

Analysis of Oligosaccharide Negative Charge by Anion-Exchange Chromatography

UNIT 17.20

This unit presents the analysis of negative charge on labeled N- or O-linked oligosaccharides. These protocols may be used in the initial screening of oligosaccharides to detect negative charge, for analytical or preparative separation of oligosaccharides based on their negative charge, or to analyze the type of negative charge found on the oligosaccharides. The Basic Protocol describes the use of anion-exchange (QAE-Sephadex) chromatography with stepwise elution for estimating the number of negative charges on an oligosaccharide sample derived from glycosidase treatment of a glycoprotein. In the Alternate Protocol, gradient elution is used for the preparative separation of oligosaccharides based on negative charge. The Support Protocol describes a method for measuring loss of or change in negative charge after treatment of the oligosaccharide sample with mild acid and/or phosphatases.

SEPARATION AND ANALYSIS OF ANIONIC OLIGOSACCHARIDES BY QAE-SEPHADEX CHROMATOGRAPHY WITH STEPWISE ELUTION

**BASIC
PROTOCOL**

Negative charges on peptide-free radioactive N- or O-linked oligosaccharides can be detected by binding the molecules to the strong anion exchanger QAE-Sephadex (see also UNIT 10.10). Stepwise batch elution (as described in this protocol) or gradient elution (see Alternate Protocol) with salt separates the bound molecules according to the approximate number of negative charges. Loss or change of negative charge following treatment with sialidases (UNIT 17.12), solvolysis (UNIT 17.23), mild acid (UNIT 17.16), or phosphatase (see Support Protocol) can also be monitored (see Fig. 17.20.1). Because of charge-to-mass effects, this method gives only a general estimate of the number of negative charges on an oligosaccharide. If more precise analysis is desired, protocols using high-performance liquid chromatography (HPLC; UNIT 17.21) are recommended.

Materials

Radiolabeled mixture of oligosaccharides released from glycoprotein
(UNITS 17.12-17.17)

Equilibrated QAE-Sephadex chromatography resin (see recipe)

2 mM Tris base

Elution buffers (see recipe)

1- to 2-ml Pasteur pipets plugged with glass wool *or* 1- to 2-ml disposable plastic columns

Sintered-glass funnel

Additional reagents and equipment for metabolic radiolabeling (UNIT 17.4) and autoradiography (APPENDIX 3A)

1. Pour 0.75-ml column of equilibrated QAE-Sephadex in a 1- to 2-ml Pasteur pipet plugged with glass wool or in a 1- to 2-ml disposable plastic column. Wash with 5 ml of 2 mM Tris base.

It is acceptable to let the top of the column bed go dry during washing.

2. Dilute or dissolve the sample in 0.75 ml of 2 mM Tris base and load onto the column. Wash with seven 0.75-ml aliquots of 2 mM Tris base and collect 1.5-ml fractions.

Try not to disturb the top of the column bed during application. Allow the top of the column bed to go dry between aliquots.

The concentration of salts in the applied sample should be <5 mM and the pH must be >7. If the original sample contains acidic buffers or other salts, it may be necessary

