SEPARATION OF MALTODEXTRINS BY CHARCOAL CHROMATOGRAPHY*

DEXTER FRENCH, JOHN F. ROBYT, MELVIN WEINTRAUB AND PATRICIA KNOCK
Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa (U.S.A.)
(Received February 16th, 1966)

INTRODUCTION

The use of charcoal columns for the chromatographic separation of homologous oligosaccharides is a method of great potential in that it is capable of handling relatively large amounts of material fairly rapidly with fair to good resolution. The chromatographic support is inexpensive, and eluting solvents are available in a state of high purity. The method has been shown[1-9] to be capable of resolving starch and cellulose oligosaccharides up through six or more D-glucose units. Both stepwise and gradient elution have been used.

In the present study, we have automated the monitoring of the carbohydrate content of the effluent from charcoal columns by using a continuous recording polarimeter or by analyzing fractions using the Technicon Autoanalyzer. This has enabled us to examine the effect of some experimental variables such as the ratio of sample size to weight of charcoal, column size, steepness of gradient, use of *n*-BuOH** and *tert.*-BuOH as eluants, and pretreatment of the charcoal by various acids. While the handling of charcoal columns is still to a large degree an art, and not exactly reproducible, we usually have been able to separate linear starch oligosaccharides up through G_{11} or G_{12} and occasionally through G_{15}.

From amylase digests of amylopectin, we have also been able to effect clean separations of branched oligosaccharides in the range of 4 to 8 D-glucose units.

Use of *n*-BuOH or *tert.*-BuOH has consistently given superior results in comparison with EtOH. *tert.*-BuOH was selected as being the highest monohydric aliphatic alcohol completely miscible with water. The concentration of *n*- or *tert.*-BuOH required was very much less than that of EtOH for elution of a given oligosaccharide. In our experience with EtOH, it has usually been found that, for each successively higher oligosaccharide, an increase of 5% in EtOH concentration is required. Such increases rather rapidly lead to such high EtOH concentrations that the oligosaccharides become relatively insoluble. On the other hand, with *tert.*-BuOH, the increases were approximately 1% of alcohol for each higher homolog. Still smaller concentrations of *n*-BuOH were required.

* Journal Paper No. J-5302 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project No. 1116. Supported in part by grants from NIH (GM 08822) and the Corn Industries Research Foundation. Presented at the Winter meeting of the American Chemical Society Divisions of Carbohydrate Chemistry and Cellulose, Wood and Fiber Chemistry, Phoenix, Arizona, January 1966; Abstracts page D 1.

** The following abbreviations are used: G_2, G_3, etc. = maltose, maltotriose, etc.; B_6, etc. = branched hexasaccharide, etc.; EtOH = ethanol; *n*-BuOH, *tert.*-BuOH = normal and tertiary butyl alcohol, respectively.

Deactivation of the charcoal by prior treatment with acid was very effective in improving the resolution and preventing tailing of the peaks. TAYLOR AND WHELAN[7] have emphasized the importance of using acidic conditions (0.01 *N* formic acid in the eluting solvent). In our first trials, 0.01 *N* HCl was equally effective. However, stearic acid pretreatment of the charcoal, as suggested by ALM *et al.*[1] has given superior results when *tert.*-BuOH was used as eluant. With *n*-BuOH as eluant, we have obtained good recovery and superior resolution, even without using the stearic acid treatment. However, a much lower concentration of *n*-BuOH is required in the gradient-developing system, and in some cases, we have resorted to a 3-chamber gradient system.

The ratio of sample size to amount of charcoal (1 %) recommended by WHISTLER AND DURSO[3] has given satisfactory results. However, resolution of the higher compounds has been slightly improved by using a 0.7 % ratio.

In view of the successful extension of the method into the megalosaccharide range, we wish to present our preliminary results, even though much work remains to be done in improving the methods.

