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THE SEPARATION OF SUGARS AND OF POLYOLS ON CATION-EXCHANGE RESINS IN THE CALCIUM FORM*†

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ABSTRACT

Chromatography on a column of a cation-exchange resin in its calcium form, with water as the eluant, is a convenient method for the separation of many sugars and polyols and their derivatives. The advantages and limitations of this method are discussed. Addition of methanol to the eluant and lowering of the temperature improve the separation.

INTRODUCTION

The separation of sugars and of polyols on cation-exchange resins in their calcium or barium form ("calcium columns", "barium columns") is not a new development. The method was fortuitously discovered by Felicetta *et al.*[2] in 1959. When separating the sugars of spent sulfite liquors from the lignin sulfonates by the use of ion-exchange resins, they found that L-arabinose was separated from D-xylose. In 1960, J. K. N. Jones and Wall[3] then studied the retention of several sugars and alditols on a column of Dowex 50-W X-8 (Ba^{2+}) ion-exchange resin, and separated a number of synthetic mixtures and some plant extracts into their components. A particularly good separation was that of the main products from the epimerization of D-galactose, namely, D-tagatose and D-talose.

Despite these successful separations, the method was but little used, either by J. K. N. Jones or by others, in the succeeding years; a few examples are to be found in ref. 4. There have been some industrial applications; *e.g.*, by this method, D-glucose is separated from D-fructose, and D-glucitol from D-mannitol[5,6]. However, in the past few years, the method has been widely used in Bratislava by Fedoroňko *et al.*[7] and Bílik *et al.*[8–12], and by our group in Sydney[1,13–17].

The reason for the neglect of this technique was probably a lack of understand-

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ing of how it operates, and so, it was not possible to predict what separations, if any, would be obtained. Yet, this is one of the best methods for separating sugars and related compounds, and the mechanism of the separation is now understood[18]. The main advantages of separating sugar derivatives on calcium columns are as follows. (*i*) Separations are rapid and clean, and the recovery is nearly quantitative; (*ii*) the eluant is water only; (*iii*) in favorable cases, the capacity of the column is large; (*iv*) the column is stable for long periods of time, and it need not be regenerated: once all of the components have been eluted, it is ready for another separation; and (*v*) the sequence of the emergence of components from the column can be predicted from theoretical considerations (see later), and, even better, by paper electrophoresis of the mixture[19].

Some details regarding the application of the method are given herein.

DISCUSSION AND RESULTS

It is now clear that separation of polyols on a calcium column is caused by the differing extent of complex-formation between the polyols and the calcium ion[20]. Polyols that form such complexes[18] are retained by the column, whereas those that do not, emerge rapidly. The best evidence for this mechanism of separation is the close similarity between the separation obtained on a calcium column and that observed on paper electrophoresis in a solution of calcium acetate[19]. In the latter procedure, the movement of polyols is caused by their complexing, and hence migrating, with the calcium ion. The correspondence between these two methods is not complete; whereas the movement of a polyol down the chromatographic column will depend only on the stability constant of its calcium complex, the rate of migration in electrophoresis will also depend on the ionic mobility of the complex cation, which will be affected by its size and shape. Hence, occasionally, two compounds are separated on the columns, but not on electrophoretograms, or *vice versa*. (For another exception, see the last paragraphs of this article.)

The calcium column is, therefore, essentially a column of immobilized calcium ions; the main function of the ion-exchange resin is to hold the ions in position.

Polyols form reasonably strong complexes with calcium ions (K=1–6 mol^{-1}) if, in their most stable conformation, they contain three hydroxyl groups in such an arrangement that they can form bonds of approximately equal length with the cation[18]. The most common complexing arrangement is a sequence of an axial, an equatorial, and an axial hydroxyl group (*a*,*e*,*a*) on a six-membered ring, or a *cis*–*cis* sequence of three hydroxyl groups on a five-membered ring. Somewhat weaker complexes are formed if the required geometrical arrangement of three oxygen atoms is only obtained by a conformational change, *e.g.*, ring inversion in a six-membered ring, or rotation around a carbon–carbon bond in an acyclic polyol[21]. If one of the hydroxyl groups is replaced by a methoxyl group, complexing is weakened[22]. Two *cis*-hydroxyl groups on a five-membered ring form a weak complex; two *cis*-hydroxyl groups on a six-membered ring afford an even weaker one. Thus, a considerable range of complexing ability is

found in various sugars and polyols, thereby enabling the separation of many mixtures. For example, the ready separation of D-galactose, D-tagatose (D-*lyxo*-2-hexulose), and D-talose from each other is predictable: D-talopyranose-$^4C_1$ contains an *a,e,a* sequence in both anomers; D-tagatopyranose in its (less stable) β-anomeric $^5C_2$ form only; and D-galactopyranose, no such sequence. Other geometrical arrangements that lead to complex-formation are discussed elsewhere[19].

