The Nature of Reducing Compounds Formed from Sucrose by *Erwinia carotovora* var. *atroseptica*

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SUMMARY

When grown in a medium containing 2 to 4 % sucrose, *Erwinia carotovora* var. *atroseptica* formed two reducing compounds which accumulated in the culture medium. These compounds had the properties of the disaccharides 6-*o*- α -D-glucopyranosyl-D-fructofuranose (palatinose, isomaltulose) and 1-*o*- α -D-glucosyl-fructose.

INTRODUCTION

When strains of *Erwinia carotovora* var. *atroseptica* were grown in a medium containing 2 % (w/v) sucrose, a compound accumulated which reduced 2,3,5-triphenyltetrazolium chloride (Moustafa & Whittenbury, 1970). The ability to produce this reducing material when grown in a sucrose medium has subsequently been used to differentiate *E. carotovora* var. *atroseptica* from *E. carotovora* var. *carotovora* (Graham, 1971). The reducing substance was not glucose, fructose or 2-keto-gluconate (Moustafa & Whittenbury, 1970), and D. C. Graham (personal communication) demonstrated by thin-layer chromatography that two reducing compounds were formed. Neither compound was 3-ketosucrose, which is produced by *Agrobacterium tumefaciens* (Bernaerts & De Ley, 1963). Graham suggested that they were tri- or tetra-saccharides. This paper concerns the nature of these reducing compounds.

METHODS

Sugars. The following sugars were used, or are referred to, as reference compounds: glucose, fructose, β -D-fructofuranosyl- α -D-glucopyranose (sucrose), 3-*o*- α -D-glucopyranosyl-D-fructose (turanose), 4-*o*- α -D-glucopyranosyl-D-fructose (maltulose), 5-*o*- α -D-glucopyranosyl-D-fructopyranose (leucrose), 6-*o*- α -D-glucopyranosyl-D-fructofuranose (palatinose, isomaltulose), 4-*o*- α -D-glucopyranosyl-D-glucopyranose (maltose), 4-*o*- β -D-glucopyranosyl-D-glucopyranose (cellobiose). Glucose, fructose, sucrose, turanose, palatinose, maltose and cellobiose were obtained from the Sigma Chemical Co., London. A sample of leucrose, used for mass spectrometry, was obtained from Professor E. J. Bourne.

Enzymes. Yeast α -glucosidase and almond β -glucosidase were obtained from the Sigma Chemical Co.

Bacteria. *Erwinia carotovora* var. *atroseptica* strain G120 (Lund & Nicholls, 1970) was originally obtained from Dr D. C. Graham. Other strains were laboratory isolates from potatoes. *E. carotovora* var. *carotovora* was a laboratory isolate from cauliflower.

Production of culture liquid containing reducing compounds. The bacteria were grown in shaken flasks for 3 days at 25 °C in a medium containing: peptone (Difco), 1 % (w/v); beef

extract powder (Lab. Lemco), 0.4 % (w/v); sucrose, 4 % (w/v); (Graham, 1971). The bacteria were removed by centrifugation and the culture liquid was stored at -25°C .

Paper chromatography. Descending chromatography on Whatman 3MM paper was used with the following solvent systems: (i) isopropanol–water (4:1, v/v); (ii) *n*-butanol–pyridine–water (5:3:2, by vol.); (iii) *n*-butanol–acetic acid–water (12:3:5, by vol.). The developing times were 48 to 54 h for solvents (i) and (ii) and 17 h for solvent (iii). Disaccharides from the crude culture liquid were purified by band chromatography on Whatman 3MM paper using solvent system (ii); two successive separations were made, washed paper being used for the second to minimize impurities from the paper. Results were expressed as R_g values, where R_g is the distance travelled by the compound/distance travelled by glucose.

Paper electrophoresis. The apparatus used was supplied by Locarte Co., 24 Emperor's Gate, London, SW7. Electrophoresis was on a strip of Whatman 3MM paper 10 cm wide, with 0.1 M-sodium borate buffer, pH 10, as electrolyte (Avigad, 1959; Foster, 1953). One thousand volts were applied (20 V/cm) for 5 h, giving a starting current of 50 to 60 mA. Sugars were detected by spray reagent D (see below). The apparent distances of migration were corrected for movement due to electroendosmosis by reference to movement of 2,3,4,6-tetramethylglucose; under the conditions employed the true distance of migration of glucose was about 20 cm. Results were expressed as M_g values, where M_g is the distance travelled by the compound/distance travelled by glucose.

