Some Nonfermentable Free Carbohydrates in the Leaves of Canary Grass (*Phalaris tuberosa*)

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Stachyose, raffinose, sucrose, glucose, and fructose were removed from extracts of *Phalaris* leaves by yeast fermentation. The nonfermentable carbohydrates were isolated and identified in all probability by paper chromatography as follows: galactinol (D-1-*O*-α-D-galactopyranosyl-*myo*-inositol), *myo*-inositol, melibiose, D-*glycero*-D-*manno*-octulose, D-galactose, D-mannitol, D-*manno*-heptulose, D-*altro*-heptulose (sedoheptulose), and L-arabinose. Melibiose was also identified by x-ray powder diffraction, and D-mannitol was unambiguously identified by a series of procedures. In addition, a ketose-reactive material was separated, but not identified.

Changes in carbohydrate composition occur at all stages of plant development and vary according to environment and mineral nutrition. Derwyn *et al.* (1967) studied the interrelation of carbohydrate metabolism, seedling development, and growth rate in *Phalaris*, but did not attempt delineation of individual carbohydrates.

Our investigation of the effects of environment and mineral nutrition on accumulation of some toxic, biogenic amines in a range grass, *Phalaris tuberosa*, required a definition of the pool of free carbohydrates so that the dynamics of the biogenic amines and of the carbohydrates could be followed under varying nutritional and climatic conditions. In the study, emphasis was placed upon the nonfermentable carbohydrates because, although frequently present in low concentrations, they may be important intermediates in photosynthetic pathways, such as the pentose phosphate pathway.

Palatability has also been correlated with total sugar content (Bland and Dent, 1962). In this connection it is of importance to know the carbohydrate content in relation to the preference of grazing animals for *Phalaris*, and to the possible correlation with the level of toxic, biogenic amines.

Qualitative information is also valuable in separating endogenous plant carbohydrates from metabolic products produced by ruminal microflora or silage. Harwood (1954) has pointed out the necessity when conducting silage experiments of distinguishing between mannitol produced by silage and that present in the herbage.

EXPERIMENTAL

The carbohydrates and other methanol soluble compounds were extracted from the dry leaves of field-grown plants of *Phalaris tuberosa* in a Soxhlet with absolute methanol for approximately 8 hr. After the extract was concentrated, the yeast fermentation and chromatographic separation were done as previously described by Rendig *et al.* (1964). Since under some conditions a ketoheptose can be synthesized during yeast fermentation of hexose or ketose monosaccharides (Robison *et al.*, 1938), or a hexitol can be produced by certain yeasts (Onishi and Suzuki, 1968) using similar substrates, we investigated the possibility that metabolites of yeast fermentation might be synthesized and be considered as *Phalaris* carbohydrates. When a substrate containing raffinose, sucrose, glucose, fructose, and mannitol was fermented, only melibiose and mannitol remained. Melibiose is not fermented by baker's yeast, and is the expected product of invertase action on raffinose. In addition, neither *myo*-inositol nor galactose was detected when D-1-*O*-α-D-galactopyranosyl-*myo*-inositol (galactinol) or galactinol plus glucose was fermented.

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RESULTS AND DISCUSSION

Chromatographic evidence indicated that the pool of free sugars before yeast fermentation was composed primarily of stachyose, raffinose, sucrose, glucose, and fructose.

The chromatographic characteristics of the nonfermentable fractions (a–j) are shown in Table I, along with similar data for various authentic carbohydrates. The following data were obtained to substantiate the identity of the carbohydrates in the isolated fractions.

Isolates—Galactinol (a) and *myo*-Inositol (b). The R_G values of isolates (a) and (b) were identical to those of authentic galactinol and *myo*-inositol. Each reacted with silver nitrate only, which is characteristic of these polyols. Brown and Serro (1953) first isolated galactinol from sugar beet juice. Later, its structure was determined by Kabat *et al.* (1953). Senser and Kandler (1967) have since found galactinol to be widely distributed in plant leaves and to be associated with oligosaccharides of the raffinose family. Galactinol has also been isolated from vetch seeds (Petek *et al.*, 1966) and identified in potato tubers (Pressey and Shaw, 1969). Tanner (1969) has recently presented evidence indicating that the *myo*-inositol glycoside may function as a galactosyl donor in the biosynthesis of raffinose sugars. The detection of *myo*-inositol was not surprising, as it is the inositol most commonly found in plants and animals (Lohmar, 1957). In addition, its presence might be expected because *myo*-inositol is a part of the galactinol moiety. Previously *myo*-inositol was identi-

Table I. Comparison of Chromatographic Data for the Nonfermentable Sugars Isolated from *Phalaris* with Data for Some Authentic Carbohydrates

Fractions Isolated from *Phalaris*	R_G[a]	Color Developed By: Orcinol	Aniline	Silver[b] Nitrate
a	0.16			Gray
b	0.33			Black
c	0.48		Brown	
d	0.86	Crimson		
e	0.88		Brown	
f	0.96			Black
g	1.02	Blue-green		
h	1.22	Blue		
i	1.16		Pink	
j	1.62	Yellow[c]		
Authentic Carbohydrates				
Stachyose	0.06	Yellow		
Galactinol	0.16			Gray
myo-Inositol	0.33			Black
Raffinose	0.35	Yellow		
Melibiose	0.48		Brown	
Sucrose	0.79	Yellow		
D-*glycero*-D-*manno*-Octulose	0.84	Crimson		
Galactose	0.88		Brown	
D-Mannitol	0.97			Black
Glucose	1.00		Brown	
D-*manno*-Heptulose	1.02	Blue-green		
Fructose	1.19	Yellow		
D-*altro*-Heptulose	1.21	Blue		
Arabinose	1.20		Pink	
Ribose	1.47		Pink	

[a] $R_G(R_{glucose})$ in ethyl acetate-pyridine-water (8:2:1 v/v). [b] Only those compounds which react with silver nitrate only. [c] Faint yellow turning to pink upon aging of the chromatogram.

fied as a constituent carbohydrate in alfalfa (Rendig *et al.*, 1964).

