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Note

Formation of D-*arabino*-hexosulose-containing oligosaccharides during borate ion-exchange chromatography of lactose*

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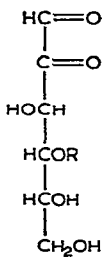
Transformation of lactose to its keto isomer, lactulose (4-*O*-β-D-galactosyl-D-*arabino*-hexulose), was observed in our laboratory[1] during borate ion-exchange chromatography at room temperature Other examples of anomalous elution profiles from these columns have been reported[2–4] Since reducing sugars can be profoundly affected by alkalis, even under mild conditions[5,6], the use of alkaline eluting solutions at or above room temperature deserves special consideration

When lactose was subjected to ion-exchange chromatography on columns of Dowex 1 (X-8 borate form) resin maintained at 25°, in addition to the peaks of lactose and lactulose eluted with 5mM and 15mM borax respectively, a third sugar-containing peak was eluted by 0 1M sodium hydrogen carbonate After partial purification of the latter peak by removal of carbonate and borate ions, and acidic sugars, the mixture of neutral sugars was fractionated by paper chromatography, t l c, and Sephadex gel-filtration into mainly disaccharides and tetrasaccharides Small amounts of monosaccharides, mostly D-galactose and traces of both D-fructose and D-*arabino*-hexosulose (glucosone) (**1**) were also present Acid or enzymic (β-D-galactosidase) hydrolysis of the oligosaccharides yielded a mixture of galactose and **1**



1 R = H
2 R = β-D-galactopyranosyl
3 dimer of 2

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The latter, as well as the oligosaccharides which contain it, gave elongated spots on paper chromatograms These spots gave characteristic colors, purple with aniline–diphenylamine[7] and yellow with aniline hydrogen phthalate[8] These sugars were all quite unstable in aqueous solution Attempts to concentrate solutions of D-*arabino*-hexosulose by lyophilization yielded a syrup and resulted in some decomposition The aqueous solutions of the oligosaccharides, on the other hand, could be concentrated by lyophilization to yield white hygroscopic powders

The identity of D-*arabino*-hexosulose was established by (a) its extreme lability to alkali, (b) its failure to produce typical colors with anthrone, phenol, and 3,5-dinitrosalicylic acid reagents, (c) its strong reducing properties at 25°, (d) its conversion into D-fructose by reduction with sodium borohydride, (e) its conversion into the respective D-glucosazone derivatives upon reaction with phenylhydrazine or dinitrophenylhydrazine at 25°, (f) its major products of alkaline treatment (*i e* D-arabinonic acid with sodium hydroxide and D-mannonic acid with calcium hydroxide), and (g) its behavior in t l c , paper chromatography, and ionophoresis which was indistinguishable from that of the authentic compound

Identification of D-*arabino*-hexosulose as the reducing residue of the D-galactose-containing disaccharide, after hydrolysis by β-D-galactosidase, suggested that the disaccharide is 4-*O*-β-D-galactopyranosyl-D-*arabino*-hexosulose (lactosone) (**2**) When the trimethylsilyl ether derivative of **2** was subjected to g l c , a major peak with a retention time of 17 4 min (retention time of lactose, 17 8 min) was observed Two additional, small peaks of unknown identity having retention times of 23 6 and 25 6 min, respectively, were also present The disaccharide **2** exhibited the same strong reducing properties as the monosaccharide **1**, and sodium borohydride treatment of **2** under controlled conditions yielded lactulose When examined by t l c , paper chromatography, and ionophoresis, authentic lactosone was indistinguishable from disaccharide **2** Spots of **2** gave the typical purple and yellow colors observed with **1**, and in addition, such reagents as 3,5-dinitrosalicylic acid and *p*-anisidine, which did not react with **1**, gave with the D-galactosyl moiety of **2** brown and green colors, respectively, identical to those given by standard D-galactose

A compound (**3**) was also isolated from the mixture of neutral sugars by column chromatography on Sephadex G-15 Since this compound is also composed of D-galactose and **1** and is eluted from the Sephadex column in the same position as stachyose, it probably represents a product of dimerization of **2** The chemical properties of **3** were similar to those of the disaccharide **2** Both compounds gave a positive reaction with Fehling[9] and Park–Johnson[10] reagents at 25°, but proportionately less color was produced by **3** Similarly, the intensity of the purple color yielded by **3** on chromatograms treated with aniline–diphenylamine was also smaller than that observed when the same amount of **2** was deposited on paper Colors produced with other carbohydrate-detection reagents were identical for both compounds In view of these findings and of the fact that glycosuloses are known to condense very easily[11], polymerization of **2**, involving the hexosulose portion of the molecule, appears to be a plausible explanation for the formation of the observed

product 3 of tetrasaccharide size The product obtained by borohydride reduction of 3 could not be readily identified and the compound was not characterized further.

