The Relationship Between the Uptake of Glucose and 3-*O*-Methylglucose and Soluble Carbohydrate and Polysaccharide in the Fungus *Dendryphiella salina*

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SUMMARY

When mycelium of *Dendryphiella salina*, pre-incubated in D-[1-¹⁴C]mannitol such that this is the only major labelled soluble carbohydrate present, absorbs glucose or the non-metabolized sugar 3-*O*-methylglucose, there is a specific stimulation of incorporation of ¹⁴C into alkali-insoluble, trichloroacetic-acid-soluble (1 → 4)-α-glucan, probably glycogen. There is also a net increase in the amount of glucan caused by stimulation of synthesis and inhibition of breakdown. Addition of 3-*O*-methylglucose results in the loss of mannitol into the medium. This loss and the reduced rate of replenishment from the glucan is important in the osmotic regulation of the hyphae. If osmotic adjustment does not occur, the hyphae do not show the specific incorporation of ¹⁴C into the glucan.

INTRODUCTION

Studies of glucose transport into cells often involve the use of non-metabolizable analogues of glucose. However, one cannot assume that such compounds have no effect on cell metabolism over short periods. Thus, Jennings & Austin (1973) showed that uptake of 3-*O*-methylglucose by the marine hyphomycete *Dendryphiella salina* resulted in a fall in the intracellular levels of mannitol, arabitol and glucose while the total ethanol-soluble carbohydrate content (including 3-*O*-methylglucose) remained constant. At the same time, the amount of insoluble material increased. The decline in mannitol content was more marked than that of arabitol or glucose. In contrast, uptake of glucose did not appear to cause such changes. When mycelium preloaded with D-[1-¹⁴C]mannitol accumulated 3-*O*-methylglucose, radioactivity was transferred from the ethanol-soluble to the insoluble material.

These results imply that the uptake of 3-*O*-methylglucose caused a reduction in the level of mannitol, with at least some of the polyol being converted to polysaccharide. Jennings & Austin (1973) hypothesized that the uptake of each 3-*O*-methylglucose molecule caused the conversion of a mannitol (or arabitol) molecule to polysaccharide or other insoluble material, thus avoiding a fluctuation in hyphal osmotic pressure.

The investigations reported here were undertaken to provide further data on metabolic changes caused by the uptake of 3-*O*-methylglucose. In particular, is there a direct conversion of mannitol to polysaccharide, and how do the metabolic events occurring during uptake of glucose compare with those which occur during uptake of 3-*O*-methylglucose?

METHODS

Growth of fungus. Stock cultures of *Dendryphiella salina* were maintained for 2 to 3 weeks in sea water containing glucose (5.0 g l⁻¹) and tryptone (3.0 g l⁻¹). Subculturing was by transfer of homogenized mycelium to fresh medium (Allaway & Jennings, 1970a). Cultures for experiments were prepared by transferring mycelial homogenate (20 ml) to fresh medium (150 ml) in 250 ml conical flasks. After 24 h incubation at 20 °C on a rotary shaker, the mycelium was again transferred to fresh medium. Cultures (consisting of approximately 2 mm mycelial aggregates) were harvested after a further 24 h incubation.

Experimental procedure. Mycelium was washed and resuspended in 10 mmol l⁻¹ calcium chloride and incubated with D-[1-¹⁴C]mannitol (50 µCi; 60 mCi mmol⁻¹) for up to 2 h. The preloaded mycelium was separated by filtration and washed three times with 10 mmol l⁻¹ calcium chloride. Identical samples of preloaded mycelium (2 to 5 g wet wt) were weighed into 250 ml conical flasks containing 140 ml 10 mmol l⁻¹ calcium chloride, and 10 ml glucose or 3-O-methylglucose solution was added. A sample of preloaded mycelium was usually extracted without incubation (zero-time mycelium). Control mycelium (zero-substrate mycelium) was incubated without glucose or 3-O-methylglucose. All treatments were carried out using a rotary shaker at 20 °C.

After incubation, mycelium was transferred to an extraction funnel with a sintered-glass base, rapidly filtered under vacuum, and then washed quickly with 10 mmol l⁻¹ calcium chloride. The funnel was then placed in a 50 ml conical flask and hot ethanol (80 %, v/v) was added.

In some experiments, the calcium chloride solution was replaced throughout by either distilled water or sea water containing 20 mmol l⁻¹ calcium chloride.

Extraction and analysis of mycelial soluble carbohydrates. This was as described by Holligan & Jennings (1972a).

Fractionation of ethanol-extracted mycelium. After ethanol-extraction, the mycelial residue was extracted as detailed in Fig. 1. Initially, fewer fractions were taken but the procedure was extended in later experiments to include the extraction with 0.1 M-trichloroacetic acid and the third extraction with 2.0 M-sodium hydroxide. The first two alkali-soluble fractions were pooled, neutralized and, if necessary, diluted before assay of ¹⁴C.

Assay of radioactivity. Mycelial extracts (up to 1 ml) were assayed for radioactivity in toluene/Triton scintillator cocktail [10 ml of a solution containing: toluene (667 ml); Triton-X100 (333 ml); PPO (5.0 g); POPOP (0.1 g)]. Solid samples (thin-layer chromatography absorbent or crushed mycelial residue) were counted as suspensions with 0.4 g fumed silica in 10 ml scintillator cocktail. All samples were counted in a Packard Tri-Carb scintillation counter.

Detection of radioactivity. Radioactive compounds were detected on paper chromatograms or thin-layer chromatography (t.l.c.) plates with a Spark Chamber Imaging system (Birch-over Instruments, Hitchin, Hertfordshire). Exposure time was normally about 30 min, depending on the radioactivity present.

Desalting. When necessary, samples were deionized by passage through columns of Amberlite IR-45 (OH⁻) and IR-120 (H⁺) ion-exchange resins.

Paper chromatography. Soluble sugars were separated by descending chromatography using (system A) butan-2-one/acetic acid/water saturated with boric acid (9:1:1, by vol.) (Holligan & Jennings, 1972a). Chromatograms were either run for a few hours to check for ¹⁴C-labelled compounds near the solvent front, or were developed overnight up to three times to effect optimum separation of monosaccharides and polyols.

