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ENZYMOLGY OF STACHYOSE SYNTHESIS
AND DEGRADATION IN LEAVES OF
CUCURBITA PEPO.

by

© Pierre-Richard Gaudreault (M. Sc.)

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ABSTRACT

An enzyme synthesizing stachyose, galactinol: raffinose galactosyltransferase (E.C. 2.4.1.67), has been purified 40 fold from mature leaves of Cucurbita pepo using ammonium sulphate precipitation, Sephadex gel filtration and DEAE Sephadex gel chromatography. The purified enzyme fraction was separated from all but 2% of the total α -galactosidase activity extracted from the tissue. The enzyme was optimally active at pH 6.9 and displayed high specificity for the donor galactinol (K_m 7.7mM) and the acceptor raffinose (K_m 4.6mM) and was unable to effect synthesis of any other member of the raffinose family of galactosyl-sucrose oligosaccharides. Co^{2+} , Hg^{2+} , Mn^{2+} and Ni^{2+} ions were particularly inhibitory; no metal ion promotion was observed and 5mM EDTA was ineffective. Myo-inositol was strongly inhibitory (K_i 2mM), melibiose weakly so. Galactinol hydrolysis occurred in the absence of the acceptor raffinose but there was no hydrolysis of either raffinose or stachyose in the absence of the donor galactinol. The reaction was readily reversible and exchange reactions were detected between substrates and products. It is proposed that the synthesis of stachyose in mature leaves of C. pepo proceeds via this galactosyltransferase and not via α -galactosidase.

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I would like to thank Dr John A. Webb for his advice and encouragement throughout the work. I would also like to express my appreciation to Mr. Hank Datema and Mr. Art Goodenough for their assistance in growing the various plants used in these studies.

To my wife and parents.

A fourth molecular form of α -galactosidase (E.C. 3.2.1.22) was discovered in leaves of Cucurbita pepo. The enzyme, designated L_{IV} , was purified 165 fold by ammonium sulphate precipitation, DEAE Sephadex gel chromatography, Sephadex gel filtration and hydroxyapatite gel chromatography. L_{IV} hydrolyzed p-nitrophenyl- α -D-galactopyranoside (PNPG; K_m 1.4mM) and stachyose (K_m 4.5mM) optimally at pH 7.5 and raffinose (K_m 36.4mM at pH 7.5) optimally at pH 6.5-7.0. L_{IV} displayed high specificity for α -linkages and hydrolyzed stachyose ten times faster than raffinose. The hydrolysis of PNPG catalyzed by L_{IV} was strongly inhibited by galactose (K_i 6.4mM) and stachyose (K_i 15.2mM) but weakly so by melibiose (K_i 70mM), galactinol (K_i 70mM) and raffinose (K_i 244mM). Zn^{2+} , Ni^{2+} , Hg^{2+} , Cu^{2+} , Ag^{2+} , Fe^{3+} and Co^{2+} were completely inhibitory at 3mM concentrations; no metal ion promotion was observed but EDTA, citrate, malate and pyruvate were found to stimulate L_{IV} -catalyzed hydrolysis of both PNPG and stachyose. The specific activity of L_{IV} in leaves of C. pepo decreased 6-fold through the transition phase during which the leaf changes from an importing to an exporting organ. A search for alkaline α -galactosidase activity (L_{IV}) in the plant kingdom revealed that L_{IV} was restricted to the family Cucurbitaceae and a similar enzyme (C_{II}) was partially purified from cotyledons of Cucumis sativus. The facts that L_{IV} activity is restricted to species in which

stachyose is the major sugar translocated and that its specific activity in these species is 6 to 8 fold higher in immature leaf tissue suggest that L_{IV} may play a major role in the hydrolysis of stachyose imported into immature leaves. The presence of galactokinase, UDP-galactose pyrophosphorylase and UDP-galactose-4-epimerase in leaves of C. pepo suggest that galactose assimilation in this tissue proceeds according to the pathway proposed by Maretzki and Thom. (1978).

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INTRODUCTION

The Raffinose Family of Oligosaccharides.

I.- Nomenclature and Classification.

An oligosaccharide (from Greek, oligos, a few) is defined as a compound that, upon hydrolysis, gives monosaccharide units only, in relatively small number per molecule. According to the number of monosaccharide residues per mole the oligosaccharides are classified as disaccharides, trisaccharides, tetrasaccharides, and so forth (Wolfson, 1963; Kandler and Hopf, 1980). It is generally agreed that a carbohydrate consisting of two to ten monomeric residues linked by O-glycosidic bonds is classed as an oligosaccharide.

Oligosaccharides are grouped into two classes: the primary and the secondary oligosaccharides. The synthesis of primary oligosaccharides is catalyzed in vivo by glycosyltransferases. In these reactions, a glycosyl residue is transferred from a glycosyl donor to a mono- or oligosaccharide acceptor. Primary oligosaccharides are found in significant amounts in plants and are an integral part of the cell or the plant metabolism where they may act as energy storage materials, translocates, frost resistance agents or serve as primers. Their number is rather limited when compared with the total number of known oligosaccharides. Secondary oligosaccharides are produced by hydrolysis of higher oligosaccharides, polysaccharides, glycoproteins and glycolipids in vivo or in vitro and they function as structural components (Kandler and Hopf, 1980).

Sucrose (O- α -D-glucopyranosyl-(1 $\rightarrow$ 2)-D-fructofuranoside) is the most widespread and abundantly occurring disaccharide amongst higher plants. The metabolism of this sugar is well documented (ap Rees, 1974; Gander, 1976; Pontis, 1977). Sucrose is a major product of photosynthesis in higher plants and is generally the main form of sugar translocated from mature leaves to other developing organs. Sucrose is also a major storage carbohydrate in plants. It is broken down by sucrose synthase and this provides a readily available source of UDP-glucose (Gander, 1976). UDP-Glucose can then be converted to α -D-glucose-1-phosphate under conditions where UDP-glucose pyrophosphorylase activity favors pyrophosphorylation (Gander, 1976). The net result of the action of these two enzymes therefore links the metabolism of sucrose with polysaccharide and energy metabolism. This sequence of reactions conserves the energy of the glycosidic bond in sucrose and provides an overall efficiency that is relatively high (Gander, 1976).

Next in abundance to sucrose in the plant kingdom are the oligosaccharides which constitute the so-called raffinose family (French, 1954). This series of homologous oligosaccharides (Table I) is based upon the trisaccharide raffinose (O- α -D-galactopyranosyl-(1 $\rightarrow$ 6)-O- α -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-fructofuranoside) and, as shown in Figure 1, the series is increased by the addition of further galactosyl residues via α 1-6 linkages, giving rise to stachyose, verbascose, ajugose and other,

Table I - The Raffinose Series of Oligosaccharides
and Related Sugars.

Raffinose	$O-\alpha-D-Gal-(1\rightarrow6)-O-\alpha-D-Glc-(1\rightarrow2)-\beta-D-Fru$
Stachyose	$O-\alpha-D-Gal-(1\rightarrow6)-Raffinose$
Verbascose	$O-\alpha-D-Gal-(1\rightarrow6)-Stachyose$
Ajugose	$O-\alpha-D-Gal-(1\rightarrow6)-Verbascose$
Melibiose	$O-\alpha-D-Gal-(1\rightarrow6)-O-\alpha-D-Glc$
Manninotriose	$O-\alpha-D-Gal-(1\rightarrow6)-Melibiose$
Verbascotetraose	$O-\alpha-D-Gal-(1\rightarrow6)-Manninotriose$

Gal: galactose Glc: glucose Fru: fructose

Figure 1 - Structure of the Raffinose Series of Oligosaccharides and Related Sugars (after Kandler and Hopf, 1980).

unnamed, longer chain oligosaccharides up to nonasaccharide (Kandler and Hopf, 1980; Candy, 1980).

The trivial names of the members of the raffinose family were assigned before their complete structures were known and they are derived from the plant or the plant material in which they were first discovered. Thus, the name of the tetrasaccharide stachyose is derived from Stachys tubrifera where it was first found by Planta and Schulze (1890) and the name of the trisaccharide raffinose is derived from its first isolation from refined (French, raffiner) sugar beet molasses by Loiseau (1876). Similarly, the pentasaccharide verbascose was first isolated from the roots of Verbascum thapsus by Bourquelot and Bridel (1910) whereas the hexasaccharide ajugose was originally detected by Murakami (1941) in the roots of Ajuga nipponensis. The heptaose, octaose and nonaose of this series have also been isolated from the roots of V. thapsus (Hérissey et al., 1954). The trivial names give little information about the structure of the oligosaccharides but will be used throughout since they are more convenient than systematic names. Reference is made to Table I and Figure 1 for details of their structure.

II:- Occurence.

Raffinose is the most widespread trisaccharide in the plant kingdom and it is quite likely that a careful examination might reveal its presence in all higher plants. Raffinose has therefore little taxonomic significance

(Jeremias, 1962). Jeremias (1962) detected its presence in 220 species of 55 families as against 165 species of 46 families for which stachyose has been reported. The next higher homolog, verbascose, has been reported for only 24 species of 7 families and ^dajugose for 6 species of 3 families (Jeremias, 1962). Raffinose occurs only at low concentrations in the leaves of plants but accumulates in high concentrations in seeds during their formation where it is virtually always accompanied by stachyose and at least traces of verbascose. The higher homologs are usually restricted to the seeds but may be formed in small amounts in leaves (Kandler and Hopf, 1980).

Stachyose is often the quantitatively dominating sugar in seeds but species-specific variations occur. As reported in Kandler and Hopf (1980), O. Preuss, W. Tanner and O. Kandler (unpublished results) found that in the seeds of 55 species or varieties belonging to 36 genera of the Fabaceae, stachyose was the dominating oligosaccharide in 30 genera whereas verbascose dominated in 4 and raffinose in only 2. Ajugose was only present in significant amounts when verbascose was the dominant sugar or was at least present in large amounts.

Courtois and co-workers (1971) observed that, in higher plants, the abundance (occurrence) of the oligosaccharides of the raffinose family is inversely proportional to their molecular weight. However, when seeds accumulate these oligosaccharides, this relationship may not hold (e.g. Table II; Dey, 1980).

Table II - Raffinose Family of Oligosaccharides
Present in Some Food Legumes Seeds^a
and Leaves of Different Plants^b.

<u>Legume Seeds</u>	<u>Raffinose</u>	<u>Stachyose</u>	<u>Verbascose</u>	<u>Galactinol</u>
<i>Cicer arietinum</i> L.	1.1	2.5	-	-
<i>Phaseolus vulgaris</i> L.	0.2	1.2	4.0	-
<i>Lens esculenta</i> L.	0.9	2.7	1.4	-
<i>Pisum sativum</i> L.	0.6	1.9	2.2	-
<i>Glycine max</i>	0.8	5.4	-	-
<u>Leaves</u>				
<i>Lamium maculatum</i>	0.663	0.683	-	0.479
<i>Origanum vulgare</i>	0.726	1.792	-	0.943
<i>Andromeda japonica</i>	0.122	0.107	-	0.069
<i>Oenothera pumila</i>	0.414	0.869	-	0.884
<i>Buddleia davidii</i>	0.219	0.160	-	0.180
<i>Catalpa bignonioides</i>	0.858	3.611	-	2.519
<i>Cucurbita pepo</i> ^c	0.214	0.839	0.066	0.209

^a Dey, 1980; expressed as % of dry matter.

^b Senser and Kandler, 1967a; expressed as mg/g fresh weight.

^c Webb, unpublished data.

Many nutritionally important food-legumes are known to cause flátulence in man and other animals (Steggerda, 1968; Calloway et al., 1966). These legumes are rich in proteins and constitute the major source of dietary protein for a large percentage of the world's population. The content of the raffinose sugars was shown to vary among the food-legumes (Table II) and significant differences were also observed within varieties of the same species (Rao and Belavady, 1978). Flatulence was correlated to the presence of raffinose oligosaccharides in these legumes when it was discovered that the intestinal mucosa in mammals lack α -galactosidase activity (Gitzelmann and Auricchio, 1965) and that the intestinal wall was impermeable to these sugars (Taeufel et al., 1967). Consequently, the raffinose sugars reach the lower intestinal tract intact where they are metabolized by the resident microflora, resulting in methane, hydrogen and carbon monoxide production (Cristofaro et al., 1974). Various approaches have been considered in order to lessen the flatulence factors of food-legumes including genetic manipulation in order to produce varieties with low levels of the oligosaccharides, removal of the oligosaccharides from the legume products using solvents, soaking and germinating the seeds, and the use of hydrolases specific to the oligosaccharides (Dey, 1980).

The accumulation of large amounts of raffinose and stachyose in leguminous seeds also bears implications

as to product sweetness. In particular instances, nearly 50% of the total sugars in peas was found to be stachyose (Shallenberger and Moyer, 1961). It is estimated that stachyose is only about one tenth as sweet as sucrose, and it is understandable why total sugar determinations may bear uncertain relation to organoleptic evaluations of pea sweetness. Maximal sweetness is found in young peas where sucrose is the dominating sugar. As the pea matures sucrose content decreases as stachyose increases (Shallenberger and Moyer, 1961).

Sugar beets are a commercially important source of sugar in many countries. However, the concentration of raffinose in molasses increases during the manufacturing process, resulting in a considerable loss in crystallinity and yield of sucrose. Treatment of beet-sugar molasses with α -galactosidase from the mold Mortierella vinacea hydrolyzed the contaminating raffinose (Yamane, 1971). This method has the additional advantage that the enzyme is bound to the mycelia of the mold and can be readily be removed by filtration and re-used.

III.- Biosynthesis and Degradation.

In his studies on the degradation of raffinose, Neuberg (1907) assumed that the synthesis of raffinose occurs by the addition of galactose to sucrose. In fact, several workers later synthesized raffinose from sucrose and different α -D-galactosides with the help of the

enzyme α -D-galactosido galactohydrolase (α -galactosidase) (Li and Shetlar, 1964; Courtois and Petek, 1966; Williams et al., 1977; Dey, 1979). The yields of raffinose, however, were low compared to the amount of galactose formed by the hydrolysis of the donor and presumably these reactions are unimportant in vivo.

The first good evidence that the synthesis of raffinose resulted from the galactosylation of sucrose in vivo was presented by Rast et al. (1963) who used detached spruce twigs in their investigations of the distribution of ^{14}C in raffinose after photosynthesis in $^{14}\text{CO}_2$ and after the assimilation of labelled monosaccharides. Raffinose formed photosynthetically had a relatively low amount of ^{14}C in its galactose moiety and seemed to be synthesized by galactosylation of uniformly labelled sucrose from a donor which was not as heavily labelled as sucrose. The authors suggested that the galactose must be diluted in a pool which was probably not free galactose since it is seldom found in the living tissues of higher plants. (After photosynthesis in $^{14}\text{CO}_2$, the twigs were kept in normal air for 19 hours. This was not taken into consideration by the authors when it probably resulted in a strong dilution of the galactose moiety of raffinose.) Raffinose formed by feeding labelled glucose or galactose through the cut end of the twigs was most heavily labelled in the galactose moiety. If raffinose were synthesized from melibiose and fructose,

an unequal labelling of fructose and glucose would have been expected.

The formation of raffinose from sucrose and uridine diphosphogalactose (UDP-Gal) was demonstrated with raw enzyme preparations from Vicia faba seeds (Pridham and Hassid, 1965; Bourne et al., 1965). Thus, raffinose synthesis appeared to be linked to the general pathway of oligosaccharide synthesis in higher plants where nucleoside diphosphate sugar derivatives play a major role as donors of glycosyl residues. However, Kandler and his collaborators (see Kandler, 1967) later accumulated much physiological evidence that suggested that the galactosyl donor may not be UDP-Gal.

of chromatograms
Radioautographs of leaf extracts from plants of different taxonomical position such as Buddleia davidii, Thladiantha dubia, Scutellaria baicalensis and Forestiera neomexicana that had been allowed to photosynthesize in the presence of $^{14}\text{CO}_2$ showed spots corresponding to raffinose and stachyose (Kandler, 1967). However, along with these, a third spot was evident, the identity of which was unknown. The hydrolysis of the unknown spot yielded myo-inositol and galactose. Brown and Serro (1953) isolated a compound of the same composition from sugar beet molasses and named it galactinol. Its structure was elucidated by Kabat et al. (1953) as 1L-1-O- α -D-galactopyranosyl-myo-inositol and is shown in Figure 2 (Angyal and Anderson, 1959; IUPAC, 1968). In order to definitely

Figure 2 - Structure of Galactinol.

identify the inositol-galactoside present in leaves, it was isolated from several kg of Lamium maculatum leaves by preparative column chromatography and repeated recrystallization from ethanol. The melting point, as well as the infrared spectrum, of this preparation were identical with authentic galactinol (Senser and Kandler, 1967a).

After isolation by paper chromatography and hydrolysis, the amount of galactinol and the other galactosides in leaves was determined quantitatively using galactose dehydrogenase (Senser and Kandler, 1967). Galactinol was found in amounts similar to those of raffinose and stachyose and at first sight the data suggested that it was another reserve substance (Table II). However, the fact that galactinol occurred only in plants containing the raffinose family of oligosaccharides suggested that some biosynthetic relationship existed between the two.

Labelling kinetics confirmed this hypothesis. The distribution of ^{14}C in extracts of Catalpa leaves after different periods of photosynthesis in $^{14}\text{CO}_2$ revealed that the labelling of galactinol was faster than that of raffinose and stachyose (Senser and Kandler, 1967). Raffinose and stachyose were labelled at a similar rate. Slowly rising curves, typical of reserve substances, were obtained for up to three hours. In contrast, the percentage of ^{14}C in galactinol rose steeply and reached

a maximum after about 30 minutes, as would be expected of an intermediary substance. The character of galactinol as an intermediary compound was demonstrated especially well in pulse-chase experiments where a period of photosynthesis in $^{14}\text{CO}_2$ was followed by one in $^{12}\text{CO}_2$. In this case, the percentage of ^{14}C in raffinose and stachyose continued to increase but that in galactinol decreased. Further evidence against galactinol being a reserve substance comparable to the members of the raffinose family came from the observation that it is not translocated (Senser and Kandler, 1967).

All these findings indicated that the galactose moiety of galactinol is derived from the photosynthetic carbon cycle and is quickly transferred to other galactosides. The de novo synthesis of myo-inositol, on the other hand, was minimal as determined by the very slow rate of incorporation of ^{14}C in this compound, suggesting a carrier function. Thus, the main function of galactinol appeared to be that of a galactosyl donor.

Frydman and Neufeld (1963) showed that, in pea seeds, galactinol is synthesized by UDP-galactose:L-myo-inositol galactosyltransferase according to:

The enzyme was found to require Mn^{2+} for maximal activity at pH 5.6 and showed low specificity for galactosyl transfer to dextro-, levo-, and scyllo-inositol.

In other experiments with mature leaves of various plants, the oligosaccharides of the raffinose family were also observed to be strongly labelled after photosynthesis for a few minutes in $^{14}\text{CO}_2$ (Webb and Gorham, 1964 & 1965; Hendrix, 1968; Kandler, 1967; Beitler and Hendrix, 1974; Webb, 1970; King and Zeevaart, 1974; Webb and Burley, 1964). This indicated that they were formed in the leaf from sugar phosphates of the photosynthetic cycle. In the mature leaves of Cucurbita pepo, the D-galactosyl residues of stachyose become labelled more readily than either the D-glucosyl or D-fructosyl residues of the molecule (Hendrix, 1968). A large proportion of the total label in the ethanol soluble fraction appeared in stachyose very early in photosynthesis and after 37 seconds stachyose contained more than three times the activity of any other sugar (Beitler and Hendrix, 1974). The degree of labelling was stachyose > raffinose > sucrose (35%, 11% and 2%, respectively). The extent of labelling in stachyose decreased rapidly with time and, at 5 minutes, was lower (8%) than that of sucrose (17%).

Similarly, when leaves of Lamium maculatum were allowed to photosynthesize in $^{14}\text{CO}_2$ for different periods of time, and the distribution of the radioactivity within raffinose and stachyose determined, ^{14}C -galactose predominated over the evenly labelled sucrose in raffinose (Senser and Kandler, 1967). Only after one hour was raffinose uniformly labelled. In stachyose the terminal galactose contained the bulk of ^{14}C , whereas the raffinose

portion reflected the distribution found in the free raffinose. These findings also suggested that raffinose and stachyose are synthesized from a D-galactose-containing intermediate derived from the photosynthetic cycle.

In addition to all the physiological evidence accumulated by Kandler and his co-workers, sufficient in vitro evidence is now available to describe the biosynthesis of the raffinose series as a sequential action of a series of α -D-galactosyltransferases catalyzing the following reactions:

Galactinol:sucrose-6-galactosyltransferase (GST) from Vicia faba seeds and from wheat germ has been isolated and characterized (Lehle et al., 1970; Lehle and Tanner, 1972 & 1973). The enzyme, purified 40 fold, demonstrated a high specificity towards the galactosyl donor at a pH optimum around 7.0. In addition to the galactosyl residue of galactinol, only that of p-nitrophenyl- α -D-galactopyranoside and raffinose could be transferred to any significant extent. The reaction with raffinose as a donor has been described as an exchange reaction between raffinose and sucrose as shown in Equation VI and was

first reported by Moreno and Cardini (1966). These authors could not detect raffinose synthesis with their preparation and it was later found to be due to the fact that the exchange reaction of GST is much more stable than its ability to synthesize raffinose, the latter activity being distinctly sensitive to oxygen and requiring strict protection by sulfhydryl reagents (Lehle and Tanner, 1973).

Uridine diphosphate-D-galactose did not serve as a galactosyl donor for GST despite previous reports of its ability to promote raffinose synthesis in enzyme extracts from V. faba seeds (Bourne et al., 1965; Pridham and Hassid, 1965) or in isolated chloroplasts (Imhof, 1973). Lehle and Tanner (1972, 1973) postulated that in these earlier experiments, where relatively impure enzyme preparations were used, raffinose synthesis could have resulted from the sum of the following two equations:

This would, however, require the addition of myo-inositol to the reaction mixture unless this compound was already present in a bound form in the enzyme preparation. The preparations from V. faba were thoroughly dialyzed and no myo-inositol was added in the assay. Thus, this controversy still remains. On the other hand, raffinose synthesis in similar crude enzyme preparations of wheat germ or peas could not be promoted by the addition of UDP-galactose (Moreno and Cardini, 1966; Frydman and Neufeld, 1963).

GST showed a high acceptor specificity when tested with ^{14}C -galactinol. Of ten substrates assayed, including raffinose and stachyose, only a transfer to sucrose could be observed (Lehle and Tanner, 1973). The enzyme is therefore unable to carry on the polymerization beyond the trisaccharide level. It was noted that during the incubation of ^{14}C -galactinol some free ^{14}C -galactose was released due to the hydrolysis of galactinol. However, in the presence of sucrose, the amount of galactose transferred was nearly five times greater than the amount of galactinol hydrolyzed. In the absence of any acceptor, considerable more galactose was set free. This was interpreted as a competitive effect between sucrose and water (Lehle and Tanner, 1973).

The synthesis of higher homologs is catalyzed by a series of α -D-galactosyltransferases exhibiting acceptor affinity only to raffinose or to raffinose and one or more of its higher homologs, but not to sucrose (Lehle and Tanner, 1972). A 20-30 fold purification of the first member of this series, galactinol:raffinose-6-galactosyltransferase (GRT_1), from seeds of P. vulgaris was achieved by Tanner and Kandler (1968). It was also later found in leaves of Cucurbita pepo (Webb and Pathak, 1970). GRT_1 was found to catalyze the following two exchange reactions in addition to the synthesis of stachyose:

Stachyose + ^{14}C -Raffinose $\rightleftharpoons$ ^{14}C -Stachyose + Raffinose (VIII)

No significant exchange was found to occur between galactinol and free ^{14}C -galactose.

GRT₁ from P. vulgaris showed high substrate specificity. Only galactinol and neither UDP-galactose nor ADP-galactose was utilized as a donor. Slight acceptor activity was observed with melibiose, glucose, galactose and lactose but neither sucrose nor stachyose served as acceptors (Tanner and Kandler, 1966). The latter specificity is in agreement with the absence of verbascose in the seeds of P. vulgaris. On the other hand, the transferase (GRT₂) from Vicia faba seeds, which contain verbascose as the main oligosaccharide, exhibited a significant affinity not only for raffinose but also for stachyose as an acceptor and synthesized verbascose in vitro (Tanner et al., 1967). A strong mutual inhibition of raffinose and stachyose was observed when both substrates were present, indicating that one and the same enzyme catalyzed both the synthesis of stachyose and of verbascose (Behle and Tanner, 1972).

Only a slow synthesis of ajugose could be observed when verbascose was supplied as acceptor. Ajugose synthesis was distinctly more rapid with enzyme preparations from the seeds of Pisum sativum or Vicia sativa which contain more ajugose than do seeds of V. faba (Preuss et al., unpublished results described in Kandler and Hopf, 1980), thus indicating the presence of a third transferase (GRT₃).

The formation of the higher homologs of the raffinose series may therefore be expressed by:

where n may be any number from one to five. The GRT_{1-5} catalyzing these reactions differ with respect to their acceptor specificity depending on the species from which they are isolated. Even in vitro they do not transfer galactosyl residues from galactinol to any homolog of raffinose of an order higher than that actually found in the plant from which the enzyme has been purified (Kandler and Hopf, 1980).

Although GST and GRT_{1-5} exhibit some hydrolytic activity, they differ clearly from α -galactosidases, for which much in vitro and in vivo evidence has been accumulated as to their role in the degradation of raffinose-type sugars (Dey and Pridham, 1972; Pridham and Dey, 1974; Dey, 1980).

α -Galactosidases are involved in the breakdown of the raffinose sugars during seed germination by splitting off the terminal galactosyl residues, thus giving rise to free galactose and the next lower homologs in the series. Tanner and co-workers (1968) showed that galactose and raffinose are accumulated to a small extent during germination of beans when stachyose, the main storage material besides starch, is broken down. Since GRT_1 activity did not increase during germination and myo-inositol concentrations

increased, the reversal of equation III could also be involved in stachyose breakdown, yielding raffinose and galactinol followed by the subsequent hydrolysis of galactinol by α -galactosidase. However, galactinol was not even temporarily accumulated but decreased in content immediately after germination was initiated. This suggested that GRT₁ is not significantly involved in the mobilization of stachyose.

α -Galactosidases (α -D-galactoside galactohydrolases; E.C. 3.2.1.22) catalyze the following reaction:

The hydroxylic acceptor molecule, R'OH, is commonly water, although R and R' can also be aliphatic or aromatic groups. Therefore, the enzyme may hydrolyze a variety of simple α -D-galactosides as well as more complex molecules, such as oligosaccharides and polysaccharides. In addition, O-transfer of α -D-galactosyl residues to various alcohol derivatives may be effected. Under special conditions, de novo synthesis can occur using D-galactose (R=H) as the donor (Dey and Pridham, 1972).

α -Galactosidases have been reported to occur widely in microorganisms, plants and animals; consequently, the kinetics and specificity of purified preparations from a variety of sources have been reported (Table III; see

Table III - Number of molecular forms, molecular weights and pH optima of α -galactosidases from various sources.

<u>Organism</u>	<u>Molecular Weight</u> ¹	<u>pH</u> ²	<u>References</u>
Diplococcus pneumoniae	I -	5.6-6.0 ^a	Li et al., 1963; Li & Shetlar, 1964.
Mortierella vinacea	I,II,III ³ -	4.0-6.0 ^a	Suzuki et al., 1970.
Saccharomyces carlsbergensis	I 300000	4.0-5.0 ^{bcd}	Lazo et al., 1977; Lazo et al., 1978.
Poterioochromonas malhamensis	I 360000	7.0 ^e	Dey & Kauss, 1981.
Acer pseudoplatanus (seeds)	I ^a 167000 II 50000	- ^f	Barham et al., 1971.
Cajanus indicus (seeds)	I 87000	- ^f	Barham et al., 1971.
Coffea sp. (seeds)	I ^a - II -	5.3 ^g 6.3 ^g	Courtois & Petek, 1966.
Coffea canephora (seeds)	I ^b 28000 II 36500	6.0 ^b 6.0 ^b	Carchon & De Bruyne, 1975.
Coffea sp. (seeds)	I 26300	- ^f	Barham et al., 1971.
Cucurbita pepo (leaves)	L ³ L ^{II} L ^{III}	5.7 ^b 4.6 ^b 5.6 ^b	Thomas & Webb, 1977.
Cucumis sativus (leaves)	I ^a 25000 II 50000	5.5 ^b 5.2 ^b	Smart & Pharr, 1980.
Ficus sp. (Ficin)	I -	2.0-5.5 ^b	Hakomori et al., 1971.
Helianthus annuus (seeds)	I ^a 159000 II 23000	- ^f	Barham et al., 1971.
Medicago sativa (seeds)	I 38000	- ^f	Barham et al., 1971.

Table III (continued)

<u>Organism</u>	<u>Molecular Weight</u> ¹	<u>pH</u> ²	<u>References</u>
<i>Medicago sativa</i> (seeds)	I 57000	4.0 ^b -5.5 ^c	Itoh et al., 1979.
<i>Phaseolus aureus</i> (seeds)	I ^α 209000	- ^f	Barham et al., 1971.
	II 38000		
<i>Phaseolus vulgaris</i> (seeds)	I ^α 125900	- ^f	Barham et al., 1971.
	II 39800		
<i>Phaseolus vulgaris</i> (seeds)	I -	6.5-6.7 ^b	Agrawal & Bahl, 1968.
<i>Pisum sativum</i> (seeds)	I ^α 121600	6.0 ^b	Barham et al., 1971.
	II 32300	4.0 ^b	
<i>Prunus amygdalus</i> (seeds)	I 32400	- ^f	Barham et al., 1971.
<i>Prunus amygdalus</i> (seeds)	I 33000	5.5-5.7 ^b	Malhotra & Dey, 1967; 1967a; Dey & Malhotra, 1969.
<i>Spinacia oleracea</i> (leaves)	I -	5.3 ^b	Gatt & Baker, 1969.
<i>Stachys affinis</i> (tubers)	I 42000	5.2 ^b	Ueno et al., 1980.
<i>Trifolium repens</i> (seeds)	I ^β 41000	3.8-4.2 ^{ac}	Williams et al., 1977;
	II 41000	4.5-5.8 ^a	Williams et al., 1978.
	III 41000	4.5-5.8 ^a	
<i>Trifolium repens</i> (seeds)	IV ^β 41000	4.5-5.8 ^a	Williams et al., 1978.
	V -	-	
<i>Vicia dumerotum</i> (seeds)	I ^α 195000	- ^f	Barham et al., 1971.
	II 57000	- ^f	

Table III (continued)

<u>Organism</u>	<u>Molecular Weight</u> ¹	<u>pH</u> ²	<u>References</u>
Vicia faba (seeds)	I ^α 209000 II 38000	3.5-5.5 ^c 4.0 ^c	Dey & Pridham, 1968; 1969; 1969a.
Vicia sativa (seeds)	I 30000	6.3 ^{be}	Petek et al., 1969.
Mytilus edulis (hepatopancreas)	I ^δ 70000 II 5000	4.5 ^b 3.5 ^b	Pernas et al., 1981.
Mouse (liver)	I 150000	4.2 ^e	Lusis & Paigen, 1976.
Man (liver)	A ^δ - B -	4.3-4.6 ^e 4.3-4.8 ^e	Dean & Sweeley, 1979; 1980.

