Soluble Carbohydrates in Legumes and Nodulated Nonlegumes

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The soluble carbohydrates in leguminous and nodulated nonleguminous plants were compared to determine if there were differences or similarities that might relate to the ability of microsymbionts to fix nitrogen. Gas–liquid chromatography of the trimethylsilyl derivatives was used to quantitate the 80% ethanol soluble carbohydrates and the identities of component peaks were verified by mass spectrometry. The sugars glucose, fructose, and sucrose and the cyclitols *myo*-inositol and *chiro*-inositol were present in varying quantities in all tissues of all species. Trehalose was present only in nodules of all species. Pinitol (1D-3-*O*-methyl-*chiro*-inositol) was a major component in all legumes but was not detected in the nodulated nonlegumes. (–)-Viburnitol (1L-1,2,4/3,5-cyclohexanepentol) was a major component in the nodulated nonlegumes but was not detected in the legumes.

Symbiotic nitrogen fixation in root nodules is dependent on a constant supply of energy from the photosynthetic portion of the plant (Bach et al., 1958). It has been estimated that between 4 and 10 g of carbon is needed to fix 1 g of nitrogen (Tjepkema and Winship, 1980). The exact nature of the organic compounds that serve as in vivo energy sources for nitrogenase activity has not been fully elucidated, but carbohydrates have been considered important for many years (Allison, 1935). The importance of the sugars, sucrose, glucose, and fructose in plant metabolism has long been recognized (Bach et al., 1958). More recently, high concentrations of cyclitols have been found in some plants, particularly legumes (Smith and Phillips 1980; Phillips et al., 1982). While the soluble carbohydrate content of plant tissues varies considerably within and among species and is influenced by age, maturity, and environmental conditions, the cyclitols, primarily pinitol, are important in all legumes (Phillips et al., 1982; Smith and Phillips, 1982). The presence of pinitol in legumes and its absence in grasses is the basis of a procedure to determine the composition of grass–legume mixtures (Smith, 1982). Although the significance of cyclitols in metabolic activities is not well understood (Anderson and Wolter, 1966), there is evidence that they may play a role in nitrogen fixation (Streeter and Bosler, 1976; Streeter, 1980).

Microsymbionts in nonleguminous nodulated plants require approximately the same energy level to fix nitrogen as those in legumes (Tjepkema and Winship, 1980). The source of this energy in nonlegumes has not been investigated extensively. The objectives of the study reported here were to (1) analyze various tissues of different species of legumes and nodulated nonlegumes for their soluble carbohydrate content and (2) ascertain discernible differences or similarities in soluble carbohydrate profiles among the species.

MATERIALS AND METHODS

The following species were included in the study: legumes—white clover (*Trifolium repens* L.), soybean [*Glycine max* (L.) Merr.], kudzu (*Pueraria thunbergiana* L.), silk tree (*Albizia julibrissin* L.)—and nonlegumes—European alder [*Alnus glutinosa* (L.) Gaertn.], and Russian olive (*Elaeagnus angustifolia* L.). Leaf, root, and nodule tissues from all species were collected from field-grown plants. The tissue was excised and freeze-dried immediately or frozen and then freeze-dried. The dried tissue was ground with a Wiley mill (A. H. Thomas Co., Philadelphia, PA), Wig-L-Bug grinder (Crescent dental Co., Lyons, Ill.), or mortar and pestle, depending on the kind and quantity of tissue being ground.

Soluble carbohydrates were extracted from the tissues with 80% ethanol and then lyophilized. Their trimethylsilyl ether (Me_3Si) derivatives were analyzed by gas–liquid chromatography as previously described (Phillips and Smith, 1973). Methyl nonadecanoate was used as the internal standard.

