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Separation of sugars by ion-exclusion chromatography on a cation-exchange resin

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ABSTRACT

A method for the separation of N-acetylmannosamine and N-acetylglucosamine is described, which consists of chromatography of the two sugars on a column (30 × 1 cm) of the cation-exchange resin, Dowex 50W-X2, in borate buffer at pH 7.8. N-Acetylmannosamine is eluted near the void volume, while N-acetylglucosamine emerges in a more retarded position. It is postulated that the separation occurs as a result of the combined effects of ion exclusion and gel permeation. Thus, in borate solution, N-acetylmannosamine presumably exists largely as a negatively charged complex and is therefore excluded from the sulfonated polystyrene matrix, while N-acetylglucosamine occurs mainly as the free sugar in the equilibrium mixture and, being a neutral compound, has free access to the porous resin. The proposed mechanism for the separation was supported by the finding that glucose and glucose 6-phosphate could also be separated on a column of the same resin, with water as the eluent.

INTRODUCTION

Established procedures for the separation of neutral monosaccharides include a group of methods, which are based on the formation of sugar–borate complexes and subsequent fractionation by various techniques originally developed for the separation of charged compounds [1,2]. This approach has been particularly valuable in the analysis of mixtures of monosaccharides that are otherwise difficult to separate, *e.g.*, N-acetylmannosamine and N-acetylglucosamine. Thus, a mixture of these two sugars can easily be resolved by paper electrophoresis in borate buffer [3,4] or by chromatography on an anion-exchange resin in borate form [5]☆. N-Acetylmannosamine migrates faster than N-acetylglucosamine on electrophoresis and binds more tightly to the resin during ion-exchange chromatography, indicating that it forms a borate complex more readily than does N-acetylglucosamine. Yet another expression of the relative strengths of the borate complexes is the difference in R_F values observed on chromatography of the two sugars on borate-treated paper [3,4,7]. N-Acetylmannosamine migrates more slowly than N-acetylglucosamine, presumably because the ionic complexes are less soluble in the mobile phase than the free sugars and N-acetylmannosamine is complexed to a greater extent than N-acetylglucosamine at equilibrium.

We have recently found that N-acetylmannosamine and N-acetylglucosamine may also be separated by chromatography on Sephadex G-15 in borate buffer [8]. Since the borate complexes are larger than the free sugars and N-acetylmannosamine forms the strongest complex, we expected that N-acetylmannosamine would emerge first and that N-acetylglucosamine would be eluted in a more retarded position. This was

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☆ The procedure described by Johnson *et al.* [5] was used for fractionation of disaccharides containing N-acetylglucosamine and N-acetylmannosamine but is also applicable to the separation of the two monosaccharides [6].

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indeed the case. When this experiment was carried out, however, we had not realized that the difference in size between the N-acetylmannosamine–borate complex (M_r 269 for the sodium salt and 246 for the complex ion) and free N-acetylglucosamine (M_r 221) is so small that there had been no reason to expect that complete separation would occur under the conditions chosen. This conclusion was reinforced by the observation that glucose and maltose (M_r 180 and 342, respectively) were separated only partially on the column (117 × 1.5 cm) used in these experiments. We therefore speculated [8] that, besides the molecular mass difference between free N-acetylglucosamine and the N-acetylmannosamine–borate complex, two additional factors contributed to the observed separation: (a) the presence of a hydration shell around the charged borate complex, which increased its apparent molecular mass, and (b) an ion-exclusion phenomenon resulting from complexing of the borate in the buffer with the glucose-containing Sephadex matrix and repulsion of the N-acetylmannosamine–borate complex from the negatively charged beads. If the latter effect were operative, separation should also occur when the two N-acetylhexosamines are chromatographed on a *bona fide* cation-exchange resin with a sufficiently porous matrix that allows entry of the free N-acetylglucosamine into the beads. In the present communication, we report that N-acetylmannosamine and N-acetylglucosamine may indeed be separated by chromatography on the cation-exchange resin, Dowex 50W-X2, in borate buffer.

MATERIALS AND METHODS

N-Acetylglucosamine was obtained from Pfanstiehl Labs. (Waukegan, IL, USA). N-Acetylmannosamine, glucose, glucose 6-phosphate, N-acetylglucosamine 6-phosphate, *p*-dimethylaminobenzaldehyde, phenol and Dowex 50W-X2-400 (200–400 mesh) were from Sigma (St. Louis, MO, USA). N-Acetyl-[1,6-^{3}H]glucosamine (sp. act., 30–60 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA) and Blue Dextran from Pharmacia LKB Biotechnology (Piscataway, NJ, USA). ^3H_2O (sp. act., 5 Ci/g) was from DuPont/New England Nuclear. Other reagents were of analytical grade and were purchased from Fisher Scientific (Atlanta, GA, USA).

