CHAPTER 17
Preparation and Analysis of Glycoconjugates

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ABSTRACT
Whereas DNA, RNA, and proteins are linear polymers that can usually be directly sequenced, glycans show substantially more complexity, having branching and anomic configurations (α and β linkages). The biosynthesis of glycans, termed glycosylation, is extremely complex, is not template-driven, varies among different cell types, and cannot be easily predicted from simple rules. This overview discusses the stereochemistry of mono- and oligosaccharides and provides diagrammatic representations of monosaccharides (Fisher projections and Haworth representations) and formulas for representation of glycan chains. A glossary of terms used in glycobiology is also provided. Curr. Protoc. Mol. Biol. 88:17.0.1-17.0.12. © 2009 by John Wiley & Sons, Inc.

Keywords: glycan ● monosaccharide ● glycan analysis ● sugar symbols ● glycoconjugate ● glycobiology

INTRODUCTION
The modern revolution in molecular biology was driven to a large extent by advances in methods for analysis and manipulation of DNA, RNA, and proteins. Although oligosaccharides (sugar chains or glycans) are also major macromolecules of the typical cell, they did not initially share in this molecular revolution. The reasons for this were to a large extent technical. Whereas DNA, RNA, and proteins are linear polymers that can usually be directly sequenced, oligosaccharides show substantially more complexity, having branching and anomic configurations (α and β linkages). Thus, whereas three amino acids or nucleotides can be combined into six possible sequences, three hexose monosaccharides can theoretically generate 1056 possible glycans. In addition, the syntheses of DNA, RNA, and proteins are template-driven, and the sequence of one can generally be predicted from that of another. In contrast, the biosynthesis of oligosaccharides, termed glycosylation, is extremely complex, is not template-driven, varies among different cell types, and cannot be easily predicted from simple rules.

It is clear that glycans have important, albeit varied, effects upon the biosynthesis, folding, solubility, stability, subcellular trafficking, turnover, and half-life of the molecules to which they are attached. These are matters of great importance to the cell biologist, protein chemist, biotechnologist, and pharmacologist. On the other hand, the successful growth of several glycosylation mutants as permanent tissue culture cell lines indicates that the precise structure of many glycans is not critical for the growth and viability of a single cell in the protected environment of the culture dish. Thus, until recently, it was possible for many researchers working with in vitro single-cell systems to ignore the existence of glycans. However, with the increasing emphasis on studying cell-cell interactions in normal development, tissue morphogenesis, immune reactions, and pathological conditions such as cancer and inflammation, the study of glycan structure and biosynthesis has become very important.

The term “glycobiology” has found acceptance for denoting studies of the biology of glycoconjugates in both simple and complex systems. Many technical advances have occurred in the analysis of glycans, making it now feasible to study them in detail. These advances include the development of sensitive
and specific assays and the availability of numerous purified enzymes (glycosidases) with high degrees of specificity.

In spite of all these advances, many analytical techniques in glycobiology remained in the domain of the few laboratories that specialized in the study of glycans. Likewise, published compendia of carbohydrate methods were designed mainly for use by experts. This chapter attempts to place some of this technology within easy reach of any laboratory with basic capabilities in biochemistry and molecular biology. The techniques described here include modern versions of time-honored methods and recently developed methods, both of which have widespread applications. However, it is important to emphasize that the protocols presented here are by no means comprehensive. Rather, they serve as a starting point for the uninitiated scientist who wishes to explore the structure, biosynthesis, and biology of glycan chains. In most cases, further analysis using more sophisticated techniques will be required to obtain final and definitive results. Nonetheless, armed with results obtained using the techniques described here, the typical researcher can make intelligent decisions about the need for such further analyses.

The appearance of many commercial kits for analysis of glycoconjugates is another sign that the technology has arrived and that many laboratories have developed an interest in glycobiology. It is worth noting that although some of these kits are designed to simplify the use of well-established techniques, others employ methodologies that have been newly developed by the companies themselves. Experience with the latter methodologies in academic scientific laboratories may be limited, and the techniques in question may therefore not be represented in this chapter. However, although the admonition caveat emptor is appropriate, some kits may well become useful adjuncts to the methods presented here.

Different types of glycosylation are perhaps best defined by the nature of the

Figure 17.0.1 Common glycan-linkage regions on animal cell glycoconjugates. The most common types of glycans found in animal glycoconjugates are shown, with an emphasis upon the linkage region between the glycan and the protein or lipid. Other rarer types of linkage regions and free glycans that can exist naturally are not shown (reproduced with permission from figure 1.6 in Varki et al., 2009). For the color version of this figure go to http://www.currentprotocols.com/protocol/mb1700.
linkage region of the oligosaccharide to a lipid or protein (Fig. 17.0.1). Although the linkage regions of these molecules are unique, the sugar chains frequently tend to share common types of outer sequences. It is important to note that this chapter deals only with the major forms of glycosylation found in “higher” animal glycoconjugates—N-acetylglucosamine (GlcNAc)-N-Asn-linked, N-acetylgalactosamine (GalNAc)-O-Ser/Thr-linked glycans on glycoproteins, xylose-O-Ser-linked glycosaminoglycans on proteoglycans, ceramide-linked glycosphingolipids, phosphatidylinositol-linked glycosphospholipid anchors, and O-linked N-acetylglucosamine (GlcNAc-O-Ser). These structures are depicted in Figure 17.0.1. Other less common forms of glycosylation may need to be considered, especially if prior literature suggests their existence in a given situation. Examples of rarer sugar chains include (1) O-linked glucose, mannose, and fucose; (2) N-linked glucose; (3) glucosyl-hydroxylysine; and (4) GlcNAc-P-Ser (phosphoglycosylation).

Likewise, this chapter deals only with the most common forms of shared outer sequences (e.g., sialylated and fucosylated lactosamines, poly-lactosamines, O-glycosaminoglycan chains, and blood group sequences), and does not deal with rarer sequences (e.g., bisecting xylose residues, N-linked glycosaminoglycans, and β-linked GalNAc residues on N-linked glycans). For further information, the reader is directed to the Key References section at the end of this chapter introduction.

**CHOICE OF TECHNIQUES**

For the novice experimenter in glycoconjugate analysis, the greatest difficulty is in deciding which protocols are applicable to the question at hand, are sensitive enough to yield results, and are most likely to give useful answers. The following tables are therefore provided as a general guide to glycoconjugate analysis. Suggestions are made for the protocols that are most likely to be useful based upon the questions being asked (Table 17.0.1), the amount of material that is available for analysis (Table 17.0.2), and the type of glycoconjugate that is being studied (Table 17.0.3). The user is advised to select protocols based on the information in these tables and then to consult the commentary section of the selected protocols for further information.