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Supplement 32

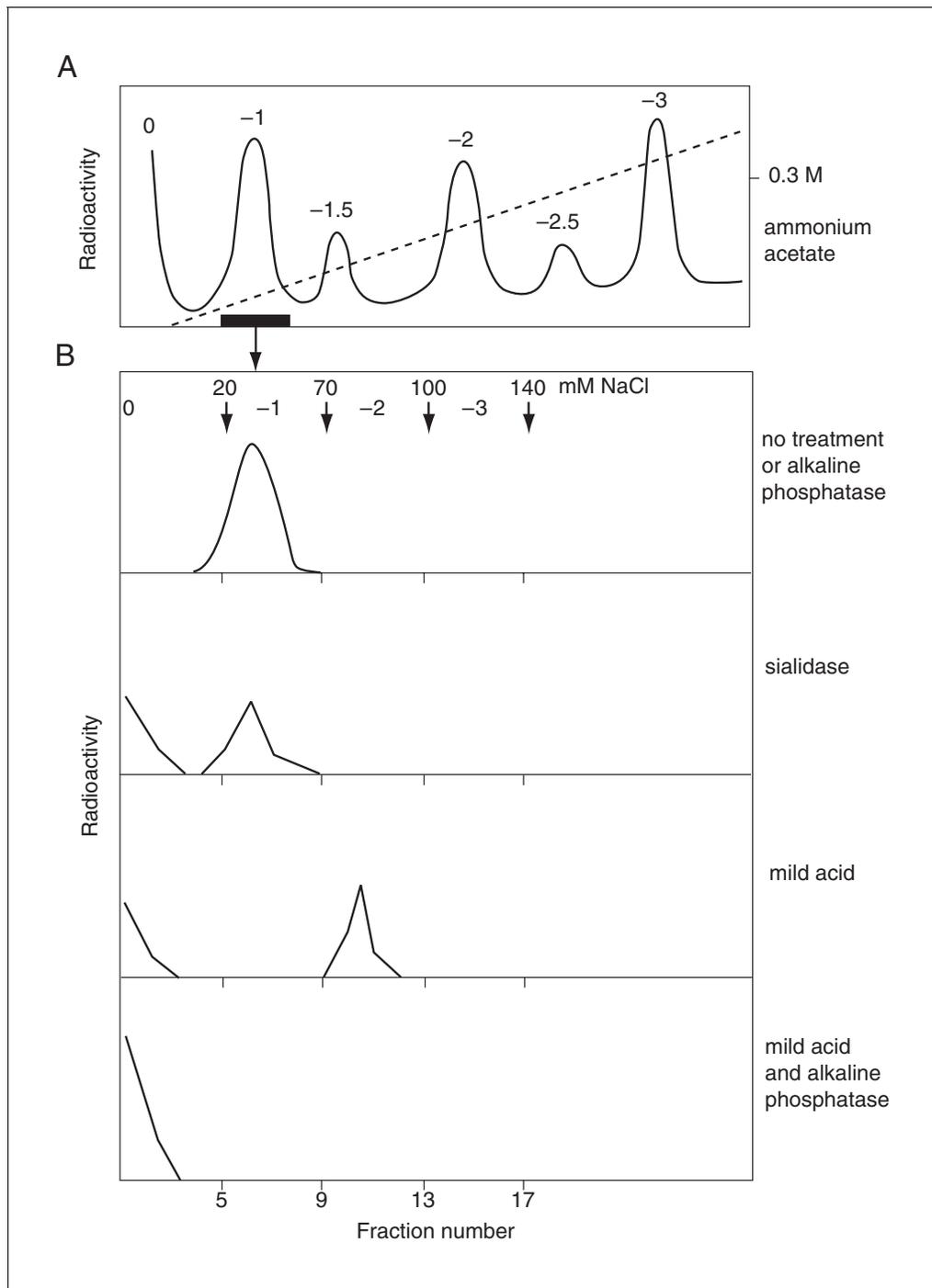


Figure 17.20.1 Schematic examples of QAE-Sephadex analysis (based on Varki and Kornfeld, 1983). Numbers in italics indicate the charges on the high mannose-type oligosaccharides eluting at that position. **(A)** Gradient fractionation of mixture of N-linked oligosaccharides with different combinations of sialic acid phosphomonoesters or phosphodiester. **(B)** Peak marked “-1” consists of a mixture of oligosaccharides with one negative charge due to either one sialic acid residue (acid- or sialidase-sensitive) or one phosphodiester (increased negative charge after mild acid, sensitive to alkaline phosphatase only after mild acid treatment). The procedures used to evaluate oligosaccharide mixtures are listed on the right with examples of resulting shifts in the elution peaks for each type of treatment illustrated on the left.

to neutralize with Tris base and/or dilute the sample to >0.75 ml to reduce the salt concentration. In these cases, the first wash-through tube will contain >1.5 ml fluid.

3. Elute sample from the column with elution buffers containing increasing concentrations of salt (i.e., 20, 70, 100, 140, 200, 400, and 1000 mM NaCl) in 2 mM Tris base. For each salt concentration, use eight 0.75-ml aliquots and collect four 1.5-ml fractions.

The salt elution series suggested above was devised for analysis of phosphorylated high mannose-type oligosaccharides from lysosomal enzymes. Salt concentrations may need to be adjusted to optimize the separation of other types of oligosaccharides (see Critical Parameters).

4. Monitor elution of the oligosaccharides by analyzing aliquots of the collected fractions (or the entire fractions) for radioactivity. In the latter case, collect the eluate directly into large-sized scintillation vials. If the first wash fraction is >1.5 ml (see step 2 annotation), monitor only an aliquot.

When comparing different treatments of the same oligosaccharide—e.g., sialidase (UNIT 17.12), mild acid, and alkaline phosphatase (see Support Protocol)—it is convenient to run all samples and controls in parallel. Approximately ten columns can be easily run at the same time. A repeating pipettor makes the process more reproducible and less taxing.