Quantitative analysis of N-acetylhexosamines was carried out by the method of Reissig *et al.* [9]. Glucose and glucose 6-phosphate were analyzed by the phenol–sulfuric acid method [10]. Radioactivity was measured by liquid scintillation spectrometry in a Packard Model 2450 instrument, using 0.5 ml of aqueous sample and 4.5 ml of Scintiverse BD (Fisher Scientific).

Chromatography was carried out on Econo-Columns (Bio-Rad Labs., Richmond, CA, USA) as follows. The cation-exchange resin was converted to the Na^+ form by washing successively with 4 *M* HCl, water, 2 *M* NaOH, and water again. A column (30 × 1 cm; bed volume, 23.5 ml) was packed and equilibrated with 0.27 *M* sodium borate, pH 7.8, which had been prepared from H_3BO_3 and NaOH [5]. Elution was carried out with this buffer at a flow-rate of 10–12 ml/h, unless otherwise indicated, and 0.5- to 1.0-ml fractions were collected. V_0 was determined by chromatography of a 200-μl sample (4 mg) of Blue Dextran, and V_t was estimated by chromatography of ^3H_2O (50 000 cpm). In some experiments, shorter columns (5.9–6.5 × 1.5 cm) were employed, which had been packed in used PD-10 columns (Pharmacia LKB Biotechnology).

RESULTS

Calibration of the standard Dowex 50W-X2-400 column (30 × 1 cm; Na^+ form) with Blue Dextran and tritiated water as markers for V_0 and V_t gave values of 10 and 23 ml, respectively (Fig. 1A). When a mixture of N-acetylmannosamine and N-acetylglucosamine was chromatographed on this column with water as eluent, no separation occurred, and the two sugars emerged as a single peak close to V_t, indicating that they had entered the resin beads (Fig. 1B). In contrast, elution with borate buffer gave two completely separated peaks, with effluent volumes of 14 and 18 ml, respectively (Fig. 1C). Since equal weights of the two N-acetylhexosamines had been applied to the column and

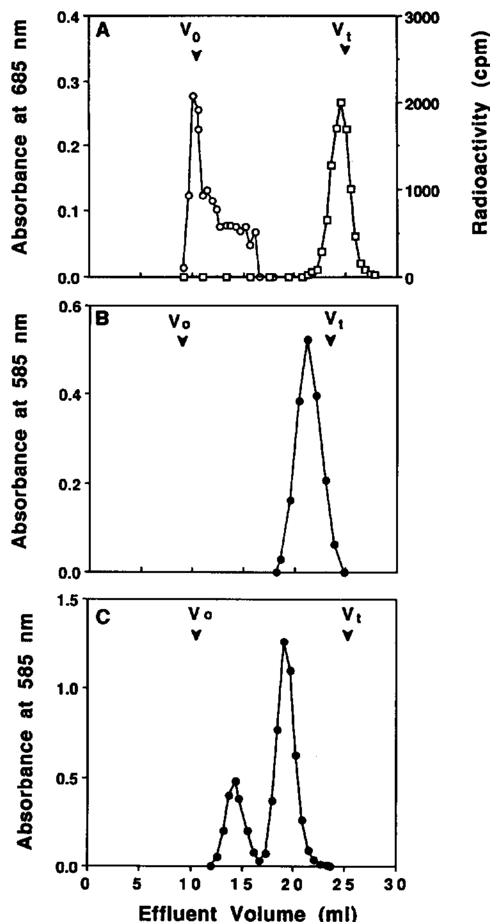

Fig. 1. Separation of N-acetylmannosamine and N-acetylglucosamine on Dowex 50W. A column (30 × 1 cm) of Dowex 50W-X2 (Na^+; 200–400 mesh) was used, which was eluted at room temperature at a flow-rate of 10–12 ml/h. (A) After equilibration of the column with 0.27 *M* borate, pH 7.8, a mixture of Blue Dextran (4 mg) and ^3H_2O (56 000 cpm) in 0.22 ml of buffer was applied, and the column was eluted with the same buffer. Absorbance at 685 nm (○) and radioactivity (□) in the effluent fractions were measured. (B) After equilibration of the column with water, a mixture of N-acetylglucosamine (0.1 mg) and N-acetylmannosamine (0.2 mg) in 0.3 ml of water was applied to the column, and elution was carried out with water. Fractions were analyzed by the Morgan–Elson procedure (●). (C) After equilibration of the column with borate buffer, a mixture of N-acetylglucosamine (1 mg) and N-acetylmannosamine (1 mg) in 0.2 ml of buffer was applied, and the column was eluted with the same buffer. Fractions were analyzed by the Morgan–Elson procedure (●).

