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Sugar and Organic Acid Constituents in White Clover[1]

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ABSTRACT

Major ethanol-soluble carbohydrate and organic acid constituents of white clover (*Trifolium repens*) have been identified by use of high-performance liquid chromatography and gas chromatography. In leaves, petioles, roots, and nodules, pinitol (3-*O*-methyl *chiro*-inositol) is the predominant sugar, with sucrose present in lower concentration. In leaves and petioles there are significant levels of α- and β-methyl glucosides, linamarin, glucose, and fructose. In the nodules glucose is rarely present at detectable levels. The concentration of pinitol is generally greater than 25 millimolar in each tissue examined whereas the level of sucrose varies depending on the time of day. Sucrose is the major sugar significantly labeled during 1 hour administration of $^{14}CO_2$ and accounts for more than 99% of all the radioactivity detected in the nodules at early times. Between 3 and 7 hours after labeling, 6% of the radioactivity is found in the organic acids fraction and 5% in the basic fraction of nodules. Malonic acid does not appear to be present in unusually high concentrations in either leaves or nodules of white clover.

Considerable effort has been expended to identify the major ethanol-soluble carbohydrates (hereafter referred to as sugars) and organic acid constituents of soybean, an economically important legume (5–7, 13, 14, 17, 19–22). Much less has been done with other legumes (1, 18). We have examined white clover (*Trifolium repens* L.) because it is a convenient test organism for studies of the fast-growing *Rhizobium trifolii*. It has been much easier to induce expression of nitrogenase *ex planta* with the slow-growing brady rhizobia (16) than with the fast growers, and the fast growers may have different requirements for symbiotic induction that can be met only by particular species of legumes. We suspected that clover might contain a specific carbon substrate needed for expression of nitrogenase activity by *R. trifolii*.

Phillips and Smith (17) reported that soybean (*Glycine max* [L.] Merr.) and other legumes contained high levels of a methyl inositol. Streeter (19, 20) showed that this compound was D-pinitol (3-*O*-methyl *chiro*-inositol) and studied its seasonal appearance and disappearance in soybeans. Other sugars present in significant quantities included *myo*-inositol, sucrose, fructose, and glucose. Smith and Phillips (18) reported the presence of 1-*O*-methyl β-D-glucopyranoside in leaves and petioles of white clover. Linamarin, a cyanogenic glucoside, is also present in these tissues (12).

Stumpf and Burris examined the organic acid constituents of soybean and found that malonic acid was a major constituent (21). Lower levels of the Krebs cycle acids also were present and readily detected in leaves and nodules. Malonic acid was rapidly labeled by feeding $^{14}CO_2$ to root nodules (22).

In agreement with Phillips and Smith (17), we have found the methyl inositol, pinitol, to be present at high concentrations in white clover. We also found β-methyl glucose (18) and linamarin (12) in leaves and petioles. We have not found significantly higher levels of malonic acid than other organic acids in white clover nodules or leaves.

MATERIALS AND METHODS

Separation of Neutral Sugars. Separation and identification of aldoses and alditols by HPLC on Dionex DA×8-11 with borate buffer was previously described by Barr and Nordin (2). Replacing the copper-bicinchoninate reagent with periodate allowed detection of all sugars containing vicinal hydroxyl groups. This method has been described in detail (15). The effluent from the postcolumn reactor at 100°C was monitored with a Hitachi model 100-40 variable wavelength HPLC detector at either 270 nm with an alkaline reagent (pH 8.6) for sucrose levels of 50 to 500 nmol or at 260 nm with an acid reagent (pH 5) for sucrose at 5 to 50 nmol. Sensitivity could be increased at least 100-fold by decreasing the reagent concentration and increasing the sensitivity of the detector and recorder (4). Mobilities of sugars are expressed as a percentage of that of glucose which elutes at 87 min in this system. Some R_{Glc} values are: α-methyl-glucoside = 11; β-methyl-glucoside = 12; linamarin = 12; sucrose = 13; pinitol = 30; and fructose = 44.

