396. *The Separation of Reducing Carbohydrates as their N-Substituted Glycosylammonium Ions.*

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A rapid method for the paper-ionophoretic separation of a homologous series of reducing oligosaccharides as their *N*-benzylglycosylammonium ions is described. The rates of migration of such ions can be used to determine the molecular sizes of oligosaccharides.

ONE of the problems encountered in the paper-chromatographic separation of oligosaccharides was their low R_F values in many solvents. In attempts to overcome this, the rate of flow of solvent has been increased by running the chromatograms [1] at 37° and a wide range of solvents has been investigated.[2] Separation of oligosaccharides as their benzylamine derivatives [3] is more rapid, taking only 18 hr. for derivatives containing up to five glucose units, but is still lengthy in comparison with the separation of certain oligosaccharides as their borate complexes by paper ionophoresis.[4] Although the latter affords some evidence as to the linkage of the reducing sugar it is not usually possible to determine the molecular size of an unknown sugar. Thus the homologous series of glucosaccharides containing 1 : 3- or 1 : 6-links can be separated but the series containing 1 : 4- (and presumably 1 : 2)-links show negligible separation.

We now report fully [5] a method whereby any reducing oligosaccharide, pentose, hexose, or heptose can be speedily separated by paper ionophoresis of its *N*-benzylglycosylammonium ion. These ions move at rates inversely related to their molecular weights and, to the first approximation, independent of the stereochemistry and mode of linkage of the sugar. Attempts are also described to use other amines of lower molecular weight in order to increase the relative separation of a homologous series.

EXPERIMENTAL

Standard Method of Separation of N-*Benzylglycosylammonium Ions.*—Each reducing sugar and reference sugar (5 μl. of 3% solution) was spotted along a line drawn on a strip (22·5 × 5 in.) of Whatman No. 3 paper and a benzylamine–methanol–10N-formic acid mixture (1 : 9 : 5; 5 μl.) superimposed. After the papers had been kept at 95° for 5 min. the *N*-benzylglycosylammonium ions were separated by ionophoresis at 600 v for 6 hr. (or 500 v for 15 hr. for higher saccharides) in an electrolyte (pH 1·8) composed of 5% aqueous sodium hydroxide (600 c.c.) and 90% formic acid (400 c.c.). After drying, the sugars and their complexes were detected with alkaline silver nitrate [6] or periodate followed by benzidine.[7] The mobility (M) of any component was expressed as the ratio of the distances separating (*a*) the *N*-benzylglycosylammonium ion from the unchanged sugar and (*b*) the *N*-benzylglycosylammonium ion from unchanged glucose.

[1] Hough, Jones, and Wadman, *J.*, 1950, 1702.
[2] Jeanes, Wise, and Dimler, *Analyt. Chem.*, 1951, **23**, 415.
[3] Bayly and Bourne, *Nature*, 1953, **171**, 385.
[4] Foster, *J.*, 1953, 982.
[5] Cf. Barker, Bourne, Grant, and Stacey, *Nature*, 1956, **177**, 1125.
[6] Trevelyan, Proctor, and Harrison, *ibid.*, 1950, **166**, 444.
[7] Cifonelli and Smith, *Analyt. Chem.*, 1954, **26**, 1132.

TABLE 1. *Mobilities of* N-*benzylglycosylammonium ions.*

Sugar	Mobility (*M*)	Ratio (*X*) *	Sugar	Mobility (*M*)	Ratio (*X*) *
D-Arabinose	1·14	1·13	Laminaribiose	0·79	0·63
L-Arabinose	1·15	1·13	Maltose	0·75	0·63
D-Ribose	1·11	1·13	Melibiose	0·77	0·63
D-Xylose	1·09	1·13	Nigerose	0·78	0·63
L-Xylose	1·12	1·13			
			Cellotriose	0·57	0·46
D-Galactose	1·00	1·00	Isomaltotriose	0·62	0·46
D-Glucose	1·00	1·00	Laminaritriose	0·63	0·46
3-deoxy-	1·04	1·06	Maltotriose	0·61	0·46
3-*O*-methyl-	0·95	0·95	Nigerian trisaccharide	0·60	0·46
2 : 3-di-*O*-methyl-	0·92	0·91	Panose	0·59	0·46
2 : 3 : 6-tri-*O*-methyl-	0·90	0·87			
2 : 3 : 4 : 6-tetra-*O*-methyl-	0·88	0·83	Laminaritetraose	0·51	0·36
D-Mannose	1·00	1·00	Maltotetraose	0·49	0·36
L-Rhamnose	1·02	1·06			
			Maltopentaose	0·42	0·29
D-*glycero*-D-*gala*Heptose	0·91	0·90			
			Maltohexaose	0·33	0·25
Cellobiose	0·71	0·63			
3-(1-β-Glucosyl)xylose	0·80	0·67	D-Fructose	1·00	1·00
Isomaltose	0·78	0·63	L-Sorbose	1·00	1·00
Lactose	0·72	0·63	D-*gluco*Heptulose	0·90	0·90