**Table 17.0.1 Protocol Choice for Glycoconjugate Analysis Based on Question Being Asked**

<table>
<thead>
<tr>
<th>Question</th>
<th>Suggested protocols (unit no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Is there anything special about purifying my glycoconjugate?</td>
<td>17.1, 17.2, 17.3</td>
</tr>
<tr>
<td>Is my protein glycosylated?</td>
<td>17.1, 17.2, 17.4, 17.5, 17.6, 17.7, 17.8, 17.9, 17.10, 17.12, 17.13, 17.17, 17.18</td>
</tr>
<tr>
<td>How much of my glycoconjugate consists of sugar chains?</td>
<td>17.9, 17.10, 17.12, 17.13, 17.17, 17.18</td>
</tr>
<tr>
<td>What monosaccharides are in my glycoconjugate, and in what ratio?</td>
<td>17.4, 17.9, 17.12, 17.16, 17.18, 17.19</td>
</tr>
<tr>
<td>How many glycosylation sites are there on my protein?</td>
<td>17.10, 17.13, 17.14B</td>
</tr>
<tr>
<td>Can I specifically label the sugar chains on my glycoconjugate?</td>
<td>17.4, 17.5, 17.6</td>
</tr>
<tr>
<td>Does my antibody recognize sugar chains on the glycoconjugate?</td>
<td>17.7, 17.8, 17.12, 17.13, 17.17</td>
</tr>
<tr>
<td>Can I selectively release the sugar chains from my glycoconjugate?</td>
<td>17.8, 17.13, 17.15, 17.17</td>
</tr>
<tr>
<td>Does my protein have a glycosphospholipid anchor?</td>
<td>17.4, 17.8</td>
</tr>
<tr>
<td>What type of glycosphingolipids does my cell have?</td>
<td>17.3, 17.4, 17.7</td>
</tr>
<tr>
<td>Are there glycosaminoglycan chains on my protein?</td>
<td>17.2, 17.13, 17.17, 17.22</td>
</tr>
<tr>
<td>Can I alter glycosylation during biosynthesis?</td>
<td>17.10, 17.11</td>
</tr>
<tr>
<td>Can I alter glycosylation on the surface of intact cells?</td>
<td>17.6, 17.10, 17.12, 17.13, 17.17</td>
</tr>
<tr>
<td>Can I release and isolate intact or fragmented oligosaccharides from my glycoconjugate?</td>
<td>17.8, 17.12, 17.13, 17.14, 17.15, 17.17</td>
</tr>
<tr>
<td>What are the basic structural characteristics of the released oligosaccharides?</td>
<td>17.5, 17.6, 17.12, 17.13, 17.20, 17.21, 17.22</td>
</tr>
</tbody>
</table>

17.0.3
Table 17.0.2  Protocol Choice Based on Amount of Glycoconjugate Available

<table>
<thead>
<tr>
<th>Unit no.</th>
<th>&gt;100 nmol</th>
<th>10–100 nmol</th>
<th>1–10 nmol</th>
<th>1–0.1 nmol</th>
<th>&lt;0.1 nmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>17.4</td>
<td>—</td>
<td>P*</td>
<td>U*</td>
<td>U*</td>
<td>U*</td>
</tr>
<tr>
<td>17.5</td>
<td>U</td>
<td>U</td>
<td>U*</td>
<td>U*</td>
<td>U*</td>
</tr>
<tr>
<td>17.6</td>
<td>P</td>
<td>U*</td>
<td>U*</td>
<td>U*</td>
<td>U*</td>
</tr>
<tr>
<td>17.7</td>
<td>—</td>
<td>—</td>
<td>U</td>
<td>U</td>
<td>U</td>
</tr>
<tr>
<td>17.8</td>
<td>—</td>
<td>P</td>
<td>U</td>
<td>U</td>
<td>U*</td>
</tr>
<tr>
<td>17.9</td>
<td>U</td>
<td>U</td>
<td>P</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>17.10</td>
<td>P</td>
<td>U</td>
<td>U*</td>
<td>U*</td>
<td>—</td>
</tr>
<tr>
<td>17.11</td>
<td>P</td>
<td>U</td>
<td>U*</td>
<td>U*</td>
<td>—</td>
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<tr>
<td>17.12</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
</tr>
<tr>
<td>17.13</td>
<td>P</td>
<td>U</td>
<td>U</td>
<td>U*</td>
<td>U*</td>
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<td>17.14</td>
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<td>U</td>
<td>U*</td>
<td>U*</td>
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<td>U</td>
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<td>U*</td>
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<tr>
<td>17.16</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
</tr>
<tr>
<td>17.17</td>
<td>P</td>
<td>U</td>
<td>U</td>
<td>U*</td>
<td>U*</td>
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<tr>
<td>17.18</td>
<td>U</td>
<td>U</td>
<td>P</td>
<td>U</td>
<td>U</td>
</tr>
<tr>
<td>17.19A</td>
<td>—</td>
<td>—</td>
<td>U</td>
<td>U</td>
<td>P</td>
</tr>
<tr>
<td>17.19B</td>
<td>—</td>
<td>—</td>
<td>P*</td>
<td>U*</td>
<td>U*</td>
</tr>
<tr>
<td>17.20</td>
<td>—</td>
<td>U</td>
<td>U*</td>
<td>U*</td>
<td>U*</td>
</tr>
<tr>
<td>17.21</td>
<td>—</td>
<td>—</td>
<td>U</td>
<td>U*</td>
<td>U*</td>
</tr>
<tr>
<td>17.22A</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U*</td>
</tr>
<tr>
<td>17.22B</td>
<td>—</td>
<td>—</td>
<td>U</td>
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<tr>
<td>17.23</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U*</td>
</tr>
</tbody>
</table>

*aAbbreviations: P, possibly useful; U, useful; * radioactive samples.

*bThe average molecular weight of a monosaccharide unit of sugar chains is ~200. To start, assume the glycoconjugate contains 5 to 10 mol monosaccharide/mol glycoconjugate. Radioactive samples should have a specific activity from 0.1 to 20 Ci/mmol.

regarding practicality and applicability to a particular experimental situation. Choice of technique may also be dictated by whether the glycoconjugate to be studied is being monitored with a radioactive tracer or by direct chemical analysis, and by what fraction of a precious purified sample can be expended for oligosaccharide analysis.

The techniques in this chapter are not sufficiently inclusive to give final confirmation of the complete structure of an oligosaccharide. In such cases, additional studies of the glycoconjugate may be necessary using more advanced techniques. In some cases, if the sample is highly purified and available in large amounts, use of nondestructive methods such as NMR should be considered before committing the sample to destructive analysis. Alternatively, the information generated by the techniques described in this chapter may be sufficient to answer the biological question(s) at hand.

STEREOCHEMISTRY AND DIAGRAMMATIC REPRESENTATION OF MONOSACCHARIDES AND OLIGOSACCHARIDES

Basic Stereochemistry of Monosaccharides

In addition to this discussion, the reader is referred to Allen and Kisalius (1992) for a more detailed discussion of the principles of carbohydrate structure. The italicized terms in the discussion below are defined in the glossary at the end of this unit.
### Table 17.0.3  Protocol Choice Based on Type of Glycoconjugate Studied\(^a\)

