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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF THE ISOMERIZATION PRODUCTS OF CARBOHYDRATES

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SUMMARY

A high-performance liquid chromatographic system for the separation of complex mixtures of carbohydrates is described. The efficiency of the system is based on the application of anion-exchange resins (particle size 10–20 μm), a specially designed gradient chamber and a solution of orcinol in concentrated sulphuric acid as the detection reagent. On the basis of the chromatographic separations, a reaction mechanism for the molybdic acid-catalyzed isomerization of aldopyranoses is given.

INTRODUCTION

Column chromatographic separations of sugar isomers are among the most difficult tasks in chromatography. In connection with our work on the isomerization reactions of carbohydrates[1–7], it became necessary for our laboratory to develop an efficient and rapid separation system for this class of compounds.

More than 20 years ago, the first column chromatographic separations of carbohydrates were reported. Since then, three different principles have been applied: (1) adsorption chromatography[8–10]; (2) partition chromatography[11–13]; and (3) ion-exchange chromatography[14–17]. Ion-exchange chromatography of carbohydrates on anion-exchange resins in the borate form has been performed for years and the recent work has been surveyed in an excellent review[18].

The availability of anion-exchange resins of small particle size, the application of our specially designed gradient-generating system[17] and the use of a solution of orcinol in concentrated sulphuric acid as the detection reagent prompted us to develop an efficient and rapid separation apparatus for the separation of carbohydrates.

EXPERIMENTAL

Materials

Sugars of the highest available purity were obtained from E. Merck (Darmstadt, G.F.R.). Boric acid, sulphuric acid and orcinol were of analytical-reagent grade and were also purchased from Merck. Potassium tetraborate was obtained from Riedel-de Haen (Seelze-Hannover, G.F.R.).

For the separations, the anion-exchange resins DA-X4 and DA-X4F (Durrum, Palo Alto, Calif., U.S.A.), which are 4% cross-linked polystyrenes of particle size $20 \pm 5\ \mu m$ and $11 \pm 1\ \mu m$, respectively, were used. The pre-washing column was filled with Dowex 1-X4 resin (200–400 mesh).

Borate buffers were prepared by dissolving boric acid in doubly distilled water and adjusting the pH to the desired value with concentrated sodium hydroxide solution.

For regeneration of the resin, aqueous potassium tetraborate solution was used, 100 g of potassium tetraborate tetrahydrate being dissolved in 1 l of deionized water.

Orcinol–sulphuric acid reagent was prepared by dissolving 2.5 g of orcinol in 2.5 l of concentrated sulphuric acid.

Apparatus

Fig. 1 shows a schematic diagram of the carbohydrate analyzer constructed in our laboratory.

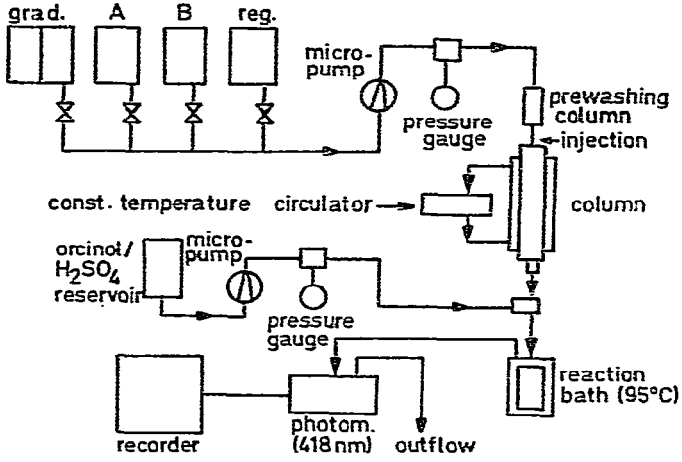


Fig. 1. Schematic diagram of the carbohydrate analyzer. grad. = Gradient-generating system; A,B = buffer reservoirs; reg. = regenerating buffer reservoir.