* X = (Mol. wt. of N-benzylglucosylammonium ion)/(mol. wt. of N-benzylglycosylammonium ion.

TABLE 2. *Mobilities of* N-*methylglycosylammonium ions.*

Sugar	Mobility (*M*1)	Ratio (*X*1) *	Sugar	Mobility (*M*1)	Ratio (*X*1) *
D-Ribose	1·17	1·18	Laminaribiose	0·65	0·54
			Maltose	0·62	0·54
D-Galactose	1·00	1·00	Melibiose	0·63	0·54
D-Glucose	1·00	1·00			
D-Mannose	1·00	1·00	Maltotriose	0·45	0·38
			Maltotetraose	0·34	0·29
D-*glycero*-D-*gala*Heptose	0·89	0·87	Maltopentaose	0·23	0·23
Cellobiose	0·61	0·54	Maltohexaose	0·20	0·19
Lactose	0·61	0·54	D-Fructose	1·00	1·00

* $X1$ = (Mol. wt. of N-methylglucosylammonium ion)/(mol. wt. of N-methylglycosylammonium ion).

TABLE 3. *Mobilities of glycosylammonium ions.*

Sugar	Mobility (*M*2)	Ratio (*X*2) *	Sugar	Mobility (*M*2)	Ratio (*X*2) *
D-Arabinose	1·25	1·20	Lactose	0·59	0·53
L-Arabinose	1·26	1·20	Laminaribiose	0·64	0·53
D-Ribose	1·22	1·20	Maltose	0·60	0·53
D-Xylose	1·21	1·20	Melibiose	0·62	0·53
L-Xylose	1·24	1·20	Nigerose	0·63	0·53
D-Galactose	1·00	1·00	Cellotriose	0·41	0·36
D-Glucose	1·00	1·00	Isomaltotriose	0·45	0·36
3-deoxy-	1·12	1·10	Laminaritriose	0·44	0·36
3-*O*-methyl-	0·95	0·93	Maltotriose	0·45	0·36
2 : 3-di-*O*-methyl-	0·89	0·87	Panose	0·43	0·36
2 : 3 : 6-tri-*O*-methyl-	0·85	0·81			
2 : 3 : 4 : 6-tetra-*O*-methyl-	0·81	0·76	Laminaritetraose	0·34	0·27
D-Mannose	1·00	1·00	Maltotetraose	0·31	0·27
			Maltopentaose	0·24	0·22
D-*glycero*-D-*gala*Heptose	0·88	0·86	Maltohexaose	0·19	0·18
Cellobiose	0·58	0·53	D-Fructose	1·00	1·00
Isomaltose	0·63	0·53	D-*gluco*Heptulose	0·88	0·86

* $X2$ = (Mol. wt. of glucosylammonium ion)/(mol. wt. of glycosylammonium ion).

Standard Method of Separating N-*Methylglycosylammonium Ions.*—The *N*-methylglycosylammonium ions were produced on Whatman No. 3 paper by superimposing on the sugar (5 μl. of 3% solution) one spot (5 μl.) of a solution obtained by passing methylamine (equivalent to 1 c.c.) into a mixture of methanol (9 c.c.) and 10N-formic acid (5 c.c.). The paper was heated for 5 min. at 90° and the *N*-methylglycosylammonium ions were separated by ionophoresis and detected as described for the *N*-benzyl derivatives. The mobility (*M*1) was expressed as the ratio of the distances separating the *N*-methylglycosylammonium ion from the unchanged sugar and the *N*-methylglucosylammonium ion from unchanged glucose.

