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Phosphorylation and metabolism of sucrose and its five linkage-isomeric α-D-glucosyl-D-fructoses by *Klebsiella pneumoniae*

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Abstract

Not only sucrose but the five isomeric α-D-glucosyl-D-fructoses trehalulose, turanose, maltulose, leucrose, and palatinose are utilized by *Klebsiella pneumoniae* as energy sources for growth, thereby undergoing phosphorylation by a phosphoenolpyruvate-dependent phosphotransferase system uniformly at O-6 of the glucosyl moiety. Similarly, maltose, isomaltose, and maltitol, when exposed to these conditions, are phosphorylated regiospecifically at O-6 of their non-reducing glucose portion. The structures of these novel compounds have been established unequivocally by enzymatic analysis, acid hydrolysis, FAB negative-ion spectrometry, and ^{1}H and ^{13}C NMR spectroscopy. In cells of *K. pneumoniae*, hydrolysis of sucrose 6-phosphate is catalyzed by sucrose 6-phosphate hydrolase from Family 32 of the glycosylhydrolase superfamily. The five 6′-O-phosphorylated α-D-glucosyl-fructoses are hydrolyzed by an inducible ($\sim$49–50 Kda) phospho-α-glucosidase from Family 4 of the glycosylhydrolase superfamily.

Keywords: Sucrose 6-phosphate; 6′-Phospho-α-D-glucosyl-D-fructoses; *Klebsiella pneumoniae*; Phospho-α-glucosidase; Sucrose 6-phosphate hydrolase; Family 4

1. Introduction

Two factors implicate sucrose (β-D-fructofuranosyl α-D-glucopyranoside) as a major contributor to the etiology of dental caries.[1] First, this dietary sugar is the precursor for synthesis of glucan that facilitates adherence of oral bacteria to the tooth surface. Second,

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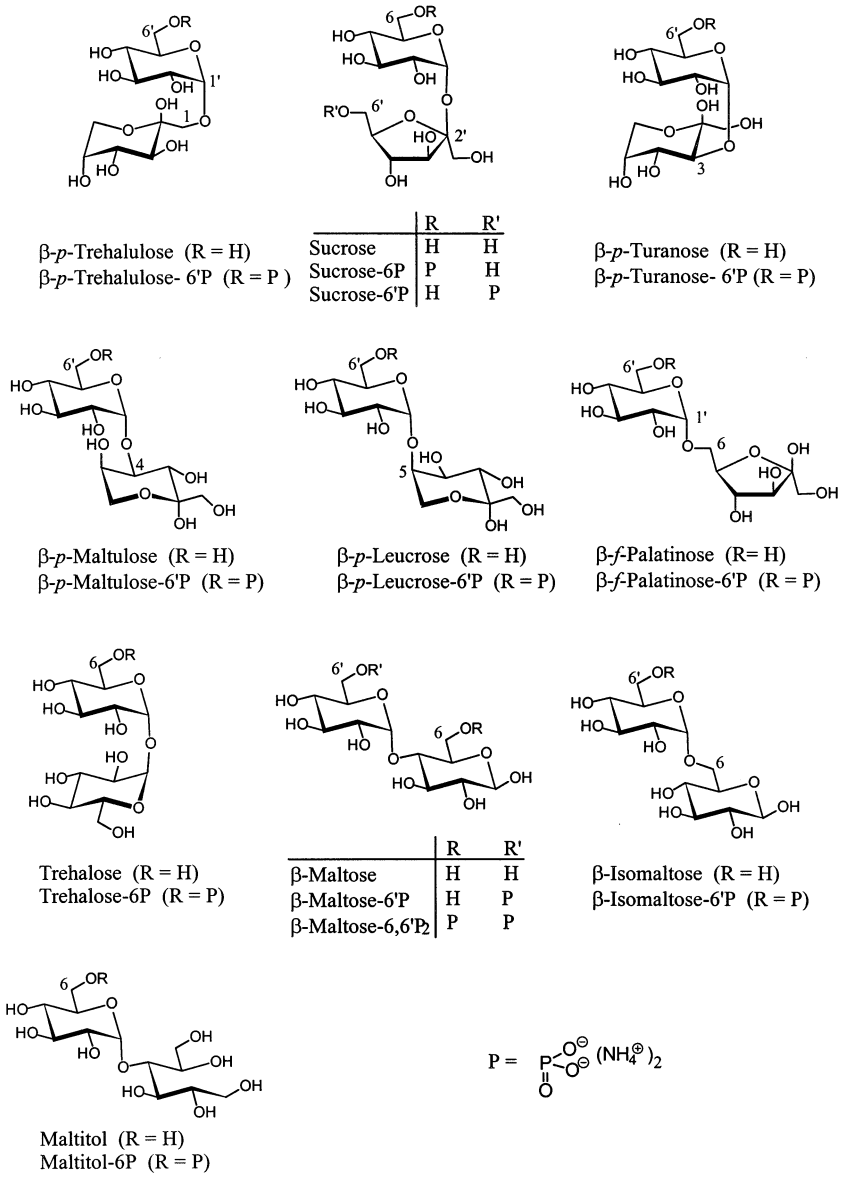

Scheme 1. Formulae of the disaccharides and their 6- resp. 6′-phosphates in the preferred ring hemiacetal (furanose or pyranose) form and anomeric configuration.

fermentation of the α-(1↔2) linked disaccharide (to lactic acid) by streptococcal species initiates the demineralization of tooth enamel. Oral streptococci, e.g., *Streptococcus mutans*[2] and *S. sobrinus*,[3] and many other microorganisms including *Lactococcus lactis*,[4] *Bacillus subtilis*,[5] *Fusobacterium mortiferum*,[6] *Escherichia coli*[7] and *Klebsiella pneumoniae*,[8] transport sucrose simultaneously with phosphorylation at O-6 of the glucosyl moiety via the multi-component phosphoenolpyruvate-dependent sugar phosphotransferase system.[9] Intracellular sucrose 6-phosphate is hydrolyzed by sucrose-6-phosphate-hydrolase,[10] and the reaction products, glucose 6-phosphate and fructose, are metabolized to lactic acid via the Embden–Meyerhof glycolytic pathway.[11]

Although *S. mutans* readily ferments sucrose, its five isomeric α-D-glucosyl-D-fructoses, i.e., trehalulose [intersaccharidic linkage: α-(1→1)], turanose [α-(1→3)], maltulose [α-(1→4)], leucrose [α-(1→5)], and palatinose [α-(1→6)] are not metabolized by mutans streptococci.[12] Indeed, to our knowledge, there are no reports of the utilization of disaccharides of the α-D-glucosyl-fructose type (Scheme 1) other than sucrose by any bacterial