For sugar analysis, an aliquot of ethanolic extract was brought to dryness under a stream of N_2 at room temperature or 50°C and redissolved in borate buffer (0.5 M, pH 8.6). Very small samples (<10 μl) in ethanol or water could be injected directly but, when injected in water, the peaks of pinitol or *myo*-inositol were sometimes split, suggesting that formation of stable borate complexes is a relatively slow process. Ethanol interferes with sucrose determination.

Analysis of Radioactive Samples. For radioactive samples, the outlet line from the detector was shortened to give a 0.5-min delay. Samples (≈0.7 ml, 1 min) were collected using a LKB 17000 Minirac into which RPI minivials were placed. Scintillation fluid (1.3 ml) consisting of 0.4% 2,5-diphenyloxazole in 2 parts toluene and 1 part Triton X-100 was then added. A turbid emulsion was obtained, but absolute counting efficiency was 77% for the acid reagent, and 70% for the alkaline reagent.

Further Characterization of Sugars. Demethylation of authentic pinitol and of a nodule sample was done by treatment with 48% HBr at 4°C for 4 d (8). Under these conditions, demethylation is not complete but other sugars are not destroyed. For acid hydrolysis, 0.5 ml ethanolic extract from the respired nodule fraction (see Table V) was dried under N_2, redissolved in 0.5 ml 2 N TFA, and heated 2 h at 100°C. It was then dried *in vacuo* at 4°C and reconstituted in buffer for analysis.

GC was done using a Hewlett-Packard HP 5880 with a column (2 mm × 2 m) of 3% ECNSS-M on Gas Chrom Q, according to the method of Conrad *et al.* (3), but using 95% ethanol as the sample solvent. Preliminary analyses were done with temperature programming. Quantitation studies were done isothermally at 190°C or 200°C. Variation between integrated peak areas for a mixture of mannitol, galactitol, glucitol, and *myo*-inositol was only

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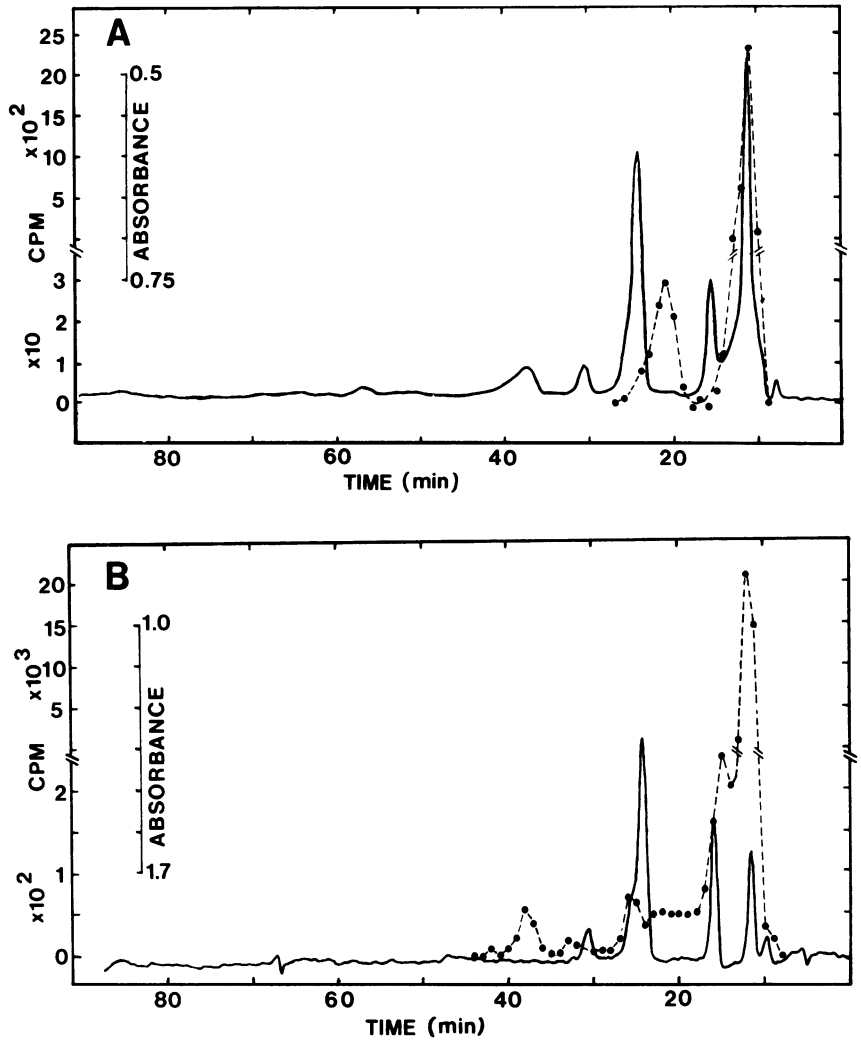