Identification of peaks was established by cochromatography with reagent-grade sugars and the cyclitols quebrachitol (L-2-*O*-methyl-*chiro*-inositol) and *myo*-inositol, which were purchased. Pinitol (1D-3-*O*-methyl-*chiro*-inositol) was extracted from soybean plants (Phillips et al., 1982); *chiro*-inositol (1D-*chiro*-inositol) was prepared by demethylation of pinitol with boiling hydroiodic acid; (–)-viburnitol (1L-1,2,4/3,5-cyclohexanepentol), (+)-quercitol (1L-1,3,4/2,5-cyclohexanepentol), and sequoyitol (5-*O*-methyl-*myo*-inositol) were supplied by Dr. Laurens Anderson (University of Wisconsin—Madison). The identities of the peaks were confirmed by the mass spectra of the Me_3Si derivatives by procedures described previously (Phillips and Smith, 1974; Smith and Phillips, 1981).

RESULTS AND DISCUSSION

The soluble carbohydrates found in the various tissues of the several species are shown in Table I. The major components of soybean and all cyclitols in Russian olive and alder were confirmed by gas–liquid chromatography–mass spectrometry in addition to cochromatography. The components of the other species in Table I were identified by cochromatography with known materials.

Because we are unaware of a published mass spectrum for (–)-viburnitol, the spectrum of the Me_3Si derivative is shown in Figure 1. The spectra of viburnitol from alder and Russian olive and from authenitic (–)-viburnitol were identical. The spectrum is unusual for a silylated carbohydrate in that the molecular ion (m/e 524) is prominent while the M – 15 ion is absent (Pierce, 1968). As would be expected, the M – 90 (m/e 434), M – 105 (m/e 419), and M – 180 (m/e 344) ions are abundant. The other abundant ions are common to the persilylated inositols (Sherman et al., 1970) except for m/e 331 and m/e 230, which are apparently analogous to m/e 419 and m/e 318, respectively, but contain carbon no. 6, which has no O–$Si(CH_3)_3$ group. In a preliminary report (Wilson et al., 1976), we incorrectly identified this component by cochromatography as quebrachitol.

The data indicate that, in general, the concentration of total soluble carbohydrates in all species was higher in

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Table I

plant	tissue	soluble carbohydrate concn, mg/g of dry wt							
		sugars[a]				cyclitols[a]			
		F	G	S	T	C	M	P	V
soybean	leaf	+	1.4	9.8	-	+	0.7	16.7	-
	root	2.1	2.2	9.5	-	+	0.2	4.4	-
	nodule	+	0.7	17.2	10.1	3.3	3.2	1.5	-
white clover	leaf	+	2.6	+	-	0.6	1.5	6.9	-
	root	+	7.4	9.1	-	0.8	0.6	3.4	-
	nodule	+	3.4	3.1	5.0	6.4	1.2	12.9	-
kudzu	leaf	+	4.5	25.9	-	1.5	1.0	49.5	-
	root	0.3	1.3	17.7	-	+	0.2	7.3	-
	nodule	+	1.2	4.6	0.8	0.4	0.6	1.4	-
silk tree	leaf	1.2	1.6	21.8	-	1.7	0.9	17.5	-
	root	+	+	19.0	-	+	0.1	6.6	-
	nodule	+	0.8	10.5	2.4	0.4	+	2.7	-
Russian olive	leaf	5.4	4.5	6.9	-	0.4	0.6	-	4.2
	root	1.9	1.4	6.4	-	0.1	0.2	-	1.5
	nodule	1.5	4.9	5.9	1.9	0.2	0.2	-	+
alder	leaf	4.2	4.2	6.0	-	0.6	3.5	-	6.3
	root	2.0	1.8	9.8	-	0.3	0.3	-	1.0
	nodule	3.8	4.2	8.4	+	+	0.6	-	+

[a] F, fructose; G, glucose; S, sucrose; T, trehalose; C, *chiro*-inositol; M, *myo*-inositol; P, pinitol; V, (-)-viburnitol; (+), less than 0.1 mg/g; (-), not detected.