Fig. 1. Scheme for the extraction of mycelium of *Dendryphiella salina*.

System A does not separate glucose and galactose. These sugars were separated using (system B) pyridine/ethyl acetate/water (35:60:10, by vol.) with two overnight developments.

Thin-layer chromatography. Extracts were chromatographed (system C) on thin layers of MN 300 cellulose in pyridine/ethyl acetate/acetic acid/water (36:36:7:21, by vol.) or (system D) on silica gel in acetone/water (9:1, by vol.).

Glucose assay. Glucose was assayed spectrometrically using the GOD-Perid kit.

Total carbohydrate. This was assayed with anthrone reagent (Yemm & Willis, 1954) using a glucose standard.

Incubation of mycelium with protease. Mycelial residue (approximately 0.5 g wet wt) was homogenized in a Potter homogenizer with 4 ml phosphate buffer (pH 7.4; 0.1 mol l⁻¹). The resultant slurry was incubated with non-specific protease from *Streptomyces griseus* (10 mg)

at 37 °C for 16 h. The reaction was stopped by immersion in boiling water for 10 min, and insoluble material was removed by centrifugation. The supernatant was assayed for ^{14}C , deionized and lyophilized.

Protein assay. Protein was assayed by the method of Lowry *et al.* (1951).

Acid hydrolysis. Polysaccharide samples were hydrolysed with 0.75 M-sulphuric acid at 100 °C for 2 h. Hydrolysates were neutralized with barium carbonate and supernatants were deionized before chromatography.

Incubation with amyloglucosidase. Solutions or suspensions of polysaccharides (10 mg ml^{-1}) were prepared in acetate buffer (pH 4.0; 0.1 mol l^{-1}) and incubated with 1 mg amyloglucosidase at 25 °C for various times. A few drops of toluene were added to the reaction mixture to inhibit bacterial growth. Enzyme and substrate blanks were included as controls. Samples of the reaction mixture were removed at intervals, chromatographed by t.l.c. (system C) and stained for reducing sugars.

Infrared spectroscopy. Samples were prepared in KBr discs, and spectra were recorded with a Pye Unicam SP200 infrared spectrophotometer.

Osmotic potentials of incubation and growth media were measured using a Fiske osmometer (Fiske, Uxbridge, Massachusetts, U.S.A.).

Materials. Insoluble laminarin was obtained from the Seaweed Research Institute, Musselburgh, Midlothian, Scotland. D-[1- ^{14}C]mannitol was obtained from The Radiochemical Centre, Amersham; GOD-Perid glucose assay kit and amyloglucosidase were from Boehringer; glycogen (from rabbit liver) and routine chemicals were from BDH; and non-specific protease (type VI from *Streptomyces griseus*) was from Sigma. (1 $\rightarrow$ 3)- β -glucanase from a thermophilic *Streptomyces* species was the kind gift of Dr A. T. Bull, University of Canterbury, Kent.

RESULTS

When *D. salina* was incubated with D-[1- ^{14}C]mannitol, ^{14}C was rapidly incorporated into both the ethanol-soluble and insoluble fractions. Analysis of the ethanol-soluble fraction by paper chromatography (system A) showed that there was transfer of ^{14}C from mannitol to an unknown compound after 2.5 h incubation. The unknown compound had similar R_f values to glycerol in several solvents. The chosen duration of preloading with D-[1- ^{14}C]mannitol was therefore a compromise between adequate labelling of the mycelium and transfer of ^{14}C . In our experiments, preloading time was never more than 2 h and in each instance it was confirmed by chromatography that mannitol was essentially the only labelled soluble compound present.

Mycelium preloaded with D-[1- ^{14}C]mannitol was incubated with glucose or 3-*O*-methylglucose. After ethanol-extraction, the mycelium was initially fractionated as detailed in Fig. 1, but without the trichloroacetic acid (TCA) extraction and the third NaOH extraction. On incubation, preloaded cells lost a considerable amount of ethanol-soluble ^{14}C , i.e. mannitol, irrespective of whether or not external sugar was present (Table 1). Approximately 45 % of the labelled material lost from the ethanol-soluble fraction was transferred to insoluble material; the remainder presumably either leaked into the medium and/or was broken down by respiration and other metabolic processes. We know from other studies that leakage of ethanol-soluble labelled material can occur. All fractions of the insoluble material, except the minor water-soluble one, increased in ^{14}C content.

Uptake of either glucose or 3-*O*-methylglucose increased the loss of [^{14}C]mannitol from the soluble fraction relative to the control without substrate; the losses were similar for both sugars. In both cases, uptake was accompanied by an increase in the amount of ^{14}C trans-

Table 1. *The effect of glucose and 3-O-methylglucose on the distribution of ^{14}C among various fractions of mycelium of Dendryphiella salina*

Mycelia were preloaded with D-[1- ^{14}C]mannitol in 10 mmol l $^{-1}$ CaCl $_2$ for 1 h, and incubated with or without glucose or 3-O-methylglucose (both at 1 mmol l $^{-1}$) in 10 mmol l $^{-1}$ CaCl $_2$ for 30 min.

	10 $^{-8} \times ^{14}\text{C}$ content of fraction (d min $^{-1}$)				
	EtOH	H $_2$ O	NaOH*	H $_2$ SO $_4$	Residue
Zero-time mycelium	805.8	7.5	13.3	55.0	24.4
Zero-substrate mycelium	571.8	5.0	39.1	120.9	46.6
3-O-Methylglucose	512.9	4.7	37.4	165.7	47.8
Glucose	514.8	4.6	33.6	178.0	48.5
Loss of ^{14}C from EtOH fraction during uptake of	$\left\{ \begin{array}{l} 3\text{-O-methylglucose} = 58.9 \times 10^8 \text{ d min}^{-1} \\ \text{glucose} = 57.0 \times 10^8 \text{ d min}^{-1} \end{array} \right.$				
Gain of ^{14}C by H $_2$ SO $_4$ fraction during uptake of	$\left\{ \begin{array}{l} 3\text{-O-methylglucose} = 44.8 \times 10^8 \text{ d min}^{-1} \\ \text{glucose} = 57.1 \times 10^8 \text{ d min}^{-1} \end{array} \right.$				

* This includes pooled hot and cold alkali-soluble material.

ferred from the soluble to the insoluble material, but the increase was only in the alkali-insoluble, H $_2$ SO $_4$ -soluble fraction.