¹Molecular forms are denoted as in the original references; molecular forms were obtained by various methods: α: gel filtration; β: aluminum oxide column; γ: Sephadex DEAE A-50 column; δ: hydroxyapatite column.

²Substrates used: (a) O-nitrophenyl-α-D-galactopyranoside; (b) p-nitrophenyl-α-D-galactopyranoside; (c) raffinose; (d) melibiose; (e) 4-methylumbelliferyl-α-D-galactopyranoside; (f) activity assayed at pH 5.0 with substrate (b); (g) α-phenyl galactopyranoside.

also Dey and Pridham, 1972; Pridham and Dey, 1974; Dey, 1980). As can be seen in Table III, one of the most striking physical property of α -galactosidases is the common occurrence of two or more molecular forms of the enzyme. Separation of the different forms by gel filtration on the basis of molecular weight (e.g. Barham et al., 1971) is not always feasible. Thus, Thomas and Webb (1977) resolved three molecular forms of α -galactosidase on DEAE-Sephadex after the activity eluted as a single peak from Sephadex G-100. Similarly, two molecular forms of α -galactosidase from coffee beans were obtained after chromatography on aluminum oxide (Courtois and Petek, 1966) but only one by gel filtration (Barham et al., 1971), whereas the three forms of α -galactosidase found in M. vinacea were detected only after chromatography using Sephadex-DEAE columns (Suzuki et al., 1970).

Some evidence for the in vitro interconversion of multimolecular forms of α -galactosidase from V. faba seeds has been described by Dey and Pridham (1972). The specific activity of a purified preparation of the lower molecular weight form, II (Table III), was found to increase rapidly over a period of days upon storage at pH 5.5 and 4 °C. Examination by Sephadex gel filtration revealed that the activity of enzyme II was decreasing at the expense of a higher molecular weight enzyme with an elution pattern identical to that of enzyme I. The interconversion was prevented if the preparation of enzyme II was first passed through Sephadex G-100. The picture was further complicated

when enzyme II itself was found to resolve into two active and four inactive protein peaks after chromatography on CM-cellulose (Pridham and Dey, 1974). Analytical data comprising studies on the molecular weight, carbohydrate and potassium content of the α -galactosidases of V. faba suggest that α -galactosidase II may be composed of an equimolar mixture of two active proteins, II¹ and II², of similar size together with other (4?) enzymatically inactive components. The amino acid analyses also suggested that there is a close relationship between II and I and Pridham and Dey (1974) proposed that monomers of II associate to form I and that in vitro there may be different degrees of association. Dey et al. (1971) also obtained evidence that K⁺ ions may be involved in the polymerization of enzyme II into enzyme I. Balasubramian et al. (1976) and Lusis and Paigen (1976) have also reported a salt and pH mediated interconversion of the molecular forms of α -galactosidase from coconut and mouse liver, respectively in vitro.

Examination of the Sephadex patterns and total activity of α -galactosidases in maturing V. faba seeds showed that in small, immature green beans only enzyme II is present and that this stage is followed by a marked increase in specific activity as the seeds mature. This increase could be related to the appearance of enzyme I and a decrease in the activity of enzyme II (Pridham and Dey, 1974). Although the changes in concentration of the

molecular forms of α -galactosidase could result from de novo synthesis of I coupled with the proteolysis of II, the analytical data suggested that there is a structural relationship between the two forms of α -galactosidase and investigations have shown that, in vitro, association of units of II may result in I or an enzyme closely resembling I in its physical and kinetic properties (Pridham and Dey, 1974). There are indications that some compartmentation of α -galactosidase activity in organelles, in the form of spherosomes, occurs in V. faba seeds; this may explain the simultaneous increase in the levels of both α -galactosidase and its substrates during seed maturation (Dey, 1978).

In Cucurbita pepo, immature importing leaves have an extremely active system that hydrolyzes raffinose, stachyose and verbascose entering their tissue from the phloem and are incapable of any detectable synthesis or accumulation of these sugars. In contrast, mature exporting leaves demonstrate both a synthesis and a negligible in vivo hydrolysis of these oligosaccharides (Turgeon and Webb, 1973; Webb, 1971; Webb and Gorham, 1964). Mature leaves contain three molecular forms of α -galactosidase (Thomas and Webb, 1977) and, therefore, loss of the α -galactosidase system was rejected as the reason for the difference in metabolism of these sugars. An attempt was then made to discover whether the three forms are distributed in other tissues of C. pepo and to relate their presence to the utilization

of the galactosyl oligosaccharides in these tissues (Thomas and Webb, 1978). These results produced no direct evidence to explain the difference between immature and mature leaf tissues toward hydrolysis of the raffinose sugars.

Considerable amounts of all three molecular forms of α -galactosidase were present in both tissues. Thomas and Webb (1979) have presented evidence that the three molecular forms of α -galactosidase, L_I , L_{II} and L_{III} , are located in the apoplast and suggested that a symplastic pathway for vein loading in mature leaves and an apoplastic step in vein unloading in immature leaves could provide an efficient mode of separation between the galactosyl oligosaccharides and α -galactosidase activity.

The level of the raffinose family has been shown to decrease during the germination of seeds of various species, including cotton (Shiroya, 1963), coffee (Shadaksharaswamy and Ramachandra, 1968), fenugreek (Sioufi et al., 1970; Reid, 1971), lucerne, guar, carob and soybean (McCleary and Matheson, 1974), lupin (Matheson and Saini, 1977), bambarra groundnut (Amuti and Pollard, 1977), pea (Lee and Shallenberger, 1969; Marbach et al., 1978) and broad bean (Pridham et al., 1969). Plant galactomannans are reserve polysaccharides composed of linear chains of (1 $\rightarrow$ 4) linked β -D-mannopyranosyl residues having single stubs of α -D-galactopyranosyl groups joined by (1 $\rightarrow$ 6) linkages along the chain and are commonly found in the endosperm of seeds of the members of the family Legumihoseae rich in oligosaccharides

of the raffinose family. Their level has also been shown to decrease sharply upon germination (Dey, 1978). The depletion of α -D-galactosidic, reserve carbohydrates in germinating seeds is accompanied by a concomitant increase in α -galactosidase activity. The specificity of α -galactosidase varies widely; the enzymes from Vicia sativa (Petek et al., 1969) and Mortierella vinacea (Suzuki et al., 1970) hydrolyze substrates of small molecular weight with marked differences in their respective rates of hydrolysis (e.g. Vicia sativa: phenyl- α -D-galactopyranoside > raffinose > stachyose > galactinol), but do not act on larger substrates such as galactomannans. However, the enzymes from Phaseolus vulgaris (Agrawal and Bahl, 1968) and coffee beans (Courtois and Petek, 1966) hydrolyze both groups of substrates. It seems apparent that the specificity and function of an α -galactosidase are related to the nature of the α -D-galactosidic compounds that may occur in a given source. Thus, only 20-40% of the galactose present in guar galactomannans could be released by α -galactosidase from Aspergillus niger (Bahl and Agrawal, 1969), Phaseolus vulgaris (Agrawal and Bahl, 1968) or coffee beans (Dey, 1978) after prolonged incubation periods whereas, interestingly, the α -galactosidase isolated from guar seeds removed all of the D-galactose residues from guaran, leaving a residue of water-insoluble D-mannan (Dey, 1978).

Dry fenugreek seeds have a high level of α -D-galactosidase in the embryo, but only negligible

amounts in the endosperm (Reid and Meier, 1973). However, during germination, whereas the enzyme level in the embryo remains constant, it increases sharply in the endosperm; this process parallels the degradation of galactomannan. Similar observations have been made on the incubation of dry-isolated embryos and of endosperms under "germinating conditions". In the in vivo experiments, the increase in enzyme level was strongly diminished by cycloheximide which prevented protein synthesis in the aleurone layer. Seiler (1977) has actually shown the synthesis of α -galactosidase by demonstrating the in vivo incorporation of L-¹⁴C-serine into the enzyme protein during the germination of carob beans. Similarly, the involvement of protein synthesis in the levels of the multimolecular forms of α -galactosidase in germinating V. faba seeds was demonstrated by Dey and his co-workers (see Dey, 1980) with the use of cycloheximide. There is also evidence that, in lettuce seeds, the increase of α -galactosidase activity associated with germination is a phytochrome controlled phenomenon (Leung and Bewley, 1981). Reid and Meier (1973) suggested that galactomannan is not the natural substrate for the embryo α -galactosidase. Rather, the enzyme may be responsible for the hydrolysis of the raffinose sugars. It has been found that α -galactosidase from germinated fenugreek seeds can be resolved into two active peaks (Dey, 1978) but the substrate specificities of the enzymes from the embryo and the endosperm have not been determined as of yet.

McCleary and Matheson (1974) showed the presence of multimolecular forms of α -galactosidase (designated A, B and C) in germinated seeds of carob, guar, lucerne and soybean. Forms A and C were common to all species examined; carob and soybean had a third form, B. If seeds were separated into endosperms and cotyledons plus embryo, α -galactosidase A and B were found only in the embryo-cotyledon fraction. In the endosperm, only α -galactosidase C was present. Form A was also present in the dry seeds and its level did not increase significantly on germination. It was suggested that forms A and B are mainly responsible for the hydrolysis of the raffinose family of sugars. On the other hand, the level of form C was found to increase rapidly during germination, except in soybean, which has a very low content of galactomannan. Enzyme C was shown to be highly specific for the hydrolysis of lucerne galactomannan.

Raffinose and its higher homologs can also be attacked by β -fructofuranosidase (invertase) in vitro. Melibiose, the splitting product of raffinose, has in fact been found in storage organs and even in leaves which contain large amounts of raffinose. Although this observation indicates that invertase also attacks raffinose in vivo, this enzyme is not involved in the mobilization of oligosaccharides of the raffinose series during germination since neither melibiose nor manninotriose

or verbascotetraose, the respective breakdown products of stachyose or verbascose, have been found in germinating seeds as of yet (Kandler and Hopf, 1980).

There is now little doubt that α -galactosidases function as hydrolytic agents in the utilization of galactosyl oligosaccharides as sources of energy and cell metabolites. The enzymes may also play an important role in the metabolism of galactolipids. Sastry and Kates (1964), for example, have shown that runner bean leaves possess all the necessary enzymes, including α -galactosidase, for the complete hydrolysis of these compounds to fatty acids, glycerol and galactose. Such a role is well exemplified in humans in the case of Fabry's disease, an X-linked glycosphingolipidosis characterized by the accumulation of glycolipids containing terminal α -galactosyl residues (Johnson and Desnick, 1978). Two forms of α -galactosidase activity, designated A and B, have been reported to occur in normal human tissues. Patients with Fabry's disease are deficient in α -galactosidase A activity while the B form occurs in normal or even somewhat elevated levels in these patients (Beck, 1980).

Chloroplast membrane galactolipids may also be affected by α -galactosidases. Bamberger and Park (1966), studying the Hill reaction of isolated chloroplasts, noted that a crude preparation containing galactolipases and galactosidases changed the physiological activity of the organelles. In this connection it is interesting to note.

that α -galactosidase has been detected in spinach (Gatt and Baker, 1970) and sugar cane (Dey and Pridham, 1972) chloroplasts.

Since free galactose is never present in seed or leaf tissues, an efficient system must be present to assume the conversion of the galactose liberated upon hydrolysis of the various substrates of α -galactosidases into a form that can be readily utilized by other biosynthetic mechanisms operating in the cells. There is now considerable evidence that galactose is incorporated into other cell constituents by the sequential action of galactokinase, UDP-galactose pyrophosphorylase and UDP-galactose-4-epimerase (Pridham et al., 1969; Sioufi et al., 1970; Maretzki and Thom, 1978; Dey, 1980) as shown in Figure 3. The sugar nucleotides thus formed can then serve as substrates for the enzymes involved in the numerous interconversions leading to glycosidic derivatives which, in turn, take part in various biosynthetic processes.

IV.- Function.

Oligosaccharides of the raffinose series are important components of the carbohydrate reserves in the vegetative storage organs and seeds of many plants. They also play an important role in sugar translocation in some plants. Thus, several workers have demonstrated the translocation of ^{14}C -labelled raffinose sugars from the leaves to the stem and roots following a period of

Fig. 3 - Metabolism of D-Galactose in Plants (from Maretzki and Thom, 1978)

photosynthesis in $^{14}\text{CO}_2$ (Webb and Gorham, 1964; Senses and Kandler, 1967; Hendrix, 1968).

The low molecular weight members of the raffinose series also play a role in cold acclimation and drought resistance. It is generally believed that the concomitant increase in content of galactosyl sucrose oligosaccharides and decrease in starch content which is observed prior to winter throughout the plant kingdom has a functional significance (Alden and Herman, 1971). Santarius (1973) showed that raffinose, sucrose and glucose (in decreasing order) protect the electron transport activity and cyclic photophosphorylation from drought and frost. Santarius and Milde (1977) also demonstrated that the de-hardening process in cells of frost-resistant cabbage leaves is accompanied by a decrease in sucrose and raffinose concentration, especially in chloroplasts. The uncoupling of photophosphorylation from the electron transport system by heat injury (Emmet and Walker, 1969) was also retarded by these sugars (Santarius and Milde, 1977). It has been suggested that the affinity of the hydroxyl groups of a sugar for water may influence the micro-environment of the labile, membrane-bound proteins, thus stabilizing them against different stress conditions (Santarius and Milde, 1977).

In Picea excelsa, both photoperiod and temperature proved to be important factors not only for the regulation

of cold acclimatation but also for the induction of raffinose synthesis (Kandler et al., 1979). The observation that raffinose synthesis and cold acclimatation are strictly coupled even under the influence of unnatural combinations of different photoperiods and temperature regimes further strengthen the hypothesis that the accumulation of these sugars is involved in cold acclimatation, although this may not be the only or not even the decisive cause of frost resistance.

Raffinose has also been related to the viability of seeds. Corn seeds that initially had a high content of moisture showed a lower concentration of the oligosaccharides after prolonged storage. The decrease in the level of oligosaccharides was especially large in seeds that had lost viability. Raffinose and sucrose were totally absent from the non-viable seeds (Dey, 1980).

Stachyose is the major sugar translocated from mature leaves of Cucurbita pepo to other parts of the plant (Webb and Gorham, 1964; Hendrix, 1968). However, very little is known about the properties of the enzyme catalyzing its synthesis in leaf tissue. Similarly, stachyose breakdown in importing tissues is presumably carried out by the three molecular forms of α -galactosidase described by Thomas and Webb (1977, 1978). However, no direct evidence was given to explain the difference in hydrolytic behaviour between mature and immature leaf tissue towards stachyose

based either on specific activity of total α -galactosidase or variation in the specific activities of the individual molecular forms. Therefore, the enzymology of stachyose synthesis and breakdown in leaf tissue was investigated.

Materials and Methods

I- Purification and Properties of Galactinol:Raffinose Galactosyltransferase (E.C. 2.4.1.67).

A- General Procedures.-

1.- Protein Determinations.

Protein determinations were made according to Bradford (1976) using crystalline BSA as the standard.

2.- Separation of Sugars by Paper Chromatography.

Separation of reaction products from enzyme assays and standard sugar solutions was carried out by descending paper chromatography on Whatman paper #4 for 24 hours or Whatman paper #1 for 3 x 24 hours using n-propanol:ethyl acetate:water (7:1:2 v/v) unless otherwise stated. Radioactive peaks on individual strips were detected using a Packard Radiochromatogram Scanner, cut out, placed in vials containing 15 ml of scintillation grade toluene and 0.5% PPO (w/v), and counted in a Packard Scintillation Spectrometer. Identification of the radioactive peaks was done by co-chromatography with strips of a standard sugar solution visualized with AgNO_3 -NaOH (Trevelyan et al., 1950).

3.- Separation of Sugars by Thin Layer Chromatography.

TLC was carried out on "Baker TLC" plates (20 x 20 cm) precoated with 250 microns of Silica Gel 60-F. Standard sugar solutions in water and products of enzyme assays were spotted on a line one cm from the edge and the plates developed unidimensionally by ascending chromatography in glass chambers which were saturated with the appropriate solvents. The solvents were:

- (1) Ethyl acetate:acetic acid:methanol:water (60:15:15:10 v/v) (Lato et al., 1969; Ghebregzabher et al., 1979);
- (2) Methyl ethyl ketone: acetic acid:methanol (6:2:2 v/v) (Jeffrey et al., 1969);
- (3) Isopropanol:water:ethyl acetate (54:26:20 v/v) (Mezzetti et al., 1971);
- (4) n-Butanol:ethyl acetate:isopropanol:acetic acid:water (35:100:60:35:30 v/v) (Lato et al., 1968);
- (5) Methyl ethyl ketone:benzene:isopropanol:acetone:0.5 M isopropylammonium benzoate (35:15:35:5:16 v/v) containing 2.0% (w/v) phenylboronic acid (Ghebregzabher et al., 1979);
- (6) n-Butanol:acetic acid:water (50:40:10 v/v) (Lato et al., 1969).

Solvents (1) and (5) were used for single developments of 10 cm, solvent (2) for single developments of 15 cm and solvents (1), (3), (4) and (6) for two consecutive developments of 10 cm where the plates were taken out after the first run and dried in a stream of hot air

supplied by a hair dryer for 5-10 minutes. The sugars were detected using four methods:

- (1) The plates were sprayed with a solution containing 3 g phenol plus 5 ml concentrated H_2SO_4 in 95 ml ethanol and heated 10-15 minutes at 110 °C (Sherma and Zweig, 1972);
- (2) The plates were sprayed with 0.2% naphtoresorcinol in ethanol and concentrated H_2SO_4 (mixed 1:0.04 v/v before use) and then heated at 100 °C for 5 minutes (Ghebregzabher et al., 1976).
- (3) The plates were sprayed with a freshly prepared solution of 2.0% diphenylamine in acetone, 2.0% aniline in acetone and 85.0% H_3PO_4 (mixed 5:5:1 v/v) and then heated at 100 °C for 10 minutes (Ghebregzabher et al., 1976);
- (4) Fleur's reagent was used to detect myo-inositol. Mercuric oxide (10 g) was dissolved in 10 g concentrated HNO_3 and added to 200 ml of water. The mercuric oxide solution was then diluted 1:1 with water before use (see A). One part of 10% aqueous barium acetate and 10 parts glacial acetic acid were mixed to prepare solution B. The plates were sprayed with solution A, heated 10 minutes at 95 °C, then sprayed with solution B and heated 10-30 minutes at 95 °C (Sherma and Zweig, 1972).

4.- Gel Electrophoresis.

Polyacrylamide gel electrophoresis was performed at room temperature using 7.5% medium pore gels (pH 8.9) at

4 mA per gel as described by Maurer (1971). The gels were stained with Coomassie Blue R (Maurer, 1971). Polyacrylamide gel electrophoresis was performed at 2-4 °C when recovery of enzyme activity from the gels was desirable. In such cases, the gels were frozen on dry ice after electrophoresis, sliced into 1 mm sections which were then individually incubated with the appropriate substrates. Separation and quantitation of reaction products was then carried out following the usual procedures described below for the respective enzymes.

5.- Chemicals.

All sugars were purchased commercially except galactinol which was purified from mature leaves of Cucurbita pepo essentially according to Brown and Serro (1953) (Webb, unpublished results) and manninotriose which was prepared by mild hydrolysis of stachyose (0.1N HCl; 2 hours at 45 °C) and isolated chromatographically pure by descending PC on Whatman #1, 5 x 24 hours in n-butanol:pyridine:water (6:4:3 v/v). The identity of galactinol was confirmed by co-chromatography with authentic galactinol obtained from Calbiochem. Galactose-labelled ^{14}C -galactinol was prepared by incubating a partially purified galactinol synthetase preparation from mature C. pepo leaves (Webb, unpublished data) with UDP- ^{14}C -galactose (New England Nuclear) and myo-inositol

and isolating the products by descending PC on Whatman paper #1, 3 x 24 hours in propanol:ethyl acetate:water (7:1:2 v/v). The identity of galactinol was confirmed as described above. ^{14}C -Raffinose was obtained from leaves exposed to $^{14}\text{CO}_2$ for 15 minutes. The leaves were extracted in alcohol and a neutral fraction obtained from which raffinose was isolated by descending PC on Whatman paper #1, 3 x 24 hours in propanol:ethyl acetate:water (7:1:2 v/v), and rerun to obtain the raffinose chromatographically pure. The identity of ^{14}C -raffinose was confirmed by co-chromatography with authentic raffinose obtained from Fisher. Over 90% of the radioactivity was located in the galactose moiety as estimated by acid hydrolysis.

B- Raffinose and Stachyose Synthesis in Leaves of C. pepo.

1.- Plant Material.

Seeds of Cucurbita pepo L. var. melo pepo f. torticolis Bailey (Early Prolific Straight-Neck Squash from W.A. Burpee, Seed growers, PA., U.S.A.) were germinated in perlite and grown in a controlled environment cabinet (Turgeon and Webb, 1973). Mature leaf blades from plants 3-5 week old were used for the enzyme extraction.

2.- Preparation of Crude Extracts.

All extraction procedures were carried out at 2-4 °C. Individual leaves were extracted in various buffers (100mM Tris pH 7.3; 100mM sodium phosphate (NaPi) pH 6.9; 50mM 2-(N-morpholino)ethane sulfonic acid (MES) pH 6.2; 50mM 3-(N-morpholino)propane sulfonic acid (MOPS) pH 7.0; 50mM imidazole pH 7.0) using an ice-cold pestle and mortar and a ratio of 4 ml buffer per g fresh weight. The effect of adding the following compounds to the individual buffers on raffinose and stachyose synthesis was studied: 5% (w/v) insoluble polyvinylpyrrolidone (PVP), 10mM diethyldithiocarbamic acid (DIECA), 5mM dithiothreitol (DTT) and 20mM 2-mercaptoethanol (BME). The homogenates were squeezed through 4 layers of cheesecloth and centrifuged 10 minutes at 30,000 g. The pellets were discarded and 1.5 g fresh weight equivalent desalted by chromatography on Sephadex G-25 columns (23 x 1.8 cm) previously equilibrated with the appropriate buffers. The 30,000 g supernatants and the desalted extracts were termed the crude (CR) and G-25 fractions, respectively. Aliquots of 20 μ l from both fractions were assayed for raffinose and stachyose synthesis by incubating with 10 μ l of the appropriate substrate in water for 30 minutes at 30 °C. The substrate for raffinose synthesis contained 5.7mM 14 C-galactinol (0.075 μ Ci/ μ mole) and 25mM sucrose while the substrate for stachyose synthesis contained 6.6mM 14 C-galactinol (0.033 μ Ci/ μ mole) and 25mM raffinose.

The reaction was terminated by adding 200 μ l anhydrous ethanol, the solution evaporated to dryness in an air stream, the products redissolved in 50 μ l 50% aqueous ethanol and separated on Whatman paper #4. The unit of activity was expressed in μ moles of product formed per mg of protein per hour and one unit corresponded to the incorporation of ca. 167,000 CPM into raffinose and 67,000 CPM into stachyose.

C- Purification and Properties of Galactinol:Raffinose Galactosyltransferase.

1.- Plant Material.

Mature leaves from Cucurbita pepo plants grown as described above were used for all enzyme extractions.

2.- Extraction and Enzyme Fractionation.

All purification procedures were carried out at 2-4 °C using (unless otherwise stated) 100mM NaPi buffer pH 6.9 containing 20mM BME. Leaves, ca. 50 g fresh weight, were homogenized in a blender for 1 minute with 200 ml of NaPi buffer. The homogenate was filtered through 4 layers of cheesecloth and centrifuged (20,000 g x 20 minutes). The pellet was discarded and the supernatant brought up to 35% saturation with a saturated solution of $(\text{NH}_4)_2\text{SO}_4$. The precipitated protein was removed by centrifugation (10,000 g x 20 minutes) and the supernatant brought up

to 55% saturation with the saturated $(\text{NH}_4)_2\text{SO}_4$ solution and centrifuged (10,000 g x 20 minutes). The supernatant was discarded and the precipitated protein redissolved in 10 ml NaPi buffer. The 35-55% $(\text{NH}_4)_2\text{SO}_4$ fraction was then applied to a Sephadex G-200 column (60 x 2.2 cm) and eluted with NaPi buffer at a flow rate of 0.25 ml per minute. Fractions of 5 ml were collected and assayed for both galactinol:raffinose galactosyltransferase (GRT) and α -galactosidase activity. Active GRT fractions were pooled, made up to 100mM NaCl, applied to a DEAE Sephadex A-50 column (15 x 1.5 cm) previously equilibrated with NaPi buffer containing 100mM NaCl and eluted with a 150 ml linear gradient of 0.1M to 0.5M NaCl in NaPi buffer at a flow rate of 0.22 ml per minute. Fractions of 4.4 ml were collected and all even-numbered fractions assayed for both GRT and α -galactosidase activity. The fractions containing GRT activity were pooled and the volume reduced to about 5 ml by ultrafiltration under N_2 (Amicon PM-10 filter); 20 ml of NaPi buffer was then added and the volume again reduced to about 5 ml. This procedure was repeated two more times. The concentrated fractions were made up to 100mM NaCl and applied to a DEAE Sephadex A-50 column (15 x 1.5 cm) previously equilibrated with NaPi buffer containing 100mM NaCl. The sample was eluted with a 150 ml linear gradient of 0.1M to 0.3M NaCl in NaPi buffer at a flow rate of 0.18 ml per minute. Fractions of 3.6 ml were collected and all even-numbered fractions

assayed for both GRT and α -galactosidase activity. The fractions containing GRT activity were pooled and concentrated by ultrafiltration as described above. The enzyme was stored at 4 °C in 100mM NaPi buffer pH 6.9 containing 20mM BME and used as the enzyme source for the study of the properties of GRT.

3.- Enzyme Assays.

All assays of galactinol:raffinose galactosyltransferase activity were carried out at 30 °C.

a) Assays during Enzyme Fractionation.

GRT activity in the crude extracts, $(\text{NH}_4)_2\text{SO}_4$, G-200 and DEAE fractions was detected by incubating 40 μl of enzyme solution with 10 μl of a standard assay solution containing 20mM raffinose and 4.3mM ^{14}C -galactinol (0.26 $\mu\text{Ci}/\mu\text{mole}$) in water. The reactions were terminated by adding 200 μl anhydrous ethanol, etc., as described above. The products were separated on Whatman paper #4 and, occasionally, on Whatman paper #1. The unit of enzyme activity was defined as the quantity that synthesized 1 μmole of stachyose per hour under the above conditions. Specific activities are expressed in mU per mg of protein. One mU corresponded to the incorporation of about 600 CPM into stachyose.

Fractions from the Sephadex G-200 and DEAE columns were also assayed by incubating 40 μl of enzyme with 10 μl of substrate solution (5mM raffinose and 1.5mM

galactinol in water). The reaction was terminated by the addition of 200 μ l anhydrous ethanol, the solution was evaporated to dryness in an air stream, the products were redissolved in 50 μ l 50% ethanol and 35 μ l spotted on TLC plates which were then developed 2 x 10 cm in solvent (1) followed by detection of the sugars using the phenol-sulfuric acid spray reagent.

Assays for α -galactosidase activity were done by incubating 100 μ l of suitably diluted enzyme solution with 200 μ l of 200mM sodium acetate buffer (pH 5.0) containing 0.3% (w/v) p-nitrophenyl- α -D-galactopyranoside for 20 minutes. The reaction was stopped with 3 ml of 5% Na_2CO_3 and the p-nitrophenol released determined at 400 nm. The unit of activity was defined as the quantity that hydrolyses 1 μ mole of substrate per minute under the above conditions.

b) pH Optimum.

The pH activity curve for GRT was determined by incubating 20 μ l of GRT solution with 20 μ l of McIlvaine (1920) buffers over the pH range 3.5 to 6.0 (McIlvaine buffers used here and in all other experiments were prepared by mixing appropriate volumes of 100mM citric acid and 200mM Na_2HPO_4 to give the desired pH), 100mM NaPi buffers over the range 6.5 to 7.0, 100mM glycine-NaOH buffer at pH 7.5 and 100mM 2-amino-2-methyl-1,3-propanediol buffers over the pH range 8.0 to 8.5 for

10 minutes. The reaction was started by the addition of 10 μ l of standard assay solution and the reaction products were separated on Whatman paper #1.

c) Km Determinations.

The Km value for raffinose was determined by incubating 20 μ l of GRT solution and 20 μ l of 2.5 or 12.5mM 14 C-galactinol (0.12 μ Ci/ μ mole) in NaPi buffer with 10 μ l of either 10, 20 or 40mM raffinose in water. The graphical procedure of Florini and Vestling^o (1957) was used to plot the results. The Km value for galactinol was determined by incubating 20 μ l of GRT solution with 5 μ l of 70mM raffinose in water and 10 μ l of 0.3 to 3.0mM 14 C-galactinol (0.12 μ Ci/ μ mole) in water. The graphical procedure of Lineweaver and Burk (Dixon and Webb, 1979) was used to plot the results. The Km value for galactinol was also determined by incubating 20 μ l of GRT solution and 10 μ l of 5, 10, 20 or 40mM raffinose in water with 20 μ l of either 2.5 or 12.5mM 14 C-galactinol (0.12 μ Ci/ μ mole) in NaPi buffer. In this case, the graphical procedure of Florini and Vestling (1957) was used to plot the results. The Km value for the hydrolysis of galactinol was determined by incubating 20 μ l of GRT solution with 20 μ l of NaPi buffer and 10 μ l of 0.3 to 2.5mM 14 C-galactinol in water. The graphical procedure of Lineweaver and Burk (Dixon and Webb, 1979) was used to plot the results. The Km value for melibiose was determined by incubating

20 μ l of GRT solution with 20 μ l of 2.5mM 14 C-galactinol (0.12 μ Ci/ μ mole) and 10 μ l of 20, 40, 60 and 80mM melibiose. The graphical procedure of Lineweaver and Burk (Dixon and Webb, 1979) was used to plot the results.

d) Inhibitory Studies.

The K_i value for myo-inositol was determined by preincubating 20 μ l of GRT solution with 20 μ l of myo-inositol at 10-40mM concentrations in NaPi buffer for 10 minutes and starting the reaction by adding 10 μ l of a substrate solution containing 50mM raffinose and 5mM 14 C-galactinol (0.12 μ Ci/ μ mole) in water. Other inhibitory studies were carried out by preincubating 20 μ l of GRT solution with 20 μ l of inhibitor solutions in NaPi buffer for 10 minutes and starting the reaction by adding 10 μ l of standard assay solution. All reaction products were separated on Whatman paper #1.

e) Effect of Metal Ions.