<table>
<thead>
<tr>
<th>Unit no.</th>
<th>(N\text{-GlcNAc-linked glycoprotein})</th>
<th>(O\text{-GalNAc-linked glycoprotein})</th>
<th>(O\text{-Xylose-linked proteoglycan})</th>
<th>Glyco-sphingolipid</th>
<th>Glyco-phospholipid anchor</th>
<th>O-linked GlcNAc</th>
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<tbody>
<tr>
<td>17.4</td>
<td>(U)^b</td>
<td>(U)</td>
<td>(U)</td>
<td>(U)</td>
<td>(U)</td>
<td>(P)</td>
</tr>
<tr>
<td>17.5</td>
<td>(U)</td>
<td>(U)</td>
<td>(____)</td>
<td>(____)</td>
<td>(P)</td>
<td>____</td>
</tr>
<tr>
<td>17.6</td>
<td>(U)</td>
<td>(U)</td>
<td>(____)</td>
<td>(____)</td>
<td>(P)</td>
<td>(U)</td>
</tr>
<tr>
<td>17.7</td>
<td>(U)</td>
<td>(U)</td>
<td>(____)</td>
<td>(U)</td>
<td>(P)</td>
<td>(U)</td>
</tr>
<tr>
<td>17.8</td>
<td>(____)</td>
<td>(____)</td>
<td>(____)</td>
<td>(____)</td>
<td>(____)</td>
<td>____</td>
</tr>
<tr>
<td>17.9</td>
<td>(U)</td>
<td>(U)</td>
<td>(____)</td>
<td>(P)</td>
<td>(P)</td>
<td>____</td>
</tr>
<tr>
<td>17.10</td>
<td>(U)</td>
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<td>(____)</td>
<td>(____)</td>
<td>(____)</td>
<td>____</td>
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<tr>
<td>17.11</td>
<td>(____)</td>
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<td>(U)</td>
<td>(____)</td>
<td>(____)</td>
<td>____</td>
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<td>17.12</td>
<td>(U)</td>
<td>(U)</td>
<td>(____)</td>
<td>(U)</td>
<td>(P)</td>
<td>____</td>
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<tr>
<td>17.13</td>
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<td>(____)</td>
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<td>17.14A</td>
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<td>____</td>
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<td>17.14B</td>
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<td>(P)</td>
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<td>(____)</td>
<td>(P)</td>
<td>____</td>
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<tr>
<td>17.15</td>
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<td>17.16</td>
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<td>(U)</td>
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<td>(P)</td>
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<td>(U)</td>
<td>(U)</td>
<td>(P)</td>
<td>(P)</td>
<td>(P)</td>
<td>(P)</td>
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<tr>
<td>17.19A</td>
<td>(U)</td>
<td>(U)</td>
<td>(____)</td>
<td>(U)</td>
<td>(P)</td>
<td>(P)</td>
</tr>
<tr>
<td>17.19B</td>
<td>(____)</td>
<td>(____)</td>
<td>(____)</td>
<td>(____)</td>
<td>(____)</td>
<td>____</td>
</tr>
<tr>
<td>17.20</td>
<td>(U)</td>
<td>(P)</td>
<td>(____)</td>
<td>(____)</td>
<td>(____)</td>
<td>____</td>
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<td>17.21</td>
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<td>(P)</td>
<td>(____)</td>
<td>(____)</td>
<td>(____)</td>
<td>____</td>
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<td>17.22A</td>
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<td>(P)</td>
<td>(____)</td>
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<tr>
<td>17.22B</td>
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<tr>
<td>17.23</td>
<td>(U)</td>
<td>(U)</td>
<td>(P)</td>
<td>(____)</td>
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<td>____</td>
</tr>
</tbody>
</table>

\(^a\)This chapter deals only with the most common forms of glycosylation on glycoconjugates.

\(^b\)Abbreviations: \(P\), possibly useful; \(U\), useful.

Glyceraldehyde, the simplest monosaccharide, has a single chiral (asymmetric) carbon (C-2). Therefore, it is a chiral molecule that shows optical isomerism; it can exist in the form of two nonsuperimposable mirror images called **enantiomers** (see Fig. 17.0.2). These enantiomers have identical physical properties, except for the direction of rotation of the plane of polarized light (\(-\), left hand; \(+\), right hand). Historically, the \((+)-glyceraldehyde\) was arbitrarily assigned the prefix \(D\) (for dextrorotatory), and the \((-)-glyceraldehyde\), the prefix \(L\) (for levorotatory). The pair of enantiomers also have identical chemical properties, except toward optically active reagents. This fact is particularly important in biological systems, because most enzymes and the compounds they work on are optically active.

The configuration of the highest-numbered asymmetric carbon atom in the carbon chain of a higher monosaccharide is determined by comparison with the configuration of the chiral (or asymmetric) carbon of glyceraldehyde. Thus, a prefix \(D\)- is added to the name of each monosaccharide having the configuration of \(D\)-glyceraldehyde at the highest-numbered asymmetric carbon, and the prefix \(L\)- to those having the configuration of \(L\)-glyceraldehyde at the highest-numbered asymmetric carbon. The direction in which the compound rotates the plane of polarized light needs to be determined experimentally, and is indicated...
Figure 17.0.2 Enantiomers of glyceraldehyde.

D–(+)–glyceraldehyde

L–(−)–glyceraldehyde

Figure 17.0.3 Formation of cyclic structures: open and ring forms of galactose.

by a − or + sign, in parentheses, immediately after the prefix D or L [e.g., D–(+)-glucose]. In biological systems, where stereochemical specificity is the rule, D molecules may be completely active and L molecules completely inert, or vice versa. All known naturally occurring monosaccharides in animal cells are in the D configuration, except for fucose and iduronic acid, which are in the L configuration.

Each aldose sugar is usually present in a cyclic structure, because the hemiacetal produced by reaction of the aldehyde group at C-1 with the hydroxyl group at C-5 gives a six-membered ring, called a pyranose (see Fig. 17.0.3). When five-membered rings are formed by reaction of the C-1 aldehyde with the C-4 hydroxyl, they are called furanoses. Hexoses commonly form pyranosidic rings, and pentoses form furanosidic rings, although the reverse is possible. Similarly, ketoses (e.g., sialic acids) can form hemiketals by reaction of the keto group at C-2 with the hydroxyl group at C-6.

These ring forms are the rule in intact oligosaccharides. The formation of the ring produces an additional chiral center at C-1 (or C-2 for keto sugars). Thus, two additional isomers are possible, which are called anomers. These anomers are designated α and β. Because glycosidic linkages (see above) occur via anomic centers, they are called α linkages and β linkages.
Diagrammatic Representations of Monosaccharides

Monosaccharides are conventionally presented using the Fisher projection or the Haworth representation. Some examples are shown in Figure 17.0.4.

The Fisher projection

The carbon chain of a monosaccharide is written vertically with carbon atom number 1 at the top. The numbering of carbon atoms follows the rules of organic chemistry, so the aldehyde carbon is C-1. The horizontal lines represent bonds projecting out from the plane of the paper, whereas the vertical lines represent bonds projecting behind the plane of the paper. When the hydroxyl group at the highest-numbered asymmetric carbon is on the right, the monosaccharide belongs to the D series. When the hydroxyl group at the highest-numbered asymmetric carbon is on the left, the monosaccharide belongs to the L series.

The Haworth representation

The six-membered ring is represented with the oxygen at the upper right corner, approximately perpendicular to the plane of the paper, and with the groups attached to the carbons
above or below the ring. All groups that appear to the right in a Fisher projection appear below the plane of the ring in a Haworth representation. The $\alpha$-series hexoses have the carboxymethyl group (C-6) above the ring. The reverse is true for the $\beta$ series. Therefore, the only difference between the $\alpha$ and $\beta$ anomers of a given hexose would be the relative position of the hydroxyl and the hydrogen at C-1. In the $\alpha$ anomer, the hydrogen is below the ring for the D-series (above the ring for the L-series); in the $\beta$ anomer, the hydrogen is above the ring for the D-series (below it for the L-series).

Conformation of Monosaccharides in Solution

The Haworth representation does not depict the real conformation of these molecules. The preferred conformation in solution of the six-membered ring monosaccharides is what is known as a chair conformation. In this conformation, each different group can adopt either an equatorial or an axial position.

There are two possible chair conformations called conformers that exist in equilibrium. The position of this equilibrium differs from one monosaccharide to another depending on the relative positions of hydroxyl groups or other substituents. The preferred conformation is that with the lowest number of bulky groups in an axial position (Fig. 17.0.5).

Formulas for Representation of Oligosaccharide Chains

Full and correct representation of the formula of an oligosaccharide chain requires notation describing the complete stereochemistry of the component monosaccharides. Thus, the simple tetrasaccharide commonly called sialyl-Lewisx (attached to an underlying oligosaccharide, R) would be written as follows:

$$\alpha$-D-Neu5Ac-2-3$\beta$-D-Galp1-4(\alpha$-L$-Fucp1-3)$-\beta$-D-GlcNAc-R$

In more common practice, the D and L configurations and the nature of ring structures are assumed, and the formula is written in one of the ways shown in Figure 17.0.6. There is also increasing use of a simplified symbol set to conveniently represent the common monosaccharides of vertebrate glycans (Fig. 17.0.7).