The increase in the ^{14}C content of H $_2$ SO $_4$ -soluble material was equal to the glucose-induced loss from the ethanol-soluble fraction. For 3-O-methylglucose, the gain in the H $_2$ SO $_4$ -soluble fraction was less than the induced loss from the ethanol-soluble fraction. This discrepancy may be due to loss of ^{14}C from the mycelium either by leakage or respiration. As previous work (Jennings & Austin, 1973) has indicated that 3-O-methylglucose has no effect on the respiration rate in *D. salina*, leakage is a more likely explanation.

Paper chromatography (systems A and B) of deionized H $_2$ SO $_4$ -soluble material showed that it consisted mainly of glucose with galactose as a minor component. Other insoluble material remaining at the origin of the chromatograms was presumably unhydrolysed oligosaccharide or polysaccharide. Glucose contained most of the radioactivity present; galactose was apparently unlabelled. Thus there appeared to be a close relationship between glucose or 3-O-methylglucose uptake and the metabolism of [^{14}C]mannitol to H $_2$ SO $_4$ -soluble polysaccharide.

Investigations of the wall components of *Neurospora crassa* (Mahedevan & Tatum, 1965) and *Aspergillus nidulans* (Bull, 1970) have shown that sulphuric acid, while removing a significant amount of wall material, produces a mixture of sugars and oligosaccharides. Mortimer (1963) used TCA (0.1 M; 100 °C; 2 h) to fractionate polysaccharides from beet leaf extracts without apparent hydrolysis. A similar extraction with TCA was introduced into the chemical fractionation of *D. salina* mycelium to differentiate possible components of the H $_2$ SO $_4$ -soluble material and to decrease, if possible, the degree of hydrolysis. The alkali-insoluble residue was therefore extracted with TCA immediately before treatment with H $_2$ SO $_4$.

Similar experiments to those described above, but including an extraction with TCA, produced similar results. Uptake of glucose or 3-O-methylglucose increased the loss of labelled material from the ethanol-soluble fraction and enhanced the transfer of ^{14}C to a specific insoluble fraction. In this case, the TCA-soluble material was the recipient of the specific transfer of ^{14}C during sugar uptake; the remainder of the H $_2$ SO $_4$ -soluble fraction showed no increase. Further experiments confirmed this specificity of transfer from soluble carbohydrate to TCA-soluble polysaccharide material.

Table 2. *The effect of different incubation media with or without glucose (1 mmol l⁻¹) on the distribution of ¹⁴C among various fractions of mycelium of Dendryphiella salina*

Mycelia were preloaded with D-[1-¹⁴C]mannitol for 2 h, and incubated with glucose for 1 h.

Medium; treatment	10 ⁻³ × ¹⁴ C content of fraction (d min ⁻¹)							10 ⁻³ × Total ¹⁴ C incorporated (d min ⁻¹)
	EtOH	H ₂ O	NaOH	TCA	H ₂ SO ₄	NaOH(3)	Residue	
H ₂ O; control	201.4	37.4	271.1	89.1	165.9	85.6	207.3	1057.7
H ₂ O; glucose	188.3	36.2	272.2	89.2	166.3	108.4	131.9	992.5
CaCl ₂ ; control	3047.3	257.8	2147.1	918.2	731.1	615.4	432.8	8149.8
CaCl ₂ ; glucose	3246.7	242.1	1831.1	932.3	680.4	611.3	391.3	7935.1
CaCl ₂ /sea water; control	10832.2	141.6	1055.2	442.9	224.6	274.1	72.9	13043.4
CaCl ₂ /sea water; glucose	10252.6	137.3	1044.7	718.9	208.5	254.1	75.3	12691.5

For mycelium in CaCl₂/sea water:

Loss from EtOH fraction during glucose uptake = 579.6×10^3 d min⁻¹

Gain by TCA-soluble fraction = 276.0×10^3 d min⁻¹

i.e. 47.6 % of ¹⁴C in EtOH fraction was transferred to TCA fraction.

Experiments in which 10 mmol l⁻¹ calcium chloride was used as the preloading and incubation medium were unpredictable; transfer of ¹⁴C from soluble to insoluble material was sometimes small and occasionally absent. Possibly hyphae of *D. salina* might be adversely affected by the transfer from sea-water growth medium to an incubation medium of lower osmotic potential and different ionic composition.

To test this hypothesis, mycelium was preloaded and incubated in either distilled water, or 10 mmol l⁻¹ calcium chloride solution, or sea water containing 20 mmol l⁻¹ calcium chloride. The last solution was chosen because it has a similar ionic composition to the growth medium, the additional calcium chloride raising the osmolarity to that of glucose/tryptone/sea water medium. After ethanol-extraction, the mycelial residue was fractionated as described previously but with an additional extraction with sodium hydroxide (2 M; 1 h; 20 °C) after treatment with sulphuric acid. We wished to examine the possibility that there might be an uptake-induced transfer of ¹⁴C specifically to this fraction which was being masked by a simultaneous drop in the radioactivity of the final residue fraction.

The composition of the preloading/incubation media had a marked effect on both the total incorporation of ¹⁴C by the mycelia and the transfer of radioactivity from ethanol-soluble to insoluble material during glucose uptake (Table 2). Mycelium incubated in distilled water showed the lowest total incorporation of ¹⁴C and the uptake of glucose had little effect on transfer of ¹⁴C except for a slight increase in the amount in the acid-insoluble, alkali-soluble fraction. No transfer of ¹⁴C to the TCA fraction was observed; indeed this fraction exhibited a slight decline.

In calcium chloride solution, the mycelium incorporated approximately eight times more total radioactivity than mycelium in distilled water and the radioactivity in the TCA fraction increased slightly during glucose uptake. All other fractions showed a decrease in radioactivity during uptake of the sugar.