Jones and Wall[3] used Dowex 50-W resin in its barium form, and the workers in Bratislava[7–12] followed his example. In Sydney, we have used the calcium and, occasionally, the strontium form. We have compared the performance of the calcium and barium columns with the following mixtures: D-fructose and D-glucose, D-allose and D-altrose, D-galactose and D-talose, and the methyl D-allopyranosides, and in all instances, the calcium column was found to be superior as far as separation and tailing are concerned; hence, we have standardized with calcium columns.

With polyols, lanthanum forms stronger complexes than the alkaline-earth metals, but its higher atomic weight makes it less convenient to use, because 3.5 times as much lanthanum as calcium is needed to saturate a column. Cation-exchange resins have frequently been used in their Li^+,Na^+, Rb^+, and Mg^{2+} forms[23] for the separation of sugars. These metals form only weak complexes with polyols[22], and the separation is then caused, not by complex-formation, but by ion exclusion, adsorption, or partition. The capacity of these columns for separations of polyols (in contrast to those of calcium columns) is small, and diastereomers are usually not well separated; *e.g.*, glucose and talose are not separated[20], although the latter has an *a,e,a* sequence and the former has not. Until recently, the separation achieved on calcium and barium columns was also ascribed to adsorption[23].

A cation-exchange resin in the Cu^{2+} form has also been used for the separation of alditols; with a few exceptions, reducing sugars are not retained on this column[24]. The eluant was a solution of copper acetate in water, and hence, the copper had to be removed before isolation of the polyols.

The principal applications of calcium columns are as follows.

(*i*) Separations of two-component mixtures, one of which forms a complex and the other does not; a good example is the separation of D-talose from D-galactose. D-Talose, until recently a rare sugar, is now readily available by the excellent method of Bílik and co-workers[9], namely, epimerization catalyzed by molybdic acid. The simplicity of the method is enhanced by the subsequent ready separation of talose from unchanged galactose on a calcium column. Another good application is the separation of D-psicose from D-fructose, acetals of both of which are formed in the reduction of 1,2-*O*-isopropylidene-β-D-*erythro*-2,3-hexodiulo-2,6-pyranose[15].

(*ii*) Separation of complexing material from impurities: commercial D-tagatose, contaminated by D-sorbose (which could not be removed by recrystallization), was readily obtained pure by the use of a calcium column[15].

(*iii*) Separation of complexing material from inorganic compounds. Some sugars (*e.g.*, D-gulose) and sugar derivatives (*e.g.*, methyl β-D-mannofuranoside[25]) are usually isolated as their crystalline, calcium chloride complexes. The traditional

method of liberating them consists in treatment with silver oxalate[25]; a simpler method is by the use of a calcium column from which the salt is eluted first, followed by the sugar, which is free from calcium ions.

(*iv*) Isolation of sugars from natural sources: examples were given by Jones and Wall[3].

(*v*) Separation of alditols from each other. The separation is based on the different energy required by each alditol in order to bring it into the conformation required for complex-formation. The electrophoretic mobilities of many alditols in calcium acetate have been determined[21]; they serve as indicators as to the separation that may be expected on a calcium column. For example, allitol, glucitol, and mannitol can be separated from each other, and *epi*-inositol has been separated from some of its isomers[26].

(*vi*) In favorable cases, all of the methyl glycosides of a sugar can be separated from each other[1,13] (see also, the discussion of the methyl D-allosides, given later). A better separation is usually obtained on a column of an anion-exchange resin in its hydroxide form[27], but such columns are not permanent and have to be freshly prepared.

Preparation of the column. The calcium column is usually made up from a cation-exchange resin, in its H^+ form, that has been treated with an excess of calcium chloride solution, and then washed until the discharge is neutral. Under these conditions, a small proportion of the resin will remain in the acid form; this may be a useful feature when free sugars are separated, because it will accelerate mutarotation; *e.g.*, the presence of acid in the column is regarded as essential for the separation of glucose from fructose[6]. The latter is probably retained on the column as the β-furanose form (*cis*-hydroxyl groups on a five-membered ring), and rapid mutarotation is essential in order to prevent tailing. For non-mutarotating compounds, however, a neutral column is just as effective as an acidic one. When acid-sensitive compounds (*e.g.*, acetals) are separated, the column is made neutral by washing it with a solution of calcium hydroxide[14], or is made up with calcium acetate solution.

