*Separations of Carbohydrates on Charcoal Columns in the Presence of Borate.**

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Borate complexes of sugars are eluted more readily from charcoal columns than are the sugars themselves. Use is made of this fact in separations of isomeric disaccharides and trisaccharides, and in the fractionation of a homologous series of oligosaccharides. The effect of pH on the efficiency of the separations has been examined, and other applications of the new technique are suggested.

WHISTLER and DURSO's method (*J. Amer. Chem. Soc.*, 1950, **72**, 677) for the fractionation of mixtures of sugars on charcoal columns is now being applied extensively to oligosaccharides (cf. Bailey, Whelan, and Peat, *J.*, 1950, 3692); elution with water containing increasing concentrations of ethanol enables good separations to be effected between mono-, di-, and tri-saccharides, etc., but separations of isomeric saccharides are usually much more difficult to achieve by this method. On the other hand, paper electrophoresis, in the presence of borate buffer (pH 10) (Foster, *J.*, 1953, 982), will separate, on a small scale, mixtures of isomeric saccharides having appropriate structural differences; thus, a glucosaccharide in which the reducing unit is linked through position 2 or position 4 migrates much more slowly than an isomeric glucosaccharide with the reducing unit linked through position 3 or position 6. The purpose of this communication is to report the development of a method for the large-scale separation of isomers, based on the incorporation of borate in the solvent in the usual charcoal-column method and, as a consequence, the extension of the useful range of charcoal columns to cover higher saccharides (of certain structural types) than has been possible hitherto.

The hypothesis from which the new method was developed was that a carbohydrate–borate complex, which has ionic groups present and at the same time carries a smaller proportion of non-polar regions than the carbohydrate itself, might be expected to assume some of the characteristics of inorganic salts and to move more readily down the column than the parent carbohydrate. The affinity of the carbohydrate for borate was expected to be reflected in the relative times which it spent in the free and the combined state, and hence in the conditions required to remove it from the column, so that a separation between isomeric carbohydrates with different affinities for borate was expected. In addition, it was envisaged that two carbohydrates having similar affinities per molecule for borate, but different molecular weights, might still be separated by virtue of their different affinities for charcoal, the advantage conferred by the borate in this case being that both components would pass more readily through the column than the free sugars. The experiments described below amply confirm these ideas.

In the first group of experiments, the fractionation of a melibiose–maltose mixture was studied, with particular reference to the effect of pH on the efficiency of the separation

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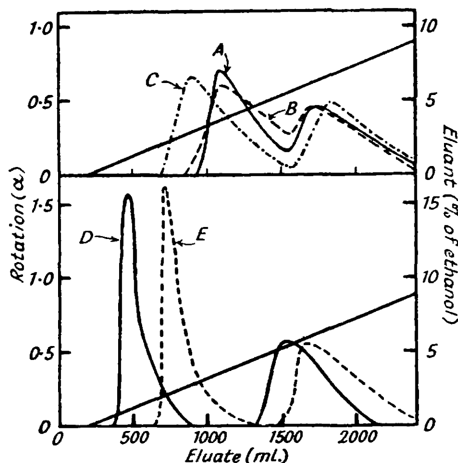
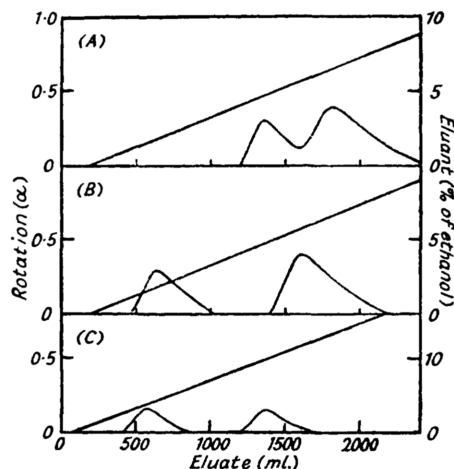
(see Fig. 1). The melibiose, which forms a much stronger borate complex than does maltose, emerged at progressively decreasing concentrations of ethanol (2·7, 2·0, and 0·8%, respectively) as the pH of the borate was increased from 5·3 to 8·5 and then to 10·0; in the absence of borate 3·2% of ethanol was required. The effect of borate on the maltose was much smaller; whereas this disaccharide was eluted with about 5·3% of ethanol in the absence of borate, 4·6% was still required at pH 10·0 in the presence of borate. This pH-dependence of complex formation is in keeping with that found by other methods (cf. Consden and Stanier, *Nature*, 1952, **169**, 783). In the above cases, as in the others described below, the column was always washed with the appropriate buffer before being used for the fractionation; the reason for this was that the charcoal–" Celite " mixture markedly reduced the pH of the buffer until sufficient buffer had been passed through for an equilibrium state to be reached. The beneficial effect of the prewashing of the column, when borate buffer at pH 10·0 was being used, can be seen in Fig. 1.