FIG. 1. Profile of sugars (——) and radioactivity (-●-) obtained from clover nodules after incorporation of $^{14}CO_2$ into leaves. A, Fifty μl of the sample taken at 4.25 h was reduced to dryness then dissolved in 100 μl borate buffer and chromatographed. Twenty μl was taken for counting and 50 μl applied to the column. Alkaline periodate reagent was used for high sensitivity to sucrose. Fifteen nmol of sucrose and 19 nmol of pinitol were detected. Note change of scale for radioactivity. Counts (not shown) for points beyond 27 min were not above background. The largest peak eluting at fraction 11 corresponds to sucrose with a R_{Glc} = 13. The peak at fraction 15 is unknown R_{Glc} = 17, the peak at fraction 25 is pinitol, the peak at fraction 30 is unknown R_{Glc} = 34 and the peak at fraction 37 is presumed to be fructose. B, Samples taken at 3, 4.25, 5, 7 h after beginning of a 1-h labeling period were combined and treated by ion exchange chromatography as described by Stumpf and Burris (5) but omitting lipid extraction. The effluent from Dowex 1 was brought to dryness and redissolved in 100 μl borate buffer and chromatographed as described in "Materials and Methods." A 10-μl portion was used for counting and 50 μl was applied to the column (full loop technique). Acidic periodate reagent was used to reduce the sensitivity for sucrose (160 nmol) relative to pinitol (175 nmol). See part A for peak identification.

±2%. A sample of nodule extract spiked with galactitol yielded 85% of the expected recovery of galactitol. A fructose standard gave only one-quarter the alditol response, and browned as did many nodule samples. Sucrose which is not eluted from this column produced no peaks, indicating that it was not hydrolyzed under the conditions of acetylation. Relative retention times for various sugars are: α-methyl glucoside, 0.64; β-methyl glucoside, 0.78; pinitol, 1.0; mannitol, 1.15; galactitol, 1.30; glucose, 1.26 and 1.35; glucitol, 1.5; inositol, 1.8; linamarin, 2.6.

Organic Acid Identification. Organic acids prepared according to Stumpf and Burris (21) were detected at 210 nm with the same HPLC system used above but with a Bio-Rad HP-87H column (7.8 × 300 mm) and were eluted with 0.01 N H_2SO_4. The residue after chromatography and removal of HCOOH (21) was redissolved in water and 50 μl injected into the HPLC system by the full-loop injection technique (11). Quantities were estimated from standards run at comparable concentrations. Changing the concentration of acid or adding sodium sulfate did not improve the resolution of the acids though it changed the migration positions of some of them.

Growth and Labeling of Plants. White clover (*Trifolium repens* L.) plants, inoculated with *R. trifolii* 0403, were grown under fluorescent lights at room temperature in sterilized vermiculite, moistened with a plant growth medium lacking combined nitrogen (23). Samples were taken 1 to 5 months after planting. Incorporation of $^{14}CO_2$ was done using a clover clone selected for having relatively large nodules, weighing several milligrams each. A representative part of this clone (designated 12-6) was transferred to a 0.5-L plastic box 9 d prior to study. Incorporation of $^{14}CO_2$ at 21°C was begun 1.5 h after the lights were turned on and lasted for 1 h. One to three nodules were picked at 1-h intervals and immediately dropped into 300 μl 80% ethanol in small plastic tubes, crushed with a stainless steel rod, centrifuged, and kept in a freezer until analysis.