To study the effect of metal ions, 4 ml of the GRT solution was dialyzed against one liter of 50mM MOPS buffer (pH 6.9) containing 20mM BME for 24 hours. Twenty μ l of the dialyzed GRT solution were then preincubated for 10 minutes with 20 μ l of 7.5mM solutions of the metal ions in 50mM MOPS buffer (pH 6.9) containing 20mM BME and the reaction started by adding 10 μ l of the standard assay solution. All reaction products were separated on Whatman paper #1.

f) Acceptor and Donor Specificity.

Acceptor specificity was determined by incubating 20 μ l of GRT solution with 20 μ l of 2.5mM 14 C-galactinol (0.12 μ Ci/ μ mole) in NaPi buffer and 10 μ l of 20mM solutions of various substrates in water. Donor specificity was determined by incubating 20 μ l of GRT solution with 10 μ l of 15.5mM 14 C-raffinose (0.70 μ Ci/ μ mole) in water and 20 μ l of NaPi buffer solutions containing one of the following: 25mM UDP-galactose, 10mM PNPG, 40mM melibiose or no addition. Other reactions catalyzed by the GRT preparations were studied by incubating 20 μ l of GRT solution with (1) 20 μ l of 20mM stachyose in NaPi buffer and 10 μ l of 20mM 14 C-myo-inositol (0.12 μ Ci/ μ mole) in water; (2) 10 μ l of 20mM 14 C-myo-inositol (0.12 μ Ci/ μ mole) in water and 5 μ l of 15mM galactinol in water; (3) 10 μ l of 20mM stachyose in NaPi buffer and 20 μ l of 15.5mM 14 C-raffinose (0.70 μ Ci/ μ mole) in water. All reaction products were separated on Whatman paper #1 with n-propanol:ethyl acetate: water (7:1:2 v/v) except when manninotriose (Whatman paper #1; n-butanol:pyridine:water (6:4:3 v/v) 5 x 24 hours) and stachyose (Whatman paper #1; n-propanol:ethyl acetate: water (10:1:8 v/v) 3 x 24 hours) were tested as acceptors.

II- Multimolecular Forms of α -Galactosidase in C. pepo.

A- General Procedures.

1.- Protein Determinations.

Protein determinations were made according to Bradford (1976) using crystalline BSA as the standard or by extinction at 260 and 280nm using the method of Warburg and Christian (1941).

2.- Separation of Sugars by TLC.

All TLC was carried out on Silica Gel 60-F using double developments of 10 cm in solvent (1) (ethyl acetate: acetic acid:methanol: water 60:15:15:10 v/v) and the sugars detected with the phenol-sulfuric acid spray reagent.

3.- Chemicals.

p-Nitrophenyl- α -D-galactopyranoside and all other glycoside derivatives of nitrophenol were from Sigma Chemical Corporation. Stachyose, 1-O-methyl- α/β -D-galactoside, N-acetyl-D-galactosamine, hydroxyapatite, β -galactose dehydrogenase (D-galactose:NAD⁺ 1-oxidoreductase; E.C. 1.1.1.48) and UDP-glucose dehydrogenase (UDP-glucose:NAD⁺ 6-oxidoreductase; E.C. 1.1.1.22) were also Sigma preparations. Radioactive sucrose, galactose and galactose-1-phosphate were purchased from New England Nuclear. Galactinol was prepared as described in Section I.A.5.

B- Acid and Alkaline α -Galactosidase Activity in *C. pepo*.

1.- Extraction and Enzyme Fractionation.

Mature leaf blades from *Cucurbita pepo* plants 3-5 weeks old were used for the enzyme extractions. All purification procedures were performed at 2-4 °C using 100mM NaPi buffer pH 7.0 containing 20mM BME. Leaves, ca. 25 g fresh weight, were homogenized in a blender for 2 minutes with 100 ml of NaPi buffer. The homogenate was filtered through 4 layers of cheesecloth and centrifuged (40,000 g x 30 minutes). The pellet was discarded and the supernatant was brought to 30% saturation with a saturated solution of $(\text{NH}_4)_2\text{SO}_4$ adjusted to pH 7.0 and the precipitated protein was removed by centrifugation (10,000 g x 10 minutes). The supernatant was brought to 60% saturation and centrifuged (10,000 g x 10 minutes). The precipitated protein was redissolved in NaPi buffer and passed through a Sephadex G-25 column (30 x 2.2 cm). The desalted 30-60% $(\text{NH}_4)_2\text{SO}_4$ fraction was then applied to a Sephadex DEAE A-50 column (15 x 2.2 cm) previously equilibrated with NaPi buffer and eluted with a 200 ml linear gradient (0-0.5M NaCl) in NaPi buffer at a flow rate of 0.25 ml per minute. Fractions of 4.5 ml were collected and assayed for α -galactosidase activity at pH 5.4 and 7.5 by incubating 100 μ l of enzyme solution in 400 μ l of McIlvaine buffer pH 5.4 or 7.5 for 10 minutes at 30 °C. The reaction was started by the addition of 100 μ l 0.9% PNPG in water and terminated by addition of 2.4 ml 5% Na_2CO_3 . The

p-nitrophenol released was determined at 400 nm. The unit of enzyme activity was defined as the quantity that hydrolyzes 1 μ mole of substrate per minute under the above conditions. The active fractions at pH 5.4 and 7.5 were pooled, concentrated by ultrafiltration under N_2 (Amicon PM 10 filter) and used as the source of enzyme to characterize the molecular forms (L_I , L_{II} , L_{III} and L_{IV}) thus obtained.

2.- pH Optimum.

The pH optimum for PNPG hydrolysis was determined by incubating 100 μ l of enzyme solution with 400 μ l of McIlvaine buffers over the pH range 3.0 to 8.0 or with 400 μ l of 100mM triethanolamine-HCl (TEA) buffers over the range pH 7.5 to 8.5 for 10 minutes at 30 °C. The reaction was started and terminated as described above. The pH optimum of L_{IV} for raffinose and stachyose hydrolysis was also determined by incubating 50 μ l of enzyme solution with 400 μ l of the buffers described above for 10 minutes at 30 °C. The reaction was started by adding 50 μ l of 100mM raffinose or stachyose in water and terminated by adding 3 ml of freshly prepared 100mM Tris buffer at pH 8.7 containing 4mM glutathione, 0.15mM NAD and 0.075 unit of β -galactose dehydrogenase. The amount of galactose released was determined by change in absorbance at 340 nm after 1 hour at 30 °C.

3.- Km Determinations.

The Km values for PNPG were determined by incubating 50 μ l of enzyme solution with 450 μ l of McIlvaine buffer at pH 5.4 (L_I , L_{II} and L_{III}) or pH 7.5 (L_{IV}), for 10 minutes at 30 °C. The reaction was started by adding 100 μ l of a range of concentrations of PNPG in water (0.6 to 12mM). The reaction was terminated by adding 2.4 ml of 5% Na_2CO_3 .

4.- Inhibitory Studies.

The effect of various sugars upon PNPG hydrolysis at pH 5.4 or pH 7.5 by the molecular forms of α -galactosidase was studied by incubating 50 μ l of enzyme with 250 μ l of McIlvaine buffer at pH 5.4 or 7.5 containing the sugars being studied at concentrations of 16 or 40mM for 10 minutes at 30 °C. The reaction was started by adding 100 μ l of 0.9% (w/v) PNPG in water and terminated by adding 2.6 ml of 5% Na_2CO_3 .

5.- Sugar Hydrolysis.

The hydrolysis of some sugars by the molecular forms of α -galactosidase was studied by incubating 20 μ l of enzyme solution with 60 μ l of McIlvaine buffer at pH 5.4 or 100mM NaPi buffer at pH 7.0 containing raffinose, stachyose or galactinol at concentrations of 5, 5 or 2.5mM, respectively, for 1 hour at 30 °C. The reaction was terminated by boiling for 2 minutes and the products separated by TLC using 25 μ l aliquots from each assay.

6.- Galactosyltransferase Activity.

The molecular forms of α -galactosidase were studied for their ability to synthesize raffinose, stachyose or verbascose at pH 5.4 or 7.0 by incubating 40 μ l of enzyme solution with 10 μ l of substrate solution in water for 1 hour at 30 °C. For the assays at pH 5.4, 1.0 ml of enzyme solution was adjusted to pH 5.4 with 500mM H_3PO_4 and used as the enzyme source. The substrate solutions for the assays contained 20mM of the appropriate acceptor (sucrose, raffinose or stachyose) and 4.3mM ^{14}C -galactinol (0.26 μ Ci/ μ mole). The reaction was stopped and the products separated on Whatman paper #1 as described above. In another series of assays, the substrates consisted of 5mM of the appropriate acceptors and 1.5mM galactinol. The reaction was terminated and the products separated on TLC as described above.

C- Purification and Characterization of L_{IV}.

1.- Extraction and Enzyme Fractionation.

The alkaline α -galactosidase L_{IV} was purified further in order to study its properties more closely. The original amount of leaf material was pushed up to 100 g fresh weight and 250 ml of NaPi buffer used for the first extraction step. The sequence followed thereafter was the same as described above in Section II.B.1. The active fractions from the DEAE Sephadex A-50 column were

then concentrated by ultrafiltration under N_2 (Amicon PM 10 filter) into 50mM NaPi buffer at pH 7.0 containing 20mM BME and placed on a Sephadex G-100 column (85 x 2.2 cm) previously equilibrated with 50mM NaPi buffer at pH 7.0 containing 20mM BME. The column was run at a flow rate of 0.24 ml per minute. Fractions of 5 ml were collected and assayed for α -galactosidase activity at pH 7.5 as described above. The active fractions were then pooled and placed on an hydroxyapatite column (15 x 2.2 cm) previously equilibrated with 50mM NaPi buffer containing 5mM BME. The protein was then eluted from the hydroxyapatite column with a 300 ml linear sodium phosphate gradient (50 to 250mM) at pH 7.0 and in the presence of 5mM BME. The column was run at a flow rate of 0.24 ml per minute. Fractions of 5 ml were collected and assayed for α -galactosidase activity at pH 7.5 as described above. The active fractions were pooled and concentrated by ultrafiltration under N_2 (Amicon PM 10 filter) into McIlvaine buffer at pH 7.5.

2.- pH Optimum.

The pH optimum for PNPG, raffinose and stachyose hydrolysis was determined as described above in Section II.B.2.

3.- Km Determinations.

Km values were determined by incubating 200 μ l of adequately diluted enzyme in McIlvaine buffer pH 7.5 for 10 minutes at 30 °C. The reaction was started by adding

50 μ l of a range of concentrations-(0.5 to 20mM) of PNPG, 0-nitrophenyl- α -D-galactopyranoside or m-nitrophenyl- α -D-galactopyranoside in water. The reaction was terminated by adding 2.5 ml of 5% Na_2CO_3 . Km values for raffinose and stachyose were determined by incubating 100 μ l of enzyme in McIlvaine buffer at pH 7.5 with 100 μ l of McIlvaine buffer (pH 7.5) containing raffinose (10-50mM) or stachyose (5-40mM) for 1 hour at 30 °C. The reaction was terminated and the galactose released determined with β -galactose dehydrogenase as described above.

4.- Substrate Specificity.

The hydrolysis of sucrose, melibiose, raffinose, stachyose and galactinol by L_{IV} was studied by incubating 25 μ l of enzyme with 25 μ l of 10mM aqueous solutions of the individual sugars at 30 °C. Aliquots of 10 μ l were taken at 15, 30, 45 and 60 minutes and spotted directly on a TLC plate to stop the reaction. The products were then separated with solvent (1) 2 x 10 cm. The hydrolysis of various glycoside derivatives of nitrophenol by L_{IV} was also studied by incubating 100 μ l of 0.9% (w/v) aqueous solutions of the glycoside derivatives with 200 μ l of the enzyme for 30 minutes at 30 °C. The reaction was stopped by adding 2.5 ml of 5% Na_2CO_3 .

5.- Inhibitory Studies.

The K_i values of some sugars were determined by adding 100 μ l of enzyme (preincubated at 30 °C) to 400 μ l of McIlvaine buffer (pH 7.5) containing 7.5mM PNPG and a range of concentrations (0-25mM) of the sugars being tested. The reaction was terminated by adding 2.5 ml of 5% Na_2CO_3 .

6.- Effect of Metal Ions.

To study the effect of metal ions on L_{IV} , the enzyme was first transferred into 10mM triethanolamine-HCl (TEA) buffer at pH 7.5 or 10mM HEPES-NaOH buffer at pH 7.5 by ultrafiltration under N_2 (Amicon PM 10 filter). The reaction was started by adding 100 μ l of enzyme (preincubated at 30 °C) to 200 μ l of 10mM TEA or HEPES buffer (pH 7.5) containing 15mM PNPG and 4.5mM of the metal ions under study. The reaction was terminated by adding 2.4 ml of 5% Na_2CO_3 .

7.- Effect of Organic Acids and EDTA.

For this study, the active fractions from the hydroxyapatite column were pooled and divided into 4 samples of about 4 ml each which were then dialyzed individually against 2 L of McIlvaine buffer, 2 L of 10mM NaPi buffer, 2 L of 10mM TEA buffer or 2 L of 10mM HEPES buffer for 72 hours. All buffers were adjusted to pH 7.5. The activity in each buffer (controls) was then

determined by incubating 25 μ l of each enzyme solution with 200 μ l of the appropriate buffer for 10 minutes at 30 °C and the reaction started by adding 75 μ l of 0.9% (w/v) PNPG in water. The reaction was terminated by adding 2.4 ml of 5% Na_2CO_3 . The effect of various organic acids and EDTA on the activity of the enzyme was determined by incubating 25 μ l of each enzyme solution with 200 μ l of the appropriate buffer containing a range of concentrations of the compounds under examination (0.375 to 15mM) for 10 minutes at 30 °C and the reaction started and terminated as in the controls. The effect of organic acids and EDTA on stachyose hydrolysis was also studied. In such cases, the reaction was started by addition of 75 μ l of 33.3mM stachyose in the appropriate buffer instead of PNPG. The galactose released was determined with β -galactose dehydrogenase as described above.

D- Distribution of L_{IV} in the Plant Kingdom.

1.- Extraction and Distribution of L_{IV} in C. pepo.

Cucurbita pepo plants grown as described above were used for all experiments. Leaf blades and petioles were harvested when the plants were between 4-6 weeks old. Roots were obtained by excising the entire rooting system from 3 week old plants. Seed extracts were prepared from whole seeds imbibed where appropriate on filter paper in

glass Petri dishes kept in a controlled environment cabinet.

For determination of total α -galactosidase activity, source material was rapidly weighed and homogenized with an ice-cold pestle and mortar at 2-4 °C using about 3 ml of 100mM NaPi buffer (pH7.0; 20mM BME) per g fresh weight in the presence of acid-washed silver sand. For material of less than 1 g fresh weight, 3 ml of buffer was used. In all cases the pestle and mortar were washed with 2 ml of buffer after the first extraction. The extract and the wash were combined and centrifuged at 40,000 g for 30 minutes at 2 °C. The supernatant was then assayed for α -galactosidase activity at pH 5.4 and 7.5 by incubating 100 μ l of enzyme with 400 μ l of McIlvaine buffer at pH 5.4 or 7.5 for 10 minutes at 30 °C. The reaction was started by addition of 100 μ l of 0.9% (w/v) PNPG in water and terminated by adding 2.4 ml of 5% Na_2CO_3 . The nitrophenol released was determined at 400 nm. The unit of enzyme activity (U) was defined as the quantity that hydrolyzes 1 μ mole of substrate per minute under the above conditions.

2.- I-IV Activity in Developing Leaves of C. pepo.

The morphological age of young, developing leaves was determined by a leaf plastochron index (Erickson and Michelini, 1957) based on a petiole length of 30 mm (Turgeon and Webb, 1973). Extraction and determination of α -galactosidase activity in these leaves was carried out as described in the previous section.

3.- Survey of the Plant Kingdom for L_{IV} Activity.

Leaf blade extracts of the following plants were also assayed for alkaline α -galactosidase activity:

spinach (Spinacea oleracea, var. Bloomsdale Longstanding Dark Gree; family Chenopodiaceae), tobacco (Nicotiana tabaccum, var. unknown; family Solanaceae), tomato (Lycopersicon esculentum, var. Big Girl; family Solanaceae), corn (Zea mays, var. Seneca Chief; family Graminae), wheat (Triticum aestivum, var. Frederick; family Graminae), soybean (Glycine max, var. unknown; family Papilionaceae), pea (Pisum sativum, var. Alaska; family Papilionaceae), cucumber (Cucumis sativus, var. Straight Eight; family Cucurbitaceae), pumpkin (Cucurbita maxima, var. unknown; family Cucurbitaceae), Catalpa bignonioides (family Bignoniaceae), Tabebuia rosea (family Bignoniaceae), Markhamia sessilis (family Bignoniaceae) and Lamium galeobdolon variegatum (family Lamiaceae).

Cucumber and pumpkin plants were grown under the same conditions as the squash plants in controlled environment cabinets. Catalpa plants were grown in soil in controlled environment cabinets while all other plants were grown in soil in greenhouses. The plants grown in soil were fed a solution of 20-20-20 all purpose fertilizer (Genstar Chemicals Ltd, Toronto) weekly. The differentiation between mature and immature leaf tissue for spinach, cucumber, pumpkin, Catalpa and Lamium was

done according to leaf size where the smallest leaves that could be harvested on individual plants were considered immature and fully expanded leaves considered mature. Extraction and determination of α -galactosidase activity was carried out following the procedure described above for Cucurbita pepo.

4.- Alkaline α -Galactosidase Activity in Developing Leaves of Cucumis sativus and Cucurbita maxima.

Leaf blades at different stages of development were obtained from 4-6 week old plants which were grown in perlite in controlled environment cabinets as described above for C. pepo. The stage of development for individual leaves of C. sativus and C. maxima were determined on the basis of fresh weight. Extraction and determination of α -galactosidase activity was carried out as described above for C. pepo.

5.- Partial Purification of Alkaline α -Galactosidase from Cucumis sativus.

Eight day old cotyledons (about 25 g fresh weight) from seeds of Cucumis sativus, var. Boston Picklers, were extracted with a blender in 100 ml of 100mM NaPi buffer pH 7.0 containing 20mM BME at 2-4 °C for 2 minutes. The procedure used for the separation of the molecular forms of α -galactosidase in C. pepo as described in Section II.B.1 was then followed. The active fractions from the DEAE

Sephadex A-50 column were pooled and used directly as the enzyme source. The pH optimum and the inhibition of PNPG hydrolysis by stachyose were determined as described in Sections II.C.2 and II.C.4, respectively.

E- Galactose Metabolism in Cucurbita pepo.

1.-/Preparation of Extracts.

Mature (petiole length > 90 mm) and immature (petiole length < 30 mm) leaf blades from Cucurbita pepo plants 6 weeks old were extracted in 100mM Tris buffer pH 7.4 containing 20mM BME at 2-4 °C with an ice-cold pestle and mortar in the presence of acid-washed sand. A ratio of 3 ml of buffer per g fresh weight was used. The pestle and mortar were washed with 2 ml of the same buffer, the wash combined with the homogenates and centrifuged at 40,000 g for 30 minutes at 2 °C. Two grams fresh weight equivalent of the supernatant were then desalted on a Sephadex G-25 column (25 x 2.2 cm) previously equilibrated with 10mM Tris buffer pH 7.4 containing 20mM BME. The desalted supernatant fraction was used as the source of enzyme for all subsequent assays.

2.- Enzyme assays.

a) α -Galactosidase.

The reaction was started by the addition of 100 μ l of 0.9% (w/v) PNPG in water to 100 μ l of enzyme which had been incubated at 30 °C for 10 minutes in 400 μ l of 200mM

sodium acetate buffer pH 5.0 or 400 μ l of McIlvaine buffer pH 7.5. The reaction was terminated by the addition of 2.4 ml of 5% Na_2CO_3 . Raffinose and stachyose hydrolysis was determined by incubating 100 μ l of enzyme with 200 μ l of 200mM sodium acetate buffer pH 5.0 or 200 μ l of McIlvaine buffer pH 7.5 for 10 minutes at 30 °C. The reaction was started by adding 100 μ l of 100mM raffinose or stachyose solutions in the appropriate buffers and the galactose released was determined as described above with β -galactose dehydrogenase.

b) Galactokinase

Galactokinase activity was determined by adding 100 μ l of enzyme preequilibrated to 30 °C to 100 μ l of 40mM Tris buffer pH 7.4 containing 20mM BME, 1.0mM EDTA, 4mM MgCl_2 , 2mM ATP and 1.0mM ^{14}C -galactose (0.2 μCi). The reaction was stopped by boiling for 90 seconds, the protein was precipitated by centrifugation at 450 g for 5 minutes and the supernatant spotted on Whatman paper #1. The reaction products were then separated by descending paper chromatography with 1M ammonium acetate pH 3.8:ethanol (2:5 v/v) as the solvent for 18 hours. The radioactive peaks present on individual strips were detected and counted as described in Section I.A.2.

c) UDP-Galactose Pyrophosphorylase.

UDP-galactose pyrophosphorylase activity was determined by adding 100 μ l of enzyme preequilibrated

at 30 °C to 100 µl of 40mM Tris buffer pH 8.5 containing 20mM BME, 1.0mM UTP, 4mM MgSO₄ and 0.4mM ¹⁴C-galactose-1-phosphate (0.1 µCi). The reaction was stopped by boiling for 90 seconds and the protein removed by centrifugation at 450 g for 5 minutes. The reaction products were then separated on Whatman paper #1 as described for the galactokinase assays.

d) UDP-Galactose-4-Epimerase.

UDP-galactose-4-epimerase activity was determined by adding 100 µl of enzyme preequilibrated at 30 °C to 100 µl of 100mM glycine-NaOH buffer pH 10.0 containing 20mM BME and 100µM UDP-galactose. The reaction was terminated by boiling for 90 seconds and the protein precipitated by centrifugation at 450 g for 5 minutes. The UDP-glucose formed was determined by incubating 100 µl of the assay supernatant with 3 ml of 200mM glycine-NaOH buffer pH 8.7 containing 1mM NAD and 0.014 units of UDP-glucose dehydrogenase at 30 °C for one hour. The change of absorbance was determined at 340 nm

e) Galactose-1-Phosphate Uridyltransferase.

Galactose-1-phosphate uridyltransferase activity was determined by adding 100 µl of enzyme preequilibrated at 30 °C to 100 µl of 100mM glycine-NaOH buffer pH 9.0 containing 20mM BME, 2mM MgCl₂, 0.5mM UDP-glucose and

0.5mM ^{14}C -galactose-1-phosphate (0.1 μCi). The reaction was stopped by boiling for 90 seconds and the protein precipitated by centrifugation at 450 g for 5 minutes. The reaction products were separated on Whatman paper #1 as described above for the galactokinase assays.

RESULTS

I- Raffinose and Stachyose Synthesis in Leaves of C. pepo.

Many instances of improved efficiency of extraction reported in the literature are attributable to removal of endogenous tannins and/or preventing formation of oxidation products of endogenous phenolics (Loomis and Battaille, 1966; Anderson and Rowan, 1967; Anderson, 1968; Van Sumere et al., 1975). Most plant tissues contain a wide range of phenolic compounds which are oxidized by copper-containing enzymes, broadly classified as phenol oxidases. The most active of these enzymes in plant tissues is O-diphenol:O₂ oxidoreductase (E.C. 1.10.3.1; trivial name O-diphenoloxidase) which oxidizes O-diphenols to the corresponding quinones. Quinones and the brown pigments formed from them by non-enzymatic polymerization reactions inhibit many enzymes. Condensed tannins, which have a lower molecular weight than brown pigments, also form quinones and inhibit enzymes (Van Sumere et al., 1975). Adsorption of phenolic substrates for O-diphenoloxidase by PVP or inhibition of O-diphenoloxidase activity itself by DIECA and other thiol compounds are all commonly used methods of preventing the formation of oxidation products of O-diphenoloxidase activity. Neither BME nor DTT have been investigated in relation to their effect on the

activity of O-diphenoloxidase but they are thought to function by preventing irreversible oxidation in air of sulfhydryl groups associated with enzyme activity (Cleland, 1964; Anderson, 1968).

It is apparent from the results presented in Table IV that raffinose synthesis in leaf homogenates of C. pepo is strictly dependent upon the presence of a protective reagent for sulfhydryl groups. When raffinose synthesis was detected a small amount of radioactivity was usually also incorporated into stachyose. In such cases, the measure of raffinose synthesis was calculated from the incorporation of radioactivity into both compounds. Desalting the crude homogenates on a Sephadex G-25 column also appeared to increase the rate of raffinose synthesis, suggesting the presence of some inhibitory substance in the crude extracts. Raffinose synthesis could not be detected in leaf homogenates using NaPi buffer containing PVP or DIECA alone. These compounds did not have a stimulatory effect on raffinose synthesis when they were studied in combination with DTT or BME.

Stachyose synthesis did not appear to be stimulated significantly by the addition of either compounds tested nor by the desalting step. The best rates of stachyose synthesis were obtained with extracts in NaPi buffer containing 20mM BME and, therefore, all further studies on galactinol:raffinose galactosyltransferase were carried out under these conditions.

Table IV - Raffinose and Stachyose Synthesis in Leaf Homogenates of Cucurbita pepo Extracted Under Various Conditions.

Extraction Buffer	Raffinose Synthesis ^a		Stachyose Synthesis ^a	
	CR	G-25	CR	G-25
100mM Tris pH 7.3	0	ND ^b	ND	ND
100mM NaPi pH 6.9	0	0	18.1	ND
50mM MES pH 6.2	0	0	ND	ND
50mM Imidazole pH 7.0	0	0	8.5	22.3
100mM NaPi + 5mM DTT	5.8	14.3	17.3	19.7
50mM MES + 5mM DTT	1.4	2.3	22.1	18.0
50mM MOPS pH 7.0 + 5mM DTT	2.5	14.4	11.3	17.8
50mM Imidazole + 5mM DTT	1.7	8.5	9.8	17.5
100mM NaPi + 20mM BME	14.6	22.3	24.8	22.7

^a Activity expressed in μ moles of product formed per hour per mg of protein $\times 10^{-2}$. The average value of two different experiments is given in each case.

^bND: Not Determined.

II - Separation of Sugars by Thin Layer Chromatography.

The detection of galactosyltransferase activity in Sephadex G-200 and DEAE Sephadex A-50 column fractions using ^{14}C -galactinol assays takes at least 24 hours and separation of stachyose from galactinol on Whatman paper #4 is often poor. Therefore, an attempt was made to develop a faster assay method using TLC on Silica gel.

The Rf values of the sugars which might be produced in galactosyltransferase assays are given in Table V using the best six solvents investigated.

Detection of the sugar spots was good with all three methods used. The phenol-sulfuric acid spray was eventually adopted because of its simplicity of preparation, its ability to retain its effectiveness over a storage period of one month and its white background (sugars appeared as brown spots) which allowed for greater sensitivity than the other two methods. Fleur's reagent was used only to determine the Rf value of myo-inositol and did not react with the other sugars except with relatively large amounts of galactinol (25-30 μg). Using this spray, inositols were detected as orange spots on a white background. Myo-inositol did not react with the phenol-sulfuric acid spray and, therefore, did not interfere with the detection of the other sugars.

Table V - Rf Values of Some Sugars
Chromatographed on Silica
Gel Thin Layers.

Sugar	Solvents (see Materials and Methods)						
	(1) ^a	(1) ^b	(2) ^c	(3) ^a	(4) ^a	(5) ^b	(6) ^a
Stachyose	0.12	0.06	0.07	0.32	0.04	0.04	0.17
Raffinose	0.24	0.15	0.22	0.45	0.14	0.16	0.32
Galactinol	0.20	0.12	0.13	0.26	0.07	0.08	0.27
Sucrose	0.45	0.30	0.46	0.60	0.27	0.28	0.50
Myo-inositol	0.38	ND ^d	ND	0.30	ND	NR ^e	0.46
Galactose	0.53	0.35	0.56	0.55	0.31	0.40	0.54
Glucose	0.59	0.42	0.58	0.59	0.39	0.35	0.58
Fructose	0.59	0.42	0.58	0.59	0.39	0.35	0.58
Melibiose	0.31	0.20	ND	ND	ND	ND	ND

^aDouble developments of 10 cm each.

^bSingle development of 10 cm.

^cSingle development of 15 cm.

^dND: Not Determined.

^eNR: No Reaction with Fleur's reagent.

Good separation of the key sugars (stachyose, raffinose, sucrose and galactinol) was obtained with solvent 2. However, pronounced tailing of the raffinose spot interfered with the detection of small quantities of stachyose. Using solvent 4, the sugars were detected as diffuse spots and galactinol interfered with the detection of stachyose. However, raffinose synthesis could easily be assayed using solvent 4.

The results obtained with solvent 5 are shown in Figure 4a. This solvent was excellent to assay raffinose synthesis (Fig. 4a; compare row #5 with row #7) but galactinol interfered with the detection of small quantities of stachyose (Fig. 4a; compare row #6 with row #8).

As shown in Figure 4b, a good separation of stachyose from the other sugars was obtained with solvent 1 (single development of 10 cm) but galactinol interfered with the assay of raffinose. This solvent was investigated further by trying double developments of 10 cm. The results obtained were excellent as shown in Figure 4c. When raffinose and galactinol were incubated with galactinol:raffinose galactosyltransferase the appearance and intensity of the spot corresponding to stachyose was clearly related to the length of the incubation period. Similarly, the intensity of the spot corresponding to stachyose was directly related to enzyme concentration and to the radioactivity incorporated into

Figure 4a - Separation of Some Sugars Using TLC on Silica Gel and Solvent 5. From left to right: (1) 10 μ g galactose; (2) 10 μ g stachyose; (3) 10 μ g galactinol; (4) 10 μ g raffinose; (5) 10 μ l of substrate for raffinose synthesis: 10mM sucrose and 7.2mM galactinol; (6) 10 μ l of substrate for stachyose synthesis: 10mM raffinose and 7.2mM galactinol; (7) same as (5) plus 5 μ g raffinose; (8) same as (6) plus 4 μ g stachyose; (9) 10 μ g glucose; (10) 10 μ g sucrose.

Figure 4b - Separation of Some Sugars Using TLC on Silica Gel and Solvent 1 (single development of 10 cm). Spots from left to right identical to those described for Fig. 4a.

Figure 4c - Separation of Some Sugars Using TLC on Silica Gel and Solvent 1 (double development of 10 cm). From left to right: (1) to (4): reaction products obtained after 10 μ l of galactinol:raffinose galactosyltransferase solution were incubated with 10 μ l 15mM raffinose and 1.5mM galactinol for 5, 10, 20 and 30 minutes, respectively; (5) control: 10 μ l 15mM raffinose and 1.5mM galactinol; (6) 10 μ g sucrose; (7) 10 μ g galactinol; (8) 10 μ g stachyose; (9) 10 μ g galactose; (10) 10 μ g glucose.

Figure 4d - Separation of Some Sugars Using TLC on Silica Gel and Solvent 3. Spots from left to right identical to those on Fig. 4c.