- 5a. The first time the experiment is done, and anytime it is possible that some of the oligosaccharides may not elute with the highest concentration of salt, add 1.5 ml of 2 mM Tris base to the column and transfer the contents of the column into a scintillation vial. Add scintillation fluid and monitor for any remaining radioactivity.
- 5b. Otherwise, discard the column contents (do not reuse).

SEPARATION AND ANALYSIS OF ANIONIC OLIGOSACCHARIDES BY QAE-SEPHADEX CHROMATOGRAPHY WITH GRADIENT ELUTION

**ALTERNATE
PROTOCOL**

After the initial studies of a mixture of oligosaccharides on a QAE-Sephadex column, it may be desirable to preparatively separate the oligosaccharides with better resolution. Although HPLC methods are preferable (see UNIT 17.21), the following protocol can be used if an HPLC unit or column is not available or if a large capacity is needed.

Additional Materials (also see Basic Protocol)

5- to 20-ml disposable plastic column
Gradient mixer

Additional materials and equipment for salt gradient preparation (UNIT 10.10)

1. Prepare a 3- to 10-ml column of QAE-Sephadex (see Basic Protocol, step 1, but use 5- to 20-ml disposable plastic column). Wash column with 10 column volumes of 2 mM Tris base.
2. Prepare and load the oligosaccharide sample (see Basic Protocol, step 2).
3. Place 50 ml of 2 mM Tris base in the first chamber of the gradient mixer; place 50 ml of an appropriate concentration of NaCl dissolved in 2 mM Tris base in the second chamber (choose salt concentration based on the results of the Basic Protocol).

An ammonium acetate gradient at pH 5.3 can be used to fractionate phosphomonoesters and phosphodiester (see Critical Parameters).

The concentration of NaCl or ammonium acetate in the second chamber is selected based on prior knowledge of the concentration required to elute the most anionic species in the mixture.

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**SUPPORT
PROTOCOL**

4. Elute the sample from the column with the selected linear gradient regimen. Collect 1-ml fractions and monitor for radioactivity.

**DETECTION AND REMOVAL OF PHOSPHODIESTERS OR
PHOSPHOMONOESTERS**

Oligosaccharide samples thought to contain phosphodiester and phosphomonoester (e.g., released from lysosomal enzymes) can be treated with mild acid and alkaline phosphatase to detect and remove them. Phosphomonoester-containing molecules will lose negative charge upon phosphatase treatment. Phosphodiester-containing molecules are resistant to alkaline phosphatase alone, but will increase in negative charge following mild acid treatment because of the generation of phosphomonoesters. A combination of mild acid and phosphatase treatment will neutralize phosphodiester; the mild acid conditions used here will also result in removal of sialic acids (see Fig. 17.20.1).

Additional Materials (also see *Basic Protocol*)

Radiolabeled mixture of oligosaccharides released from glycoprotein (*UNITS* 17.12-17.17), desalted and lyophilized
10 U/ml *E. coli* alkaline phosphatase
2 M acetic acid
200 mM Tris·Cl, pH 8.0 (*APPENDIX 2*)

Strong 10-ml conical glass tubes *or* 1-ml Reacti-Vials (Pierce)
Water bath or heating block 80°C

1. Dissolve the oligosaccharide sample in 0.5 ml of 2 M acetic acid in a strong 10-ml conical glass tube or 1-ml Reacti-Vial. Tightly cap or stopper the tube and heat 120 min at 80°C.

This step is designed to detect phosphodiester. A second, identical sample should be prepared and processed starting with step 3; if phosphodiester are present, the acid-treated sample will show a higher negative charge than this non-acid-treated control upon analysis.

2. Flash-freeze and lyophilize the sample.
3. Dissolve in 20 µl water, using a pipettor to wash the walls of the tube. Vortex well and centrifuge briefly at room temperature to get sample to bottom of tube.
4. Remove a 10-µl aliquot of sample and dilute to 1.5 ml with 2 mM Tris base. Apply to a 0.75-ml QAE-Sephadex column for analysis (see *Basic Protocol*).
5. To the remaining sample, add 10 µl of 200 mM Tris·Cl (pH 8.0) and mix. Add 1 µl of 10 U/ml *E. coli* alkaline phosphatase (10 mU). Incubate 1 hr at 37°C.
6. Dilute sample to 1.5 ml with water and apply to a 0.75-ml QAE-Sephadex column (see *Basic Protocol*).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.