Table I. *Concentrations of Sucrose and Pinitol in Clover Nodules*

Nodule samples 0 to 5 were collected beginning at 9 AM, 45 min after lights were turned on. Samples 6 and 7 were prepared 6 weeks later using clone 12-6.

Sample No.	Sucrose	Pinitol	Sucrose: Pinitol
	μmol/g fresh wt		*ratio*
0—9 AM	6	27	0.22
1—11 AM	13	42	0.31
2—11 AM	16	50	0.32
3—11 AM	8	31	0.26
4—4 PM	20	47	0.43
5— respired 24 h	1.5	25	0.06
6—8 AM	7.3	22	0.33
7—7 PM	8.8	16	0.55

Larger samples of nodules were picked from several plants at one time, weighed, and immediately put into ~15 volumes of 80 or 95% ethanol. They were then crushed thoroughly, centrifuged, and stored in a freezer until analysis. Respired samples were kept moist on root systems at room temperature. Leaves and petioles were prepared as follows. Ten g tissue was extracted with 50 ml 95% ethanol for 2 h at room temperature and then reextracted with 50 ml boiling 80% ethanol for 10 min. The residue was further rinsed with 12 ml 80% ethanol. Combined extracts were kept at 4°C.

RESULTS

The method of Nordin (15) applied to clover nodules yielded a simple profile, with two major sugars, shown below to be sucrose and pinitol (Fig. 1). Two unknown sugars were present in relatively constant proportion to pinitol. From their response to changing temperature and pH of the periodate reaction, we deduce that they are similar to pinitol in reactivity, and neither one is a reducing sugar (no response to the bicinchoninate reagent). Their mobilities do not correspond to any of the sugars listed by Nordin (15) and they may be other methyl inositols.

Identity of Major Sugars. To show that the fast-moving major peak was sucrose and not trehalose or an unknown reducing sugar which might run at nearly the same rate in HPLC, two experiments were done. First, it was shown by using the bicinchoninate reagent that there was no reducing sugar in the position corresponding to sucrose. Second, treatment of a nodule extract with invertase caused complete loss of sucrose and appearance of equal amounts of both fructose and glucose.

In the HPLC system used here, pinitol is clearly resolved from *chiro*-inositol but unresolved from *myo*-inositol. Demethylation and HPLC analysis of the authentic pinitol sample (19) or nodule extract yielded *chiro*-inositol. GC of acetate derivatives showed clearly that the peak with $R_{Glc} = 30$ (Fig. 1) contained predominantly pinitol. Peak areas for *myo*-inositol and pinitol were in the ratio of about 1:30 (7 samples, range of 1:25–1:35). With leaf material, the ratio was 1:8. Nodules consistently yielded a material which migrated the same as glucitol in GC.

During HPLC of nodule extracts, pinitol and the unknown with $R_{Glc} = 17$ remained in constant proportion from one sample to the next while concentrations of sucrose relative to pinitol varied from one experiment to another and with the time of day, generally increasing during the light period (Table I). When nodules were picked from root systems of small plants that had been used for studies of acetylene reduction for 4 h, the sucrose level was markedly lowered relative to pinitol when compared to freshly picked nodules (data not shown). When nodules attached to portions of root were kept for 24 h, sucrose was depleted to a very low level (Table I).