N-acetylmannosamine gives only about half as much color as N-acetylglucosamine in the Morgan–Elson procedure [7], it was concluded that the smaller, earlier peak was N-acetylmannosamine and that the more retarded peak was N-acetylglucosamine.

To establish the identities of the two peaks more directly, a sample of radioactive N-acetylglucosamine was chromatographed in borate buffer, separately and in mixture with non-radioactive N-acetylmannosamine (Fig. 2). As shown in Fig. 2A, a major radioactive peak was observed in the same position as that of the putative N-acetylglucosamine peak in Fig. 1C. In addition, however, at least three minor impurities were present, one emerging with the

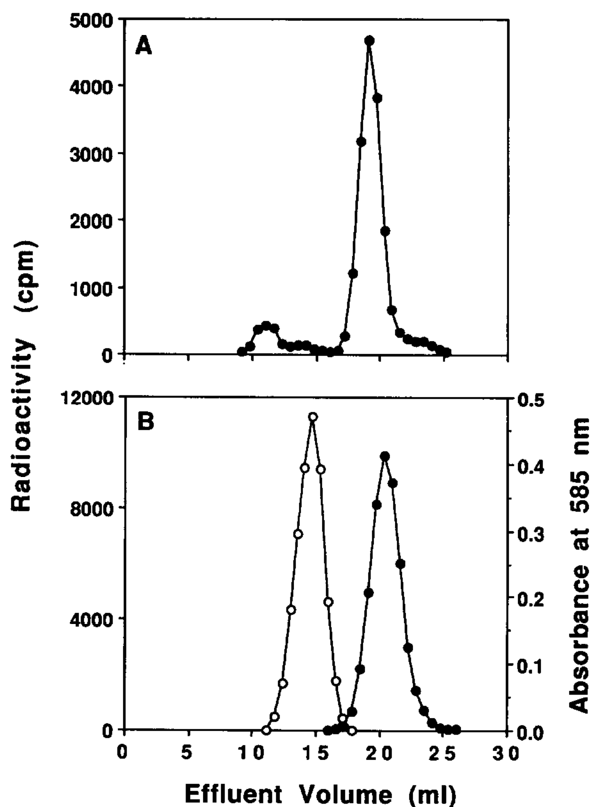

Fig. 2. Analysis of N-acetyl-D-[1,6-^{3}H]glucosamine by chromatography on Dowex 50W. (A) A sample of N-acetyl-D-[1,6-^{3}H]glucosamine (0.2 ml; *ca.* 100 000 cpm) was applied to the column after equilibration with 0.27 *M* borate, pH 7.8. Elution was carried out with the borate buffer, and fractions were analyzed for radioactivity (●). (B) A 1.0-ml sample containing N-acetylmannosamine (2 mg) and purified N-acetyl-D-[1,6-^{3}H]glucosamine (70 000 cpm) was applied to the column after equilibration with borate buffer. Elution was carried out with the same buffer, and fractions were analyzed for N-acetylhexosamine (○) and radioactivity (●).

void volume, a second in the same position as the presumed N-acetylmannosamine peak in Fig. 1C, and a third at the tail end of the major radioactive peak. It is not known whether these impurities were present in the material supplied by the manufacturer or had been formed during storage. After isolation of the major component by fractionation of a larger batch of the radioactive sugar, addition of non-radioactive N-acetylmannosamine and repeated chromatography gave the results shown in Fig. 2B. It is seen that N-acetylmannosamine was eluted first, as indicated by analysis with the Morgan–Elson procedure, and was completely separated from the radioactive N-acetylglucosamine, which now gave a symmetrical peak and showed no signs of the presence of any contaminants. It may be noted that the effluent volume of the N-acetylglucosamine peak in Fig. 2B was slightly larger than that of the major radioactive peak in Fig. 2A; this was probably due mainly to the larger sample volume applied to the column (1.0 *vs.* 0.2 ml).