The finer the resin, the more effective it will be, because a higher proportion of the calcium ions will be near its surface; resin particles of 200–400 mesh are therefore used. For compounds that have a strongly complexing arrangement (such as an *a*, *e*, *a* sequence), such a column has a very large capacity. For the separation of talose from galactose, Bilik and co-workers[9] used a column containing about 12 mol of barium ions for each mol of talose; the excellent separation obtained shows that the column was larger than necessary. In fact, we have found that 3 mol of calcium ions is sufficient for the initial retention of talose: 14 g of D-talose and 20 g of D-galactose were completely separated on a column (4 × 50 cm) of Dowex AG 50-W X-2 (Ca^{2+}) ion-exchange resin. For compounds that complex only weakly, the capacity of the column is, of course, smaller.

Reducing sugars. Difficulties arise in the chromatography of allose and gulose. For these sugars, the α-pyranose form has the complexing, *a*, *e*, *a* arrangement, but the β-pyranose, the preponderant form, has not. Mutarotation is slow and, therefore,

there is enrichment in the β form at the front, and in the α form at the rear, of the sugar descending the column. Hence, the sugars do not emerge in a narrow zone, but "tail"; in paper electrophoresis, they[19] "streak". The hydrogen ions attached to the resin do not accelerate mutarotation sufficiently; this difficulty was overcome by the addition of trimethylamine to the eluant, raising its pH to ~8.5, and the sugars then emerged in narrow zones. Calcium ions are, however, eluted during this process, and the fractions contain some material that, after evaporation and extracting the residues with anhydrous ethanol, leaves behind some amorphous solid.

D-Allose is readily obtainable, whereas D-altrose is still difficult to synthesize; hence, the epimerization of the former to the latter by the Bílik reaction[11] is useful, provided that the two sugars can readily be separated. Working on a small scale, with water as the eluant, the two sugars were found to emerge together from a calcium column. (Altrose complexes weakly, owing to the presence of substantial proportions of the furanoses[28], and rapid mutarotation[29].) In the presence of trimethylamine, however, the two sugars were separated. Bílik[11] obtained partial separation on a large column by collecting fractions during a period of 30 h, thereby allowing sufficient time for mutarotation. (Lessening the flow-rate improves the separation, in any case.)

Similarly, with water as the eluant, glucose and gulose were eluted together, but were separated in the presence of trimethylamine. The same problem does not arise with ribose, or with the ketoses, because they mutarotate rapidly.

The effect of temperature and solvent. Another way in which to increase the rate of mutarotation during chromatography would be to work at a higher temperature. At 60°, D-allose was eluted by water in a compact zone; however, D-allose and D-altrose were not separated. It was noted that the elution volumes were smaller than at room temperature, and hence, separations were conducted at 0°; the fractions were then "wider" but the elution volumes were also larger, and the separations were better than at room temperature (see Table I). It may not be worth while to conduct the separations at a low temperature by the use of a jacketed column but, if a cool room is available, it is recommended that the column be set up there.

In complex-formation between polyols and cations, the polyol displaces the

TABLE I

SEPARATION OF D-GALACTOSE FROM D-TALOSE[a]

Eluant	*Temp.*	*Galactose eluted in fractions*	*Talose*
H_2O	ambient	9–13	16–26
H_2O	0°	9–16	21–40
30% MeOH	ambient	11–17	22–46
30% MeOH	0°	11–21	34–70
50% MeOH	ambient	12–23	36–60

[a]One g of each on a column (1.5 × 30 cm) of Dowex AG 50-W X-2 (Ca^{2+}) ion-exchange resin; 4.5-mL fractions.

solvent from the solvation shell of the cation. If the solvent is less strongly solvating than water, complex-formation with the polyol will be enhanced; *e.g.*, in ethanol, the stability constants of the complexes will be greater. Unfortunately, most of the polyols are insoluble in alcohols, but the addition of even a limited proportion of an alcohol to the aqueous solution has a noticeable effect on complex-formation. Addition of 30% of methanol will usually not precipitate sugars, even from concentrated, aqueous solutions. Table I shows the improvement in the separation of galactose from talose under these conditions. Cooling the column to 0°, or increasing the concentration of methanol to 50%, causes further improvement. Similar improvement was shown in the separation of glucose from fructose under the conditions specified in Table I, namely, with water at ambient temperature, the sugars were found in fractions 9–16 and 12–19, respectively (overlap of 5 fractions); with 30% methanol, 9–16 and 15–22 (overlap of 2 fractions); and with 30% methanol at 0°, 10–18 and 18–34 (practically no overlap, and very little substance in fraction 18).