In the leaves of white clover (Fig. 2), both fructose and glucose were readily detectable and there was an unknown compound similar to galactitol ($R_{Glc} = 80$). GC of the acetylated nodule extract showed that it could not be galactitol. Both the β- and α-methyl glucoside were present in leaf tissue (18) eluting immediately before sucrose in HPLC. GC of the acetates yielded relative peak areas about 20% and 2% that of pinitol which was ~22 mM. Linamarin gave the same peak area as the less abundant α-methyl glucoside. In petioles, β-methyl glucoside was about two-thirds as abundant as pinitol which was ~7 mM. The unknown $R_{Glc} = 17$ (Fig. 1), was present but relatively less in leaves than in nodules. Leaf, petiole, and root tissues yielded the characteristic pair of glucose isomers in GC and no detectable glucitol-like or galactitol derivatives. The peak migrating as glucitol in GC of nodules may be unknown $R_{Glc} = 34$, but it cannot be glucitol on the basis of HPLC chromatography.

Ethanolic extracts of nodules, leaves, and petioles were treated by Dowex ion-exchange chromatography for preparation of organic acids. Portions of the column effluent were analyzed for sugar content to insure that all of the HPLC peaks in the original ethanol extracts corresponded to simple sugars and not to lipid, acidic, or basic derivatives. The HPLC patterns of treated extracts were essentially the same as for the untreated materials. Thus, all the observed periodate reactive peaks correspond to neutral, nonlipid sugars.

Radioactive Labeling of Ethanol-Soluble Materials. Although it is clear that methyl inositols are present in clover, as in other legumes (17), it is presently unknown whether they are significantly metabolized in nodules. We therefore carried out an experiment using $^{14}CO_2$ to determine the relative rate of labeling of various ethanol-soluble materials in the clover plant and to monitor their rate of appearance in nodules. Table II provides a summary of results for concentration of [^{14}C]sucrose in nodules as a function of time. Some radioactivity was found to chromatograph in the general region of pinitol (though not coinciding with any periodate-detectable compound) when ethanolic extracts were used directly without removal of acidic or basic material (Fig. 1A). Therefore, a pooled sample was subjected to clean-up by ion-exchange chromatography as described by Stumpf and Burris (21) but omitting chloroform extraction. The material passing through both Dowex 50 and Dowex 1 was collected, taken to dryness, redissolved in borate and chromatographed. Results are summarized in Table III (see also Fig. 1B). It is clear from Figure 1B that only a very small amount of radioactivity was present in pinitol and the peak of radioactivity observed prior to ion-exchange chromatography represents a nonneutral compound. The increased amount of radioactivity applied in Figure 1B revealed a small shoulder on the sucrose peak which may correspond to material with $R_{Glc} = 17$. In Figure 2, for leaf material, this unknown was significantly labeled.

Radioactively labeled leaf material was analyzed and results for the leaf are shown in Table IV and Figure 2. This fraction was neither lipid-extracted nor fractionated by ion-exchange chromatography, so that there may be an appreciable background of nonneutral ethanol-extractable substances in the chromatogram.

Organic Acid Determination. Identifications of organic acids were made relative to known compounds as described in "Materials and Methods." Table V gives a summary of the estimated concentrations of organic acids in leaves and nodules. There was no marked elevation of malonic acid above that of the other acids of the Krebs cycle.

DISCUSSION

This study was carried out primarily to identify the ethanol-soluble carbohydrates and organic acids present in and rapidly labeled in white clover nodules, so that we might more effectively provide nodule constituents to *R. trifolii ex planta*. Pankhurst (16)