Selective spray reagents. The spray reagents used were: A, silver nitrate–ammonia reagent (Partridge, 1948) for detection of reducing sugars; B, urea–phosphoric acid (Wise, Dimler, Davis & Rist, 1955) for detection of ketoses; C, aniline–diphenylamine–phosphoric acid for distinguishing between 1,4-linked and 1,6-linked glucosaccharides (Schwimmer & Bevenue, 1956); D, aniline phosphate (Frahn & Mills, 1959; Hough, Jones & Wadman, 1950) for reducing sugars and sucrose; E, glucose oxidase reagent for blood sugar determination (Boehringer Co. Ltd) to confirm the identity of glucose on chromatograms.

Acid hydrolysis of sugars. Hydrolysis was effected by heating in a boiling water bath in 0.5 N-sulphuric acid for 2 h. After neutralizing, the hydrolysate was evaporated to dryness under reduced pressure and the sugars extracted with hot ethanol.

Enzymic hydrolysis of sugars. Susceptibility to α - and β -glucosidase was tested by superimposing enzyme solution on the sugar spot on Whatman 3MM paper and subsequently separating the hydrolysis products by chromatography (Williams & Bevenue, 1951).

Reduction of sugars. A 10 ml sample of crude culture liquid was adjusted to pH 8, mixed with 2 ml of 5 % (w/v) sodium borohydride solution and placed overnight at 1°C (Bragg & Hough, 1957). Completion of reduction was checked by acidifying a sample to pH 5, to decompose residual borohydride, and testing the ability of the residual sample to give a precipitate when heated with an equal volume of Benedict's reagent. When reduction was complete, the solution was adjusted to pH 5 to decompose the borohydride. Sodium ions were removed by treatment with Dowex 50W-X8 (H^+), the liquid was evaporated to dryness under reduced pressure and borate removed by repeated evaporation of methanol from the residue which was finally redissolved in distilled water.

Mass spectra. Compound II (see Results and Discussion) was purified by elution from paper chromatograms. Reference compounds were used as supplied, without further purification. Per(trimethylsilyl)ethers of sugars were prepared as follows: to 0.1 to 1.0 mg of sugar was added 50 to 100 μl of Tri-Sil 'Z' reagent, containing 1.5 m-equiv. *N*-trimethylsilylimidazole/ml in silylation-grade pyridine (Phase Separations Ltd, Deeside Industrial Estate, Queensferry, Flintshire); the reaction was allowed to proceed for 1 h at room temperature in a sealed ampoule. Mass spectra were recorded on an AEI MS 902 double-focusing mass spectrometer

at an ionizing potential of 70 eV and an ion chamber temperature of 220 °C. Intensities, expressed relative to the peak at m/e 361 (Kamerling, Vliegthart, Vink & de Ridder, 1971; 1972) are given in parenthesis for the molecular ion (M^+), for peaks above m/e 360 with relative intensities greater than 1 %, and for peaks between m/e 200 and m/e 360 with relative intensities greater than 10 %.

RESULTS AND DISCUSSION

In preliminary work, when samples of the culture liquid from *Erwinia carotovora* var. *atroseptica* strain G120 were analysed by paper chromatography in solvent (i) for 18 h and spraying with reagent A, only one reducing compound was detected. In subsequent work longer developing times were used (see Methods) resulting in resolution of two reducing compounds, I and II. After chromatography for 18 h in solvent (i), R_g values were as follows: fructose, 1.1; I+II (not resolved), 0.68; lactose, 0.41; α -oxoglutarate, 0.29. Chromatography for 17 h in solvent (iii) gave R_g values: fructose, 1.1; I+II (not resolved), 0.56; lactose, 0.45; α -oxoglutarate, 2.0. Since the R_g values of I+II in contrast to α -oxoglutarate were similar in the neutral and the acidic solvents, it was concluded that compounds I and II were not acidic. Chromatography for 54 h in solvent (i) resolved compounds I and II and showed that they differed in mobility from the reducing compound, 3-ketosucrose, present in the culture liquid of sucrose-grown *Agrobacterium tumefaciens*, confirming D. C. Graham's unpublished report.