Mycelia preloaded and incubated in the sea water/calcium chloride medium showed a higher incorporation than any previously observed, approximately 78 % of the available D-[1-¹⁴C]mannitol being absorbed. The radioactivity in all ethanol-insoluble fractions, other than the TCA-soluble fraction, remained essentially unchanged during glucose uptake.

Fig. 2. The uptake of 3-*O*-methylglucose by mycelium of *Dendryphiella salina* in 15 min as a function of the external concentration of the sugar.

Fig. 3. The gain of ¹⁴C in 15 min by the TCA-soluble fraction in mycelium of *Dendryphiella salina* in relation to the amount of 3-*O*-methylglucose absorbed.

However, the ¹⁴C content of the TCA fraction increased by about 75 %. Again, the transfer of ¹⁴C to polysaccharide accounted for only a proportion (47.6 %) of the loss of radioactivity from the ethanol-soluble fraction, indicating that soluble sugars can be lost from the hyphae into the medium.

The acid-insoluble, alkali-soluble fractions apparently have no role in the uptake-induced transfer of ¹⁴C from the ethanol-soluble fraction to polysaccharides; the slight gain observed in the mycelium incubated in distilled water is considered to be insignificant or a product of abnormal circumstances. The increased proportion of ¹⁴C present in the alkali-soluble fraction (before H₂SO₄ extraction) arose because we discontinued dialysis of these extracts before ¹⁴C assay. Despite the increased content of radioactivity, this fraction plays no part in the transfer of ¹⁴C from ethanol-soluble to insoluble material during sugar uptake.

The reason for the effects of the different media is unknown but both osmotic and ionic factors are probably involved. Various ions, including Na⁺, K⁺ and Ca²⁺ have been reported to affect sugar transport by, and loss of polyols from, *D. salina* (Allaway & Jennings, 1970*a, b*). Subsequent incubations in sea water/calcium chloride medium did prove to be reproducible, resulting in a high total incorporation and consistent transfer of material to the TCA fraction during glucose or 3-*O*-methylglucose transport.

If transfer of soluble sugars to polysaccharide is part of an osmoregulatory system as

Table 3. *The effect of different concentrations of 3-O-methylglucose on the transfer of ^{14}C from ethanol-soluble to TCA-soluble material in the mycelium of Dendryphiella salina*

Mycelia were preloaded with D-[1- ^{14}C]mannitol in CaCl_2 /sea water for 2 h, and incubated for 15 min in the same medium.

	^{14}C in TCA fraction (d min^{-1})	Gain of $^{14}\text{C}^*$ (d min^{-1})
Zero-time mycelium	171.3	
Zero-substrate mycelium	211.8	
3-O-Methylglucose (mmol l^{-1})		
0.25	214.3	2.5
0.5	219.9	8.1
1	235.2	23.4
2	263.3	51.4
4	291.9	80.1
8	299.1	87.3
16	317.7	105.9
32	287.7	75.9
64	273.5	61.7

* Gain of ^{14}C = (^{14}C content of TCA fraction) - (^{14}C content of TCA fraction in zero-substrate mycelium).

suggested by Jennings & Austin (1973), one would expect the amount transferred to be related to the uptake of sugar and therefore to the external concentration of glucose or 3-O-methylglucose. This was examined by measuring the uptake of 3-O-methylglucose over a range of external concentrations of the sugar, using a short incubation time (15 min) and a large medium volume to mycelium weight ratio, to reduce any change in external sugar concentration during uptake.

As expected, 3-O-methylglucose uptake increased with increasing external concentration (Fig. 2). The graph of uptake against external concentration has a discontinuity which might indicate that more than one transport system is involved in uptake. Transfer of ^{14}C from the ethanol-soluble fraction to the TCA fraction increased non-linearly with external concentration of 3-O-methylglucose up to 16 mmol l^{-1} but decreased at higher sugar concentrations (Table 3). However, the transfer of ^{14}C to the TCA fraction increased linearly with uptake up to a level corresponding to an external concentration of 16 mmol l^{-1} , but above this it decreased (Fig. 3). Thus the transfer of material to the TCA fraction appears to be directly proportional to 3-O-methylglucose uptake until some critical internal concentration is reached, after which it declines with increasing concentration.

Calculations based on the specific activity of mannitol (nearly all of the ^{14}C in the ethanol-soluble material was in mannitol) and on the amount of ^{14}C transferred to the TCA fraction show that only a very small amount (maximum 30 μg) of material was transferred to the polysaccharide during uptake. Assays, by the anthrone method and by gas-liquid chromatography, of the carbohydrate in hydrolysates of the TCA fractions confirmed this; no significant differences were found. Whereas the amount of mannitol showed little change during the experiment, only increasing slightly at the highest concentrations of 3-O-methylglucose used, the total soluble carbohydrate rose steadily with 3-O-methylglucose accumulation (Table 4). Similar results have been obtained in other experiments of short duration (approx. 60 min). This suggests there is a time delay between the uptake of soluble sugar and adjustment of the total soluble carbohydrate content.

To test this possibility, mycelia were incubated for either 1 or 5 h in one of three concen-

Table 4. *The effect of different concentrations of 3-O-methylglucose on the amounts of ethanol-soluble carbohydrates in the mycelium of Dendryphiella salina*

Experimental details as in Table 3.					
	Amount of soluble carbohydrate (μg)				Intracellular concn of 3- <i>O</i> -methylglucose* (mmol l ⁻¹)
	Arabitol	Mannitol	3- <i>O</i> -Methyl- glucose	Total	
Zero-time mycelium	1175	1500	—	2675	—
Zero-substrate mycelium	1340	1800	—	3140	—
3- <i>O</i> -Methylglucose (mmol l ⁻¹)					
0.25	990	1200	400	2590	0.010
0.5	1065	1275	500	2840	0.013
1	1015	1140	740	2895	0.019
2	1031	1206	940	3177	0.024
4	1100	1100	1240	3440	0.032
8	1140	1290	1815	4245	0.047
16	1000	1140	2365	4505	0.061
32	1390	1700	5750	8840	0.148
64	1465	2200	11550	15215	0.298

