Invertases. Primary Structures, Functions, and Roles in Plant Development and Sucrose Partitioning

Arnd Sturm*

Friedrich Miescher Institute, Maulbeerstrasse 66, CH–4058 Basel, Switzerland

One of the key features of plants is their ability to reduce carbon dioxide in the presence of sunlight and water to sugars, and the subsequent transport of assimilated carbon to the nonphotosynthetic tissues (sink tissues). In most plants, the transported sugar is Suc, a nonreducing disaccharide, in which Glc and Fru are linked (α1→β2). Depending on the physiological activities and the biochemical needs of the heterotrophic tissues, Suc is channeled into various pathways in different subcellular compartments. It may enter glycolysis and the tricarboxylic acid cycle for the production of ATP and NADH. The carbon of the disaccharide may also be used for the biosynthesis of primary metabolites important for tissue growth and development. Likewise, Suc may become converted into polymers such as starch, triacyl glycerides, or polypeptides for long-term storage, or into secondary compounds, enabling plants to cope with predators and pests or other environmental challenges. Many of these processes may take place in the same cell at the same time and, thus, allocation of Suc to these different processes requires efficient and precise control mechanisms.

Utilization of Suc as a source of carbon and energy depends on its cleavage into hexoses, and in plants either Suc synthase (EC 2.4.1.13) or invertase (EC 3.2.1.26) catalyzes this reaction. Suc synthase is a glycosyl transferase, which converts Suc in the presence of UDP into UDP-Glc and Fru. Invertase is a hydrolase, cleaving Suc into the two monosaccharides. Suc synthase is a cytoplasmic enzyme and, in most plants, two closely related isoforms have been identified (for summary, see Sturm et al., 1999). Invertase exists in several isoforms with different biochemical properties and subcellular locations (Sturm et al., 1996; Tymowska-Lalanne and Kreis, 1998). The specific functions of the different invertase isoforms are not clear, but they appear to regulate the entry of Suc into the different utilization pathways. Because sugars in plants are not only nutrients but also important regulators of gene expression (Koch, 1996), invertases may be indirectly involved in the control of cell differentiation and plant development.

**PLANT CELLS HAVE VACUOLAR, EXTRACELLULAR (CELL WALL), AND CYTOSOLIC INVERTASES**

Most plant species contain at least two isoforms of vacuolar invertase, which accumulate as soluble proteins (soluble acid invertases) in the lumen of this acidic compartment. Likewise, several isoforms of extracellular invertase (cell wall invertases) that are ionically bound to the cell wall have been detected. Vacuolar and cell wall invertases share some biochemical properties, e.g. they cleave Suc most efficiently between pH 4.5 and 5.0 and attack the disaccharide from the Fru residue. Thus, these so-called acid invertases are β-fructofuranosidases and also hydrolyze other β-Fru-containing oligosaccharides such as raffinose and stachyose. Additionally, plants have at least two isoforms of cytoplasmic invertase with pH optima for Suc cleavage in the neutral or slightly alkaline range. Neutral and alkaline invertases are less well characterized but, in contrast to the acid invertases, these enzymes appear to be Suc specific.

**VACUOLAR AND EXTRACELLULAR INVERTASES HAVE SIMILAR ENZYMATIC PROPERTIES**

Acid invertases have been purified from several plant species (for summary, see Unger et al., 1992). The enzymes have a $K_m$ for Suc in the low-millimolar range. Activity is inhibited by heavy metal ions such as Hg$^{2+}$ and Ag$^{+}$, suggesting the presence of a sulfhydryl group at the catalytic site. Acid invertases are also inhibited by their reaction products, with Glc acting as a non-competitive inhibitor and Fru as a competitive inhibitor. The mature polypeptides are N-glycosylated and the majority have molecular masses between 55 and 70 kD. Analysis of some of the purified proteins on denaturing SDS gels under reducing conditions revealed the presence of proteolytic fragments. A 70-kD monomeric form of vacuolar invertase of mung bean hypocotyls was found to be split into a 30-kD N-terminal and a 38-kD C-terminal fragment (Arai et al., 1991). Likewise, the 68-kD monomer of isoform I of vacuolar invertase from carrot was fragmented into N- and C-terminal polypeptides of 43 and 25 kD, respectively (Unger et al., 1992, 1994). Under native conditions, these fragments appear to be tightly associated and, in a complex, possess enzyme activity. Fragmentation does not appear to be an artifact of protein purification, but instead seems to be under developmental control. For example, the full-length proteins predominate in very young hypocotyls of bean and seedlings of carrot, whereas with increasing hypocotyl and seedling age the fragments were more abundant. Whether fragmentation has a physiological function is not clear.