Further studies of the properties of these compounds (Table 1) were made after purification by chromatography. The R_g values indicated that I and II were probably disaccharides. Both were ketose-containing compounds which gave a blue colour with spray reagent B and, after acid hydrolysis, released glucose and fructose in approximately equal amounts as judged by intensity of spots on chromatograms and comparison with standards. That glucose was released on hydrolysis was confirmed by spraying the chromatogram with glucose oxidase reagent, spray D. After reduction of the crude culture liquid containing I and II, followed by acid hydrolysis, glucose was the only reducing hexose detectable on paper chromatograms; thus fructose probably provided the terminal reducing group of these compounds. Both I and II were hydrolysed by α -glucosidase to give glucose and fructose. They were not attacked by β -glucosidase under conditions which hydrolysed cellobiose, indicating that I and II were α -glucosyl-fructoses.

Paper chromatography and reactions with spray reagents distinguished I from 1- α -glucosylfructose (Avigad, 1959), sucrose, turanose, maltulose (Avigad, 1959; Täufel, Ruttloff & Täufel, 1967), and leucrose (Wise *et al.* 1955; Stodola, Sharpe & Koepsell, 1956) (Table 1). Compound I showed the same R_g value as palatinose by paper chromatography in solvent systems (i) and (ii) and the same M_g value as palatinose by paper electrophoresis. With the aniline-diphenylamine-phosphoric acid reagent, spray C, both I and palatinose gave a yellow-green colour similar to that reported for 1,6-linked glucosaccharides. These properties indicate that I was palatinose, this conclusion being supported by the value of its optical rotation. The measured optical rotation of I was slightly lower than that of the crystalline reference compound, probably because the sample of I retained a slight impurity and was not crystallized.

By chromatography and its reactions with spray reagents, II could be distinguished from sucrose, turanose, maltulose, leucrose, and palatinose (Table 1). With the aniline-diphenylamine-phosphoric acid reagent, spray C, II gave a red colour, which has previously been reported as being characteristic of 1- α - and 1- β -glucosylfructose, but not of other α -glucosylfructoses (Avigad, 1959). The chromatographic mobility of II was less than

Table 1. *Properties of unknown compounds I and II and reference compounds*

Compound	Linkage	Chromatography R_f^*		Electro-phoresis $M_f^\dagger$	Colour with spray $B^\ddagger$	Colour with spray $C^\ddagger$	α_D (H ₂ O)	
		Solvent (i)	Solvent (ii)				Measured§	Values given in the literature
Glucose	—	I	I	I	N.R.	—	—	—
Fructose	—	I·I	I·I	—	Blue	—	—	—
1- α - α -Glucosyl fructose	α -D-(1 $\rightarrow$ 1)	—	—	—	(Blue)	(Red)	—	+49° (Avigad, 1959)
Sucrose	Glc $\rightarrow$ Fru	0·85	0·83	0·35	Blue	Slate	—	+66·5° (Pazur, 1970)
Turanose	Glc $\rightarrow$ Fru	0·84	0·83	0·75	Blue	—	—	+75° (Avigad, 1959)
Maltulose	α -D-(1 $\rightarrow$ 3)	—	—	—	(Blue)	(Violet)	—	+52 to +60° (Staneck <i>et al.</i> 1965) (Täufel, <i>et al.</i> 1967).
Leucrose	α -D-(1 $\rightarrow$ 5)	—	—	—	(Red-brown)	—	—	—8° (Avigad, 1959)
Palatinose	Glc $\rightarrow$ Fru	0·75	0·75	0·58	Blue	Yellow-green	+96°	+94 to +103° (Staneck <i>et al.</i> 1956; Avigad, 1959)
Maltose	Glc $\rightarrow$ Fru	0·61	0·70	0·48	N.R.	Violet	—	+130° (Pazur, 1970)
Compound I	α -D-(1 $\rightarrow$ 4)	0·75	0·75	0·57	Blue	Yellow-green	+91·5°	—
Compound II	Glc $\rightarrow$ Glc	0·67	0·62	0·75	Blue	Red	+45 to +49°	—

N.R., no reaction.

