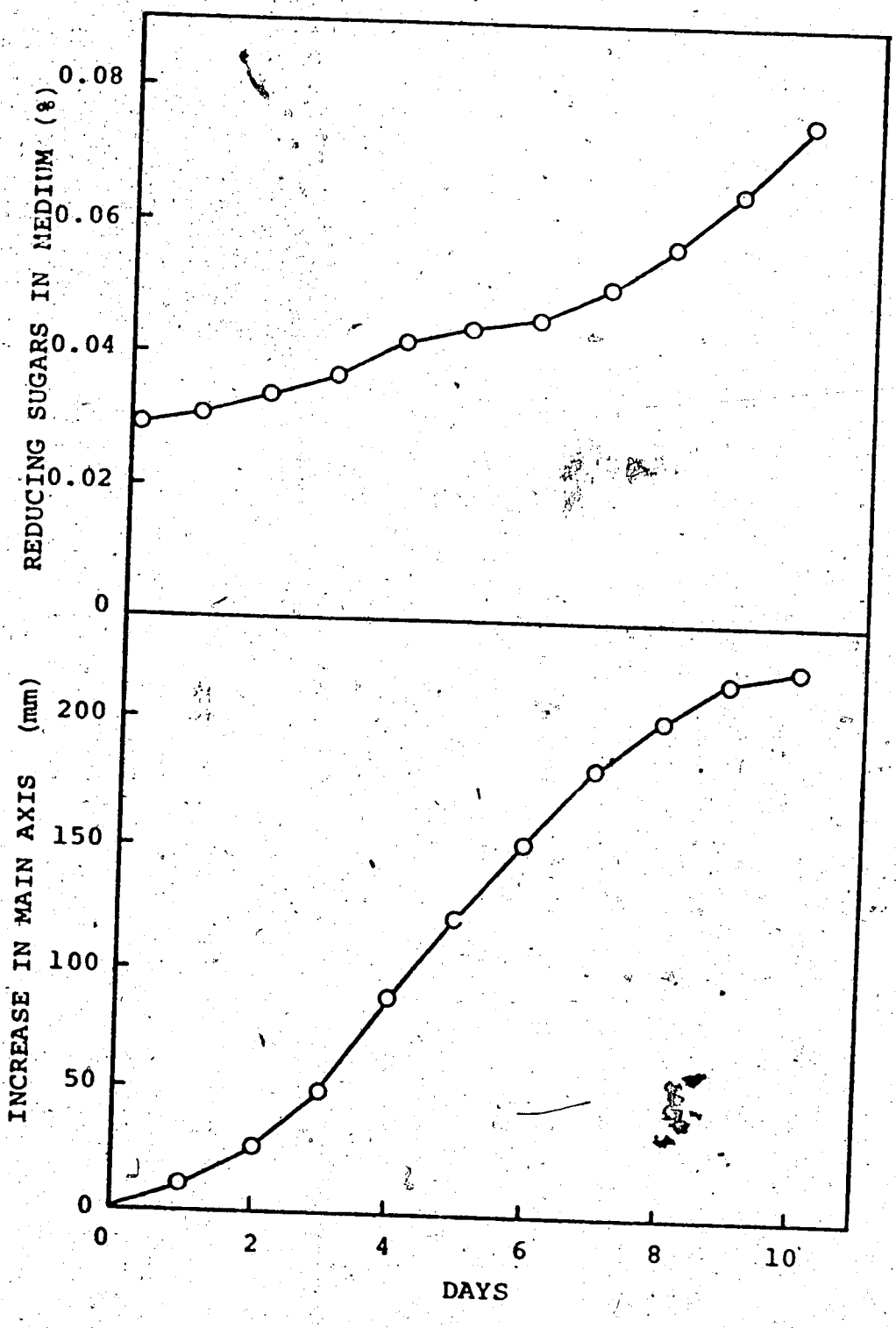

REDUCING SUGARS IN MEDIUM (%)
0.08
0.06
0.04
0.02
0
INCREASE IN MAIN AXIS (mm)
200
150
100
50
0
0
2
4
6
8
10
DAYS

TABLE 10

Effect of Glucose and Fructose on Activity of Extracted Tomato Root Invertases

Concentration of Sugars	Invertase Activity mg hexose/mg protein/2 hrs	
	Soluble	Cell Wall
5% sucrose	2.94	1.34
5% sucrose + 0.02% glucose	2.90	1.32
5% sucrose + 0.02% fructose	2.60	1.32
1.5% sucrose	1.07	0.40
1.5% sucrose + 0.05% glucose	1.04	0.39
1.5% sucrose + 0.05% fructose	1.02	0.39

Reaction system consisted of 90 µmoles sodium phosphate-citric acid buffer, pH 4.8, 1 ml substrate, and enzyme preparation (0.1 mg protein). Mixture was incubated at 28°C for 2 hours.

B. The effect of different carbon sources on growth and invertase levels

The correlation of growth and invertase activity was studied by growing roots in different carbon sources, *viz.* sucrose, glucose, fructose and raffinose. Table 11 shows that roots grown in sucrose had the highest growth rate and highest invertase activity; roots grown in glucose, fructose and raffinose medium had lower growth rates and invertase activities. The results thus corroborate those obtained in Section IV showing that growth and invertase activities were related.

C. The effect of transferring tomato roots from standard to 1.5% glucose medium

The effect of glucose on growth and invertase development was studied by transferring the roots grown in standard medium for 7 days to 1.5% glucose medium. Figure 12 shows that growth and invertase activity declined after transfer. The half-lives of soluble and cell wall invertases in glucose medium could be estimated as 5 and 6 days, respectively.

D. The effect of mixtures of sugars on growth and invertase activity

The question of the glucose repression of invertase synthesis was further tested by growing roots in mixtures of sucrose and glucose. The results of the experiment (Table 12) show that the addition of glucose to sucrose medium caused an increase in growth and invertase activity. Thus the presence of glucose clearly did not repress the synthesis of invertases.

TABLE 11

Effects of Four Sugars as Carbon Sources on Growth and Invertase Activity

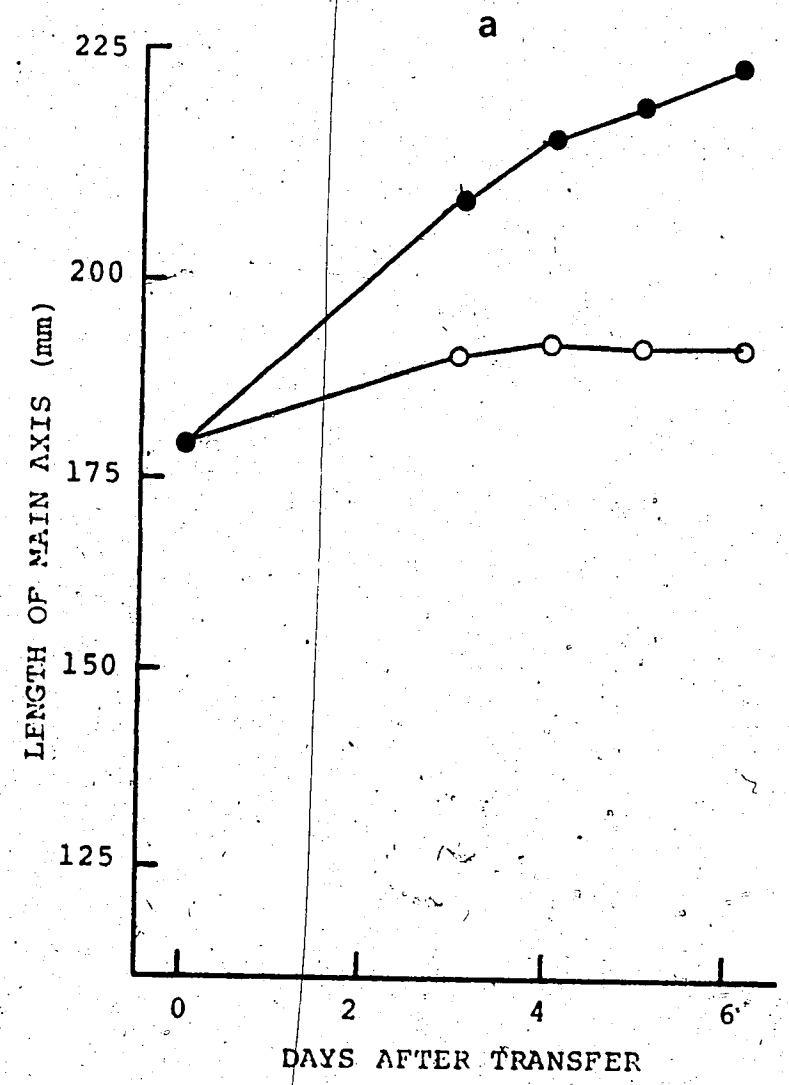
Carbon source	Increase in Main Axis (mm)	Invertase Activity mg hexose/mg protein/2 hrs	
		Soluble	Cell Wall
1.5% sucrose	180	2.93	1.45
1.5% glucose	83	1.24	0.65
1.5% fructose	86	1.67	0.73
1.5% raffinose	28	1.29	0.54

Roots were grown in different carbon sources for 7 days, then growth was measured and invertases extracted, as described in 'Methods'. Reaction system consisted of 290 µmoles sucrose, 90 µmoles sodium phosphate-citric acid buffer, pH 4.8, and enzyme preparation (0.1 mg) in a final volume of 2 ml. Mixture was incubated at 28°C for 2 hours.

FIGURE 12

Effects of transferring excised tomato roots from standard to glucose medium on (a) growth and (b) invertase activities

At zero time 7-day-old roots were transferred to fresh standard medium (closed figures) or 1.5% glucose medium (open figures).


a
225
200
175
150
125
LENGTH OF MAIN AXIS (mm)
0
2
4
6
DAYS AFTER TRANSFER

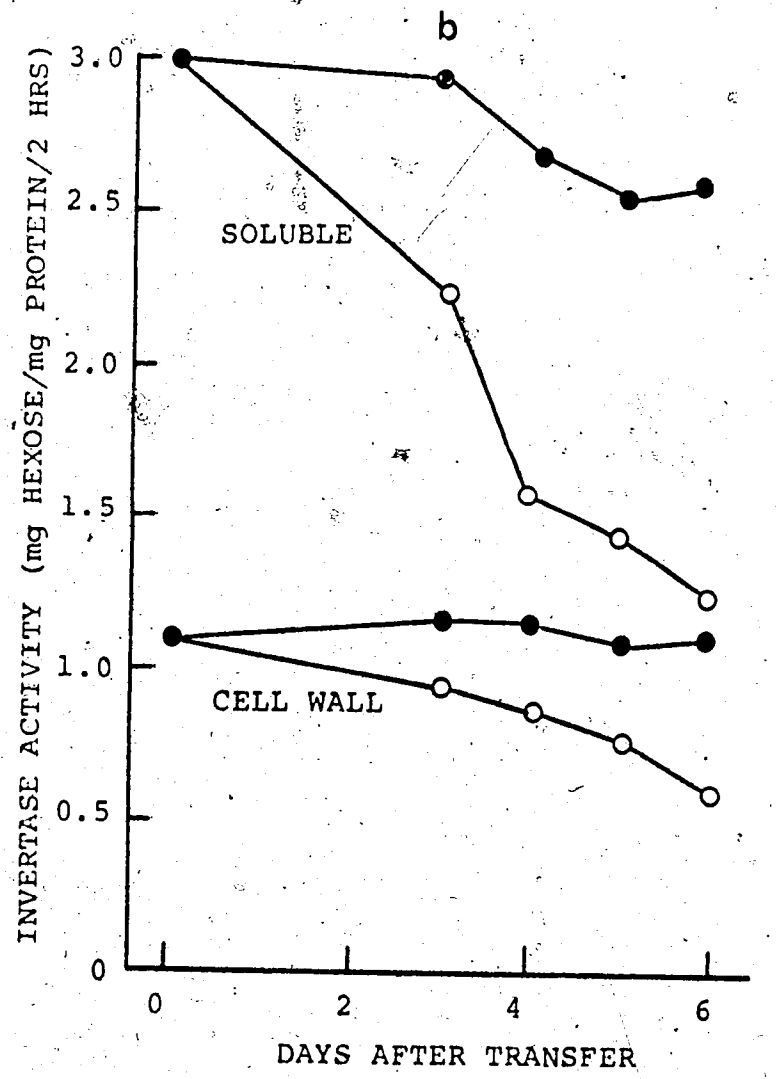

b
3.0
2.5
2.0
1.5
1.0
0.5
0
INVERTASE ACTIVITY (mg HEXOSE/mg PROTEIN/2 HRS)
SOLUBLE
CELL WALL
0
2
4
6
DAYS AFTER TRANSFER

TABLE 12

Effect of Carbon Sources on Growth and Invertase Activity

Sugars	Fresh weight g/100 roots	Invertase Activity mg hexose/mg protein/2 hours	
		Soluble	Cell Wall
1.5% sucrose	1.645	3.44	1.47
[redacted]	0.952	2.67	1.18
[redacted]ucose	1.304	3.02	1.13
[redacted]	0.817	2.59	1.15
[redacted]cose	1.291	2.94	1.27
[redacted]sucrose	0.434	2.24	1.11
0.6% sucrose + 0.9% glucose	0.644	2.59	1.13

Roots were grown in different carbon sources for 7 days, then growth was measured, and invertases extracted as described in 'Methods'. Reaction system consisted of 290 µmoles sucrose, 90 µmoles sodium phosphate-citric acid buffer, pH 4.8, and enzyme preparation (0.1 mg protein) in a final volume of 2 ml. Reaction mixture was incubated at 28°C for 2 hours.

E. The effect of sucrose concentration of growth and invertase activity

To further study the correlation of growth and invertase activity of excised tomato roots, the growth of the roots was altered by varying the sucrose concentration of the medium. Table 13 shows that once again the invertase activity and growth of the roots shows reasonable correlation.

F. The effect of starvation on invertase activity and growth

The findings that the presence of glucose did not repress the synthesis of invertases and that invertase activities directly varied with sucrose concentration suggest that the synthesis of invertases may be induced by sucrose. Experiments were, therefore, carried out to deprive the roots of sucrose to determine the effect on invertase activity. Roots were grown in standard medium for 7 days and then washed twice in sucrose-free medium prior to incubation in this medium. Figure 13 shows that root-growth ceased immediately after transfer, and both soluble and cell wall invertase activity declined. The decline of invertase activities in sucrose-free medium thus favors the theory that invertase synthesis in excised tomato roots is induced by sucrose.

G. The effect of reducing growth with low temperature on invertase activities

Varying the carbon sources always resulted in parallel alteration in growth and invertase activities, making it very difficult to identify which is the primary effect of the carbon source. It was, thus, decided to modify the growth of excised tomato roots with other

TABLE 13

Effect of Sucrose Concentrations on Growth and Invertase Activity

Sucrose Concentration	Fresh weight g/100 roots	Invertase Activity mg hexose/mg protein/2 hrs	
		Soluble	Cell Wall
1.5%	1.82	3.47	1.49
1.2%	1.02	2.83	1.24
1.0%	0.91	2.67	1.11
0.8%	0.79	2.52	1.09
0.6%	0.48	2.08	0.91

Roots were grown in media of different sucrose concentrations for 7 days, then growth was measured, and invertase extracted, as described in 'Methods'.

Reaction system consisted of 290 µmoles sucrose, 90 µmoles sodium phosphate-citric acid buffer, pH 4.8, and enzyme preparation (0.1 mg protein) in a final volume of 2 ml. Reaction mixture was incubated 28°C for 2 hours.

FIGURE 13

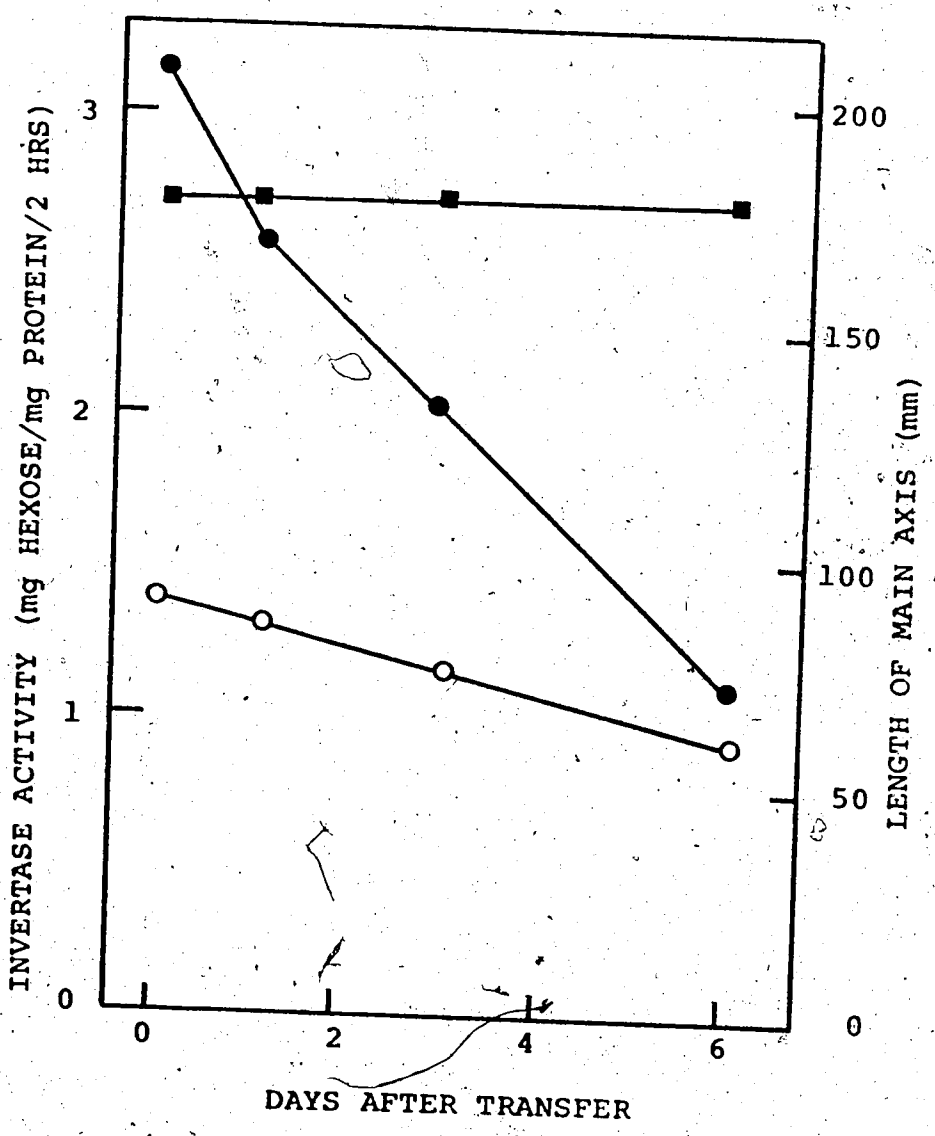
Effect of starvation on growth and invertase activity

At zero time 7-day-old roots were transferred to minus sugar medium.