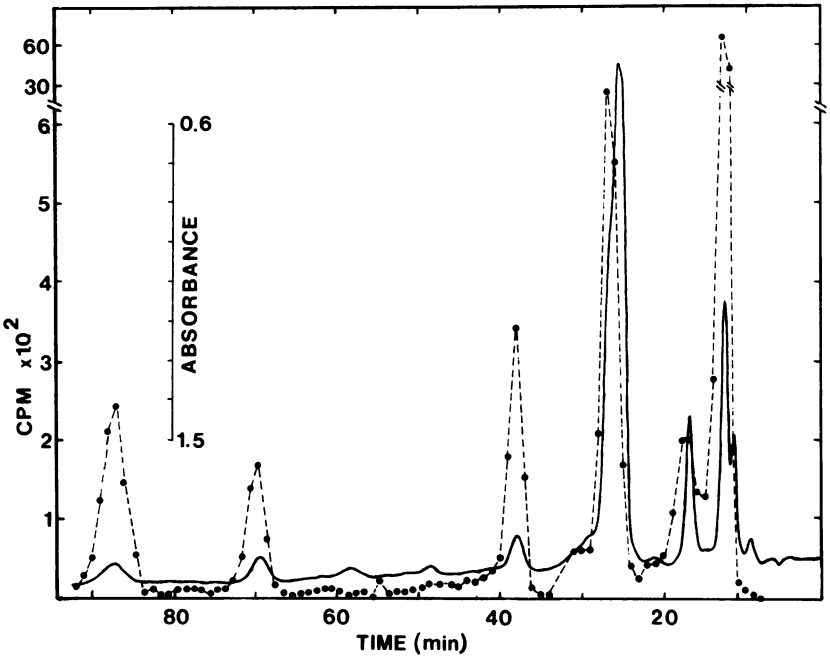

FIG. 2. Chromatographic profile of sugars (——) and radioactivity (-●-) from clover leaf after 1 h incorporation of $^{14}CO_2$. From 2 ml extract, 100 μl was reduced to dryness, dissolved in 100 μl borate buffer, and 50 μl chromatographed. Alkaline periodate was used for detection. Peak identities are as in Figure 1, with unknown R_{Glc} = 80 at fraction 70 and glucose at fraction 87.

Table II. *Specific Activity of Clover Nodule after $^{14}CO_2$ Labeling*

Nodule samples were prepared as described in "Materials and Methods," and analyzed by HPLC.

Time	Sucrose
h	*cpm/nmol*
1	30
2	115
4.25	229
5	298
6	250
7	179
12	120
24	33
Pooled 3, 4, 5, 7	225

Table III. *Radioactivity in Sugar Constituents of Clover Root Nodules*

Data for pooled samples 3, 4, 5, 7 of Table I. See Figure 1 and "Materials and Methods" for details. Concentrations of sugars were determined from standards assuming unknown R_{Glc} = 17 responds the same as inositol and unknown R_{Glc} = 34, the same as fructose. 38,000 cpm were recovered from the columns, 92% of the applied radioactivity. No fractions beyond fructose were collected and its concentration was unmeasurably low.

Compound	Relative Specific Activity	Total	% Total
	cpm/nmol	*cpm*	
Sucrose	225	36,665	>98
Unknown R_{Glc} 17	<20	<20	0.5
Pinitol	1.2	190	0.5
Unknown R_{Glc} 34	5	30	0.1
Fructose		110	0.3

has shown that, for induction of nitrogenase in several (brady) rhizobia, very high concentrations of carbon substrates are needed for optimum expression of activity. It might be speculated that the methyl inositols, which appear to be relatively inert, metabolically, are useful to provide an osmoticum for stabilization of the bacteroid state of the rhizobium symbiont within the plant cell. We have found the level of pinitol to be relatively high throughout several months in the vegetative development of the clover plant. Streeter (20) had previously found high levels of pinitol in soybeans and noted that its concentration was much more constant than that of sucrose.

Labeling with $^{14}CO_2$ showed that sucrose was very quickly labeled in the leaf and quickly appeared in the nodule. It was the only neutral sugar significantly labeled in 1 h and found at reasonable specific activities in the nodule. A portion of the radioactive label was found in acidic and basic compounds after several hours and progressively less of the radioactive label was found in the sucrose. Respiration experiments (nonradioactive) showed that sucrose was rapidly degraded while pinitol appeared relatively stable in the nodules. From the extent of labeling in leaf and nodule, it is possible to estimate that pinitol must be synthesized at a rate at least 20 times slower than that for sucrose and translocated 10 times more slowly, and hence must be metabolized much more slowly during normal conditions.

