

Identification of Glucitol (Sorbitol) and Ribitol in a Rust Fungus, *Puccinia graminis* f. sp. *tritici*

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SUMMARY

An examination of the soluble carbohydrates of the wheat stem rust fungus, *Puccinia graminis* Pers. f. sp. *tritici* Erikss. & E. Henn., showed the presence of glucitol (sorbitol), ribitol, fructose, and traces of xylitol, as well as confirming the presence of mannitol, arabitol, trehalose, inositol and erythritol. Ribitol and glucitol were major components in glucose-grown mycelium, and appeared to be the major components in mycelium parasitic on wheat leaves, but not in germinated or ungerminated uredospores. It is suggested that glucitol and ribitol may be intermediates (or by-products) of glucose utilization, whereas mannitol, arabitol and trehalose represent storage carbohydrates.

INTRODUCTION

Rust fungi, in common with many other fungi, accumulate much of their carbon reserves as acyclic sugar alcohols (polyols). D-Mannitol and D-arabitol have been reported most commonly in fungi and, together with erythritol and glycerol, have been identified in germinated and ungerminated uredospores of the wheat stem rust fungus *Puccinia graminis* Pers. f. sp. *tritici* Erikss. & E. Henn. (Prentice *et al.*, 1959; Reisener *et al.*, 1962). The presence of these polyols, together with the disaccharide trehalose, has been confirmed by many subsequent workers using paper and gas-liquid chromatography (e.g. Lewis & Smith, 1967*a*; Daly, Knoche & Wiese, 1967; Mitchell & Shaw, 1968; Mitchell & Roberts, 1973; Holligan *et al.*, 1974). However, with one possible exception (Miersch, 1973), ribitol has not been identified as a product of the metabolism of glucose in fungi (Holligan & Lewis, 1973) and glucitol has not been identified in rust fungi.

We have examined the soluble carbohydrates of vegetative mycelium (glucose-grown axenic cultures), and of germinated and ungerminated uredospores of *Puccinia graminis tritici*, and of wheat infected with this fungus; and have confirmed and extended a preliminary finding (Maclean, 1971) that the vegetative mycelium contains high concentrations of glucitol and ribitol.

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METHODS

Growth of the fungus. Strains VIB, V3A and V3B isolated from race 126-Anz-6,7 of *Puccinia graminis* Pers. f. sp. *tritici* Erikss. & E. Henn. were grown in Petri dishes on a cellulose film over medium IV solidified with agar, for 4 weeks at 23 °C (Maclean, 1974). Medium IV contained Czapek's mineral salts, 1% (w/v) peptone (Evans Medical, Speke, Liverpool), 4% (w/v) D-glucose, 0.3% (w/v) trisodium citrate, final pH 6.0; no growth occurred if glucose was omitted from the medium. The mycelium was lifted from the cellophane surface and blotted gently with filter paper; about 0.5 to 1 g fresh wt /9 cm Petri dish was obtained.

Preparation of uredospores and infected wheat. *Puccinia graminis tritici* race 21 (kindly supplied by Dr R. Johnson, Plant Breeding Institute, Cambridge) and 'Little Club' wheat were used. Uredial cultures were maintained on wheat as described by Williams, Scott & Kuhl (1966). Uredospores were collected from infected plants on which pustules had been open for about one week; the uredospores were used for the extraction of carbohydrates or for the preparation of sporelings within one week of collection. Infected wheat for carbohydrate analysis was prepared by dusting the first leaf of six-day-old wheat with sufficient uredospores to form 80 or more pustules per leaf; the leaves were harvested 5 days later, at which stage the mycelium was just beginning to form a macroscopically-visible bulge beneath the abaxial epidermis of the leaves. Sporelings were prepared by germinating uredospores on distilled water (2 ml/mg uredospores) for 6 h at 21 °C in the dark (Daly *et al.*, 1967).

Preparation of neutral extracts for analysis of soluble carbohydrates. Healthy wheat leaves, rust-infected wheat leaves, and vegetative mycelium of the fungus were macerated with 10 or more vols of 80% (v/v) ethanol for 1 min in a rotary-blade homogenizer, and the macerate was boiled for at least 10 min. Uredospores and sporelings were boiled directly in 80% ethanol for 30 min. All extracts were cooled, filtered, evaporated to dryness at 40 to 50 °C in a rotary evaporator, dissolved in water, extracted with chloroform, and the aqueous fraction was deionized by shaking with a mixed resin (Amberlite IRA-400 bicarbonate form, Zeokarb 225 hydrogen form). The cleared extract was evaporated to dryness, weighed and made up to a known volume with water.