Figure 4e - Separation of Some Sugars Using TLC on Silica Gel and Solvent 6. Spots from left to right identical to those on Fig. 4c.

stachyose using ^{14}C -galactinol assays.

Using solvent 3, the synthesis of stachyose was clearly reflected by a change in the intensity of the spot corresponding to stachyose (Fig. 4d). However, galactinol migrated to a position similar to that of stachyose and interfered with the positive identification of small quantities of stachyose.

Solvent 6 was perhaps the best solvent which could be used in the detection of stachyose synthesis (Fig. 4e) but preference was eventually given to solvent 1 (double development) because total development time with the latter (including enzyme assays, spotting, development and sugar detection) usually took only 3 hours as compared to 5 hours with solvent 6.

III- Purification and Properties of Galactinol:Raffinose Galactosyltransferase.

The pellet remaining after centrifugation of the leaf homogenate was unable to synthesize any detectable amount of stachyose. The galactinol:raffinose galactosyltransferase (GRT) activity was precipitated with ammonium sulphate at between 35-55% saturation of the homogenate. High concentrations of ammonium sulphate appeared to have an inhibitory effect on GRT activity when compared to the crude homogenate (Table VI); however, desalting was not investigated at this stage of the purification. For assays of the ammonium sulphate fractions, the reaction products were passed through 1 ml strong anion (acetate) exchange resin to improve their subsequent chromatographic separation on Whatman paper #1 or #4. No GRT activity remained in the supernatant of the 35-55% ammonium sulphate pellet while, occasionally, traces of GRT activity could be detected in the 0-35% ammonium sulphate pellet.

Assaying 5 ml fractions from the Sephadex G-200 column, GRT emerged as a single peak of activity between fractions 20-26. α -Galactosidase activity in the eluate, assayed with PNPG as substrate, began to emerge at fraction 23 with maximum activity in fractions 27-28 and terminating in fraction 35. Thus, considerable separation of α -galactosidase activity from GRT was achieved at this stage.

2

Table VI - Typical Purification Sequence for Galactinol:Raffinose Galactosyltransferase from 50 g of Mature Leaves.

Purification Step	Volume (ml)	Total Enzyme Activity (mU) ^a	Total Protein (mg)	Sp. Act. (mU/mg)	Recovery (%)	Purification fold
Extract in phosphate buffer	177	34520	1184	29.2	-	-
Ammonium sulphate (35-55% pellet) ^c	12.8	6850 ^c	463	14.8	19.8	-
Sephadex G-200	40	25300.	77.5	326.0	73.3 ^e	11.2 ^d
DEAE Sephadex A-50 (0.1-0.5M NaCl)	5	11030	16.5	668.0	43.6	22.9
DEAE Sephadex A-50 (0.1-0.3M NaCl)	5.8	9800	8.8	1113.0	88.8	38.1

^aUnit of activity, 1 μ mole stachyose per hour. One mU corresponded to the incorporation of about 600 CPM into stachyose.

^bRecovery is calculated relative to the previous purification step. Overall recovery: 28.4%.

^cStrong inhibition noted.

^dCalculated from the activity of the phosphate buffer extract.

^eRecovery calculated relative to activity present in phosphate buffer.

A further separation of the two enzyme activities was achieved with the first DEAE Sephadex A-50 column (0.1-0.5M NaCl). As indicated in Figure 5 most of the remaining α -galactosidase activity was eluted in the salt gradient before the single peak of GRT emerged. With the second DEAE Sephadex A-50 column (0.1-0.3M NaCl) a single peak of GRT activity emerged between fractions 12-18 which contained only 1-2% of the original α -galactosidase activity. The stages of the purification procedure are summarized in Table VI.

To reduce the time spent on assaying fractions from the G-200 and DEAE Sephadex A-50 columns a TLC method was devised (see above). Good separation of the reaction products was achieved in 3 hours. Figures 6a and 6b illustrate the results obtained with the TLC method when fractions from the first and second DEAE Sephadex A-50 were assayed for GRT activity. In inactive fractions (e.g. Fig. 6a, fractions # 16, 18 and 20), only raffinose and galactinol appeared as a single large band. In fractions containing GRT activity, stachyose appeared as a band of varying intensity below raffinose (Fig. 6a, fractions # 22, 24, 26, 28 and 30; Fig. 6b, fractions # 12, 14 and 16). Fractions 8 to 14 of the first DEAE Sephadex A-50 column (Fig. 6a) were also shown to contain invertase activity. In these fractions, melibiose ran slightly above the raffinose

Figure 5 - Separation of α -galactosidase activity
(closed circles) from galactinol:raffinose
galactosyltransferase activity (open circles)
on DEAE Sephadex A-50 (0.1-0.5M NaCl). Solid
line represents transmittance at 254 nm and
280 nm; broken line represents NaCl
concentration.

Figure 6a - Reproduction of TLC Plate Showing Galactinol:Raffinose Galactosyltransferase Activity in Fractions Collected from the First DEAE Sephadex A-50 Column (0.1-0.5M NaCl). Even-numbered fractions were assayed; from left to right: fractions 8 to 30. Dark bands represent substrate (raffinose + galactinol) and open bands represent products (melibiose and glucose in #8, 10, 12 & 14; stachyose in # 22, 24, 26 & 28).

Figure 6b - Reproduction of TLC Plate Showing Galactinol:Raffinose Galactosyltransferase Activity in Fractions Collected from the Second DEAE Sephadex A-50 Column (0.1-0.3M NaCl). Even-numbered fractions were assayed; from left to right: fractions 4 to 28. Dark bands represent substrate (raffinose + galactinol) and open bands represent product (stachyose in # 12, 14 & 16).

A

Glucose

Melibiose
Raffinose
Stachyose

B

Raffinose
Stachyose

band while fructose migrated further up. The presence of invertase activity in these fractions was confirmed by following the hydrolysis of ^{14}C -sucrose under the same conditions and separating the products on Whatman paper #4.

The active fractions from the second DEAE Sephadex A-50 column were concentrated by ultrafiltration and stored at 4 °C. No loss of activity was observed up to 45 days of storage. Polyacrylamide gel electrophoresis of the GRT preparation stained with Coomassie Blue revealed nine visible protein bands (Fig. 7). An attempt was made to locate the position of the GRT by incubating 1 mm slices of the gel in the assay medium but no activity could be recovered. α -Galactosidase activity, which can be readily located in such gel slices using PNPG as substrate, could not be detected in these gels. Furthermore, no hydrolysis of either raffinose or stachyose could be detected when these substrates were incubated along with GRT under the standard assay conditions at pH 6.9 for 30 minutes or at pH 5.4 where α -galactosidase activity is optimally active with PNPG as substrate (Thomas and Webb, 1977).

The pH optimum for stachyose biosynthesis appeared to plateau between pH 6.5 and 7.0 with ca. 50% reduction at pH 5.4 and 7.6 (Fig. 8). Tris buffer at 0.1M inhibited stachyose synthesis by approximately 50%.

+

Figure 7 - Reproduction of
a Polyacrylamide
Gel of the
Galactinol:Raffinose
Galactosyltransferase
Preparation Purified
40 Fold.

Figure 8 - Galactinol:Raffinose Galactosyltransferase Activity as a Function of pH.

GRT hydrolyzed galactinol when the acceptor raffinose was either absent or present at very low concentrations. Thus, during K_m determinations for raffinose at low substrate concentrations, free ^{14}C -galactose was released up to a maximum representing about 10-12% hydrolysis of the ^{14}C -galactinol. This reaction was almost completely eliminated at saturation levels of raffinose (Fig. 9). In the absence of raffinose, a K_m of 0.33mM for galactinol hydrolysis was obtained from a standard Lineweaver-Burk plot (Fig. 10). As stated above, there was no detectable hydrolysis of raffinose in the absence of galactinol. For stachyose synthesis, a K_m of 7.25mM was obtained for galactinol in the presence of 10mM raffinose (Fig. 11). Using the graphical procedure of Florini and Vestling (1957) for determining enzyme constants for a two substrate reaction at non-saturating substrate concentrations, a K_m of 7.7mM was obtained for galactinol (Fig. 12). Using the same procedure, a K_m of 4.6mM was obtained for raffinose in the presence of non-saturating concentrations of galactinol (Fig. 13). Both melibiose and myo-inositol showed inhibitory effects on the synthesis of stachyose. Lineweaver-Burk plots indicated that melibiose was a non-competitive inhibitor of raffinose while myo-inositol was a competitive inhibitor (Fig. 14). In the presence of 10mM raffinose and 1mM galactinol, a K_i for myo-inositol

Figure 9 - Distribution of ^{14}C into stachyose (open circles) and galactose (closed circles) when GRT is incubated with $0.86\text{mM } ^{14}\text{C}$ -galactinol and various concentrations of raffinose. Inset: Lineweaver-Burk plot for the determination of the K_m for raffinose under these conditions.

Figure 10 - Lineweaver-Burk determination of the K_m for galactinol hydrolysis in the absence of raffinose.

Figure 11 - Lineweaver-Burk determination of the K_m for galactinol in the presence of 10mM raffinose.

Figure 12 - Florini-Vestling determination of the K_m for galactinol at non-saturating raffinose concentrations. Upper graph: A and B correspond to concentrations of 1.06 and 5.2mM galactinol, respectively.

Figure 13 - Florini-Vestling determination of the K_m for raffinose at non-saturating galactinol concentrations. Upper graph: A, B and C correspond to concentrations of 2, 4 and 8mM raffinose, respectively.

Figure 14 - Lineweaver-Burk determination of the K_m for raffinose in the presence of 0.86mM galactinol ($\circ$ — $\circ$) and the effect of 20mM melibiose ($\bullet$ — $\bullet$) and 20mM myo-inositol (Δ — Δ).

of 2mM was determined using the graphical procedure of Dixon for competitive inhibitors (Fig. 15). At a final concentration of 8mM melibiose in the standard assay, the rate of stachyose formation decreased by about 13% compared to the control. Neither stachyose nor galactose were inhibitory when present in concentrations of up to 40mM in the standard assay.

GRT appeared to be highly specific for raffinose. Thus, there was no detectable transfer of the galactosyl group from ^{14}C -galactinol to any of the following sugars replacing raffinose at 4mM concentrations in otherwise standard assays: fructose, glucose, cellobiose, lactose, gentiobiose, melezitose, sucrose, trehalose, maltotriose and manninotriose. In all the above assays, about 10% of the galactinol was hydrolyzed over 30 minutes, resembling the results reported above for assays in the absence of the acceptor raffinose. On the other hand, incubation of GRT with 4mM melibiose replacing raffinose as the galactosyl acceptor in the standard assay system, resulted in the synthesis of manninotriose but at a rate ten times less than with 4mM raffinose as the acceptor. A K_m of 5.2mM was obtained for melibiose in the presence of 1mM galactinol (Fig. 16). In the presence of 4mM galactose as acceptor, two minor ^{14}C -labelled products, $R_{\text{galactose}}$ 0.33 and 0.53, respectively, were observed on the

Figure 15 - Dixon plot of GRT activity in the presence of 10mM raffinose, 1.0mM galactinol and varying concentrations of myo-inositol.

Figure 16 - Lineweaver-Burk determination of the K_m for melibiose in the presence of 1.0mM galactinol.

chromatograms but their identities were not investigated. When the galactose concentration was brought up to 16mM, less than 5% incorporation of the total radioactivity into these two compounds occurred. When both raffinose (4mM) and galactose (16mM) were incubated together with ^{14}C -galactinol and GRT, stachyose was the only product observed. No verbascose synthesis could be detected in the assay system 30 minutes after incubation of GRT with up to 40mM concentrations of stachyose as acceptor. In this latter assay, some ^{14}C -stachyose was formed in addition to the production of free ^{14}C -galactose. The labelling of stachyose may have been caused either by a galactosyl exchange between ^{14}C -galactinol and the terminal galactosyl residue of stachyose or, and perhaps more likely, by the initial hydrolysis of ^{14}C -galactinol by GRT followed by labelling of the terminal ^{14}C -galactosyl group in stachyose through equilibration of the forward and back reactions in the synthesis of stachyose by GRT. The reversibility of GRT was readily demonstrated. A rate of 0.91 $\mu\text{moles } ^{14}\text{C}$ -galactinol per hour per mg of protein was observed in the presence of 8mM stachyose and 4mM ^{14}C -myo-inositol. Further exchange reactions occurred when the enzyme was incubated in the presence of 2.1mM galactinol and 5.7mM ^{14}C -myo-inositol in NaPi buffer at pH 6.9, producing ^{14}C -galactinol at ca. 2.7 $\mu\text{moles per hour per}$

mg of protein, and also when incubated in the presence of 6.2mM ^{14}C -raffinose (galactose labelled) and 4mM stachyose, producing ^{14}C -stachyose at ca. 0.4 μmoles per hour per mg of protein.

Neither UDP-galactose (10mM) or melibiose (16mM) could replace galactinol as the galactosyl donor in the standard assay system. Incubating the enzyme with raffinose alone did not produce stachyose. However, it was found that substituting 1mM galactinol with 4mM p-nitrophenyl- α -D-galactopyranoside in the standard assay system produced a rate of stachyose synthesis ca. 50% that was obtained with 1mM galactinol.

Because several of the metal ions studied were precipitated in NaPi buffer at pH 6.9, the galactosyl-transferase was transferred into MOPS buffer pH 6.9 where, under otherwise standard conditions, the activity was about 70% that obtained in NaPi buffer. The order of inhibition for 3mM final concentrations of the metal ions was $\text{Co}^{2+} > \text{Hg}^{2+} > \text{Zn}^{2+} > \text{Mn}^{2+} > \text{Ni}^{2+} \gg \text{Mg}^{2+} \gg \text{Ca}^{2+} \gg \text{Ag}^{2+} \gg \text{Cu}^{2+}$ while 3mM MoO_4^{2-} , K^+ , Na^+ , Fe^{3+} and up to 5mM EDTA had no effect on enzyme activity (Table VII). Concentrations of up to 20mM $(\text{NH}_4)_2\text{SO}_4$ were not inhibitory although, as stated above, high concentrations used in the extraction procedure were suspected to have caused considerable inhibition.

Table VII - Effect of Metal Ions on
Galactinol:Raffinose
Galactosyltransferase.

Assay Conditions	Relative Activity ^a	Percent Inhibition ^b
NaPi buffer pH 6.9	100	-
MOPS buffer pH 6.9	70.1±3.6	-
MOPS buffer +		
3mM AgNO ₃	57.9±4.1	17.5
3mM CaCl ₂	55.8±4.1	20.4
3mM CoCl ₂	15.5±1.2	77.8
3mM CuNO ₃	60.0±6.2	14.4
5mM EDTA	69.8±3.0	-
3mM FeCl ₃ -EDTA	70.2±3.1	-
3mM HgCl ₂ -EDTA	22.4±0.7	68.1
3mM KCl	72.9±6.5	-
3mM MgCl ₂	54.4±4.7	22.5
3mM MnCl ₂	34.2±3.6	51.2
3mM MoO ₄	68.5±0.1	-
3mM NiCl ₂	36.7±1.4	47.6
3mM ZnCl ₂	26.5±0.7	62.2

^a Average calculated from two series of experiments using different GRT preparations. Duplicate assays were carried out with each preparation.

^b Percent inhibition when compared to relative activity in MOPS buffer.

IV - Multimolecular Forms of α -Galactosidase in *C. pepo*.

The elution pattern of the multimolecular forms of α -galactosidase present in mature leaves of *Cucurbita pepo* is shown in Figure 17. A clear separation occurred between the acid forms L_I , L_{II} and L_{III} as previously reported by Thomas and Webb (1977; 1978). The assays for α -galactosidase activity at pH 7.5 also distinguished L_I and L_{III} from L_{II} . L_I and L_{III} showed residual activity at pH 7.5 whereas L_{II} did not. The residual activity present in fractions containing L_I was especially helpful in determining which fractions should be pooled to obtain a preparation of L_I essentially free of L_{II} activity since both enzymes eluted very close to one another, L_I appearing as a small shoulder on the large peak of L_{II} activity (Fig. 17).

A fourth molecular form of α -galactosidase was detected and named L_{IV} . This enzyme came off the DEAE Sephadex A-50 column at the end of the salt gradient and differed strikingly from L_I , L_{II} and L_{III} by displaying activity only when assayed at pH 7.5. Single experiments where 8-days old cotyledons, mature petioles (longer than 80 mm) or young developing leaves (petiole length of 30-40 mm) were used as starting material also revealed the presence of L_{IV} in those tissues.

Fractions containing L_I (5-8), L_{II} (10-20), L_{III} (24-30) and L_{IV} (36-44) were pooled and concentrated

Figure 17 - Elution pattern of the molecular forms of α -galactosidase from Cucurbita pepo on DEAE Sephadex A-50. Enzyme activity was determined at pH 5.4 (closed circles) and pH 7.5 (open circles). Broken line shows NaCl concentration.

by ultrafiltration. A typical purification scheme is presented in Table VIII. Less than 5% of the α -galactosidase activity recovered in the supernatant of the NaPi buffer extract was found to be associated with the pellet for both acid and alkaline α -galactosidase. The overall recovery of acid α -galactosidase activity was about 40% after ammonium sulphate fractionation and ion-exchange chromatography. L_I and L_{III} each accounted for ca. 10-15% of this activity and L_{II} for ca. 70%.

Over 50% of the alkaline α -galactosidase activity, L_{IV} , was lost during the ammonium sulphate fractionation step. The protein precipitated between 0-30% saturation with $(NH_4)_2SO_4$ and the supernatant of the 30-60% fraction contained about 8-12% of the total activity found at pH 7.5 in the NaPi buffer homogenate. None of this activity was due to residual activity of L_I and L_{III} at pH 7.5 since no α -galactosidase activity could be detected at pH 5.4 in these fractions. This indicates that L_I , L_{II} and L_{III} were completely precipitated between 30-60% saturation with ammonium sulphate and that the alkaline α -galactosidase activity found in the 0-30% $(NH_4)_2SO_4$ saturated precipitate and the supernatant of the 30-60% fraction corresponds to L_{IV} activity only.

Desalting NaPi extracts with Sephadex G-25 gave 100% recovery of α -galactosidase activity assayed

Table VIII - Typical Purification Sequence for L_I, L_{II}, L_{III} and L_{IV}
from 25 g of Mature Leaves.

Purification Step	Volume (ml)	Total Enzyme Activity (mU) pH 5.4	Total Enzyme Activity (mU) pH 7.5	Total Protein (mg)	Specific Activity (mU/mg) pH 5.4	Specific Activity (mU/mg) pH 7.5
Extract in phosphate buffer	104	8506	5779	305	27.9	19.0
Ammonium sulphate (30-60% pellet)	6.2	6776	2752	255	26.6	10.8
DEAE Sephadex A-50 and Ultrafiltration						
L _I	5.3	424 ^a	-	6.0	70.8 ^c	-
L _{II}	6.7	2474 ^a	-	12.0	206.2 ^c	-
L _{III}	5.4	480 ^a	-	14.7	32.8 ^c	-
L _{IV}	5.2	-	1463 ^b	12.8	-	114.3 ^c

^a Overall recovery of acid α -galactosidase activity: 39.7%. L_I, L_{II} and L_{III} accounted for 12.6, 73.2 and 14.2% of the recovered activity, respectively.

^b Overall recovery of alkaline α -galactosidase activity: 25.3%.

^c Purification fold 2.5, 7.4, 1.2 and 6.0 for L_I, L_{II}, L_{III} and L_{IV}, respectively.

at pH 7.5. The possibility that the loss of activity encountered during the ammonium sulphate fractionation might represent loss of an activator (metallic or other) is therefore unlikely and these results suggest that L_{IV} is readily denatured in the presence of high salt concentrations. Further evidence of the sensitivity of L_{IV} came from the observation that the enzyme experiences an irreversible 85% loss of activity after incubation at pH 5.6 for 30 minutes at 22 °C as tested with PNPg as substrate. The overall recovery of alkaline α -galactosidase activity after the ion-exchange chromatography step was usually 20-25%. When aliquots of the L_{IV} preparation were submitted to polyacrylamide gel electrophoresis, the activity recovered from the gels corresponded to a sharp discrete protein band on gels of a similar run stained with Coomassie Blue. The gels also revealed that L_{IV} quickly migrated towards the anode. This and the elution pattern of L_{IV} on DEAE Sephadex A-50 indicated that L_{IV} has a strong negative charge.

α -Galactosidase activity was also detected in fractions 31-35 from the DEAE Sephadex A-50 column (Fig. 17). These fractions were found to contain the bulk of the GRT activity which eluted from the Sephadex A-50 column. These fractions were therefore pooled, concentrated by ultrafiltration and the enzyme preparation thus obtained referred to as the GRT fraction and its

properties studied along with those of the L_I , L_{II} , L_{III} and L_{IV} fractions.

The pH optima for PNPG hydrolysis of L_I , L_{II} , L_{III} , L_{IV} and GRT are shown in Figure 18. L_I displayed maximal activity between pH 5.4 and 6.0 with 50% residual activity remaining at pH 4.4 and 7.0. L_{II} showed maximal activity at pH 5.5 with 50% residual activity remaining at pH 3.1 and 6.1. The shoulder observed between pH 3.5 and 4.5 was always reproducible using L_{II} fractions from different extractions. L_{III} showed maximal activity at pH 5.4 with 50% residual activity remaining at pH 3.0 to 4.0 and pH 6.8. L_{IV} displayed maximal activity at pH 7.5 with 50% residual activity remaining at pH 6.5 and 8.4. It is interesting to notice that very little overlap in activity occurs between L_{II} and L_{IV} , the two molecular forms of α -galactosidase which are responsible for most of the recovered hydrolytic activity. The GRT fraction did not show a distinct pH optimum and its activity over the pH range studied remained relatively constant.

The pH optimum for raffinose and stachyose hydrolysis was also determined for L_{IV} as shown in Figure 19. Stachyose hydrolysis was maximal at pH 7.5 with two minor peaks at pH 4.0 and 5.0. Raffinose hydrolysis was maximal at pH 5.0 but over 80% as much activity was observed at pH 4.0 and pH 6.5 to 7.0. No hydrolysis of raffinose or stachyose could be detected

Figure 18 - Hydrolysis of PNPG by L_I , L_{II} , L_{III} , L_{IV} and GRT as a function of pH.

Figure 19 - Hydrolysis of PNP (○—○), raffinose (□—□) and stachyose (△—△) by L_{IV} as a function of pH. Open symbols: McIlvaine buffer; closed symbols: triethanolamine buffer.

when L_{IV} was incubated in 100mM Tris buffer at pH 8.7. It is interesting to notice that L_{IV} hydrolyzed stachyose far more readily than raffinose. This is a significant reversal of the usual substrate preference of acid forms of α -galactosidase (e.g. Dey and Pridham, 1972; Williams et al., 1977 and 1978; Smart and Pharr, 1980).

Km values of 0.20, 0.48 and 0.42mM for PNPG hydrolysis were obtained from a standard Lineweaver-Burk plot with L_I , L_{II} and L_{III} , respectively, at pH 5.4 (Fig. 20a). A Km value of 0.60mM was obtained with L_{IV} at pH 7.5 and a Km value of 0.27mM was obtained with the GRT fraction at pH 7.0 (Fig. 20b). The fact that the Km value determined for L_I was highly reproducible from one preparation to another indicates that L_I was essentially free of L_{II} activity. The Km values obtained with L_{II} and L_{III} were also reproducible from one preparation to another. However, the Km value obtained with GRT showed considerable variation (± 0.1 mM) as did the Km value determined for L_{IV} (± 0.11 mM).

The effect of various sugars on the hydrolysis of PNPG at pH 5.4 by L_I , L_{II} and L_{III} , and at pH 7.5 by L_{IV} and at pH 6.9 by the GRT fraction was studied. (Preparations of galactinol:raffinose galactosyltransferase, purified 40-fold as described in Section I.C.2 in Materials and Methods, hydrolyzed both galactinol and PNPG; therefore, the effect of the same sugars on the α -galactosidase

Figure 20 - Lineweaver-Burk determinations of the K_m values for PNPg hydrolysis by L_I , L_{II} , L_{III} , L_{IV} and GRT.

activity present at pH 6.9 in these preparations (GRT-40) was also investigated.) The results are presented in Table IX.

Galactinol inhibited L_I , L_{II} , L_{III} and L_{IV} to about the same degree (35-40%) while galactose was the most potent inhibitor of these enzymes and approximately only 50% residual activity was observed in the presence of 10mM concentrations of this sugar. Melibiose, myo-inositol, raffinose and stachyose inhibited L_I to similar degrees (ca. 20%). Melibiose appeared to be the most inhibitory to L_{II} (after galactose and galactinol) while myo-inositol, raffinose and stachyose were weak inhibitors at 10mM concentrations (ca. 5%). Characteristic of L_{III} and L_{IV} was a lack of inhibition by myo-inositol. L_{III} was further characterized by ca. 15% inhibition in the presence of 10mM melibiose and stachyose and little inhibition (ca. 6%) by 10mM raffinose. On the other hand, L_{IV} displayed a characteristic pattern of inhibition where 10mM melibiose was a weak inhibitor (ca. 9%) and 10mM raffinose was without any detectable inhibitory properties. Strikingly, L_{IV} was inhibited to a large degree by stachyose which appeared to be the second most potent inhibitor of this enzyme after galactose, a position held by galactinol in the case of L_I , L_{II} and L_{III} .

Table IX - The Effect of Some Sugars on the Hydrolysis of PNPB by L_I, L_{II}, L_{III}, L_{IV}, GRT and GRT-40

Sugars	Concentration (mM)	Percent Inhibition ^{ab}				
		L _I	L _{II}	L _{III}	L _{IV}	GRT-40
Galactinol	25	33.3	44.3	45.5	30.8	37.0
Galactose	25	75.6 ^c	63.0 ^d	69.4 ^d	77.6 ^d	45.3 ^d
	10	69.4	45.4	50.6	53.2	28.7
Melibiose	25	25.7 ^c	15.8 ^c	33.0 ^d	31.6 ^d	+ 6.2 ^d
	10	18.3	14.2	18.4	9.1	0
Myo-inositol	25	24.3 ^d	15.1 ^d	0	0	+ 40.4 ^c
	10	19.3	4.2	0	0	+ 32.8
	1	ND ^e	ND	ND	ND	+ 96.4 ^d
Raffinose	25	20.9 ^c	8.1 ^c	16.9 ^d	7.6 ^d	+ 32.0 ^d
	10	17.2	5.4	6.0	0	+ 8.1
	1	ND	ND	ND	ND	+ 61.0 ^d
Stachyose	25	18.2 ^c	10.7 ^d	35.2 ^d	55.2 ^d	42.0 ^d
	10	21.5	5.8	14.7	36.4	24.0

^a L_I, L_{II} and L_{III} were assayed at pH 5.4, L_{IV} at pH 7.5 and GRT and GRT-40 at pH 6.9.

^b Stimulation of enzyme activity was observed with some sugars and indicated by a + sign.

^c Difference between 25 and 10mM not significant at the 0.5 level of confidence as determined using an F test (Runyon and Heber, 1971). Each value is the average of 6 determinations.

^d Difference between 25 and 10mM significant at the 0.5 level of confidence as determined using an F test (Runyon and Heber, 1971). Each value is the average of 6 determinations.

A comparison of the inhibition pattern of GRT and GRT-40 by various sugars unveiled many similarities as well as some differences. Most striking was the stimulatory effect brought about by melibiose, myo-inositol and raffinose upon PNPG hydrolysis in these two fractions and the lack of inhibition of GRT-40 by galactose. The stimulatory effect observed with melibiose and raffinose can be explained by the properties of galactinol:raffinose galactosyltransferase and could have been predicted. It has been shown that GRT-40 catalyzes the following reactions:

PNPG can replace galactinol as donor and the following reactions were also found to occur:

In the presence of an acceptor, the release of p-nitrophenol by GRT-40 is therefore increased since hydrolysis of the donor (whether it is galactinol or PNPG) is greatly increased as a result of the transferring nature of the enzyme. The stimulatory effect observed is greater with raffinose than melibiose as would be expected since galactosyl transfer proceeds faster with the prime acceptor, raffinose.

Myo-inositol had a stimulatory effect on PNPG hydrolysis and this can again be explained by the properties of GRT-40 which catalyzes the following exchange reaction:

If PNPG replaces galactinol, the reaction becomes:

Therefore, in the presence of myo-inositol, additional p-nitrophenol is released and a stimulatory effect upon PNPG hydrolysis is observed. Similarly, in addition to competition effects between PNPG and galactinol, the inhibition of GRT-40 by galactinol is presumably partly due to the following reaction:

resulting in less p-nitrophenol being free to react with Na_2CO_3 .

Myo-inositol, melibiose and raffinose also had a stimulatory effect upon the hydrolysis of PNPG by the GRT fraction but to a lesser degree. This may be due to the fact that the hydrolytic activity present in this fraction might not result from GRT action alone. This is particularly apparent when the effect of galactose on GRT and GRT-40 is compared. GRT-40 was not inhibited by galactose while GRT was inhibited considerably, suggesting that the latter fraction is contaminated with possibly L_{III} or L_{IV} . If either one of these enzymes are present

in the GRT fraction, the stimulatory effect of melibiose and raffinose on GRT would be partly masked by their inhibitory effect on the contaminating enzymes.

Stachyose strongly inhibited both GRT and GRT-40. This was rather unexpected since stachyose can neither be used as an acceptor nor inhibit the transfer reaction catalyzed by GRT. The following reactions could possibly explain these results:

Thus, the release of p-nitrophenol in the presence of stachyose would be nullified by the synthesis of PNPG from stachyose and p-nitrophenol. Reactions (XII) and (XVI) were shown to proceed readily when galactinol was used instead of PNPG.

Sucrose, arabinose and fructose did not have any effect on the hydrolysis of PNPG by L_I , L_{II} , L_{III} , L_{IV} , GRT and GRT-40 at concentrations of 25mM.

Concentrations of 25mM glucose inhibited L_{II} and L_{III} 4.4 and 7.4%, respectively, but had no effect on the other enzymes. Concentrations of 10mM glucose did not inhibit any of the enzymes.