■ : growth

● : soluble invertase

○ : cell wall invertase


INVERTASE ACTIVITY (mg HEXOSE/mg PROTEIN/2 HRS)
LENGTH OF MAIN AXIS (mm)
DAYS AFTER TRANSFER
0
1
2
3
0
50
100
150
200
0
2
4
6

factors. Preliminary studies showed that it was possible to lower the growth rate by lowering the temperature of incubation without altering the medium. Lowering the temperature to 5°C stopped the growth of the roots. However, growth resumed if the temperature was brought back to 28°C showing that the roots were not killed by the low temperature.

To alter the growth with incubation temperature, roots grown in 1.0 and 1.5% sucrose were incubated at 5° and 28°C for 7 days, and the growth rate and invertase activity then determined. The results (Table 14) show that roots grown at different temperatures, in spite of having radically different growth rates, had similar invertase activities. On the other hand roots grown in different sucrose concentrations at the same temperature had different invertase activity. These data strongly suggest that the factor controlling the invertase activity of excised tomato roots was the sucrose concentration and not the growth rate. These results together with those of previous sections indicate that growth may be dependent upon invertase activity and not the reverse.

H. The effect of transferring the tomato roots from one sucrose concentration to another

If the sucrose concentration and not growth rate is the primary factor affecting the invertase activity of tomato roots then a sudden change in the sucrose concentration of the medium may be expected to cause a change in invertase activity earlier than a change in growth rate. Roots were, therefore, grown in a low sucrose concentration (0.5%) and on the fourth day half of them transferred to standard medium (1.5% sucrose). The invertase activities and growth rates of

TABLE 14

Effect of Temperature on Growth and Invertase Activity

Sucrose Concentration	Incubation Temperature °C	Increase in main axis (mm)	Invertase Activity mg hexose/mg protein/2 hrs	
			Soluble	Cell Wall
1.5%	5	7	3.40	1.44
1.5%	28	177	3.43	1.49
1.0%	5	5	2.67	1.02
1.0%	28	171	2.78	1.09

Seven-day-old roots were used in growth and invertase activities determinations.

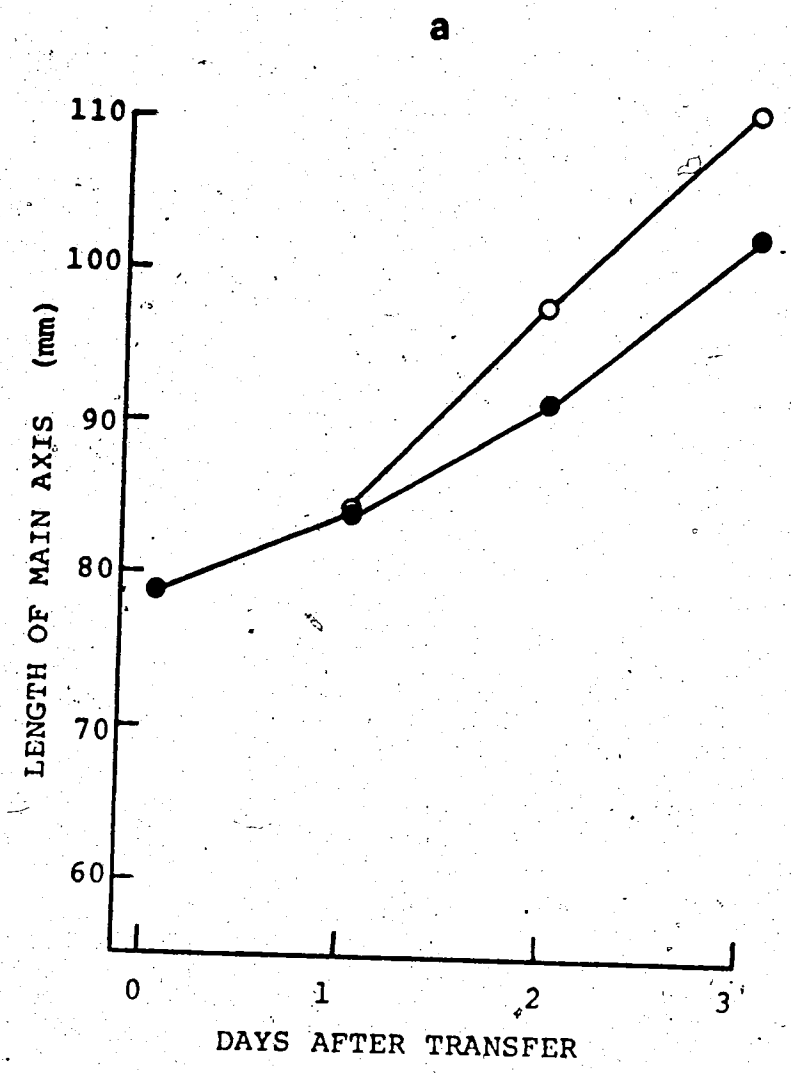
these roots were followed. Figure 14 shows that both solubie and cell wall activities showed an increase over the control within the first day of transfer. However, in the same period of time the growth of the transferred roots was the same as the control. From the second day onwards while the invertase activities of the transferred roots continued to increase, they began to grow faster than controls. The results, therefore, show that the increase in invertase activity preceded the increase in growth rates.

Figure 15 shows the invertase activites and growth rates of roots grown continuously in 1.5% sucrose and of those transferred on the fourth day from 1.5 to 0.5% sucrose medium. The invertase activities of the roots transferred from 1.5 to 0.5% dropped to lower than in controls within the first day of transfer, whereas growth did not. The invertase activities of the experimental roots dropped further below that of the controls on the second day and only from this time was growth slower than the controls. Thus the results depicted in Figures 14 and 15 demonstrated that treatments affecting both invertase activity and growth caused effects on invertase activity earlier than on growth. These results are consistent with the theory that growth is dependent upon invertase activity, and that alteration in the latter is the primary effect of altering the sucrose concentration.

FIGURE 14

Effects of transferring excised tomato roots from 0.5% to 1.5% sucrose medium on (a) growth and (b) invertase activities

At zero time 4-day-old roots grown in 0.5% sucrose were transferred to fresh 0.5% sucrose (closed figures) or fresh medium (open figures).


a
LENGTH OF MAIN AXIS (mm)
110
100
90
80
70
60
0
1
2
3
DAYS AFTER TRANSFER

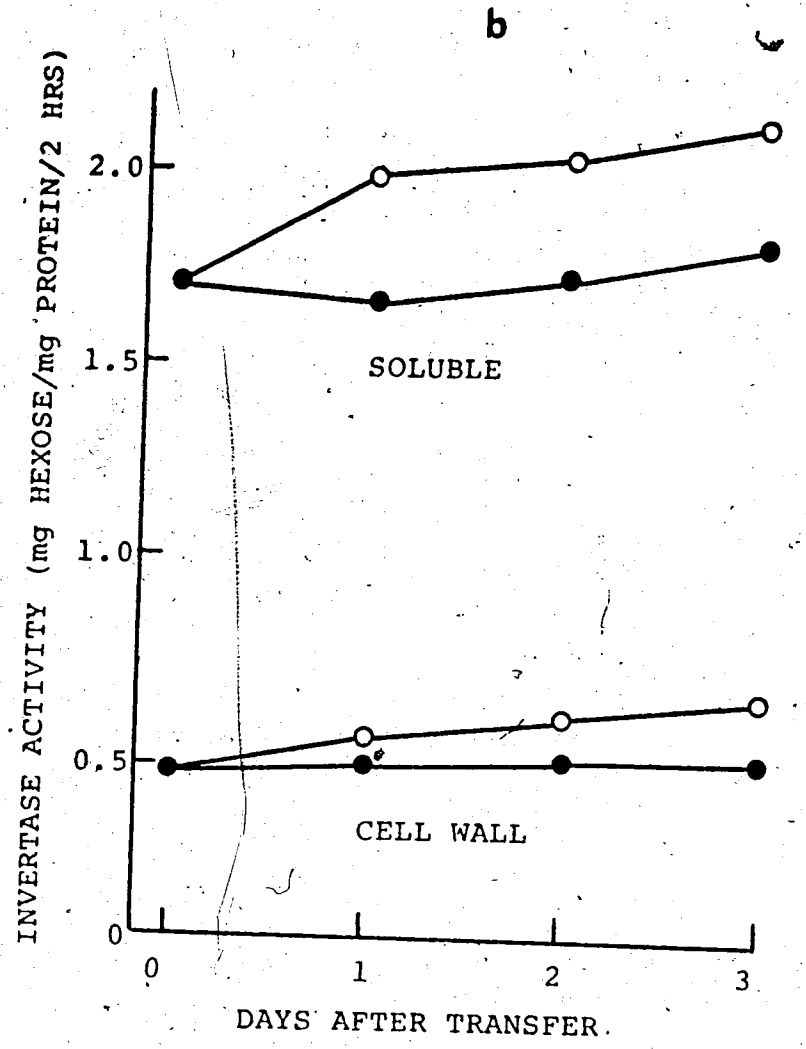
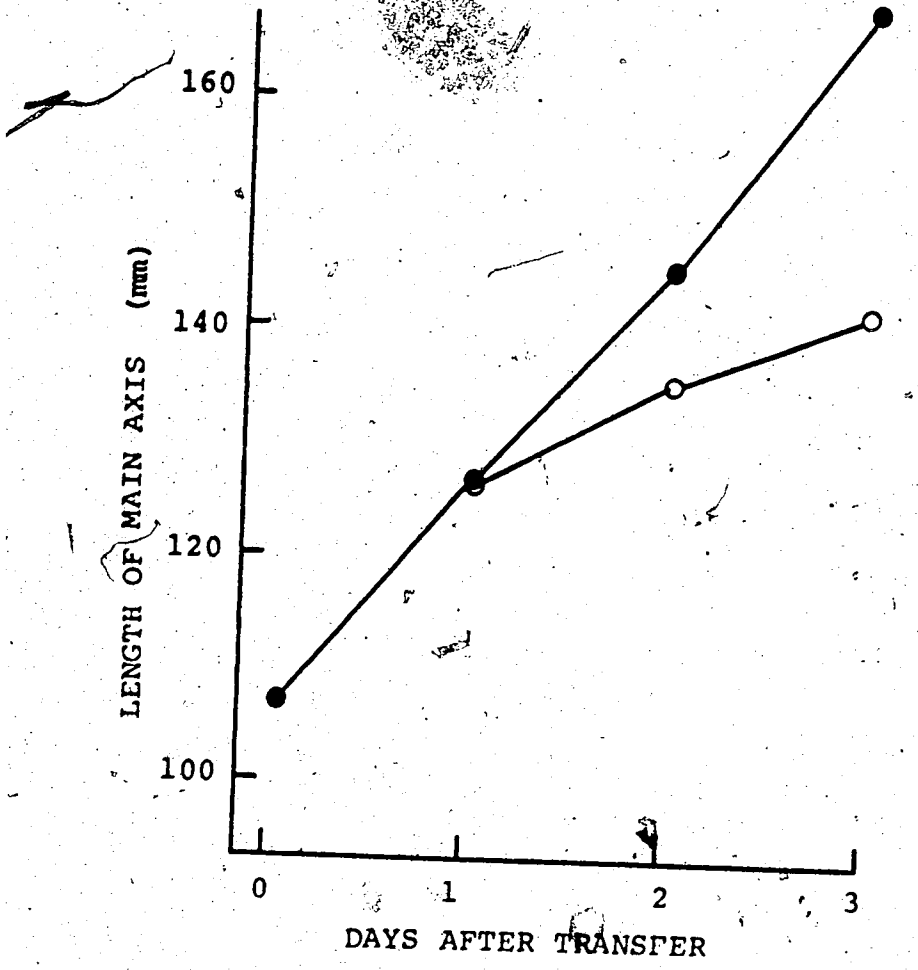
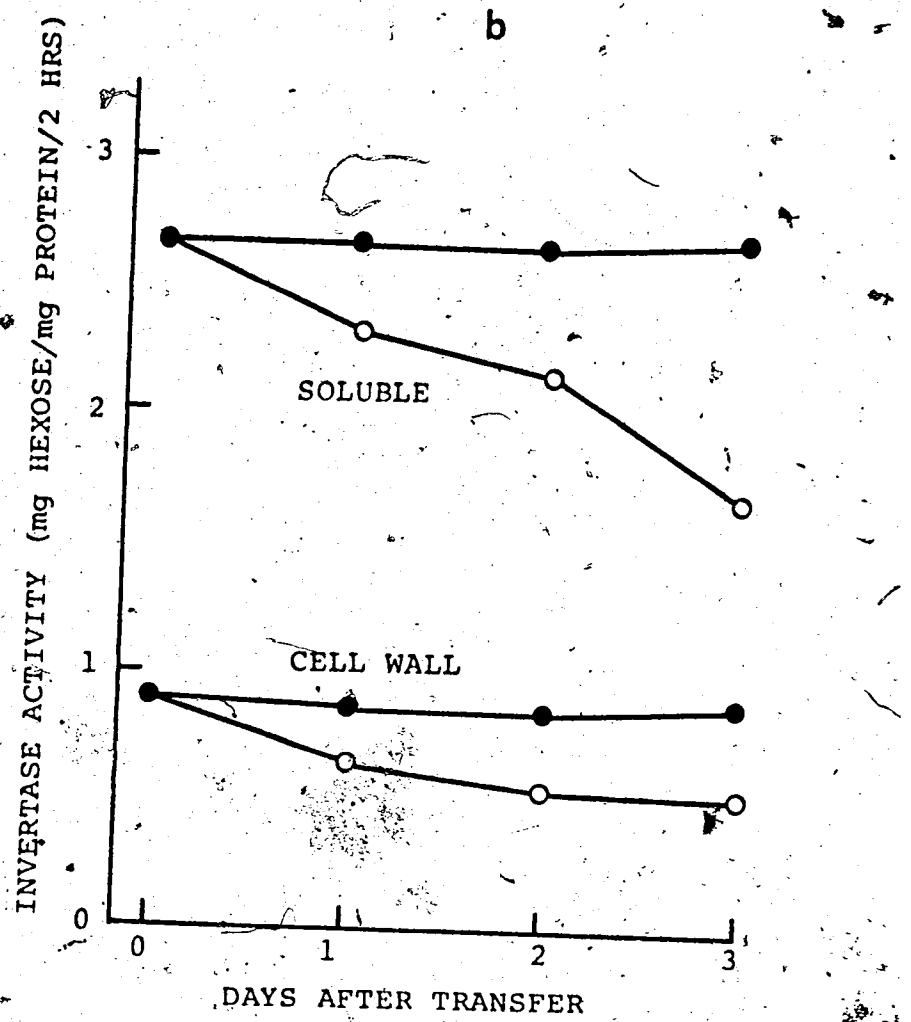

b
INVERTASE ACTIVITY (mg HEXOSE/mg PROTEIN/2 HRS)
2.0
1.5
1.0
0.5
0
SOLUBLE
CELL WALL
0
1
2
3
DAYS AFTER TRANSFER.

FIGURE 15

Effe[redacted] transferring excised tomato roots from 1.5% to 0.5% sucrose medium on (a) growth and (b) invertase activities

At zero time 4-day-old roots grown in 1.5% sucrose were transferred to fresh 1.5% sucrose (closed figures) or fresh 0.5% sucrose medium (open figures).


a
LENGTH OF MAIN AXIS (mm)
160
140
120
100
0
1
2
3
DAYS AFTER TRANSFER


b
INVERTASE ACTIVITY (mg HEXOSE/mg PROTEIN/2 HRS)
3
2
1
0
SOLUBLE
CELL WALL
0
1
2
3
DAYS AFTER TRANSFER

SECTION VI.

Effects of Plant Growth Regulators on Growth and Invertase Activity

A. The effects of plant growth regulators on the activity of extracted soluble and cell wall invertase

The effects of various plant growth regulators on the activity of extracted soluble and cell wall invertase were studied. None of the growth regulators tested showed appreciable effects (Table 15). Thus excised tomato invertases appear to differ from yeast invertase which was weakly inhibited by ABA (Saunders and Poulson, 1968).