Higher-Order Structures in Glycans

The presence of monosaccharides with different types of glycosidic linkages in oligo- and polysaccharides creates further complexity, causing the development of secondary and tertiary structures in these molecules. The approximate shape adopted in solution by a given carbohydrate chain can be predicted according to the type of glycosidic linkages involved. These higher-order structures may be critical for the biological roles of glycoconjugates.

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**Figure 17.0.5** D-Mannose chair conformation.

**Figure 17.0.6** Two common short forms for denoting glycan structures. The tetrasaccharide known as sialyl-Lewisx, is shown attached to an underlying oligosaccharide, R.
GLOSSARY

The following definitions are presented for the terms most commonly used throughout this chapter.

**aldose:** monosaccharide with a carbonyl group at the end of the carbon chain (aldehyde group); the carbonyl group is assigned the lowest possible number (i.e., carbon 1).

**carbohydrates:** polyhydroxyaldehydes or polyhydroxyketones, or compounds that can be hydrolyzed to them.

**diastereoisomers:** compounds with identical formulas that have a different spatial distribution of atoms (e.g., galactose and mannose).

**enantiomers:** nonsuperimposable mirror images of any compound (e.g., D- and L-glucose).

**epimers:** two monosaccharides differing only in the configuration of a single chiral carbon.

**Fuc (L-fucose):** type of deoxyhexose (see also types of monosaccharides below).

**Gal (D-galactose):** type of hexose (see also types of monosaccharides below).

**GalNAc (N-acetyl-D-galactosamine):** type of hexosamine (see also types of monosaccharides below).

**ganglioside:** anionic glycolipid containing one or more units of sialic acid.

**Glc (D-glucose):** type of hexose (see also types of monosaccharides below).

**GlcNAc (N-acetyl-D-glucosamine):** type of hexosamine (see also types of monosaccharides below).

**glyceraldehyde:** simplest monosaccharide (an aldotriose; i.e., containing three carbon atoms).

**glycoconjugate:** natural compound in which one or more monosaccharide or oligosaccharide units are covalently linked to a noncarbohydrate moiety.

**GIUA or GIA (D-glucuronic acid):** type of uronic acid (see also types of monosaccharides below).

**glycolipid or glycosphingolipid:** glycan attached via glucose or galactose to the terminal primary hydroxyl group of ceramide, which is composed of a long-chain base (i.e., sphingosine) and a fatty acid. Glycolipids can be neutral or anionic (negatively charged).

**glycosphingolipid anchor:** glycan bridge between phosphatidylinositol and a phosphoethanolamine in amide linkage to the C terminus of a protein; constitutes the sole membrane anchor for such proteins.

**glycoprotein:** glycoconjugate in which a protein carries one or more glycan chains covalently attached to a polypeptide backbone via N-GlcNAc- or O-GalNAc-linkages.

**glycosaminoglycan:** linear copolymers of disaccharide repeating units, each composed of a hexosamine and a hexose or hexuronic acid; these are the glycan chains that define the proteoglycans. The type of disaccharide unit can define the glycosaminoglycans as chondroitin or dermatan sulfate, heparan or heparin sulfate, hyaluronic acid, and keratan sulfate. The glycosaminoglycans (except hyaluronic acid) also contain sulfate esters substituting either hydroxyl or amino groups (N- or O-sulfate groups).

**glycosidic linkage:** linkage of a monosaccharide to another residue via the anomic hydroxyl group.

**IdUA or IdA (L-iduronic acid):** type of uronic acid (see also types of monosaccharides below).

**ketose:** monosaccharide with a carbonyl group in an inner carbon; the carbonyl group is assigned the lowest possible number (i.e., carbon 2).

**Man (D-mannose):** type of hexose (see also types of monosaccharides below).

**monosaccharide:** carbohydrate that cannot be hydrolyzed into simpler units (see also types of monosaccharides below).

**mucin:** large glycoproteins that contain many (up to several hundred) O-GalNAc-linked glycan chains that are often closely spaced.

**N-linked oligosaccharide:** glycan covalently linked to an asparagine residue of a polypeptide chain in the consensus sequence -Asn-X-Ser/Thr. Although there are many different kinds of N-linked glycans, they have certain common features: (1) a common core pentasaccharide containing two mannosyl residues α-linked to a third mannosyl unit, which is in turn β-linked to a chitobiosyl group and (2) a chitobiosyl group that is β-linked to the asparagine amide nitrogen. The N-linked glycans can be divided into three main classes: high-mannose type, complex type, and hybrid type.

**Neu5Ac (N-acetyl-D-neuraminic acid):** type of sialic acid (Sia; see also types of monosaccharides below).

**O-linked oligosaccharide:** glycan linked to the polypeptide via N-acetylgalactosamine (GalNAc) to serine or threonine. Note that other types of O-linked glycans also exist (e.g., O-GlcNAc) but that the O-GalNAc linkage, being the most well known, is often described by this generic term.
**oligosaccharide:** branched or linear chain of monosaccharides attached to one another via glycosidic linkages (see also polysaccharides below).

**polylactosaminoglycan:** long chain of repeating units of the disaccharide β-Gal(1-4)-GlcNAc. These chains may be modified by sialylation, fucosylation, or branching. When sulfated, they are called keratan sulfate (see glycosaminoglycans above).

**polysaccharides:** branched or linear chain of monosaccharides attached to each other via glycosidic linkages that usually contain repetitive sequences. The number of monosaccharide residues that represents the limit between an oligosaccharide and a polysaccharide is not defined. A tetrafucosylated, sialylated, tetraantennary carbohydrate moiety containing eighteen monosaccharide units, present in a glycoprotein, is considered an oligosaccharide. On the other hand, a carbohydrate composed of eighteen glucose residues linked β-(1-4) present in a plant extract is considered a polysaccharide.

**polysialic acid:** homopolymer of sialic acid selectively expressed on a few vertebrate proteins and on the capsular polysaccharides of certain pathogenic bacteria.

**proteoglycan:** glycoconjugate having one or more O-xylose-linked glycosaminoglycan chains (rather than N-GlcNAc- or O-GalNAc-linked glycans) linked to protein. The distinction from a glycoprotein is otherwise arbitrary, because some proteoglycans can have both glycosaminoglycan chains and N- or O-linked oligosaccharides attached to them.

**reducing sugar:** sugar that undergoes typical reactions of aldehydes (e.g., is able to reduce Ag⁺ or Cu⁺ + ). Mono-, oligo-, or polysaccharides can be reducing sugars when the aldehyde group in the terminal monosaccharide residue is not involved in a glycosidic linkage.

**saccharide modifications:** hydroxyl groups of different monosaccharides can be subject to phosphorylation, acetylation, sulfation, methylation, or fatty acylation. Amino groups can be free, N-acetylated, or N-sulfated. Carboxyl groups are occasionally subject to lactonization to nearby hydroxyl groups.

**Sia (sialic acid):** generic name for a family of acidic nine-carbon monosaccharides (e.g., N-acetyllneuraminic acid, Neu5Ac; and N-glycolylneuraminic acid, Neu5Gc).