EXPERIMENTAL

Carbohydrates

In the initial work, a mixture of chromatographically pure D-glucose and commercial "C-P" maltose was used. The maltose contained appreciable quantities of maltotriose and other oligosaccharides which were frequently resolved. The carbohydrate sample for most of the work has been a digest of amylose ("Superlose", Stein-Hall Mfg. Co.) with crystalline *Aspergillus oryzae* α-amylase (Taka A)[10]. Ten grams of Superlose were suspended in 100 ml of dimethyl sulfoxide and heated to 90° until a clear solution was obtained; water was then slowly added with stirring to a volume of 900 ml; 100 ml of 200 m*M* acetate buffer pH 5.7 were added; 15 units* of *A. oryzae* α-amylase were added to effect the reaction, which was allowed to proceed at 40° (*ca.* 2 h.) until the reducing value indicated 25 % apparent maltose ($\overline{DP} = 8$). The reaction was stopped by adding 200 ml of glacial acetic acid; after a period of 2 h, the acid was neutralized to pH 5.4 with 200 ml of concentrated ammonium hydroxide. The digest was concentrated in a rotary vacuum evaporator to 500 ml. Paper chromatography of such a digest showed linear starch oligosaccharides from G_2 up through at least G_{11}.

Branched oligosaccharides were obtained by the action of crystalline human salivary α-amylase[11] on waxy maize starch. Waxy maize starch (25 g) was suspended in 1.5 l of water; 20 ml of 3 *M* sodium hydroxide was added, and the suspension was warmed in a boiling water bath until solution occurred; it was then cooled and neutralized (pH 6.5); 120 ml of 500 m*M* citrate buffer pH 6.5, and 300 ml of 1 *M* sodium chloride were added; the mixture was then diluted to 3 l. The reaction was effected by 1000 units* of crystalline human salivary α-amylase. The reaction was allowed to proceed at 37° under toluene for 72 h; it was terminated by boiling for 5 min and the reaction mixture was concentrated under vacuum to 200 ml. The bulk of the glucose, G_2 and G_3 were removed by a crude preliminary separation on a large charcoal column without attempting to resolve the individual branched components.

* Units are expressed as micromoles of glucosyl bonds cleaved per minute at pH 5.7 and 40°

Apparatus

The apparatus is indicated schematically in Fig. 1. The sintered glass plate indicated in Fig. 1 can be replaced by glass wool, which is more satisfactory especially with small columns. A solvent gradient was formed by allowing solvent (*e.g.* 10% *tert.*-BuOH) from a reservoir to flow into a large mixing chamber (initially containing pure water). The mixed solvent was conducted into the charcoal column. The effluent passed first through a flow cell in a continuous recording polarimeter (Model 143A,