Borate complexes of carbohydrates specifically interact with anion-exchange resins and are therefore separable on columns filled with such material. A Milton Roy Dosapro micro-pump pumps borate buffer from a gradient-generating system (grad., Fig. 1)[7] and borate buffer reservoirs (A, B, Fig. 1) via a pre-washing column on a Biotronik (Frankfurt, G.F.R.) glass-jacketed high-performance liquid chromatographic column, filled with DA-X4 anion-exchange resin. To the effluent from the column, orcinol–sulphuric acid reagent is added and the mixture is passed through a 20 m × 0.7 mm PTFE coil that is situated in a heated (98–100°) water-bath. Under these conditions, sugars from furan derivatives react with orcinol to give a red-yellow dye which has an absorption maximum at 420 nm. A Biotronik BT 6620 spectrophotometer with an interference filter at 420 nm is used for photometric detection (path length of the optical cell, 1 cm; extinction ranges, 0.1, 0.2, 0.5, 1.0 and 2.0 absorbance units).

Preparation of the sugar mixtures

Pure carbohydrate (1 g) is dissolved in 100 ml of water or in 100 ml of methanol–water (30:70). To these solutions, 10 mg of molybdic acid are added and then the mixtures are heated for different periods of time. The reaction mixtures are cooled to room temperature, filtered and deionized with Dowex 1-X4 (OH^-).

RESULTS AND DISCUSSION

Chromatographic separations

The efficiency of our system[7] is demonstrated by the 16-component standard chromatogram in Fig. 2. The buffer gradient (Fig. 2, broken line) is produced by a specially designed two-chamber gradient genetaror. One of these chambers is filled with the diluted borate buffer A (0.1 mole/l) of pH 8.0 and the other with the concentrated borate buffer B (0.5 mole/l), adjusted to pH 10. Fig. 2 shows that a mixture of 16 mono- and disaccharides are well separated in about 5 h.

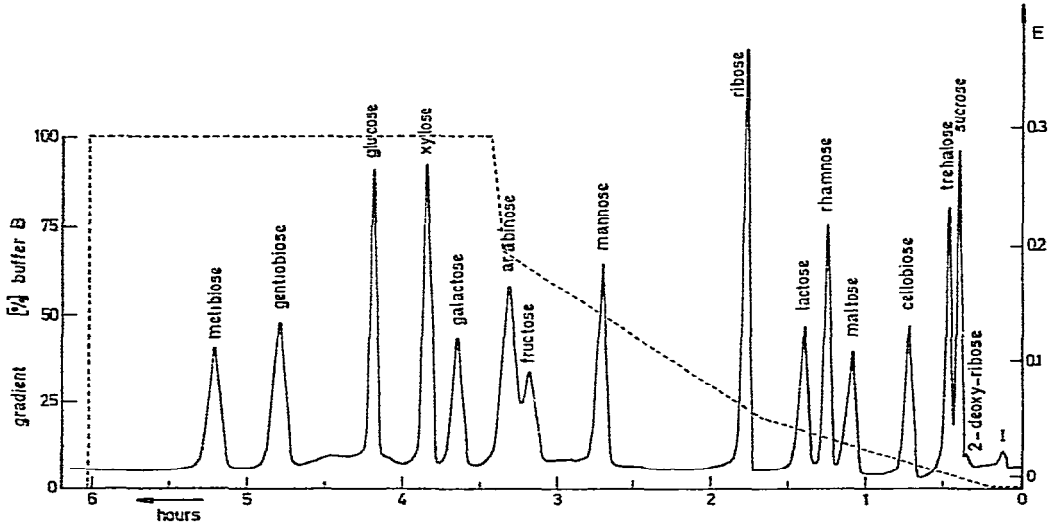


Fig. 2. Chromatogram of a 16-component standard. Gradient, 0.1–0.5 mmole/l H_3BO_3, pH 8.0–10.0; 280 × 6 mm column; DA-X4 resin; flow-rate, 1 ml/min; temperature, 50°; recorder range, 0–0.5 A. Each peak represents 80 nmole of monosaccharide or 40 nmole of disaccharide. I = Injection peak.

Separations with one-buffer elutions are more convenient. Using resins of small particle size, many of the liquid chromatographic problems in carbohydrate chemistry can be solved in a minimum of time, as demonstrated by Fig. 3. A mixture of six common monosaccharides is separated in less than 1 h by applying a 0.4 *M* H_3BO_3 buffer of pH 9.2 and a DA-X4F resin of particle size 11 ± 1 μm.