* Based on a ratio of intracellular water to dry wt of 2.54 (Slayman & Tatum, 1964).

trations of 3-O-methylglucose (0.1, 1 and 10 mmol l⁻¹) (Table 5). Uptake of 3-O-methylglucose increased with increasing external concentration and, at any one concentration, the internal level rose with time. Uptake of 3-O-methylglucose caused a loss of ¹⁴C from the ethanol-soluble fraction and a rise in the ¹⁴C content of the TCA fraction. These changes were enhanced by increases in incubation time or in the external 3-O-methylglucose concentration. In some cases, the increase in ¹⁴C content of the TCA fraction occurred when the actual amount of TCA-soluble material decreased (cf. the control mycelium lacking 3-O-methylglucose), indicating that synthesis and hydrolysis of TCA-soluble polysaccharide were occurring at the same time. The specific activity (d min⁻¹ μ mol⁻¹) of TCA-soluble material also increased with uptake of 3-O-methylglucose and with time of incubation.

Uptake of 3-O-methylglucose caused a decrease in the concentration of mannitol inside the mycelium, the fall being directly proportional to the uptake of 3-O-methylglucose. This effect was more clearly seen after the 5 h incubation than after 1 h. Calculation of the ratio of mannitol lost to 3-O-methylglucose uptake indicates that there is a delay in the response to 3-O-methylglucose uptake. The ratio increased from 0.1 to 0.2 at 1 h to 0.72 (mean value) after 5 h. The ratio of the increase in material in the TCA fraction to 3-O-methylglucose uptake varied little. The total amount of soluble carbohydrate showed some variation after 1 h but after 5 h it was essentially the same in all treatments. There was apparently no link between uptake of 3-O-methylglucose and the intracellular level of arabitol, except for a significant drop in arabitol in mycelium incubated in 10 mmol l⁻¹ 3-O-methylglucose for 5 h.

The concentrations of mannitol in the control mycelium incubated without 3-O-methylglucose was greatly reduced after 1 h but, after 5 h incubation, it had recovered to the zero-time level. The amount of TCA-soluble polysaccharide in the same mycelium had decreased after 5 h by an amount (5.92 μ mol glucose equivalents) which would be sufficient to restore the mannitol to the zero-time level.

The amount of material or ¹⁴C gained by the TCA fraction (compared with that in control mycelium incubated without 3-O-methylglucose) was approximately proportional to the uptake of 3-O-methylglucose (Fig. 4). However, the relationship between the amount of ¹⁴C

Table 5. Changes in the amount of ^{14}C in mannitol and TCA-soluble material, and in the amounts of soluble carbohydrate and TCA-soluble material in mycelium of *Dendryphiella salina* after treatment with different concentrations of 3-O-methylglucose for 1 or 5 h

Mycelia were preloaded with D-[1- ^{14}C]mannitol in CaCl_2 /sea water for 1.25 h.

	$10^{-5} \times ^{14}\text{C}$ content (d min $^{-1}$)		Amount of soluble carbohydrate (μmol)				TCA fraction (μmol glucose equiv.)	$10^{-3} \times$ Specific activity (d min $^{-1}$ μmol^{-1})		Mannitol loss*/ 3-O-Methyl- glucose uptake	TCA gain*/ 3-O-Methyl- glucose uptake
	Mannitol	TCA fraction	Mannitol	3-O-Methyl- glucose	Arabitol	Total		Mannitol	TCA fraction		
Zero-time mycelium	34.7	0.39	17.0	—	16.05	33.05	11.23	232	3.48	—	—
1 h incubation with 3-O-methylglucose (mmol l $^{-1}$)											
None	34.7	0.70	10.83	—	11.36	22.19	9.98	320	7.01	—	—
0.1	34.2	0.87	10.56	2.45	12.17	25.18	10.78	324	8.08	0.11	0.33
1.0	33.2	1.63	11.67	10.10	12.34	34.11	12.18	284	13.35	—	0.22
10	30.0	3.39	7.96	13.90	12.21	34.07	14.38	377	23.56	0.21	0.32
5 h incubation with 3-O-methylglucose (mmol l $^{-1}$)											
None	0.89	0.89	17.22	—	15.39	32.61	5.31	176	16.81	—	—
0.1	1.38	1.38	13.09	4.64	12.63	30.36	6.49	157	21.18	0.89	0.25
1.0	4.12	4.12	6.64	17.97	12.17	36.76	10.82	313	35.05	0.59	0.31
10	6.23	6.23	2.50	21.49	6.84	30.83	15.37	536	40.55	0.68	0.47

* Relative to corresponding control sample incubated without 3-O-methylglucose.

Fig. 4. The increase in ^{14}C (○) and in glucose content (●) in the TCA-soluble fraction in mycelium of *Dendryphiella salina* in relation to the amount of 3-*O*-methylglucose absorbed.

Fig. 5

Fig. 5. The loss of ^{14}C (○) from mannitol and the loss of mannitol (●) from within mycelium of *Dendryphiella salina* in relation to the amount of 3-*O*-methylglucose absorbed. Broken line, 1 h incubation; continuous line, 5 h incubation. Figures at top of graph refer to the initial concentration of the sugar in the medium.

Fig. 6

Fig. 6. The difference between loss of ^{14}C from the ethanol-soluble fraction and gain in ^{14}C by the TCA-soluble fraction in mycelium of *Dendryphiella salina* in relation to the amount of 3-*O*-methylglucose absorbed: ○, 1 h incubation; ●, 5 h incubation.

lost by mannitol or the loss of mannitol itself and 3-*O*-methylglucose uptake is much less clear (Fig. 5). Little was lost at low concentrations of 3-*O*-methylglucose or at any of the concentrations after only 1 h incubation, but after 5 h incubation there was a marked increase in the loss particularly in 10 mmol l⁻¹ 3-*O*-methylglucose. It has been shown previously that the TCA fraction is the sole insoluble recipient of ^{14}C from mannitol and as 3-*O*-methylglucose uptake does not affect respiration, the differences between the loss of ^{14}C from the ethanol-soluble fraction and the gain in ^{14}C by the TCA fraction must be proportional to the loss of [^{14}C]mannitol to the medium. It seems (Fig. 6) that loss to the medium increases with uptake of 3-*O*-methylglucose and that this effect is more pronounced after 5 h incubation than after 1 h. Results from other experiments (e.g. Table 2) confirm

Table 6. ^{14}C content of various fractions (using two different fractionation schemes; see text) of ethanol-insoluble material in the mycelium of *Dendryphiella salina*

Mycelia were preloaded with D-[1- ^{14}C]mannitol in CaCl_2 /sea water for 2 h, and incubated for 1 h with or without 3-*O*-methylglucose in the same medium.