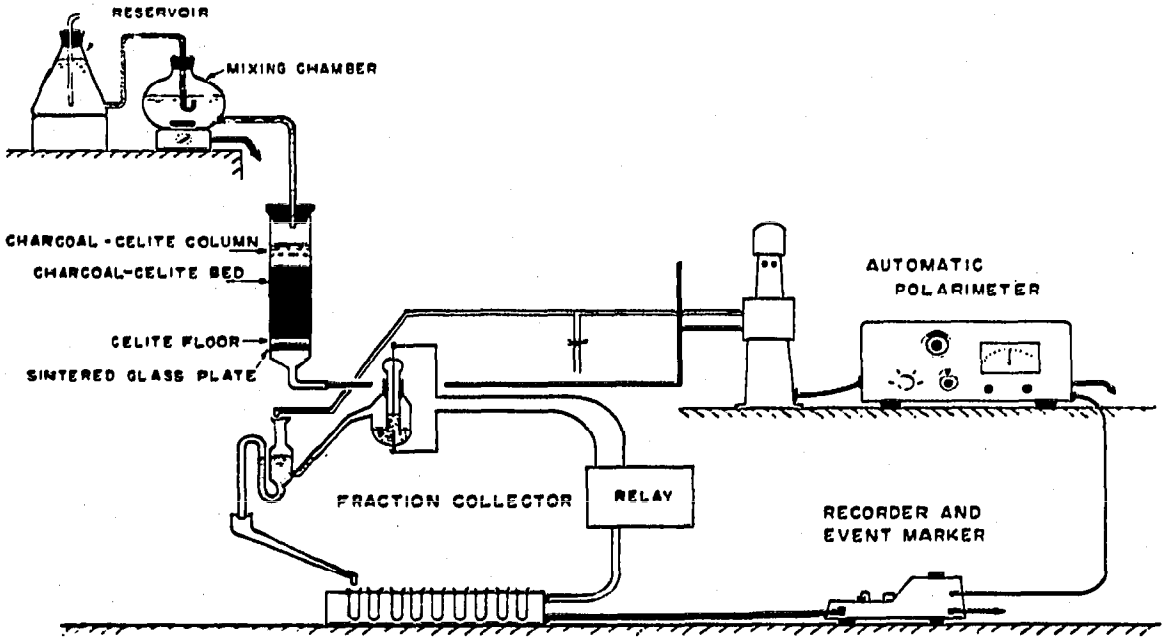


Fig. 1. Apparatus employed in the polarimetric automated charcoal columns.

Bendix Corporation, Cincinnati Division, Cincinnati, Ohio) and thence into a fraction collector. The recorder for the polarimeter was equipped with an event marker so that, at each advance of the fraction collector turntable, a signal was sent to the event marker. In this way, it was possible to obtain a record of the optical rotation of each fraction. The continuous flow cells supplied with the polarimeter were unsatisfactory for mixed solvents owing to the swelling of the gasket material and concomitant bubble collection within the cell. The bubble problem was partially remedied by using an all-glass flow cell and a simple but effective bubble trap and debubbling device. In some runs, the carbohydrate content of each tube was measured by determining the reducing value by the alkaline ferricyanide–cyanide procedure using the Autoanalyzer (Technicon Instruments Corp., Chauncey, N.Y.).

For chromatography at higher temperatures, a jacketed column, similar to that used for high temperature cellulose chromatography[12], was used.

Column preparation and chromatography

For the majority of the studies, an intermediate size column (3.6 × 40 cm) and a mixture of linear dextrins from the *A. oryzae* α-amylase digestion of amylose was used. The column packing was prepared by mixing 100 g of Darco G-60 (Atlas Chemical Industries, Wilmington, Dela.) with 75 g of Celite 560 (Johns-Manville,

Manville, N.J.) which was slurried with 1 *M* HCl and allowed to stand overnight. The slurry was filtered by suction and washed with water until chloride-free. If stearic acid treatment was used, the mixture was suspended in 666 ml of absolute EtOH which contained 16.7 g of stearic acid (2.5 % w/v). After 30 min it was filtered and resuspended in 666 ml of 50 % EtOH (v/v). It was then filtered and washed with about 1 l of water. The treated charcoal was slurried in about 400 ml of water, poured into the column, and washed with 2–5 l of water. A sample of 700 mg of carbohydrate in 100 ml of water was layered onto the top of the column and washed down with three 15 ml portions of water. After the sample and washings were absorbed onto the column, the charcoal was overlaid with about 100 ml of water and the gradient system started. At no time after making the column was it allowed to run dry. The gradient system consisted of two 6 l flasks. The first contained 6 l of 10 % *tert.*-BuOH and the second or mixing chamber 5 l of water. The flow rate was about 2.5 ml/min with a total hydrostatic head of approximately 2 m.