Relationship between peak area and sugar concentration

For several sugar analysis systems, the orcinol reagent is prepared from 70% sulphuric acid[15,19] [*e.g.*, 1 g of orcinol is dissolved in 1 l of 70% (v/v) sulphuric acid]. This orcinol reagent becomes brownish after storage for longer than 3 weeks in the

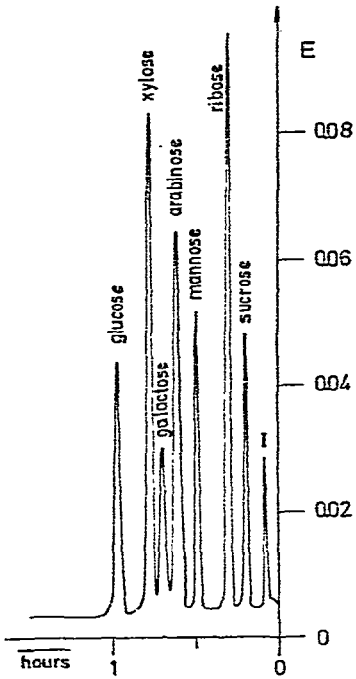


Fig. 3. Chromatogram of a seven-component standard. One-buffer elution with H_3BO_3 buffer (0.4 mole/l, pH 9.2); 190 × 6 mm column; DA-X4 resin; flow-rate, 1.3 ml/min; temperature, 60°; recorder range, 0–0.1 A. Each peak represents 1 μg per component. I = Injection peak.

cold, and higher blank values and lower colour yields are obtained with such a colorimetric reagent[19]. However, we found that the stability of the orcinol–sulphuric acid reagent can be improved considerably by dissolving 1 g of orcinol in 1 l of concentrated (95–97%) sulphuric acid. This 0.1% orcinol reagent is stable for several months without forming brownish by-products.

The coupled furan- and dye-forming reaction of carbohydrates in the presence of the ocrinol–sulphuric acid reagent is very dependent on the acid concentration, as demonstrated in Fig. 4. The highest colour yield is acieved with 50% sulphuric acid.

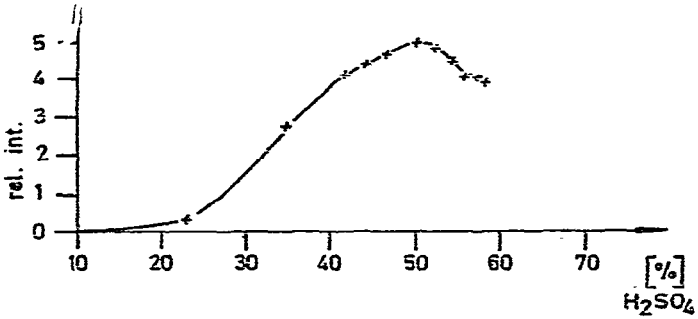


Fig. 4. Dependence of the colour yield on the sulphuric acid concentration for the reaction of sucrose with orcinol (0.1%)–sulphuric acid reagents of different sulphuric acid concentrations. The solutions were 0.01 *M* in sucrose and were measured after heating for 15 min in boiling water at a wavelength of 423 nm. The relative intensity refers to scale units for an optical path length of 1 cm.

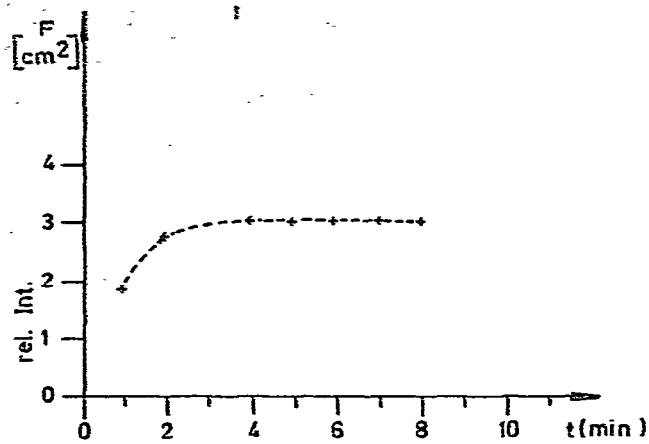


Fig. 5. Dye development for the reaction of sucrose with orcinol–sulphuric acid reagent in the sugar analyzer as a function of reaction time. Reaction-bath temperature, 100° (boiling water); borate buffer flow-rate, 60 ml/h; orcinol (0.1 %)–sulphuric acid (95–97 %) flow-rate, 66 ml/h; variation of the length of the mixing coil, 5.5–44 m; internal diameter of the mixing coil, 0.7 mm; t = flow-time of the sugar reagent mixture through the coil; rel. int. = relative intensity of the calculated peak areas, relative to $A = 0.0$–1.0.