---

* E-mail sturm@fmi.ch; fax 41–61–697–39–76.
VACUOLAR AND EXTRACELLULAR INVERTASES HAVE SOME COMMON MOLECULAR FEATURES BUT DIFFER IN IMPORTANT RESPECTS

Like plant invertase, yeast invertase exists in different isoforms with discrete subcellular locations (Carlson and Botstein, 1982). A secreted form of the enzyme is highly N-glycosylated and its expression is regulated by Glc repression. Cytoplasmic yeast invertase is a nonglycosylated polypeptide that is constitutively expressed at low levels. The two isoforms are encoded by the same gene and the two transcripts originate from differential splicing of a heteronuclear mRNA. In contrast, cDNA cloning of plant acid invertases revealed that each isoform is encoded by a different gene (Sturm, 1996; Tymowska-Lalanne and Kreis, 1998). The polypeptides encoded by these genes can be divided into two main classes with different properties. One class consists of cell wall invertase polypeptides with a basic pI, and the second class comprises vacuolar invertase polypeptides with an acidic pI.

The first cloned plant acid invertase was cell wall invertase from carrot (Sturm and Chrispeels, 1990). Since its isolation in 1990, more than 20 sequences of cDNAs for acid invertases from mainly dicot species have been deposited into the databases (Tymowska-Lalanne and Kreis, 1998). The cDNA-derived amino acid sequences all belong to a large protein family and are related to the sequences of invertases from yeast and bacteria. Common features are the pentapeptide NDPNG (βF-motif), which is close to the N terminus of the mature proteins, and a Cys residue and its neighboring amino acids, which are located closer to the C terminus (Fig. 1). It can be assumed that these conserved sequences are important constituents of the catalytic domain of acid invertases.

Comparison of the amino acid sequences derived from the cDNAs for acid invertases with the N-terminal sequences of the mature polypeptides revealed the presence of N-terminal domains up to 100 amino acid residues long, which most likely consist of a signal peptide and an N-terminal propeptide (Fig. 1) (Sturm and Chrispeels, 1990; Unger et al., 1994). The function of the putative propeptides is not clear, but in analogy to other “proenzymes,” they may play a role in protein folding (Klionsky et al., 1988), protein targeting (Klionsky et al., 1988), or in the control of enzyme activity (Hasilik and Tanner, 1987).

In comparison with the sequences of plant cell wall invertases, those of the vacuolar invertases appear to have short C-terminal extensions (Fig. 1) (Unger et al., 1994). The corresponding domains of isoform I and II of vacuolar invertase of carrot contain short hydrophobic amino acid stretches, which, in analogy to studies on barley lectin and tobacco chitinase, may form the critical cores of vacuolar sorting signals (Bednarek and Raikhel, 1992).

THE BIOCHEMICAL PROPERTIES OF NEUTRAL AND ALKALINE INVERTASES CLEARLY DIFFER FROM THOSE OF THE ACID INVERTASES

Because the plant invertases with pH optima between 7.0 and 7.8 are extremely labile and enzyme activity is rapidly lost after tissue homogenization, their purification turned out to be very difficult. Only in a few cases were polypeptides of apparent electrophoretic homogeneity obtained (for summary, see Ross et al., 1996). With the exception of alkaline invertase from carrot (Lee and Sturm, 1996), the native polypeptides are homotetramers composed of subunits with a molecular mass of 54 to 65 kD. The polypeptides are not N-glycosylated and preferentially hydrolyze Suc with a Km of 10 mM. They are strongly inhibited by Glc, Fru, and Tris, but not by heavy metal ions, suggesting marked differences between the catalytic sites of neutral/alkaline and acid invertases.