B. The effect of GA on growth and invertase activity of excised tomato roots

To study the effect of GA on growth and invertase activity roots were grown in 0.5% sucrose medium and on the fourth day transferred to fresh 0.5% sucrose medium or 0.5% sucrose medium supplemented with GA. The results (Figures 16a and 16b) show that concentrations from 2.5 to 10 µM GA reduced both growth and invertase activity slightly. The promotion of growth reported by Butcher and Street (1959) was not observed. As in the studies of Butcher and Street, GA was added at the beginning but not on the fourth day of the growth cycle. The GA experiment was repeated by treating the roots with GA at the beginning of the growth cycle. In addition, a much wider range of GA concentrations (16 µM to 40 mM) were used. The results show that high GA concentration inhibited growth as well as invertase activity (Figures 17a and 17b).

TABLE 15

Effects of Various Plant Growth Regulators on Activity of Soluble and Cell Wall Invertases

Plant growth regulators	Final Concentration (μM)	Invertase Activity mg hexose/mg protein/2 hrs.	
		Soluble	Cell Wall
GA	0	3.59	1.49
	2.5	3.60	1.45
	5.0	3.49	1.44
	10.0	3.60	1.48
NAA	0	3.45	1.44
	0.16	3.47	1.45
	0.32	3.45	1.44
	0.64	3.41	1.38
Kinetin	0	3.59	1.49
	46	3.56	1.47
	92	3.59	1.49
ABA	0	3.32	1.31
	0.75	3.33	1.36
	1.5	3.36	1.35
	3.0	3.27	1.31

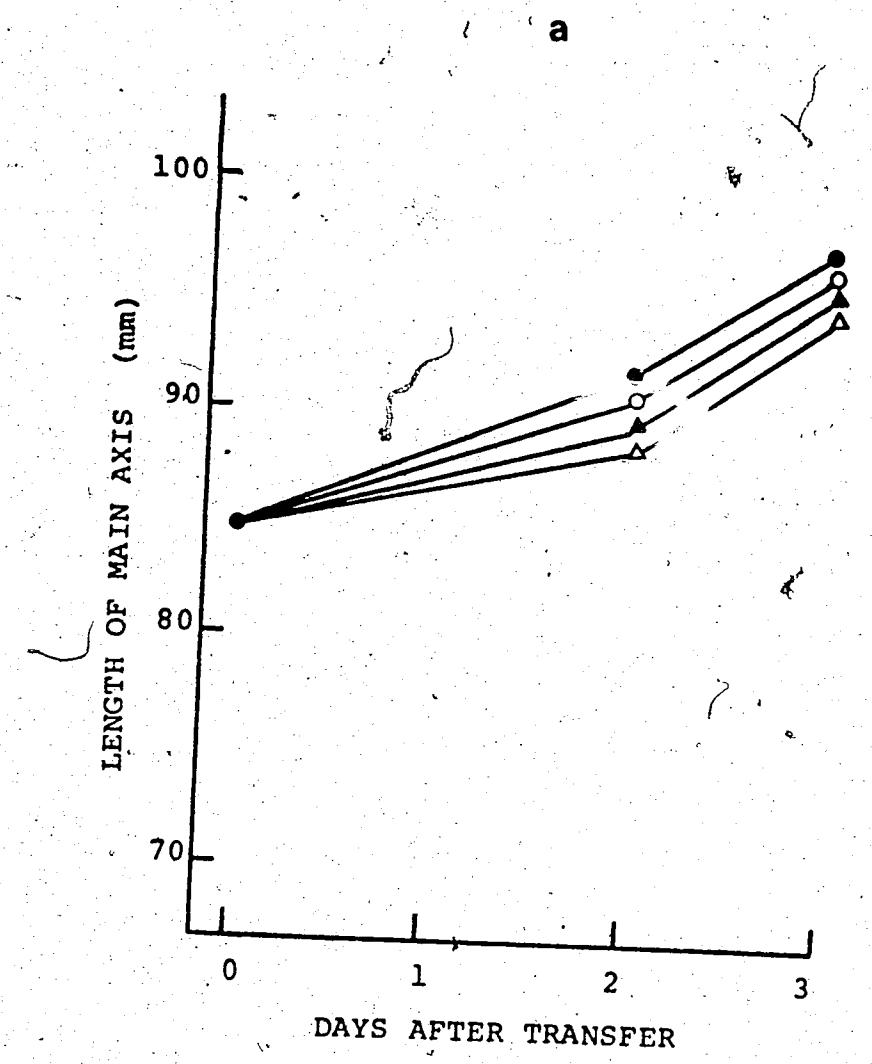
Reaction system contained 290 μmoles sucrose, sodium phosphate-citric acid buffer, pH 4.8 containing various concentrations of plant growth regulators and enzyme preparation (0.1 mg protein) in a final volume of 2 ml.

Reaction mixture was incubated at 28°C for 2 hours.

FIGURE 16

Effects of GA on (a) growth and (b) invertase activities of excised tomato roots

At zero time roots grown in 0.5% sucrose medium were transferred to fresh sucrose medium (●) or 0.5% sucrose medium containing 2.5 µM (○), 5 µM (▲), or 10 µM (△) GA.


a
100
90
80
70
LENGTH OF MAIN AXIS (mm)
0
1
2
3
DAYS AFTER TRANSFER

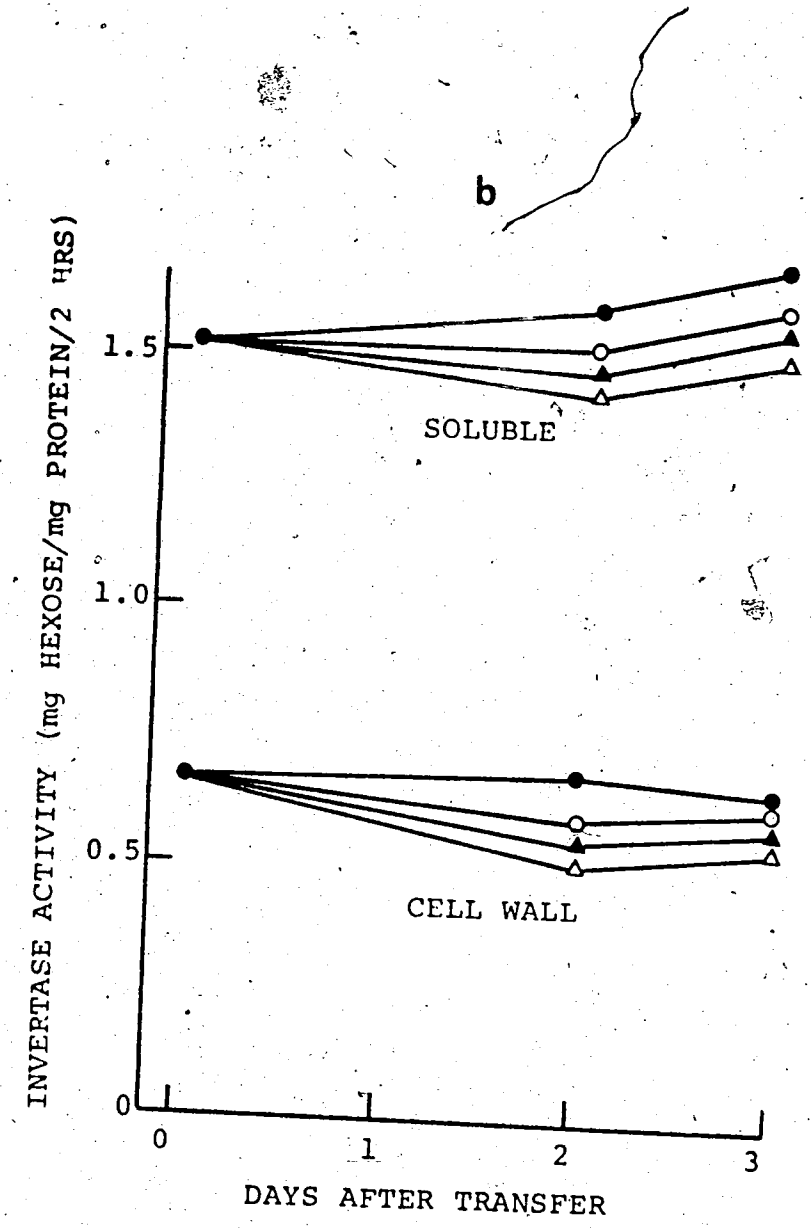
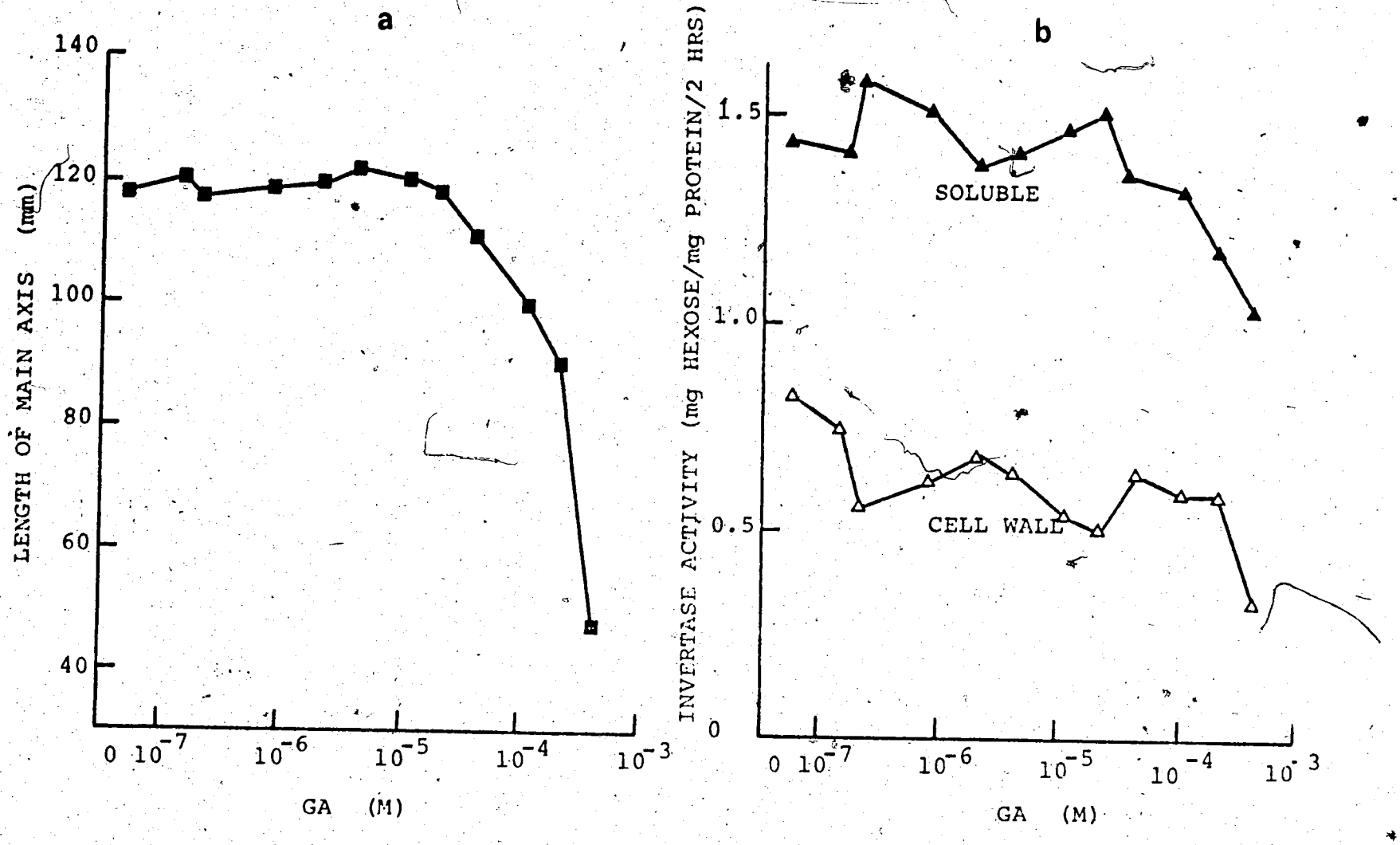

b
1.5
1.0
0.5
0
INVERTASE ACTIVITY (mg HEXOSE/mg PROTEIN/2 HRS)
SOLUBLE
CELL WALL
0
1
2
3
DAYS AFTER TRANSFER

FIGURE 17

Effects of GA on (a) growth and (b) invertase activities of excised tomato roots

Roots were grown in media containing varying amounts of GA for 7 days and then the growth rate and invertase activities of the roots determined.


a
b
LENGTH OF MAIN AXIS (mm)
140
120
100
80
60
40
0 10^-7
10^-6
10^-5
10^-4
10^-3
GA (M)
INVERTASE ACTIVITY (mg HEXOSE/mg PROTEIN/2 HRS)
1.5
1.0
0.5
0
SOLUBLE
CELL WALL
0 10^-7
10^-6
10^-5
10^-4
10^-3
GA (M)

C. The effect of NAA on growth and invertase activity of excised tomato roots

In the studies of the effect of NAA on growth and invertase activity roots were grown in 0.5% sucrose medium for 4 days prior to incubation in fresh 0.5% sucrose medium containing 0.16 µM, 0.32 µM or 0.64 µM NAA. Both the soluble and cell wall invertase activities were found to be lower but growth was not appreciably nor consistently affected by NAA treatment (Figures 18a and 18b). Thus these results indicate that a reduction in invertase activity did not necessarily always cause a reduction in growth.

D. The effect of kinetin on growth and invertase activity of excised tomato roots

To test the effect of kinetin roots were grown in 0.5 or 1.5% sucrose for 4 days prior to incubation in fresh medium with various amounts of kinetin added. No significant effect of kinetin on growth or invertase activity of roots grown in 1.5% sucrose medium were found (Figures 19a and 19b). There was an effect of kinetin on 0.5% sucrose-grown roots, however. The results (Figure 20b) show that while the cell wall invertase activity was raised by kinetin, the soluble invertase activity was unchanged. Also the growth (Figure 20a) of roots was very slightly reduced even though the cell wall invertase activity was higher.

E. The effect of ABA on growth and invertase activity of excised tomato roots

To study the effect of ABA, roots were cultured in 1.5% sucrose

FIGURE 18

Effects of NAA on (a) growth and (b) invertase activities of excised tomato roots

At zero time 4-day-old roots grown in 0.5% sucrose medium were transferred to fresh medium (●) or fresh medium containing 0.16 µM (○), 0.32 µM (▲), or 0.64 µM (△) NAA.