Table 1
Rates of disaccharide utilization (in nmol min^{-1} mg dry weight of $cells^{-1}$) in the fermentation of sucrose and its isomeric α-D-glucosyl-D-fructoses by washed cells of *K. pneumoniae*

Cells grown on	Disaccharide present in fermentation assay [a]					
	Trehalulose	Sucrose	Turanose	Maltulose	Leucrose	Palatinose
Trehalulose	48.0	75.2	27.1	42.4	34.1	45.4
Sucrose	[b]	38.8	[b]	[b]	[b]	[b]
Turanose	58.8	92.7	53.4	51.1	39.0	53.4
Maltulose	40.3	58.5	37.6	48.6	45.1	44.9
Leucrose	35.5	67.6	28.6	42.7	33.6	36.0
Palatinose	45.1	50.6	38.3	43.6	41.1	39.7

[a] Disaccharide concentration in fermentation assay: 10 mM.
[b] No detectable metabolism.

species. It was, therefore, unexpected to find that all five sucrose analogs supported growth of *K. pneumoniae*. It was equally surprising to discover that whereas sucrose-grown organisms readily fermented sucrose, the same cells failed to metabolize any of the isomeric glucosyl-fructoses. Conversely, washed cells of *K. pneumoniae* grown previously on the individual isomers, rapidly fermented sucrose and all other isomeric compounds. In this communication, besides offering explanations for these findings, we present a simple procedure for the regiospecific phosphorylation of sucrose, and its five linkage-isomeric α-D-glucopyranosyl-D-fructoses at their glucosyl-O-6 position, in a quantity sufficient for enzymatic assays and unequivocal structural proof.

2. Results

Fermentation of sucrose and isomers.—Cells of *K. pneumoniae* ATCC 23357 utilized sucrose and each of its five isomeric α-D-glucosyl-D-fructoses as energy sources for growth. Furthermore washed cells, grown previously on any of these glucosyl-fructoses, readily metabolized sucrose and all of its isomeric disaccharides (Table 1), whereas sucrose-grown cells fermented only sucrose.

Of several explanations for these unexpected findings, we considered it plausible that sucrose 6-phosphate hydrolase present in *K. pneumoniae* was unable to hydrolyze the (putative) isomeric phosphates. To address this issue, the five α-D-glucosyl-D-fructose phosphates, presumably phosphorylated at their glucosyl-C-6, and all unknown so far, had to be generated in quantity to prove their structure.

Phosphorylation of sucrose and the α-D-glucosyl-D-fructoses.—The sucrose 6- and maltose 6′-phosphates, both carrying their phosphoric ester group at the glucopyranosidic C-6 (rather than at the primary hydroxyl of the fructose resp. reducing glucose portion) have previously been prepared via the PEP-dependent phosphotransferase system in permeabilized cells of *S. mutans*[13] and *F. mortiferum*,[10f,14] respectively. Modification of these procedures (by working with permeabilized cells of *K. pneumoniae* at low pH) not only permitted the ready biosynthesis of sucrose 6-phosphate, but also of a variety of other disaccharide 6′-phosphates such as those from all of the α-D-glucosyl-D-fructoses, differing from sucrose 6-phosphate only in their intersaccharidic linkage (Scheme 1), and the respective analogs derived from maltose, isomaltose, and maltitol.

Structural proof and assignment of phosphorylation sites.—The molecular formula for each of the newly prepared phosphates was secured by negative-ion FAB mass spectrometry, i.e. m/z of 421.1 for all disaccharide monophosphates (corresponding to $C_{12}H_{22}O_{14}P$), 423.2 for maltitol-6-phosphate, and 501 for maltose-6,6′-diphosphate. Acid hydrolysis of maltose 6′-phosphate (1 M HCl at 100 °C for 10 min) produced glucose 6-phosphate and glucose in a 1:1 ratio. However, the non-stoichiometry of the glucose-6P:fructose ratio for

sucrose 6-phosphate and the phosphorylated α-D-glucosyl-fructoses was only 1:0.4–0.6, indicating significant loss of the ketose moiety during their acid hydrolysis. Nevertheless, these simple hydrolytic studies already provide ample evidence for the phosphate ester group invariably being situated at the primary glucosyl-O-6. This assignment is cogently substantiated by distinct ^{1}H as well as ^{13}C NMR chemical shift differences between the parent disaccharides and their 6- resp. 6′-phosphates.

With respect to the ^{1}H NMR signals, phosphorylation of a hydroxyl group in a mono- or disaccharide usually results[15–17] in a small but distinctive downfield shift of 0.2–0.5 ppm for the protons attached to the carbon carrying phosphate ester group (here the 6-CH_2), whereas hydrogens situated vicinal thereto (H-5) and in the β-position (H-4) exhibit only minor deshielding. As all other hydrogens usually resonate at or about the chemical shift values of the parent sugar, the analysis of the ^{1}H NMR chemical shift differences of a mono- or a disaccharide phosphate permits the position of phosphorylation to be determined.

In the case of the five α-D-glucosyl-D-fructoses and their 6′-phosphates, however, this approach towards proof of phosphorylation sites is complicated by adoption of up to four tautomeric forms in aqueous solution (D_2O), of which each can give rise to a set of different chemical shifts and coupling patterns, thereby grossly impeding assignments. Fortunately though, similar to D-fructose itself where the β-pyranoid form is highly preferred (72% in water at 25 °C[18]), the fructose portions of the glucosyl-fructoses elaborate tautomeric equilibria with the respective β-fructopyranose forms prepondering:[19] 71% β-*p* tautomer for trehalulose in water at 20 °C, 47% for turanose, maltulose (73%) and leucrose (98%). Only palatinose, due to carrying the glucosyl residue at O-6 of the fructose portion, can adopt only furanoid forms, featuring 80% β-furanose at 20 °C.[19] For the respective 6′-phosphates of the five glucosyl-fructoses, the distribution of tautomers in aqueous solutions were surmised to be the same or very similar, in as much as the phosphate ester group at one end of the disaccharide should have little if any effect on the anomeric carbon at the other. In fact, this turned out to be the case, since the readily identifiable anomeric protons (i.e., H-1 of the glucosyl portions) showed practically identical intensities for both phosphate derivative and parent sugar.