NEUTRAL AND ALKALINE INVERTASES ARE UNIQUE TO PLANTS AND PHOTOSYNTHETIC BACTERIA

Recently, a cDNA was obtained from poison rye grass (Lolium temulentum) that codes for a polypeptide with neutral/alkaline invertase activity (Gallagher and Pollock, 1998). The clone was isolated by a functional assay in which Escherichia coli cells were transformed with an L. temulentum cDNA library and then screened for cells capable of growth on minimal medium supplemented with Suc as the sole carbon source. In extracts of these cells, Suc was cleaved into Glc and Fru, with maximal activity in the pH range of 7.0 to 7.5 and a Km of approximately 18 mM. The enzyme is not a β-fructofuranosidase and was inhibited by Tris and Fru. The cDNA clone codes for a protein of 571 amino acids with an estimated molecular mass of approximately 63 kD.

Concomitant with the cloning of L. temulentum cDNA, a carrot cDNA was isolated by using an internal tryptic peptide sequence of the purified neutral invertase (Sturm et al., 1999). The deduced sequence shares 71% similarity (63% identity) with the sequence encoded by the L. temulentum cDNA, suggesting that the two clones code for related polypeptides with possibly similar roles in Suc metabolism. The N termini of the sequences differ markedly, whereas a domain of approximately 450 amino acid residues is highly conserved. Four amino acid residues (NDPNG) of the pentapeptide NDPNG, which is a hallmark.

Figure 1. Schematic comparison of the amino acid sequences deduced from cDNAs of carrot cell wall invertase and isoenzymes I and II of carrot vacuolar invertase (Unger et al., 1994). The mature proteins share 64% similarity and 46% identity. The amino acid sequence NDPNG is a hallmark of plant acid invertases (βF-motif; Sturm and Chrispeels, 1990). The sequence WECXDF indicates a highly conserved peptide domain consisting of a Cys residue and a few neighboring amino acids (Sturm and Chrispeels, 1990).
of the acid β-fructofuranosidases (Sturm and Chrispeels, 1990), are conserved in the L. temulentum sequence but were not found in the sequence of the carrot clone. Thus, it is unlikely that this sequence is essential for the function of cytoplasmic plant invertases.

The function of the carrot protein encoded by the cDNA was confirmed by its expression in E. coli. The deduced amino acid sequence lacks an N-terminal signal peptide and shares no similarity with the sequences of the acid invertases. The protein is Cys-rich, and homologous sequences were only detected in the genomes of photosynthetic bacteria and plants. Therefore, the protein must have evolved independently of other Suc-cleaving enzymes. Unlike acid invertase, neutral invertase is not a β-fructofuranosidase and Suc appears to be its sole substrate. Transcripts for neutral invertase were detected in all plant organs at all developmental stages analyzed, with the highest levels in rapidly developing tissues, suggesting a more general and possibly growth-related function of the enzyme in carrot Suc metabolism.

**GENES FOR ACID INVERTASES ARE REGULATED DEVELOPMENTALLY AND BY SUGARS, WOUNDING, AND PATHOGENS**

Genes for acid invertases have been isolated from tomato (Elliott et al., 1993), Arabidopsis (for summary, see Tymowska-Lalanne and Kreis, 1998), maize (Xu et al., 1995), and carrot (Ramloch-Lorenz et al., 1993; Lorenz et al., 1995; Sturm, 1996). Their structures are fairly similar and contain six to eight exons (Tymowska-Lalanne and Kreis, 1998). With the exception of the gene for the main form of carrot cell wall invertase (Ramloch-Lorenz et al., 1993), all of the other genes contain an extremely small exon (exon 2) that codes only for the core tripeptide (DPN) of the conserved F-motif NDPNG. In potato, the mini exon of one of the genes was susceptible to alternative splicing (Bourney et al., 1996). No aberrant post-transcriptional processing was observed during normal invertase gene expression in potato. In contrast, RNA processing was perturbed by cold stress, resulting in the deletion of the mini exon from some transcripts. It is not known whether the aberrant splicing of the invertase gene has a physiological role.

From carrot, five genes for acid invertases have been isolated, including the genes for the main form of the cell wall enzyme and the two isoforms of vacuolar invertase (Fig. 2; Sturm, 1996). Comparison of their 5' upstream regions showed no common sequence elements, suggesting independent modes of regulation. Analysis of isoform-specific steady-state transcript levels confirmed this finding and showed markedly different organ- and development-stage-specific expression patterns (Sturm et al., 1995). Similar results were obtained in a study of the expression of genes for one vacuolar and four cell wall invertase isoforms from tomato (Godt and Roitsch, 1997). Interestingly, both carrot (Lorenz et al., 1995) and tomato (Godt and Roitsch, 1997) contain a flower-specific gene for an acid invertase. Together, these findings suggest that plants have evolved a small family of acid invertase genes that are expressed independently at specific times and places during plant development.