We were also successful in scaling up the method by using six times the amount

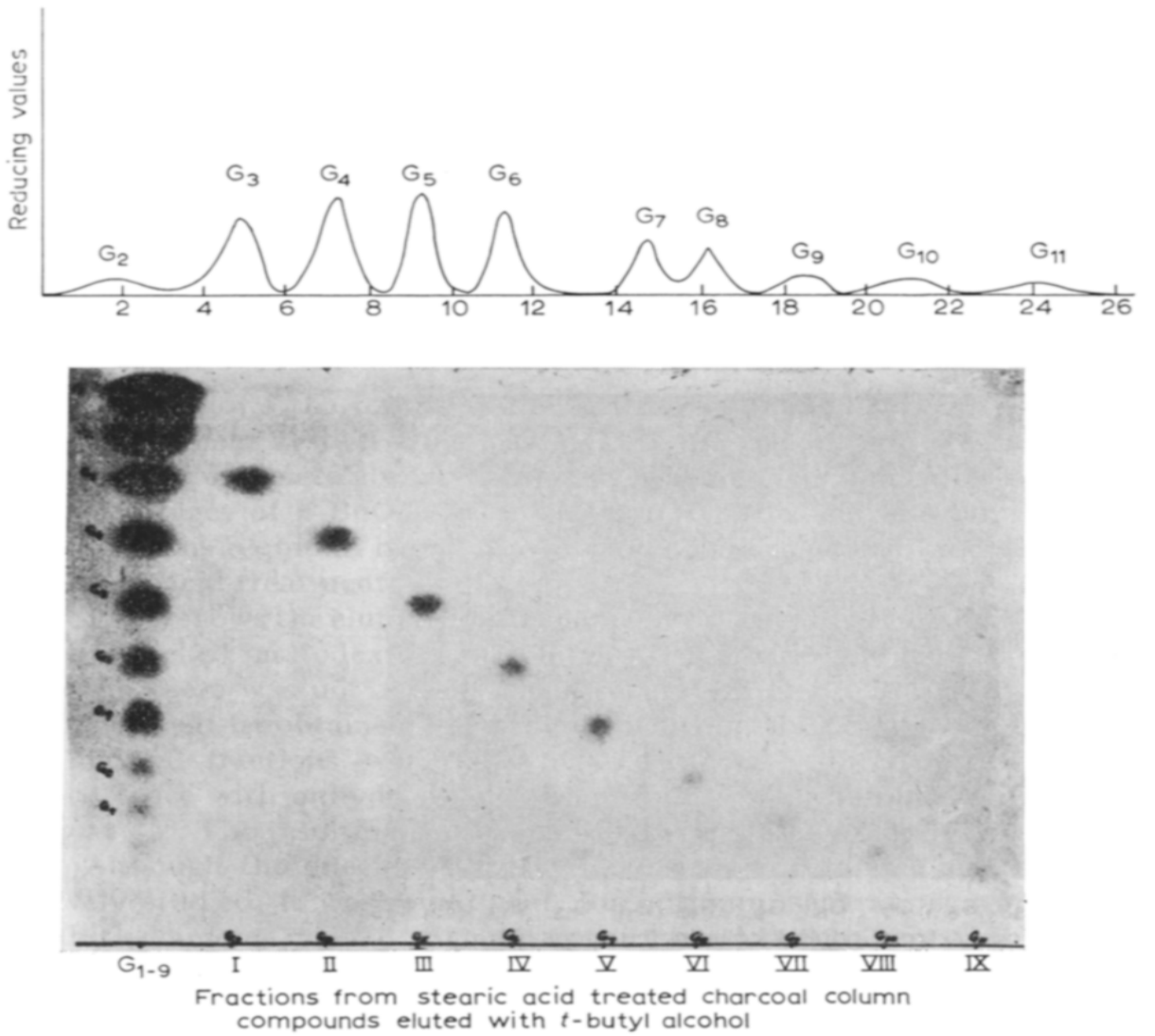


Fig. 2. Elution diagram and chromatographic analysis of linear maltodextrins (G_2–G_{11}) on a small stearic acid treated charcoal–celite column with *tert.*-butyl alcohol gradient elution.

of charcoal, Celite, sample, and stearic acid–EtOH solutions. The eluting system consisted of 20 l of 10 % *tert.*-BuOH and 15 l of water, calculated from the gradient elution formula given by ALM *et al.*[1]. The dimensions of the larger columns were 6.5 × 70 cm.