FIG. 1. *Separations of melibiose and maltose.*



A, In the absence of added borate.
B, In the presence of boric acid, pH 5·3.
C, In the presence of borate buffer, pH 8·5.
D, In the presence of borate buffer, pH 10·0, column prewashed.
E, In the presence of borate buffer, pH 10·0, column not prewashed.

FIG. 2. *Separation of* iso*maltose from maltose, and of* iso*maltotriose from maltotriose.*



A, Separation of *iso*maltose and maltose in the absence of borate.
B, Separation of *iso*maltose and maltose in the presence of borate buffer, pH 10·0.
C, Separation of *iso*maltotriose and maltotriose in the presence of borate buffer, pH 10·0.

For fractionations of the other mixtures chosen, pH 10·0 was selected. *iso*Maltose, which forms a strong borate complex, was easily separated from maltose; it emerged with 1·2% of ethanol instead of the 4·0% necessary in the absence of borate (Fig. 2). Likewise, the elution of *iso*maltotriose from the column was facilitated by borate, which reduced the alcohol concentration required from 9·3% to 2·8%, and thus enabled a good separation from maltotriose to be effected (Fig. 2).

Each component of the three mixtures separated at pH 10·0 (melibiose–maltose, *iso*maltose–maltose, *iso*maltotriose–maltotriose) was recovered in *ca.* 90% yield as a freeze-dried powder, which was shown to be pure by chromatography and ionophoresis. Slightly higher values were obtained when the yields were calculated from the areas beneath the curves shown in Figs. 1 and 2, comparison being made with the observed rotations of known weights of the sugars, examined under the same conditions.

An alternative use for the new technique is in fractionations of homologous series of oligosaccharides which form strong complexes with borate at one, or both, of the chain ends. In such a case, the normal sequence of the saccharides on charcoal is preserved, but the whole series is eluted more readily than in the absence of borate, thus enabling higher saccharides to be purified than has been possible hitherto. A typical example is to be

found in Fig. 3 where a fractionation of the dextran-type oligosaccharides is shown; nine distinct fractions were obtained by the time that the ethanol concentration reached 23%. An almost linear relation was found between the chain-length of the oligosaccharide (4—10 units) and the concentration of ethanol at which it emerged from the column (Fig. 4). It is probable that even higher saccharides in this series could be obtained, before the solubility limit is reached, by using more ethanol, so that it is not unreasonable to suppose that the new method, with appropriate modifications (*e.g.*, the use of a constant, rather than a decreasing, concentration of borate), might form a basis for the preparation of short-chain dextrans, with a small molecular-size distribution, suitable for sulphation to give heparin substitutes.

The experiments cited are sufficient to indicate that the technique of using charcoal columns with borate buffers as solvents could well find wide application in the carbohydrate field. For example, separations of suitably substituted isomeric derivatives of monosaccharides, polyols, and cyclitols should be readily accomplished. Preliminary experiments already indicate that much larger molecules, which are able to form complexes with borate at intervals along the chain (*e.g.*, those of yeast-mannan type), can be eluted from

FIG. 3. *Separation of the components of a dextran hydrolysate in presence of borate buffer,* pH 10.

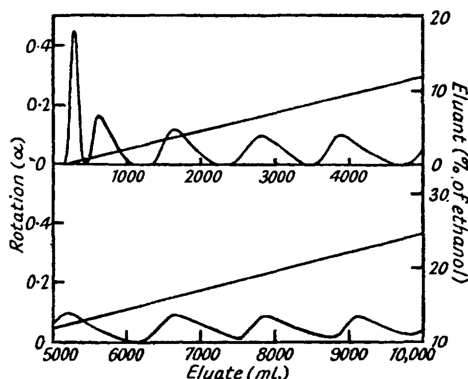
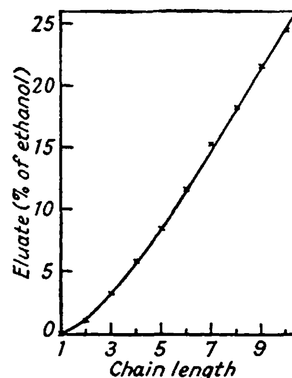


FIG. 4. *Relation between chain length and concentration of ethanol required for elution of dextran oligosaccharides.*



the column in the presence of borate, so that separation of such a polymer from another which does not form a complex is a distinct possibility.