Isolates—Melibiose (c), D-*glycero*-D-*manno*-Octulose (d) and D-Galactose (e). The presence of raffinose was detected before fermentation and, as noted, melibiose would be an expected product of yeast fermentation of raffinose. Identification was made by cochromatography, and its identity was confirmed by comparing its x-ray powder diffraction pattern with that of authentic melibiose. Evidence that the isolates (d) and (e) were D-*glycero*-D-*manno*-octulose and D-galactose, respectively, was the same as that previously described when these two compounds were isolated from alfalfa (Rendig *et al.*, 1964).

Isolates—D-Mannitol (f). This compound was initially separated as a crystalline mass which formed in the isolated syrup. The fact that it reacted only with silver nitrate indicated that it was a polyol, and not a sugar. The R_G value was similar to that of authentic mannitol. Periodate oxidation of the compound formed 32% HCHO compared with 33% for mannitol and 35% (theoretical) for a hexitol. Oxidation with *Acetobacter suboxydans* produced fructose as the predicted product of mannitol oxidation. The compound had an mp of 166–168° C and a mixed mp of 168° C with mannitol. However, the x-ray powder pattern and the ir spectrum of these crystals were not identical with those of authentic mannitol. Further recrystallization from alcohol yielded crystals which produced a third dissimilar pattern. These ambiguous x-ray patterns were not found when the pattern for the hexaacetate derivative was compared with that of authentic mannitol hexaacetate. These patterns were identical. Upon further investigation we found that, during the isolation of crystals, polymorphic forms of mannitol were formed, each producing a different x-ray pattern from that of authentic mannitol. The polymorphic nature of D-mannitol was first reported by von Zepharovich (1887). Recently, Berman *et al.* (1968) and Walter-Levy (1968) have reported on the variety and structure of the crystalline forms of D-mannitol. The optical rotation of the hexaacetate derivative was $[\alpha]_D^{26}$ + 18.5° (c, 1.0 benzene) as compared with $[\alpha]_D^{26}$ + 19.7° (c, 1.0 benzene) for D-mannitol hexaacetate, and with a reported value of $[\alpha]_{5790}^{22}$ + 18.2° (c, 2.14 in benzene) by Patterson and Todd (1929). D-Mannitol is widespread among plants. It has been isolated from perennial rye grass (Harwood, 1954), and most recently has been reported in pichi tops *Fabiana imbricata* (Richtmyer, 1970). To our knowledge, this is the first identification of D-mannitol in *Phalaris*.

Isolates—D-*manno*-Heptulose (g) and D-*altro*-Heptulose (h). These isolates chromatographed in one and two dimensions with authentic D-*manno*-heptulose and D-*altro*-heptulose. The chromatographic behavior agreed with that previously reported by Rendig *et al.* (1964) for alfalfa.

Isolates—L-Arabinose (i) and Unidentified (j). The R_G of isolate (i) corresponded closely to that of authentic arabinose in one dimension, and was identical in two-dimensional cochromatography. Arabinose was reported to be a constituent of the nonfermentable sugars in alfalfa (Rendig *et al.*, 1964), and Bonner (1950) included arabinose in the list of sugars commonly found in plants. Identity of fraction (j) was not established. The yellow ketose-reactive test which turned pink with aging is characteristic of pentuloses, however.

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LITERATURE CITED

Berman, H., Jeffrey, G. A., Rosenstein, R. D., *Acta Crystallogr.* B**24,** 442 (1968).
Bland, B. F., Dent, J. W., *J. Brit. Grassl. Soc.* **17,** 1957 (1962).
Bonner, J., "Plant Biochemistry," p. 25, Academic Press, New York (1950).
Brown, R. J., Serro, R. F., *J. Amer. Chem. Soc.* **75,** 1040 (1953).
Derwyn, R., Whalley, B., McKell, C. M., *Agron. J.* **59,** 223 (1967).
Harwood, V. D., *J. Sci. Food Agr.* **5,** 453 (1954).
Kabat, E. A., MacDonald, D. L., Ballou, C. E., Fischer, H. O. L., *J. Amer. Chem. Soc.* **75,** 4507 (1953).
Lohmar, R. L., "The Carbohydrates," W. Pigman, Ed., p. 271, Academic Press, New York (1957).
Onishi, H., Suzuki, T., *Appl. Microbiol.* **16,** 1847 (1968).
Patterson, T. S., Todd, A. R., *J. Chem. Soc.* 2870 (1929).
Petek, F., Villarroya, E., Courtois, J-E., *C. R. Acad. Sci. Paris* **263,** Ser. D., 195 (1966).
Pressey, R., Shaw, R., *Eur. Potato J.* **12,** 64 (1969).
Rendig, V. V., McComb, E. A., Hu, C-L., J. AGR. FOOD CHEM. **12,** 421 (1964).
Richtmyer, N. K., *Carbohyd. Res.* **12,** 233 (1970).
Robison, R., MacFarlane, M. G., Tazelaar, A., *Nature* (London) **142,** 114 (1938).
Senser, M., Kandler, O., *Phytochemistry* **6,** 1533 (1967).
Tanner, W., *Ann. New York Acad. Sci.* **165,** (Art. 2), 509 (1969).
Walter-Levy, L., *C. R. Acad. Sci. Paris,* Ser. C, **267,** 1779 (1968).
von Zepharovich, Z., *Kristallogr. Mineral.* **13,** 145 (1887).

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