Since the results described above were in accord with our predictions, the influence of a negative charge on the chromatographic behavior of some other sugars was also explored. When a mixture of glucose and glucose 6-phosphate was chromatographed on a column (30 × 1 cm) of Dowex 50W-X2 in Na^+ form, which was eluted with water rather than with borate buffer, complete separation occurred, as illustrated in Fig. 3A. Glucose 6-phosphate emerged with the void volume, while glucose was eluted near V_t of the column, as was established by chromatographing each sugar individually. Similar results were observed on elution with the standard borate buffer or with 0.2 *M* NaCl. Complete separation of N-acetylglucosamine 6-phosphate and N-acetylglucosamine was likewise obtained upon chromatography with water as the eluent (Fig. 3B). It should be noted that the two sugar phosphates were both eluted with the void volume, whereas N-acetylmannosamine, chromatographed in borate buffer, emerged in a more retarded position. This difference was probably an expression of the fact that a portion of the N-acetylmannosamine molecules are not complexed with borate in the equilibrium mixture and therefore are not repelled by the porous resin, leading to an overall slower migration through the column.

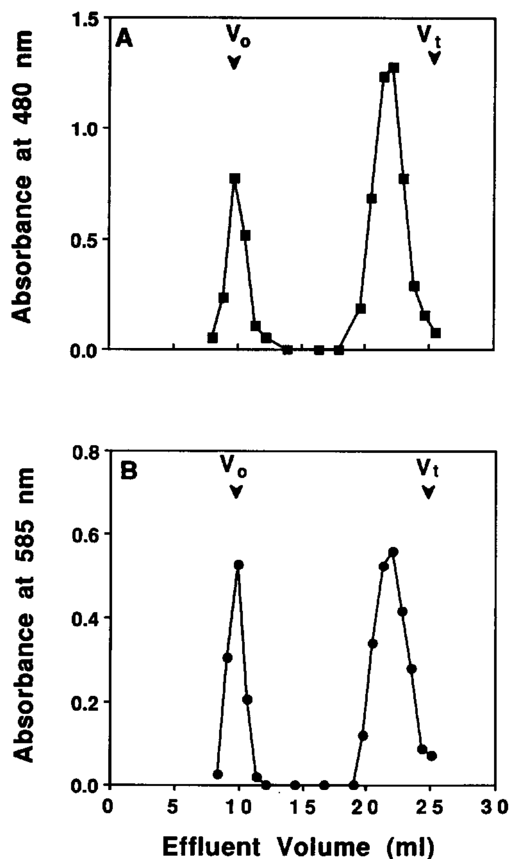

Fig. 3. Isolation of sugar phosphates by chromatography on Dowex 50W. A column (30 × 1 cm) of Dowex 50W (Na^+; 200–400 mesh) was equilibrated with water. (A) A mixture of glucose (5 mg) and glucose 6-phosphate (2.5 mg) in 0.15 ml of water was applied to the column, and elution was carried out with water. Fractions were analyzed by the phenol–sulfuric acid method (■). (B) A mixture of N-acetylglucosamine (2 mg) and N-acetylglucosamine 6-phosphate (2 mg) in 0.4 ml of water was chromatographed under the same conditions. Fractions were analyzed by the Morgan–Elson procedure (●).

In view of the excellent separation between the neutral monosaccharides and their phosphate derivatives, it was of interest to determine whether the procedure could be simplified by use of a shorter column. As shown in Fig. 4A, complete separation of glucose and glucose