For the optimum separation of the branched dextrins, less stearic acid was required for the charcoal treatment. The stearic acid concentration in the EtOH was reduced from 2.5 % to 1.0 % w/v. The volume of EtOH and the 50 % EtOH wash remained the same. Results similar to those obtained for the small columns were obtained for the branched dextrins when the method was scaled up six times.

RESULTS AND DISCUSSION

Fig. 2 shows the elution diagram and paper chromatographic analysis of the pooled fractions from a typical separation of the linear maltodextrins. Fig. 3 shows

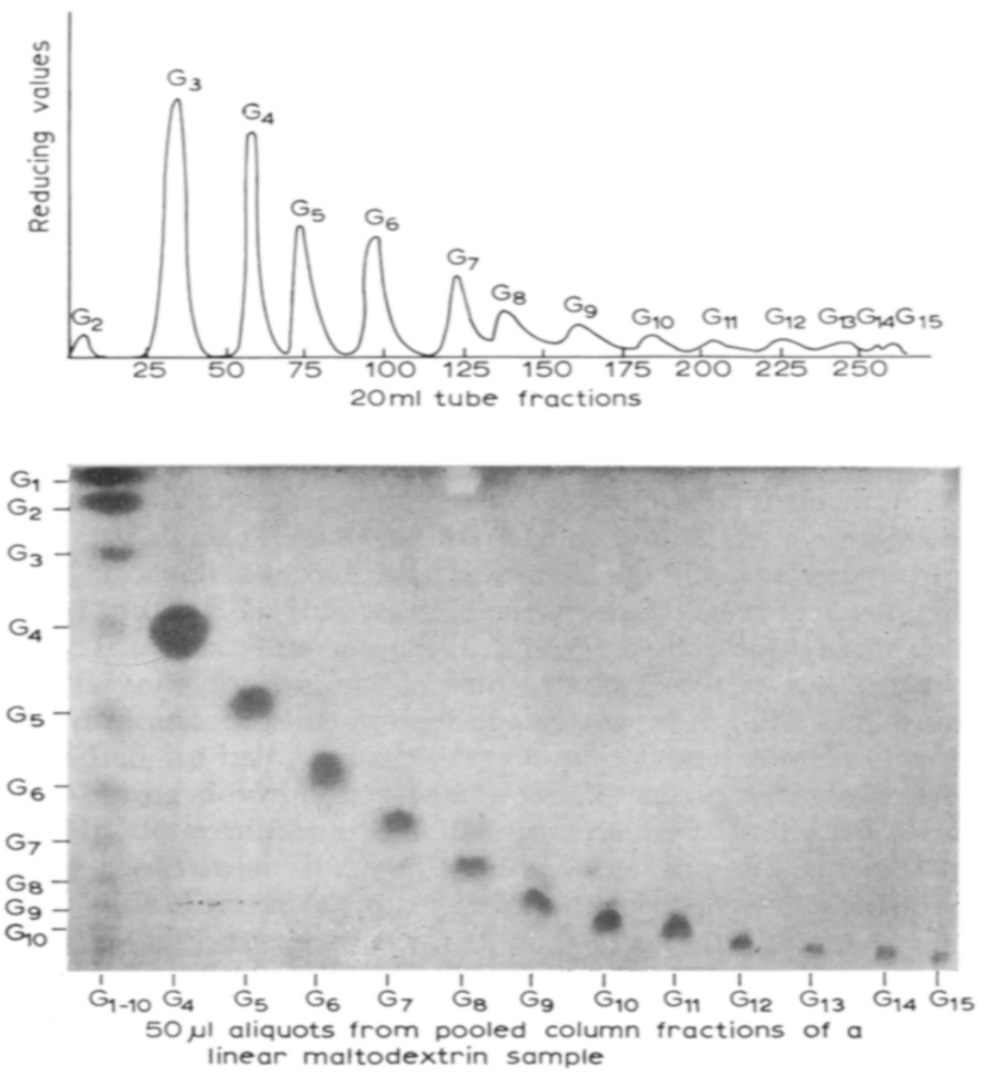


Fig. 3. Elution diagram and chromatographic analysis of linear maltodextrins on a large stearic acid treated charcoal–celite column with *tert.