The development of dye originating from sucrose as a function of time is shown in Fig. 5. The results were obtained with the sugar analyzer described above using reaction coils of different lengths. Fig. 5 shows that after a reaction time of 3–4 min the colour yields become constant.

The relationships between the peak areas and the concentrations of gentiobiose, lactose, mannose and glucose are given in Fig. 6. The results were ob-

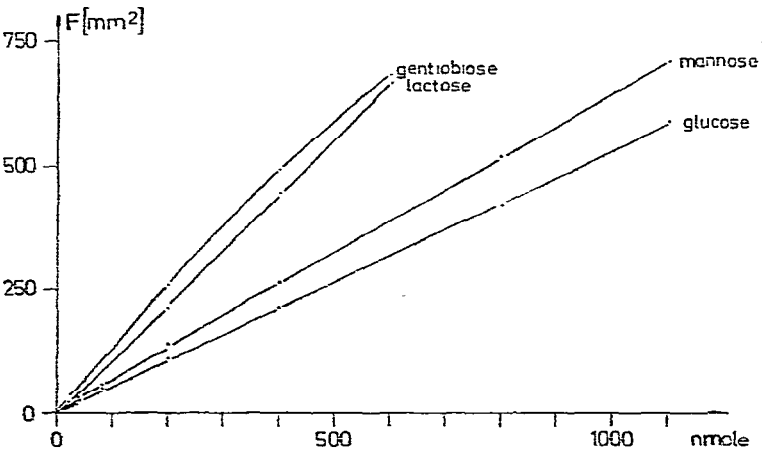


Fig. 6. Linearity of peak areas as a function of sugar concentration established with the sugar analyzer shown in Fig. 1. F = area (mm²) relative to $E = 1$; gradient, 0.1–0.5 mole/l H_3BO_4, pH 8.0–10.0; 280 × 6 mm column; DA-X4 resin; flow-rate, 1 ml/min; reagent flow-rate, 1.1 ml/min; temperature of separation column, 50°; temperature of heating bath, 100° (boiling water).

tained with the sugar analyzer, using a reaction coil of length 19 m and I.D. 0.7 mm. The limits of detection are in the range 0.1–2.0 nmole.

Under the experimental conditions, the relative elution times and the colour yields for a larger series of carbohydrates were established (Table I).

TABLE I

RELATIVE ELUTION TIMES AND MOLAR EXTINCTION COEFFICIENTS FOR SOME ORCINOL–SULPHURIC ACID DYES RESULTING FROM REACTIONS WITH SUGARS

Molar extinction coefficient $C_{HW} = HW/M$ (H = height of the peak in extinction units; W = half-width of the peak; M = amount of sugar in μmoles). Gradient, 0.1–0.5 mole H_3BO_3, pH 8.0–10.0; 280 × 6 mm column; DA-X4 resin; temperature of separation column, 60°.

Compound	*Relative elution time (glucose = 100)*	*Molar extinction coefficient*
Methyl β-arabinopyranoside	6.4	—
Methyl β-xylopyranoside	8.0	—
2-Deoxyribose	8.4	47.8
Sucrose	9.6	1056
Trehalose	11.2	912
Methyl α-mannopyranoside	12.0	—
Cellobiose	13.2	924
Methyl β-glucopyranoside	22.8	—
Maltose	26.4	864
Rhamnose	30.0	636
Lactose	33.6	1088
Erythrose	39.9	—
Ribose	42.8	963
Lyxose	42.0	—
Mannose	65.2	652
Tagatose	74.0	—
Fructose	76.4	506
Gulose	76.4	—
Lactulose	77.6	—
Allose	77.6	—
Arabinose	78.8	900
Fucose	78.8	—
Epimelibiose	82.8	—
Talose	85.3	—
Galactose	87.6	462
Altrose	89.0	—
Sorbose	89.2	—
Xylose	92.4	786
Glucose	100.0	684
Gentiobiose	114.4	1342
Melibiose	125.2	1336

Molybdic acid-catalyzed isomerization of aldopyranoses

Aldohexoses and aldopentoses from complexes with molybdate, as can be proved unequivocally by circular dichroism studies[20,21]. These sugars isomerize in the presence of catalytic amounts of molybdic acid upon heating in aqueous solutions[1,2,22].