Ammonium acetate (pH 5.3), 1 M

Dissolve 38.54 g ammonium acetate in H₂O. Add H₂O to 500 ml. Adjust pH to 5.3 with 1 M acetic acid. Store at 4°C.

Elution buffers

Prepare stock solutions of 1 M Tris base (pH unadjusted; store at 4°C) and 2 M NaCl (store at room temperature). Use these stocks to make elution buffers containing 2 mM Tris base and different concentrations of NaCl. For gradient elution, make appropriate dilutions of the 1 M ammonium acetate (pH 5.3) stock solution (see recipe). It is not necessary to check the final pH of the diluted solutions.

QAE-Sephadex chromatography resin, equilibrated

Swell QAE-Sephadex (Pharmacia Biotech) in 1 M Tris base (using 1 g dry resin/20 ml Tris) overnight at 4°C, then decant. Repeat once. Pour slurry over a sintered-glass funnel and wash with >20 vol of 2 mM Tris base. Check the pH of the final wash with pH paper to make sure it is similar to that of the Tris base; if not, wash again until appropriate pH is achieved. Store equilibrated resin in 2 mM Tris base at 4°C.

There is no need to remove “fines” (small particles) from the resin because the columns used are small and flow rates are not limiting. With storage and exposure to air, the pH of the Tris base will drift towards neutral; however, this is not of serious concern.

COMMENTARY

Background Information

Negatively charged monosaccharides and highly anionic oligosaccharides bind well to most anion-exchange resins. However, large oligosaccharides with very few negative charges have a poor charge-to-mass ratio and may fail to bind to some anion-exchange resins. The strong anion exchanger QAE-Sephadex was first used (Tabas and Kornfeld, 1980) to permit analysis of high mannose-type N-linked oligosaccharides with single phosphodiester. The column was equilibrated in 2 mM Tris base to ensure that the pH was above neutral (maximizing the deprotonation of all anions) and to limit the amount of salt competing for binding sites. The protocol described here is based upon modifications of this method (Varki and Kornfeld, 1980, 1983; Goldberg and Kornfeld, 1981).

In the time since the original description, modifications have been developed to separate a wide variety of anionic oligosaccharides, including O-linked oligosaccharides, N-linked glycopeptides, and sulfated oligosaccharides (Roux et al., 1988; Fukuda, 1989; Cummings et al., 1989). In each case, column sizes and elution conditions were adjusted to allow appropriate separations and yield. In recent years, Mono-Q FPLC columns (Pharmacia Biotech) have permitted the use of the same QAE exchanger group for more rigorous and complete separations (van Pelt et al., 1987; *UNIT 10.10*).

Critical Parameters

The QAE-Sephadex resin must be thoroughly equilibrated in 2 mM Tris base. The initial loading must be carried out in a minimum

amount of salt (<2.5 mM) to ensure that weakly charged molecules do not escape binding. This can be achieved by desalting samples thoroughly (*UNIT 10.9*) and/or diluting them sufficiently with water or 2 mM Tris base before loading. To make the dilution volume reasonable, the preceding steps (e.g., enzyme reactions) should be carried out in the minimum volumes and lowest salt concentrations possible. Acid treatments are carried out with volatile acids that can be lyophilized before analysis. The charge-to-mass ratio of an oligosaccharide affects the amount of NaCl required for elution. Thus, a high mannose-type N-linked oligosaccharide with a single mannose-6-phosphate residue will elute with 50 to 70 mM NaCl, while free mannose-6-phosphate (with the same anionic group) requires 100 to 120 mM NaCl. The amount of salt needed to elute a particular charge or size class of molecules must be determined empirically. Thus, this technique is most useful when a related series of oligosaccharides with a limited range of size and charge (e.g., phosphorylated N-linked oligosaccharides with one to four negative charges) are being separated and analyzed. However, even when there is very extensive heterogeneity in size and charge (e.g., sulfated sialylated N-linked oligosaccharides), information can be obtained by following relative shifts in the elution position of groups of molecules (Roux et al., 1988). Gradient elution gives more defined separation between individual types of molecules, and is used primarily for preparative separations. If the molecules contain mixtures of phosphomonoesters and phosphodiester, separation can be obtained with a

gradient of ammonium acetate adjusted to pH 5.3 (close to the pK_a of the second negative charge of a phosphomonoester).