Fraction	3- <i>O</i> -Methyl- glucose ...	$10^{-5} \times ^{14}\text{C}$ content of fraction (d min^{-1})		
		Mycelium A		Mycelium B
		Present	Absent	Present
Organic acid		*	*	—
Protease		*	*	3.64
TCA		5.69	2.26	1.40
H_2SO_4		1.37	1.45	1.25
$\text{NaOH}(3)$		1.87	2.15	1.70
Final residue		0.64	0.71	0.65

Protease-soluble + TCA-soluble material in mycelium B = $5.04 \times 10^5 \text{ d min}^{-1}$

Increase of ^{14}C in TCA-soluble material in mycelium A as a result of incubation in 3-*O*-methylglucose = $3.43 \times 10^5 \text{ d min}^{-1}$

* Not treated with reagent.

that uptake of glucose or 3-*O*-methylglucose increases the loss of labelled mannitol from the mycelium after 1 h but this was not always apparent after shorter periods.

Investigation of the identity of TCA-soluble polysaccharide

Although investigation of the physiological role of the TCA-soluble material was the main aim of our experiments, we carried out a preliminary study of its structure.

Identical samples of mycelium (4.5 g wet wt) were preloaded with D-[1- ^{14}C]mannitol, and incubated with 2 mmol l^{-1} 3-*O*-methylglucose for 1 h. One sample was then extracted in the normal manner (mycelium A). The other (mycelium B) was extracted as usual up to and including the second NaOH extraction, but was then treated with dilute organic acids (0.1 mol l^{-1} citric acid for 16 h at 20 °C and for 1 h at 60 °C; 0.01 M and 0.2 M-acetic acid for 1 h at 60 °C, and 0.1 M-TCA for 16 h at 20 °C) and protease, before continuing the extraction according to the normal procedure (Table 6).

Extraction with organic acids yielded negligible amounts of ^{14}C -labelled material. Homogenization of the mycelium followed by extraction with protease (three times) yielded a substantial amount of radioactivity; further treatment with protease extracted negligible radioactivity. Subsequent extraction with TCA in the normal manner solubilized other ^{14}C -labelled material. The sum of the protease-soluble and TCA-soluble ^{14}C -labelled material from mycelium B was essentially the same as that extracted by TCA from mycelium A.

Subsequent extraction of mycelium B with H_2SO_4 and NaOH yielded essentially the same levels of radioactivity as those observed in equivalent extracts of mycelium A. It is reasonable to conclude that the protease/TCA-soluble material from mycelium B is the same as the TCA fraction of mycelium A. The radioactivity extracted by protease alone ($3.64 \times 10^5 \text{ d min}^{-1}$) was similar to the difference between the radioactivity in the TCA-soluble material in mycelium A and that in mycelium not incubated with 3-*O*-methylglucose ($3.43 \times 10^5 \text{ d min}^{-1}$). This may indicate that newly-synthesized material is protein-bound.

Samples of protease-soluble (mycelium B) and TCA-soluble (mycelium A) material were hydrolysed with sulphuric acid, neutralized with barium carbonate and deionized before chromatographic analysis. Gas-liquid chromatography showed that both extracts mainly consisted of glucose (95 %) with a trace of galactose (5 %). No mannitol or other sugar was

detected. The product of hydrolysis of both samples co-chromatographed on paper (system B) with glucose markers. Areas corresponding to glucose and galactose positions were eluted and assayed for radioactivity. The results showed that only glucose was labelled. Incubation of hydrolysates with glucose oxidase and subsequent t.l.c. (systems C and D) showed that all ^{14}C was located at the position of gluconic acid.

These results indicate that the transfer of ^{14}C from ethanol-soluble to insoluble material during glucose uptake involves metabolism of mannitol to a polysaccharide whose constituent monomer is D-glucose. Paper chromatography (systems A and B) of unhydrolysed protease-treated extract and the subsequent TCA extracts revealed that TCA-extracted material contained significant amounts of free reducing sugars whereas material extracted by protease contained little. The polysaccharide appears to be acid labile, even 0.1 M-TCA causing significant hydrolysis. Protein assay of TCA-soluble material gave a value of 0.4 %. This was precipitated by adding solid TCA (5 %, w/v) before dialysis.

Samples of both the protease-soluble and the TCA-soluble extracts were dialysed and lyophilized, and each gave a creamy white, slightly fibrous, solid. Infrared spectra of this material (in KBr discs) were recorded together with those of authentic samples of glycogen, nigeran, laminarin and cellulose. The diagnostic peaks, at 930, 860–850, 760 and 705 cm^{-1} , of a glycogen-type polysaccharide were readily distinguishable. The spectrum indicated the presence of (1 $\rightarrow$ 4)- α linkages.

Dialysed samples of protease-soluble and TCA-soluble material were completely hydrolysed by amyloglucosidase. Samples of the incubation mixture were separated by t.l.c. (system C) and stained for reducing sugars. Neither sample contained free sugars before incubation but both gave a strong reducing sugar reaction after incubation for 3 h, as did the reference α -D-glucan, glycogen. Laminarin [(1 $\rightarrow$ 3)- β -glucan] and cellulose [(1 $\rightarrow$ 4)- β -glucan] gave negative results.