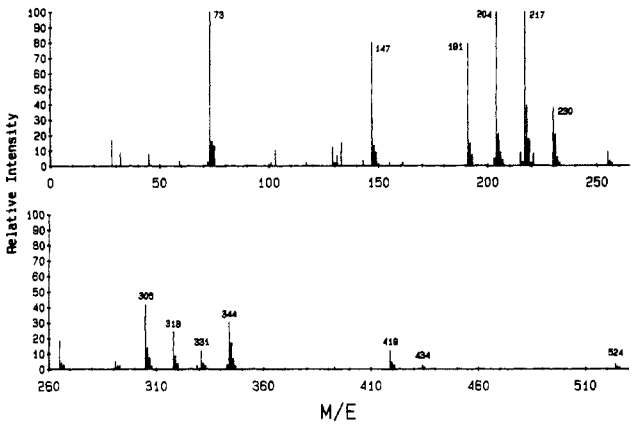

Figure 1. Mass spectrum (70 eV) of the trimethylsilyl ether of (-)-viburnitol (1L-1,2,4/3,5-cyclohexanepentol).

leaves than in roots and nodules. However, the total soluble carbohydrates within a given tissue varied substantially between species. There were also considerable differences in the concentration of individual carbohydrates both within and between tissues and species. Qualitatively the legumes all had identical sugar and cyclitol contents despite the extreme differences in growth habit of the species examined. These results also agree with previous reports of the soluble carbohydrate content of these and other legumes (Phillips et al., 1982; Smith and Phillips, 1980: Streeter, 1980). Plant age, maturity, and environmental conditions have been shown to influence the amounts of various soluble carbohydrates in plant parts (Phillips and Smith, 1974; Streeter, 1981; Smith and Phillips, 1982). Because of the wide range in growth pattern of the species used in this study, tissues from different species varied in physiological age, which may render small differences in carbohydrate concentrations between tissues or species unimportant. However, large differences may be metabolically significant.

Pinitol was present in significant quantities in all the legumes. This agrees with previous reports (Streeter, 1980; Smith and Phillips, 1980). The finding that the nonlegumes (alder and Russian olive) do not contain pinitol suggests that the presence of this cyclitol in legumes is not uniquely related to the ability to establish a symbiotic relationship with nitrogen-fixing organisms since these nonlegumes also have this capacity. However, since the nodulated nonlegumes both contained considerable quantities of the cyclitol (-)-viburnitol, perhaps the presence of a substantial quantity of some cyclitol is necessary for symbiotic nitrogen fixation.

All species examined contained the sugars glucose, fructose, and sucrose in all tissues and trehalose only in the nodules. The nonlegumes had generally somewhat lower concentrations of sucrose and somewhat higher concentrations of glucose and fructose than the legumes. There may be little significance to this distinction because sucrose is readily hydrolyzed to glucose and fructose in most biological systems.

The cyclitols *chiro*-inositol and *myo*-inositol made up rather small percentages of the soluble carbohydrates in all tissues of all species except the nodules of soybean and white clover and the leaves of alder. Sequoyitol (5-*O*-methyl-*myo*-inositol) has been identified in soybean plants but is present in very low quantities (Phillips et al., 1982). The analyses reported here were done before the presence of sequoyitol was confirmed, but no components with retention times similar to sequoyitol were present in more than trace amounts in any species examined.

The soluble carbohydrate profiles of the legumes and nonlegumes analyzed are distinctly different only with respect to pinitol and viburnitol. Pinitol is generally present in legumes and absent in nonlegumes, whereas viburnitol is generally present in the nodulated nonlegumes and absent in legumes. Pinitol is present in many species in several plant families while viburnitol is known to occur in only a few species (Anderson and Wolter, 1966; Angyal and Anderson, 1959; Elsohly et al., 1976). Russian olive and alder belong to plant families not previously known to contain viburnitol.