The ^{1}H NMR data listed in Table 2 for the five glucosyl-fructoses and their 6′-phosphates are those for the major tautomer present in solution, identified in the spectra via the intensity of the anomeric glucosyl H-1, well separated from the other protons. Detailed inspection in Table 2 of the proton chemical shifts of the fructose unit reveals practically no differences between phosphate and parent sugar, clearly indicating the fructose residues to be unsubstituted. That, in turn, all of the five α-D-glucosyl-D-fructoses are phosphorylated at O-6 of their glucosyl moiety, is evidenced by the 0.2 ppm downfield shift of the respective 6-CH_2 protons relative to their parent disaccharides, whilst all other resonances remain essentially constant. Further substantiation for this assignment is provided by the fact that sucrose 6-phosphate also showed this 0.2 ppm downfield shift as compared to sucrose,[17] and that sucrose 6′-phosphate, a biosynthetic precursor of sucrose,[20] carrying its phosphate ester group at C-6 of the fructosyl portion, reveals the downfield shift for the fructofuranosyl-6-CH_2 protons, an outcome that is similarly observed for D-fructose 6-phosphate and its 1,6- and 2,6-diphosphates (Table 2).

The products obtained by phosphorylation of trehalose, maltose, isomaltose and maltitol exhibited analogous down-field shifts for the 6-CH_2 protons of their non-reducing glucoside half (Table 3), whereas in the other glucose resp. glucitol unit the chemical shift values for phosphate and parent disaccharide are practically unchanged, findings that are clearly consistent with their phosphate ester group being attached to the primary C-6 of the non-reducing glucoside residue.

In order to get some insight into conceivable relationships between the preferred orientations of the 6-hydroxyl versus the 6-phosphate ester group (relative to the pyranoid ring), a more detailed analysis of the ^{1}H

Table 2
^{1}H Chemical shifts (500 MHz in D_2O, 25 °C, ppm from internal TSP [a]) of D-fructose, D-glucose, sucrose, and the five isomeric α-D-glucosyl-fructoses in comparison with their 6- resp. 6′-phosphates [b]

Compound	α-D-Glucopyranosyl residue						D-Fructose unit				
	H-1	H-2	H-3	H-4	H-5	6-H_2	1-H_2	H-3	H-4	H-5	6-H_2
β-*p*-D-Fructose							3.57, 3.71	3.80	3.89	3.99	3.71, 4.03
β-*f*-D-Fructose							3.52, 3.56	4.10	4.10	3.83	3.68, 3.81
β-*p*-D-Fructose-1P							3.82	3.81	3.85	4.00	3.70, 4.05
β-*f*-D-Fructose-6P							3.58	4.12	4.24	3.88	3.94
β-*f*-D-Fructose-1,6P_2							3.83	4.18	4.18	4.02	3.95
β-*f*-D-Fructose-2,6P_2							3.85, 3.94	3.98	4.18	3.94	3.94, 4.03
α-D-Glucose	5.20	3.52	3.72	3.42	3.85	3.75, 3.83					
α-D-Glucose-6P	5.20	3.55	3.73	3.52	3.91	3.99, 4.02					
β-*p*-Trehalulose	4.97	3.58	3.78	3.43	3.72	3.79, 3.86	3.47, 3.94	3.85	3.91	4.02	3.71, 4.08
β-*p*-Trehalulose-6′P	4.96	3.59	3.79	3.56	3.79	4.02	3.45, 3.95	3.87	3.90	4.02	3.71, 4.07
Sucrose [c]	5.42	3.56	3.77	3.47	3.90	3.80, 3.86	3.69	4.22	4.05	3.86	3.80, 3.86
Sucrose-6P [c]	5.41	3.61	3.77	3.58	3.95	3.95, 4.05	3.68	4.20	4.11	3.89	3.83, 3.87
Sucrose-6′P [c]	5.42	3.55	3.78	3.37	3.94	3.80, 3.88	3.75	4.21	4.21	3.95	3.94, 3.97
Turanose	5.30	3.58	3.78	3.45	3.73	3.80, 3.85	[d]				
Turanose-6′P	5.28	3.63	3.82	3.55	3.73	4.03	[d]				
β-*p*-Maltulose	5.25	3.58	3.87	3.43	4.00	3.79, 3.85	3.60, 3.78	3.85	3.98	4.17	3.73, 3.85
β-*p*-Maltulose-6′P	5.22	3.60	3.80	3.52	4.00	4.03	3.55, 3.73	3.82	3.95	4.23	3.60, 3.72
β-*p*-Leucrose	5.11	3.55	3.77	3.45	3.95	3.75, 3.85	3.56, 3.70	3.90	3.97	4.03	3.97
β-*p*-Leucrose-6′P	5.11	3.58	3.78	3.60	3.95	3.95, 4.03	3.59, 3.70	3.91	3.95	4.06	4.00
β-*f*-Palatinose	4.97	3.57	3.75	3.41	3.75	3.75, 3.88	3.57	4.12	4.20	3.96	3.75, 3.88
β-*f*-Palatinose-6′P	4.97	3.62	3.60	3.76	3.82	4.03	3.55	4.12	4.22	3.97	3.70, 3.90

[a] TSP = sodium-2,2,3,3-tetradeutero-3-trimethylsilyl-propionate.
[b] 500 MHz ^{1}H NMR are available for D-fructose,[22] and trehalulose,[23] sucrose and its 6- resp. 6′-phosphates;[17] data listed here are new recordings to ensure more reliable comparative evaluations between disaccharide and their phosphates.
[c] Data essentially identical with those observed previously,[17] only the primary CH_2 protons, which based on 1D-, 2D-TOCSY and NOESY experiments, have been assigned more exactly.
[d] Exact assignment not feasible due to high complexity.

NMR spectra was performed with respect to the $J_{5,6}$ coupling constants observed. This assessment, however, failed to give a clear-cut picture, mainly due to the fact, that the resolution of the respective 6-CH_2 protons obviously depends on the extent of *gauche–gauche* and *gauche-trans* rotamer populations[21] of 6-CH_2OH versus 6-CH_2OP, which seems to vary, as well as on the nature of the second glycosyl residue. In the case of sucrose and palatinose and their phosphates, all having the fructose moiety in the furanose form, the glucosyl-6-CH_2 protons are well separated and allow the $J_{5,6}$ values to be readily determined, whereas in the leucrose/ leucrose 6′-phosphate pair, in which the fructose moiety can only adopt the pyranoid form, the 6′-CH_2 fall into narrow multiplets (Table 2).