Some other applications are as follows.

(*i*) For the study of the conformation of D-glucitol by n.m.r. spectroscopy[21], it was necessary to synthesize D-glucitol-*4*-^2H. The method used was the reduction[15] of D-fructose-*4*-^2H by means of sodium borohydride. The mixture of D-mannitol-*3*-^2H and D-glucitol-*4*-^2H thus obtained was readily separated on a calcium column.

(*ii*) The synthesis of methyl α-L-gulopyranoside with the aid of metal complexing has been described[13]; it was separated from its isomers by the use of an anion-exchange column in its hydroxide form. The more-convenient calcium column has now been found to be equally suitable.

(*iii*) The separation of the four methyl D-allosides[30] from each other also used the hydroxide column. The separation of the four glycosides on a calcium column has now been studied; the results are shown in Table II, which also illustrates the improvement in separation obtained by the use of methanol and a lower temperature. Complete separation of the two α-glycosides was not achieved. The calcium column is suitable for the isolation of the two β-allosides and of the α-furanoside, but not of the α-pyranoside. By contrast, the four methyl ribosides are completely separated by the calcium column[13].

TABLE II

SEPARATION OF METHYL D-ALLOSIDES[a]

Eluant	*Temp.*	*β-Pyranoside*	*β-Furanoside*	*α-Pyranoside*	*α-Furanoside*
		eluted in fractions			
H_2O	ambient	11–13	14–17	20–22	20–32
30% MeOH	ambient	11–13	15–18	27–30	27–43
30% MeOH	0°	12–16	18–22	33–38	36–48
50% MeOH	ambient	14–16	18–20	36–40	36–66

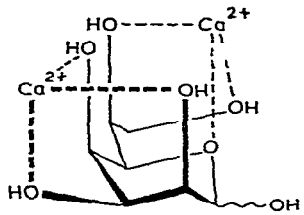
[a]See Experimental part for details.

Double complexing. It is surprising that, whereas methyl α-D-allofuranoside and methyl α-D-allopyranoside are not separated from each other on the calcium column, methyl α-L-gulofuranoside emerges much later than methyl α-L-gulopyranoside[13]. The complexing sites are the same (O-1,2,3) for both sugars, which differ only in the configuration at C-4. In paper electrophoresis in calcium acetate solution[19], the furanoside of each sugar migrates somewhat *slower* than the pyranoside.

This instance is reminiscent of the results of Bílik and co-workers[12] on the elution volumes (from a barium column) of sugars of the homologous series of galactose and of talose. The hexoses, heptoses, and octoses of the former series all have similar elution volumes; in the talose series, however, D-*glycero*-L-*talo*-heptose and D-*erythro*-L-*talo*-octose were found to be eluted much later than their isomers. Clearly, the effect is due to the side chain, because L-*glycero*-L-*talo*-heptose and D-*threo*-L-*talo*-octose do not show this increase in elution volume. That is, a side chain in which O-5 and O-6 have the *threo* configuration increases the elution volume, whereas one with O-5 and O-6 *erythro* does not. In alditols, a vicinal, *threo* arrangement favors complex-formation[21]. In the *galacto* series, however, the *threo* configuration of the side chain causes only a very slight increase in the elution volume. It appears that a weak complexing-site increases the retention on a calcium column only when there is also a strong complexing-site in the carbohydrate molecule.

Methyl α-L-gulofuranoside also possesses a side chain having *threo* oxygen atoms (at C-4 and C-5), but methyl α-D-allofuranoside has an *erythro* side chain. Only the former has a large elution volume.

To explain these observations, it is postulated that, when the carbohydrate molecule is attached to the surface of the ion-exchange resin by co-ordination with a calcium ion, a neighboring calcium ion on the surface can form an additional complex, even with a weak site. In the absence of a strong complexing-site, the weak site will have little effect. In our examples, complexing at both sites would occur on the same side of the molecule (see formulas **1** and **2**); this is probably a condition for the existence of "double complexing". In support of this hypothesis, it should be noted that this effect is not noticeable during electrophoresis (that is, in solution); D-talose, D-*glycero*-L-*talo*-heptose, and D-*erythro*-L-*talo*-octose all have practically the same electrophoretic mobility in a solution of calcium acetate[19].