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**Figure 17.0.7 (appears on following page)** Recommended symbols and conventions for drawing glycan structures. (A) Each monosaccharide class (e.g., hexose) has the same shape, and isomers are differentiated by color/black/white/shading. The same shading/color is used for different monosaccharides of the same stereoregional designation, e.g., Gal, GalNAc, and GaIA. To minimize variations, sialic acids and uronic acids are in the same shape, and only the major uronic and sialic acid types are represented. When the type of sialic acid is uncertain, the abbreviation Sia can be used instead. Only common monosaccharides in vertebrate systems are assigned specific symbols. All other monosaccharides are represented by an open hexagon or defined in the figure legend. If there is more than one type of undesignated monosaccharide in a figure, a letter designation can be included to differentiate between them. Unless otherwise indicated, all of these vertebrate monosaccharides are assumed to be in the D configuration (except for fucose and iduronic acid, which are in the L configuration), all glycosidically linked monosaccharides are assumed to be in the pyranose form, and all glycosidic linkages are assumed to originate from the 1-position (except for the sialic acids, which are linked from the 2-position). Anomeric notation and destination linkages can be indicated without spacing/dashes. Although color is useful, these representations will survive black-and-white printing or photocopying with the colors represented in different shades (the color values in the figure are the RGB color settings obtained within PowerPoint). Modifications of monosaccharides are indicated by lowercase letters, with numbers indicating linkage positions, if known (e.g., 9Ac for the 9-O-acetyl group, 3S for the 3-O-sulfate group, 6P for a 6-O-phosphate group, 8Me for the 8-O-methyl group, 9Acy for the 9-O-acyl group, and 9Lt for the 9-O-lactyl group). Esters and ethers are shown attached to the symbol with a number. For N-substituted groups, it is assumed that only one amino group is on the monosaccharide with an already known position (e.g., NS for an N-sulfate group on glucosamine, assumed to be at the 2-position). (B) Typical branched "biantennary" N-glycan with two types of outer termini, depicted at different levels of structural details. (C) Some typical glycosaminoglycan (GAG) chains (reproduced with permission from figure 1.5 in Varki et al., 2009). For the color version of this figure go to [http://www.currentprotocols.com/protocol/mb1700](http://www.currentprotocols.com/protocol/mb1700).
Figure 17.0.7 (legend appears on preceding page)
**types of monosaccharides:** monosaccharides may have a carbonyl group at the end of the carbon chain (aldehyde group) or in an inner carbon (ketone group). The carbonyl group is assigned the lowest possible number, e.g., carbon 1 (C-1) for the aldehyde group; carbon 2 (C-2) for the most common ketone groups. These two types are named aldoses and ketoses, accordingly. The simplest monosaccharide is glyceraldehyde (see Fig. 17.0.2), an aldohexose (i.e., containing three carbon atoms). Natural aldoses with different numbers of carbon atoms in their chain are named accordingly (e.g., aldohexoses, containing six carbon atoms). Two monosaccharides differing only in the configuration of a single chiral carbon are called epimers. For example, glucose and galactose are epimers of each other at C-4. The common monosaccharides present in animal glycoconjugates are (1) deoxyhexoses (e.g., L-Fuc); (2) hexosamines, usually N-acetylated (e.g., D-GalNAc and D-GlcNAc); (3) hexoses (e.g., D-Glc, D-Gal, and D-Man); (4) pentoses (e.g., D-Xyl); (5) sialic acids (Sia; e.g., Neu5Ac); and (6) uronic acids (e.g., D-Gla and L-IdA).

**Xyl (D-xylene):** type of pentose (see also types of monosaccharides above).

For further detailed information on the analysis of glycans, the reader is referred to the specific literature cited in the individual protocol units. In addition, the following sources can be used for general information, for details on specific methods, and for many additional methods that are not included in this chapter.

**LITERATURE CITED**


**KEY REFERENCES**


SPECIAL CONSIDERATIONS OF GLYCOCONJUGATES AND THEIR PURIFICATION

Because of their unique composition and structural characteristics, there are instances in which glycoconjugates can be studied in biological mixtures directly, without purification. However, as Arthur Kornberg has said, to answer a biological question the best course of action is usually to “purify, purify, purify.” Indeed, the complete elucidation of any biological system involving a glycoconjugate will require that it be purified for structural and functional characterization.

This section describes general principles for purifying three major classes of glycoconjugates—glycoproteins, glycolipids, and proteoglycans. These classes have been described in the chapter introduction and glossary. Each is a large and polydisperse family of molecules with widely ranging structural and functional characteristics. Needless to say, many of the classic principles used for purification of other macromolecules (e.g., size fractionation and selective precipitation) apply to each of these types of glycoconjugates; on the other hand, an individual situation may provide unique opportunities for selective purification of a particular molecule.

The units presented in this section take the middle road and outline the principles particularly applicable to the purification of each class of glycoconjugate, taking advantage of group-specific properties. Glycoproteins usually have sugar chains that can be recognized by specific plant lectins; thus, affinity chromatography using immobilized lectins is a powerful technique, when properly applied (UNIT 17.1). Proteoglycans are characterized by a high density of negative charge and frequently by unusual buoyant densities and sedimentation properties, which can be used to advantage in their purification (UNIT 17.2). Glycolipids as a group are more hydrophobic than the other glycoconjugates, yet have amphipathic properties that allow them to be purified away from other lipids (UNIT 17.3).

Selective purification of these groups of molecules facilitates their further fractionation and final characterization. However, it is important to realize that exceptions can be encountered (e.g., glycoproteins with unusually high negative charges or glycolipids with extremely hydrophilic properties). Also, the phenomenon of microheterogeneity in oligosaccharide structure can cause even a single glycoconjugate to manifest a range of properties. For example, if a single glycoprotein from a single cell type has heterogeneity in N-linked oligosaccharide processing, some (but not all) of the protein may be recovered during affinity chromatography on wheat germ agglutinin (WGA)-Sepharose (UNIT 17.1).

At the present time there is no generalized purification scheme that will provide good yields of all of the major classes of glycoconjugates from a single starting sample. For example, the organic extractions required for glycolipids (UNIT 17.3) irreversibly denature most glycoproteins and proteoglycans. Conversely, the glycolipids are usually irretrivable from the detergent extractions used for membrane-bound glycoproteins (UNIT 17.1). Thus, if there is a need to examine all of the major glycoconjugates in a given cell or tissue, it is advisable to purify the major classes of molecules in parallel with one another, using separate aliquots of the same batch of starting material.

Even when studying a specific molecule, it is usually worthwhile to carry out a group-specific purification as a first step—e.g., to extract total cellular lipids before purifying away a specific disialoganglioside (UNIT 17.3). If the glycoconjugate in question has been radiolabeled (see UNIT 17.4), one also has the advantage that purification to “radiometric homogeneity” is sufficient for many types of analyses. Thus, if a glycoprotein has been

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metabolically labeled with [2-3H]mannose and the intent is to study the N-linked oligosaccharides, it may not matter that various unlabeled proteins or lipids are contaminating the final preparation. In the final analysis, the broad principles outlined in UNITS 17.1-17.3 must be adapted by the investigator to the situation at hand.

**UNIT 17.1**

**Special Considerations for Glycoproteins and Their Purification**

Glycoproteins contain a variety of different sugar chains. Some glycoproteins have many large and highly charged chains while others have only a single neutral sugar residue that is easily overlooked. This enormous diversity makes it impossible to recommend any single procedure for purifying glycoproteins. Most procedures used to purify nonglycosylated proteins are equally useful for glycoproteins. However, the presence of large amounts of carbohydrate on glycoproteins often endows them with higher charge, increased density, larger apparent size, and greater solubility than nonglycosylated proteins. Decades ago, these physical properties were cleverly exploited to purify and characterize many of the most abundant glycoproteins (Gibbons, 1972). More recently, greater knowledge of carbohydrate structure and biosynthesis has led to development of techniques that rely on more subtle and specific aspects of carbohydrate structure. Some of these approaches can also be used to detect a single sugar on a few picomoles of protein (see UNIT 17.7).