The hydrolysis of galactinol, raffinose and stachyose by L_I , L_{II} , L_{III} , L_{IV} and GRT at pH 5.4 and 7.0 was studied using TLC. Galactinol was hydrolyzed

minimally by GRT at pH 7.0 where a small spot of galactose could be detected on the plates after 1 hour. No galactose could be detected with GRT at pH 5.4 nor with any of the other enzymes at either pH. The hydrolysis of raffinose by the various fractions at pH 5.4 or 7.0 is shown in Figure 21. The appearance of melibiose in assays containing L_I and L_{II} at pH 5.4 was very clear, indicating the presence of invertase in these two fractions. Sucrose was also apparent, indicating that L_I and L_{II} hydrolyze the terminal galactosyl residue of raffinose at pH 5.4. When assayed at pH 7.0, L_I showed some residual α -galactosidase and invertase activity while L_{II} showed only invertase activity. L_{III} was free of invertase activity and showed good α -galactosidase activity at pH 5.4 while at pH 7.0 the hydrolysis of raffinose was greatly reduced. The GRT fraction hydrolysed raffinose to sucrose and galactose at pH 5.4 but no hydrolysis was detected at pH 7.0. Raffinose was not metabolized to any detectable amount by L_{IV} at either pH. The pattern of stachyose hydrolysis was similar to that obtained with raffinose. The major difference was found to be with L_{IV} at pH 7.0 where both raffinose and galactose were detected. It was estimated from these experiments that α -galactosidase L_{III} metabolized raffinose ca. 5-10 times faster than L_I or L_{II} at pH 5.4 while L_{IV}

Figure 21 - Hydrolysis of Raffinose by L_I (A),
 L_{II} (B), L_{III} (C), GRT (D) and
 L_{IV} (E). The numbers indicate pH
of the assays (e.g. A5: L_I assayed
at pH 5.4; A7: L_I assayed at pH 7.0).
Standard (STD) from origin and up:
stachyose, galactinol, raffinose,
melibiose (MEL), sucrose, galactose,
glucose + fructose. Control (CTL):
raffinose alone.

STD 5 μ l	A 5	A 7	B 5	B 7	C 5	CTL	C 7	D 5	D 7	E 5	E 7	STD 10 μ l
MEL →												

metabolized stachyose at pH 7.0 about 5-10 times faster than L_I or L_{II} at pH 5.4 while L_{III} hydrolyzed stachyose minimally at pH 5.4. It is interesting to notice that raffinose and stachyose are also hydrolyzed at pH 5.4 by a very active invertase system present in preparations of L_I and L_{II} .

The ability of the four molecular forms of α -galactosidase and of the GRT fraction to synthesize raffinose, stachyose and verbascose was also investigated using the ^{14}C -galactinol assay method and the TLC assay method. No synthesis of raffinose or verbascose could be detected with any of the enzyme fractions at pH 5.4 or 7.0 with either assay method. Stachyose synthesis was only detected in assays of the GRT fraction at pH 7.0 (Fig. 22) while the results obtained with other fractions at pH 5.4 or 7.0 were similar to those obtained from assays where raffinose was incubated alone with the various enzyme fractions. Synthesis of stachyose using the ^{14}C -galactinol assay was also detected only with the GRT fraction at pH 7.0.

Figure 22 - TLC Assays of Stachyose Synthesis by
L_I (A), L_{II} (B), L_{III} (C), GRT (D)
and L_{IV} (E). The numbers indicate the
pH of the assays as described in Fig.
21. Standard (STD) as in Fig. 21.
Control (CTL): raffinose + galactinol.

STD 15 pl	A 5	A 7	B 5	B 7	C 5	CTL	C 7	D 5	D 7	E 5	E 7	STD 20 pl

V - Properties of Alkaline α -Galactosidase.

A typical purification sequence for the alkaline α -galactosidase L_{IV} is presented in Table X. The two additional steps, gel filtration on Sephadex G-100 and ion-exchange chromatography on hydroxyapatite, resulted in an overall purification fold of 165 with an overall recovery of about 5%. L_{IV} eluted off the hydroxyapatite as a single peak at a phosphate concentration of approximately 190mM. Polyacrylamide gel electrophoresis of the active fractions collected from the G-100 and hydroxyapatite columns, respectively, revealed that the hydroxyapatite step removed most of the contaminating protein present in the G-100 fractions. As shown in Fig. 23, the final preparation of L_{IV} contained one sharp protein band and two other diffuse bands. It was shown that the α -galactosidase activity present in the preparation was associated with the sharp band by slicing duplicate gels into 1mm sections and assaying for α -galactosidase activity at pH 7.5. The other bands had no α -galactosidase activity.

The hydrolysis of stachyose and PNPG was optimal at pH 7.5 while raffinose hydrolysis was optimal between pH 6.5 and 7.0. As shown in Figure 24, the minor peaks of activity which were previously observed at pH 4.0 and 5.0 with a less purified preparation of L_{IV} (see Fig. 19) were absent from this preparation.

Table X - Typical Purification Sequence for L_{IV} Isolated
from 100 g of Mature Leaves.

Purification Step	Volume (ml)	Total Enzyme Activity (mU)	Total Protein (mg)	Sp. Activity (mU/mg)	Recovery ^a (%)
Extract in phosphate buffer	450	29,508	1575	18.7	-
Ammonium sulphate (30-60% pellet)	30	11,431	1257	9.1	38.7
DEAE Sephadex A-50 (0-0.5M NaCl)	35	4,705	133	35.4	41.1
Sephadex G-100	14.5	3,974	8	498.0	26.6
Hydroxyapatite	9.4	1,452	0.47	3089.0	36.5

^a Recovery is calculated relative to the previous purification step. Overall
recovery was 4.9%. A purification fold of 165 was obtained overall.

Figure 23 - Polyacrylamide Gels of L_{IV}.
(A) Pooled G-100 fractions.
(B) and (C) Two different
preparations after the
hydroxyapatite step.
Arrows indicate the band
corresponding to alkaline
 α -galactosidase activity
as determined in unstained
duplicate gels.

A

B

C

Figure 24 - Hydrolysis of PNPg (○—○) and stachyose (△—△) by L_{IV} as a function of pH. Open symbols: triethanolamine buffer; closed symbols: McIlvaine buffer.

Km values of 1.7, 1.0 and 2.0mM for para-, ortho- and meta-nitrophenyl- α -D-galactopyranoside hydrolysis, respectively, were obtained from standard Lineweaver-Burk plots at pH 7.5 (Fig. 25). Km values of 4.5 and 36.4mM for stachyose and raffinose hydrolysis, respectively, were obtained from standard Lineweaver-Burk plots at pH 7.5 (Fig. 26). A Vmax value of 3.5 μ moles per minutes per mg with PNPG as the substrate was consistently obtained for preparations of L_{IV}. Vmax values for the other substrates expressed relative to PNPG equalling 100 were 150, 45, 9.4 and 4.0 for o-nitrophenyl- α -D-galactopyranoside, stachyose, m-nitrophenyl- α -D-galactopyranoside and raffinose, respectively. Melibiose and galactinol were also hydrolyzed by L_{IV} at pH 7.5 but the Km values were not determined. The Km and Vmax values for various substrates and the specificity of L_{IV} towards some glycosyl derivatives of nitrophenol are summarized in Table XI. L_{IV} appears to possess a high specificity towards α -linked galactosyl residues. It is also interesting to notice that L_{IV} was not inhibited by high concentrations of PNPG (up to 20mM), a situation which occurs with L_I, L_{II} and L_{III} (Thomas and Webb, 1977) and most other forms of acid α -galactosidase (Dey and Pridham, 1972).

Figure 25 - Lineweaver-Burk determination of the K_m values for meta (A)-, para (B)- and ortho (C)-nitrophenyl- α -D-galactopyranoside hydrolysis by L_{IV} at pH 7.5. Upper 1/S scale for (A) and lower 1/S scale for (B) and (C).

Figure 26 - Lineweaver-Burk determination of the K_m for raffinose (A) and stachyose (B) hydrolysis by L_{IV} at pH 7.5.

Table XI - Specificity of Alkaline α -Galactosidase
L_{IV}.

Substrate	Km (mM)	Vmax ^a
p-nitrophenyl- α -D-galactoside	1.40	100
o-nitrophenyl- α -D-galactoside	1.00	150
m-nitrophenyl- α -D-galactoside	2.00	9.4
Raffinose	36.40	4.0
Stachyose	4.50	45
Melibiose	Hydrolyzed	
Galactinol	Hydrolyzed	
p-nitrophenyl- α -D-mannoside	No hydrolysis	
p-nitrophenyl- α -D-fucoside	"	
p-nitrophenyl- α -D-glucoside	"	
p-nitrophenyl- β -D-galactoside	"	
p-nitrophenyl- β -D-glucoside	"	
o-nitrophenyl- β -D-galactoside	"	

^a Vmax values are expressed relative to p-nitrophenyl- α -D-galactoside equalling 100.

The hydrolysis of PNPG by L_{IV} at pH 7.5 was inhibited by several sugars. K_i values of 6.4, 15.6, 70.0 and 70.0mM were determined from standard Dixon plots for galactose, stachyose, melibiose and galactinol, respectively (Fig. 27). A K_i value of 244mM was obtained for raffinose. The degree of inhibition caused by each sugar thus followed the pattern previously observed with the less purified L_{IV} preparation (see Table X). Several other sugars were tested for inhibition but none showed significant inhibition at the 0.5 level of confidence (Table XII).

To study the effect of metal ions and PCMB on the hydrolysis of PNPG by L_{IV} , the enzyme was first transferred into 10mM triethanolamine-HCl (TEA) buffer pH 7.5 or 10mM HEPES buffer pH 7.5 by ultrafiltration. The activity of the enzyme in TEA buffer was ca. 70% that of the L_{IV} preparation in McIlvaine buffer pH 7.5 and the activity of the enzyme in HEPES was ca. 60% that of the preparation in McIlvaine buffer pH 7.5. L_{IV} was found to be sensitive to several ions (Table XIII). Na^+ and K^+ were not inhibitory to the enzyme in either TEA or HEPES buffer. Mn^{2+} , Ca^{2+} and Mg^{2+} inhibited L_{IV} to various degrees. L_{IV} in TEA buffer retained ca. 60% residual activity in the presence of 3mM concentrations of Mn^{2+} and Ca^{2+} and ca. 45% in the presence of Mg^{2+} . L_{IV} in HEPES buffer retained ca. 75% residual activity in the presence of

Figure 27 - Dixon plot determination of the K_i values for galactose (A), stachyose (B) and galactinol and melibiose (C) for L_{IV} at pH 7.5 in the presence of 6.0mM PNPG.

Table XII - Inhibition of Alkaline α -Galactosidase
 L_{IV} -catalyzed Hydrolysis of PNPG by
 Sugars.

Inhibitor	K_i (mM) ^a
Galactose	6.4
1-O-methyl- α -D-galactoside	ND ^b
1-O-methyl- β -D-galactoside	ND
N-acetyl-D-galactosamine	ND
Arabinose	ND
Glucose	ND
Fucose	ND
Mannose	ND
Xylose	ND
Myo-inositol	ND
Stachyose	15.6
Melibiose	70.0
Galactinol	70.0
Raffinose	244.0

^a K_i values correspond to the concentration of inhibitor which gave 50% inhibition in the presence of 6mM PNPG.

^b ND: No inhibition detected at final concentration 20mM.

Table XIII - Effect of Metal Ions and PCMB on the
Hydrolysis of PNP_G by L_{IV} in HEPES
and Triethanolamine Buffers at pH 7.5

Metal Ions	Activity ^a	
	Triethanolamine	HEPES
3mM Na ⁺ , K ⁺	100	100
3mM Mn ²⁺	62	77
3mM Ca ²⁺	58	35
3mM Mg ²⁺	43	72
3mM MoO ₄ ²⁻	ND ^b	42
3mM Co ²⁺ , Fe ³⁺	9	0
3mM Ni ²⁺ , Zn ²⁺	6	0
3mM Hg ²⁺ , Cu ²⁺ , Ag ²⁺	0	0
100 uM PCMB	0	0

^a Activity values expressed relative to control
equalling 100.

^b ND: Not determined.

3mM concentrations of Mn^{2+} and Mg^{2+} while Ca^{2+} (and MoO_4^{2-}) inhibited the enzyme by ca. 60%. The other ions tested inhibited L_{IV} completely or by more than 90% at 3mM concentrations, depending on the ion and the buffer used. L_{IV} was completely inhibited in the presence of 100uM PCMB in either buffer.

The effect of EDTA and organic acids on the activity of L_{IV} in TEA buffer was also investigated and the results presented in Table XIV. It was observed that the inhibition caused by the transfer of the enzyme from McIlvaine buffer into TEA buffer by ultrafiltration could be relieved to various degrees by the addition of EDTA and some organic acids. In the case of PNPG hydrolysis, the 30% loss in activity experienced after transfer of L_{IV} into TEA buffer could be almost completely relieved by the addition of 3mM EDTA and significant increases in activity were also observed in the presence of citrate, pyruvate and malate. Stachyose hydrolysis was also found to increase significantly in the presence of EDTA, citrate, pyruvate and malate. The effect of citrate on PNPG and stachyose hydrolysis is particularly interesting when the composition of McIlvaine buffer is examined. The McIlvaine buffer routinely used in these studies was prepared by mixing appropriate volumes of 200mM Na_2HPO_4 and 100mM citric acid to the desired pH. It was estimated

Table XIV - Effect of Organic Acids and EDTA on the Hydrolysis of PNPG and Stachyose by L_{IV} at pH 7.5.

Addition	Relative Enzyme Activity	
	PNPG Hydrolysis	Stachyose Hydrolysis
Control ^a	100	Not Determined
Control ^b	68.1	100
3mM Citrate	85.1	460
3mM Pyruvate	79.7	260
3mM Malate	79.7	260
3mM Succinate	68.1	100
3mM EDTA	96.0	460

^a Relative enzyme activity in McIlvaine buffer before L_{IV} was transferred into 10mM triethanolamine buffer by ultrafiltration.

^b Relative enzyme activity after transfer of L_{IV} into 10mM triethanolamine buffer.

that McIlvaine buffer at pH 7.5 contains a final concentration of approximately 8mM citrate. Therefore, the 30% loss of activity observed in TEA buffer could be the result of removing an activator (e.g. citrate) rather than inhibition by the buffer itself.

The effect of buffer composition, EDTA and organic acids on L_{IV} activity was then examined more closely. Fractions containing L_{IV} activity were collected from an hydroxyapatite column, the fractions were pooled and equal volumes dialyzed 72 hours against 10mM HEPES, 10mM TEA, 10mM NaPi or McIlvaine buffer, all adjusted to pH 7.5. L_{IV} activity in the various buffers was then determined and the results are presented in Table XV. When L_{IV} was dialyzed against McIlvaine buffer, almost all the activity was recovered. The 6.5% loss of activity observed could easily be attributable to physical loss of enzyme during the dialysis procedure. On the other hand, the activity recovered when L_{IV} was dialyzed against NaPi and TEA buffer was greatly reduced while no activity could be detected when L_{IV} was assayed in HEPES buffer.

The effect of EDTA, citrate, malate, pyruvate and succinate on L_{IV} activity in TEA, HEPES or NaPi buffer was then determined and the results are presented in Figure 28A, 28B and 28C, respectively. L_{IV} activity in TEA buffer increased sharply in the presence of either 0.25mM citrate or EDTA while its response to pyruvate,

Table XV - Effect of Buffer Composition on the
Hydrolysis of PNP_G by L_{IV} at pH 7.5.

Buffer	Enzyme Activity ^a (mU)	Percent Recovery ^b
McIlvaine	253.5	93.5
10mM NaPi	110.9	40.9
10mM Triethanolamine	23.6	8.7
10mM HEPES	0	0

^a The activity present after dialysis was determined in the same buffer system against which the enzyme was dialyzed.

^b Activity present before dialysis was determined in McIlvaine buffer and the value obtained (271 mU) used to calculate percent recovery of activity after dialysis.

Figure 28 - Effect of EDTA, citrate, malate, pyruvate and succinate (0.25 to 10mM) on the hydrolysis of PNPG by L_{IV} at pH 7.5 in 10mM triethanolamine (A), 10mM HEPES (B) or 10mM NaPi buffer (C).

malate and succinate was not as marked although 10mM concentrations of these latter organic acids also caused significant increases in enzyme activity. A similar pattern was observed with L_{IV} in HEPES and NaPi buffer. The highest amount of PNPG hydrolysis was obtained with L_{IV} in NaPi buffer containing 0.75mM EDTA or 10mM citrate. L_{IV} activity in the three buffers in the presence of optimal concentrations of EDTA and organic acids was determined and the values thus obtained were used to calculate the percentage recovery of activity in each buffer as compared to the activity originally present before dialysis (Table XVI). The only combination where no stimulation was observed was L_{IV} in NaPi buffer and up to 10mM concentrations of succinate. It is interesting to notice that recovery of L_{IV} activity in NaPi buffer containing citrate (conditions which mimic the composition of McIlvaine buffer at pH 7.5) was ca. 95 to 110% over the range of citrate concentrations tested (0.25-10mM). The recovery of L_{IV} activity in TEA and HEPES buffer containing optimal concentrations of citrate was 67 and 81%, respectively. These results are remarkable when one considers that L_{IV} activity in these two buffers was hardly detectable in the absence of citrate. On the other hand, the failure of citrate to completely restore L_{IV} activity in TEA or HEPES buffer suggests that these buffers might themselves be somewhat inhibitory to the enzyme.

Table XVI - Recovery of L_{IV} Activity in 10mM TEA, HEPES and NaPi Buffer in the Presence of Optimal Concentrations of Organic Acids and EDTA Using PNPg as Substrate.

Activator	Percent Recovery of Enzyme Activity ^a					
	TEA Buffer	Activator (mM)	HEPES Buffer	Activator (mM)	NaPi Buffer	Activator (mM)
None	8.7	-	0	-	40.9	-
Citrate	67.4	2.5	80.8	1.5 ^b	111.4	10.0
Malate	22.6	10.0	40.0	10.0	51.3	10.0
Pyruvate	56.5	10.0	62.9	10.0	72.4	10.0
Succinate	13.9	10.0	24.2	10.0	40.9	10.0
EDTA	85.6	0.25	85.6	0.25	111.4	0.75

^a The activity present before dialysis was determined in McIlvaine buffer and the value obtained (271 mU) used to calculate percent recovery of activity as assayed in TEA, HEPES or NaPi buffer in the presence of EDTA and organic acids.

The lack of L_{IV} activity in TEA and HEPES buffer after dialysis was surprising since L_{IV} retained ca. 70 and 60% residual activity when it was transferred into TEA and HEPES buffer by ultrafiltration, respectively. A possible explanation may be that traces of citrate (from the McIlvaine buffer) remained in the enzyme solutions after ultrafiltration. Further experiments with citrate revealed that 0.016mM concentrations of this compound were sufficient to cause 48 and 40% recovery of L_{IV} activity dialyzed against TEA and HEPES buffer, respectively. Concentrations of 0.1mM citrate restored 70 and 63% of L_{IV} activity in TEA and HEPES buffer, respectively. It is therefore conceivable that this discrepancy might have resulted from differences in the effectiveness of the two dialysis procedures used.

Another major difference between the two procedures was noted. When L_{IV} was transferred into TEA or HEPES buffer by ultrafiltration, the enzyme had been in McIlvaine buffer and, therefore, in contact with citrate. On the other hand, when L_{IV} was dialyzed against TEA or HEPES buffer, the enzyme had never been in contact with citrate before since the fractions from the hydroxyapatite column were used directly for these experiments. Binding of citrate to L_{IV} might be involved in the process of activation and the possibility that it might not be readily reversed by dialysis remained to be investigated. Therefore, 1 ml aliquots of L_{IV} solution

which had been dialyzed against TEA or NaPi buffer were incubated with 100 μ l of 15mM citrate for 30 minutes at 4 °C and then dialyzed 24 hours against 2 liters of the appropriate buffers. The activity of the enzyme solutions after treatment with citrate was determined before and after dialysis. It was observed that the stimulation in enzyme activity caused by citrate was not completely eliminated by dialysis. Thus, L_{IV} activity present after the citrate treatment and dialysis was about 1.4 and 3 times higher than in controls in NaPi and TEA buffer, respectively. If L_{IV} activity was then assayed in the presence of 10mM citrate, both the controls and the treated enzyme solutions displayed the same levels of activity originally observed. L_{IV} therefore appears to be highly sensitive to citrate and accurate determination of L_{IV} activity requires careful monitoring of this compound.

The effect of EDTA and organic acids on the hydrolysis of stachyose by L_{IV} was also investigated using the enzyme which had been dialyzed against NaPi buffer. The activity of the L_{IV} solution before dialysis was determined in McIlvaine buffer and the value obtained (104 mU) used to calculate the percent recovery of activity after dialysis. The dialyzed L_{IV} solution was found to contain about 52% (54 mU) of the original activity when assayed in NaPi buffer. The effect of

EDTA and organic acids on stachyose hydrolysis was then investigated. As shown in Figure 29, a large increase in L_{IV} activity occurred in the presence of EDTA, citrate and pyruvate while malate and succinate were weak activators. L_{IV} activity in the presence of optimal concentrations of EDTA and organic acids was determined and the values thus obtained were used to calculate the percent recovery of activity as compared to the activity present before dialysis (Table XVII). The results obtained were similar to previous observations using an L_{IV} preparation that had been transferred into 10mM TEA buffer by ultrafiltration (see Table XIV). In both cases, the addition of EDTA or citrate resulted in rates of stachyose hydrolysis that were about 5 to 6 times greater than in controls. This large increase in activity also resulted in total activity values that were significantly higher than the total activity values that were obtained before dialysis or ultrafiltration while assaying in McIlvaine buffer. For example, as shown in Table XVII, although only 104 mU of L_{IV} were originally detected in McIlvaine buffer before dialysis, the addition of citrate to L_{IV} in 10mM NaPi buffer resulted in the detection of 243 mU or about 2.3 times more activity. On the other hand, although the hydrolysis of PNPG was also stimulated by EDTA and citrate, the total activity detected in the presence of these compounds did not significantly exceed that originally determined before dialysis or ultrafiltration. These

Figure 29 - Effect of EDTA, citrate, malate, pyruvate and succinate on the hydrolysis of stachyose by L_{IV} in 10mM NaPi buffer pH 7.5.

Table XVII - Recovery of L_{IV} Activity in 10mM NaPi Buffer in the Presence of Optimal Concentrations of EDTA and Organic Acids Using Stachyose as Substrate.

Activator	Enzyme Activity ^a (mU)	Percent Recovery ^b
None	54	51.7
0.75mM EDTA	281	270.0
2.5mM Citrate	243	234.0
10mM Pyruvate	232	223.0
10mM Malate	98	94.2
10mM Succinate	54	51.7

^a The activity present after dialysis was determined in 10mM NaPi buffer to which various organic acids or EDTA was added.

^b The activity present before dialysis was determined in McIlvaine buffer and the value obtained (104 mU) used to calculate percent recovery of activity after dialysis.

results suggest that the high concentrations of Na_2HPO_4 present in McIlvaine buffer at pH 7.5 might inhibit stachyose hydrolysis by L_{IV} but not that of PNPG. The inhibition of L_{IV} by Na_2HPO_4 would be lifted when the enzyme is transferred into 10mM buffers and the full potential of EDTA and citrate as activators of stachyose hydrolysis can then be appreciated.

VI - Studies on the Distribution of Alkaline α -Galactosidase.

In Cucurbita pepo, mature, exporting leaves synthesize the raffinose family of oligosaccharides in large amounts and little hydrolysis of these compounds occurs prior to their export. On the other hand, immature leaves do not synthesize these sugars but rapidly hydrolyze the galactosyl portion of imported raffinose, stachyose and verbascose molecules. Thomas and Webb (1978) found L_I , L_{II} and L_{III} to be present in both mature and immature leaves and could not obtain satisfactory evidence that fluctuations in the specific activity of one of the forms was responsible for the difference in the hydrolytic behaviour of the two tissues. The discovery of a fourth molecular form of α -galactosidase in leaves of Cucurbita pepo, alkaline α -galactosidase L_{IV} , raised the possibility that this enzyme might be responsible for the different pattern of utilization of galactosyl sucrose oligosaccharides by mature and immature leaves. The distribution of L_{IV} activity in Cucurbita pepo was therefore investigated.

Acid and alkaline α -galactosidase activity in different parts of C. pepo was determined at pH 5.4 and 7.5, respectively. These two pH values were selected on the basis of the pH optima of the four molecular forms of α -galactosidase present in squash. As shown earlier in Figure 18, L_I , L_{II} and L_{III} hydrolyzed PNPG optimally at pH 5.4 while L_{IV} showed optimal activity at pH 7.5.

L_I and L_{III} showed about 8 and 26% residual activity at pH 7.5 but L_{II} , which accounted for more than 70% of the total acid α -galactosidase activity recovered from DEAE Sephadex A-50 columns, was totally inactive at pH 7.5. L_{IV} showed less than 10% residual activity at pH 5.4. The selection of pH 5.4 and 7.5 for the determination of α -galactosidase activity was further validated when the hydrolysis of PNPG by crude extracts was determined over the pH range 3.0 to 8.0 using McIlvaine buffers. Optimal hydrolysis in the acid range occurred at pH 5.5 to 6.0 while optimal hydrolysis in the basic range occurred at pH 7.5 (Fig. 30). The pH curve obtained with crude extracts was strikingly similar to the pH curve one could construct by juxtaposition of the individual pH curves of L_{II} and L_{IV} described in Figure 18. Finally, the hydrolysis of PNPG by crude extracts at pH 7.5 was positively correlated with the hydrolysis of stachyose as shown in Figure 31. Raffinose hydrolysis was minimal at pH 7.5 while at pH 5.4 the degree of raffinose hydrolysis followed that of PNPG. Stachyose hydrolysis at pH 5.4 was minimal.

The distribution of acid and alkaline α -galactosidase activity in different parts of Cucurbita pepo plants is shown in Table XVIII. Mature leaves contained about 2.5 times more acid α -galactosidase

Figure 30 - Hydrolysis of PNPG by crude extracts of *Cucurbita pepo* leaves as a function of pH.

Figure 31 - Relationship between stachyose and PNPG hydrolysis in crude extracts of *Cucurbita pepo* leaves at pH 7.5.

Table XVIII - Distribution of α -Galactosidase
Activity in Different Parts of
Cucurbita pepo.

Tissue	mU/mg protein		mU/g fresh weight	
	pH 5.4	pH 7.5	pH 5.4	pH 7.5
Mature Leaf	54	20	598	221
Immature Leaf	52	75	527	769
Mature Petiole	90	118	82	107
Immature Petiole	98	332	165	559
Roots	43	82	66	125
Seeds (Dry)	7	1	121	18
Seeds (Imbibed 24 hrs)	9	1	142 ^a	17 ^a
Seeds (Imbibed 48 hrs)	14	1.3	152 ^a	14 ^a

^a Fresh weight prior to imbibition.

than alkaline α -galactosidase activity. In mature petioles, the α -galactosidase activity detected at pH 7.5 was slightly higher than the activity detected at pH 5.4. In roots, the total alkaline α -galactosidase activity was about twice as high as the total acid α -galactosidase activity. Imbibition of seeds with water up to 48 hours did not change the levels of alkaline α -galactosidase activity. The low levels of alkaline α -galactosidase present in the seeds might represent residual activity from the acid α -galactosidases and the presence of L_{IV} in seeds was not confirmed by DEAE Sephadex A-50 chromatography of 30-60% ammonium sulphate fractions. Whereas at least triplicate experiments were used to determine the values given in Table XVIII for the α -galactosidase activity present in mature leaf and petiole tissue, roots and seeds, the values given for immature leaf and petiole tissue are the result of single experiments. A large degree of variability was observed in total alkaline α -galactosidase activity extracted from immature tissue. A leaf plastochron index (LPI) based on a petiole length of 30 mm (Turgeon and Webb, 1973) was used to determine the morphological age of the leaves more accurately. Young, developing leaves between LPI -2.0 to 0.2 were net importing organs and the blades had expanded their surface areas up to about 10% of their final size.

Between LPI 0.2 and 1.3, the capacity to import was lost progressively, beginning with the leaf tip and continuing basipetally along the leaf length. At about LPI 1.5 the leaf had become a net exporting organ and was 50% expanded (Turgeon and Webb, 1973). In Figure 32, the specific activities of acid and alkaline α -galactosidase present in crude homogenates of leaves are plotted against leaf plastochron index. While the results previously reported by Thomas and Webb (1978) were confirmed and the level of acid α -galactosidase activity remained constant throughout leaf development to maturity the level of alkaline α -galactosidase activity changed very markedly. In immature, importing leaves total alkaline α -galactosidase activity was about 3 fold higher than total acid α -galactosidase. A sharp decline in total alkaline α -galactosidase activity was observed with increasing leaf maturity (from LPI -0.5 to +1.0). In mature, exporting leaves (LPI > 1.5) total alkaline α -galactosidase activity amounted to about 60% of total acid α -galactosidase activity. Overall, total alkaline α -galactosidase activity underwent a 6 fold decrease between LPI -1.5 and +1.5.

A search for alkaline α -galactosidase activity in leaf extracts of different plants was then carried out. The plants selected fell into three distinct groups.

Figure 32 - Relationship between α -galactosidase activity and developmental stages of *Cucurbita pepo* leaves. Acid α -galactosidase (closed circles) and alkaline α -galactosidase (open circles) specific activities are plotted against leaf plastochron index.

The same results are plotted in a different way in Fig. 42 in the Addendum on page 240.

One group consisted of plants which do not synthesize raffinose-type sugars in their leaves as determined by analysis of the products from $^{14}\text{CO}_2$ labelling experiments (Webb, unpublished data). These included spinach, corn, tobacco, wheat, pea, soybean and tomato. The second group consisted of plants which are members of the Cucurbitaceae family. These plants synthesize raffinose-type sugars in their leaves and included squash, pumpkin and cucumber. The third group consisted of plants which synthesize raffinose-type sugars in their leaves but are not members of the Cucurbitaceae family. These included Catalpa bignonioides, Tabebuia rosea and Markhamia sessilis (all members of the Bignoniaceae family), and Lamium galeobdolon variegatum, a member of the family Lamiaceae. The synthesis of raffinose and stachyose in Lamium and Catalpa leaves was confirmed by $^{14}\text{CO}_2$ labelling experiments (Webb, unpublished data). The presence of raffinose and stachyose in leaves of Markhamia and Tabebuia was not confirmed in this study. The results of this survey are presented in Table XIX. Acid α -galactosidase activity was found in the leaves of all the plants examined. On the other hand, alkaline α -galactosidase activity appeared to be restricted to members of the Cucurbitaceae. In the other two groups, the activity observed at pH 7.5 was negligible in most

Table XIX - α -Galactosidase Activity in Leaf
Tissue of Various Plants.