In addition to the organ- and development-specific regulation of gene expression, alterations in sugar composition and concentration markedly affect some of the acid invertase genes, e.g. those for vacuolar invertases from maize (Xu et al., 1996). Two gene classes with contrasting sugar responses were identified. One class is up-regulated by increasing carbohydrate supply, whereas a second class in the same gene family is repressed by sugars and up-regulated by depletion of this resource. Responses to Glc, Suc, and other metabolizable sugars were similar. In contrast, in cells of Chenopodium rubrum, the expression of genes for soluble acid invertase was not affected by sugars, whereas the expression of the gene for cell wall invertase was enhanced (Roitsch et al., 1995). Interestingly, in plants such as carrot, no sugar regulation of acid invertase genes was found. Why these fundamental differences exist in the responsiveness of acid invertase genes to sugars in different plant species is not clear. One possibility is that in plants such as carrot, which store high concentrations of sugars, regulation of acid invertase genes by sugars may not be beneficial and, therefore, either did not develop or has been lost during evolution. In these plants, short-term physiological changes only lead to minor sugar concentration changes, which may not be large enough to efficiently alter regulation of gene expression.

An increase in acid invertase activity in response to hormones such as auxins (Morris and Arthur, 1984), GAs (Wu et al., 1993), or cytokinins (Ehness and Roitsch, 1997) was observed in several plant species. It is not clear whether these effects are due to direct regulation of invertase genes by plant hormones or via stimulated cell proliferation creating new sinks for Suc. In cultured cells of C. rubrum, the increase in cell wall invertase mRNA by cytokinin was paralleled by an increase in transcripts for a Glc transporter (Ehness and Roitsch, 1997), most likely resulting in a higher carbohydrate supply of hormone-stimulated cells.

Genes for acid invertases have also been found to be regulated by wounding. For example, the activity of solu-
ble acid invertase was markedly increased in aging slices of sweet potato tuber (Matsushita and Uritani, 1974), rapidly reached a maximum at 18 h, and decreased thereafter. Furthermore, expression of a gene for carrot cell wall invertase was markedly altered by mechanical wounding of tap roots (Sturm and Chrispeels, 1990). A transient increase of the steady-state mRNA level with a maximum of 12 h after the treatment was followed by an increase in enzyme activity. Induction of gene expression was not systemic but was restricted to the wound site.

A correlation between increased acid invertase activity and infection of plants with various pathogens has been reported several times (for summary, see Sturm and Chrispeels, 1990). In carrot tap roots, the response to infection with the bacterial pathogen Erwinia carotovora was extremely fast and transient (Sturm and Chrispeels, 1990). Maximal levels of transcripts were reached 1 h after first contact with the pathogen and rapidly declined thereafter. Again, induction of gene expression by pathogen infection appears not to be systemic, but restricted to the site of infection (Benhamou et al., 1991).

**ACID INVERTASES ARE REGULATED BY A PROTEINOUS INHIBITOR**

During the purification of cell wall invertase from a suspension culture of tobacco, a small polypeptide of 17 kD that inhibits enzyme activity in a pH-dependent manner was identified (Weil et al., 1994). The highest inhibition was found at pH 4.5, the optimal pH for cell wall invertase activity. The inhibitor polypeptide was shown to colocalize with the enzyme in the cell wall, suggesting an important physiological role in the regulation of invertase activity. However, the finding that binding of the inhibitor to invertase is inhibited by fairly low concentrations of Suc (half-maximum activity at 1.3 mM) is puzzling and raises questions about its postulated function.

A cDNA clone containing partial sequences of the purified inhibitor protein was isolated (Greiner et al., 1998). Its deduced amino acid sequence has no homology to a protein with known function, but several related sequences were identified in the genomes of other plant species, including Arabidopsis. A His-tagged recombinant fusion protein was shown to inhibit the activities of several cell wall and vacuolar plant invertases in vitro but no inhibitory activity was found toward invertases from fungi (Greiner et al., 1998). The cDNA for the inhibitor was also used for expression studies, and a comparison with the steady-state mRNA levels for cell wall invertase revealed that the expression of both proteins is not always coordinate.