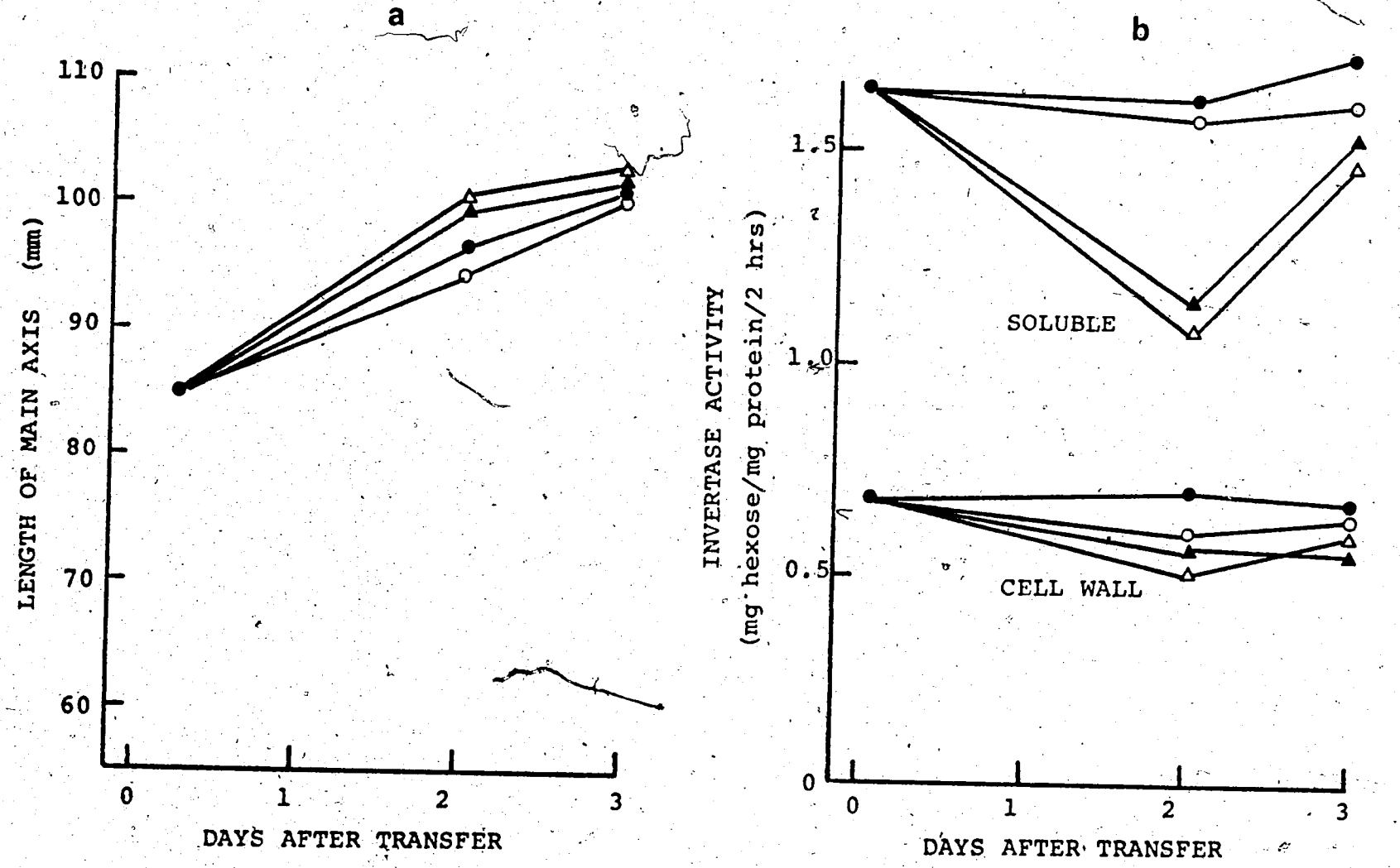
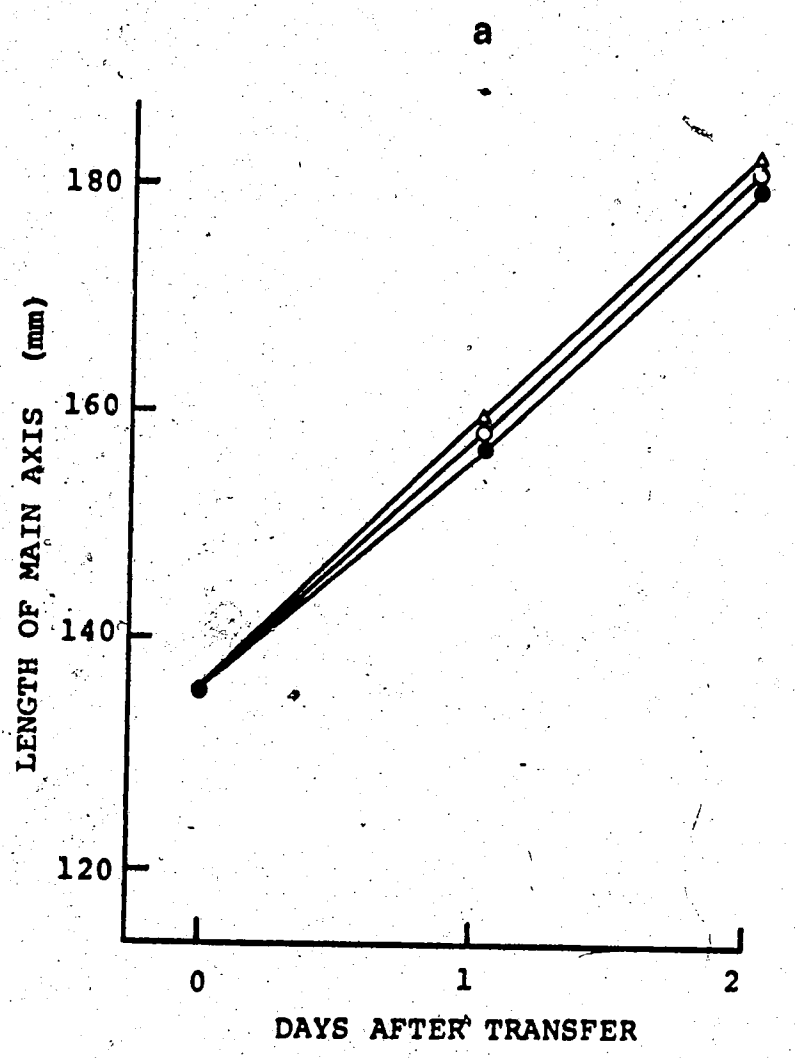

a
LENGTH OF MAIN AXIS (mm)
110
100
90
80
70
60
0
1
2
3
DAYS AFTER TRANSFER
b
INVERTASE ACTIVITY
(mg hexose/mg protein/2 hrs)
1.5
1.0
0.5
0
SOLUBLE
CELL WALL
0
1
2
3
DAYS AFTER TRANSFER

FIGURE 19

Effects of kinetin on (a) growth and (b) invertase activities of excised tomato roots grown in 1.5% sucrose medium

At zero time 4-day-old roots grown in standard medium were transferred to fresh medium (●) or fresh medium containing 46 μM (○), or 92 μM (△) kinetin.


a
LENGTH OF MAIN AXIS (mm)
180
160
140
120
0
1
2
DAYS AFTER TRANSFER

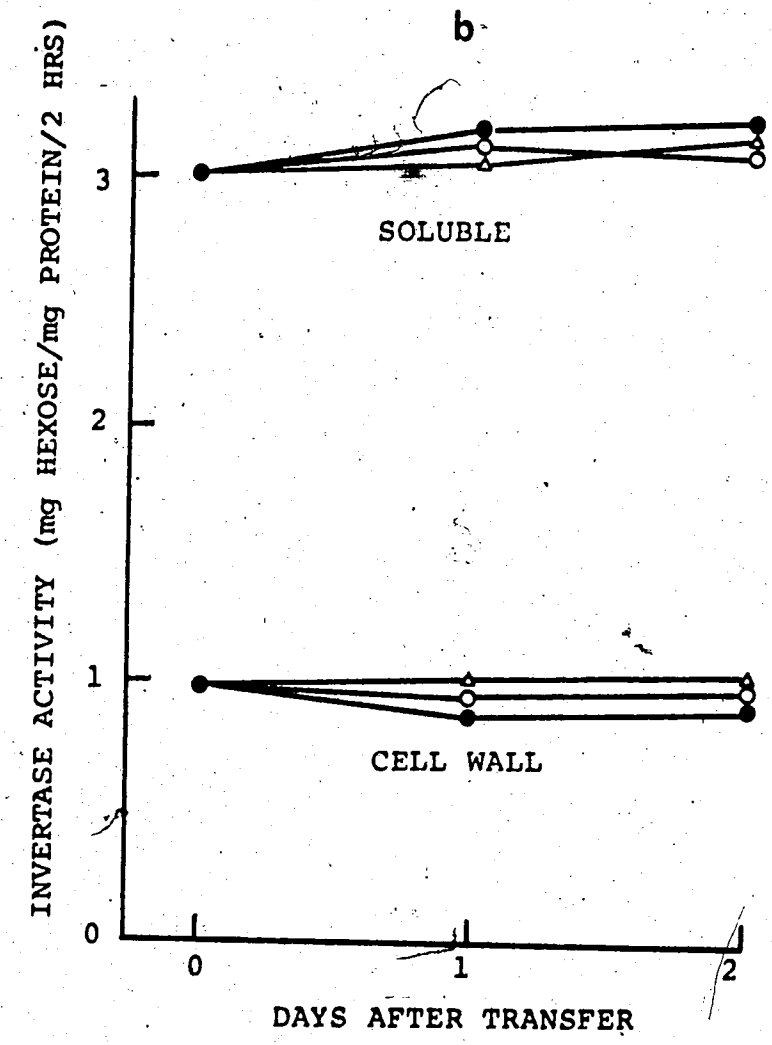
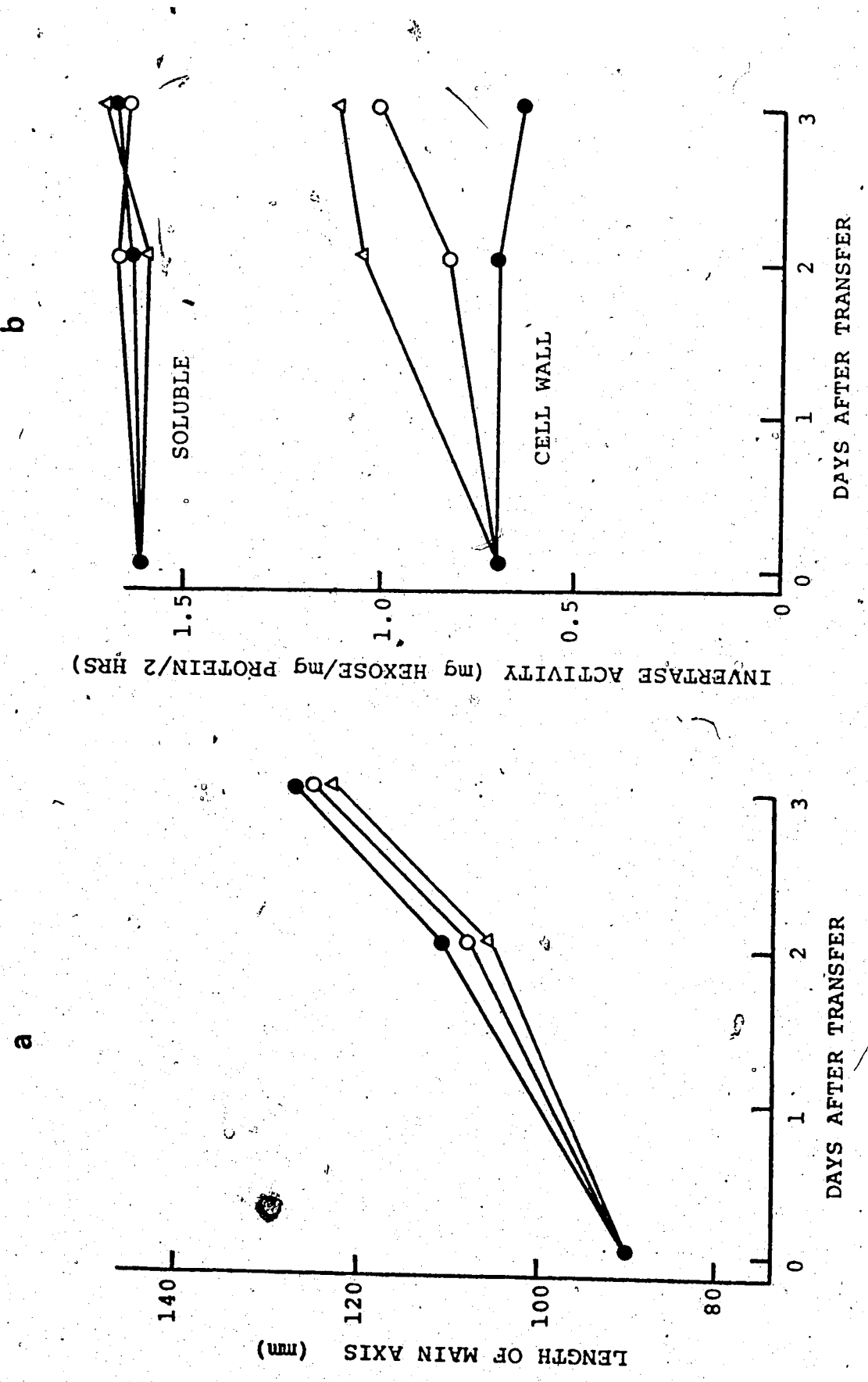

b
INVERTASE ACTIVITY (mg HEXOSE/mg PROTEIN/2 HRS)
3
2
1
0
SOLUBLE
CELL WALL
0
1
2
DAYS AFTER TRANSFER

FIGURE 20

Effects of kinetin on (a) growth and (b) invertase activities of excised tomato roots grown in 0.5% sucrose medium

At zero time 4-day-old roots grown in 0.5% sucrose medium were transferred to fresh medium (●), or fresh medium containing 46 μM (○) or 92 μM (△) kinetin.

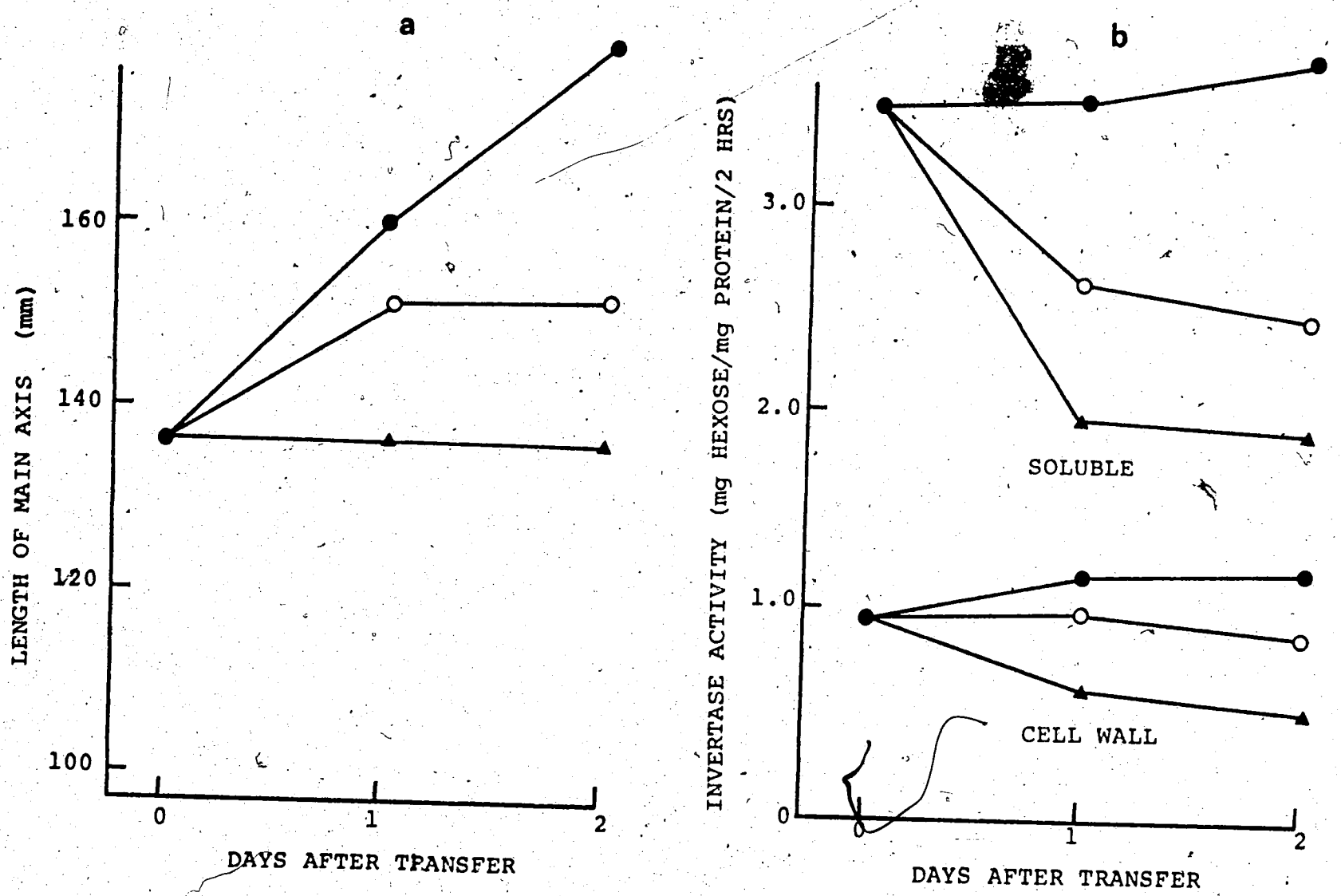

a
LENGTH OF MAIN AXIS (mm)
140
120
100
80
0
1
2
3
DAYS AFTER TRANSFER
b
INVERTASE ACTIVITY (mg HEXOSE/mg PROTEIN/2 HRS)
SOLUBLE
CELL WALL
1.5
1.0
0.5
0
0
1
2
3
DAYS AFTER TRANSFER

medium and on the fourth day transferred to fresh medium with 0.75 and 1.5 µM ABA added. The roots transferred to medium containing 0.75 µM ABA showed a reduced growth rate during the first day of the transfer and ceased to grow from this point onward (Figure 21a). The soluble and cell wall invertase activities both declined after the transfer (Figure 21b). In 1.5 µM ABA roots ceased to grow during the first day and both the soluble and cell wall invertase activities declined faster than in the 0.75 µM ABA treatment. A correlation between invertase activity and growth thus appears to exist.

FIGURE 21

Effects of ABA on (a) growth and (b) invertase activities of excised tomato roots.

At zero time 4-day-old roots grown in 1.5% sucrose medium were transferred to fresh medium (●), or fresh medium containing 0.75 µM (○), or 1.5 µM (▲) ABA.


a
LENGTH OF MAIN AXIS (mm)
160
140
120
100
0
1
2
DAYS AFTER TRANSFER
b
INVERTASE ACTIVITY (mg HEXOSE/mg PROTEIN/2 HRS)
3.0
2.0
1.0
0
SOLUBLE
CELL WALL
0
1
2
DAYS AFTER TRANSFER

SECTION VII

Partial Purification and Characterization of Invertases of Excised Tomato Roots

A. Solubilization of cell wall invertase

To solubilize the cell wall invertase, the cell wall fraction was treated with carbowax 4,000, Tween 20, Tween 80, Triton X-100, deoxycholate, and borate buffer. None of the treatments was found effective in removing significant amounts of invertase from the wall. The inability of the detergents Tween 20, Tween 80, Triton X-100 and deoxycholate to solubilize the enzyme suggests that the cell wall activity was not the result of contamination with membrane fragments.

The effect of the pH of extraction buffer on the distribution of invertase activity in soluble and cell wall fractions was studied with sodium phosphate-citric acid buffer of varying pH. The results (Table 17) show that the higher the pH of the extraction buffer the greater the invertase activity recovered in the soluble fraction. A reasonable interpretation of the above results is that fraction with low pH leaves a moiety of soluble invertase adsorbed to the wall. If this is the case then alteration of the pH of the extraction medium after extraction but before centrifugation should alter the distribution of the enzyme. Table 18 shows that this is indeed the case, indicating that at least some of the cell wall invertase was adsorbed to the wall by ionic force.