This unit begins by describing some properties of glycoproteins—e.g., subcellular location and solubility—that may be useful in determining which purification techniques to try. This discussion is followed by two protocols describing preparative glycoprotein purification using lectin-affinity chromatography, as well as an outline for a small-scale pilot procedure designed to check lectin binding and elution conditions. Lectins are often used for purifying glycoproteins because, in contrast to conventional purification procedures (e.g., gel filtration and ion-exchange chromatography; UNITS 10.9 & 10.10) that exploit general physical properties of glycoproteins, lectins recognize specific three-dimensional structures created by a cluster of sugar residues. Conventional purification procedures are generally tried before applying lectin-affinity chromatography.

**SPECIAL CONSIDERATIONS IN THE PURIFICATION OF GLYCOPROTEINS**

**Subcellular Location of Glycosylated Proteins**

Most protein glycosylation occurs in the lumen of the endoplasmic reticulum and the Golgi apparatus, but certain types of glycosylation also occur in the cytoplasm (see UNIT 17.13). Secreted proteins, membrane proteins, and proteins that enter vesicles such as lysosomes are good candidates for having some type of carbohydrate component. The most common types of sugar chains are found in N- and O-linkages to proteins and as components of glycosphospholipid anchors. Each of these types of glycosylation can occur individually or in any combination. The physical properties they impart can be effectively used for some purifications. Knowledge of the type of glycosylation is thus necessary in order to determine the optimal purification method. The methods outlined below may be helpful for determining glycosylation type.

**Secreted glycoproteins.** It is relatively simple to determine if the protein is secreted by analyzing conditioned medium used to culture the cells. This may require dialysis and concentration of the conditioned medium. If the cells must be grown in the presence of added serum components during the time when the protein is secreted, it may be very difficult to use any specific glycoprotein purification approach to enrich for your protein because competing serum glycoproteins will probably be in great excess. Thus it is important to determine if the target molecule can be secreted into serum-free medium.

**Cytoplasmic glycoproteins.** If the protein is not secreted, cells can be ruptured in an iso-osmotic medium (e.g., sucrose) and centrifuged to separate insoluble membranes and vesicles.
from soluble cytoplasmic components (UNIT 17.6). Glycoproteins are not commonly found in the soluble fraction. If the protein of interest is detected in the soluble fraction and is later found to be glycosylated, it may have been proteolyzed from a membrane. This is the most likely explanation for N-linked glycoproteins that are found in the soluble fraction. It is also possible that the protein is one of the recently described cytoplasmic or nuclear glycoproteins that contain O-linked N-acetylgalactosamine (GlcNAc), in which case it may bind to wheat germ agglutinin (WGA, from *Triticum vulgaris*; alternate protocol).

**Organelle glycoproteins.** If the protein of interest is not present in the soluble cytoplasmic fraction, the membrane and vesicle pellet is then resuspended in the presence of protease inhibitors and briefly exposed to a hypo-osmotic medium (or frozen and thawed) to lyse sensitive organelles and release their soluble contents. Membranes are again sedimented by centrifugation. Glycoprotein solubilized by this procedure will be accompanied by lysosomal enzymes. These proteases are potentially troublesome, but protease inhibitors will lessen or eliminate their effect. Various exo- and endoglycosidases may also be released by this procedure, but they are usually present at such a low concentration that they do not degrade the sugar chains on other glycoproteins.

**Membrane-bound and glycosphospholipid-anchored proteins.** If the protein of interest is not solubilized by any of the above procedures, it is probably a peripheral or integral membrane protein, or it is anchored to glycosphospholipid. Different nonionic detergents or increasing concentrations of a single detergent can be used to solubilize these proteins. Detergents that have high critical micelle concentrations (CMC) and can be easily dialyzed are preferred, e.g., hexyl-, heptyl-, and octyl-glucoisides or glucamide detergents (MEGA-8, Calbiochem). However, a glucose-based detergent such as octyl-glucoside may block protein binding on some lectin columns such as Concanavalin A (Con A)–Sepharose, or may produce a very high background in direct sugar analysis (UNIT 17.9) even after extensive dialysis. If the protein is glycosphospholipid-anchored, the lipid component will cause it to partition into the detergent phase of a Triton X-114/water two-phase system (UNIT 17.8) and to interact strongly with hydrophobic matrices such as phenyl-Sepharose. In some instances, the lipid portion of the anchor may be removed by digestion with phosphatidylinositol-specific phospholipase C (UNIT 17.8). This cleavage leaves the carbohydrate portion of the anchor associated with the protein and converts the lipophilic protein into a soluble, more hydrophilic protein. Phospholipase digestion reduces the protein’s affinity for hydrophobic matrices and results in its extraction into the aqueous phase of Triton X-114/water mixture (UNIT 17.8).

**Solubility Properties**

The solubility properties of a glycoprotein with a small amount of carbohydrate (~5%) will not be influenced much by the sugar component. If the carbohydrate is part of a glycosphospholipid anchor, detergent solubilization may be required. Standard ammonium sulfate or ethanol precipitation (UNIT 10.9) works well for many glycoproteins, but those with high sugar content may remain soluble, even in relatively high concentrations of ethanol. Very anionic proteins with abundant sialic acid or sulfate esters may be resistant to precipitation with trichloroacetic acid or perchloric acid (APPENDIX 2). This unusual solubility is sometimes exploited as a purification step because most other proteins will be precipitated under these conditions. However, a combination of 10% trichloroacetic acid and 2% phosphotungstic acid will precipitate even highly charged proteins. Prolonged exposure to low pH may cause partial loss of sialic acid and deamination of Asn and Gln, resulting in unwanted protein microheterogeneity. Extremes of pH should be avoided because of the possibility of hydrolysis of labile groups such as phosphodiesters or acetyl esters.

Nonglycosylated proteins band in cesium chloride gradients at ~1.3 g/ml and polysaccharides at ~1.6 to 2.0 g/ml. This substantial difference in density can be used to separate very highly glycosylated proteins from those containing lesser amounts of carbohydrate. This treatment may permanently denature or inactivate some proteins, and is useful only if the protein is stable, is active in the presence of CsCl₂, or renatures once the CsCl₂ is removed.

**Chromatographic and Staining Properties**

**Gel-filtration columns and SDS-PAGE.** Proteins with high carbohydrate content (>20 to 30%) elute much earlier on gel-filtration columns (UNIT 10.9) than nonglycosylated proteins of similar size. If the amount of carbohydrate is variable, it may broaden the peak as well. Migration of proteins in SDS-PAGE (UNIT 10.2) is also affected by the extent of glycosylation. A protein with multiple anionic sugar
chains, each with a variable number of sialic acids or sulfate esters, can broaden a single protein band into a smear on gels. Closely clustered, short, O-linked sugar chains can appear to contribute as much as ten times their actual mass to the apparent molecular weight of a protein.

Very anionic glycoproteins may not stain with the normal Coomassie brilliant blue or silver staining procedures \( \text{UNIT 10.6} \). Some glycoproteins may even be seen as negative bands against the brownish background of silver-stained gels. Other stains, such as the Coomassie-based ProBlue stain (Integrated Separation Systems) or the cationic dyes toluidine blue and alcian blue, are often used for staining proteoglycans and other highly anionic proteins.

**Ion-exchange and isoelectric focusing chromatography.** The presence of sialic acids and sulfate esters makes some glycoproteins bind well to ion-exchange columns (e.g., DEAE-Sephadex, DEAE-Sephacel; \( \text{UNIT 10.10} \)). High-salt solutions elute these glycoproteins well after most other proteins have been eluted. The variable number of negative charges can also give multiple separate or very broad and poorly resolved protein peaks (\( \text{UNITS 10.5 & 10.10} \)). Multiple peaks or broad bands are also encountered in isoelectric focusing gels. Sialidase or endo-glycosidase digestions can sometimes simplify these patterns by removing negative charges.