In any individual case, it is necessary to bear in mind the alkali-lability of the components when choosing the pH value for the system; the exclusion of oxygen might be advantageous in preventing degradation in some cases. In our experiments, pH 10·0 was usually employed, since the sugars under investigation were relatively stable to alkali. It is known that even such alkali-labile sugars as nigerose and laminaribiose are stable during paper ionophoresis at this pH (otherwise discrete spots would not be obtained), although they are less stable when not adsorbed on the paper (Barker, Bourne, and O'Mant, unpublished results). For this reason, the sugars were not placed in contact with the buffer before they were adsorbed on the charcoal, and the fractions were acidified immediately they emerged from the column. We believe that, under these conditions, alkaline degradation was negligible, as was indicated by the purities of the products, and by the failure of any product to give a ketose reaction with naphtharesorcinol. In addition, the pH of the system may be an important factor controlling the order of elution of two sugars from the column, since Consden and Stanier (*loc. cit.*) have shown that the electrophoretic mobilities of two sugar–borate complexes may be reversed at two different pH values.

Separations of sugars have already been achieved by elution of their borate complexes from strongly basic anion-exchange resins (Khym and Zill, *J. Amer. Chem. Soc.*, 1952, **74**, 2090; Zill, Khym, and Cheniae, *ibid.*, 1953, **75**, 1339). The fundamental difference

between the two methods is that complex formation decreases the rate of movement of a sugar down a resin column, but increases it on a charcoal column. One result of this is that the use of borate on a resin enables very good fractionations between unsubstituted monosaccharides to be made, whereas borate cannot effect much improvement on charcoal columns since monosaccharides already move very rapidly and are eluted with water alone. However, with mixtures of higher saccharides, the charcoal–borate technique offers advantages because of the contribution to the fractionation made by the charcoal itself. Moreover, the charcoal method seems to be more flexible, as regards both the range of pH values available, and the possibility of using different solvents at different stages in the elution of a single column; for example, it is possible to use a solvent free from borate early in the process to separate the smaller components, and then to introduce borate in the later stages, or in other cases, the borate may be applied initially to remove those saccharides which form complexes, fractionation of the residual sugars being achieved subsequently with a borate-free solvent. To a large extent the two methods are complementary and together they are applicable to a wide variety of problems.

EXPERIMENTAL

Preparation of the Column.—The procedure was similar to that described by Lindberg and Wickberg (*Acta Chem. Scand.*, 1954, **8**, 569). Equal parts of activated charcoal (B.D.H.) and " Celite " (No. 545) were treated with concentrated hydrochloric acid for 1 hr. and then washed thoroughly with water. The mixture was left in ethanol for 1 hr., filtered, washed with water, and dialysed against running tap water for two days. The slurry was kept under vacuum for 30 min. to remove most of the air, and then poured on to thin layers of cellulose powder and " Celite," supported by a porcelain disc, to form a column (*l*, 40 cm.; diam., 2·5 cm.). A new column of the same batch of charcoal–" Celite " was prepared for each fractionation.

Fractionation Procedure.—When the eluting solvent contained borate buffer or boric acid, the column was thoroughly washed with this first (except where otherwise stated) until the pH values of the solutions entering and leaving the column were the same. Small amounts of water (30 ml.) were run into the column before and after the addition of the mixture of carbohydrates in aqueous solution (10% w/v). The column was then washed with the appropriate solvent mixture, using the linear gradient technique described by Lindberg and Wickberg (*loc. cit.*). The fractions (15—20 ml.) were collected in tubes which, when borate buffer was being used, already contained *ca.* 10 drops of 5N-hydrochloric acid; the pH values of the fractions were then 3—5. The separation was followed polarimetrically (2 dm. tube) and the purity of the fractions checked by paper chromatography, with the organic phase of a mixture of butan-1-ol (40%), ethanol (10%), water (49%), and ammonia (1%) as the solvent, and by paper ionophoresis in borate buffer of pH 10·0 (Foster, *loc. cit.*).