B. Purification of soluble invertase

The soluble fraction was extracted from 7-day-old roots and purified with ammonium sulphate fractionation and Sephadex G-100 column

TABLE 16

Effects of Various Treatments on Solubilization of Cell Wall Invertase

Treatment	Invertase Activity mg hexose/g fr wt/2 hrs	% Change
Control	7.8	
6% Carbowax 4000	7.8	0
10% Carbowax 4000	7.6	-2
1% Tween 20	7.8	-0
5% Tween 20	7.5	-4
1% Tween 80	7.7	-1
2% Tween 80	7.5	-4
0.1% Triton X-100	7.5	-4
0.2% Triton X-100	7.1	-8
0.5% Deoxycholate	7.4	-5
1% Deoxycholate	7.4	-5
0.1 M Borate buffer (pH 7.0)	7.4	-5

The cell wall preparation was centrifuged at 12,000 x *g* for 10 minutes and the pellet resuspended in borate buffer or 0.05 M sodium phosphate-citric acid buffer containing indicated amounts of various compounds for 1 hour. Then the cell wall was centrifuged at 12,000 x *g* for 10 minutes, and the pellet washed twice with 0.05 M sodium phosphate-citric buffer (pH 7.0). The final sediment was resuspended in 0.05 M sodium phosphate-citric acid buffer (pH 7.0).

TABLE 17

Effect of pH of Extraction Buffer on Distribution of Tomato Root Invertase

pH of Extraction medium	Invertase Activity mg hexose/gm fr wt/2 hrs		Total Activity	Cell Wall Activity as % of total
	Soluble	Cell Wall		
4.8	15.24	9.33	24.57	38.0
6.0	18.00	7.80	25.80	30.2
7.0	24.48	6.28	30.76	20.6
8.0	25.60	6.02	31.62	19.0

Invertases were prepared as described in 'Methods' except that buffers of varying pH were used. Reaction system contained 90 µmoles sodium phosphate-citric acid buffer, pH 4.8, 290 µmoles sucrose and 0.1 ml enzyme preparation in a total volume of 2 ml. Reaction mixture was incubated at 28°C for 2 hours.

TABLE 18

Effect of Altering pH after Extraction on Distribution of Invertase Activity

Treatment	Invertase Activity mg hexose/gm fr wt/2 hrs				Cell Wall Activity as % of total
	Homogenized at pH 4.8		Homogenized at pH 7.0		
	Soluble	Cell Wall	Soluble	Cell Wall	
Control	15.24	9.33			38
pH changed from 418 to 7.0 with Na_2PO_4			24.48	6.28	20
pH changed from 7.0 to 4.8 citric acid	18.09	3.48			16
			16.64	8.84	35

*Activity assayed at pH 4.8.

Seven-day-old roots were homogenized with 0.05 M sodium phosphate-citric acid buffer, pH 4.8 or 7.0. The pH of half of the preparations was then adjusted with Na_2PO_4 or citric acid as indicated and then separated into cell wall and soluble fractions as described in 'Methods'.

Assay system contained 90 µmoles sodium phosphate-citric acid buffer, pH 4.8, 290 µmoles sucrose, and 0.1 ml enzyme in a total volume of 2 ml. Reaction mixture was incubated at 28°C for 2 hours.

chromatography as described in the 'Methods'. The results of the purification are presented in Table 19. The soluble invertase activity was resolved by Sephadex G-100 filtration into two peaks called invertase I and invertase II (Figure 22). The Sephadex G-100 filtration also successfully separated the α-glucosidase from the two invertases. The fractions containing each enzyme were pooled and used in the later studies.

C. Specificity

The specificity of invertase I, II and α-glucosidase was tested with sucrose, raffinose, maltose and trehalose. Results (Table 20) show that invertase I and II attacked sucrose and raffinose but not trehalose and maltose, indicating that they were genuine β-fructofuranosidases and attacked only substrates with a free β-fructofuranosyl end. The α-glucosidase attacked sucrose, maltose and trehalose, all of which possessed a free α-glucosyl group, but not raffinose which did not. However, α-glucosidase did not attack sucrose, maltose, and trehalose at the same rate; it was approximately 3.5 times more active on maltose than on sucrose.

D. Heat stability of invertases

The heat stability of the two invertases was examined by incubating the enzymes at various temperatures for 12 minutes prior to the determination of activity. As indicated in Figure 23 invertase II was stable below 35°C; at temperatures at and above 40°C it rapidly lost activity. Invertase I was stable up to 50°C; it lost its activity completely after 12 minutes incubation at 75°C. The cell wall enzyme

TABLE 19

Purification of Soluble Enzyme

Fraction	Total Enzyme Units*	Total Protein (mg)	Specific Activity**
Crude extract	4117	438	9.4
Ammonium sulphate	2749	49	56.1
Sephadex G-100			
Invertase I	1639	7	234.2
Invertase II	423	3.2	132.2
α-glucosidase	78	1.2	65.3

Enzyme activity was determined as described in 'Methods'. Sucrose was used as substrate in invertase assay, and trehalose was used in α-glucosidase assay.

* One enzyme unit is defined as the amount of enzyme which catalyzes the hydrolysis of 1 μmole of substrate in 2 hours.

** Specific activity is expressed in units/mg protein.

FIGURE 22

Fractionation of invertase and α-glucosidase on Sephadex G-100

2 ml of ammonium sulphate enzyme containing approximately 50 mg protein were layered on Sephadex G-100 column (2.5 x 50 cm). The column was eluted with 0.05 M sodium phosphate-citric acid buffer, pH 7.0. Fractions of 2.5 ml were collected. The protein content, invertase activity, and α-glucosidase activity, in each fraction, were determined.

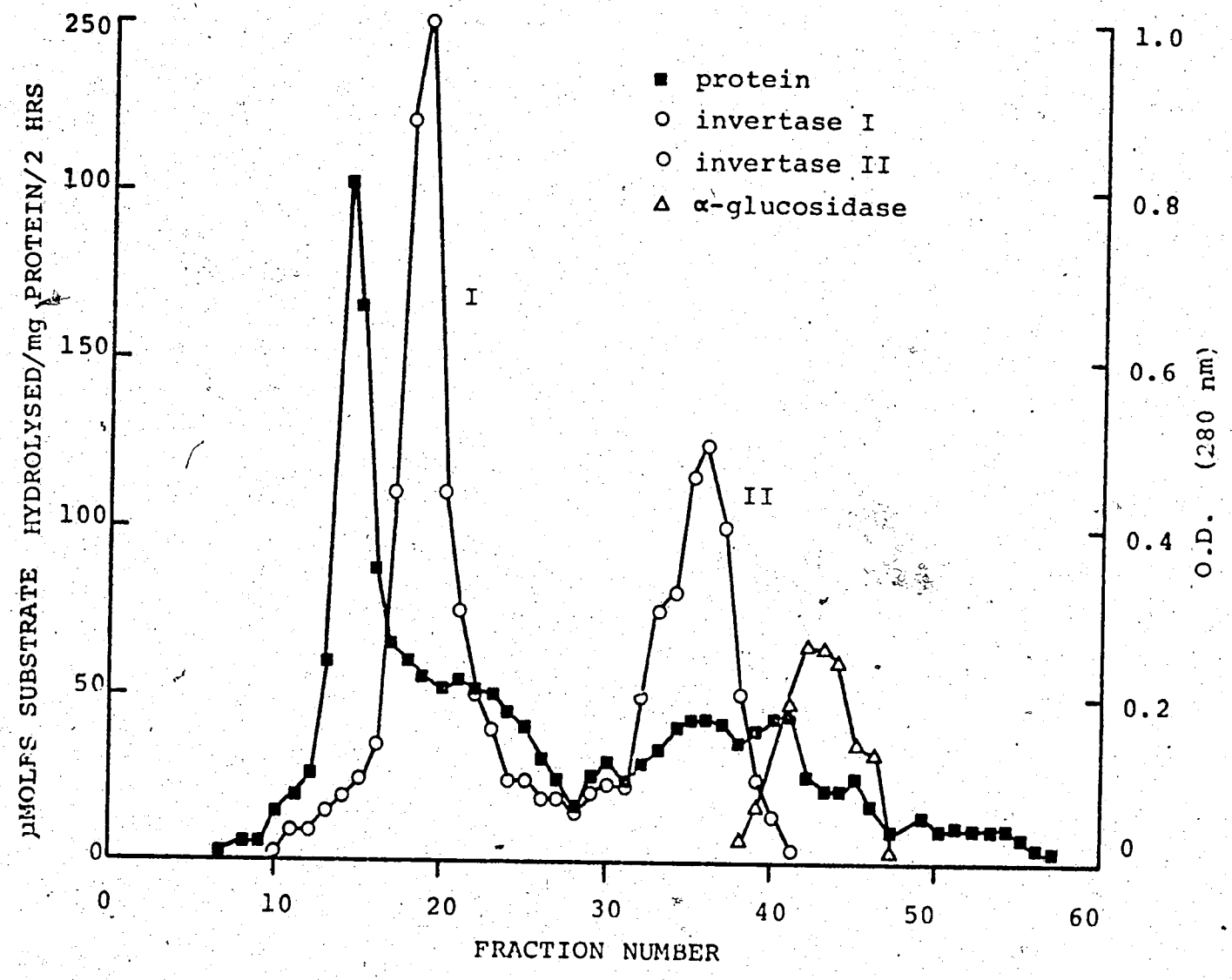

250
100
150
100
50
0
µMOLES SUBSTRATE HYDROLYSED/mg PROTEIN/2 HRS
protein
invertase I
invertase II
α-glucosidase
I
II
1.0
0.8
0.6
0.4
0.2
0
O.D. (280 nm)
0
10
20
30
40
50
60
FRACTION NUMBER

TABLE 20

Specificity of α-glucosidase and Invertases

Substrate	Enzyme Activity µmoles substrate hydrolyzed/mg protein/2 hrs		
	Invertase I	Invertase II	α-Glucosidase
Sucrose	93.7	68.7	12.7
Raffinose	70.8	41.6	0
Maltose	0	0	43.7
Trehalose	0	0	32.0

Reaction mixture contained 120 µmoles of substrate, 90 µmoles sodium phosphate-citric acid buffer, pH 4.8, and Sephadex G-100 treated enzyme (50 µg protein) in a final volume of 2 ml. Reaction mixture was incubated at 28°C.

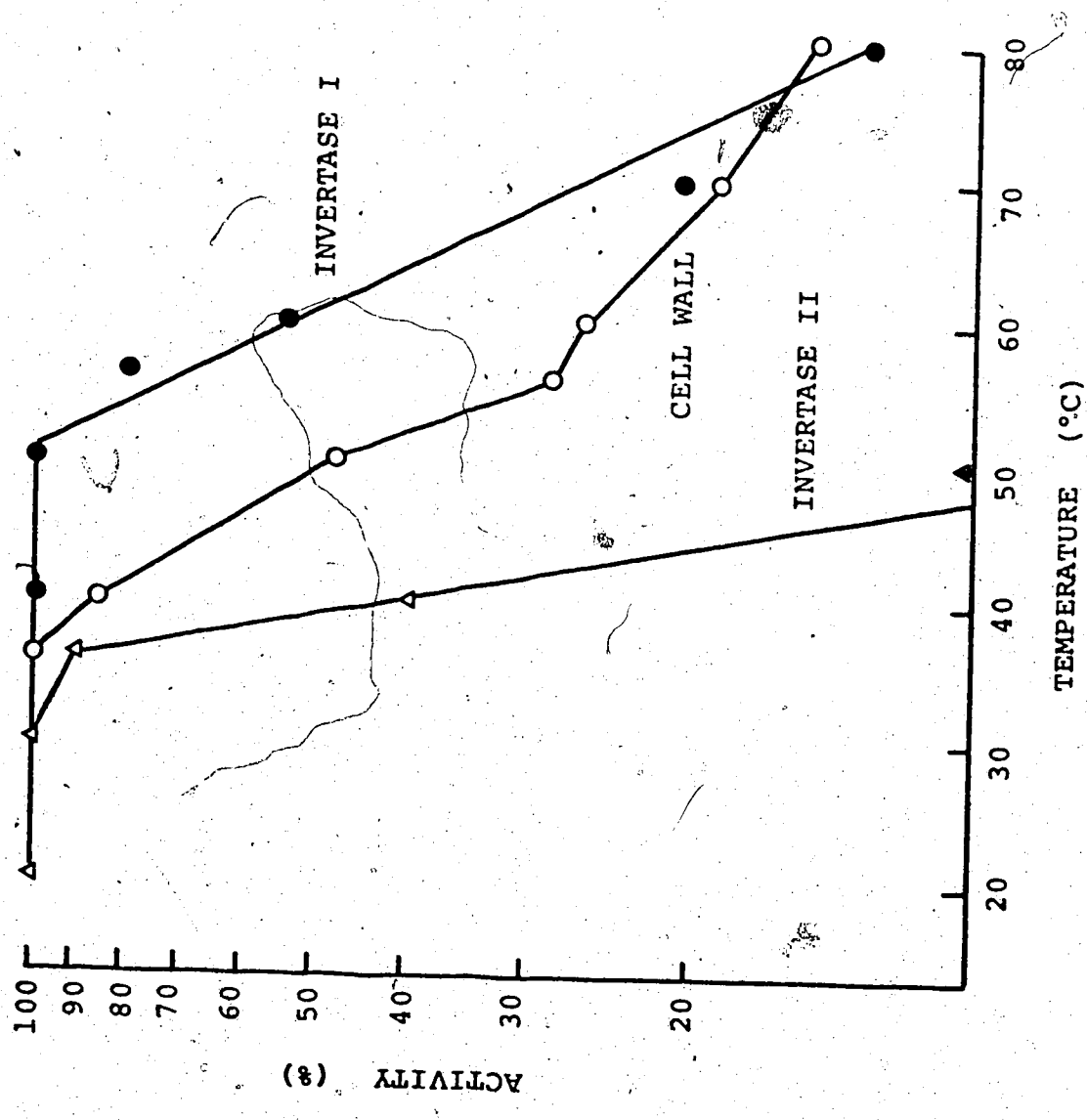
FIGURE 23

Effects of temperature on the stability of invertase I, invertase II, and cell wall invertase

Invertases were incubated at various temperatures for 12 minutes and then the activities determined.

The reaction mixtures contained 290 µmoles sucrose, 90 µmoles sodium phosphate-citric acid buffer, pH 4.8, and treated enzyme preparation in a final volume of 2 ml.

Reaction mixtures were incubated at 28°C for 2 hours.


INVERTASE I
CELL WALL
INVERTASE II
ACTIVITY (%)
100
90
80
70
60
50
40
30
20
20
30
40
50
60
70
80
TEMPERATURE (°C)

began to lose its activity at 45°C and after 12 minutes at 85°C it lost its activity completely.

To study the time course of heat inactivation of invertase, enzyme was incubated at 50°C and at intervals aliquots were removed and tested. Figure 24 shows that invertase II lost its activity in only 6 minutes. Invertase I lost activity at a rate slower than that of invertase II and at the end of 6 minutes it still retained 60% of its activity. The kinetics of inactivation of invertase I and II were essentially first order reaction. The inactivation of cell wall invertase, however, deviated from first order kinetics, suggesting that it might consist of a mixture of more than one enzyme.

E. The effect of pH on activity of invertase I and II.

The effects of pH on invertase I and II was studied with sodium phosphate-citric acid buffer. The pH curves of the two enzyme were very similar, both with pH optimum at 4.8 (Figure 25).

F. Effect of sucrose concentration on activity of invertase

The effects of sucrose concentrations from 20 to 100 mM on invertase I and II are shown in Figures 26 and 27, respectively. The Lineweaver-Burk plots for both invertases show a straight line. The apparent Michaelis constant calculated from the Lineweaver-Burk plot was 15 mM for invertase I and 18 mM for invertase II.

The effect of sucrose concentration of cell wall invertase was also determined (Figure 28). The Michaelis constant estimated from the Lineweaver-Burk plot as 25 mM.

FIGURE 24

Time course of heat inactivation of invertases

Invertases were incubated at 50°C and at 3 minute intervals aliquots were tested for activities.

The reaction mixtures contained 290 µmoles sucrose, 90 µmoles sodium phosphate-citric acid buffer, pH 4.8, and treated enzyme preparation in a final volume of 2 ml.

Reaction mixtures were incubated at 28°C for 2 hours.