Paper chromatography. All chromatography was carried out on Whatman 3MM paper (56 cm long) which had previously been washed by descending chromatography using distilled water (24 h) followed by absolute ethanol (24 h), and then dried. Depending on the resolution required, descending chromatograms were developed for up to 80 h in the solvent systems described in Table 1 (Hough & Jones, 1962; Lewis & Smith, 1967*b*; Holligan & Lewis, 1973).

Detection of sugars and polyols on chromatograms. Aldoses were detected with an aniline hydrogen phthalate reagent (Nemec, Kefurt & Jary, 1967) and ketoses (either free or combined) with a *p*-anisidine reagent (Lewis *et al.*, 1972).

Aldoses, ketoses, oligosaccharides, and polyols were detected using an alkaline silver oxide method (Trevelyan, Procter & Harrison, 1950), modified as follows to give even spot development. Silver nitrate reagent was prepared by dissolving 1 g silver nitrate in 3 ml water, and adding 100 ml aldehyde-free acetone. Potassium hydroxide reagent was prepared a few hours before use by mixing 5 ml 10 M-potassium hydroxide (aqueous) with 40 ml absolute ethanol (AR grade), adding 55 ml re-distilled diethyl ether, and allowing the solution to clear: the upper layer was used. Chromatograms were dipped in the silver nitrate reagent, allowed to dry for about 10 min, dipped with an even motion in the potassium hydroxide

Table 1. *Chromatographic mobility of carbohydrates in five solvent systems*

Mobilities are expressed relative to the mobility of glucose (R_{glu}) in each solvent.

Carbohydrate	Solvent system*				
	A	B	C	E	F
<i>m</i> -Inositol	0.33	0.16	0.12	0.19	0.37
Trehalose	0.26	0.10	0.10	0.25	0.60
Sucrose	0.46	—	—	—	0.77
D-Glucose	1.00	1.00	1.00	1.00	1.00
Galactitol	1.18	2.52	0.71	smear	—
D-Mannitol	1.23	2.65	0.86	smear	—
D-Glucitol	1.19	3.12	0.81	smear	—
D-Fructose	1.43	1.75	1.52	smear	—
Xylitol	1.90	4.94	1.40	0.90	—
D-Arabitol	1.90	4.44	1.55	1.25	—
Ribitol	1.90	5.26	1.84	1.85	1.27
Erythritol	2.62	6.00	2.90	2.23	1.48

* Solvent A: ethyl acetate/acetic acid/formic acid/water (18:3:1:4, by vol.). Solvent B: ethyl methyl ketone/acetic acid/water saturated with boric acid at 20 °C (9:1:1, by vol.). Solvent C: ethyl acetate/pyridine/water (70:25:5, by vol.). Solvent E: ethyl acetate/pyridine/water saturated with boric acid at 20 °C (60:25:10, by vol.). Solvent F: iso-propanol/n-butanol/water (14:2:4, by vol.).

reagent, and dried with a stream of air in a fume hood for 6 to 8 min to allow the spots to develop; the chromatograms were then soaked for 30 min in 5% (w/v) sodium thiosulphate, washed in running water, and dried. All chromatograms developed in solvents containing boric acid were stained by the silver nitrate method after pretreatment with 4% (w/v) hydrofluoric acid in acetone to remove the boric acid (Britton, 1959).

Strips cut from chromatograms stained by the silver method were quantified by using a transmission densitometer and measuring the area under the peak: results were within about 10% for duplicate samples, with a maximum loading of 5 to 30 µg per spot depending on the sugar and the period of chromatographic development. Quantitative results were more accurate if the spot intensity was reduced by soaking the chromatogram for 1 h in 5% (w/v) sodium thiosulphate acidified with acetic acid (to convert silver to silver sulphide) followed by washing and drying. Standards were run on all chromatograms.