**INVERTASES FUNCTION IN METABOLISM AND OSMOREGULATION**

In connection with the various roles Suc plays in plants (nutrient, osmoticum, and signal molecule), invertases may have several different functions (Fig. 3). Most likely, invertases cleave Suc into hexoses to provide cells with fuel for respiration and with carbon and energy for the synthesis of numerous different compounds. Invertases may also be involved in the long-distance transport of Suc by generating the necessary Suc concentration gradient between sites of phloem loading and unloading (Eschrich, 1980). Cleavage of Suc into Glc and Fru could greatly increase the osmotic pressure of cells, suggesting a possible function of invertases in cell elongation and plant growth (Gibeaut et al., 1990).

In numerous studies, invertase functions were deduced from a correlation of invertase activity with physiological processes such as tissue growth or utilization and storage of sugars in sink organs. High acid invertase activity was found in rapidly growing tissues such as developing roots of carrot (Ricardo and Ap Rees, 1970) or elongating stems of bean (Morris and Arthur, 1985), in which the Suc content was low or declining rapidly. Thus, the function of acid invertases in these tissues is to hydrolyze Suc under conditions where there is a high demand for hexoses.

**Figure 3.** Subcellular locations and proposed functions of plant invertases. Plant cells may accumulate different invertase isoforms in the apoplast, cytoplasm, and vacuole. Hexoses generated by the activities of the different enzymes most likely have different fates and functions (a few important examples are indicated in italics).
levels of reducing sugars in opening carnation flowers as important factors in the regulation of carnation petal growth, which is mainly caused by cell enlargement. The source of these reducing sugars was thought to be the import and subsequent rapid hydrolysis of Suc.

In sugar-storing sink organs such as fruit, high acid invertase activity appears to be correlated with the accumulation of hexoses. In the Suc-storing fruit of wild tomato, including *Lycopersicon chinense*, invertase activity is greatly reduced, whereas in the hexose-storing fruit of domestic tomato (*Lycopersicon esculentum*), invertase activity increases at the onset of fruit ripening (Klann et al., 1993). The reduction of invertase activity by an antisense approach (Klann et al., 1996) or gene suppression (Ohyama et al., 1995) led to the conversion of hexose-storing into Suc-storing fruit, demonstrating that in tomato a soluble invertase controls sugar composition. Similar conclusions were drawn from a study of sugar accumulation during cold storage of mature potato tubers. Reduction of soluble acid invertase activity by an antisense approach was correlated with a decrease in the hexose to Suc ratio but did not alter the total amount of released sugars (Zrenner et al., 1996). Thus, a soluble acid invertase functions in this post-harvest process as a regulator of sugar composition.

**INVERTASES AND SUC ALLOCATION**

A more controversial question is whether invertases are involved in Suc metabolism in actively filling sink organs such as seeds, tubers, or roots. In developing seeds of lima bean and tubers of potato, Suc synthase was found to be the prominent Suc breakdown activity, and saccharolyis via invertase was low and secondary (Sung et al., 1989). This conclusion is supported by a study of endosperm development in wheat (Riffkin et al., 1995). The authors reported that Suc synthase had consistently higher activity than invertase throughout endosperm development, and that invertase activity did not change appreciably. Weber et al. (1995) studied the molecular physiology of photosynthetic unloading and partitioning during seed development of fava bean and reached the opposite conclusion. During the prestorage phase, they found that high levels of hexoses in the cotyledons and the apoplastic endospermal space were correlated with high levels of cell-wall-bound invertase in the seed coat. They proposed a model for an invertase-mediated unloading process during early seed development, in which cell wall invertase contributes to establishing sink strength in young seeds. A similar conclusion was reached by Miller and Chourey (1992), who studied the genetic defect in a natural mutant of maize called *Miniature-1* (*Mn1*). In this mutant, the seeds have only one-fifth of the normal weight. The authors showed that the *Mn1* seed locus encodes an endosperm-specific isozyme of cell wall invertase. The lack of invertase activity in the mutant causes an early degeneration and withdrawal of maternal cells from the endosperm, and thereby an interruption of the transport of photoassimilates into the developing kernel.