The conclusions reached on the basis of the ^{1}H NMR chemical shift differences between disaccharide phosphate and parent sugar are borne out even more convincingly by their ^{13}C NMR data listed in Tables 4 and 5. In accord with previous observations on a number of phosphates derived from lactose,[15] trehalose,[16] and sucrose,[17] the ^{13}C NMR shift-effects induced by phosphorylation are most pronounced for the α-carbon resonance, with the primary C-6 of the glucose residue involved. Indeed, throughout, C-6 is shifted downfield on phosphorylation by a margin of 3 ppm on the average, i.e., +4.6 ppm for C-6 in sucrose versus +2.5–3.5 ppm for C-6′ in the others (Tables 4 and 5), providing further unambiguous proof for the respective sites of phosphorylation. The respective differences found for the β- and γ-carbons (C-5 and C-4, resp.) are comparatively small, not uniform throughout and, hence, have not been relied on for structural assignments.

Table 3
^{1}H NMR data (500 MHz in D_2O, 25 °C, ppm from internal TSP) [a] of α-D-glucosyl-glucoses and maltitol as compared with those of their 6- resp. 6′-phosphates

Compound [a]	α-D-Glucopyranoside residue						Reducing glucose residue					
	H-1	H-2	H-3	H-4	H-5	6-H_2	H-1	H-2	H-3	H-4	H-5	6-H_2
β-*p*-Maltose	5.39	3.59	3.68	3.42	3.72	3.85	4.65	3.20	3.76	3.62	3.60	3.74, 3.91
β-*p*-Maltose-6′P	5.35	3.63	3.69	3.59	3.78	3.95, 4.03	4.65	3.29	3.63	3.59	3.78	3.75, 3.93
β-*p*-Maltose-6,6′P	5.46	3.60	3.75	3.54	3.86	4.07	4.70	3.30	3.78	3.68	3.98	3.96, 4.15
β-*p*-Isomaltose	4.96	3.55	3.77	3.42	3.75	3.76, 3.85	4.66	3.26	3.45	3.51	3.65	3.78, 3.96
β-*p*-Isomaltose-6′P	4.96	3.57	3.76	3.74	3.80	4.02, 4.07	4.68	3.27	3.48	3.53	3.62	3.80, 3.98
							second α-D-glucose residue					
Trehalose	5.20	3.65	3.85	3.45	3.85	3.76, 3.88	5.20	3.65	3.85	3.45	3.85	3.76, 3.88
Trehalose-6P	5.18	3.68	3.88	3.62	3.91	3.96, 4.03	5.22	3.65	3.85	3.46	3.89	3.76, 3.85
							D-glucitol residue					
Maltitol	5.12	3.58	3.74	3.44	3.88	3.79, 3.88	3.65, 3.70	3.87	3.86	3.87	3.99	3.68, 3.79
Maltitol-6P	5.12	3.63	3.75	3.57	3.98	4.02, 4.04	3.65, 3.70	3.89	3.92	3.87	4.04	3.68, 3.79

[a] 500 MHz ^{1}H NMR data are available for trehalose,[24] its 6-phosphate,[16] and isomaltose.[24] The data listed here are new recordings to allow reasonable chemical shift comparisons with their 6- resp. 6′-phosphates.

Table 4
^{13}C Chemical shifts (125 MHz in D_2O, 25 °C, ppm from internal TSP) of D-fructose, D-glucose, sucrose, and the five isomeric α-D-glucosyl-fructoses in comparison with their 6- resp. 6′-phosphates

Compound	α-D-Glucopyranosyl unit						Fructose moiety					
	C-1	C-2	C-3	C-4	C-5	C-6	C-1	C-2	C-3	C-4	C-5	C-6
β-*p*-D-Fructose							64.7	99.1	68.4	70.5	70.0	64.1
β-*f*-D-Fructose							63.6	102.6	76.4	75.4	81.6	63.2
β-*p*-D-Fructose-1P							69.2	100.8	70.8	72.2	71.9	66.2
β-*f*-D-Fructose-6P							65.7	104.3	78.3	77.4	82.7	67.3
β-*f*-D-Fructose-1,6P_2							68.8	103.6	78.8	77.1	82.2	68.0
β-*f*-D-Fructose-2,6P_2							65.9	106.9	80.6	78.6	83.0	68.5
α-D-Glucose	94.8	77.0	74.4	72.2	73.5	63.4						
α-D-Glucose-6P	95.0	77.9	74.3	72.0	73.3	66.2						
β-*p*-Trehalulose [a]	101.3	74.2	77.2	72.5	75.8	63.3	72.4	100.7	70.7	72.4	72.4	66.4
β-*p*-Trehalulose-6′P	101.3	74.4	75.5	74.4	74.3	66.4	71.9	100.6	72.5	70.7	72.0	65.7
Sucrose	95.0	73.8	75.3	72.0	75.0	63.1	64.3	106.5	79.4	76.8	84.1	65.1
Sucrose-6P	94.9	74.1	75.0	72.0	74.9	65.7	64.3	106.5	79.3	77.0	84.1	65.4
Sucrose-6′P	95.2	74.2	75.4	72.7	75.3	63.4	64.1	106.6	79.2	76.9	83.3	67.0
Turanose	103.7	74.2	75.5	72.2	75.5	63.2	[b]					
Turanose-6′P	100.6	75.2	75.1	73.1	75.1	66.2	[b]					
β-*p*-Maltulose	103.3	74.0	75.2	72.5	72.0	63.3	66.7	104.7	74.7	80.8	72.3	66.3
β-*p*-Maltulose-6′P	103.6	74.8	74.9	72.2	69.7	66.6	66.8	104.6	74.8	81.6	71.8	65.5
β-*p*-Leucrose	103.0	74.7	75.7	72.4	70.8	63.5	66.7	100.8	74.8	72.5	81.7	64.9
β-*p*-Leucrose-6′P	103.3	74.9	75.4	71.9	74.5	65.8	66.8	100.9	70.8	74.3	81.9	65.0
β-*f*-Palatinose	100.9	74.1	74.6	73.3	75.8	63.3	65.6	104.4	77.7	77.3	81.6	70.5
β-*f*-Palatinose-6′P	101.2	72.8	74.7	74.3	74.3	65.9	65.7	104.5	77.4	78.3	81.8	70.7

[a] ^{13}C-NMR data at 125 MHz for trehalulose[23] have already been reported. To allow for more reliable comparisons of chemical shift differences between the carbons of the disaccharide and its monophosphate, the data given here are new recordings; they differ from those reported within 0.05 ppm at the most.

[b] Exact assignment not feasible due to high complexity.