Similar incubations with a (1 $\rightarrow$ 3)- β -glucanase preparation from *Streptomyces griseus* indicated that only a trace amount of reducing sugar was released from protease-soluble and TCA-soluble extracts. The reference (1 $\rightarrow$ 3)- β -glucan, laminarin, gave a strong reducing sugar reaction. Glycogen gave a negative result. The (1 $\rightarrow$ 3)- β -glucanase preparation has slight activity against (1 $\rightarrow$ 3)- α and (1 $\rightarrow$ 6)- α glucosidic linkages but it is not active against (1 $\rightarrow$ 4)- α (cellulosic) linkages (A. T. Bull, personal communication). The trace amount of hydrolysis detected in protease and TCA extracts could possibly indicate a small proportion of (1 $\rightarrow$ 3)- α glucosidic linkages in the polysaccharide.

Extraction and anthrone assay of TCA-soluble material from ethanol-extracted mycelium of known dry weight indicated that the TCA-soluble polysaccharide constituted approximately 15 % of the ethanol-extracted mycelium. This could presumably vary according to cultural conditions.

Once extracted from the mycelium, the TCA-soluble polysaccharide was readily soluble in cold aqueous solutions including 2 M-NaOH. Its insolubility in alkali *in situ* must therefore be due to it being bound in some way, possibly by protein, or to it being unable to pass through the wall.

DISCUSSION

Our experiments show that the uptake of glucose or 3-O-methylglucose by *Dendryphiella salina* causes a reduction in the level of ethanol-soluble carbohydrates and an increase in one particular polysaccharide fraction, which cannot be extracted with alkali but is extracted by trichloroacetic acid, and which is predominantly a (1 $\rightarrow$ 4)- α -glucan, probably glycogen.

Both glucose and 3-O-methylglucose cause ^{14}C to be transferred from mannitol to the

TCA-soluble fraction. This indicates that it is a normal response of glucose uptake rather than an abnormal one due to uptake of a non-metabolizable analogue. Lowe & Jennings (1975) provided evidence that mannitol can act as an intermediate in the incorporation of radioactivity from glucose into polysaccharide hydrolysable by 5 M-sulphuric acid. However, in our experiments, we are not observing polysaccharide biosynthesis due to an increased level of glucose in the mycelium because the transfer of ^{14}C into (1 $\rightarrow$ 4)- α -glucan is brought about by the non-metabolizable sugar, 3-*O*-methylglucose. Further, whereas mycelium preloaded with D-[1- ^{14}C]mannitol continued to show a general synthesis of polysaccharide material when incubated in the absence of glucose or 3-*O*-methylglucose, when either of these two sugars was being absorbed there was a specific increase in the production of TCA-soluble polysaccharide. This increase occurred with only a slight decrease, if any, in the production of other insoluble fractions, and therefore was not due to a diversion of precursors but to an increase in total polysaccharide synthesis channelled to a specific fraction.

The transfer of ^{14}C from mannitol to the TCA-fraction increased linearly with external 3-*O*-methylglucose concentrations up to 4 mmol l $^{-1}$ but then showed little further increase with increasing concentration (Table 3). However, the transfer of ^{14}C increased linearly with internal 3-*O*-methylglucose concentration up to a level corresponding to an external concentration of 16 mmol l $^{-1}$. Thus, the uptake of 3-*O*-methylglucose could be the rate-limiting step in the transfer of ^{14}C to the TCA fraction during uptake under these conditions. At 3-*O*-methylglucose concentrations above 16 mmol l $^{-1}$, the transfer of ^{14}C to the TCA fraction fell sharply.

As noted earlier, there was no evidence for any significant transfer of material (as opposed to ^{14}C) from mannitol to the TCA-soluble fraction in mycelium incubated for 30 min or less. Although ^{14}C transfer to the TCA fraction increased linearly with respect to the intracellular levels of 3-*O*-methylglucose, this does not mean that 3-*O*-methylglucose uptake was accompanied by an immediate synthesis of polysaccharide or loss of mannitol on a 1:1 basis to compensate for uptake of the sugar. The most probable sequence of events is an initial increase in turgor with sugar uptake followed by a more gradual adjustment of the total content of soluble carbohydrates. When the uptake of 3-*O*-methylglucose is too great (as at 32 or 64 mmol l $^{-1}$), the adjustment is inadequate and the hyphal tips burst.

When mycelia were incubated for 1 or 5 h there was detectable biosynthesis of TCA-soluble polysaccharide and loss of mannitol. The data from this experiment allow several conclusions to be drawn. The ratio of the increase in TCA-soluble polysaccharide to the uptake of 3-*O*-methylglucose was essentially constant with time and was reflected in the approximately linear relationship between gain of ^{14}C or carbohydrate by the TCA fraction and 3-*O*-methylglucose uptake. Thus there was apparently no time lag between uptake of 3-*O*-methylglucose and polysaccharide biosynthesis.

In contrast, the ratio of the loss of mannitol from the ethanol-soluble fraction to the uptake of 3-*O*-methylglucose increased from 0.1 to 0.2 at 1 h to 0.72 at 5 h, indicating that there was a delayed response to the uptake of 3-*O*-methylglucose. Figure 6 indicates that the long-term response to uptake of 3-*O*-methylglucose was an increased loss of mannitol into the medium. This was true even though the amount of 3-*O*-methylglucose in the mycelium in some treatments (those with 10 mmol l $^{-1}$ 3-*O*-methylglucose) was greater at 1 h than that in mycelium in other treatments (in 0.1 mmol l $^{-1}$ 3-*O*-methylglucose) after 5 h. There was no constant ratio between the loss of mannitol and carbohydrate gained by the TCA fraction. Long exposure of mycelium to 3-*O*-methylglucose clearly increased leakage of soluble material. These conclusions about leakage of mannitol from the mycelium are not based on direct measurements.

Calculation of the amounts of mannitol transferred to the TCA fraction based on values of the specific activity of mannitol and the amount of ^{14}C gained by the fraction show that the increases in TCA-soluble carbohydrates cannot be accounted for by the transfer of mannitol unless there is preferential utilization of unlabelled molecules. Although initially there may be some compartmentation of unlabelled mannitol already in the mycelium and ^{14}C -mannitol absorbed during preloading, a similar argument cannot be used to explain the rise in specific activity of the TCA fraction, even in cases where the polysaccharide content is greatly reduced during incubation. The data are best explained on the basis of the inhibition of polysaccharide breakdown by 3-*O*-methylglucose.