In order to obtain greater insight into the reaction mechanism of these isomerizations, we investigated the reaction products with our carbohydrate analysis system.

Heating glucose solutions with catalytic amounts of molybdic acid causes epimerization and mannose can be isolated from the reaction mixture[23]. Fig. 7 shows a gradient chromatogram of an aqueous glucose solution treated for 14 days with catalytic amounts of molybdic acid. According to the peak intensities, about one third of the glucose has epimerized to mannose, but in addition small amounts of altrose, allose and disaccharides could also be detected.

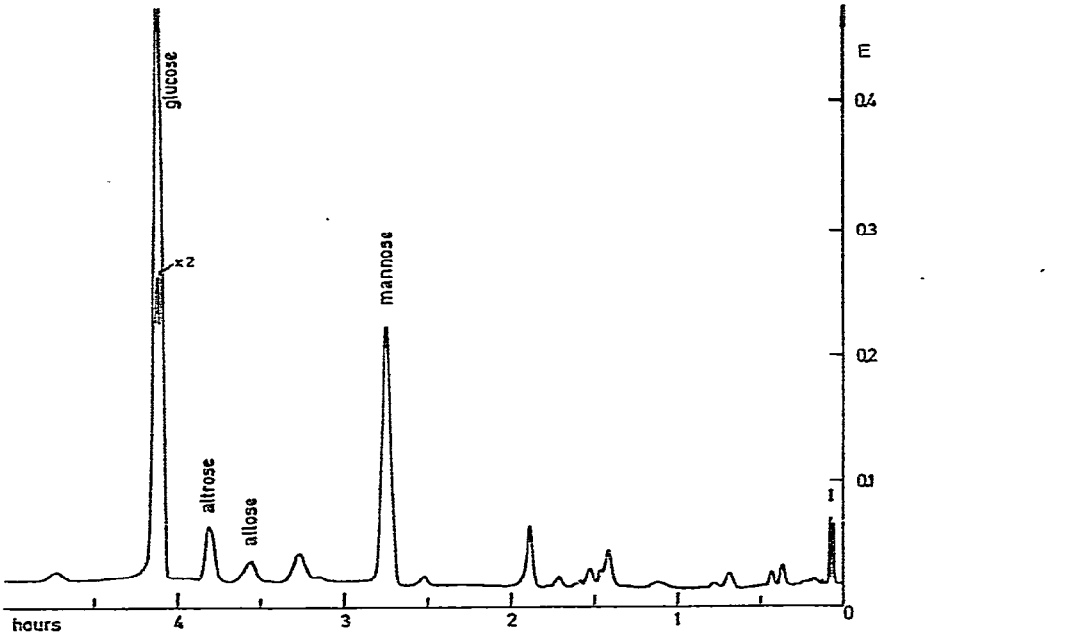
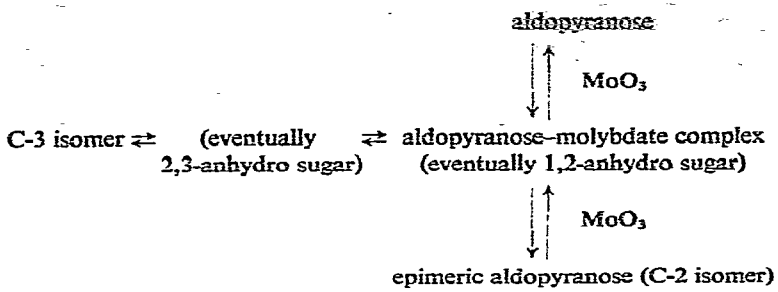


Fig. 7. Gradient chromatogram of the reaction mixture from an aqueous glucose solution heated for 14 days at 90° in the presence of catalytic amounts of molybdic acid. Gradient, 0.1–0.6 mole/l H_3BO_3, pH 8.0–10.0; 280 × 6 mm column; DA-X4 resin; flow-rate, 1 ml/min; temperature, 55°; injection of 50 μg. I = Injection peak.