Plant		mU/mg protein		mU/g fresh wt		Ratio 7.5/5.4
		pH 5.4	pH 7.5	pH 5.4	pH 7.5	
Spinach	(M) ^a	12.4	6.0	64	31	0.48
	(I)	10.2	5.1	94	47	0.50
Tobacco	(M)	75	2.8	182	13.7	0.04
Corn	(M)	60	8	247	33	0.13
Wheat	(M)	36.1	14.4	274	109	0.40
Pea	(M)	16.4	0	107	0	0
Soybean	(M)	7.1	0	108	0	0
Tomato	(M)	14.7	0	160	0	0
Squash	(M)	54	20	598	221	0.37
	(I)	52	75	527	769	1.44
Cucumber	(M)	25.6	16.1	463	290	0.63
	(I)	183	246	1611	2162	1.34
Pumpkin	(M)	32.1	23.2	609	440	0.72
	(I)	75.4	115.8	1342	2061	1.54
Lamium sp.	(M)	171	126	1053	768	0.74
	(I)	117	107	1287	1196	0.91
Catalpa sp.	(M)	46	0	132	0	0
	(I)	26.5	0	245	0	0
Markhamia sp.	(M)	25.5	3.1	148	18	0.12
Tabebuia sp.	(M)	6.7	0	95	0	0

^a M: Mature leaves I: Immature leaves

instances. Spinach, wheat and Lamium were the only plants where considerable activity was observed at pH 7.5. A pH curve of the crude extracts of spinach and Lamium is shown in Figure 33. It appears that α -galactosidase activity in these two species was optimal at pH 6.0 and that the activity observed at pH 7.5 was residual activity. It was also observed that the ratio of total alkaline α -galactosidase activity over total acid α -galactosidase activity did not differ significantly between mature and immature leaf tissue of spinach and Lamium. This also suggests that the same enzyme(s) is responsible for both acid and alkaline α -galactosidase activity. On the other hand, a significant change in the ratio of alkaline α -galactosidase activity over acid α -galactosidase activity was noticed in immature and mature leaf tissue of plants belonging to the family Cucurbitaceae. However, unlike squash, the total acid α -galactosidase activity in immature leaf tissue of cucumber and pumpkin was significantly higher than the activity present in mature leaf tissue.

α -Galactosidase activity in cucumber and pumpkin was then studied in more detail. It was first observed that pH curves of leaf extracts of both plants displayed two distinct peaks of activity at pH 5.5 and 7.5 (Fig. 34). Acid and alkaline α -galactosidase

Figure 33 - Hydrolysis of PNPase by leaf extracts of Lamium (open circles) and spinach (closed circles) as a function of pH.

Figure 34 - Hydrolysis of PNPase by leaf extracts of *Cucumis sativus* (A) and *Cucurbita maxima* (B) as a function of pH.

Text complete; leaf 160 omitted in numbering

activity in leaves at different stages of development was then determined at pH 5.4 and 7.5. In pumpkin, total alkaline α -galactosidase activity was significantly higher than total acid α -galactosidase activity until the leaves reached a fresh weight value of about 0.2 g (Fig. 35). During this period of growth, both alkaline and acid α -galactosidase activity dropped rapidly. The level of acid α -galactosidase activity then seemed to stabilize while alkaline α -galactosidase activity kept decreasing but at a much slower rate. Overall, alkaline α -galactosidase activity decreased 5 to 6 fold while acid α -galactosidase activity decreased 2 to 3 fold. In young leaves (fresh weight < 0.2 g) alkaline α -galactosidase activity was about 1.5-2.0 fold higher than acid α -galactosidase activity. In older leaves (fresh weight > 1.0 g) acid α -galactosidase activity was about 1.3 fold higher than alkaline α -galactosidase activity. A similar pattern was observed in developing leaves of cucumber (Fig. 36). However, the higher levels of alkaline α -galactosidase activity present in the youngest leaves were only observed until individual leaves reached a fresh weight value of 0.1 g. The levels of alkaline and acid α -galactosidase activity then reached similar values until, in older leaves (fresh weight > 1.0 g), acid α -galactosidase activity was about 1.5 fold higher than alkaline α -galactosidase

Figure 35 - Relationship between α -galactosidase activity and developmental stages of Cucurbita maxima leaves. Acid α -galactosidase (closed circles) and alkaline α -galactosidase (open circles) specific activities are plotted against fresh weight of individual leaves.

Figure 36 - Relationship between α -galactosidase activity and developmental stages of

Cucumis sativus leaves. Acid α -galactosidase (closed circles) and alkaline α -galactosidase (open circles) specific activities are plotted against fresh weight of individual leaves.

activity. Overall, alkaline α -galactosidase activity decreased 7 to 8 fold while acid α -galactosidase activity decreased only 3 to 4 fold. When acid and alkaline α -galactosidase activity on a protein basis was plotted against fresh weight of individual leaves, the pattern observed did not differ since the proportion of the protein component of the leaf's fresh weight soluble in NaPi buffer did not alter during leaf development (Fig. 37).

Finally, the presence of alkaline α -galactosidase in cucumber was confirmed when the physical separation of acid and alkaline α -galactosidase activity was achieved. Cucumber cotyledons were extracted in NaPi buffer and the procedure used to separate L_I , L_{II} and L_{III} from L_{IV} in squash leaves was followed. Two peaks of α -galactosidase activity were eluted off a DEAE Sephadex A-50 column with a 200 ml 0-0.5M NaCl linear gradient (Fig. 38). The first peak (C_I) consisted of acid α -galactosidase while the second peak (C_{II}) consisted of alkaline α -galactosidase. The residual α -galactosidase activity observed at pH 7.5 in fractions 8 to 16 suggest that C_I might contain two forms of α -galactosidase. Nevertheless, fractions 10 to 20 were pooled for acid α -galactosidase, C_I , and fractions 51 to 55 for alkaline α -galactosidase, C_{II} .

Figure 37 - Relationship between fresh weight and protein in *Cucumis sativus* (open circles) and *Cucurbita maxima* (closed circles).

Figure 38 - Separation of acid α -galactosidase (closed circles) and alkaline α -galactosidase (open circles) activity from *Cucumis sativus* on DEAE Sephadex A-50. Solid line: % transmittance at 254 nm and 280 nm.

The pH optima of both enzymes was then determined. C_I hydrolyzed PNPG optimally at pH 5.5 while raffinose and stachyose were hydrolyzed optimally at pH 5.0 (Fig. 39). PNPG was hydrolyzed ca. 6.5 times faster than raffinose and ca. 20 times faster than stachyose when these substrates were assayed at their pH optima. C_{II} hydrolyzed PNPG optimally at pH 7.5 while stachyose hydrolysis was optimal between pH 7.5 and 8.0 (Fig. 40). Raffinose hydrolysis was barely detectable at pH 7.0 and 7.5. None of the three substrates were hydrolyzed below pH 6.0. PNPG was hydrolyzed about 3.5 times faster than stachyose at pH 7.5. Like in squash, a composite of the pH curve for PNPG hydrolysis by the two forms of α -galactosidase present in cucumber strikingly resembled the pH curve of the crude extract (see Fig. 34A). The hydrolysis of PNPG (3.0mM) by C_{II} was inhibited 57% in the presence of 10mM stachyose at pH 7.5.

Figure 39 - Hydrolysis of PNP (open circles), raffinose (closed circles) and stachyose (triangles) by C_I as a function of pH.

Figure 40 - Hydrolysis of PNP (open circles), raffinose (closed circles), and stachyose (triangles) by C_{II} as a function of pH.

VII - Galactose Metabolism in Leaves of Cucurbita pepo.

A possible pathway for galactose metabolism in plants was suggested by Maretzki and Thom (1978) (see Fig. 3). The enzymes involved in the assimilation of the free galactose released by α -galactosidase-catalyzed reactions are galactokinase, UDP-galactose pyrophosphorylase and UDP-galactose-4-epimerase. The activity of these three enzymes in immature and mature leaf tissue of Cucurbita pepo was therefore investigated along with α -galactosidase activity. The results are presented in Table XX.

As expected, alkaline α -galactosidase activity as determined with PNPG as the substrate in mature and immature leaf tissue was about 0.6 and 3.7 times that of acid α -galactosidase activity, respectively. The rate of hydrolysis of raffinose and stachyose by acid α -galactosidase was not significantly different between mature and immature leaf tissue while the rate of raffinose and stachyose hydrolysis by alkaline α -galactosidase was about 2.5 and 5.0 times higher in immature tissue, respectively. It was also observed that in mature leaf tissue the rate of raffinose hydrolysis by acid and alkaline α -galactosidase was not significantly different but that the rate of stachyose hydrolysis by alkaline α -galactosidase was about twice as high as that obtained with acid α -galactosidase. It thus appears that although

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Table XX - Comparison of the Levels of Activity of Enzymes Involved in Galactose Metabolism in Mature and Immature Leaves of *Cucurbita pepo*.^a

	Mature Leaf Tissue	Immature Leaf Tissue
Acid. α -Galactosidase		
PNPG	19.9 $\pm$ 2.6	30.5 $\pm$ 3.4
Raffinose	10.5 $\pm$ 1.2	6.6 $\pm$ 1.2
Stachyose	3.3 $\pm$ 0.7	2.5 $\pm$ 2.1
Alkaline α -Galactosidase		
PNPG	12.8 $\pm$ 1.2	114.3 $\pm$ 29.1
Raffinose	8.5 $\pm$ 0.5	21.2 $\pm$ 5.8
Stachyose	7.2 $\pm$ 1.5	37.4 $\pm$ 5.9
Galactokinase	13.4 $\pm$ 1.3	27.0 $\pm$ 5.9
UDP-Gal Pyrophosphorylase	21.2 $\pm$ 1.7	54.3 $\pm$ 3.2
UDP-Gal-4-Epimerase	Present	Present
Gal-1-P Uridyltransferase	0 -	0 -

^a All enzyme activities are expressed in nmoles of product per minute per mg of protein.

total acid α -galactosidase activity is higher than total alkaline α -galactosidase activity in mature tissue (as determined with PNPG), the lesser amounts of alkaline α -galactosidase nevertheless hydrolyzed stachyose significantly faster than the higher amounts of acid α -galactosidase present. This is further demonstrated in the rates of hydrolysis of raffinose and stachyose in immature leaf tissue where total alkaline α -galactosidase activity was about 3.7 fold higher than acid α -galactosidase activity (as determined with PNPG as substrate). In this tissue, raffinose and stachyose hydrolysis catalyzed by alkaline α -galactosidase was about 3.0 and 15.0 fold higher than that catalyzed by acid α -galactosidase.

Galactokinase and UDP-galactose pyrophosphorylase activity was detected in both mature and immature tissue. These two enzymes were assayed routinely with fixed substrate concentrations and the minimal amount of protein which resulted in maximal rates of conversion under the conditions used was not determined. Therefore, although immature leaf tissue appeared to contain higher levels of these enzymes (Table XX), the significance of the quantitative differences observed is questionable since the saturation point for these reactions was not determined accurately between replicate experiments. UDP-galactose-4-epimerase activity was also present in mature and immature

leaf tissue. However, under the assay conditions used in this study, the epimerase activity was very low with values ranging from about 1.0 to 6.0 nmoles UDP-glucose per minute per mg of protein. These values corresponded to changes in O.D. 340 nm of only 0.01-0.03 unit of absorbance and are therefore highly unreliable. Only in one instance was a large amount of epimerase activity observed. A change in O.D. 340 nm of 0.12 unit of absorbance was observed using an extract of mature leaf tissue and corresponded to a rate of 36 nmoles UDP-glucose per minute per mg of protein. The absence of galactose-1-phosphate uridylyltransferase activity suggests that the pathway of galactose assimilation shown in Fig. 3 is predominant in Cucurbita pepo leaves.

DISCUSSION

The isolation of active enzymes from plant tissues present all the usual difficulties resulting from the interactions of proteins with other protoplasmic constituents such as lipids and nucleic acids. In addition to these, plant enzymes usually require protection from the vacuole acids and phenolic compounds (Loomis and Battaile, 1965). The medium for extracting enzymes from plants must therefore be carefully formulated in order to obtain optimum activity in cell-free extracts (Anderson, 1968).

Hydrogen-bonding plays a very important role in the reversible binding of phenols to protein but more stable covalent bonds are also formed between quinones and several substituents (amine, α -amino, imino and thiol groups) present in proteins. The effect of such reactions on enzyme activity is indirectly demonstrated by the fact that many successful studies in the field of plant enzymology have been carried out with material such as spinach or leguminous seedlings which contain low levels of phenolic compounds (Neish, 1968). Polyvinylpyrrolidone (PVP) has been frequently added to extraction media and found to increase the recovery of enzyme activity in several cases (Loomis and Battaile, 1965). It is generally believed that PVP exerts its protective action by

effectively competing with the peptide bond of proteins for the unionized hydroxyl groups of phenols which are responsible for hydrogen-bond formation between phenols and proteins. The oxidation of phenols to quinones by O-diphenyloxidase is usually prevented by addition of copper-chelating agents to the extraction medium. The frequency of irreversible covalent binding of quinones to proteins is thus greatly reduced and higher enzyme activity in cell-free extracts is usually observed (Loomis and Battaille, 1966; Anderson, 1968).

Galactinol:sucrose galactosyltransferase (GST) activity in leaf homogenates of Cucurbita pepo could not be detected at pH 6.2, 6.9 or 7.3 (Table IV). The addition of PVP or the copper-chelating compound diethyldithiocarbamate (DIECA) did not result in detectable GST activity, suggesting that the enzyme's primary mode of inhibition was not due to interaction with phenolics. Lehle and Tanner (1973) purified GST from Vicia faba seeds and found that enzyme activity was strictly dependent upon the presence of sulfhydryl group-protecting reagents. The addition of such compounds to the extraction medium also proved necessary for the detection of GST activity in leaf homogenates of C. pepo. The use of dithiothreitol (DTT) as a protective reagent for sulfhydryl groups was first described by Cleland (1964). 2-Mercaptoethanol (BME) is another widely used protective reagent (Königsberg, 1972).

The sulfhydryl groups of proteins are readily oxidized in air to disulfides. DTT and BME regenerate these sulfhydryl groups by reducing the disulfide bridges (Cleland, 1964; Konigsberg, 1972). Both compounds also protect enzyme activity by competing with the sulfhydryl groups of proteins for other reversible -SH blocking compounds such as heavy metals and quinones (Barron, 1951; Konigsberg, 1972). The expression of GST activity in leaf homogenates of C. pepo therefore appears to be dependent upon the intactness of the sulfhydryl groups present in the enzyme molecule. Desalting crude extracts by gel filtration also appeared to result in higher levels of GST activity. This procedure removed quinones, metal ions and other small molecular weight compounds from the medium and, presumably, more efficient protection of the sulfhydryl groups was achieved by decreasing the competition between thiol groups and these compounds for DTT and BME. The possibility also remains that an indigenous inhibitor of GST present in crude extracts was removed during the desalting step.

On the other hand, galactinol:raffinose galactosyltransferase (GRT) activity did not appear to be significantly stimulated by any of the compounds added to the extraction medium, and especially when NaPi buffer was used. Although the addition of BME did not appear to increase the recovery of GRT activity, all further studies on this enzyme were carried out in NaPi buffer containing

20mM BME for precautionary measures and also to help in the detection of GST activity in fractions containing GRT during the various purification steps.

The separation of carbohydrates on silica gel thin-layers usually requires impregnation of the layer with some inorganic salts. Such additives as sodium mono- and diphosphate, boric acid, tungstic acid and hydrogen acetate have been used extensively in the TLC separation of carbohydrates (Ghebregzabher et al., 1976). These inorganic salts are usually incorporated into the silica gel matrix by preparing slurries in aqueous solutions of these compounds. However, the use of pre-coated silica gel plates presents many advantages over home-made layers: (1) better reproducibility of the results; (2) higher sensitivity of the sugars to the detecting reagents; (3) silica gel layers of commercial origin do not become friable after single or multiple elution and treatment with the spray reagents (Ghebregzabher et al., 1979).

In this laboratory, preliminary experiments with home-made plates were highly unsatisfactory due to reasons mentioned above. In addition to these problems, it was found to be extremely difficult to spot the sugar solutions along a sharp straight line as the silica gel became highly friable after spotting minimal volumes of solution, resulting in diffusion of the samples around the origin. Pre-coated silica gel plates were therefore used and

good separation of the sugars of interest (galactinol, sucrose, raffinose, stachyose and melibiose) was observed with several solvent systems, even in the absence of inorganic salts (Fig. 4A-E; Table V). Solvent 1 (ethyl acetate:acetic acid:methanol:water 60:15:15:10 v/v) was eventually adopted and used to assay GRT activity. Solvent 1 also proved satisfactory for studies on the hydrolysis of galactosyl-sucrose oligosaccharides by α -galactosidase.

To the best of the author's knowledge this is the first report of a partial purification of galactinol:raffinose galactosyltransferase (GRT) from leaf tissue. The demonstration that GRT can be separated from α -galactosidase by ion-exchange chromatography (Fig. 5) suggests that the latter enzyme may not be involved in stachyose biosynthesis as suggested by several authors (Li and Shetlar, 1964; Courtois and Petek, 1966; Dey, 1979), at least not in the leaf tissue of C. pepo. Thus, in the presence of 16mM raffinose as the acceptor, the amount of ^{14}C -galactose incorporated into stachyose was more than 25 times the amount of ^{14}C -galactose released as a result of ^{14}C -galactinol hydrolysis (Fig. 9). On the other hand, α -galactosidases do not catalyze significant transfer of galactose unless acceptor concentrations are at least 100mM to 1M and, even under such artificial conditions, the amount of galactose

transferred is usually less than the amount set free by hydrolysis (e.g. Dey, 1979). It is also very common for α -galactosidases to transfer galactose to and from a wide variety of sugars (Courtois and Petek, 1966) whereas GRT appeared to be highly specific for the donor galactinol and the acceptor raffinose. Thus, GRT was unable to synthesize either raffinose or verbascose in vitro, the two homologs immediately adjacent to stachyose in the $[O-\alpha-D\text{-galactosyl-(1}\rightarrow\text{6)}]_n$ sucrose series. Furthermore, GRT lacked the ability to hydrolyze either raffinose or stachyose at pH 5.4 or 6.9 and high concentrations of galactose failed to inhibit its galactosyltransferase activity at pH 6.9. A similar separation of these two enzymes was achieved from seeds of V. faba and P. vulgaris (Lehle and Tanner, 1972; Tanner and Kandler, 1968).

A comparison of the total activity of GRT (Table VI) with data previously reported for both the photoassimilation of CO_2 by mature leaves and the rate of ^{14}C -incorporation into stachyose (Turgeon and Webb, 1973 and 1975) indicates that a more than sufficient amount of the enzyme is present in a mature leaf to account for the rate of stachyose biosynthesis. Thus, the GRT activity present in one g of leaf tissue (0.69 $\mu\text{moles stachyose formed hr}^{-1} \text{ g fresh weight}^{-1}$; Table VI) is sufficient to synthesize the total amount of stachyose present in mature leaves (1.26 $\mu\text{moles stachyose per g fresh weight}$; Webb, unpublished data) in less than two hours. It is also

important to realize that the standard assay conditions used to determine the specific activity of GRT at the various steps of purification is by no means the maximal rate of stachyose synthesis that could be obtained with these enzyme solutions. Although great care was taken to insure that the rate of synthesis value used to determine specific activity was linear with time, there is no doubt that higher concentrations of raffinose and especially galactinol would yield much superior rates. Thus, the values given in Table VI have to be considered as minimum rates of synthesis. The low concentration of galactinol used in the standard assay system was due to the restricted amounts available.

¹⁴C-Tracer studies have failed to detect any synthesis of galactosyl-sucrose oligosaccharides in immature leaves (Turgeon and Webb, 1975). Their synthesis is first detectable at the semi-mature stage of leaf development (Turgeon and Webb, 1975). These results were confirmed in so far that GRT activity could not be detected in crude extracts from immature leaves. The fact that α -galactosidase activity remains approximately constant at all stages of leaf development (Thomas and Webb, 1978) again suggests that α -galactosidase is not involved in stachyose biosynthesis.

A Km value of 7.25mM was determined for galactinol in the presence of 10mM raffinose and GRT from C. pepo (Fig. 11). This value is almost identical with that

Tanner and Kandler (1968) reported for GRT in seeds of *P. vulgaris* (7.3mM). The graphical procedure of Florini and Vestling (1957) is recommended (Michal, 1978) when it is difficult to achieve high saturating concentrations of one of the substrates. In this study, the method of Florini and Vestling was first used to determine the K_m value of galactinol in the presence of varying concentrations of raffinose and it was compared to the value obtained from a standard Lineweaver-Burk plot. The K_m value determined using the Florini-Vestling method (7.7mM) compared favorably with the K_m value obtained using the Lineweaver-Burk method (7.25mM). A K_m value of 4.6mM was determined for raffinose using the Florini-Vestling method. K_m values of 0.84mM and 9.3mM were reported for raffinose by Tanner and Kandler (1968) in the presence of 0.30 and 8.30mM galactinol, respectively. These authors suggested that galactinol had some affinity towards the raffinose site and thereby acted as a competitive inhibitor. On the other hand, the lower K_m value obtained for galactinol hydrolysis (0.14mM) in the absence of an acceptor was interpreted to mean that raffinose could also bind to the same site as galactinol. This explanation is however very simple and no sound experimental evidence was provided to support it. The kinetics of two-substrate reactions are usually very complex and more knowledge of the effects the binding of one substrate has on the

transient state of the enzyme molecule and how this might affect the affinity of the second substrate for its binding site is required before such a statement can be made. On the other hand, sufficient experimental evidence is available to speculate about a possible reaction mechanism for galactinol:raffinose galactosyltransferase.

Transferases are classified according to the nature of the group transferred (Dixon and Webb, 1979). Such reactions can be written in the general form shown in Equation XVII where, in the case of GRT, AX would be

galactinol (where A represent myo-inositol and X the galactosyl moiety), B, raffinose, and BX, stachyose.

Two questions need to be answered before a reaction mechanism can be suggested. First, there is the need to know which bond in the glycosyl donor is broken so that one can define the structure of the transferred group X. Secondly, one wishes to know if the reaction proceeds in a single step by the direct transfer of X from A to B or if it proceeds by a two step transfer. In the latter case, the first step results in the formation of a glycosylated enzyme complex, EX, with concomittant release of A, followed by a second separate step where X is transferred from the enzyme to B (Koshland, 1953; Glaser, 1966; Dixon and Webb, 1979).

Although no experimental evidence was presented here that would answer the first question, the results of several workers strongly suggest that cleavage occurs between the anomeric carbon (i.e. the carbon atom bearing a carbonyl group or potential carbonyl group through which cyclic forms and glycosides can be formed; e.g. C-1 of galactose) and oxygen and comes on the side of the oxygen atom which is closest to the part of the molecule for which the enzyme is most specific. In other words, it occurs as closely as possible to the group which is being transferred (Koshland and Stein, 1954; Glaser, 1966; Dixon and Webb, 1979) and, in the case of galactinol (see Fig. 2), cleavage by GRT presumably occurs to the left of the oxygen which forms the glycosidic bond between galactose and myo-inositol. This distinction is also made by hydrolases since these enzymes can usually catalyze transfer reactions by reversal of the hydrolytic reaction.

Kinetic studies can usually help to differentiate one step transfer from two step transfer reactions (Cleland, 1963 and 1977). Thus, Florini-Vestling plots which show intersecting or parallel patterns are commonly representative of one step and two step reactions, respectively. However, the limited number of experimental points determined in this study using the Florini-Vestling graphical procedure (Fig. 12 and 13) strongly forbids any valuable interpretation.

Koshland (1953) has pointed out that the configuration of the anomeric carbon in the glycosyl donor and the product must be considered in determining the reaction mechanism of a glycosyltransferase. If the reaction involves only one transfer of the group, optical inversion occurs; if it involves two successive transfers, the effects will cancel and no inversion will be observed. The fact that the configuration of the anomeric carbon C-1 of galactose is preserved in stachyose therefore suggests that GRT catalyzes a two step transfer reaction similar to that described by Koshland (1953). Such a mechanism is described in Equations XVIII and XIX.

In these two equations, an electron-seeking group was designated as A^+ , galactinol as C-X (where C corresponds to the anomeric carbon C-1 of the galactosyl moiety, X to myo-inositol, and r, s and t to the C-O, C-H and C-C bonds formed by C-1 when galactose is in the pyranose form, respectively), Y represents the acceptor raffinose and C-Y the product stachyose. Koshland (1953) suggested that a group on the enzyme surface with an unshared pair of

electrons initiates the reaction by nucleophilic attack on the backside of the anomeric carbon of galactose. The affinity of the substrate for the enzyme surface will place the electron donor group on the enzyme surface in a favorable position for reaction resulting in an enzyme-substrate intermediate of inverted configuration (Equation XVIII). The proton donor, A, might come either from the solution or might be an electron-seeking site on the enzyme surface. The enzyme-substrate interaction keeps the enzyme on the backside of the substrate molecule and allows the approach of Y only from the front. In the second step of the reaction, raffinose (Y) makes an electron sharing attack, displaces the enzyme and forms the product stachyose with the α -configuration (Equation XIX).

The study of isotope exchange reactions catalyzed by transferase enzymes constitute another powerful method for ~~d~~istinguishing between one-step and two-step transfer reactions (Glaser, 1966; Dixon and Webb, 1979). If the reaction catalyzed by GRT occurs by a two step mechanism, the first step (Equation XVIII) only requires the presence of galactinol. If galactinol is added to the enzyme in the complete absence of raffinose, a small amount of free myo-inositol will be reversibly formed. Consequently, if ^{14}C -myo-inositol is incubated in the presence of GRT and

galactinol, the reverse reaction of Equation XVIII should result in the formation of isotopically labelled galactinol (see Equation VII). In other words, in the absence of the acceptor raffinose, the enzyme will catalyze an isotopic exchange reaction involving that part of the substrate molecule which is not transferred to the enzyme. Similarly, if stachyose is incubated with labelled raffinose and GRT in the absence of myo-inositol, an isotopic exchange of raffinose will be catalyzed. Both exchange reactions have been found to occur readily in these studies and also by Tanner and Kandler (1968). Further evidence for a two step mechanism and a galactosyl-enzyme intermediate was obtained by Tanner and Kandler (1968) who could not detect any isotopic exchange between ^{14}C -galactose and galactinol when these were incubated alone in the presence of GRT from V. faba seeds.

The inhibition of GRT by 20mM concentrations of myo-inositol and melibiose was found to be competitive and non-competitive with raffinose, respectively (Fig. 14). Under the conditions used, the inhibition of myo-inositol was most probably caused by shifting the equilibrium of the reaction to the left. Myo-inositol therefore competed effectively with raffinose for the galactosyl-enzyme intermediate, resulting in synthesis of galactinol instead of stachyose. It is unlikely that myo-inositol is involved

in regulating GRT activity in vivo. Regulation of polysaccharide synthesis in plants usually occurs at the level of the nucleotide diphosphate sugar formation (Preiss and Kosuge, 1976; Candy, 1980) and there is no reason to believe that stachyose synthesis would be regulated by fluctuations in the concentration of an ubiquitous compound such as myo-inositol rather than at the galactinol synthesis level (see Equation I). Webb (unpublished results) has obtained some evidence that such control occurs. The synthesis of galactosyl-sucrose oligosaccharides would then be controlled early in the biosynthetic pathway and undesired accumulation of intermediate metabolites would be prevented. Several metabolic pathways in plants have been shown to be regulated in such fashion (Preiss and Kosuge, 1976).

The non-competitive pattern of inhibition observed with melibiose is harder to explain since alternate substrates usually inhibit in a competitive manner with the natural (physiological) substrate (Dixon and Webb, 1979). Since only one set of assay conditions were used to determine the type of inhibition caused by melibiose, more kinetic studies are needed to confirm this result unequivocally.

Galactose did not seem to inhibit the transferring activity of GRT even though it served as a substrate to a minimal extent. These observations, coupled with the fact that galactose did not inhibit the hydrolytic activity

of GRT as tested with ^{14}C -galactinol and PNPG, suggest that the myo-inositol moiety of galactinol plays an important role in binding of galactinol to the enzyme and the subsequent formation of the galactosyl-enzyme complex. On the other hand, the failure of stachyose to serve as substrate and the small rates of galactosyl transfer observed using melibiose and galactose as substrates suggest that the formation of the galactosyl-enzyme complex imposes very strict structural requirements on the acceptor molecule. This also implies that a third enzyme, galactinol:stachyose galactosyltransferase must be present in the leaves of C. pepo to catalyze the synthesis of verbascose.

Several metal ions inhibited GRT (Table VII). This suggests that sulfhydryl groups are required for the expression of GRT activity. Besides Fe^{3+} , Cu^{2+} and Mn^{2+} , which form unstable complexes and act as catalysts for the oxidation of sulfhydryl groups, Co^{2+} , Ni^{2+} , Zn^{2+} , Hg^{2+} , Mg^{2+} and Ag^{2+} are all known to combine with -SH groups and form mercaptides of different degrees of reversibility (Barron, 1951). Most of the heavy metals which form mercaptides also combine with the carboxyl, amino and imidazolium groups of proteins (Simpson, 1961; Barron, 1951). Williams (1953) reported that the stability of metal ions complexes with any of these residues

(except -SH) lie in the general order $Mn^{2+} < Fe^{3+} < Co^{2+} < Ni^{2+} < Cu^{2+} < Zn^{2+}$ for members of the first transition series. However, the large number of different co-ordinating groups available in proteins make the interpretation of results difficult and, clearly, the order of inhibition observed with the various ions tested does not reflect their ability to form stable complexes. Tanner and Kandler (1968) observed that GRT from P. vulgaris seeds was strongly inhibited in the presence of the sulfhydryl poison p-chloromercuribenzoate.

The presence of multimolecular forms of α -galactosidase in leaf tissue of C. pepo has been reported before (Thomas and Webb, 1977) and is of common occurrence in the plant kingdom (Dey, 1972). Multimolecular forms have also been found for several other hydrolases, including invertase (Fleischmacher et al., 1980), α -glucosidase (Yamasaki and Suzuki, 1980 and 1980a) and α -L-fucosidase (Chien and Dawson, 1980). A fourth molecular form of α -galactosidase was discovered in leaves of C. pepo. This enzyme, L_{IV} , presumably escaped detection until now due to its properties which differ strikingly from the other three forms, L_I , L_{II} and L_{III} . Thus, L_{IV} was found to hydrolyze PNPG and stachyose optimally at pH 7.5 while raffinose hydrolysis was best achieved at pH 6.5-7.0 (Fig. 19). As shown in Table III, all α -galactosidases previously reported showed optimum activity at acidic pH values. The introduction of the

terms alkaline and acid α -galactosidase therefore appears appropriate at this stage to differentiate the two activities. Similar terminology is currently used for other enzymes such as acid and alkaline invertase and acid and alkaline phosphatase (Dixon and Webb, 1979).

L_{IV} also differed from acid α -galactosidases by its apparent lack of inhibition by high concentrations of the artificial substrate PNPG. Such an effect is commonly observed with acid α -galactosidases and Dey and Pridham (1969) speculated that it might result from the association of two molecules of substrate with the enzyme, giving rise to an inactive complex, ESS. Carchon and DeBruyne (1975) suggested that ESS is a dead-end complex based on the observation that the rate of hydrolysis of p-chlorophenyl- α -D-galactopyranoside by α -galactosidase from coffee bean kept decreasing with increasing concentrations of the substrate.

Another major difference between alkaline and acid α -galactosidase from leaves of C. pepo is the ability of L_{IV} to breakdown stachyose at a faster rate than raffinose. The rate of hydrolysis of homologous series of α -D-galactosides by acid α -galactosidases has been shown to diminish with increasing chain length (Dey and Pridham, 1972; Williams et al. 1978; Smart and Pharr, 1980).

The inhibition of PNPG hydrolysis by partially purified preparations of L_{IV} by various sugars was also

compared to that of L_I , L_{II} and L_{III} preparations (Table IX). High levels of inhibition were observed in all cases with galactose, a powerful and competitive inhibitor of α -galactosidases (Dey and Pridham, 1972; Gatt and Baker, 1969; Thomas and Webb, 1977).