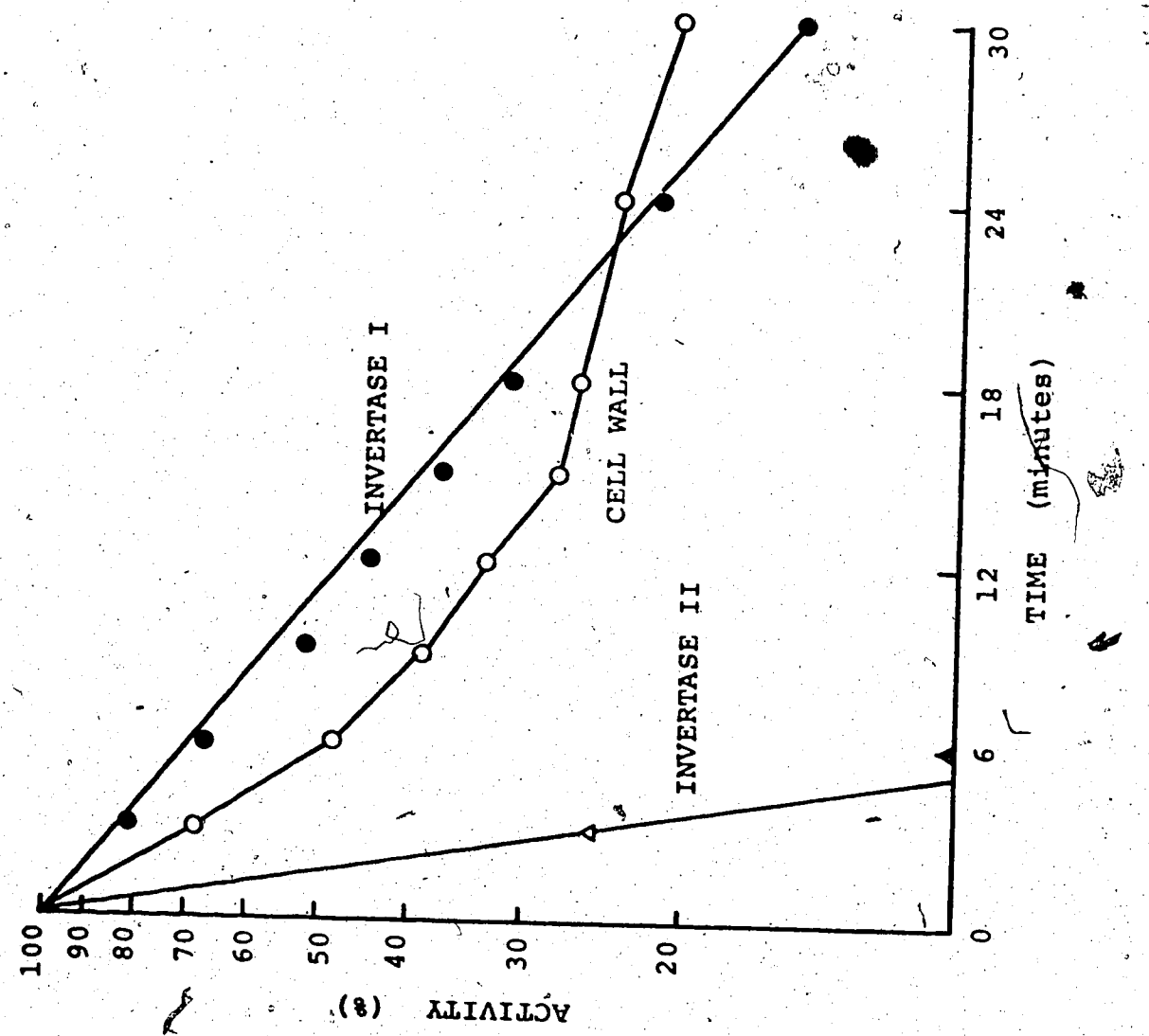

INVERTASE I
CELL WALL
INVERTASE II
ACTIVITY (%)
TIME (minutes)
100
90
80
70
60
50
40
30
20
0
6
12
18
24
30

FIGURE 25

Effect of pH on the activity of invertase I and invertase II

The reaction mixtures contained 290 µmoles sucrose, 90 µmoles sodium phosphate-citric acid buffer and enzyme preparation (0.05 mg protein) in a final volume of 2 ml.

Reaction mixtures were incubated at 28°C for 2 hours.

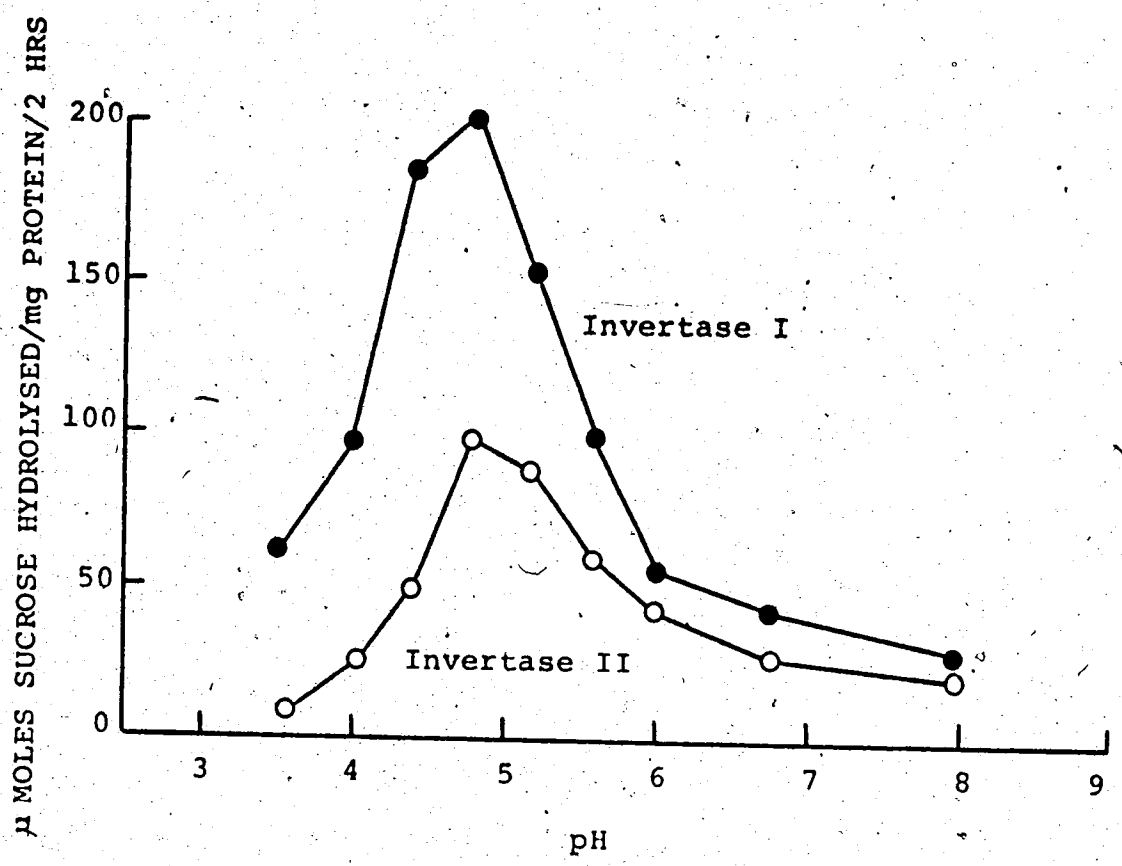

μ MOLES SUCROSE HYDROLYSED/mg PROTEIN/2 HRS
200
150
100
50
0
3
4
5
6
7
8
9
pH
Invertase I
Invertase II

FIGURE 26

Effect of sucrose concentration on invertase I activity

The reaction mixtures contained 90 µmoles sodium phosphate-citric acid buffer, pH 4.8, enzyme preparation (0.05 mg protein) and varying amounts of sucrose in a final volume of 2 ml.

The reaction mixtures were incubated at 28°C for 2 hours.

Inset: Lineweaver-Burk plot of 1/[sucrose] versus 1/v.

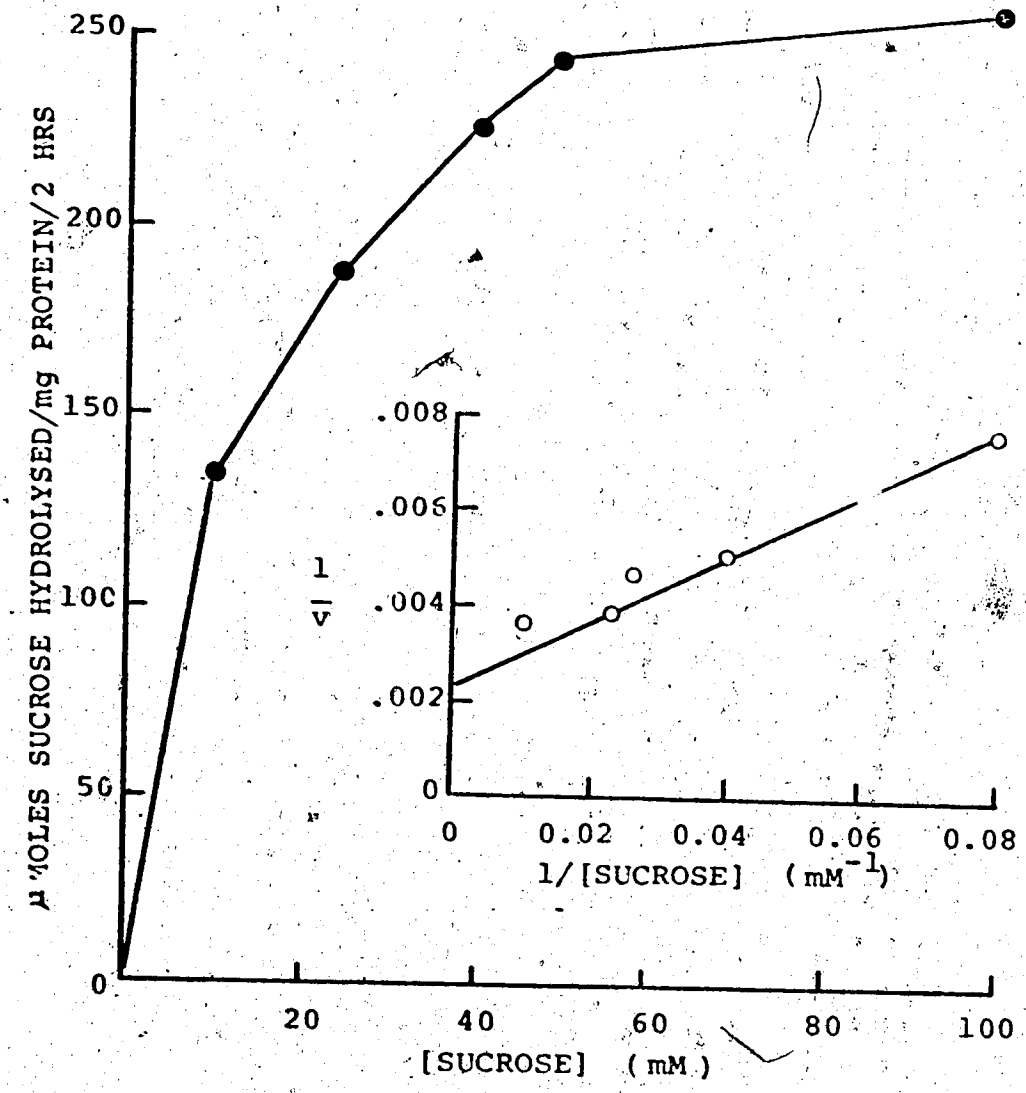

250
200
150
100
50
0
µ MOLES SUCROSE HYDROLYSED/mg PROTEIN/2 HRS
20
40
60
80
100
[SUCROSE] (mM)
.008
.006
.004
.002
0
1/v
0
0.02
0.04
0.06
0.08
1/[SUCROSE] (mM⁻¹)

FIGURE 27

Effect of sucrose concentration on invertase II activity

The reaction mixtures contained 90 µmoles sodium phosphate-citric acid buffer, pH 4.8, enzyme preparation (0.05 mg protein) and varying amounts of sucrose in a final volume of 2 ml.

The reaction mixtures were incubated at 28°C for 2 hours.

Inset: Lineweaver-Burk plot of 1/[sucrose] versus 1/v.

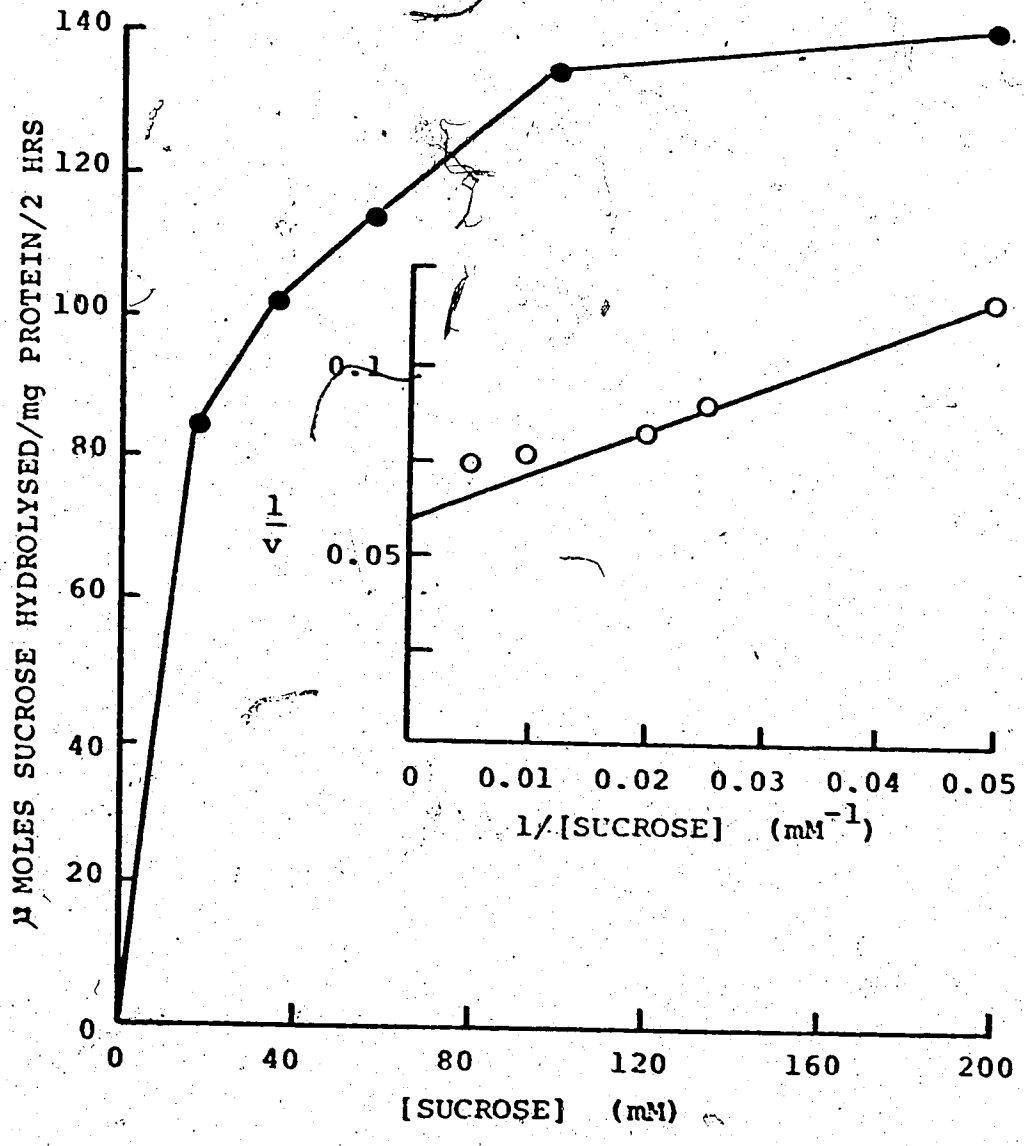

140
120
100
80
60
40
20
0
μ MOLES SUCROSE HYDROLYSED/mg PROTEIN/2 HRS
0
40
80
120
160
200
[SUCROSE] (mM)
0.1
0.05
1/v
0
0.01
0.02
0.03
0.04
0.05
1/[SUCROSE] (mM⁻¹)

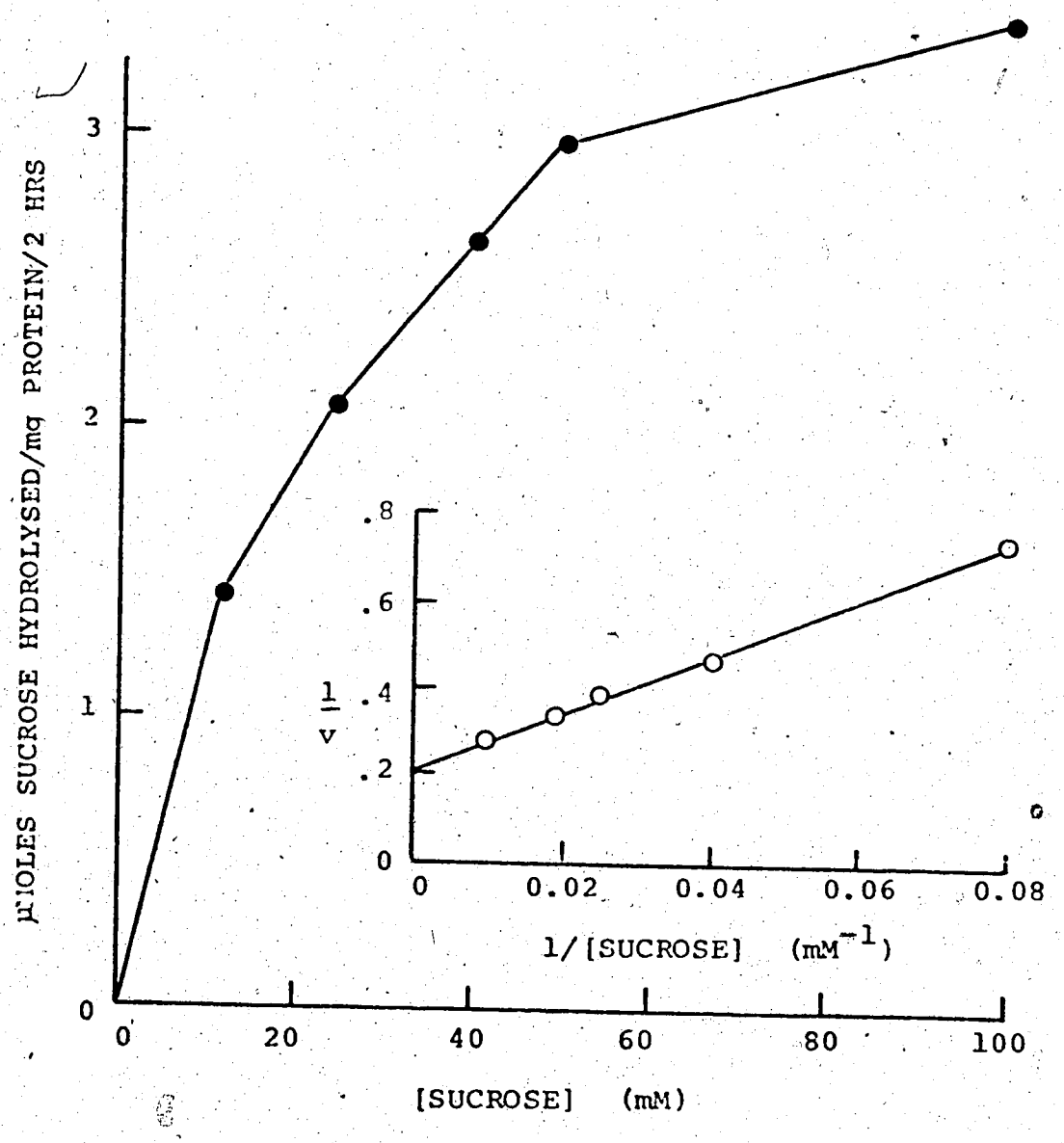
FIGURE 28

Effect of sucrose concentration on cell wall invertase activity

The reaction mixtures contained 90 µmoles sodium phosphate-citric acid buffer, pH 4.8, enzyme preparation (0.1 mg protein) and varying amounts of sucrose in a final volume of 2 ml.