Fig. 8 shows chromatograms of aqueous glucose and arabinose solutions heated with catalytic amounts of molybdic acid and obtained after 1–2 h and 140–190 h, respectively. The chromatograms demonstrate clearly that catalytic amounts of molybdic acid first cause epimerization and, after longer reaction times, isomerization at the C-3 position of aldopyranoses occurs. This conclusion was confirmed by the determination of the concentrations of the main products for several isomerization reactions as a function of time (Fig. 9).

On the basis of these chromatographic separations, the following reaction mechanism is suggested for the molybdic acid-catalyzed isomerization reaction:



The formation of oligosaccharides can be explained by the reaction of the intermediate with a second carbohydrate molecule.

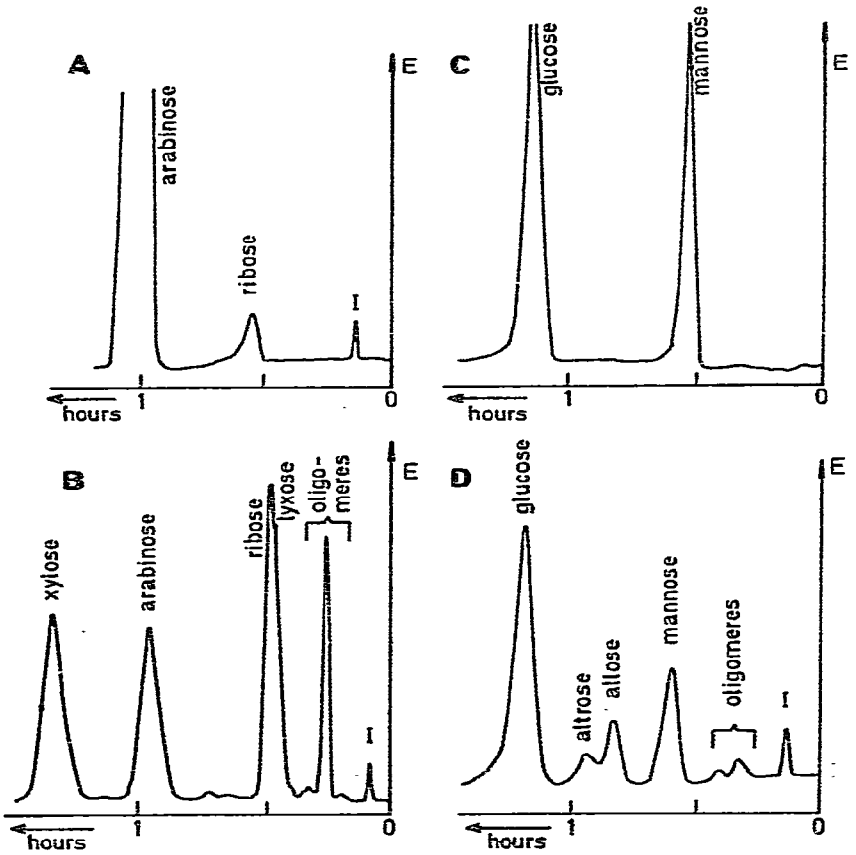


Fig. 8. Chromatograms of aqueous glucose and arabinose solutions heated with catalytic amounts of molybdic acid. A, starting material arabinose, reaction time 1.5 h; B, starting material arabinose, reaction time 188 h; C, starting material mannose, reaction time 2 h; D, starting material mannose, reaction time 141 h; one-buffer elution with H_3BO_3 buffer (0.4 mole/l, pH 9.0); 280 × 6 mm column (A and B)or 190 × 6 mm column (C and D); DA-X4 resin; flow-rate, 1 ml/min; temperature, 50°.