*-butyl alcohol gradient elution. Column: 600 g charcoal–450 g celite, treated with 2.5 % stearic acid. Sample: 4.2 g (G_2–G_{15}). Elution gradient: 10 % *tert.*-butyl alcohol (20 l) into H_2O (15 l). Flow rate: *ca.* 4 ml/min.

the elution diagram and chromatographic analysis of the pooled fractions from a large column separation of the linear maltodextrins. For the separation of the linear maltodextrins, the *tert.*-BuOH gradient system with a 2.5 % stearic acid treatment of Darco G-60 charcoal gave the best separation.

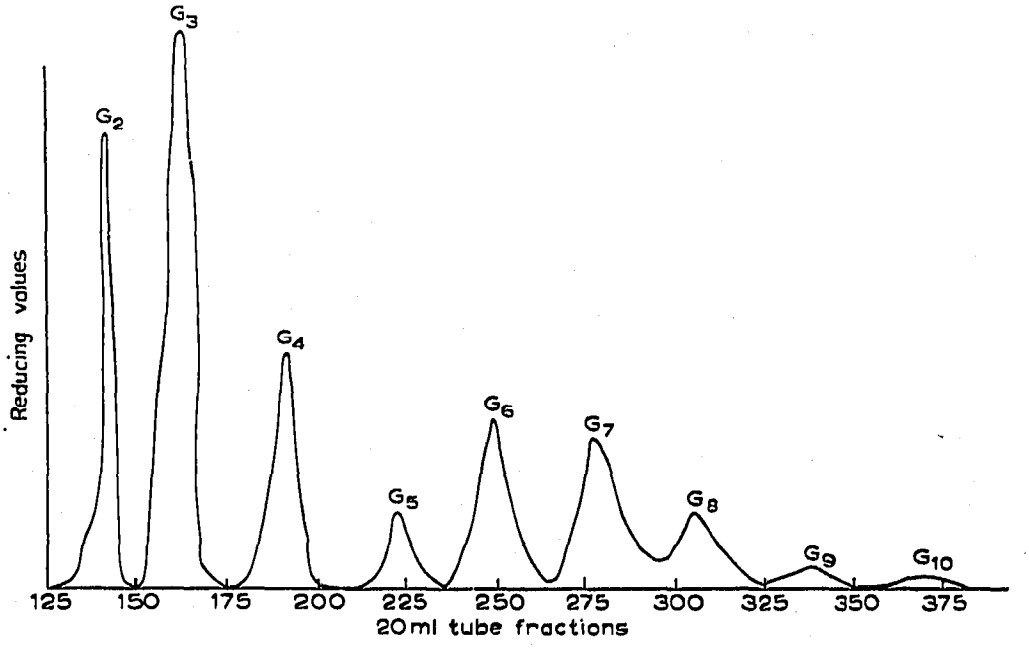


Fig. 4. Elution diagram of linear maltodextrins on a charcoal–celite column with *n*-butyl alcohol gradient elution. Column: 100 g charcoal–75 g celite. Sample: 700 mg maltodextrin (G_2–G_{10}). Elution gradient: 6 l 3 % *n*-butyl alcohol into 5 l water, followed by 6 l 3.5 % *n*-butyl alcohol. Flow rate: *ca.* 2.5 ml/min.