Purification and isolation of polyols. Pentitols were initially separated from hexitols by applying bands (1 to 2 mg cm⁻¹ or less) of cleared extract to sheets of washed Whatman 3MM paper and developing for 30 to 35 h in solvent A; using the silver method polyols were located on strips cut from the chromatogram. Aldoses and ketoses were removed from the original cleared extract, and from the pentitol and hexitol fractions after the initial separation, by heating aqueous solutions with Amberlite IRA-400 resin (hydroxide form) for 15 to 20 min; this treatment did not cause isomerization of polyols. The pentitol fraction was resolved by rechromatography with solvent E, and the hexitol fraction by rechromatography with solvents B and C. After chromatography with solvents B or E, boric acid was removed as the volatile methyl ester by evaporating to dryness at 50 °C four to five times after sequential additions of absolute methanol, followed by treatment with Amberlite IRA-400 (hydroxide form). It was often necessary to re-chromatograph preparations of some polyols, because the heavy loadings necessary for large-scale separations sometimes resulted in small but significant amounts of one polyol being 'carried' in a dense band of another polyol. Polyols were eluted from chromatograms with water.

Identification of polyols. Authentic polyols (from BDH and Sigma), and rust polyols

tentatively identified by their chromatographic mobilities, were recrystallized from the following solvents: ribitol from ethanol, D-glucitol from methanol containing acetone, D-mannitol from aqueous methanol, D- and L-arabitol and xylitol from methanol, and galactitol from water. Infrared spectra were obtained from discs prepared by compressing mixtures of the polyol and potassium bromide; the discs were scanned with a Unicam SP1000 infrared spectrophotometer. The polyols listed above, except for D- and L-arabitol, were readily distinguished from each other by their infrared spectra.

RESULTS

Table 2 summarizes the sugars and polyols detected in extracts of various materials. Each component of a fraction gave the expected R_{gin} value in at least two solvent systems (Table 1) and gave the same reaction as authentic standards with specific stains.

Identity of polyols in vegetative mycelium

About 4% (w/w) of the fresh wt of vegetative mycelium was recovered as soluble neutral compounds; this represents about 20% on a dry wt basis (mycelium is about 20% dry matter). The hexitol fraction from all strains (V1B, V3A, V3B) contained glucitol and mannitol but no trace of galactitol. The pentitol fractions contained mostly ribitol, with smaller arabitol components and traces of xylitol.

Fractionation of V3B mycelium yielded 10 mg recrystallized mannitol, 20 mg glucitol and 10 mg ribitol; insufficient arabitol and xylitol were obtained for recrystallization. Recrystallized compounds gave identical melting points to authentic compounds: ribitol 100 to 101 °C, mannitol 166 to 167 °C, glucitol sintered over the range 85 to 100 °C. The infrared spectra of mycelial polyols and authentic standards were identical over the range 3500 to 625 cm^{-1} , except for some very minor variations with mannitol which are attributed to traces of impurities or slight differences in the preparation of the potassium bromide discs. Although insufficient xylitol was obtained for physical measurements, both authentic and mycelial xylitol developed spots more rapidly than other pentitols when chromatograms were stained by the silver method.

Polyols in healthy and rust-infected wheat

Extracts of healthy and rust-infected wheat leaves were digested with hot Amberlite IRA-400 resin (hydroxide form) to remove aldoses and ketoses (glucose, fructose and minor monosaccharides) before analysis of the polyols. Rust-infected wheat contained considerably larger amounts of polyols than healthy wheat; low levels of compounds with the chromatographic mobilities of ribitol, arabitol and mannitol were detected on chromatograms very heavily loaded with extracts of healthy wheat, but these compounds could not be detected quantitatively in extracts of healthy leaves equivalent to the quantities used for analyses of infected leaves (Table 2). Presumably, the polyols in infected leaves were mostly of fungal origin.

In rust-infected wheat, the relative amounts of mannitol, glucitol, arabitol and ribitol as a percentage of total content of acyclic polyols were within the range found in two strains of the fungus (V1B, V3B) grown on glucose medium, except that the proportion of arabitol was somewhat higher in the infected wheat. This similarity of polyol proportions is consistent with the fungus having similar pathways of carbohydrate utilization whether growing parasitically on wheat, or axenically on glucose medium.

Table 2. Soluble carbohydrates in *Puccinia graminis* f. sp. *tritici*The carbohydrate content is expressed as mg (g fresh wt)⁻¹.