Kouchi (10) used $^{13}CO_2$ isotopic labeling of soybeans to follow the time course of appearance of several compounds in nodules, after labeling leaves. Both *myo*-inositol and sequoyitol (4-*O*-methyl *myo*-inositol) were significantly labeled in leaves after 3 h exposure. In nodules, pinitol and *myo*-inositol were much less extensively labeled than *chiro*-inositol even after 24 h, and all together they accumulated much less label than sucrose. Antoniw and Sprent (1) examined fractionated *Phaseolus vulgaris* nodules after $^{14}CO_2$ labeling of leaves and found the highest levels of labeling in sucrose. Malic acid was relatively highly labeled and in bacteroids a compound resembling phosphogluconate was labeled. However, the long fractionation times required in their procedure may have allowed conversion of label, or leakage from bacteroids, etc., so that quantitative interpretations are not possible. They found label in arabinose (R_{Glc} = 50 in HPLC) whereas we did not detect either labeled or unlabeled arabinose.

Transport of radioactively labeled sugars has been studied in

Table IV. *Radioactivity in Leaf Sugars of White Clover*

See Figure 2 and "Materials and Methods" for details. Concentrations of sugars were determined relative to standards assuming unknown R_{Glc} = 17 gives the same response as inositol and unknown R_{Glc} = 80 gives the same response as glucose. A total of 15,200 cpm were recovered from the column, 94% of the applied radioactivity.

Compound	Relative Specific Activity	% Total
	cpm/nmol	*cpm*
Linamarin		
Sucrose	750	64
Unknown R_{Glc} 17	<20	<2
Pinitol	<40	11
Fructose	135	5
Unknown 2 R_{Glc} 80	80	3
Glucose	250	5.5

Table V. *Organic Acid Constituents of White Clover Tissues*

All results shown are from single samples prepared as described (18). For nodules, two other samples gave a variability of ±25% in the relative peak heights.

Acid	Leaf	Petiole	Nodule
		μmol/g fresh wt	
Succinate	2.2		2.9
Fumarate	0.1	0.3	0.14
Malate	5.2	12.5	0.19
Malonate	2.8	7.6	1.4
Citrate	4.8	1.0	2.0
Oxaloacetate	1.5	0.8	2.6

soybean where it is proposed that there is active transport of sucrose down the stem (5–7, 13, 14). In the experiments cited, fructose and glucose were not actively transported (5, 14) and sucrose transport was not due to bulk flow with water (TOH) or chloride ion (7). Our results with radioactive labeling would be consistent with this. Although radioactivity was found associated with pinitol (or *myo*-inositol) in the leaf to the extent of 10% of the amount found in sucrose, very little labeled cyclitol was observed in the nodules. In an actively growing plant, there must be synthesis of the cyclitols to the extent of several percent of the pool per day in order to double their mass with that of the whole plant, even if they are relatively inert.

Based on the absolute specific activity of the input CO_2 and container volume, we can estimate the expected specific radioactivity in the leaf during a 1-h labeling period. The observed specific radioactivity was 750 cpm/nmol sucrose (1.35 bq/nmol carbon) while the input CO_2 was 39.4 bq/nmol. With about 15 μmol CO_2 available for incorporation, a leaf mass of 4 g (estimated by sampling the plant) and sucrose concentration of 5 mM (measured), the CO_2 would be diluted ~15-fold if all were incorporated. There are probably two pools of sucrose in the leaf, one for rapid transport and one relatively static (13). The observed specific activity dilution of 29-fold in the leaf is not too far from the expected level, if only part of the input CO_2 was incorporated or if a pool of nonlabeled sucrose was present in the leaf, while newly labeled material was exported to the root system. Based on the rate of increase and decrease in sucrose-specific radioactivity in nodules (Table II), we can estimate its half-life is between 3 and 5 h, consistent with our observations on respired nodules and root systems.