In the case of “mucins” (highly charged glycoproteins with closely clustered or extended O-linked GalNAc chains) or proteoglycans, ion-exchange columns may need to be run in 6 M urea to prevent protein aggregation.

**Preparation of Protein Samples for Carbohydrate Analysis**

**Carbohydrate contamination.** A highly purified protein that gives only a single band by SDS-PAGE analysis may still be unsuitable for carbohydrate analysis. Glucose is only very rarely found on sugar chains of proteins. On the other hand, it is almost always a readily available contaminant. DEAE- and CM-cellulose, agarose, Sephadex, Kimwipes, sucrose, glycerol, starch powder used on latex gloves, and glucoside-based detergents are all carbohydrates and can make a relatively large contribution to the sugar content of a sample, depending upon exactly how the material is analyzed. If proteins are purified on sucrose gradients, extracted with glucose-based detergents, or normally stored in glycerol, these sugar contaminants must first be completely removed by dialysis. Acrylamide-based gels (e.g., Bio-Gel series) are preferred over the dextran-type gels (Sephadex) for gel-filtration purification because the latter can contribute fragments of beads as soluble but nondialyzable material \( \text{UNIT 10.9} \). As a final precaution, protein-containing solutions should be filtered through a 0.2-µm filter to remove any particles that may have come from ion-exchange or affinity columns. If glucose is found by compositional analysis even after taking these precautions, it is most likely to be a contaminant and not a component of the glycoprotein.

**Lectin-affinity chromatography.** The most frequently used specific purification procedure for glycoproteins is lectin-affinity chromatography. Dozens of plant lectins have been identified and in many cases their sugar specificity is known. This sugar specificity is the basis for the separation and structural analysis of many individual oligosaccharides and glycopeptides described in this chapter, and for the identification of lectin-binding glycoproteins \( \text{UNIT 17.7} \). Glycoproteins often contain multiple chains of a given sugar type and multiple interactions with the immobilized lectin may make it difficult to elute some of the proteins from the lectin column. The presence of multiple low-affinity sugar chains can still give substantial interaction with a lectin-containing column even though the individual sugar chains may themselves have a low affinity. Thus, one should be careful not to interpret too much about sugar chain structure from a protein’s binding to a lectin column. This by no means belittles their usefulness for glycoprotein purification; lectin-affinity chromatography may be used to partially purify glycoproteins and to provide rather limited qualitative information about the nature of their carbohydrate components. Other preparative purification steps, such as ammonium salt precipitation \( \text{UNIT 11.3} \) and gel-filtration and ion-exchange chromatographies \( \text{UNITS 10.9 & 10.10} \), are usually done prior to using lectins, but this is not necessary.

Many different lectins are commercially available in an immobilized form suitable for glycoprotein purification, but some can be rather expensive. Con A—Sepharose is the most commonly used lectin for glycoprotein purification. It is relatively inexpensive, it is stable, and it can bind to many different glycans. Bound proteins can be eluted with α-methyl-D-mannoside (αMM). WGA is the next most popular lectin for the same reasons although it is somewhat more expensive.
Other notable lectins used to purify glycoproteins include Ricinus communis lectin (RCA I) for proteins that carry Gal-terminated sugar chains, and pea or lentil lectins for those N-linked oligosaccharides that have a fucose residue in the chitobiosyl core region. The utility of these lectins for characterizing glycopeptides is described in UNIT 17.7. Limited availability, higher price, limited track records, and, in some cases, the requirement for exotic sugars to elute bound proteins make some of these lectins less appropriate for routine use in glycoprotein purification. However, the principles of Con A–Sepharose and WGA-agarose lectin-affinity chromatography should apply to these other lectins as well. A broad range of immobilized lectins is available from E-Y Laboratories, Pharmacia Biotech, Vector Labs, and Sigma (APPENDIX 4).

**Examples of lectin-affinity chromatography purifications.** The basic and alternate protocols presented below describe the use of lectins for preparative glycoprotein purification. Con A–Sepharose and WGA-agarose were chosen for convenience and availability. The support protocol describes a small-scale pilot procedure to test for lectin binding and to determine elution conditions. There are many variations on the basic procedure in the literature, but all use the same principles: bind the protein to immobilized lectin through its sugar chain, wash away unbound protein, and elute bound protein with a simple sugar that resembles the sugar ligand of the bound protein. Because many proteins have sugar chains that can bind to a specific lectin, this procedure seldom yields a pure protein.

**CON A–SEPHAROSE AFFINITY CHROMATOGRAPHY**

Con A–Sepharose chromatography is used to partially purify glycoproteins that contain terminal mannose or glucose residues; the steps presented below are typical conditions for this type of chromatography, although a variety of approaches have been utilized. In this protocol, bound glycoproteins are eluted with α-methyl-D-mannoside (αMM) after the column is first washed to remove unbound and weakly bound proteins. Before proceeding, it is advisable to conduct a pilot study to test the protein of interest for lectin binding and elution conditions (see support protocol).

**Materials**

- 10 mg/ml Con A–Sepharose (Pharmacia Biotech or Sigma)
- Column buffer
- 0.5 M αMM in column buffer
- Protein sample in column buffer
- Glass wool
- 1.5 × 30–cm glass or disposable chromatographic column

**NOTE:** This procedure should be carried out at room temperature if the protein to be isolated will tolerate this condition. If not, carry it out in a cold room, and prechill all solutions to maintain temperature.

1. Gently resuspend 50 ml settled Con A–Sepharose (10 mg lectin/ml packed resin) in 50 ml column buffer to make a slurry. Degas the slurry.

   *This volume of lectin beads should be sufficient to bind ~100 mg glycoproteins. If a 1.5 × 30–cm column is not available, either the dimensions of the column or the total amount of resin can be modified. A 1.0 × 30–cm column will bind ~50 mg total glycoprotein. If the amount of glycoprotein in the sample is considerably less than 50 to 100 mg, decrease the volume of the column accordingly. The ratio of input protein to lectin does not seem to matter as long as the column is not overloaded.*
2. Pack a glass wool plug over a scinterned glass or polypropylene frit at the bottom of the column and pour the degassed slurry into the column.

   The glass wool is important to prevent the beads from clogging the frit and slowing the flow rate.

3. Continue packing the column until the desired level is reached; for a 50-ml volume this is \( \sim 28 \) cm. Wash the gel with 2 to 3 column volumes of column buffer to remove any loosely bound or degraded Con A.

4. Wash with 2 to 3 column volumes of 0.5 M of \( \alpha \)MM in column buffer or the highest concentration of \( \alpha \)MM that will be used.

5. Wash the column with >5 column volumes of column buffer without \( \alpha \)MM to reequilibrate.

   It is important to prewash the column with the eluting sugar and then to reequilibrate the column to remove any materials which might have previously bound to the column. Check the completeness of washing by the phenol-sulfuric acid assay (UNIT 17.9). Estimate the amount of residual sugar using \( \alpha \)MM as a standard. An acceptable level is <0.1 mM or \( \sim 20 \mu g/ml \).

6. Slowly load the protein sample on the column to permit binding without disturbing the surface. A flow rate of \( \sim 1 \) ml/min (\( \sim 0.5 \) ml/min per cm\(^2\) of area) is desirable.

7. Wash column with column buffer and monitor the flowthrough and subsequent wash fractions by measuring the \( A_{280} \) until it approaches baseline value.

   If the \( A_{280} \) does not rapidly return to baseline, it may indicate weak interaction of some proteins with the lectin, or overloading of the column.