Analysis of glycopeptides by QAE-Sephadex chromatography is less satisfactory, because the peptide portion of the molecules can carry variable amounts of negative and/or positive charge. However, if complete proteolytic cleavage is performed (see UNIT 17.14A), each glycopeptide should have only one carboxyl group and separations can be made on the basis of the additional negative charges on the oligosaccharide. Molecules with very high charge density (e.g., heparin chains) are not well resolved on QAE columns because they are difficult to elute.

Troubleshooting

If molecules known to be anionic do not bind to the QAE-Sephadex column, the most likely problems are failure to properly equilibrate the column in 2 mM Tris base and the presence of excessive salt in the sample. If peaks are broad and slurred, the most likely problem is excessive disturbance of the top of the column bed during the application of elution buffers.

Anticipated Results

The method presented in this unit can be used for the following analyses.

1. *Initial screening of oligosaccharides for the presence of negative charge.* The sample is applied and the column washed and then directly eluted with 1 M NaCl in 2 mM Tris base. As a control, a known neutral oligosaccharide and an anionic oligosaccharide should be run under exactly the same conditions to obtain background numbers. A small quantity of the anionic oligosaccharide (<5%) can be expected to escape binding to the column, and a small amount of label from the neutral molecule (<2%) will be nonspecifically found in the salt eluate.

2. *Analytical separation of oligosaccharides by negative charge.* A mixture of oligosaccharides is applied and sequentially eluted batchwise with a defined series of salt elution steps (e.g., 20, 70, 100, 140, 200, 400, and 1000 mM NaCl), and the profile is monitored.

3. *Preparative separation of oligosaccharides by negative charge.* This is based upon the profile obtained by analytical separation. Gradient elution using NaCl or ammonium acetate may be preferred. Peaks are pooled, dried, and desalted on columns of Sephadex G-25 in water.

4. *Analysis of the type of negative charge on oligosaccharides.* Individual peaks obtained by preparative separation are analyzed before and after treatment or combinations of treatments with mild acid (see Support Protocol), with sialidase (UNIT 17.12), by solvolysis (UNIT 17.23), and/or with alkaline phosphatase (see Support Protocol). Fig. 17.20.1 schematically depicts an example of such analyses.

Time Considerations

The initial setup of small columns should take 10 to 20 min. The setup for gradient elution takes somewhat longer. Stepwise elutions of approximately ten samples can usually be carried out in parallel in ~1 hr. If sample preparation for scintillation counting is performed during time gaps in the elution procedure, the entire process can be completed in 2 to 3 hr. The time for scintillation counting will depend upon the amount of radioactivity available in each sample.

Literature Cited

- Cummings, R.D., Merkle, R.K., and Stults, N.L. 1989. Separation and analysis of glycoprotein oligosaccharides. *Methods Cell Biol.* 32:141-183.
- Fukuda, M. 1989. Characterization of O-linked saccharides from cell surface glycoproteins. *Methods Enzymol.* 179:17-29.
- Goldberg, D.E. and Kornfeld, S. 1981. The phosphorylation of β -glucuronidase oligosaccharides in mouse P388D1 cells. *J. Biol. Chem.* 256:13060-13067.
- Roux, L., Holoyda, S., Sundblad, G., Freeze, H.H., and Varki, A. 1988. Sulfated N-linked oligosaccharides in mammalian cells I: Complex-type chains with sialic acids and O-sulfate esters. *J. Biol. Chem.* 236:8879-8889.
- Tabas, I. and Kornfeld, S. 1980. Biosynthetic intermediates of β -glucuronidase contain high mannose oligosaccharides with blocked phosphate residues. *J. Biol. Chem.* 255:6633-6639.
- van Pelt, J., Damm, J.B., Kamerling, J.P., and Vliegthart, J.F.G. 1987. Separation of sialyl-oligosaccharides by medium pressure anion-exchange chromatography on Mono Q. *Carbohydr. Res.* 169:43-51.
- Varki, A. and Kornfeld, S. 1980. Structural studies of phosphorylated high mannose-type oligosaccharides. *J. Biol. Chem.* 255:10847-10858.
- Varki, A. and Kornfeld, S. 1983. The spectrum of anionic oligosaccharides released by endo- β -N-acetylglucosaminidase H from glycoproteins. Structural studies and interactions with the phosphomannosyl receptor. *J. Biol. Chem.* 258:2808-2818.

Key Reference

Varki, A. and Kornfeld, S. 1980. See above.

Figures in the miniprint section of this paper provide several examples of the types of analyses described here.

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