Table 5
^{13}C Chemical shifts (125 MHz in D_2O, 25 °C, ppm from internal TSP) disaccharides in comparison with their 6- resp. 6′-phosphates [a]

Compound	α-D-Glucopyranoside unit						Reducing glucose portion					
	C-1	C-2	C-3	C-4	C-5	C-6	C-1	C-2	C-3	C-4	C-5	C-6
β-*p*-Maltose	101.1	73.1	74.3	70.8	74.0	62.0	97.2	75.4	77.5	78.6	76.0	62.2
β-*p*-Maltose-6′P	102.7	75.2	76.0	71.8	77.5	65.5	98.6	76.4	75.1	80.3	75.1	63.5
β-*p*-Maltose-6,6′P	101.5	75.3	76.4	71.9	75.9	66.0	98.6	77.0	78.9	79.0	76.5	66.9
β-*p*-Isomaltose	100.8	72.3	75.9	72.4	74.3	63.4	98.9	78.8	76.9	75.9	74.3	68.6
β-*p*-Isomaltose-6′P	101.0	72.0	75.9	75.6	74.1	64.9	98.9	77.2	78.9	72.2	74.2	68.7
							second α-D-glucose unit					
Trehalose	96.0	73.6	75.1	72.3	74.5	63.4	96.0	73.6	75.1	72.3	74.5	63.4
Trehalose-6P	96.3	74.1	75.0	72.0	75.3	65.5	96.2	73.8	75.1	72.6	74.7	63.4
							D-glucitol residue					
Maltitol	103.3	74.4	75.7	72.2	73.3	63.3	65.1	74.3	75.3	84.6	75.5	65.6
Maltitol-6P	103.5	74.5	75.4	72.0	74.2	65.8	65.7	74.6	75.4	84.7	75.6	65.2

[a] The NMR recordings for the disaccharides are new to facilitate reliable shift comparisons. Differences with 125 MHz literature data for trehalose and its 6-phosphate[16] are 0.05 ppm or less.

Enzymatic hydrolysis of α-glucoside 6-phosphates.—Sucrose-grown cells of *K. pneumoniae* are induced for sucrose 6-phosphate hydrolase,[8a] and a cell extract readily hydrolyzed sucrose 6-phosphate. However, there was no detectable hydrolysis of the phosphorylated α-D-glucosyl-fructoses, or of the other disaccharide 6′-phosphates tested (Table 6). Furthermore, inclusion of the sucrose-isomeric 6′-phosphoglucosyl-fructoses in the assay caused no significant change in the hydrolysis rate of sucrose 6-phosphate; thus, they are apparently neither substrates, nor inhibitors of the sucrose-6-phosphate-hydrolase from *K. pneumoniae*. Cells grown on the sucrose isomers (by forming fructose, the intracellular inducer) are also induced for sucrose-6P-hydrolase and extracts from palatinose- and leucrose-grown organisms catalyzed the hydrolysis of sucrose 6-phosphate (Table 6). Although growth on maltitol does not elicit an expression of sucrose-6P-hydrolase by *K. pneumoniae*, an extract from such cells also hydrolyzed sucrose 6-phosphate, albeit at significantly lower rate. Importantly, the three extracts hydrolysed all α-glucoside phosphates, except the 6,6′-diphosphate of maltose. These findings indicated in the three extracts the presence of an enzyme (distinct from sucrose-6P-hydrolase) with the capacity to hydrolyze the 6′-phosphoglucosyl-fructoses.

Expression of 6-phospho-α-glucosidase.—Comparative analysis of the protein composition of cell extracts by 2D-PAGE, revealed a high level expression of a polypeptide of $M_r \sim 49-50$ Kda during growth of *K. pneumoniae* on the five sucrose isomers. This protein was also induced by growth on other α-glucosides, including maltose and maltitol, but was not expressed during growth of the organism on sucrose.[25] Electroelution and microsequence analysis of the induced protein provided the unambiguous sequence of the first 25 residues from the N-terminus: **MKKFSVVIAGGGSTFTPGIVLMLLA**. When used as a probe to search the non-redundant protein sequence database with the BLAST program,[26] this amino acid sequence showed ~85% identity with the N-termini of 6-phospho-α-glucosidase from *B. subtilis*[27a]) and *F. mortiferum*.[28] [In the SWISS-PROT database[40c] these enzymes are designated GlvA (P54716) and MalH (O06901), respectively]. The antibody purified 6-phospho-α-glucosidase from *F. mortiferum*,[29] cross-reacted specifically with a polypeptide ($M_r \sim 49-50$ Kda) that was present in extracts prepared from cells grown on the sucrose isomers (Fig. 1, lanes 3–6). The

Table 6
Hydrolysis rates of disaccharide 6-phosphates by cell extracts of *K. pneumoniae*, expressed as nmoles of glucose-6P formed per min per mg of protein [a]

Substrate [b]	Cells grown previously on			
	Sucrose	Palatinose	Leucrose	Maltitol
Trehalulose-6′P	[c]	64.4	36.9	15.0
Sucrose-6P	549.5	783.3	687.5	21.1
Turanose-6′P	[c]	154.9	116.4	53.9
Maltulose-6′P	[c]	93.9	71.6	27.8
Leucrose-6′P	[c]	16.9	12.5	4.5
Palatinose-6′P	[c]	55.8	34.3	12.3
Maltose-6′P	[c]	36.8	30.8	11.7
Maltose-6,6′P_2	[c]	[c]	[c]	[c]
Isomaltose-6′P	[c]	46.3	32.7	15.3
Maltitol-6P	[c]	167.3	124.7	51.7
Trehalose-6P	[c]	27.5	24.1	19.5

[a] Values are average of two separate assays.
[b] Substrate concentration in the assay: 1 mM.
[c] No detectable hydrolysis.

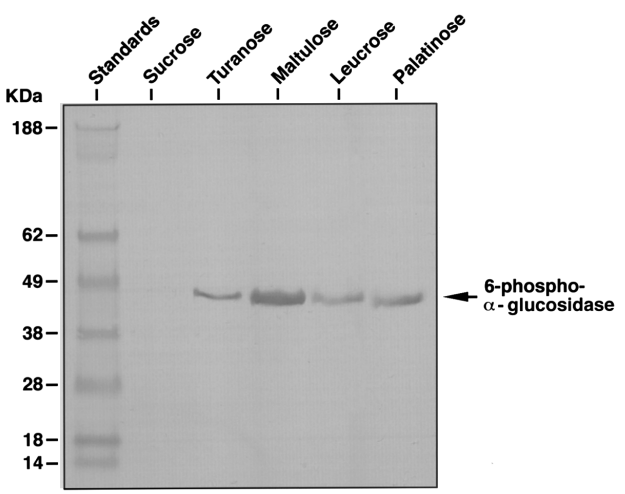

Fig. 1. Western blot showing immuno-reaction between the induced protein ($\sim$49–50 Kda) in cell extracts from *K. pneumoniae* grown on disaccharides isomeric with sucrose, and antibody prepared against phospho-α-glucosidase from *F. mortiferum*.[29] Approx. 25 μg of protein were applied per lane. (Although not shown in this figure, an extract prepared from trehalulose-grown cells also contained the induced, immuno-reactive polypeptide).

immunoreactive protein was not present in sucrose-grown cells (Fig. 1, lane 2). The enzyme induced by growth of *K. pneumoniae* on the α-D-glucosyl-D-fructoses was thus identified as being a 6-phospho-α-glucosidase.