The legumes used in this study were selected to represent several diverse genera within this large plant family. The two nonlegumes were selected because their abilities to establish symbiotic relationships with nitrogen-fixing organisms are well established (Bond, 1977) and because they were available in the area.

Streeter and Bosler (1976) and Streeter (1980) have correlated pinitol concentration with nitrogen fixation in soybean. Since most legumes contain pinitol and also form symbioses with nitrogen-fixing rhizobia, this correlation may be valid for all legumes. In the two nonlegumes,

viburnitol has replaced pinitol as the major cyclitol, and the microsymbiont involved in nitrogen fixation in both Russian olive and alder is an actinomycete in the genus *Frankia* (Silvester, 1977).

It is possible this difference in cyclitol composition may reflect a basic difference in the metabolic pathways involved in symbiotic nitrogen fixation where *Rhizobium* is the microsymbiont (legumes) as compared to nonlegumes where the microsymbiont *Frankia* sp. is functioning. Evidence for the validity of any such hypothesis will have to await the examination of a larger number of legume and nonlegume species.

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Registry No. Glucose, 50-99-7; fructose, 57-48-7; sucrose, 57-50-1; *myo*-inositol, 87-89-8; *chiro*-inositol, 643-12-9; trehalose, 99-20-7; sinitol, 10284-63-6; (−)-viburnitol, 488-76-6.

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Isolation and Identification of a Branched Quercetin Triglycoside from *Ribes rubrum* (Saxifragaceae)

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Quercetin 3-*O*-(2″-*O*-α-L-rhamnopyranosyl-6″-*O*-α-L-rhamnopyranosyl)-β-D-glucopyranoside was isolated from leaves of red current (*Ribes rubrum*) by means of preparative HPLC. Almost complete identification of the glycoside was carried out with a 2-mg sample after benzoylation of the partially hydrolyzed triglycoside and separation of the benzoates by normal-phase HPLC. Total identification by spectroscopic methods such as UV, IR, ^{1}H NMR, ^{13}C NMR, and FAB-MS verified the results obtained after hydrolysis and determined the branched structure of the saccharide.

In the past the isolation of flavonol glycosides was carried out by column chromatographic and paper chromatographic methods (Mabry et al., 1970; Harborne and Mabry, 1982). Disadvantages of these methods are the loss of time, an often inadequate separation, and therefore an insufficient purity of the isolated glycoside. Preparative high-performance liquid chromatography (HPLC) is a time-saving and more efficient method and is also qualified for the isolation of sensitive substances. The preparative column efficiency is comparable with that of an analytical column, filled with the same stationary phase. Hence, each analytical separation can be transferred to preparative dimension. Only the flow rate must be adapted.

In the past the identification was performed after isolation by means of the UV spectra in methanol and after addition of shift reagents (Markham, 1982). Acid and enzymatic hydrolyses were attached (Mabry et al., 1970; Markham, 1982). The gas chromatograph is used more and more in analyses of the structural members of flavonols (Harborne and Mabry, 1982).

The determination of sugars by HPLC is difficult, because the indication sensitivity is often insufficient when the available quantity is too low. The benzoylation of sugars (Galensa, 1984) and its liquid chromatographic separation are a suitable method. Each derivated hydroxyl group leads to a considerable increase in sensitivity (about a 1000-fold for each hydroxyl group; e.g., sorbitol, with its six hydroxyl groups, gains a 6000-fold increase in indication sensitivity). Also, flavonol glycosides are simultaneously determinable in small amounts (rutin, e.g., has ten hydroxyl groups). The nonpolar derivates may be separated isocratically on normal and reverse phase in the case of sugars and flavonol aglycons. For the separation of mono-, di- and triglycosides on normal phase, a mobile-phase system was developed, which allows the determination of sugars, aglycons, and the above-mentioned glycosides in a period of 20 min (see Materials and Methods). Separation of glycosides on nonpolar reverse phase was possible

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