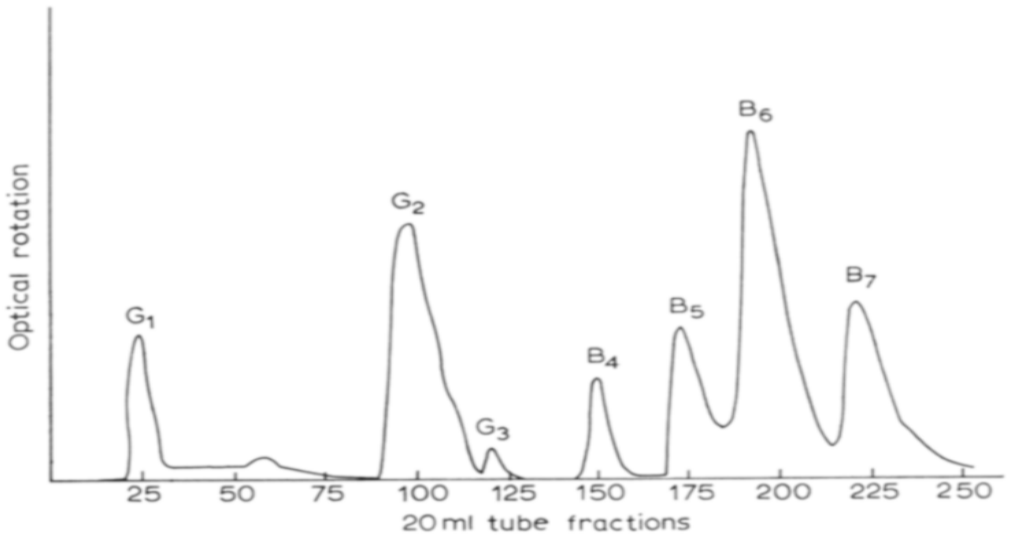
Fig. 4 shows the results of the separation of the linear maltodextrins for the *n*-BuOH gradient system using Darco G 60 charcoal without stearic acid treatment. Although not completely worked out, this system offers some interesting possibilities. The advantages of *n*-BuOH were: better resolution and less tailing, lower alcohol concentrations required, better recovery of oligosaccharides, and the elimination of the stearic acid treatment.

Fig. 5 shows the elution diagram and chromatographic analysis of the separation of the branched maltodextrins on a large 1.0 % stearic acid treated charcoal column. Although there was not a really clean separation of B_6, B_7 and B_8, relatively pure fractions could be obtained by judiciously cutting the peaks.

Pooled fractions were concentrated under reduced pressure, lyophylized, treated twice with anhydrous acetone and once with absolute EtOH, and dried *in vacuo* at 50°. The resulting specimens were white, easily handled powders.

Although the effect of column dimensions and sample size has not been extensively studied, it was found that, for optimum separation, a minimum column length to diameter ratio of 10:1 was required and that the maximum sample size for the stearic acid treated columns was 0.7 % the weight of the charcoal.

In some cases, use of a higher temperature (50°) has improved resolution and recovery, and reduced tailing. Flow rates at higher temperatures are much faster than at room temperature.