Carbohydrate	Glucose-grown mycelium		Infected wheat	Uredospores	Sporelings	Diffusates from sporelings
	Strain V1B	Strain V3B				
Inositol	0.12	0.16	0.06	0.7	+	+
Trehalose	2.4	4.6	0.16†	3.8	+	?
Glucose	1.4	4.8	0†	0	+	+
Fructose	1.3	1.0	0†	0	+	0
Mannitol	19.8	8.8*	0.20	50	+	+
Glucitol	4.5	15.4*	0.29	0	0	0
Arabitol	1.0	0.7	0.09	49.5	+	+
Ribitol	1.8	6.0*	0.16	0.5	0	0
Xylitol	trace	≈ 0.14	0	trace(?)	0	0
Erythritol	+	+		+		
Total hexitol	24.3	24.2	0.49	50		
Total pentitol	2.8	6.7	0.25	50		

+, Present but amount not estimated; 0, not detected (i.e. absent from the extract).

* Identity confirmed by melting point and infrared spectrum; † destroyed (or partially destroyed) by alkaline digestion.

Polyols in uredospores, sporelings, and diffusates from sporelings

The predominant polyols in preparations of germinated and ungerminated uredospores were mannitol and arabitol (Table 2). Ribitol was present as a very minor component of uredospores, but could not be detected in sporelings or diffusates from sporelings; glucitol could not be detected in any of these materials.

DISCUSSION

Ribitol, glucitol, and probably xylitol, can now be added to the list of acyclic polyols identified in rust fungi. The inability of other workers to detect these compounds in vegetative mycelium (e.g. plant material infected with a rust fungus) could be due to the failure of many chromatographic systems to separate the isomeric hexitols from each other, and the isomeric pentitols from each other. Ribitol and xylitol are both *meso* compounds; the glucitol is presumed to be the D form because D-glucitol (but not L-glucitol) has often been identified as a natural product (Touster & Shaw, 1962; Lewis & Smith, 1967*a*); we did not isolate sufficient glucitol to determine its optical rotation. The presence of ribitol is of particular mycological interest, because previous reports of ribitol as a product of the metabolism of glucose in fungi have not been confirmed (Holligan & Lewis, 1973), except for one possibly novel situation in a yeast which overproduces riboflavin (Miersch, 1973).

The precise role of free polyols in intermediary metabolism or parasitic associations of rust fungi is not understood (Lewis & Smith, 1967*a*; Smith, Muscatine & Lewis, 1969). Whereas mannitol, arabitol and the disaccharide trehalose are present in both uredospores and vegetative mycelium, glucitol and ribitol are present in appreciable quantities only in vegetative mycelium. This suggests that glucitol and ribitol may be intermediates (or by-products) of the uptake and utilization of exogenous carbohydrates such as glucose, and that mannitol, arabitol and trehalose represent reserve or storage carbohydrates. The presence of significant pools of fructose (0.1 % fresh wt, Table 2) in glucose-grown mycelium

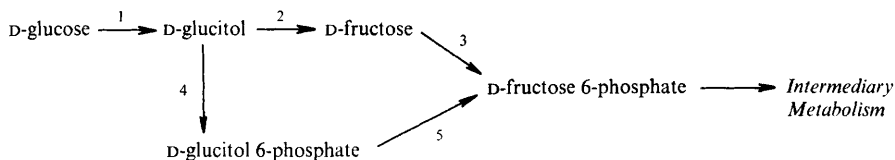


Fig. 1. Possible scheme(s) for the uptake and utilization of glucose by *Puccinia graminis* f. sp. *tritici*

is consistent with a pathway for the conversion of glucose to fructose via non-phosphorylated intermediates, as is known in some animal systems (Hers, 1960; Touster & Shaw, 1962). A possible scheme for the uptake and utilization of glucose by rust fungi (Fig. 1) shows D-glucitol as the first product of glucose utilization, followed by two alternative pathways to fructose 6-phosphate. Preliminary studies on cell-free extracts provide evidence for reaction 1 (NADP-dependent oxidation of D-glucitol), reaction 2 (slow NAD-dependent oxidation of D-glucitol) (Maclean, 1971), and reaction 5 (NADH-dependent reduction of fructose 6-phosphate) (Hendrix, Daly & Livne, 1964). Further work is required to define clearly enzyme systems which may participate in the above scheme and to follow the incorporation of labelled substrates into metabolic intermediates by the living fungus.

Note added in proof: Since this work went to press, we have become aware of the work of McComb & Rendig (1970), who found that D-mannitol produces polymorphic crystals which differ in infrared spectra and X-ray powder patterns. The differences we observed between the infrared spectra of mannitol isolated from the rust fungus, and the spectra of authentic D-mannitol, are presumably due to differences in the relative proportion of each crystal type after each recrystallization, or after various periods of storage prior to running spectra.

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