The conditions of the ion-exchange chromatography (*i e*, mild alkaline pH, room temperature, and the presence of air and of borate ions) probably provide an optimal environment for a partial oxidation of disaccharides and, at the same time, they might retard degradation of the products by stabilizing the hexosulose-containing oligosaccharides through the formation of borate complexes It is unlikely, that resin impurities may catalytically affect the oxidative process because, although the manufacturer's assay listed 4% ash for Dowex 1-X-8 resin (commercial grade) and 0 06% ash for Bio-Rad AG 1-X-8 resin (analytical grade), the production of hexosulose-containing oligosaccharides (Table I) was equally effective with either resin On the other hand, since the sodium tetraborate solutions also contain trace amounts of Ca^{2+}, Pb^{2+}, and Fe^{3+}, the possible catalytic role of impurities cannot be ruled out

TABLE I

YIELD OF D-*arabino*-HEXOSULOSE-CONTAINING OLIGOSACCHARIDES FORMED DURING BORATE ION-EXCHANGE CHROMATOGRAPHY OF LACTOSE

Resin[a]	*Column temp* (°)	*Yield* (%)[b]
I	25	8 2
I	25	8 4
I	25	9 6
I	25	4 8
II	25	1 0
II	25	1 4
II	25	5 4
II	37	7 0
II	37	3 4
III	25	6 2
III	25	4 0

[a]Resin I was commercial grade Dowex 1 (X-8, Cl^-, 50–100 mesh) resin (J T Baker Chemical Co , Phillipsburg, N J 08865) Resin II was the same commercial-grade resin type from Bio-Rad Laboratories, Inc Richmond, Ca 94804 Resin III was an analytical-grade resin designated AG 1 (X-8, Cl^-, 50–100 mesh) from Bio-Rad Laboratories, Inc [b]Yields are expressed as percent of initial dry weight of lactose The analyses were performed by the anthrone reaction with D-galactose as the standard, and the results are not corrected for the presence of the anthrone-negative sugar residues

The practical implications of the findings described in this paper are that column ion-exchange chromatography methods, particularly those employing alkaline borate solutions and elevated temperatures, must be used with caution because of the alterations in sugar structure which have been shown to occur

EXPERIMENTAL

Chromatographic procedures — Paper chromatograms were developed by descending irrigation on Whatman No 1 paper with the following solvent systems

(v/v). *A*, 3 3 1 ethyl acetate–acetic acid–water, *B*, 5 3 2 butanol–pyridine–water; *C*, 4 1 1 butanol–acetic acid–water, *D*, 4 1 5 butanol–acetic acid–water (upper phase), *E*, 4 1 phenol–water, *F*, 4.1 1 butanol–95% ethanol–water, and *G*, 6 3.1 ethyl acetate–95% ethanol–water. T l c was performed on glass plates (20 × 20 cm) precoated with a 250-μm thickness of silica gel (Mann Research Laboratories, Orangeburg, N Y 10962). The plates were developed with solvent *G* or with water-saturated butanol (solvent *H*) Ionophoresis experiments were performed on Whatman No 3 MM paper with 0 05M sodium tetraborate, pH 9 2, at 375 V for 4 h The spots were detected with *a*, aniline–diphenylamine[7], *b*, aniline hydrogen phthalate[8], and *c*, *o*-biphenylamine oxalate[12]

G l c. of the per(trimethylsilyl) ethers[13] was performed with a Perkin–Elmer 881 gas chromatograph equiped with a glass column packed with 3% SE-30 on Gas-Chrom Q, 100–120 mesh (Applied Science Labs, Inc , State College, Pa 16801) and a hydrogen-flame, ionization detector The column was maintained at 140° for the separation of aldonic acids and at 210° for the separation of disaccharides

Borate ion-exchange chromatography — Columns (1 × 18 cm) were packed with Dowex 1 (X-8, Cl^-, 50–100 mesh) resin pretreated with hot 4M hydrochloric acid[14], or with Bio-Rad AG 1 (X-8, Cl^-) resin without pretreatment The resins were converted into the borate form with 0 1M sodium tetraborate (borax, 15 column-vol), and the columns were equilibrated with 5mM sodium tetraborate at a flow rate of 60 ml per h Samples (0 5 g) of α-lactose (Fisher Scientific Co , Pittsburgh, Pa 15219) were dissolved in 100 ml of 5mM sodium tetraborate and the solutions were allowed to equilibrate for 2 h at 25° prior to application to the column Elution was performed at 25° by the method previously described[1 15] but with sodium tetraborate instead of potassium tetraborate Fractions of 100 ml each were collected and analyzed for hexose by the anthrone reaction[16] Nontransformed lactose was eluted with 5mM sodium tetraborate and lactulose was eluted with 15mM sodium tetraborate (*ca* 2 l of each solution) A third peak of sugars was eluted with 0 1M sodium hydrogen carbonate, the elution was completed usually after 200–300 ml of effluent had been collected The fractions from the third peak were pooled and treated batchwise with Dowex 50W (X-8, H^+, 20–50 mesh) resin to remove sodium ions and to facilitate evolution of carbon dioxide. The solution was then concentrated and passed through a column (2 9 × 35 cm) of the same resin, the effluent was evaporated to dryness, and then treated with methanol to remove boric acid as methyl borate The final residue, dissolved in water (25 ml) gave a clear, colorless acidic solution (pH 3) Contaminating acidic components were removed on a column (2 9 × 35 cm) of Bio-Rad AG 1 (X-2, HCO_2^-, 200–400 mesh) resin, and formic acid was evaporated from the effluent by repeated evaporation at 40°. The final residue, dissolved in water (25 ml), gave a solution of pH 6