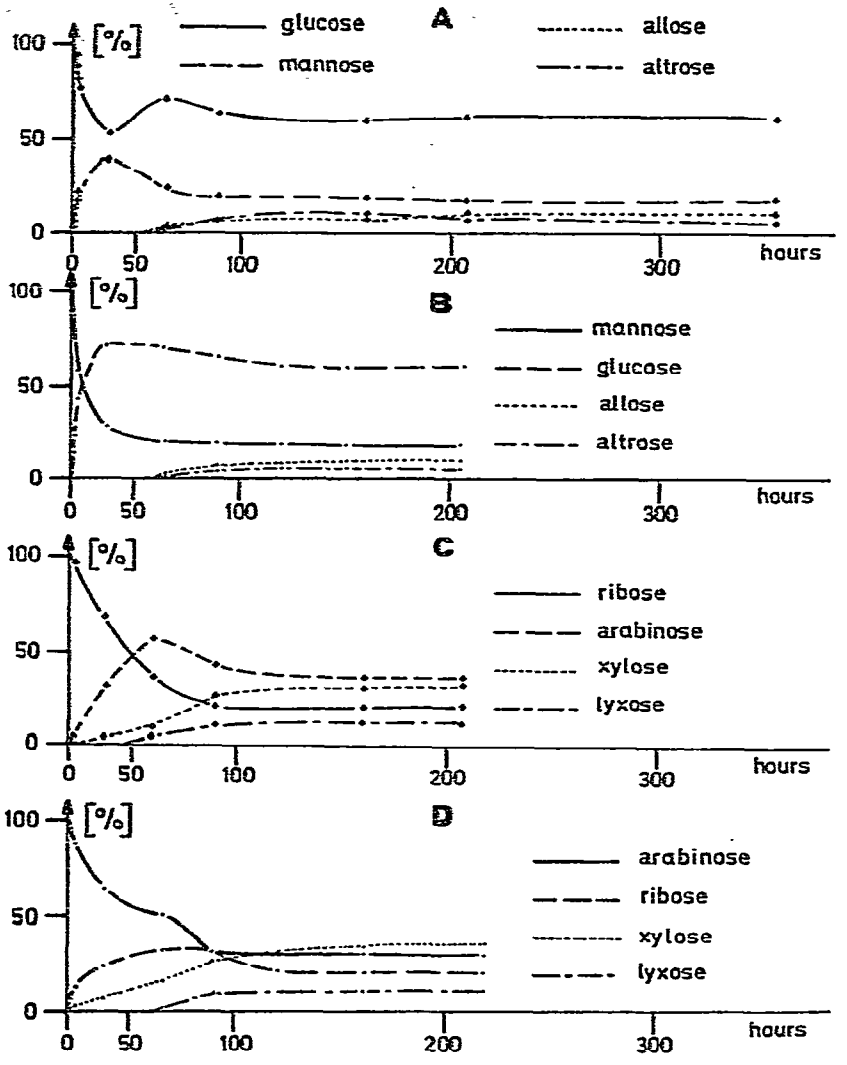


Fig. 9. Concentration of the components in molybdic acid-catalyzed isomerization reactions as a function of reaction time. Starting materials: A, glucose; B, mannose; C, ribose; D, arabinose. For separation technique, see legend to Fig. 8.

In the presence of methanol, the active intermediate forms a series of different methyl derivatives, which so far have not been identified. Fig. 10 shows the gradient chromatogram of the reaction mixture of glucose after heating for 14 days in methanol–water (3:7) at 90° in the presence of catalytic amounts of molybdic acid. The methylated carbohydrates are eluted from the column within 1–2 h.