L-Arabinose, a structural analog of D-galactose (see Fig. 41), was ineffective in most cases. D-Glucose, D-fructose and D-xylose were also generally non-inhibitory. Mixed results have been published concerning the inhibition of α -galactosidase by these sugars. Thus, L-arabinose and D-glucose did not inhibit α -galactosidase from V. faba (Dey and Pridham, 1969) and T. repens (Williams et al., 1977) but inhibited α -galactosidase from S. carlsbergensis (Lazo et al., 1978) and M. vinacea (Suzuki et al., 1970). Inhibitory studies by Li and Shetlar (1964a) suggested that attachment of α -D-galactosides to α -galactosidase has very strict requirements for the configuration of D-galactose and that C-1, C-2, C-4 and C-6 of D-galactose are involved in the binding to the enzyme. Similarly, Dey and Pridham (1969) provided evidence that the affinity of α -galactosidase for p-nitrophenyl- α -D-glycosides was largely dependent on the structural changes in the glycone moiety. Affinity decreased in the order α -D-galactoside > α -D-fucoside < β -L-arabinoside and these authors suggested that one of the specific points of binding of the substrate with α -galactosidase may be through the primary alcohol group of the galactosyl moiety (see Fig. 41).

α -D-Galactose

α -D-Glucose

α -L-Fucose

α -D-Mannose

N-Acetyl-D-Galactosamine

α -D-Xylose

α -L-Arabinose

myo-Inositol

Figure 41 -- Structural Analogs of Galactose.

Galactinol inhibited all four molecular forms of α -galactosidase to a considerable degree (Table IX). This compound has been reported to be a poor substrate for α -galactosidase from V. faba (Dey and Pridham, 1969), V. sativa (Petek et al., 1969), M. vinacea (Suzuki et al., 1970) and T. repens (Williams et al., 1977). The hydrolysis of this sugar alcohol by the molecular forms of α -galactosidase from C. pepo also appeared to be minimal based on results obtained with TLC analysis of its hydrolytic products. Dey and Pridham (1969) found that galactinol had the highest affinity for the binding site of α -galactosidase I from V. faba but that the rate of hydrolysis of this compound was one of the lowest among several substrates tested. Therefore, galactinol inhibited α -galactosidase effectively by its high affinity for the enzyme's active site, coupled with a low rate of hydrolysis, resulting in a monopolizing effect on the concentrations of the free form of the enzyme.

No significant effect on L_{III} and L_{IV} was observed when myo-inositol was added to the assay system whereas both L_I and L_{II} were inhibited (Table IX). Sharma (1971) provided evidence that myo-inositol is a highly selective inhibitor of α -galactosidases from various plant sources. His results indicated that the orientation of hydroxyl groups at C-1 and C-4 in the D-galactopyranoside ring and the conformation together with the orientation

of hydroxyl groups in myo-inositol are critical. The orientation of hydroxyl groups at C-2, C-3 and C-4 in myo-inositol is identical to that of C-4, C-3 and C-2 or C-1, C-2 and C-3 in D-galactose (Fig. 41). α -Galactosidase possesses a specificity site and a catalytic site (Kelemen and Whelan, 1966; Dey and Pridham, 1972). The specificity site determines the carbohydrate which will be hydrolyzed and the catalytic site of the enzyme determines the nature of the bond which will be hydrolyzed. If it is assumed that configuration at C-4 and C-1 in galactosyl residues is involved in binding of substrate at specificity and catalytic sites, respectively, then the structural similarity between myo-inositol and D-galactose might allow myo-inositol to prevent enzyme-substrate interactions by competing for both sites on the enzyme (Sharma, 1971). The regulation of α -galactosidase activity by product inhibition is unlikely since galactose is generally not encountered in the free state in plants (Maretzki and Thom, 1978; Dey, 1980) and the galactose liberated by hydrolysis of various substrates in vitro does not inhibit the enzyme significantly (Williams et al, 1978; Dey and Pridham, 1972). In vivo, galactose is quickly metabolized to galactose-1-phosphate (Shiroya, 1963; Pridham et al., 1969; Maretzki and Thom, 1978) and this compound is itself a poor inhibitor of α -galactosidase activity (Dey and Pridham, 1969). Sharma (1971) suggested

that myo-inositol may play a role in galactose metabolism in plants. However, other mechanisms must exist since two of the molecular forms of α -galactosidase from C. pepo and α -galactosidase from T. repens (Williams et al., 1977) were not affected by this compound.

Melibiose, raffinose and stachyose inhibited the four molecular forms of α -galactosidase from C. pepo to various degrees (Table IX). Dey and Pridham (1969) have shown that these sugars are competitive inhibitors of α -galactosidases and can all serve as substrates. The hydrolysis of raffinose and stachyose by L_I , L_{II} , L_{III} and L_{IV} was confirmed by TLC. It was also observed that these oligosaccharides were hydrolyzed by an invertase system present in preparations of L_I and L_{II} . Invertase has not been studied in leaves of C. pepo but the results obtained with L_I and L_{II} warrant a closer examination of its properties and the role it may play in the breakdown of raffinose oligosaccharides in vivo.

The inhibition of the hydrolytic activity of GRT and GRT-40 by various sugars was also examined as a matter of interest. The results obtained (Table IX) were described and discussed in the Results section. It may be added that the stimulatory effect in PNPG hydrolysis observed in the presence of the acceptor raffinose, the alternate acceptor melibiose and the product myo-inositol provides confirmatory evidence that the transferring and hydrolytic properties of the GRT-40 preparation are

catalyzed by the same enzyme and that contamination of GRT-40 by α -galactosidase is unlikely.

The alkaline α -galactosidase L_{IV} was purified to a high level of purity in order to examine its properties more closely. The pH optima for raffinose, stachyose and PNPG hydrolysis were confirmed (Fig. 24). The minor peaks of activity which were observed with less purified preparations of L_{IV} were completely eliminated. An α -galactosidase with a pH optimum of 7.0 was purified from the golden-brown alga P. malhamensis (Dey and Kauss, 1981; see Table III). This enzyme appeared to have several properties similar to L_{IV} from C. pepo: it was quickly denatured during fractionation with ammonium sulphate, it was very unstable in acidic pH, the enzyme was inhibited by PCMB and $HgCl_2$ and was not inhibited by high concentrations of PNPG. On the other hand, this "alkaline" α -galactosidase was highly specific for its physiological substrate, isofloridoside (O- α -D-galactopyranosyl-(1 $\rightarrow$ 1) $\rightarrow$ glycerol), and no hydrolysis of galactinol, raffinose, melibiose and stachyose could be observed. This enzyme is apparently involved in the osmoregulation of P. malhamensis (Kreuzer and Kauss, 1980). As stated above, all the remaining α -galactosidases isolated from higher plants display pH optima in the acidic range (Table III).

Substrate specificity studies revealed that alkaline α -galactosidase was highly specific for α -linkages (Table XI), suggesting that the configuration at C-1 of the substrate is important. On the other hand, contrary to acid α -galactosidase from V. faba seeds (Dey and Pridham, 1969), L_{IV} did not cleave p-nitrophenyl- α -D-fucoside indicating that the structure at C-6 of the galactosyl moiety of an α -galactoside is also important in the binding of the substrate to alkaline α -galactosidase. Similarly, the lack of hydrolysis of p-nitrophenyl- α -D-glucoside by L_{IV} suggests that the configuration of the hydroxyl group at C-4 is also critical to the expression of enzyme activity.

Both K_m and V_{max} were affected when the position of the nitro substituent of phenyl- α -D-galactosides was changed (Table XI). Similar results were observed for acid α -galactosidase (Dey and Pridham, 1969) and it was suggested that several factors affect the affinity of aromatic α -D-galactosides including position and size of the substituent groups on the phenyl ring, its electronic effect and its degree of hydration (Dey and Pridham, 1972). Carchon and DeBruyne (1975) did not observe any differences on the rate of hydrolysis of 8 para-substituted phenyl- α -D-galactopyranosides by coffee bean α -galactosidase except for nitro groups. Therefore, it seemed highly unlikely that the release of the phenyl aglycon group

constitutes the rate-limiting step of the enzyme reaction mechanism. They proposed a pathway for hydrolysis which involves at least two intermediates as shown in Equation XX. After the formation of the enzyme-substrate complex,

ES, the aglycon group (P_1) is released with the simultaneous formation (glycosylation step) of an enzyme-galactosyl complex (ES') which then reacts with water, yielding galactose (P_2) and free enzyme (deglycosylation). The rate of hydrolysis will be independent of the aglycon group if it is assumed that, for phenyl- α -D-galactopyranosides or substrates with a good leaving aglycon group, the deglycosylation step is rate-limiting. The results presented here and by Dey and Pridham (1969) suggest that the position of nitro-substituents on the aglycone group has a significant effect on the rate of hydrolysis. Since the affinity of L_{IV} for para-, ortho- and meta- α -D-galactopyranoside is not significantly different, the position of the nitro group must somehow affect the galactosylation step in the proposed reaction mechanism of α -galactosidase (Equation XX). The reason why raffinose and stachyose do not show substrate inhibition is unknown but probably lies in the large structural differences between aryl galactosides and oligosaccharides.

Determination of K_m and V_{max} values for stachyose and raffinose hydrolysis by the highly purified L_{IV} preparation at pH 7.5 also confirmed previous observations made with the partially purified enzyme. Thus, stachyose was hydrolyzed about 10 times faster than raffinose as well as having a much higher affinity for the binding site of L_{IV} (Table XI).

The inhibition of L_{IV} by several sugars was also reinvestigated with the highly purified preparation (Table XII). The K_i values for galactose, stachyose, melibiose, galactinol and raffinose in the presence of 6.3mM PNPG reflected the pattern of inhibition previously observed with the less purified L_{IV} preparation (see Table IX) and, in the case of the oligosaccharides, also reflected the order of affinity observed when they were used as substrates (see Table XI). Pridham and Dey (1969) and Lazo et al. (1978) reported that when PNPG is used as substrate to determine acid α -galactosidase activity, the inhibition observed with other substrates is competitive. Both groups of workers also observed that the K_i values determined for various sugars were in agreement with the K_m values obtained for these sugars when used as substrates. Dey and Pridham (1969) therefore suggested that all substrates for α -galactosidase are bound to the same active site on the enzyme molecule and that the K_m is almost a true representation of the dissociation constant, K_s , of the enzyme-substrate complex. However, in the case of L_{IV} , the K_i values

obtained with stachyose and raffinose were considerably higher than their respective K_m values. This discrepancy is due to the fact that the K_i value of the sugars was determined at only one PNPG concentration and it is quite likely that proper determination of the inhibitory constants using several PNPG concentrations would lower the individual K_i s to values similar to the K_m values of the various sugars.

The structure of C-2 on the galactosyl ring also appeared to be important for binding since N-acetyl-D-galactosamine (Fig. 41) was not inhibitory (Table XII). Similar results were reported by Suzuki et al. (1970). Roeser and Legler (1981) observed that β -glucosidase from Aspergillus wentii could not cleave 4-methylumbelliferyl- β -D-N-acetylglucosamine and that the hydroxyl group at C-2 had a very pronounced effect on the catalytic ability of this enzyme. They suggested that interaction of the hydroxyl group with β -glucosidase is required to have the substrate deformed towards the transition state and to induce the optimal orientation of the catalytic groups with respect to C-1 of the glycosyl oxygen. Presumably, the introduction of the acetyl group at C-2 of galactose strongly prohibits binding of N-acetyl-D-galactosamine to α -galactosidase. N-Acetyl glycosamines are common constituents of the carbohydrate moiety of glycoproteins (Candy, 1980) and

hydrolases highly specific for these glycopyranosyl groups and the anomeric configuration of the glycosidic bond are present in plants (Agrawal and Bahl, 1968; Neely and Beevers, 1980). Therefore, it may not be too surprising to find a high degree of specificity between the two types of hydrolases with respect to their involvement in different physiological processes and potential regulation of their respective activities.

Finally, the lack of inhibition caused by 1-O-methyl- α -D-galactopyranoside suggest that alkaline α -galactosidase has strict structural requirements as to the nature of the galactose-carrying moiety. The low rates of hydrolysis observed with raffinose also support this assumption. Presumably, the raffinose moiety of the stachyose molecule plays an important role in the orientation of the substrate on the enzyme prior to its cleavage and galactosylation of the enzyme.

Complete inhibition of L_{IV} activity was observed in the presence of several metal ions and the sulfhydryl poison PCMB (Table XIII), suggesting the involvement of thiol groups in enzyme catalysis. Dey and Pridham (1969) could not detect thiol groups in purified acid α -galactosidase from V. faba seeds even though the enzyme was strongly inhibited by Hg^{2+} , Ag^{2+} and Cu^{2+} . As stated earlier in the case of GRT, the inhibition of enzyme activity by a metal ion is rarely specific and is usually dependent upon the ability of a particular ion

to react with the several different groups present in proteins (Williams, 1953). For example, Hg^{2+} is known to react with carboxyl, amino and imidazolium groups in addition to sulfhydryl groups and peptide linkages (Simpson, 1961). The reactivity of Hg^{2+} with different groups is also known to vary with pH (Dey and Pridham, 1972). More importantly, none of the ions tested were found to stimulate L_{IV} activity. Various ions have been shown to weakly activate acid α -galactosidase activity in a few cases (Pernas et al., 1981; Petek et al., 1969; Dey and Pridham, 1972) but most acid α -galactosidases are indifferent to monovalent and divalent cations. The effect of buffer on the degree of metal ion inhibition also appears to be a factor to consider (Table XIII).

EDTA and various organic acids had a stimulatory effect on L_{IV} activity using PNPG (Table XIV, XVI and Fig. 28) or stachyose (Table XIV, XVII and Fig. 29) as substrate. This effect was itself affected by the type of buffer in which L_{IV} was assayed. The results obtained when L_{IV} was transferred from one buffer to another by ultrafiltration instead of dialysis were slightly different. The discrepancies observed were given in the Results section and discussed. The experimental conditions were more carefully controlled in the study where dialysis was used and this discussion will deal mostly with these results.

The following important facts about L_{IV} were thus determined: (1) buffer composition greatly influences the rate of hydrolysis of PNPG (Table XV) and stachyose by L_{IV} ; (2) chelating compounds stimulate L_{IV} activity and their presence or absence in various buffers appear to be responsible to a large extent for the buffer effects on enzyme catalysis (Fig. 28); (3) high concentrations of dibasic sodium phosphate appear to inhibit the hydrolysis of stachyose but not PNPG, suggesting that observations made using one substrate do not necessarily indicate that the same reactions are occurring when using another substrate. This last observation is reminiscent of results presented by Dey and Pridham (1969a) where α -galactosidase from V. faba seeds was reported to hydrolyze PNPG optimally around pH 6.8 while raffinose hydrolysis was optimal around pH 4.5.

It is not clear from the experimental evidence given how the stimulatory effect caused by EDTA and citrate is brought about. The best explanation might reside in looking at these two compounds as stabilizing agents rather than activators. L_{IV} preparations were stable for at least a week in McIlvaine buffer at pH 7.5 (which contains a final concentration of 8mM citrate). However, the stability of L_{IV} in NaPi, TEA and HEPES buffer was never determined for extended periods of time and can be questioned when the following observations are considered. Thus, the low overall recovery value

(about 5%) obtained for L_{IV} activity after the various steps of the purification procedure could be attributed to instability of alkaline α -galactosidase in NaPi buffer and to a gradual loss of activity. It is however evident from the results presented in Table XVI that the loss of activity observed in NaPi buffer is readily reversible by the addition of the stabilizing agents EDTA and citrate. It is also apparent from the results presented in Table XV that L_{IV} is more stable in NaPi buffer than either TEA or HEPES buffer. The stability of L_{IV} in TEA and HEPES buffer could also explain the differences observed in the activity of the enzyme when it was transferred into these buffers by ultrafiltration or by dialysis. When ultrafiltration was used, 60 to 70% residual activity could be found after transfer. This procedure took usually only one hour and the enzyme preparation thus obtained used immediately after to study the effect of metal ions. On the other hand, dialysis was allowed to proceed for 72 hours before the enzyme was used for the assays. If the recovery of activity from TEA and HEPES buffers is time dependent, one can clearly understand why ultrafiltration yielded higher levels of activity. It should also be noticed that the loss in L_{IV} activity observed in TEA or HEPES buffer is readily reversible (Table XV) and that the stabilizing effect of EDTA and citrate does not require prolonged preincubation

of the enzyme with these compounds.

The demonstration that restoration of activity is accomplished rapidly, that the levels of activity recovered can be shown to be dependent upon the concentration of EDTA and citrate and that the stimulatory (stabilizing) effect is not easily abolished by dialysis suggest physical interaction between the enzyme and the chelating compounds. It was also observed that EDTA and citrate enhanced the hydrolysis of stachyose to a much greater degree than PNPG hydrolysis in the absence of high concentrations of dibasic sodium phosphate. Therefore, it would be interesting to measure raffinose hydrolysis under conditions which are optimal for stachyose hydrolysis to see if the rate of hydrolysis of raffinose by L_{IV} could also be increased.

Citrate holds several key physiological functions. It is an intermediate in the later stages of carbohydrate metabolism where it is oxidized in the mitochondria leading to the production of energy, it supplies acetyl groups for biosynthetic pathways and it serves as a hydrogen donor for cytosolic syntheses. Citrate also has a controlling action (activating or inhibiting) on several enzymes, including acetyl CoA carboxylase and phosphofructokinase (Glusker, 1980; Dyson, 1978; Lehninger, 1970; Preiss and Kosuge, 1976). Glusker (1980) has suggested that the reaction mechanism of enzymes such as aconitase and citrate synthase involves chelation of citrate to enzyme-bound metal ions. Finally, Boyer and

Preiss (1979) described the stimulation of corn starch synthase I activity by citrate and showed that it was not specific when EDTA and malate were also found to stimulate the enzyme.

The discovery of L_{IV} raised the possibility that this enzyme might be responsible for the different patterns of utilization of galactosyl-sucrose oligosaccharides by mature and immature leaves. The specific activity of L_{IV} in different parts of C. pepo was determined and the results indicated that alkaline α -galactosidase activity was present at significantly higher levels in immature petiole and leaf tissue than in their mature counterparts (Table XVIII). The specific activity of alkaline α -galactosidase also appeared to be higher than that of acid α -galactosidase in importing organs (roots, immature petioles and immature leaves), suggesting that L_{IV} may play a major role in the hydrolysis of stachyose.

This was further substantiated when L_{IV} was found to undergo a 6 fold decrease in specific activity over the developmental period during which immature, importing leaves become net exporting organs (Fig. 32). Stachyose is the major translocated sugar in squash and high levels of L_{IV} activity in importing leaves represent an obvious advantage. On the other hand, the presence of significant, but much lower, levels of L_{IV} in mature leaves suggest that some degree of spatial separation

between L_{IV} and the site of stachyose synthesis must be taking place. The decrease in the specific activity of L_{IV} during leaf development is reminiscent of the changes observed in the specific activity of several hydrolases during seed germination. For example, acid α -galactosidase activity has been found to increase in germinating seeds of several plant species and this increase was invariably accompanied by a decrease in galactomannan and galactosyl-sucrose oligosaccharides content (Pridham and Dey, 1974; McLeary and Matheson, 1974; Shiroya, 1963; Dey, 1980). The regulation of starch breakdown in plants also usually occurs by regulation of enzyme levels in the tissues. Thus, in cereal seeds, large increases in α - and β -amylase activity are observed after the initiation of germination (Candy, 1980; ap Rees, 1976).

The presence of acid and alkaline α -galactosidase activity in leaves of C. pepo is also reminiscent of acid and alkaline invertase activity in sugar cane (ap Rees, 1976; Fleischmacher et al, 1980; Vattuone et al., 1981). Acid invertase is known to occur in the cell wall and intracellularly. Cell wall acid invertases are apparently involved in the translocation of sucrose from the apoplast to the symplast of sugar cane (Hawker and Hatch, 1965; Glasziou and Gayler, 1972). However, in other plant species, sucrose uptake was observed to occur without prior hydrolysis in the free space

(ap Rees, 1976). The suggestion has been made that cell wall acid invertase in these plants may regulate sucrose concentrations in the free space and play a role in differentiation (Jeffs and Northcote, 1966 and 1967; ap Rees, 1976). The general role of intracellular acid invertase appears to be mobilization of the sucrose stored in the cell vacuole (ap Rees, 1976; Gayler and Glasziou, 1972). The role of alkaline invertase is not clear but could also involve sucrose mobilization in the cytoplasm of cells that store sucrose (these cells usually contain low levels of intracellular acid invertase) (ap Rees, 1976). The separation of the stored sucrose from the cytoplasmic alkaline invertase would permit considerable sucrose storage despite the presence of the enzyme. At the same time, the cell would retain the ability to hydrolyze sucrose and meet its demands for hexoses.

Thomas and Webb (1979) have presented some evidence for an exocellular localization of acid α -galactosidase in C. pepo. One of their experiments also suggests that alkaline α -galactosidase is located in the cytoplasm. In this experiment, exocellular acid α -galactosidase activity was assayed in vivo over the pH range 3.0 to 7.2 by incubating leaf strips in McIlvaine buffers of appropriate pH containing PNPG

and the nitrophenol released was determined at 400 nm to calculate enzyme activity (Thomas and Webb, 1979).

Exocellular α -galactosidase activity decreased between pH 6.5 and 7.2, suggesting that alkaline α -galactosidase is located in the cytoplasm since pH curves of leaf homogenates show a sharp increase in α -galactosidase activity between pH 6.5 and 7.5 (see Fig. 30). Thomas and Webb (1979) suggested that the galactosyl-sucrose oligosaccharides synthesized by mature leaves might avoid hydrolysis by exocellular acid α -galactosidase prior to their translocation if they were channelled symplastically from their site of synthesis to the minor veins. The presence of a highly active alkaline α -galactosidase in the cytoplasm would seem to contradict this hypothesis. However, the cytoplasm is highly compartmentalized and spatial separation between L_{IV} and the site of stachyose synthesis is a possibility that remains to be investigated. Moreover, L_{IV} activity in leaf tissue appears to be under some type of control and its specific activity in various parts of the plant reflects the physiological need of the tissue for such an enzyme. Thus, importing organs have higher levels of L_{IV} than exporting organs.

The observation that alkaline α -galactosidase activity in leaves of higher plants is restricted to the family Cucurbitaceae whereas acid α -galactosidase activity is ubiquitous (Table XIX) also suggest that L_{IV} is involved

in an entirely different physiological process in these species. On the other hand, the presence of acid α -galactosidase activity in the leaves of plants which do not synthesize nor translocate galactosyl-sucrose oligosaccharides suggest that this enzyme must have some other function in leaves.

The specific activity of alkaline α -galactosidase in cucumber and pumpkin was also shown to decrease as the leaves matured (Fig. 35 and 36). However, unlike the situation in squash, it was observed that acid α -galactosidase also decreased in specific activity in these species. The significance of this latter result is opened to speculations since it is not known if the specific activity of acid α -galactosidase in leaves of other plants is also dependent upon the developmental stage of the leaf. It would be especially interesting to study the occurrence of such a phenomenon in leaves of plants that do not synthesize or translocate galactosyl-sucrose oligosaccharides since, in these plants, acid α -galactosidase might fulfill a physiological need other than the hydrolysis of these sugars. In other words, whereas the decrease in the specific activity of alkaline α -galactosidase in maturing leaves of squash, cucumber and pumpkin has obvious physiological implications in terms of galactosyl-sucrose oligosaccharide metabolism,

the decrease in the specific activity of acid α -galactosidase in cucumber and pumpkin is harder to explain on the same physiological basis since it is known not to occur in squash.

Gross and co-workers (1981) provided some evidence that acid α -galactosidase is located exocellularly in cucumber callus cultures. They also observed that cucumber callus required an adaptative period prior to optimal growth on stachyose and raffinose. However, the lag in growth of cucumber calli on these sugars did not involve induction of acid α -galactosidase activity. It would be interesting to determine if the lag period observed could be due to induction of alkaline α -galactosidase. If so, it would strongly suggest that alkaline α -galactosidase in cucurbits plays an important role in the metabolism of galactosyl-sucrose oligosaccharides.

Two forms of α -galactosidase were resolved from cucumber cotyledons (Fig. 38). The first peak (C_I) hydrolyzed PNPG, stachyose and raffinose optimally between pH 5.0 and 5.5 (Fig. 39). C_I probably consisted of two forms of acid α -galactosidase with one form displaying residual activity at pH 7.5 while the other did not (Fig. 38). This elution pattern is similar to that of L_I and L_{II} in squash. Smart and Pharr (1980) have reported that cucumber leaves contain two forms

of acid α -galactosidase with pH optima of 5.5 and 5.2 using PNPg as substrate but they did not detect any alkaline α -galactosidase activity.

The second peak of α -galactosidase activity (C_{II}) had properties similar to that of L_{IV} . Thus, C_{II} hydrolyzed stachyose optimally between pH 7.5 and 8.0 while the hydrolysis of PNPg showed a sharp pH optimum at 7.5. Raffinose hydrolysis was barely detectable at pH 7.0 and 7.5. C_{II} eluted off the ion-exchange column late in the NaCl gradient, suggesting that it also has a strong negative charge. Finally, stachyose was found to be a potent inhibitor of PNPg hydrolysis by C_{II} .

Galactose assimilation in leaves of C. pepo follows the pathway suggested by Maretzki and Thom (1978) (Fig. 3). High levels of galactokinase and UDP-galactose pyrophosphorylase activity were present in both mature and immature leaf tissue (Table XX). UDP-galactose-4-epimerase activity was also detected. Evidence that galactose assimilation in the seeds of various plants also proceeds by this pathway has been reported by several workers (Shiroya, 1963; Amuti and Pollard, 1977; Dey, 1980). The presence of galactokinase, UDP-galactose pyrophosphorylase and UDP-galactose-4-epimerase in fruit peduncles was also confirmed for Cucumis sativus, Citrullus lanatus, Cucurbita moschata, Cucumis melo and Phaseolus vulgaris (Gross et al., 1979; Gross and Pharr, 1981).

CONCLUSIONS

Galactinol:raffinose galactosyltransferase (GRT) partially purified from leaves of Cucurbita pepo has properties very similar to those of GRT from Phaseolus vulgaris seeds (Tanner and Kandler, 1968). However, unlike GRT from seeds of Vicia faba (Lehle and Tanner, 1972), the enzyme from C. pepo is unable to catalyze the synthesis of verbascose, the next higher homolog after stachyose in the galactosyl-sucrose series. This indicates that three distinct galactosyltransferases which catalyze the synthesis of raffinose, stachyose and verbascose, respectively, are present in leaves of C. pepo. Isotope exchange studies and other evidence presented in this study suggest that stachyose synthesis proceeds via a two step transfer of the galactosyl moiety of galactinol to raffinose according to the mechanism proposed by Koshland (1953). The effect of various sugars of the hydrolysis of p-nitrophenyl- α -D-galactopyranoside (PNPG) by GRT suggest that the α -galactosidase activity present in preparations of GRT is due to the transferring nature of GRT itself and does not result from contamination of the preparation with one of the molecular forms of α -galactosidase found in leaves of C. pepo.

A fourth molecular form of α -galactosidase was discovered in leaves of C. pepo. The properties of this enzyme, designated L_{IV}, differ strikingly from those of all other forms of α -galactosidases found in the plant kingdom (Dey and Pridham, 1972). These include (1) a pH optimum for the hydrolysis of PNPG, raffinose and stachyose in the alkaline range; (2) the ability of L_{IV} to hydrolyze stachyose much more efficiently than raffinose; (3) a lack of inhibition by high concentrations of the artificial substrate PNPG; and (4) the stimulation of L_{IV} activity by chelating agents such as citrate and EDTA.

L_{IV} experienced a 6-fold decrease in specific activity over the growth period during which immature, importing leaves of C. pepo become net exporting organs while the specific activity of acid α -galactosidase remained constant. Alkaline α -galactosidase activity was restricted to members of the Cucurbitaceae family in which stachyose is the major sugar translocated. These observations suggest that alkaline α -galactosidase might play a major role in the metabolism of galactosyl-sucrose oligosaccharides in leaves of cucurbits and that acid α -galactosidase, which is ubiquitous in leaves throughout the plant kingdom, might be involved in some other physiological process in leaf tissue.

REFERENCES

- 1 - Agrawal, K.M.L. and Bahl, O.P. (1968) J. Biol. Chem. 243:103-111. Glycosidases of *Phaseolus vulgaris*. II. Isolation and General Properties.
- 2 - Alden, J. and Hermann, R.K. (1971) Bot. Rev. 37:37-142. Aspects of the Cold-Hardiness Mechanism in Plants.
- 3 - Amuti, K.S. and Pollard, C.J. (1977) Phytochem. 16: 533-537. The Metabolism of Galactose and the Raffinose Oligosaccharides by Germinating Bambarra Groundnut Seeds.
- 4 - Anderson, J.W. (1968) Phytochem. 7:1973-1988. Extraction of Enzymes and Subcellular Organelles from Plant Tissues.
- 5 - Anderson, J.W. and Rowan, K.S. (1967) Phytochem. 6: 1047-1056. Extraction of Soluble Leaf Enzymes with Thiols and Other Reducing Agents.
- 6 - Angyal, S.J. and Anderson, L. (1959) Adv. Carbohydrate Chem. 14:135-212. The Cyclitols.
- 7 - ap Rees, T. (1974) "Pathways of Carbohydrate Breakdown in Higher Plants." in Plant Biochemistry, Vol. 11, D.H. Northcote (ed.), Butterworths, London, pp. 89-127.
- 8 - Balasubramian, K., Day, P.M. and Pridham, J.B. (1976) Phytochem. 15:1445-1446. α -Galactosidase from Coconut Kernels.

- 9 - Bamberger, E.S. and Park, R.B. (1966) Plant Physiol. 41:1591-1600. Effect of Hydrolytic Enzymes on the Photosynthetic Efficiency and Morphology of Chloroplasts.
- 10 - Barham, D., Dey, P.M., Griffiths, D. and Pridham, J.B. (1971) Phytochem. 10:1759-1763. Studies on the Distribution of α -Galactosidases in Seeds.
- 11 - Barron, E.S.G. (1951) Adv. Enzymology, Vol. XI:201-264. Thiol Groups of Biological Importance.
- 12 - Beck, G. (1980) Schweiz. Med. Wschr. 110:1190-1201. Beitrag zur Fabryschen Krankheit.
- 13 - Beitler, G.A. and Hendrix, J.E. (1974) Plant Physiol. 53:674-676. Stachyose: An Early Product of Photosynthesis in Squash Leaves.
- 14 - Bourne, E.J., Walter, M.W. and Pridham, J.B. (1965) Biochem. J. 97:802-806. The Biosynthesis of Raffinose.
- 15 - Bourquelot, E. and Bridel, M. (1910) C. R. Acad. Sci. 151:760-762. New Sugar Verbascose Obtained from Mullein Root.
- 16 - Boyer, C.D. and Preiss, J. (1979) Plant Physiol. 64:1039-1042. Properties of Citrate-stimulated Starch Synthesis Catalyzed by Starch Synthase I of Developing Maize Kernels.