Standard Method of Separation of Glycosylammonium Ions.—These ions were formed on Whatman No. 3 paper by superimposing on the sugar (5 μl. of 3% solution), one spot (5 μl.) of a solution of ammonium formate (1 g.) in methanol (10 c.c.). The papers containing hexoses and oligosaccharides were heated for 5 min. at 85° and those containing pentoses for 3 min. at 85°. The glycosylammonium ions were separated by ionophoresis and detected as described above for the *N*-benzyl derivatives. The mobility (*M*2) was expressed as the ratio of the distances separating the glycosylammonium ion from the unchanged sugar and the glucosylammonium ion from unchanged glucose.

Discussion

Initial experiments showed that the *N*-benzylglycosylamines produced by the paper-chromatographic method of Bayly and Bourne [3] could be detected by alkaline silver nitrate,[6] periodate,[7] and aniline hydrogen phthalate [8] in addition to ninhydrin. The suitability of these sprays for detection of the *N*-benzylglycosylammonium ions under the various conditions used in ionophoresis is commented upon in the appropriate sections.

Choice of Electrolyte.—The known stability [9] of glycosylammonium ions at pH 2 led to the employment of an acidic electrolyte and initial experiments were carried out with hydrochloric acid at pH 2. The proportion of *N*-benzylmaltosylammonium ion to unchanged maltose was low under these conditions but was increased by the addition of sodium chloride to the electrolyte. The amount that could be added was limited because the increased conductivity gave rise to extra heat, which it was difficult to dissipate. Also, the high chloride content of the paper reduced the sensitivity of the alkaline silver nitrate method of detection. Streaking of spots was encountered when acetic acid (8N and 2N), acetate (pH 3·1), or formic acid (pH 2) was used, but a mixture of 5% aqueous sodium hydroxide (600 c.c.) and 90% formic acid (400 c.c.) gave satisfactory results. The pH of the electrolyte stayed constant at 1·8 during ionophoresis. The formic acid was removed by heating the paper, and the residual sodium formate did not appreciably reduce the sensitivity of the silver nitrate method of detection.

Formation of the Sugar–Benzylamine Complex.—With maltose as a guide, the optimum conditions for the formation of the benzylamine complex were found to be 95° for 5 min. It was desirable to commence ionophoresis directly the *N*-benzylglycosylamine had been formed. It was appreciated that the presence of excess of benzylamine initially would increase the pH near the spot. This was overcome by adding 10N-formic acid (5 c.c.) to the benzylamine (1 c.c.) in methanol (9 c.c.) which was superimposed on the sugar. The optimum heating conditions remained the same but there was considerably higher conversion (*ca.* 70%) of the sugar into the *N*-benzylglycosylammonium ion. It may be that the formic acid catalysed it.[10]

Detection of the N-*Benzylglycosylammonium Ion.*—A method of detection based on the sugar part of the molecule was desirable because the sensitivity would then be largely independent of the molecular size of the oligosaccharide. Although the silver nitrate method [6] was sensitive enough in most cases, it failed to detect benzylamine complexes of 2-methyl derivatives of glucose. These were, however, located by using a fluorescent

[8] Partridge, *Nature*, 1949, **164**, 443.
[9] Isbell and Frush, *J. Amer. Chem. Soc.*, 1950, **72**, 1043.
[10] Cameron, *ibid.*, 1927, **49**, 1759; Bayly, Bourne, and Stacey, *Nature*, 1952, **169**, 876.

screen to detect the ultraviolet light absorption.[11] Aniline hydrogen phthalate [8] showed only a slight reaction with *N*-benzylglycosylammonium ions. Detection by periodate followed by benzidine [7] was found to be suitable providing the amount of the ion present was known approximately.

Choice of Support.—Whatman No. 1 paper can be substituted for Whatman No. 3 with the advantage that a lower current results for a given voltage. An attempt was made to use fibreglass paper. Unfortunately there is a large endosmotic flow towards the cathode which markedly decreases the absolute distance of migration of a cation which can be achieved on a given length of paper. Also the fibreglass holds more electrolyte than paper and thus becomes more difficult to cool.