3. Discussion

There are numerous reports of sucrose fermentation by microorganisms, but there are few (if any) descriptions of bacterial utilization of the five linkage-isomers of this disaccharide. In the case of *S. mutans*, sucrose is rapidly fermented to caries-inducing lactic acid, but the isomeric glucosyl-D-fructoses are not metabolized by this, or other species of oral streptococci.[12] Palatinose and leucrose are produced on an industrial scale[30,31] and, by virtue of their non-cariogenicity and comparative sweetness, the two analogs attract attention as potential substitutes for dietary sucrose.[32] It was unexpected, but of considerable interest to find excellent growth of *K. pneumoniae* on all sucrose isomers, and three significant advances emerged from these observations. First, the PEP-dependent phosphotransferase activity of permeabilized palatinose-grown cells permitted the stereospecific synthesis and preparation (in substrate amount) of the 6′-phospho- derivatives of all sucrose isomers. Second, growth of *K. pneumoniae* on the five glucosylfructoses induced a high-level expression of 6-phospho-α-glucosidase. Third, the results of enzymatic analysis provide strong evidence, that: (i) sucrose-6P-hydrolase only hydrolyzes sucrose 6-phosphate and (ii) 6-phospho-α-glucosidase catalyzes the hydrolysis of a wide variety of disaccharide phosphates, including the five 6′-phosphoglucosyl-fructoses.

The extreme specificity of sucrose-6P-hydrolase for its single substrate (sucrose 6-phosphate) is noteworthy, because it suggests that their reciprocal molecular recognition, in terms of the rigid lock-and-key analogy[33] or the flexible induced-fit model,[34] as a prerequisite to interaction is optimal in contrast to the five α-D-glucosyl-fructoses, which formally are isomers of sucrose, yet have free (reducing) anomeric centers, and conceivably of higher significance, have distinctly different molecular shapes. As borne out by the graphical illustrations of Fig. 2, sucrose is a remarkably compact molecule, even in aqueous solution due to intramolecular hydrogen bonding between O-2_g and O-$1'_f$ through a water bridge;[35] in eliciting the sweetness response, it conceivably docks onto the taste bud receptor protein

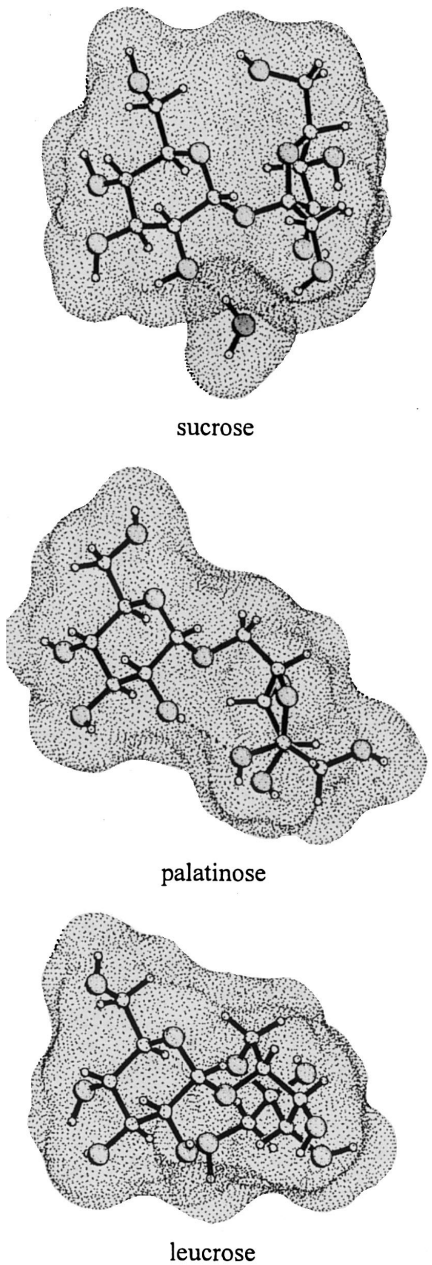

Fig. 2. Preferred molecular geometries of sucrose, here shown with the water molecule bridging the glucosyl-*O*-2 and fructosyl-*O*-1 through hydrogen bonding,[35,38] of palatinose (=isomaltulose, β-*f* anomer),[38,39] and leucrose (β-*p* anomer)[39] as evidenced by ball-and-stick models and their solvent-accessible surfaces (in dotted form). Thereby, the α-D-glucopyranosyl portions of the three disaccharides (left half) is kept in the same orientation to more distinctly reveal the differently attached furanoid (sucrose, palatinose) and pyranoid (leucrose) fructose parts.

with its hydrophobic region,[36] which on the basis of the calculated MLP (molecular lipophilicity potential) profiles encompasses the entire outer surface side of the fructose portion.[36,37] The same docking procedure is expected for sucrose 6-phosphate at the sucrose-6P-hydrolase, in as much as the fructose portion of sucrose is not likely to be substantially altered by phosphorylation at the glucosyl-O-6.

Palatinose, however, in which the fructose portion can only adopt furanose forms, with the β-*f* anomer strongly preponderating in aqueous solution (80% at 20 °C[19]), assumes, as compared to sucrose, a longish-extended molecular geometry[38,39] (Fig. 2, center). The same is to be expected for its 6′-phosphate, thus obviously being incompatible with the enzymes binding site, in as much as the MLP profile, i.e., distribution of hydrophobic and hydrophilic regions over the contact surface is distinctly different.[39] Similar considerations with respect to the molecular shape and MLP profiles of leucrose,[39] the major tautomeric form adopted in solution is that of the β-pyranose,[19] as presented in Fig. 2, bottom, clearly reveal ample differences as do, in fact, trehalulose, turanose, and maltulose, thus making the non-compatibility of their 6′-phosphates with sucrose-6P-hydrolase comprehensible.