- 17 - Bradford, M.M. (1976) Analytical Biochem. 72:248-254.
A Rapid and Sensitive Method for the Quantitation
of Microgram Quantities of Protein Utilizing the
Principle of Protein-Dye Binding.
- 18 - Brown, R.J. and Serro, R.F. (1953) J. Am. Chem. Soc.
75:1040-1042. Isolation and Identification of
O- α -Galactopyranosyl-myo-inositol and of
Myo-inositol from Juice of the Sugar Beet (*Beta
vulgaris*).
- 19 - Calloway, D.H., Colasito, D.J. and Matthews, R.D.
(1966) Nature (London) 212:1238-1239. Gases
Produced by Human Intestinal Microflora.
- 20 - Candy, D.J. (1980) Biological Functions of Carbohydrates.
Blackie, Glasgow. 197 pages.
- 21 - Carchon, H. and DeBruyne, C.K. (1975) Carb. Res. 41:
175-189. Purification and Properties of Coffee Bean
 α -Galactosidase.
- 22 - Chien, S. and Dawson, G. (1980) B.B.A. 614:476-488.
Purification and Properties of Two Forms of Human
 α -L-Fucosidase.
- 23 - Cleland, W.W. (1963) B.B.A. 67:104-137. The Kinetics
of Enzyme-catalyzed Reactions with Two or More
Substrates or Products. I. Nomenclature and Rate
Equations.

- 24 - Cleland, W.W. (1964) Biochem. 4:480-482. Dithiothreitol, a New Protective Reagent for SH Groups.
- 25 - Cleland, W.W. (1977) Adv. Enzymology 45: 273-387. Determining the Chemical Mechanisms of Enzyme Catalyzed Reactions by Kinetic Studies.
- 26 - Courtois, J.E. and Petek, F. (1966) Methods in Enzymology 8:565-571. α -Galactosidase from Coffee Beans.
- 27 - Courtois, J.E. and Percheron, F. (1971) Pages 207-229 in "Chemotaxonomy of Leguminosae", J.B. Harborne, D. Boulter and B.L. Turner (eds), Academic Press, New-York.
- 28 - Cristofaro, E., Mottu, F. and Wuhrmann, J.J. (1974) Involvement of the Raffinose Family of Oligosaccharides in Flatulence. In "Sugars in Nutrition.", H.L. Sipple and K. McNutt (eds.), Academic Press, New-York, pages 313-336.
- 29 - Dean, K.J. and Sweeley, C.C. (1979) J. Biol. Chem. 254:9994-10000. Studies on Human Liver α -Galactosidases. I. Purification of α -Galactosidase A and its Enzymatic Properties with Glycolipid and Oligosaccharide Substrates.
- 30 - Dean, K.J. and Sweeley, C.C. (1979) J. Biol. Chem. 254:10001-10005. Studies on Human Liver α -Galactosidases. II. Purification and Enzymatic Properties of α -Galactosidase B (α -N-Acetyl-galactosaminidase).

- 31 - Dey, P.M. (1978) Adv. Carb. Chem. Biochem. 35:341-376.
Biochemistry of Plant Galactomannans.
- 32 - Dey, P.M. (1979) Phytochem. 18:35-38. Transgalactosylation
Activity of Sweet Almond α -Galactosidase: Synthesis
of Saccharides.
- 33 - Dey, P.M. (1980) Adv. Carb. Chem. Biochem. 37:283-372.
Biochemistry of α -D-Galactosidic Linkages in the
Plant Kingdom.
- 34 - Dey, P.M. and Kauss, H. (1981) Phytochem. 20:45-48.
 α -Galactosidase of *Poterioochromonas malhamensis*.
- 35 - Dey, P.M. and Malhotra, Q.P. (1969) B.B.A. 185:402-408.
Kinetic Behaviour of Sweet Almond α -Galactosidase.
- 36 - Dey, P.M. and Pridham, J.B. (1968) Phytochem. 7:1737-
1739. Multiple Forms of α -Galactosidase in *Vicia*
fabae Seeds.
- 37 - Dey, P.M. and Pridham, J.B. (1969) Biochem. J. 115:
47-54. Substrate Specificity and Kinetic Properties
of α -Galactosidases from *Vicia faba*.
- 38 - Dey, P.M. and Pridham, J.B. (1969a) Biochem. J. 113:
49-55. Purification and Properties of α -Galactosidase
from *Vicia faba* Seeds.
- 39 - Dey, P.M. and Pridham, J.B. (1972) Adv. Enzymology 36:
91-130. Biochemistry of α -D-Galactosidases.
- 40 - Dey, P.M., Khaleque, A. and Pridham, J.B. (1971)
Biochem. J. 124:27P. Further Observations on the
 α -Galactosidase Activity of *Vicia faba* Seeds.

- 41 - Dixon, M. and Webb, E.C. (1979) Enzymes. Academic Press, New-York.
- 42 - Dyson, R.D. (1978) Cell Biology. A Molecular Approach. Allyn and Bacon, Inc., Boston.
- 43 - Emmet, J.M. and Walker, D.A. (1969) B.B.A. 180:424-425. Thermal Uncoupling in Chloroplasts.
- 44 - Erickson, R.O. and Michelini, F.J. (1957) Amer. J. Bot. 44:297-305. The Plastochron Index.
- 45 - Fleischmacher, O.L., Prado, F.E. and Sampietro, A.R. (1980) Plant and Cell Physiol. 21:1273-1281. Cell Wall Invertases from Apex and Callus Tissue of Sugar Cane.
- 46 - Florini, J.R. and Vestling, C.S. (1957) B.B.A. 25: 575-578. Graphical Determination of the Dissociation Constants for Two-Substrate Enzyme Systems.
- 47 - French, D. (1954) Adv. Carb. Chem. 9:149-184. The Raffinose Family of Oligosaccharides.
- 48 - Frydman, R.B. and Neufeld, E.F. (1963) Biochem. Biophys. Res. Commun. 12:121-125. Synthesis of Galactosylinositol by Extracts from Peas.
- 49 - Gander, J.E. (1976) "Mono- and Oligosaccharides." in Plant Biochemistry, J. Bonner and J.E. Varner (eds.), Academic Press, New-York, pages 337-379.

- 50 - Gatt, S. and Baker, E.A. (1969) B.B.A. 206:125-135.
Purification and Separation of α - and
 β -Galactosidases from Spinach Leaves.
- 51 - Gayler, K.R. and K.T. Glasziou (1972) Physiol. Plant.
27:25-31. Physiological Functions of Acid and
Neutral Invertases in Growth and Sugar Storage
in Sugar Cane.
- 52 - Ghebregzabher, M., Rufini, S., Monaldi, B. and Lato, M.
(1976) J. Chromatogr. 127:133-162. Thin-layer
Chromatography of Carbohydrates.
- 53 - Ghebregzabher, M., Rufini, S., Sapia, G.M. and Lato, M.
(1979) J. Chromatogr. 180:1-16. Improved Thin-
layer Chromatographic Method for Sugar Separations.
- 54 - Gitzelman, R. and Auricchio, S. (1965) Pediatrics 36:
231-236. The Handling of Soya Alpha-Galactosides
by a Normal and a Galactosemic Child.
- 55 - Glaser, L. (1966) Comprehensive Biochem. 15:112-137.
Glycosyl-Transfer Reactions. M. Florkin and E.H.
Stotz, eds., Elsevier, London.
- 56 - Glasziou, K.T. and Gayler, K.R. (1972) Bot. Rev. 38:
471-490. Storage of Sugars in Stalks of Sugar
Cane.
- 57 - Glusker, J. (1980) Acc. Chem. Res. 13:345-352. Citrate
Conformation and Chelation: Enzymatic Implications.

- 58 - Gross, K.C. and Pharr, D.M. (1981) Plant Sci. Letters, in press. A Potential Pathway for Galactose Metabolism in *Cucumis sativus* L., a Stachyose Transporting Species.
- 59 - Gross, K.C., Locy, R.D. and Pharr, D.M. (1979) Plant Physiol. 63:suppl. 108. Galactose Interconversions Catalyzed by Cucumber (*Cucumis Sativus* L.) Peduncle Extracts.
- 60 - Gross, K.C., Pharr, D.M. and Locy, R.D. (1981) Plant Sci. Letters 20:333-341. Growth of Callus Initiated from Cucumber Hypocotyls on Galactose and Galactose-containing Oligosaccharides.
- 61 - Hakomori, S., Siddiqui, B., Li, Y.T., Li, S.C. and Hellerquist, C.G. (1971) J. Biol. Chem. 246:2271-2277. Anomeric Structures of Globoside and Ceramide Trihexoside of Human Erythrocytes and Hamster Fibroblasts.
- 62 - Hawker, J.S. and Hatch, M.D. (1965) Physiol. Plant. 18:444-453. Mechanism of Sugar Storage by Mature Stem of Sugar Cane.
- 63 - Hendrix, J.E. (1968) Plant Physiol. 43:1631-1636. Labeling Pattern of Translocated Stachyose in Squash.
- 64 - Hérissé, H., Fleury, P., Wickstrom, A., Courtois, J.E. and Le Dizet, P. (1954) Bull. Soc. Chim. Biol. 36: 1507-1518. Isolement de Cinq Galactosides du Saccharose des Racines de Bouillon Blanc.

- 65 - Imhoff, V. (1973) Hoppe-Seyler's Physiol. Chem. 354: 1550-1554. Synthesis of Galactosides by Chloroplasts Isolated from Pea Leaves.
- 66 - Itoh, T., Shimura, S. and Adachi, S. (1979) Agric. Biol. Chem. 43:1499-1504. α -Galactosidase from Alfalfa Seeds (*Medicago sativa* L.).
- 67 - I.U.P.A.C. (1968) Eur. J. Biochem. 5:1-12. The Nomenclature of Cyclitols. Tentative Rules.
- 68 - Jeffrey, D.C., Arditti, J. and Ernst, R. (1969) J. Chromatogr. 41:475-480. Determination of Di-, Tri- and Tetrasaccharides in Mixtures with their Component Moieties by Thin Layer Chromatography.
- 69 - Jeffs, R.A. and Northcote, D.H. (1966) Biochem. J. 101:146-152. Experimental Induction of Vascular Tissue in an Undifferentiated Plant Callus.
- 70 - Jeffs, R.A. and Northcote, D.H. (1967) J. Cell Sci. 2:77-88. The Influence of Indol-3-yl Acetic Acid and Sugar on the Pattern of Induced Differentiation in Plant Tissue Culture.
- 71 - Jeremias, D. (1962) Ber. Deut. Bot. Ges. 75:313-322. Der Einfluss der Temperatur auf die Speicherung der Raffinose.
- 72 - Johnson, D.L. and Desnick, R.J. (1978) B.B.A. 538: 195-204. Molecular Pathology of Fabry's Disease. Physical and Kinetic Properties of α -Galactosidase A in Cultured Human Endothelial Cells.

- 73 - Kabat, E.A., MacDonald, D.L., Ballon, C.E. and Fischer, H.O.L. (1953) J. Am. Chem. Soc. 75:4507-4509. On the Structure of Galactinol.
- 74 - Kandler, O. (1967) "Biosynthesis of Poly- and Oligosaccharides During Photosynthesis in Green Plants." in Harvesting the Sun, A. San Pietro, F.A. Greer and T.J. Army (eds.), Academic Press, New-York, pp. 131-152.
- 75 - Kandler, O. and Hopf, H. (1980) "Occurrence, Metabolism and Function of Oligosaccharides." in The Biochemistry of Plants, Vol. 3, J. Preiss (ed.), Academic Press, New-York, pp. 221-270.
- 76 - Kandler, O., Dover, C. and Ziegler, P. (1979) Ber. Deut. Bot. Ges. 92:225-241. Kalteresistenz der Fichte. I. Steuerung von Kalteresistenz, Kohlehydrat und Proteinstoffwechsel durch Photoperiode und Temperatur.
- 77 - Kelemen, M.V. and Whelan, W.J. (1966) Arch. Biochem. Biophys. 117:423-428. Inhibition of Glucosidases and Galactosidases by Polyols.
- 78 - King, R.W. and Zeevaart, J.A.D. (1974) Plant Physiol. 53:96-103. Enhancement of Phloem Exudation from Cut Petioles by Chelating Agents.
- 79 - Konigsberg, W. (1972) Meth. Enzymology XXV Part B:185-188. Reduction of Disulfide Bonds in Proteins with Dithiothreitol.

- 80 - Koshland, D.E.Jr (1953) Biol. Rev. 28:416-436.
Stereochemistry and the Mechanism of Enzymatic Reactions.
- 81 - Koshland, D.E.Jr and Stein, S.S. (1954) J. Biol. Chem. 208:139-148. Correlation of Bond Breaking with Enzyme Specificity. Cleavage Point of Invertase.
- 82 - Kreuzer, H.P. and Kauss, H. (1980) Planta 147:435-438. Role of α -Galactosidase in Osmotic Regulation of *Poteroiochromonas malhamensis*.
- 83 - Lato, M., Brunelli, B., Ciuffini, G. and Mezzetti, T. (1968) J. Chromatogr. 36:191-197. Analysis of Carbohydrates in Biological Fluids by Means of Thin Layer Chromatography.
- 84 - Lato, M., Brunelli, B., Ciuffini, G. and Mezzetti, T. (1969) J. Chromatogr. 39:407-417. Thin Layer Chromatography of Carbohydrates on Silica Gel Impregnated with Sodium Acetate, Monosodium Phosphate and Disodium Phosphate.
- 85 - Lazo, P.S., Ochoa, A.G. and Gascon, S. (1977) Eur. J. Biochem. 77:375-382. α -Galactosidase from *Saccharomyces carlsbergensis*. Cellular Localization and Purification of the External Enzyme.
- 86 - Lazo, P.S., Ochoa, A.G. and Gascon, S. (1978) Arch. Biochem. Biophys. 191:316-324. α -Galactosidase (Melibiase) from *Saccharomyces carlsbergensis*: Structural and Kinetic Properties.

- 87 - Lee, C.Y. and Shallenberger, R.S. (1969) *Experientia* 25: 692-693. Changes in Free Sugar During the Germination of Pea Seeds.
- 88 - Lehle, L. and Tanner, W. (1972) *Meth. Enzymology* 8:522-530. Synthesis of Raffinose-Type Sugars.
- 89 - Lehle, L. and Tanner, W. (1973) *Eur. J. Biochem.* 38: 103-110. The Function of myo-Inositol in the Biosynthesis of Raffinose. Purification and Characterization of Galactinol:Sucrose 6-Galactosyltransferase from *Vicia faba* Seeds.
- 90 - Lehle, L. , Tanner, W. and Kandler, O. (1970) *Hoppe-Seyler's Z. Physiol. Chem.* 351:1494-1498. myo-Inositol, a Cofactor in the Biosynthesis of Raffinose.
- 91 - Lehninger, A. (1970) *Biochemistry*. Worth Publ., New-York.
- 92 - Leung, D.W.M. and Bewley, J.D. (1981) *Nature* 289:587-588. Immediate Phytochrome Action in Inducing α -Galactosidase in Lettuce Seeds.
- 93 - Li, Y.-T. and Shetlar, M.R. (1964) *Arch. Biochem. Biophys.* 108:301-313. Galactosyl Transfer Reactions Catalyzed by Pneumococcal α -Galactosidase.
- 94 - Li, Y.-T. and Shetlar, M.R. (1964a) *Arch. Biochem. Biophys.* 108:523-529. Occurrence of α -Galactosidase in Higher Fungi: Isolation of α -Galactosidase from *Calvatia cyathiformis*.

- 95 - Li, Y.-Y., Li, S.-C.C. and Shetlar, M.R. (1963) Arch. Biochem. Biophys. 103:436-442. α -Galactosidase from *Diplococcus pneumoniae*.
- 96 - Loiseau, D. (1876) Ber. der Deut. Chem. Ges. 9:732.
- 97 - Loomis, W.D. and Battaile, J. (1966) Phytochem. 5: 423-438. Plant Phenolic Compounds and the Isolation of Plant Enzymes.
- 98 - Lysis, A.J. and Paigen, K. (1976) B.B.A. 437:487-497. Properties of Mouse α -Galactosidase.
- 99 - Malhotra, O.P. and Dey, P.M. (1967) Biochem. J. 103: 508-513. Purification and Physical Properties of Sweet Almond α -Galactosidase.
- 100 - Malhotra, O.P. and Dey, P.M. (1967a) Biochem. J. 103: 739-743. Specificity of Sweet Almond α -Galactosidase.
- 101 - Marbach, I., Mayer, A.M. and Maron, R. (1978) Phytochem. 17:655-657. Galactosidases in Cultivated and Wild Peas.
- 102 - Maretzki, A. and Thom, M. (1978) Plant Physiol. 61: 544-548. Characteristics of a Galactose-adapted Sugarcane Cell Line Grown in Suspension Culture.
- 103 - Matheson, N.K. and Saini, H.S. (1977) Phytochem. 16: 59-66. Polysaccharide and Oligosaccharide Changes in Germinating Lupin Cotyledons.
- 104 - Maurer, H.R. (1971) Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis. Walter de Gruyter, Berlin.

- 105 - McCleary, B.V. and Matheson, N.K. (1974) *Phytochem.* 13: 1747-1757. α -D-Galactosidase Activity and Galactomannan and Galactosylsucrose Oligosaccharide Depletion in Germinating Legumes Seeds.
- 106 - McIlvaine, T.C. (1921) *J. Biol. Chem.* 49:183-186.
A Buffer Solution for Colorimetric Comparison.
- 107 - Mezzetti, T., Lato, M., Rufini, S. and Ciuffini, G. (1971) *J. Chromatogr.* 63:329-342. Thin Layer Chromatography of Oligosaccharides with Tungstic or Molybdic Acid as Impregnent.
- 108 - Michal, G. (1978) "Determination of Michaelis Constants and Inhibitor Constants." in *Principles of Enzymatic Analysis*, H.U. Bergmeyer (ed.), Verlag Chemie, Weinheim, pp. 30-40.
- 109 - Moreno, A. and Cardini, C.E. (1966) *Plant Physiol.* 41: 909-910. A Raffinose-Sucrose Transgalactosidase from Wheat Germ.
- 110 - Murakami, S. (1941) *Acta Phytochimica* 12:97-114.
Untersuchungen über Kohlehydrate von den Labiaten. II. Mitteilung. Über ein Neues Hexasaccharid "Ajugose" aus den Wurzeln von *Ajuga nipponensis* Makino.
- 111 - Neely, R.S. and Beevers, L. (1980) *J. Exptl Bot.* 31: 299-312. Glycosidases from Cotyledons of *Pisum sativum* L.

- 112 - Neish, A.C. (1968) "Monomeric Intermediates in the Biosynthesis of Lignin" in Constitution and Biosynthesis of Lignin, K. Freudenberg and A.C. Neish (eds.), Springer-Verlag, Berlin, p. 14.
- 113 - Neuberg, C. (1907) Biochem. Z. 3:519-534. Zur Kenntnis der Raffinose. Abbau der Raffinose zu Rohrzucker und D-Galactose.
- 114 - Pernas, R.V., Martinez, I.R., Lopez, M.R. and Rama, M.F. (1981) Comp. Biochem. Physiol. 68B:141-145. Purification and Properties of Two Isozymes of α -D-Galactosidase from Lysosomes of *Mytilus edulis* L. Hepatopancreas.
- 115 - Petek, F., Villaroya, E. and Courtois, J.E. (1969) Eur. J. Biochem. 8:395-402. Purification et Propriétés de l' α -Galactosidase des Graines Germées de *Vicia sativa*.
- 116 - Pierpont, W.S. (1969) Biochem. J. 112:619-629. O-Quinones Formed in Plant Extracts. Their Reactions with Bovine Serum Albumin.
- 117 - Pontis, H.G. (1977) "Riddle of Sucrose" in Plant Biochemistry II, Vol. 13, D.H. Northcote (ed.), University Park Press, Baltimore. pp. 79-117.
- 118 - Preiss, J. and Kosuge, T. (1976) "Regulation of Enzyme Activity in Metabolic Pathways." in Plant Biochemistry, J. Bonner and J.E. Varner (eds.), Academic Press, New-York. pp. 277-336.

- 119 - Pridham, J.B. and Hassid, W.Z. (1965) Plant Physiol. 40:984-986. Biosynthesis of Raffinose.
- 120 - Pridham, J.B. and Day, P.M. (1974) Plant Carb. Biochem. 10:83-96. The Nature and Function of Higher Plant α -Galactosidases.
- 121 - Pridham, J.B., Walter, M.W. and Worth, H.G.J. (1969) J. Exptl Bot. 20:317-324. The Metabolism of Raffinose and Sucrose in Germinating Broad-Bean (*Vicia faba*) Seeds.
- 122 - Rao, P.U. and Belavady, B. (1978) J. Agric. Food Chem. 26:316-319. Oligosaccharides in Pulses: Varietal Differences and Effects of Cooking and Germination.
- 123 - Rast, D., McInnes, A.G. and Neish, A.C. (1963) Can. J. Bot. 4:1681-1686. Synthesis of Raffinose in Spruce Twigs.
- 124 - Reid, J.S.G. (1971) Planta 100:131-142. Reserve Carbohydrate Metabolism in Germinating Seeds of *Trigonella foenum-graecum* L. (Leguminosae).
- 125 - Reid, J.S.G. and Meier, H. (1973) Planta 112:301-308. Enzymic Activities and Galactomannan Mobilization in Germinating Seeds of Fenugreek (*Trigonella foenum-graecum* L. Leguminosae). Secretion of α -Galactosidase and β -Mannosidase by the Aleurone Layer.

- 126 - Roeser, K.R. and Legler, G. (1981) B.B.A. 657:321-323.
Role of Sugar Hydroxyl Groups in Glycoside
Hydrolysis. Cleavage Mechanism of Deoxyglucosides
and Related Substrates by β -Glucosidase A₃ from
Aspergillus wentii.
- 127 - Runyon, R.P. and Heber, A. (1971) Fundamentals of
Behavioral Statistics. Addison-Wesley Publishers,
Reading, MA, U.S.A.
- 128 - Santarius, K.A. (1973) Planta 113:105-114. The Protective
Effect of Sugars on Chloroplast Membranes During
Temperature and Water Stress and its Relationship
to Frost, Desiccation and Heat Resistance.
- 129 - Santarius, K.A. and Milde, H. (1977) Planta 136:163-
166. Sugar Compartmentation in Frost-hardy and
Partially Dehardened Cabbage Leaf Cells.
- 130 - Sastry, P.S. and Kates, M. (1964) Biochem. 3:1271-
1280. Lipid Components of Leaves. V - Galactolipids,
Cerebrosides and Lecithin of Runner-Bean Leaves.
- 131 - Seiler, A. (1977) Planta 134:209-221. Galaktomannanabau
in Keimeden Johannisbrotsamen (*Ceratonia siliqua* L.).
- 132 - Senser, M. and Kandler, O. (1967) Z. Pflanzenphysiol.
57:376-388. Galactinol, ein Galactosyldonor für
die Biosynthese der Zucker der Raffinosefamilie
in Blättern.

- 133 - Senser, M. and Kandler, O. (1967a) Phytochem. 6:1533-1540. Vorkommen and Verbreitung von Galactinol in Blättern Hoherer Pflanzen.
- 134 - Shadaksharaswamy, M. and Ramachandra, G. (1968) Phytochem. 7:715-719. Changes in the Oligosaccharides and the α -Galactosidase Content of Coffee Seeds During Soaking and Germination.
- 135 - Shallenberger, R.S. and Moyer, J.C. (1961) J. Agric. Food Chem. 9:137-140. Relation between Changes in Glucose, Fructose, Galactose, Sucrose and Stachyose and the Formation of Starch in Peas.
- 136 - Sharma, C.B. (1971) Biochem. Biophys. Res. Commun. 43: 572-579. Selective Inhibition of α -Galactosidases by Myo-Inositol.
- 137 - Shiroya, T. (1963) Phytochem. 2:36-46. Metabolism of Raffinose in Cotton Seeds.
- 138 - Simpson, R.B. (1961) J. Amer. Chem. Soc. 83:4711-4717. Association Constants of Methyl-Mercury with Sulfhydryl and Other Bases.
- 139 - Sioufi, A., Percheron, F. and Courtois, J.E. (1970) Phytochem. 9:991-999. Nucleosides-diphosphate-oses et métabolisme glucidique au cours de la germination chez le fenugrec.
- 140 - Smart, E.L. and Pharr, D.M. (1980) Plant Physiol. 66: 731-734. Characterization of α -Galactosidase from Cucumber Leaves.

- 141 - Steggerda, F.R. (1968) Ann. N.Y. Acad. Sci. 150:57-66.
Gastrointestinal Gas Following Food Consumption.
- 142 - Suzuki, H., Li, S.-C. and Li, Y.-T. (1970) J. Biol.
Chem. 245:781-786. α -Galactosidase from *Mortierella*
vinacea.
- 143 - Taeufel, K., Krause, W.G., Rutloff, H. and Maune, R.
(1967) Z. Gesamte Exp. Med. Einschl. Exp. Chir.
144:54-66. Zur Intestinalen Spaltung von
Oligosacchariden.
- 144 - Tanner, W. and Kandler, O. (1966) Plant Physiol. 41:
1540-1542. Biosynthesis of Stachyose in *Phaseolus*
vulgaris.
- 145 - Tanner, W. and Kandler, O. (1968) Eur. J. Biochem. 4:
233-239. Myo-Inositol, a Cofactor in the
Biosynthesis of Stachyose.
- 146 - Tanner, W., Lehle, L. and Kandler, O. (1967) Biochem.
Biophys. Res. Commun. 29:166-171. Myo-Inositol, a
Cofactor in the Biosynthesis of Verbascose.
- 147 - Tanner, W., Seifarth, H. and Kandler, O. (1968)
Z. Pflanzenphysiol. 58:369-377. Der Umsatz der
Oligosaccharide in Reifenden und Keimenden Samen
von *Phaseolus vulgaris*.
- 148 - Thomas, B. and Webb, J.A. (1977) Phytochem. 16:203-
206. Multiple Forms of α -Galactosidase in Mature
Leaves of *Cucurbita pepo*.

- 149 - Thomas, B. and Webb, J.A. (1978) Plant Physiol. 62:
713-717. Distribution of α -Galactosidase in
Cucurbita pepo.
- 150 - Thomas, B. and Webb, J.A. (1979) Can. J. Bot. 57:
1904-1911. Intracellular Distribution of
 α -Galactosidase in Leaves of Cucurbita pepo.
- 151 - Trevelyan, W.E., Procter, D.P. and Harrison, J.S.
(1950) Nature 166:444-445. Detection of Sugars
on Paper Chromatograms.
- 152 - Turgeon, R. and Webb, J.A. (1973) Planta 113:179-191.
Leaf Development and Phloem Transport in Cucurbita
pepo: Transition from Import to Export.
- 153 - Turgeon, R. and Webb, J.A. (1975) Planta 123:53-62.
Leaf Development and Phloem Transport in Cucurbita
pepo: Carbon Economy.
- 154 - Ueno, Y., Ikami, T., Yamauchi, R. and Kato, K. (1980)
Agric. Biol. Chem. 44:2623-2629. Purification and
Some Properties of α -Galactosidase from Tubers of
Stachys affinis.
- 155 - van Sumere, C.F., Albrecht, J., Dedonder, A., de Pooter,
H. and Pé, I. (1975) "Plant Proteins and Phenolics."
in The Chemistry and Biochemistry of Plant Proteins,
J.B. Harborne and C.F. van Sumere (eds.), Academic
Press, London, pp. 211-264.

- 156 - Vattuone, M.A., Prado, F.E. and Sampietro, A.R. (1981) Phytochem. 20:189-191. Cell Wall Invertases from Sugar Cane.
- 157 - von Planta, A. and Schulze, E. (1890) Ber. Deut. Chem. Ges. 23:1692-1699. Ueber ein Neueskrystallisirbares Kohlenhydrat.
- 158 - Warburg, O. and Christian, W. (1941) Biochem. Z. 310: 384-421. Isolierung und Kristallisation des Garungsferments Enolase.
- 159 - Webb, J.A. (1970) Can. J. Bot. 48:935-942. The Translocation of Sugars in Cucurbita melopepo. V - The Effect of Leaf Blade Temperature on Assimilation and Transport.
- 160 - Webb, J.A. (1971) Can. J. Bot. 49:717-733. Translocation of Sugars in Cucurbita melopepo. VI - The Reversible Low Temperature Inhibition of ^{14}C -Movement and Cold Acclimatation of Phloem Tissue.
- 161 - Webb, J.A. and Gorham, P.R. (1964) Plant Physiol. 39: 663-672. Translocation of Photosynthetically Assimilated ^{14}C in Straight-Necked Squash.
- 162 - Webb, J.A. and Gorham, P.R. (1965) Can. J. Bot. 43: 97-103. Radial Movement of ^{14}C -Translocates from Squash Phloem.

- 163 - Webb, J.A. and Pathak, S. (1970) Plant Physiol. 46:
Suppl. 27. Biosynthesis of Stachyose in Cucurbita.
- 164 - Webb, K.L. and Burley, J.W.A. (1964) Plant Physiol.
39:973-977. Stachyose Translocation in Plants.
- 165 - Williams, J.P. (1953) Biol. Rev. 28:381-432. Metal
Ions in Biological Systems.
- 166 - Williams, J., Villaroya, H. and Petek, F. (1977)
Biochem. J. 161:509-515. Purification and
Properties of an α -Galactoside Galactohydrolase
from the Seeds of Trifolium repens (White Clover).
- 167 - Williams, J., Villaroya, H. and Petek, F. (1978)
Biochem. J. 175:1069-1077. α -Galactosidases II,
III and IV from Seeds of Trifolium repens.
- 168 - Wolfrom, M.L. (1963) J. Org. Chem. 28:281-291.
Rules of Carbohydrate Nomenclature.
- 169 - Yamane, T. (1971) Sucr. Belge 90:345-348. The
Decomposition of Raffinose by α -Galactosidase.
- 170 - Yamasaki, Y. and Suzuki, Y. (1980) Planta 148:354-361.
Two Forms of α -Glucosidase from Sugar Beet Seeds.
- 171 - Yamasaki, T. and Suzuki, Y. (1980a) Agric. Biol. Chem.
44:707-715. Two Forms of α -Glucosidase from Rice
Seeds at the Milky Stage.
- 172 - Zweig, G. and Sherma, J. (1972) Editors of Handbook
of Chromatography, Vol. II, CRC Press, Cleveland,
pp. 124-130.

ADDENDUM

Figure 42 - Relationship between α -galactosidase activity and developmental stages of Cucurbita pepo leaves. Acid α -galactosidase (closed circles) and alkaline α -galactosidase (open circles) specific activities are plotted against fresh weight of individual leaves.

END

10	5	10	9	8	4
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FIN