The reaction mixtures were incubated at 28°C for 2 hours.

Inset: Lineweaver-Burk plot of 1/[sucrose] versus 1/v.


µMOLES SUCROSE HYDROLYSED/mg PROTEIN/2 HRS
0
1
2
3
0
20
40
60
80
100
[SUCROSE] (mM)
1/v
0
.2
.4
.6
.8
0
0.02
0.04
0.06
0.08
1/[SUCROSE] (mM⁻¹)

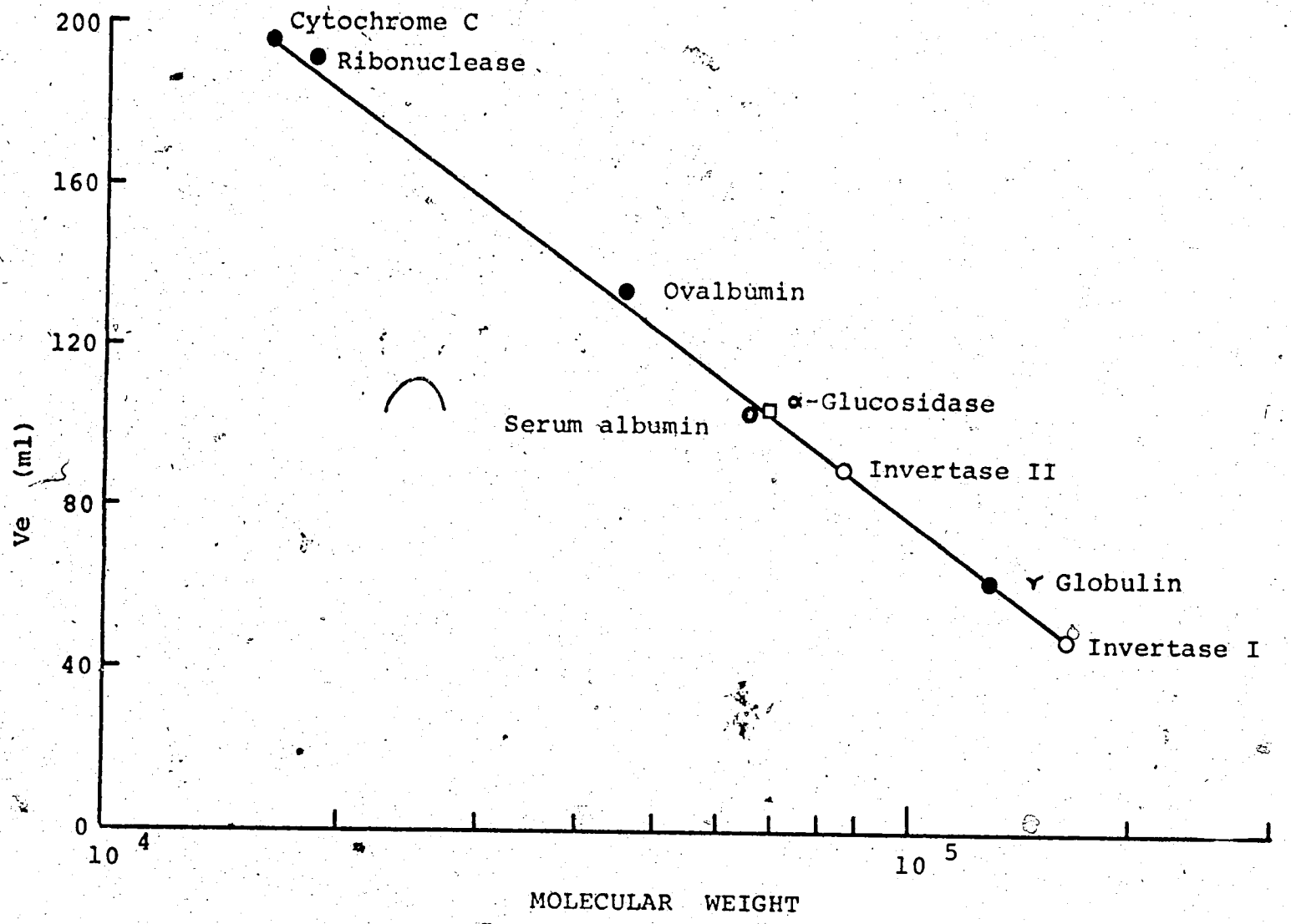
G. Molecular weight determinations

The molecular weights of the two invertases and α-glucosidase were estimated by the method of Andrews (1964) using Sephadex G-100 column (2.5 x 50 cm). Serum albumin, ovalbumin, cytochrome c, γ-globulin, and pancreatic ribonuclease were used as standards. The elution pattern of these proteins was measured from the optical extinction at 280 nm and the peaks of invertases and α-glucosidase determined from their activities. The molecular weight of invertase I was estimated to be 106,000 and that of invertase II 85,000. The α-glucosidase was estimated as 65,000 (Figure 29).

FIGURE 29

Sephadex G-100 filtration of invertases, α-glucosidase and marker proteins

Samples of invertases, α-glucosidase and marker proteins were applied to Sephadex G-100 column and eluted as described in 'Methods'. Activities of invertases and α-glucosidase were assayed. The positions of the marker proteins in the effluent were determined on the basis of extinction measurements at appropriate wavelengths (Andrews, 1964).


200
160
120
80
40
0
Ve (ml)
Cytochrome C
Ribonuclease
Ovalbumin
α-Glucosidase
Serum albumin
Invertase II
γ Globulin
Invertase I
10 4
10 5
MOLECULAR WEIGHT

DISCUSSION

There have been many studies designed to understand the preference of sucrose as carbon source for the growth of excised tomato roots. Although these studies have yet to produce a satisfactory answer, they have shown that the inability of the roots to grow well in glucose and fructose is not due to a deficiency in the absorption (Weston, 1967) or metabolism (Morgan and Street, 1959; Thomas *et al.*, 1963) of these sugars. Thomas and Weir (1967) concluded that tomato roots require a critical level of sucrose in their meristem, for a specific physiological process. Sucrose has indeed been reported to be involved in the differentiation of vascular tissue (Roberts, 1969; Wetmore and Rier, 1963; Jeffs and Northcote, 1967) and also to affect chloroplast formation (Edelman and Hanson, 1971; Edelman and Hanson, 1972). If sucrose is required by the roots for a morphogenetic process, excised tomato roots could either absorb it from the medium or synthesize it themselves. Thomas and Weir (1967) proposed that the critical level of sucrose required is established and maintained only by the provision of exogenous sucrose. Their hypothesis thus infers the absorption of undegraded sucrose and the inability of roots to synthesize sufficient quantities of sucrose.

The mechanism of sucrose absorption by higher plants varies with the tissues, *e.g.* bean endocarp (Sacher, 1966), tobacco leaves (Porter and May, 1955), and *Castor* bean endosperm (Kriedemann and Beevers, 1967) absorb sucrose intact, whereas the absorption of sucrose by sugar cane tissues requires prior hydrolysis (Hatch and Glasziou, 1963; Bowen and

Hunter, 1972). Experiments in Section I showed that when ^{14}C-fructosyl-sucrose was fed to excised tomato roots, almost all the sucrose recovered in the roots retained an asymmetrical labelling pattern. Further, the presence of large quantities of glucose or fructose during the feeding of ^{14}C-U-sucrose did not alter the uniform labelling pattern of sucrose in the roots. These data clearly indicate that prior hydrolysis was not essential, and that sucrose was absorbed intact by excised tomato roots. The results of the absorption experiments, therefore, are in favor of the hypothesis of Thomas and Weir (1967).

The validity of the hypothesis was further examined by studying the two enzymes involved in sucrose synthesis, *viz.* sucrose synthetase and sucrose phosphate synthetase. These two enzymes were detected only in glucose-grown but not in sucrose-grown roots (Section II). Thus, the synthesis of sucrose synthetase and sucrose phosphate synthetase appears to be similar to many repressible enzymes, *e.g.* tryptophan synthetase (Monod and Cohen-Bazire, 1953), asparagine synthetase (Ravel *et al.*, 1962), and glutamine synthetase (DeMars, 1958) which are repressed by the presence of high concentrations of product. The demonstration of sucrose synthetase and sucrose phosphate synthetase in glucose-grown roots appears to contradict the hypothesis of Thomas and Weir (1967). However, this contradiction can be reconciled by: (1) the possibility that the rate of sucrose synthesis by the glucose-grown roots is not fast enough to build up sufficient amount of sucrose required in the morphogenetic process, or (2) the synthesized and the absorbed sucrose are located in different pools and that only the latter is accessible to the morphogenetic process. The detection of sucrose synthetase and sucrose phosphate synthetase activities in glucose-grown roots, although not

conclusively ruling out the hypothesis of Thomas and Weir, nevertheless suggests that it is inadequate.

There is a similarity between the preference of sucrose over glucose and fructose and that of nitrate over ammonium ions by excised tomato roots. High growth rates of excised tomato roots are obtained with nitrate (Sheat *et al.*, 1959), however, when nitrate is replaced by ammonium ions at an equivalent nitrogen concentration, the growth is much inferior (Robbins and Schmidt, 1938). It has been observed that the presence of large amounts of ammonium ions tended to induce a deficiency of other cations, *e.g.* calcium, potassium, and magnesium in many plant tissues (Iwanova, 1934; Sideris and Young, 1944). An explanation for the superior growth-supporting ability of nitrate is that its utilization could allow excised tomato roots to regulate nitrogen metabolism and also avoid building up excess amounts of ammonium ions which are harmful to the roots. In a very similar way excised tomato roots prefer sucrose to glucose and fructose, which are intermediates of sucrose utilization. If glucose or fructose are in any way detrimental to excised tomato roots, and there is evidence that this is so (Weston, 1973), then, if there is a mechanism regulating the production of glucose and fructose from sucrose, these detrimental effects may be avoided by culturi ots in sucrose. This would then account for the preference for sucr e. This infers a relationship between growth and the enzymes involved in sucrose utilization. Several enzymes, *viz.* sucrose synthetase, sucrose phosphorylase, α-glucosidase, and invertase, are known to play a potential role in sucrose degradation; these enzymes in excised tomato roots were thus studied.

The reaction catalyzed by sucrose synthetase is reversible and

although the equilibrium constant in the direction of synthesis was calculated to be between 1.6 and 8, which is in favor of synthesis, (Cardini *et al.*, 1955), the enzyme is believed to involve sucrose utilization (Hawker, 1971; Grimes *et al.*, 1970). Its absence in excised tomato roots grown in sucrose medium (Section II, A), however, indicates that it is not important in sucrose utilization of these roots.

Sucrose phosphorylase was also not detected in excised tomato roots (Section III, C). Sucrose phosphorylase has been found mainly in bacteria (Doudoroff *et al.*, 1943; Kagan *et al.*, 1942; Doudoroff *et al.*, 1949) and reports on its presence in higher plants are scarce. Shukla and Prabhu (1960) and Pa[illegible]ya and Ramakrishnan (1956) reported its presence in sugar cane tissues. However, attempts by Hatch *et al.* (1963) to reproduce their results were unsuccessful, and Hassid and Doudoroff (1950) and Gibbs (1959) were unable to detect it in a number of plant tissues. In view of these results it is unlikely that this enzyme is important in sucrose utilization of higher plants.

Sucrose is hydrolyzed by two enzymes, invertase and α-glucosidase. The former attacks sucrose from the β-fructosyl end and the latter from the α-glucosyl end. In excised tomato roots, the cell wall possesses only invertase activity, whereas the cytoplasm possesses both invertase and α-glucosidase activities (Table 8) The soluble enzyme fraction attacked sucrose at a rate of 8.57 µmole sucrose hydrolyzed/mg protein/2 hours, and maltose at 0.95 µmole/mg protein/2 hours (Table 8). As α-glucosidase was approximately 3.5 times more active on maltose than on sucrose (Table 20), only three to four percent of the sucrose hydrolysis activity of the soluble fraction was accounted for by α-glucosidase activity. Thus, it is considered that for sucrose utilization in excised

tomato roots, invertase is a far more important enzyme than α-glucosidase.

As cells of different stages of development are spatially separated along the root axis, the correlation of growth and invertase activity of excised tomato roots was studied by examining the distribution of invertase activities along the root axis. A peak of cell wall invertase activity was found associated with the region of cell elongation (Figure 9), suggesting that growth and invertase activity are related. A correlation between growth and soluble invertase activity from the meristem to the zone of elongation was also observed (Figure 9). However, the high soluble invertase activity did not decline after the cells ceased to grow. The lack of correlation between growth and soluble invertase in the region where the cells ceased to grow may be accounted for as follows. The experiments with cycloheximide (Figure 10) showed that tomato root invertase was broken down only slowly; it had a half-time of degradation of 48 hours. On the other hand, the roots grew relatively rapidly; approximately 30 mm per day between day five and seven. Thus in the 0 to 15 mm segment studied, no cells were older than 12 hours and due to the long half-time of degradation of invertase no appreciable decline could be observed. This being the case, the question now arises as to how the cell wall activity fell as rapidly as it did. It is proposed that this was caused by the movement of part of it into the cytoplasm, a phenomenon observed by Vaughan and MacDonald (1967) and Copping and Street (1972), thus contributing to the maintenance of the high activity of the soluble fraction.

The growth rate and levels of invertases in excised tomato roots were affected by the source of carbon. Roots grown in sucrose had a much higher growth rate and invertase activities than those grown in

glucose, fructose, or raffinose (Table 11). When the roots were grown in sucrose the growth rate and invertase activity were related to the sucrose concentration (Table 13). At 1.5% sucrose, high growth rate and invertase activities were obtained. As the sucrose concentration declined, the growth rate and invertase activities also declined. These experiments indicated, therefore, that invertase activities and growth were related.

Invertase synthesis in yeast and sugar cane has been found to be repressed by hexoses (Dodyk and Rothestein, 1964). However, tomato roots grown in sucrose medium supplemented with glucose had slightly higher invertase activities than roots grown in the same concentration of sucrose alone (Table 12), indicating that glucose did not repress invertase synthesis in excised tomato roots. In all likelihood, invertase synthesis is probably regulated by different mechanism in different plant tissues. Thus, Copping and Street (1972) observed that in sycamore cells invertase activities were the same irrespective of whether sucrose or glucose was used as carbon source. Also, the development of acid invertase activity in both tissue cultures and disks of storage tissue of carrot was not reduced by exogenous hexose (Pricardo *et al.*, 1972).

The close correlation between growth and invertase suggests that one may be regulated by the other. Although both growth and invertase activities were affected when roots were transferred from one sucrose concentration to another, the change in invertase activities preceded the change in growth rate (Section V, H). The results suggest that sucrose first exerted its effect on invertase and that the effect of sucrose on growth was possibly mediated through invertase. The

suggestion that invertase activities were the primary effect of sucrose was also supported by the results of incubating the roots at low temperature. When roots were incubated at 5°C the growth rate was drastically reduced (Table 14), but the invertase activities of these roots were found to be similar to roots grown in the same sucrose concentration but incubated at 28°C. Thus in excised tomato roots, the presence of an optimum amount of sucrose is a critical factor for high invertase activities. The induction of enzyme synthesis by its substrate has been demonstrated for many enzymes, *e.g.* β-galactosidase of *E. coli* (Jacob and Monod, 1961), nitrate reductase in high plants (Hewitt *et al.*, 1967; Ingle, 1966), thymidine kinase in wheat embryos (Stern, 1966). As sucrose is the substrate of invertase it appears that the increase in invertase activity can be ascribed to induction by sucrose. Recently, sucrose was also found to increase the invertase levels of *Avena* internode (Kaufman *et al.*, 1973). However, raffinose, which is also a substrate of invertase (Table 20) failed to increase the invertase activities. There is a possibility that raffinose is not absorbed readily by tomato roots, as raffinose supported only a very low level of growth (Table 11) and respiration (Morgan and Street, 1959). Moreover, it is partially degraded by cell wall invertase (Straus, 1962) and may not be absorbed intact. Thus the failure of raffinose to induce the production of invertase may not be evidence against the control of invertase levels by substrate induction.