By contrast, 6-phospho-α-glucosidase exhibits little discrimination and (provided the non-reducing glucose 6-phosphate moiety is present) this enzyme tolerates considerable variation in size, and structure of the α-linked aglycon component of its substrates. The gene that encodes 6-phospho-α-glucosidase in *K. pneumoniae* has recently been cloned and sequenced,[25] but comparative alignment of the deduced amino acid sequence of this enzyme with that determined previously for sucrose-6P-hydrolase,[8b] reveals virtually no homology between the two phospho glycosyl-hydrolases. Furthermore, when classified by the amino acid sequence-based method of Henrissat et al.,[40] sucrose 6-phosphate hydrolase is assigned to the fructofuranosidase Family 32 whereas 6-phospho-α-glucosidase is included in Family 4 of the glycosylhydrolase superfamily.[27]

Our contention that 6-phospho-α-glucosidase participates in the dissimilation of sucrose isomers by *K. pneumoniae* is supported by our finding that the gene encoding this glycosylhydrolase, lies adjacent to a gene that encodes an EIICB component of the PEP-de-

pendent phosphotransferase system.[41] It is likely that the two genes are components of an operon whose products facilitate transport, phosphorylation and hydrolysis of glucosyl-fructoses and related α-glucosides by *K. pneumoniae*. The fact that neither of these genes has been found during sequencing of the chromosome of *S. mutans*, may explain the failure of this organism to utilize disaccharides isomeric with sucrose as energy sources for growth.

4. Experimental

Materials and reagents.—Sucrose and its isomers were obtained from the following sources: maltulose (TCI America); palatinose (Wako Chemicals); trehalulose (Südzucker, Mannheim/Ochsenfurt); leucrose (Fluka); turanose and sucrose (Pfanstiehl Laboratories). Glucose 6-phosphate dehydrogenase/hexokinase (G6PDH/HK) and phosphoglucose-isomerase (PGI) were obtained from Boehringer Mannheim. Thin-layer microcrystalline cellulose sheets for chromatography (Polygram cell 400) were from Macherey & Nagel. Phosphoenolpyruvate (mono Na^+ salt), $NADP^+$, D-fructose 1- and 6-phosphate, its 1,6- and 2,6-biphosphates, sucrose 6′-phosphate and trehalose 6-phosphate were purchased from Sigma Chemical Co. Maltose-6,6′-diphosphate was prepared by phosphorylation of maltose with phosphorus oxychloride in trimethyl phosphate by modification of the procedure of Wilson and Fox,[42] and purification by elution from AG1-X8 formate resin (Bio-Rad) with an increasing concentration gradient ($0 \rightarrow 0.4$ M) of NH_4HCO_3, followed by lyophilization and paper chromatography (see below).

General methods.—Negative-ion FAB spectra were obtained on a JEOL SX102 mass spectrometer operated at an accelerating voltage of -10 KV. Samples were desorbed from a glycerol matrix using 6 K eV xenon atoms. Mass measurements in FAB were performed at 10,000 resolution using electric field scans and matrix ions as the reference material. Analyses were run at low resolution for integer mass information and at high resolving power for determination of molecular formulae. ^1H and ^{13}C NMR spectra were recorded with a Bruker Avance-500 MHz instrument at 500 and 125 MHz, respectively. Chemical shifts were measured, relative to internal sodium 2,2,3,3-tetradeutero-3-trimethylsilylpropionate in a sealed concentric tube of outside diameter 5 mm with solvent D_2O. Concentration of the samples was 40 mg/mL; aqueous solutions were prepared 24 h in advance in order to ensure that equilibrium had been reached. Assignments of signals was ascertained by gradient selected (gs)-HMQC, (gs)-COSY, 2D-TOCSY and, if required, by 1D-TOCSY (HOHAHA) and (gs)-NOESY NMR experiments. To unambiguously verify the assignment of the phosphorylation site, the ^1H and ^{13}C NMR chemical shifts of the phosphates obtained were compared (Tables 2–5) with those of the parent disaccharides as well as with the NMR data for the known yet newly measured D-fructose 1- and 6-phosphate, its 1,6- and 2,6-diphosphates, and with the 6- and 6′-phosphates of sucrose.

Procedures for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, and immunodetection of cross-reactive protein to antibody raised against phospho-α-glucosidase from *F. mortiferum*, have been described previously.[29] Two-dimensional gel electrophoresis and protein microsequencing were carried out by Kendrick Labs, Inc. WI. and by the Protein Chemistry Core Facility, Columbia University, NY, respectively. Protein concentrations were determined by the BCA protein assay kit (Pierce).

Growth of cells.—*K. pneumoniae* ATCC 23357 was grown (37 °C) aerobically, and without shaking in 1 L bottles containing 800 mL each of defined medium[43] supplemented with either 0.4% palatinose or appropriate sugar. After growth overnight to the stationary phase (at 37 °C), the cells were harvested by centrifugation (13,000 × g for 10 min at 5 °C), and washed twice in 25 mM Tris-HCl buffer (pH 7.5) containing 1 mM $MgCl_2$. The cell yield was approx. 2 g wet wt. per L.

Metabolism of sucrose and its isomeric glucosyl-fructoses by K. pneumoniae.—Washed cells grown previously on the appropriate disaccharide, were added (15 mg dry wt. mL^{-1})

to 10 mL of solution containing: 0.1 M Na^+/K^+-phosphate buffer (pH 7), 5 mM $MgCl_2$ and 10 mM sucrose or isomer. The suspensions were placed on a water-bath shaker (37 °C) and, at intervals of 10 min throughout an incubation period of 1 h, samples (0.5 mL) were withdrawn, chilled quickly to 0 °C, and cells were removed by centrifugation. Mixtures of 100 μL of supernatant plus 100 μL 2 N HCl were heated for 1 h at 100 °C, cooled and neutralized by addition of 200 μL 1 N KOH. Aliquots (40 μL) of this solution were assayed for glucose (equivalent to disaccharide remaining), and fermentation rates were determined as μmoles disaccharide consumed min^{-1} mg dry wt. $cells^{-1}$.