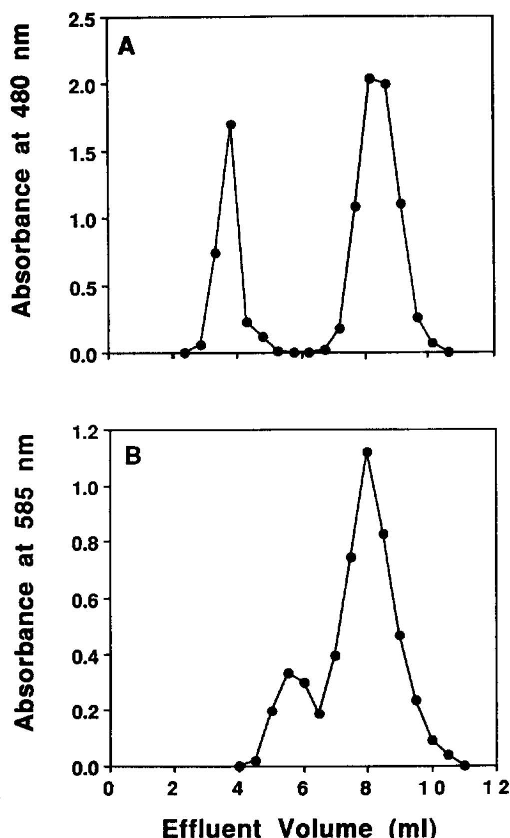

Fig. 4. Chromatography of (A) glucose and glucose 6-phosphate and (B) N-acetylglucosamine and N-acetylmannosamine on a short column of Dowex 50W. (A) A column (6.5 × 1.5 cm) of Dowex 50W-X2 (Na^+; 200–400 mesh) was equilibrated with water. A mixture of glucose (5 mg) and glucose 6-phosphate (2.5 mg) in 0.15 ml of water was applied, and the column was eluted with water. Fractions (0.5 ml) were collected and analyzed by the phenol–sulfuric acid method (●). (B) A column (5.9 × 1.5 cm) of Dowex 50W was equilibrated with 0.27 *M* borate, pH 7.8, and a sample (0.2 ml) containing N-acetylglucosamine (2 mg) and N-acetylmannosamine (2 mg) was applied. The column was eluted with borate buffer, and fractions (0.5 ml) were analyzed by the Morgan–Elson procedure (●).

6-phosphate was obtained on a 6.5 × 1.5 cm column of Dowex 50W-X2-400 in the Na^+ form, which was eluted with water. The same result was observed when resin in the H^+ form was used. N-Acetylmannosamine and N-acetylglucosamine, however, were separated only partially when chromatographed on the shorter column in borate buffer (Fig. 4B).

DISCUSSION

This study was undertaken to test the hypothesis that ion exclusion might have been a contributing factor in the previously observed separation of N-acetylmannosamine and N-acetylglucosamine, which occurs on chromatography of the two sugars on Sephadex G-15 in borate buffer [8]. The results presented here support this notion, inasmuch as the two N-acetylhexosamines could also be separated by chromatography on the cation-exchange resin, Dowex 50W-X2, provided that the elution was carried out with borate buffer. We envisage that under these conditions the separation occurs by the following process. Most of the N-acetylmannosamine molecules form negatively charged complexes with borate in the equilibrium mixture, and these are repelled by the sulfonated polystyrene matrix, resulting in early elution of the amino sugar. In contrast, the majority of the N-acetylglucosamine molecules presumably remain largely as the free sugar in the equilibrium mixture and, being neutral, have free access to the porous resin and are eluted near V_t of the column. The separation may thus be viewed as the result of a combination of ion exclusion and gel permeation. N-Acetylglucosamine emerged somewhat earlier when the column was eluted with borate rather than with water (*cf.* Fig. 1C and B), and this difference probably reflects the extent of complex formation in borate solution. It seems possible that a quantitative relationship could be established between the equilibrium constants of borate complex formation and the relative elution positions of the sugars, but no attempt to do so was made in the present study.

The observed separation is not only of theoretical interest but represents a practical, new method for the separation of the two N-acetylhexosamines in both analytical and preparative work. The usefulness of the new method is not limited to this particular fractionation, and the complete separation of glucose and N-acetylglucosamine from their respective 6-phosphate derivatives on a short resin column illustrates the broader applicability of the ion-exclusion/gel-permeation principle. In an exten-

sion of these experiments, the Dowex 50 procedure is being used in the development of an alternative assay of hexokinase activity with radioactive glucose as the substrate, and the preliminary results suggest that this approach will be successful.

It is apparent from the results of this study that the new method is well suited for use as an analytical tool. Its usefulness in preparative applications, however, may be limited by the presence of other compounds in the mixtures to be fractionated, and, *e.g.*, anion-exchange chromatography on a resin in the borate form is likely to yield better resolution of multi-component mixtures, since the N-acetylhexosamines and other sugars that form borate complexes are eluted well after the total bed volume of the resin. It should also be pointed out that the experimental conditions chosen in the present study may not be ideal for a particular application and that, *e.g.*, the use of a borate solution of lower concentration may facilitate subsequent desalting of the pooled fractions (see ref. 7).

In summary, we have developed a new method for the separation of N-acetylmannosamine and N-acetylglucosamine, which consists of chromatography on a cation-exchange resin in borate buffer. Cation exchange, however, apparently does not contribute to the observed separation. Rather, the fractionation seems to occur as a result of the combined effects of ion exclusion and gel permeation. Since the method is based on general principles and not only on the unique properties of the two N-acetylhexosamines, it is also applicable to the separation of other mixtures of neutral and negatively charged small molecules.

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