Isolation of the Component Sugars.—In the presence of borate, the solution was passed down a column of Amberlite IR-120 (H^+ form), neutralised with silver carbonate, filtered, and evaporated *in vacuo*. Methanol (50 ml.) was distilled twice from the residue, and the product was treated again with a small amount of Amberlite IR-120 and methanol, as before (cf. Zill, Khym, and Cheniae, *loc. cit.*). The purified sugar was extracted with hot methanol, filtered, concentrated, and freeze-dried from aqueous solution. All fractions were dried further for 4 hr. at 60° *in vacuo* over phosphoric anhydride.

Separation of Melibiose and Maltose.—Five mixtures, each containing melibiose dihydrate (1 g.) and maltose hydrate (1 g.), were separated on charcoal–" Celite " columns, which were washed severally by gradient elution with 0—8% aqueous ethanol (2 l.) and then with 8—12% aqueous ethanol (1 l.), each solvent containing the following aqueous solutions in place of the water used in the control experiment (*A*): (*B*) boric acid (7·45 g./l.), pH obtained 5·3; (*C*) boric acid (7·45 g./l.) and sodium hydroxide (1·13 g./l.), pH obtained 8·5; (*D*) boric acid (7·45 g./l.) and sodium hydroxide (4·00 g./l.), pH obtained 10·0; and (*E*) as (*D*), but column previously washed with water only. The separations achieved are shown in Fig. 1. In each case, chromatographic and ionophoretic analyses showed that the first peak was due to melibiose and the second to maltose, and that all the fractions were pure, except where the peaks overlapped. The recoveries of the hydrates of melibiose $\{[\alpha]_D^{19} +128{\cdot}2°$ (*c*, 2·0 in H_2O)$\}$ and maltose $\{[\alpha]_D^{19} +128{\cdot}1°$ (*c*, 2·0 in H_2O)$\}$ were 89·9 and 89·5%, respectively, from the column washed as in (*D*).

Separation of iso*Maltose and Maltose.*—Two mixtures, each containing *iso*maltose (0·25 g.)

and maltose hydrate (0·60 g.), were fractionated by gradient elution on charcoal–" Celite " columns. In one case 0—8% aqueous ethanol (2 l.), and then 8—12% (1 l.), was used as the solvent; in the other case, the water was replaced by borate buffer, pH 10·0 (see above). The separations achieved are shown in Fig. 2. Chromatographically pure *iso*maltose {yield, 91·1%; $[\alpha]_D^{19}$ +121·0° (*c*, 2·0 in H_2O)} was recovered from the fractions constituting the first peak, while pure maltose hydrate {yield, 87·1%; $[\alpha]_D^{19}$ +130·5° (*c*, 2·0 in H_2O)} was obtained from those constituting the second, when the eluting solvent contained borate buffer.

Separation of iso*Maltotriose and Maltotriose.*—A mixture of *iso*maltotriose (100 mg.) and maltotriose (100 mg.) was separated on a smaller charcoal column (*l*, 20 cm.; diam., 1·8 cm.) by gradient elution with 0—15% aqueous ethanol (2 l.) containing borate buffer, pH 10·0 (see above). The first of the trisaccharides to be eluted was *iso*maltotriose {yield, 92·4%; $[\alpha]_D^{19}$ +141·2° (*c*, 2·0 in H_2O)}; it was chromatographically pure, as also was the maltotriose {yield, 89·0%; $[\alpha]_D^{19}$ +148·2° (*c*, 2·0 in H_2O)}. The separation achieved is shown in Fig. 2.

Separation of the Homologous Series of Dextran Oligosaccharides.—An essentially unbranched dextran was partially hydrolysed with N-sulphuric acid at *ca.* 95° for 1·5 hr. After neutralisation with barium carbonate, filtration, and freeze-drying, the product was dried at 60°/12 mm. over phosphoric anhydride; it had $[\alpha]_D^{20}$ +144° (*c*, 2·0 in H_2O). A sample (3·15 g.) was separated on a charcoal–" Celite " column (*l*, 40 cm.; diam., 2·5 cm.) by gradient elution with 0—25% aqueous ethanol (10 l.) containing borate, pH 10·0 (see above). The separation is shown in Fig. 3. Portions (100 ml.) of the eluates containing the first four components, taken where the rotation exhibited was a maximum, were worked up and checked by electrophoresis and paper chromatography. Except for the first, which contained a very small amount of fructose, arising from the polysaccharide, they were shown to be pure; they behaved identically to glucose, *iso*maltose, *iso*maltotriose, and *iso*maltotetraose, severally.

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