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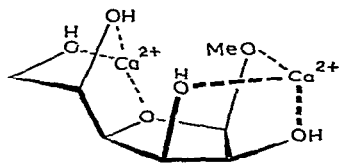

2

EXPERIMENTAL

Preparation of the column. — Dowex 50-W X-4 (H^+) ion-exchange resin (200–400 mesh) is gently stirred in a beaker with water (6 vol.), the resin is allowed to settle, the water is decanted, and the process is twice repeated, to remove fine particles. An aqueous solution of calcium chloride or calcium acetate (25%, 0.6 vol.) is added to the resin, and the mixture is gently stirred and then poured into a chromatographic column, and allowed to settle. The column is washed, first with a further 1.4 vol. of calcium chloride or acetate solution, and then with water until the effluent is free from calcium ions (test with sodium carbonate solution). If the chromatographic separation is to be conducted with aqueous methanol, the resin is now washed with that solvent mixture (5 vol.).

The resin contracts considerably when converted from the hydrogen form into the calcium form. If this conversion is performed in the chromatographic column, it is likely that the resin will break up into an uneven mass.

Separation of D-*mannitol from* D-*glucitol.* — A mixture (90 mg) of D-mannitol-*3*-^2H and D-glucitol-*4*-^2H, prepared by the reduction of D-fructose-*4*-^2H, was separated on a column (2 × 25 cm) with water as the eluant at a rate of 40–50 mL/h; 5-mL fractions were collected. Mannitol (36 mg) was obtained in fractions 11–12, and glucitol (46 mg) in fractions 14–16, uncontaminated by each other; there was an overlap of one fraction.

Separation of D-*allose from* D-*altrose.* — The mixture formed[11] by the epimerization of D-altrose (0.2 g) was de-ionized with Amberlite IRA-400 (HCO_3^-) resin, and placed on the top of a calcium column (2.8 × 38 cm); elution was performed with water at the rate of 40 mL/h, and 10-mL fractions were collected. The sugars were not separated, and the allose tailed badly. The experiment was repeated, using water containing 0.025% of trimethylamine as the eluant. Altrose was collected first, followed by allose (97 mg). After extraction with anhydrous ethanol (10 + 5 mL) to remove an insoluble calcium salt, the sugar was obtained pure.

Separation of D-*glucose from* D-*gulose.* — Using the conditions of the preceding experiments, the two sugars (0.1 g each) were completely separated. After extraction with anhydrous ethanol, 97 mg of D-glucose and 94 mg of D-gulose were obtained. When the separation was attempted with distilled water, the gulose tailed badly, and no separation was achieved.

Methyl α-L-*gulopyranoside.* — L-Gulose (3.5 g) was converted into a mixture of methyl gulosides as described[13]. The mixture of crude glycosides was chromatographed, in two equal portions, on a calcium column (3.8 × 20 cm); it was eluted with water at a flow rate of 40–50 mL/h. The β-pyranoside and inorganic compounds emerged first, followed by the β-furanoside contaminated with a little free L-gulose and α-pyranoside. The next fraction contained the α-pyranoside and a little L-gulose; it was evaporated, and the residue extracted with acetone, and evaporation of the solvent gave pure methyl α-L-gulopyranoside (1.9 g, 51%) which slowly crystallized. After recrystallization from 1-propanol, it melted at 74–78°. (Interestingly, this compound was

first isolated as its crystalline, calcium chloride complex[31].) Later fractions contained the α-furanoside.

Separation of methyl D-*allosides.* — A mixture of D-allose (2.0 g), anhydrous strontium chloride (3.5 g), methanol (40 mL), and acetyl chloride (0.15 mL) was boiled under reflux for 24 h. Sodium acetate (1.0 g) was added to the cooled solution, and the solvent was evaporated. The residue was acetylated, and the product deacetylated as described before[30]. The mixture (1.9 g) was separated on a calcium column (1.5 × 35 cm), with collection of 4.5-mL fractions. The contents of the fractions were identified by t.l.c. in 5:4:1 acetone–ethyl acetate–water, in which the four methyl allosides are well separated. When all of the fractions had been identified, their contents were recombined, and separated again under different conditions. The results are shown in Table II. After the last separation, the α-furanoside, the β-furanoside, and the β-pyranoside were isolated from the respective fractions, and were found to melt at 103–104°, 85–86°, and 155–156°, respectively (lit. m.p.[30,31] 105–105.5°, 86–88°, and 154–155°).

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