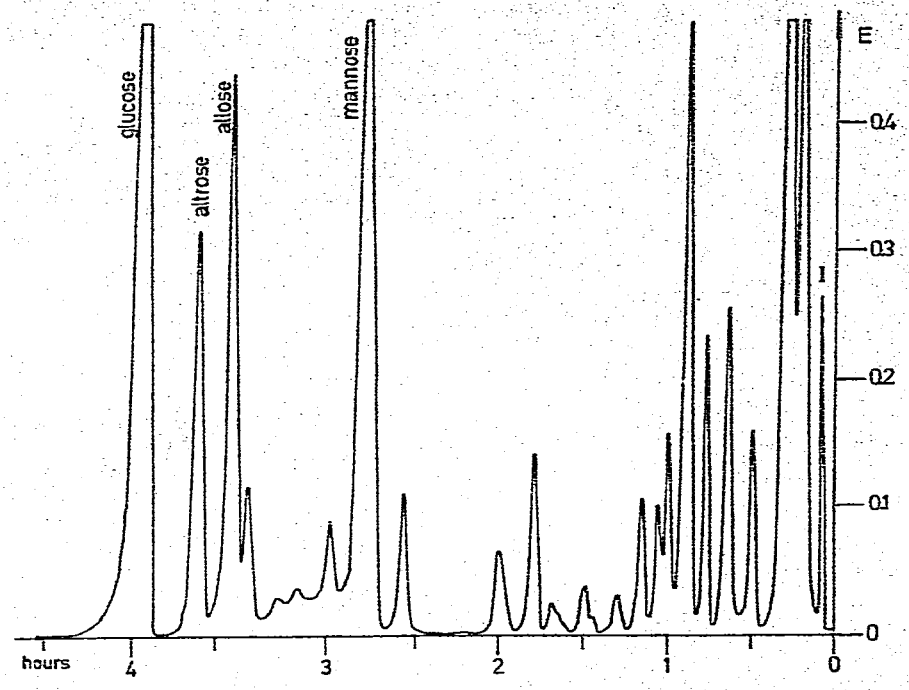


Fig. 10. Gradient chromatogram of the reaction mixture from a glucose solution heated for 14 days at 90° in the presence of catalytic amounts of molybdic acid and methanol–water (3:7). Gradient, 0.1–0.6 mole/l H_3BO_3, pH 8.0–10.0; 280 × 6 mm column; DA-X4 resin; flow-rate, 1 ml/min; temperature, 55°; injection of 50 μg. I = Injection peak.

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REFERENCES

1 V. Bilik, W. Voelter and E. Bayer, *Angew. Chem.*, 83 (1971) 967; *Angew. Chem. Int. Ed. Engl.*, 10 (1971) 909.
2 V. Bilik, W. Voelter and E. Bayer, *Justus Liebigs Ann. Chem.*, 759 (1972) 189.
3 W. Voelter (Editor), *Abstracts of the "Tübingen Seminar", Methoden zur Trennung und Strukturaufklärung von Kohlenhydraten, Z. Naturforsch. B*, 29 (1974) 1.
4 V. Bilik, W. Voelter and E. Bayer, *Justus Liebigs Ann. Chem.*, (1974) 1162.
5 W. Voelter and H. Bauer, *Tetrahedron Lett.*, (1974) 3597.
6 W. Voelter, *Chem.-Ztg.*, 98 (1974) 493.
7 H. Bauer, *Thesis*, University of Tübingen, Tübingen, 1975.
8 F. Hyashi, *J. Biochem. (Tokyo)*, 16 (1932) 1.
9 W. H. McNeely, W. W. Binkley and M. L. Wolfrom, *J. Amer. Chem. Soc.*, 67 (1945) 527.
10 R. L. Whistler and D. F. Durso, *J. Amer. Chem. Soc.*, 72 (1950) 677.
11 H. Rückert and O. Samuelson, *Sätryck Sv. Kem. Tidskr.*, 66 (1954) 337.
12 J. K. N. Jones, R. A. Wall and A. P. Pittet, *Can. J. Chem.*, 38 (1960) 2285.
13 J. K. N. Jones and R. A. Wall, *Can. J. Chem.*, 38 (1960) 2290.
14 J. X. Khym and L. P. Zill, *J. Amer. Chem. Soc.*, 73 (1951) 2399.

15 R. B. Kesler, *Anal. Chem.*, 39 (1967) 1416.
16 D. D. Chilcote, C. D. Scott and W. W. Pitt, Jr., *J. Chromatogr.*, 75 (1973) 175.
17 W. Voelter and H. Bauer, *GIT Fachz. Lab.*, 17 (1973) 846.
18 P. Jandera and J. Churáček, *J. Chromatogr.*, 98 (1974) 55.
19 Y. C. Lee, J. F. McKelvy and D. Lang, *Anal. Biochem.*, 27 (1969) 567.
20 W. Voelter, G. Kuhfittig, G. Schneider and E. Bayer, *Justus Liebigs Ann. Chem.*, 734 (1970) 126.
21 W. Voelter, G. Kuhfittig, O. Oster and E. Bayer, *Chem. Ber.*, 104 (1971) 1234.
22 V. Bílik and J. Čaplovič, *Chem. Zvesti*, 27 (1973) 547.
23 V. Bílik, *Chem. Zvesti*, 26 (1972) 183.