* Comparable values are given by Avigad (1959).

† Comparable values given by Avigad (1959) and Bourne *et al.* (1961).

‡ Results in brackets are from the literature.

§ Concentrations between 1 and 3 %; temperature 25 °C.

that of the other α -glucosylfructoses tested and was close to that of maltose; similar results were reported for 1- α -glucosylfructose by Avigad (1959) and by Chiba & Shimomura (1971). The electrophoretic mobility of II (M_g 0.75) was comparable to those reported in the same buffer for 1- α -glucosylfructose (M_g 0.70) and 1- β -glucosylfructose (M_g 0.68) by Avigad (1959) and for 1- β -glucosylfructose (M_g 0.74) by Bourne, Hutson & Weigel (1961). The optical rotation of purified II confirmed its probable identity as 1- α -glucosylfructose. An authentic sample of 1- α -glucosylfructose was not available. Further evidence for the identity of II was obtained from mass spectrometry of the per(trimethylsilyl) (TMS) derivative of this compound and of other α -glucosylfructoses. For II the most significant peaks were: m/e 918 (M^+ , 0.15), 813 (1.0), 723 (1.0), 684 (14.1), 569 (4.5), 539 (5.1), 525 (1.0), 451 (4.5), 437 (505.0), 435 (8.1), 407 (1.5), 393 (1.0), 361 (100), 319 (43.4), 307 (15.2), 305 (13.6), 231 (12.6), 230 (13.1), 217 (437), 204 (276). Most of these peaks correspond to fragment ions previously reported as characteristic of TMS-glucosyl-aldohexoses and TMS-glucosylfructoses (Kamerling *et al.* 1971; 1972). The spectrum agreed closely with that previously reported for 1- β -glucosylfructose (Kamerling *et al.* 1972), and spectra for sucrose, turanose, leucrose and palatinose were also similar to the spectra reported by these authors. A distinctive feature of the mass spectrum of II was the intense ion at m/e 437, shown by these authors to differentiate the 1 $\rightarrow$ 1 and 1 $\rightarrow$ 2 glucosylfructoses from the 1 $\rightarrow$ 3, 1 $\rightarrow$ 4, 1 $\rightarrow$ 5 and 1 $\rightarrow$ 6 hexosylfructoses. Previous tests having shown that II was not 1 $\rightarrow$ 2 glucosylfructose, the mass spectral data confirmed its identity as a 1- α -glucosylfructose.

No quantitative estimations were made of compounds I and II, but they were apparently formed in about equal amounts. Examination by paper chromatography in solvent (ii) of culture filtrates of ten further isolates of *Erwinia carotovora* var. *atroseptica* also showed reducing compounds corresponding in position to the same pair of disaccharides; these compounds were not detected in cultures of a strain of *E. carotovora* var. *carotovora*.

Production of reducing disaccharides containing glucose and fructose by α -D-glucosyl transfer from sucrose has previously been reported for several bacteria. Weidenhagen & Lorenz (1957) obtained nearly 90 % conversion of sucrose into palatinose by a red-pigmented bacterium, named *Protaminobacter rubrum*, isolated from a sugar-beet processing factory (Weidenhagen, 1961). Leucrose and palatinose have been isolated as by-products during dextran production from sucrose by *Leuconostoc mesenteroides* (Stodola *et al.* 1956; Sharpe, Stodola & Koepsell, 1960) and by *Streptococcus bovis* (Bailey & Bourne, 1959; Bourne *et al.* 1961). There are few reports of microbial production of 1- α -glucosylfructose. A mixture of palatinose and 1- α -glucosylfructose in the ratio of about 10 to 1, together with traces of other di- and tri-saccharides, was produced by the action of a haploid-yeast α -glucosidase on sucrose (Avigad, 1959). More recently, formation of a mixture of 1- α -glycosylfructose, turanose, maltulose, isomaltose and nigerose was reported when phenyl- α -glucoside and D-fructose were incubated with brewer's yeast α -glucosidase (Chiba & Shimomura, 1971).

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