The HPLC method described in this paper allows rapid sample preparation and analysis and successful identification of a large number of sugar constituents of plants in very small samples (<1 mg fresh wt). The use of acetylation and GC permits confirmation of sugar identities with minimal manipulation of the samples. Although a single HPLC column will not resolve all possible sugar isomers, it is more effective than GC for some pairs of interest (*cf.* 10, 19, 20) and provides the simplest possible sample workup. It can be used to reliably detect and quantitate sugars in the micromoles range with little difficulty. We have shown that for white clover, as for soybeans and other legumes, pinitol is a major sugar constituent of several plant parts. Its role in physiology and metabolism during the legume rhizobium symbiosis remains to be established.

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LITERATURE CITED

1. Antoniw LD, JI Sprent 1978 Primary metabolites of *Phaseolus vulgaris* nodules. Phytochemistry 17: 675–678
2. Barr J, P Nordin 1980 Microdetermination of neutral and amino sugars found in glycoproteins. Anal Biochem 108: 313–319
3. Conrad GA, P Ager-Johnson, M-L Woo 1982 Antibodies against the predominant glycosaminoglycan of the mammalian cornea, keratan sulfate I. J Biol Chem 257: 464–471
4. Crouthamel CE, HV Meek, DS Martin, CV Banks 1949 Spectrophotometric studies of dilute aqueous periodate solutions. Am Chem Soc 71: 3031–34
5. Fisher DB 1970 Kinetics of C-14 translocation in soybean I kinetics in the stem. Plant Physiol 45: 107–113
6. Fisher DB 1970 Kinetics of translocation of soybean II kinetics in the leaf. Plant Physiol 45: 114–118
7. Gage RS, S Aranoff 1960 Translocation III experiments with carbon 14, chlorine 36 and hydrogen 3. Plant Physiol 35: 53–64
8. Hough L, RS Theobold 1963 De-O-methylation. Meth Carbohydr Chem II: 203–206
9. Jordan DC 1982 Transfer of *Rhizobium japonicum* (Buchanan 1980) to *Brady rhizobium gen. nov.*, a genus of slow-growing root nodule bacteria from leguminous plants. Int J Sys Bacteriol 32: 136–139
10. Kouchi H 1982 J Chromatogr 14: 305–323
11. Lawrence JL 1981 Organic Trace Analysis by Liquid Chromatography. Academic Press, New York
12. Maher EP, MA Hughes 1971 Isolation of linamarin and lotaustratin from *Trifolium repens*. Phytochemistry 10: 3005–3008
13. Nelson CD, H Claus, DC Mortimer, PR Gorham 1961 Translocation of the products of photosynthesis in soybean. Plant Physiol 36: 581–588
14. Nelson CD, PR Gorham 1957 Translocation of radioactive sugars in the stems of soybean seedlings. Can J Bot 35: 703–713
15. Nordin P 1982 Monitoring of carbohydrates with periodate in effluents from HPLC column. Anal Biochem 131: In press
16. Pankhurst CE 1981 Nutritional requirement for the expression of nitrogenase activity by Rhizobium in agar culture. J Appl Bacteriol 50: 45–54
17. Phillips DV, AE Smith 1974 Soluble carbohydrates in soybean. Can J Bot 52: 2447–2452
18. Smith AE, DV Phillips 1981 Identification of methyl β-D-glucopyranoside in white clover foliage. J Agric Food Chem 29: 850–852
19. Streeter JG 1980 Carbohydrates in soybean nodules. II. Distribution of compounds in seedlings during the onset of nitrogen fixation. Plant Physiol 66: 471–476
20. Streeter JG 1980 Seasonal distribution of carbohydrates in nodules and stem exudate from field-grown soya bean plants. Ann Bot 48: 441–450
21. Stumpf DK, RH Burris 1979 A micromethod for the purification and quantification of organic acids of the tricarboxylic acid cycle in plant tissues. Anal Biochem 95: 311–315
22. Stumpf DK, RH Burris 1981 Biosynthesis of malonate in roots of soybean seedlings. Plant Physiol 68: 992–995
23. Wacek TJ, WJ Brill 1976 Simple rapid assay for screening nitrogen-fixing ability in soybean. Crop Sci 16: 519–523