Enzymatic analyses.—Glucose, fructose and glucose 6-phosphate were determined in an $NADP^+$-coupled enzyme assay that contained (in 1 mL): 0.1 M HEPES buffer (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) (pH 7.5), 1 mM $MgCl_2$, 1 mM $NADP^+$, 5 mM ATP and sequential additions of 2 IU of the appropriate enzymes (G6PDH, HK, and PGI). Reduction of $NADP^+$ was monitored at A_{340nm} in a Beckman DU 640 spectrophotometer, and a molar extinction coefficient for NADPH of $\varepsilon = 6{,}220\ M^{-1}\ cm^{-1}$ was assumed for calculation of NADPH generated (i.e., equivalent to glucose 6-phosphate, glucose or fructose). For determination of hydrolase activities, the respective disaccharide phosphates were present at a concentration of 1 mM, and the assay was begun by addition of 20 μl ($\sim$ 500 μg protein) of cell extract.

Preparation of disaccharide phosphates.—Palatinose-grown cells of *K. pneumoniae* were added to 5 mL of 25 mM Tris-HCl buffer (pH 8) containing 1 mM $MgCl_2$ (at 0 °C) to a density of 10 mg dry wt. per mL. Organisms were permeabilized by the addition of 50 μL of 9:1 (v/v) acetone–toluene and vigorous agitation (30 s) of the suspension on a Vortex mixer. This procedure was repeated three times. Thereafter, 1 mL of 25 mM Tris-HCl buffer (pH 8) containing 150 μmoles of α-glucoside, sucrose or the respective isomeric glucosyl-fructoses and 100 μmoles of PEP (mono Na^+ salt) was added†, and the suspension was incubated at 37 °C for 1.5 h (For preparative purposes, 15 such reactions were used for each compound). The suspensions were pooled, cells were removed by centrifugation and (after adjustment to pH 8.2) 8 mL of a 25% solution of barium acetate was added. The mixture was chilled on ice for 30 min, the heavy precipitate was removed by centrifugation, and the supernatant was filtered through a 0.45 μm pore-size membrane. Thereafter, 4 volumes of 95% ethanol (0 °C) were added and the solution was maintained at 4 °C overnight. The white flocculent precipitate (containing the ethanol-insoluble Ba^{2+} salts of the phosphorylated disaccharides and residual PEP) was collected by centrifugation, and

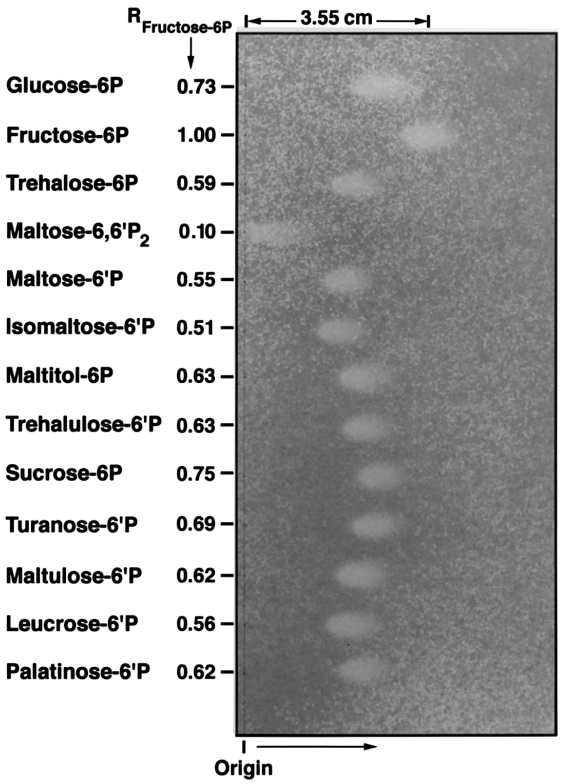

Fig. 3. TLC of the 6-phosphates of D-glucose, D-fructose, α,α-trehalose and sucrose together with the 6′-phosphates of trehalulose, turanose, maltulose, leucrose, and palatinose. The compounds ($\sim$ 0.05 μmol) were applied to a 0.1 mm layer of microcrystalline cellulose, were separated (6 h) in 5:2:3 *n*-butanol–acetic acid–water, and detected by the stain of Wade and Morgan.[44]

† It is important to note, that addition of 1 mL of sugar/PEP Na^+ salt solution lowers the pH of the permeabilized cell suspension from pH 8 to 4.2. Remarkably, at pH 4.2 there is continued operation of the PEP: PTS, but fortuitously (and essentially) this low pH inactivates enzymes(s) that at 'physiological' pH would catalyze the hydrolysis of the phosphorylated products.

Ba^{2+} was exchanged for H^+ by addition of ~2 mL of aqueous suspension of Bio-Rad AG 50W X2 (H^+-form) resin. After adjustment to pH 7.2, the solution was frozen and lyophilized. Separation of the disaccharide-phosphates from residual PEP was achieved by descending chromatography (Whatman 3 MM paper, 20 h) in a solvent containing 5:2:3 (v/v) *n*-butanol–AcOH–water, and the phosphate-containing compounds were located with Wade–Morgan stain,[44] followed by centrifugal elution with distilled water. After adjustment to pH 7.2 (with NH_4OH), the solutions were frozen and lyophilized. The respective disaccharide-phosphates were obtained as their ammonium salts in the form of white crystalline powders in 20–50 mg quantities (Fig. 3).

Physicochemical characterization of phosphorylated derivatives.—TLC of the products on 0.1 mm layers of microcrystalline cellulose with 5:2:3 *n*-butanol–AcOH–water as the running phase and detection by the Wade–Morgan stain[44] provided evidence to the purity of the preparations, by revealing a single (phosphate-containing) spot for each derivative (Fig. 3). Negative-ion FAB mass spectrometry provided $[M-H]^+$ data, and confirmed the molecular formula for each compound: $m/z = 421.1$ corresponding to $C_{12}H_{22}O_{14}P$, for sucrose 6-phosphate and the 6′-phosphates of trehalulose, turanose, maltulose, leucrose, and palatinose. Maltitol 6-phosphate gave $m/z = 423.2$ conforming to $C_{12}H_{24}O_{14}P$, maltose 6,6′-diphosphate gave $m/z = 501$, commensurate with $C_{12}H_{23}O_{17}P_2$. The ^1H and ^{13}C NMR data obtained for, the five 6′-phospho-α-D-glucosyl-D-fructoses, and the 6′-phosphates of trehalose, maltose, isomaltose, and maltitol are listed in Tables 4–6.

Acid hydrolysis (1 N HCl, 2 h at 100 °C) of maltose 6′-phosphate produced glucose 6-phosphate and glucose in a 1:1 ratio. However, the non-stoichiometry of the glucose 6-phosphate:fructose ratio for sucrose 6-phosphate and the phosphorylated glucosyl-fructoses, which were in the 1:0.4–0.6 range, indicated significant loss of the ketose moiety during their acid hydrolysis.

Acknowledgements

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