That breakdown of polysaccharide in the TCA fraction can proceed at the same time as synthesis is demonstrated in mycelium in which the ^{14}C content of the TCA fraction rises at the same time as the total amount falls.

In the absence of 3-*O*-methylglucose (zero-substrate mycelium), there is a net loss of TCA-soluble polysaccharide as breakdown apparently exceeds synthesis. After 5 h, the polysaccharide content is reduced by about 50 %. The ^{14}C content of the TCA fraction in zero-time mycelium was negligible compared with that of the ethanol-soluble fraction. Therefore breakdown of the polysaccharide to glucose, which is converted to ^{12}C -mannitol, results in dilution of the ^{14}C -labelled mannitol, reducing its specific activity. Conversely, any contemporaneous synthesis of polysaccharide in the TCA fraction leads to an increase in its ^{14}C content and specific activity. A similar situation appears to hold in mycelium incubated for 5 h with 0.1 mmol l⁻¹ 3-*O*-methylglucose. There is a greater synthesis of TCA-soluble material and the specific activity also increases, but breakdown is still sufficient to cause an overall loss of polysaccharide from the zero-time level. The loss is only slightly less than that observed in mycelium incubated in the absence of sugar. When mycelium was incubated in 1 mmol l⁻¹ 3-*O*-methylglucose for 5 h, synthesis approximately equalled breakdown as there was little change in the carbohydrate content of the TCA fraction. The ^{14}C content and the specific activity of the TCA fraction are further increased compared with mycelium incubated in the absence or in the presence of 0.1 mmol l⁻¹ 3-*O*-methylglucose. With 10 mmol l⁻¹ 3-*O*-methylglucose, synthesis significantly exceeds breakdown leading to both an overall increase in the amount as well as in the specific activity of the TCA-soluble polysaccharide.

Confirmation that hydrolysis of the TCA fraction is accompanied by synthesis of mannitol comes from results for mycelium incubated in the absence of 3-*O*-methylglucose. After 1 h, the mannitol level had dropped sharply, whereas there was only a slight decline in the carbohydrate level in the TCA fraction. After 5 h, the mannitol concentration has been restored to its initial level and the carbohydrate level in the TCA fraction had decreased by an equivalent amount.

After prolonged incubation with 3-*O*-methylglucose, the sharp increase in mannitol lost to the medium, particularly marked at 10 mmol l⁻¹ 3-*O*-methylglucose, seems more likely to be due to imbalanced metabolism in the presence of the non-metabolizable sugar than to a controlled response by the mycelium. The sudden drop in the other major soluble compound, arabinol, the level of which remained constant in other samples, seems to support this conclusion.

The overall pattern of events during 3-*O*-methylglucose uptake appears to be an initial rise in the total amount of soluble carbohydrate which is then gradually reduced by loss of mannitol to the medium and enhanced by the lack of breakdown of polysaccharide caused by inhibition of hydrolysis by the glucose analogue. Some mannitol will be continuously lost by respiration. Above a certain external concentration (16 mmol l⁻¹) hyphal tips burst and the ability to synthesize TCA-soluble polysaccharide is lost. Although the net increase in the

TCA fraction is important in reducing the soluble carbohydrate content, prolonged exposure to the non-metabolizable sugar could lead to intracellular levels of 3-*O*-methylglucose at which inhibition of polysaccharide hydrolysis is maximal and significant reduction of the soluble carbohydrate content can only be brought about by loss to the medium. The degree of control retained by the mycelium over this leakage to the medium probably varies with the intracellular level of 3-*O*-methylglucose and with time exposure. However, by a combination of net production of polysaccharide and a variable loss of soluble sugars (particularly mannitol) the mycelia maintain an essentially constant level of soluble carbohydrates. With reference to this we note that, after 5 h incubation, the ratio of (μmol mannitol lost plus μmol gained by TCA fraction) to (μmol 3-*O*-methylglucose uptake) is close to unity.

The situation in mycelia incubated with glucose is expected to differ from that encountered with 3-*O*-methylglucose particularly for prolonged incubation. Most of our experiments were carried out with 3-*O*-methylglucose and not glucose to avoid the complicating factor of the metabolism of the latter, and it is this metabolic use of glucose which would result in differences on prolonged incubation.

For periods up to 1 h, glucose and 3-*O*-methylglucose had similar effects on mycelia, causing the same transfer of ^{14}C from mannitol to the TCA fraction. We therefore conclude that glucose also inhibits polysaccharide hydrolysis. However, prolonged uptake of glucose will allow the replenishment and even an increase of the amount of mannitol in the mycelium (Holligan & Jennings, 1972*a*, *b*). Uptake of glucose would also provide a substrate for respiration which would not only remove soluble sugar but could also provide energy for other metabolic processes including, under certain circumstances, the synthesis of other polysaccharides from precursors which will also be produced from glucose.

The fact that glucose can be metabolized provides an explanation for the absence of an increase in insoluble polysaccharide when mycelium is incubated with glucose (Jennings & Austin, 1973). Under the conditions used it is probable that glucose in the medium was exhausted before the end of the 3 h incubation period. Continued metabolism, for example respiration of absorbed glucose, would lead to a decrease in mannitol which, in turn, would be replenished from TCA-soluble polysaccharide. Any inhibition of polysaccharide hydrolysis by glucose or its metabolic products would decline as the sugar was metabolized. At the end of incubation, therefore, it is not surprising that insoluble polysaccharide declined to a level similar to that in the control mycelium.

In summary, it seems that if *Dendryphiella salina* is utilizing glucose as a carbon source, uptake of the sugar from relatively dilute solutions is gradually compensated by a net gain of TCA-soluble polysaccharide if the hyphae are strong enough to survive the temporary increase in turgor. Some loss of mannitol into the medium also occurs but the amount appears to be only significant after prolonged incubation with a non-metabolizable substrate.

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