Gel filtration — Aliquots (*ca* 20–40 mg dry wt) of the mixture of neutral sugars were dissolved in water (1 ml), applied to a column (2 2 × 94 cm) of Sephadex G-15, and then eluted with degassed water at a flow rate of 6 ml per h Fractions (3 ml each) were collected and analyzed by the anthrone method The column was

previously standardized for void volume (V_o) with Blue Dextran and for sugar-elution volumes (V_e) with a mixture composed of galactose, lactose, raffinose, and stachyose (5 mg each) The V_e values for product 3 (181 ml), for 2 (210 ml), and for 1 (228 ml) corresponded closely to those of the tetrasaccharide (stachyose), the disaccharide (lactose), and the monosaccharide (galactose), respectively

Borohydride reduction — A 1 5 (w/w) proportion of sugar to sodium borohydride in 0 1M boric acid–sodium borate buffer, pH 7 6 was used After 16 h at 4°, the solution was neutralized with acetic acid, and the sodium ions were removed on a column (2 × 24 cm) of Bio-Rad AG 50W (X-8, H^+, 200–400 mesh) resin The eluate was evaporated and boric acid removed as volatile methyl borate

Alkaline treatment — Aliquots of **1** (10 mg) were treated with 0 04M sodium hydroxide and with 0 02M calcium hydroxide following essentially the experimental procedure of Lindberg and Theander[17]

Preparation of D-arabino-*hexosulose* (**1**) — Three different methods were used (a) D-Glucose or D-fructose was oxidized with cupric acetate[18] and **1** was isolated and purified by preparative paper chromatography on pre-washed Whatman No. 3 MM with solvent system *A* (b) Acid degradation of *N*-butyl-D-glucopyranosylamine[19] gave **1**, in addition to 3-deoxy-D-*erythro*-hexosulose Isolation and purification of these compounds was performed on a column (4 6 × 42 cm) of microcrystalline cellulose (Avicel)[20] with solvent system *A* (c) D-Fructose phenylosazone was cleaved with nitrous acid[21] D-*arabino*-Hexosulose obtained by these three methods gave a single, elongated spot on paper chromatograms R_G 0 55 (*A*), 1 0 (*B*), 1 2 (*C*), 1 1 (*D*), 0 77 (*E*), 1 1 (*F*), and 0 73 (*G*), in t l c R_G 0 73 (*G*) and 1 2 (*H*); in borate ionophoresis, M_G 1 06

4-O-*β*-D-*Galactopyranosyl*-D-arabino-*hexosulose* (**2**) — This compound was prepared by the cleavage of lactose phenylosazone[22], modified by use of nitrous acid[21] Attempts to prepare **2** by oxidation of lactose with cupric acetate were unsuccessful, whereas the reaction of lactose with butylamine and acetic acid gave only small amounts of **2** Authentic **2** and the disaccharide obtained during borate ion-exchange chromatography of lactose showed one spot on paper chromatograms R_G 0 33 (*A*), 0 80 (*B*), 0 55 (*C*), 0 58 (*D*), and 0 86 (*E*); in t l c R_G 0 25 (*G*) and 0 35 (*H*), in borate ionophoresis M_G 0 75.

Enzymic hydrolysis — Oligosaccharides **2** and **3** were hydrolyzed with *β*-D-galactosidase (Lactase from *Saccharomyces fragilis*, Nutritional Biochemicals Corp , Cleveland, Ohio 44128) The experimental procedure was essentially that of Caputto *et al*[23] For identification of hydrolysis products, the enzyme was precipitated with ethanol and removed by centrifugation After evaporation of the alcohol, the aqueous hydrolyzate was desalted on coupled columns (1 × 5 cm each) of Bio-Rad AG 50W (X-12, H^+) and AG 1 (X-2, HCO_2^-) resins, and then analyzed by paper chromatography

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