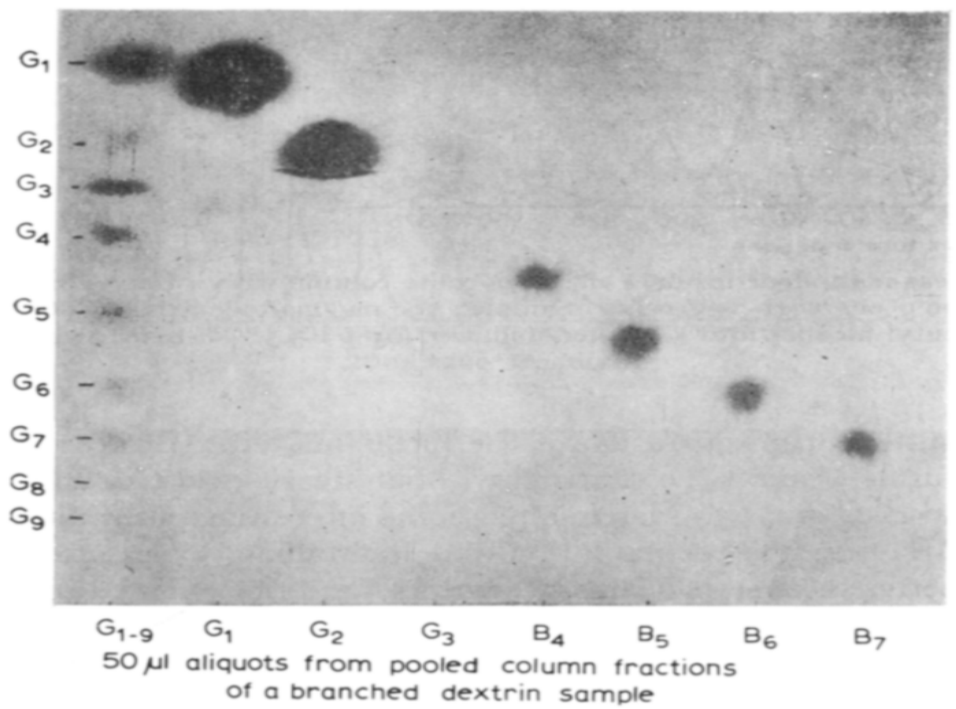


Fig. 5. Elution diagram of branched maltodextrins on a stearic acid treated charcoal–celite column with *tert.*-butyl alcohol gradient elution. Column: 600 g charcoal–460 g Celite, treated with 1.0% stearic acid. Sample: 4.0 g linear and branched maltodextrins. Elution gradient: 10% *tert.*-butyl alcohol (20 l) into H_2O (15 l).

SUMMARY

Gradient elution of charcoal columns using aqueous *tert.*-BuOH has proved very effective for separating individual starch oligosaccharides in the range up to 15 D-glucose units. Effects of sample size, steepness of gradient and pretreatment of charcoal were examined. Carbohydrate in the effluent was monitored by a continuous recording polarimeter or by analyzing fractions with a Technicon Autoanalyzer. Branched, as well as linear, oligosaccharides could be separated.

REFERENCES

1 R. S. ALM, R. J. P. WILLIAMS AND A. TISELIUS, *Acta Chem. Scand.*, 6 (1952) 826.
2 R. S. ALM, *Acta Chem. Scand.*, 6 (1952) 1186.
3 R. L. WHISTLER AND D. F. DURSO, *J. Am. Chem. Soc.*, 72 (1950) 677.
4 R. L. WHISTLER AND J. L. HICKSON, *J. Am. Chem. Soc.*, 76 (1954) 1671.
5 R. L. WHISTLER AND J. H. DUFFY, *J. Am. Chem. Soc.*, 77 (1955) 1017.
6 R. L. WHISTLER AND B. F. MOY, *J. Am. Chem. Soc.*, 77 (1955) 5751.
7 P. M. TAYLOR AND W. J. WHELAN, *Chem. Ind. (London)*, (1962) 44.
8 W. J. HOOVER, A. I. NELSON, R. T. MILNER AND L. S. WEI, *J. Food Sci.*, 30 (1965) 248.
9 G. L. MILLER, J. DEAN AND R. BLUM, *Arch. Biochem. Biophys.*, 91 (1960) 21.
10 E. H. FISCHER AND R. DEMONTMOLLIN, *Helv. Chim. Acta*, 34 (1951) 1987.
11 E. H. FISCHER AND E. A. STEIN, *Biochem. Prep.*, 8 (1961) 27.
12 J. A. THOMA, H. B. WRIGHT AND D. FRENCH, *Arch. Biochem. Biophys.*, 85 (1959) 452.