Use of Other Amines.—It was realised that use of an amine having a lower molecular weight or one that would give rise to a doubly charged ion might increase the relative separation or speed of separation of a homologous series of oligosaccharides. Methylamine formate and ammonium formate both led to the formation of their corresponding ions which showed the expected increased mobilities. Optimum conditions were determined but these ions, particularly the glycosylammonium ions, were more unstable and occasionally more than one positive ion was obtained from a given sugar.[10] Spots of D-xylose and ammonium formate (10%) in methanol were heated for various times at 85° and then submitted to ionophoresis. The amount of D-xylosylammonium ion formed increased to a maximum after 3 minutes' heating; none could be detected after heating for longer than 5 minutes. Other ions were produced after various times with mobilities 0·816 (after 2·5 min.), 0·64 (after 3 min.), and 0·15 (after 5 min.). Glucosylammonium ions, although more stable, underwent a similar decomposition giving ions with mobilities 1·00, 0·63, 0·49, and 0·12. One of the decomposition products was presumably formed by condensation to give a diglycosylammonium ion. The mobility (*M*3) of a diglycosylammonium ion was expressed as the ratio of the distances separating the diglycosylammonium ion from the unchanged sugar and the glucosylammonium ion from unchanged glucose (see Table 4). Evidence for the molecular weight of these ions was provided by the following comparisons of mobilities: diglucosylammonium 0·63, maltosylammonium 0·60; dimaltosylammonium 0·33, maltotetraosylammonium 0·31; dimaltotriosylammonium 0·24, maltohexaosylammonium 0·19.

TABLE 4. *Mobilities of diglycosylammonium ions.*

Ion	Mobility (*M*3)	Ratio (*X*3) *	Ion	Mobility (*M*3)	Ratio (*X*3) *
Diarabinosylammonium	0·82	0·64	Dimaltotriosylammonium	0·24	0·18
Diglucosylammonium	0·63	0·53	Dimaltotetraosylammonium ...	0·15	0·14
Di-D-*glycero*-D-*gala*heptosylammonium	0·53	0·45	Dimaltopentaosylammonium	0·12	0·11
Dimaltosylammonium	0·33	0·27	Dimaltohexaosylammonium ...	0·09	0·09

* *X*3 = (Mol. wt. of glucosylammonium ion)/(mol. wt. of diglycosylammonium ion).

Glucose has been condensed with dimethylamine in methanol and formic acid by heating them at 90° for 5 min. Ionophoresis with formate (pH 1·8), followed by development of the paper with alkaline silver nitrate, showed that the *N*-dimethylglucosylammonium ion was formed in good yield. Condensation of glucose with aniline in methanol and formic acid at 85° for 5 min. gave only a small amount of *N*-phenylglucosylammonium ion. 2-Aminopyridine also gave a poor conversion and the spot detected corresponded to an ion carrying only one positive charge. No positive ion was observed when using glucose and 2 : 4-dinitrophenylhydrazine, hydrazine, or hydroxylamine.

Table 5 illustrates the inverse relation between the mobility and molecular weight of some of these substituted glucosylammonium ions. The mobility (*M*4) of such an ion was the ratio of distances separating it and the *N*-benzylglucosylammonium ion from unchanged glucose.

[11] Jones and Marsh, unpublished work.

TABLE 5. *Comparative mobilities of substituted glucosylammonium ions.*

Ion	Mobility (*M*4)	Ratio (*X*4) *	Ion	Mobility (*M*4)	Ratio (*X*4) *
N-Benzylglucosylammonium ...	1·00	1·00	*N*-Dimethylglucosylammonium	1·27	1·30
N-Phenylglucosylammonium ...	1·02	1·05	*N*-Methylglucosylammonium ...	1·28	1·39
N-Pyridylglucosylammonium	1·02	1·05	Glucosylammonium	1·32	1·50

* *X*4 = (Mol. wt. of *N*-benzylglucosylammonium ion)/(mol. wt. of the ion).

Other Observations.—Non-reducing sugars, *e.g.*, sucrose, αα-trehalose, raffinose, and methyl α-D-glucoside did not react with benzylamine. When fructose was heated with benzylamine formate the spot became brown and conversion into the positive ion was only about 20%. In the paper-chromatographic method *N*-benzylfructosylamine was unstable. The *N*-benzyl-2-deoxyglucosylammonium ion was unstable but the *N*-benzyl-3-deoxyglucosylammonium ion was formed in a poor yield.

In all the above experiments the relative mobilities of the ions were reproducible ($M \pm 0{\cdot}015$) but the absolute mobilities varied owing to many factors.[12] Ions placed near the edge of the paper tended to move more slowly and in each experiment suitably placed markers were used.

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[12] Bourne, Foster, and Grant, *J.*, 1956, 4311.