It has been reported that the growth rate of excised tomato roots in a low sucrose concentration (0.5%) could be raised by the addition of appropriate amounts of GA (Butcher and Street, 1960), or NAA (Weston and Street, 1968b). As plant growth regulators have been known to affect the

production of invertase in many plant tissues (Cherry, 1968; Edelman and Hall, 1964; Sacher *et al.*, 1963; Seitz and Lang, 1968; Kaufman *et al.*, 1968) it is possible that the regulators first exert their effects on invertase production which in turn affects growth rates. In this study when the roots were treated with ABA a clear correlation between growth and invertase activities was observed (Section VI, E). A less obvious correlation was obtained with GA treatment (Section VI, B). However, no correlation between growth and invertase activities was found when roots were treated with NAA and kinetin. Plant growth regulators are capable of influencing a variety of aspects of growth, *e.g.* auxins have been found to increase the elasticity and plasticity of the cell wall (Burstrom *et al.*, 1970), enhance cytoplasmic streaming (Jackson, 1960), affect the permeability of the plasma membrane (Cocking, 1961); kinetin has been reported to affect mitosis (Guttman, 1956; Torrey, 1961), and cell enlargement (Miller, 1956; Scott and Liverman, 1956); NAA, GA, ABA and benzyladenine were found to enhance the ageing of roots cultured in 1% sucrose medium (Weston, 1973). As the effect of growth regulators on growth is multiple, their effect on other aspects of growth could conceivably disturb the correlation between invertase and growth.

The promotion of growth by GA and NAA reported by Butcher and Street (1960), and Weston and Street (1968b) was not observed in this study. Instead, a slight reduction of growth was obtained with GA treatment, and no significant effect was observed with NAA. Charles (1959) found that roots from different geographical strains of *Senecio vulgaris* differed markedly in their growth rate in a standard auxin-free medium. In addition, he showed that all of them could be raised to a

similar and very high level of growth by adding appropriate amounts of 2-naphthoxyacetic acid. Slowly-growing strains required more 2-naphthoxyacetic acid than fast growing strains, indicating that roots of different clones could possess different amounts of endogenous hormone. Although Sutton's 'Best of All' variety was used in this study and those of Butcher and Street, and Weston and Street, the roots in this study were of a different clone. The variation in results was, therefore, probably due in part, at least, to this fact. The main axis of the roots used in this study increased approximately by 100 mm in 7 days whereas those of Butcher and Street (1960) and Weston and Street (1968b) increased by only approximately 30 mm. The vast difference in growth rate strongly indicates that for the roots used in this study 0.5% sucrose did not represent a markedly sub-optimal medium; thus growth regulators did not enhance growth.

The consistent correlation between invertase activities and growth observed in many experiments in this investigation supports the contention that invertase has a role in growth. As the function of invertase is to hydrolyze sucrose into glucose and fructose, its role in growth might thus be controlling the levels of these hexoses in the roots. The detrimental effect on growth of accumulation of glucose is well known for many animal tissues (Dickens *et al.*, 1968). Therefore, the glucose concentration is properly maintained by an elaborate system (Dickens *et al.*, 1968). Recently, Weston (1973) reported a specific effect of glucose on excised tomato roots. He observed that pre-treating the roots with glucose markedly enhanced ageing of the roots on subsequent sub-culture in sucrose medium as compared to pre-treating roots with sucrose or fructose. There is no data on how fructose may affect

the metabolism of excised tomato roots. However, the very poor growth rate of roots in fructose and glucose suggests that high concentrations of hexoses are deleterious to growth. However, the results of growth experiments in a mixture of sucrose and glucose (Table 12) tend to disagree with this suggestion. It has been reported that sucrose reduced the absorption of glucose (Weston, 1973) from the mixture of the two. Thus, possibly due to the inhibition of glucose absorption by sucrose, the roots grown in a mixture of sucrose and glucose might not accumulate glucose to a harmful concentration and consequently would not impair the growth of the roots.

In other studies in which a correlation between invertase activity and growth was found, several specific functions of invertase were proposed. Hatch *et al.* (1963) and Bowen and Hunter (1972) have produced evidence that the inversion of sucrose in the free space was essential for sugar absorption. However, Weston and Street (1968a) observed that the alteration of external pH, while drastically altering the levels of glucose and fructose appearing in the medium, did not affect sucrose absorption. In the present study, it was found that sucrose was absorbed intact (Section I). Thus it is believed that, unlike sugar cane, excised tomato roots do not have to rely on invertase for sucrose absorption. Hedlebust and Forward (1962) proposed that by the inversion of sucrose, invertase could be involved in maintaining the osmotic pressure of the cell sap during growth. While the soluble invertase could have such a function, the cell wall invertase, due to its location, clearly could not play such a role. Indeed, the action of the cell wall enzyme on apparent-free-space sucrose would tend to reduce the water potential in that region and thus reduce growth. Possibly the cell wall

invertase may be involved in providing substrates for the biosynthesis of the cell wall. In the synthesis of the cell wall the pectins and hemicellulosic polysaccharides are believed to be synthesized within the membrane complex of the Golgi body and its associated vesicles (Harris and Northcote, 1971). The cellulose microfibrils are probably synthesized at the plasmalemma (Northcote, 1969). Therefore, the cell wall and cell wall invertase are not likely to be essential in the synthesis of cell wall materials. Thus the role of invertases in excised tomato roots could not be satisfactorily or adequately accounted for by the functions suggested. It is proposed that the role of invertases of excised tomato roots is to regulate the flow of carbohydrates for biosynthesis and respiration. This role could be performed by both the invertase in the cell wall and in the cytoplasm. The importance of invertase lies in the fact that a proper flow of carbohydrates could ensure an adequate supply of substrates for biosynthesis and respiration, and prevent building up intermediates that might impair growth.

Approximately 20% of the total invertase activity of excised tomato roots was consistently found in the cell wall. Hawker (1969) found that carbowax 4000, Tween 20, and borate buffer released the wall-bound invertase of grapes and concluded that cell wall invertase of grape was an artifact of extraction. The same treatments, however, had no effect on the cell wall invertase of excised tomato roots (Table 16). Thus the cell wall invertase of tomato roots cannot be due to an artifact of extraction. The invertase in the wall could conceivably be bound to membrane imbedded in the cell wall. To test this possibility the cell wall fraction was treated with Triton X-100, and deoxycholate. Triton

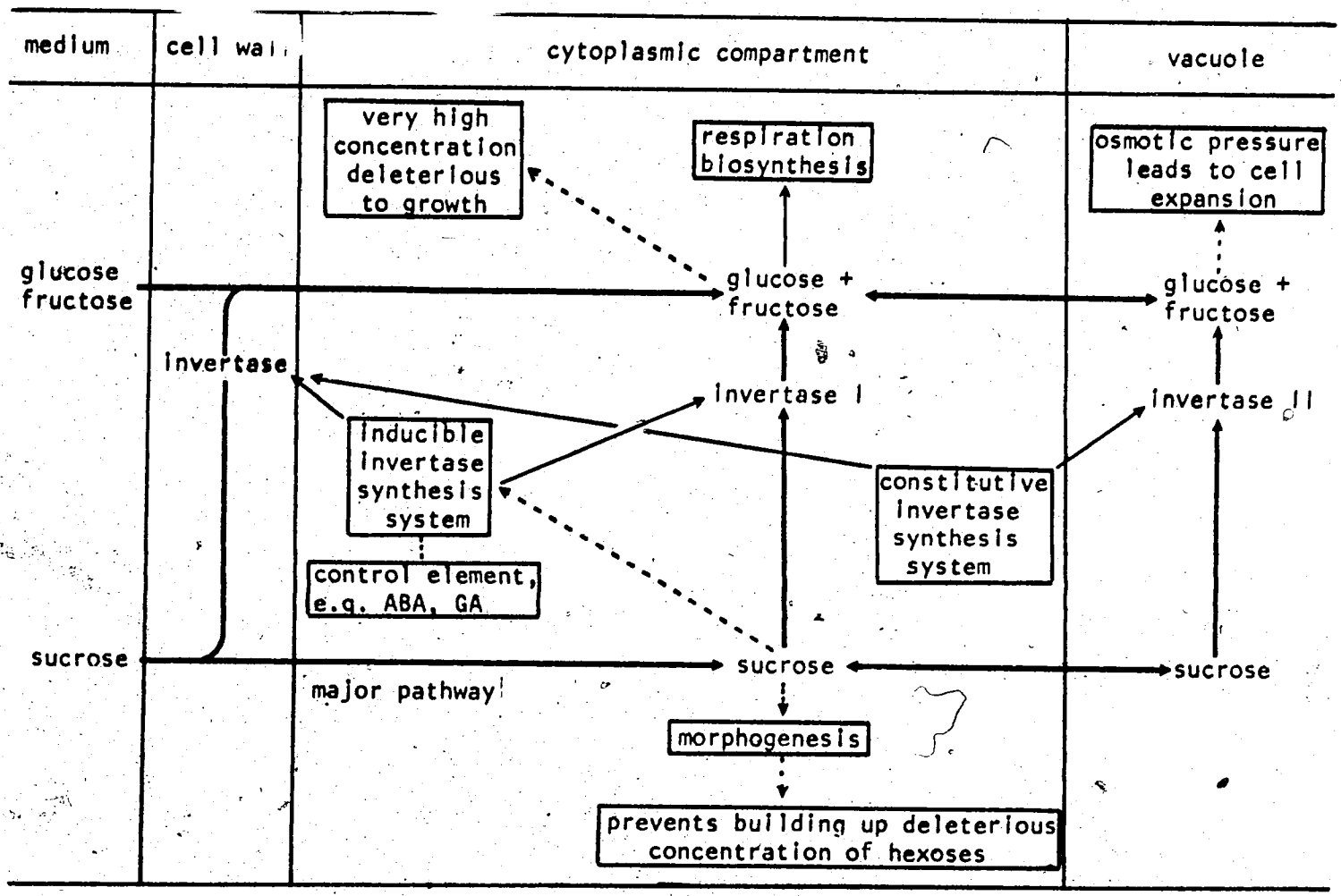
X-100 is a non-ionic detergent known to cause deterioration of cell membranes and has been used widely to solubilize membrane-bound enzymes from animal and plant tissues (Wattiaux and de Duve, 1956; Sahan, 1965; Boll, 1970). The ionic detergent deoxycholate is also used extensively to solubilize cell membranes (Matile *et al.*, 1967; Munoz *et al.*, 1970). No significant amount of invertase could be released from the wall by these two detergents (Table 16) indicating that the cell wall invertase of excised tomato roots was not the result of the presence of membrane-bound invertase.

Ricardo and Ap Rees (1970) showed that the distribution of acid invertase activity in homogenates of aged carrot disks depended on the pH of the extraction medium. A similar result was obtained in the present study. However, the effect of pH was found to be less in tomato roots than that observed with carrot disks (Table 17). The release of some invertase from the wall by high pH shows that at least some invertase was observed in the wall by ionic forces. However, no pH that has been used could remove all the invertase from the cell wall, indicating that in addition to ionic forces other forces are involved in binding the invertase to the wall.

The partial purification of the soluble fraction by ammonium sulphate fractionation and Sephadex G-100 gel filtration resolved two invertase isozymes. The two invertase isozymes attack sucrose and raffinose, but not maltose and trehalose (Table 20), indicating that they are genuine β-fructofuranosidase. These two invertase isozymes both had optimum activity at pH 4.8 and thus are acid invertases. The K_m values for sucrose for these two isozymes were quite similar; 15 mM for invertase I and 18 mM for invertase II. These values were higher

than those of the two isozymes of maize endosperm (2 mM and 10.5 mM, Jaynes and Nelson, 1971). However, they were lower than the 26 mM and 25 mM reported for yeast external (Gascon *et al.*, 1968) and internal (Gascon and Lampen, 1968) invertases respectively. Invertase I, with a molecular weight of 106,000, was smaller than the external (M.W. 270,000, Gascon *et al.*, 1968) and internal (135,000, Gascon and Lampen, 1968) invertases of yeast, and was slightly larger than the invertase of barley root (M.W. 92,000, Neville, 1972). The molecular weight of invertase II of 85,000 was smaller than that of the external and internal invertases of yeast but is close to that of the invertases of barley root. The tomato root invertase isozymes could best be distinguished by their thermostability. Invertase I remained stable at temperatures up to 50°C, whereas invertase II was stable only up to 35°C. The half-time of degradation at 50°C for invertase I was approximately 12 minutes and that for invertase II was only approximately one minute. In this respect, the two isozymes are similar to the heat-stable heavy and heat-labile light invertase of *Neurospora crassa* (Metzenberg, 1964).

Based on the results in this investigation a scheme is presented to illustrate the relationship between the sugars, plant growth regulators, invertase activities and growth of excised tomato roots (Page 148). As depicted in the scheme sucrose is either absorbed intact or in lesser extent hydrolyzed by the invertase in the cell wall and then absorbed. The absorbed sugars could remain in the cytoplasm or move into the vacuoles. It is speculated that invertase is present in both cytoplasmic and storage compartments and that invertase in these two compartments is of different species; invertase I in the cytoplasm



A scheme illustrating the relationship between the sugars, plant growth regulators, invertase activities and growth of excised tomato roots.

and invertase II in the vacuoles. Although efforts to characterize the cell wall invertase were not successful, it is suggested that cell wall invertase derives from soluble invertases and thus like soluble invertases consists of two species. Invertase activity in excised tomato roots is induced by sucrose (Section V). However, as roots grown in the absence of sucrose still possess a considerable amount of invertase activity it is proposed that the excised tomato roots have two invertase synthesis systems; one of them is inducible and the other is constitutive. The inducible system is regulated by sucrose, and some plant growth regulators, *e.g.* ABA and GA. The glucose and fructose formed by the action of invertase serve as substrates for respiration and biosynthesis. The conversion of sucrose into glucose and fructose in the vacuoles should increase the osmotic pressure and lead to cell expansion. In addition to its effect on invertase synthesis, sucrose is believed to induce morphogenesis. As a consequence, a demand for hexoses, which are required to furnish the energy and biosynthetic substrates for the morphogenesis, is created. The dual action of sucrose on morphogenesis and invertase synthesis thus prevents the levels of hexoses from building up too high. However, if hexoses are supplied to the roots directly, the action of sucrose and invertase is by-passed, and the accumulation of high concentrations of hexoses would then impair the growth of the roots.

Concluding remarks

In this study a hypothesis is put forward that regulation of carbohydrate metabolism by invertase is important to the growth of the excised tomato roots. The results of the present work appear to

support this, but, obviously more work is required to substantiate or refute this hypothesis.

It would be useful if a method could be found to specifically alter the invertase levels and then observe the effect on growth. To inhibit the invertase with inhibitors is one way to alter the invertase activity. Metal ions, *e.g.* Hg, Ag, Cu, Pb, have been found to inhibit yeast invertase (Myrback, 1960). However, these ions also affect the activity of many other enzymes (Dixon and Webb, 1967) and the use of them would not pinpoint the role of invertase in growth. Specific invertase inhibitors have been found in potato (Pressey, 1967), red beet, sugar beet and sweet potato (Pressey, 1968); they are protein molecules and therefore probably not capable of penetrating the plasma membrane. If these inhibitors also inhibit tomato root invertase they would allow the determination of the effect of the cell wall invertase on growth. Bowen and Hunter (1972) were successful in obtaining a rabbit anti-invertase serum and used it to block sucrose transport into sugar cane tissue by inactivating the cell wall invertase. Thus the rabbit anti-invertase serum might be an effective tool to study the function of cell wall invertase of excised tomato roots. To study the role of soluble invertase would require specific inhibitors of small molecular weight which could enter the tomato roots cells freely. No such specific inhibitors are known at present. A search for these specific inhibitors would be rewarding since it might lead to an understanding of the role of soluble invertase.

The invertase of excised tomato roots is apprently inducible by substrate. If a substrate of the enzyme, other than sucrose, that could penetrate readily into the cell, could be found, then it could be

used to study its effect on invertase synthesis and growth Although sucrose could raise the invertase levels, the fact that the roots still possessed a considerable amount of invertase activity in the absence of sucrose (Table 11) and that two invertase isozymes were found (Figure 22) suggested that one of them may be synthesized constitutively and the other induced by sucrose. This suggestion could be tested by purifying the soluble invertases of roots grown in glucose or fructose. If only one type of invertase was found in glucose or fructose-grown roots then this is evidence for the contention that the synthesis of one of the enzymes depends on the presence of sucrose. If the level of one of the two invertases is related to the sucrose concentration, this would show that the degree of induction is related to sucrose concentration.

Due to the inability to release the cell wall invertase from the wall, this enzyme was not purified. Probably the cell wall invertase also consists of two species. If a way could be found to release the enzyme from the wall then the purification and characterization of the cell wall enzyme should add information to the biosynthetic relationship of the soluble and cell wall invertases of excised tomato roots.

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