**INVERTASES AND DEVELOPMENT**

Marked changes in the activity of acid and alkaline invertases appear to be intimately related to the process of cell differentiation in carrot tissue cultures (Silva and Ricardo, 1992). Somatic embryogenesis was characterized by an increase in alkaline invertase and a decrease in the acid enzyme. Nonembryogenic cell lines, in contrast to embryogenic cell lines, maintained very high acid invertase activities. The authors concluded that if Suc participates in plant cell morphogenesis, the persistence of high acid invertase activity at certain developmental stages could affect differentiation by reducing the level of Suc. Thus, the concerted action of two invertases in the cell may contribute to establishing the appropriate levels of sugars, which, by interaction with other components, participate in the regulation of development.

To study the functions of acid invertases in carrot, the antisense technique was used to generate transgenic carrot plants with reduced enzyme levels (Tang et al., 1999). Phenotypic alterations appeared at very early stages of development. In somatic embryos expressing antisense mRNA for cell wall invertase, the cotyledons failed to separate. In contrast, embryos expressing antisense mRNA for vacuolar invertase had quite large cotyledons but stunted hypocotyls and roots. At the stage when control plantlets had two to three foliage leaves and one primary root, shoots of transgenic plantlets expressing antisense mRNA for either cell wall invertase or vacuolar invertase were not separated into individual leaves but consisted of several stunted, interconnected green structures.

When transgenic plantlets were grown on a mixture of Suc, G1c, and Fru instead of only Suc, the malformation was clearly alleviated and plantlets looked more or less normal. Plantlets from hexose-containing media or from transgenic lines with less severe phenotypes produced mature plants when transferred to soil. Such plants in soil expressing antisense mRNA for cell wall invertase had a bushy appearance due to the development of extra leaves. Simultaneously, tap root development was markedly delayed and reduced. Compared with control plants, the leaf to root dry-weight ratio of antisense cell wall invertase plants was shifted from 1:3 to 17:1. Plants expressing antisense mRNA for vacuolar invertase also had more leaves than control plants, but the tap root developed normally though smaller, with a leaf to root dry-weight ratio of 1.5:1. The data suggest that invertases have multiple functions. In the early stages, invertase appears to play a role in plant development, most likely via control of sugar composition and metabolic fluxes. Later, when reduction of cell wall invertase or vacuolar invertase activity have shifted development in favor of leaves, both isoenzymes appear to have important functions in Suc partitioning.

**FUTURE PROSPECTS**

During the past decade, great progress has been made in the characterization and functional analysis of plant invertases. Despite these accomplishments, numerous important questions still need to be answered. Why are there inver-
tases with different properties in different subcellular compartments and how do these enzymes cooperate? What is the function of neutral/alkaline invertase and why does it exist only in photosynthetic organisms? Under what conditions do the products of Suc hydrolysis regulate invertase gene expression and enzyme activity? What other metabolites or effector molecules modulate invertase expression? What is the role of the protease-inhibitory invertase inhibitor (Greiner et al., 1998)? What is the significance of the N-terminal propeptides of the acid invertases and the fragmentation of the mature polypeptides? Where are the regulatory elements for gene activity in the 5′, 3′, and introns of the genes (Fu et al., 1995)? What is the evolutionary and regulatory significance of the intron that encodes the DNP tripeptide? What is the physiological significance of the wound and pathogen response of gene activity? Why does Suc needs to be cleaved when plants are wounded or attacked by pathogens and are the hexoses released involved in stress signaling? And, finally, what is the evolutionary link between β-fructosidases and fructosyltransferases, enzymes that share more than 50% amino acid sequence identity (Sprenger et al., 1995; Vijn and Smeekens, 1999)?

Answers to these questions may only come from multi-disciplinary approaches, and most likely will include combined usage of physiological, biochemical, and molecular techniques. The knowledge gained will fill the gaps in our understanding of one of the most fundamental processes in plants, which may allow the successful manipulation of carbohydrate metabolism and partitioning in our future crop plants.

ACKNOWLEDGMENTS

I thank my colleagues Guo-Qing Tang and Patrick J. King (Friedrich Miescher Institute) for critical reading of the manuscript and Thomas Rausch (University of Heidelberg) for helpful discussions.

Received May 20, 1999; accepted June 1, 1999.

LITERATURE CITED