8. Assay flowthrough and wash fractions for the presence of the protein of interest.

   To check the lectin-binding properties, see support protocol below. To determine whether the column was overloaded, add a small amount of the flowthrough (1%) to a small volume of beads as described in the support protocol. If the sample is bound to the beads as determined by the appropriate assay, overloading of the original column is indicated. In this case, reapply the flowthrough with excess glycoprotein to the larger column after the bound proteins of the first run are eluted with \( \alpha \)MM and the column is reequilibrated.

9. Elute the column with 0.5 M \( \alpha \)MM in column buffer and monitor fractions for \( A_{280} \) and activity. Pool peak-activity fractions.

   Very broad peaks may result during elution with the sugar if the protein dissociates very slowly. When this happens, sample recovery can be improved by filling the column with 0.5 M \( \alpha \)MM in column buffer and allowing the column to stand for a few hours. This allows dissociation of the bound material; when the flow is started again, it should give a very sharp peak. Other possible remedies include warming a cold column to room temperature in the presence of \( \alpha \)MM, increasing the concentration of \( \alpha \)MM to 1 M, or increasing the NaCl concentration to 1 M. A combination of these may be needed to elute a tightly bound protein.

10. Regenerate the column by washing it with 10 vol column buffer or until the \( \alpha \)MM concentration is <20 \( \mu g/ml \).

   Column may now be used for another run or stored indefinitely at 4°C in column buffer containing 0.02% (w/v) NaN\(_5\). Reequilibrate in column buffer before using again.
PILOT STUDY TO DETERMINE LECTIN BINDING AND ELUTION CONDITIONS

If the target protein can be detected easily, it is worthwhile to test a small sample to establish binding and elution conditions before applying the entire sample to a large column. The easiest approach is to mix a small amount of sample with a measured amount of Con A–Sepharose beads in a series of microcentrifuge tubes. If the material in the sample binds, it can then be eluted from the washed beads with various amounts of competing αMM to determine when the activity is eluted into the supernatant. This shows whether binding occurs and what concentration of competing sugar (or other conditions) will be required to elute it from the column. An abbreviated version of this procedure can be done with a single sample to determine if the protein binds and if it can be eluted with only one concentration (0.75 M) of αMM. It is important to keep the temperature constant, because changes can affect binding and dissociation of the ligands.

Additional Materials

- Sepharose 4B (Pharmacia Biotech) or other beaded gel to fill space in the tubes
- αMM: 0, 0.1, 0.2, 0.4, 0.8, 1.0, and 1.5 M concentrations, in column buffer

1. Prepare a 50% slurry of Con A–Sepharose in column buffer. Cut ~2 to 3 mm off the small end of a 1000-µl pipet tip. Resuspend the slurry immediately before each pipetting, and use the truncated tip to dispense 200 µl of slurry into a series of seven microcentrifuge tubes. To one of these tubes—the no-lectin control—add an equal volume of 50% slurry of Sepharose 4B. Allow gel to settle to see if the dispensing was reasonably accurate.

   Gel material is needed in the controls to account for the volume changes caused by adding the gel. Other nonionic beaded gels, such as Sephadex (Pharmacia Biotech) or Bio-Gel (Bio-Rad) may be substituted.

2. Add the protein sample in 50 µl column buffer and allow 15 min for binding (shake the tube occasionally). Microcentrifuge the gel beads 1 min at 1000 × g to sediment. Remove the supernatant and save for analysis in step 6.

3. Use an appropriate assay for the protein of interest to determine if it binds to Con A–Sepharose and not to control beads.

4. Wash the gel beads with three 1.4-ml washes of column buffer; after each wash microcentrifuge the beads using conditions in step 2.

5. Resuspend gel beads in 200 µl of buffer containing 0, 0.1, 0.2, 0.4, 0.8, 1.0, or 1.5 M α-MM for each of the seven tubes containing Con A–Sepharose. Add the same volume of column buffer or 1.5 M αMM in column buffer to the control.

6. Incubate 15 min and centrifuge the gel beads as in step 2. Analyze the supernatants using the appropriate detection assay to determine if the protein has been eluted and at what concentration of competing sugar.

   Increasing the concentration of αMM should elute the target protein from the Con A–Sepharose. If it is not eluted, longer incubation times (10 hr) in the presence of αMM or higher temperature may be needed to elute the protein from the beads. Although the entire procedure can be done in the cold, binding is tighter at low temperature, and this may make elution more difficult. Once the conditions are established for a particular sample or a lectin, scaling up the preparation for the basic protocol should proceed smoothly.
WHEAT GERM AGGLUTININ (WGA)–AGAROSE AFFINITY CHROMATOGRAPHY

WGA-agarose chromatography is used to purify proteins that contain terminal N-acetylglucosamine (GlcNAc) or sialic acid residues. A protein sample is applied to the column, the column is washed to remove unbound and weakly bound proteins, and bound glycoproteins are eluted with GlcNAc. Most of the advice for purification of glycoproteins on Con A columns applies to similar columns containing immobilized WGA. It is best to test the binding of the target protein to the lectin using a pilot study like that for Con A (support protocol) before running a large column, substituting GlcNAc for the αMM.

Additional Materials

- 5 mg/ml wheat germ agglutinin (WGA)–agarose (E-Y Laboratories, Pharmacia Biotech, Sigma)
- Phosphate-buffered saline (PBS; APPENDIX 2)
- 0.1 M N-acetylglucosamine (GlcNAc) in PBS
- Protein sample in PBS
- 1.0 × 10–cm glass or disposable column

1. Resuspend 1 vol WGA-agarose in 2 to 3 vol PBS to make a slurry.
   
   One milliliter of gel should be enough to bind 1 to 2 mg glycoprotein. To be safe, assume ~5% to 10% of total cell lysate can bind. If target protein requires detergent to solubilize it, WGA is still active in 25 mM Tris·Cl (pH 7.5)/1% Lubrol PX/0.1% sodium deoxycholate. Triton X-100 or NP-40 (~2% to 3% v/v final) may also be used.

2. Place a glass wool plug over the frit at the bottom of a column whose length is 10 times its diameter. Pour the slurry into the column. Wash the column with ~2 column volumes of PBS to remove any unbound or degraded WGA, then wash with 1 column volume PBS containing 0.1 M GlcNAc. Finally, wash with 5 column volumes PBS.

   The length of the column should be ~10 times its diameter so that weakly bound proteins can be eluted by prolonged PBS wash in the absence of any competing sugar. This will separate them from proteins that do not interact at all, and differs from using Con A where continued buffer wash in the absence of αMM does not usually elute weakly bound protein. The choice of these column dimensions for WGA and for most other lectins is based on the successful fractionation of individual glycopeptides by serial lectin-affinity chromatography.

3. Add protein sample in ~0.1 column volumes at a flow rate of ~2 ml/cm² per hour, and wash the column with PBS until the A₂₈₀ returns to baseline.

   With a small sample volume it is easier to detect proteins that do not bind to the column at all and elute promptly as a sharp peak in the flowthrough. Proteins that bind weakly to the column are gradually washed off by continued elution with PBS alone; these proteins would “smear” as a trailing peak. If a preliminary test run of the target protein shows that it binds strongly to the lectin, and if the column is not overloaded, the sample can be added to the column in a larger volume. The flow rate can be varied. Sometimes the protein of interest can be absorbed to the lectin simply by gently mixing the two components for several hours before pouring the loaded gel into the column. The protein can then be eluted after washing as determined by the pilot study.

4. Elute the column with 2 to 3 column volumes of 0.1 M GlcNAc in PBS, and assay the fractions for the protein of interest.

   Generally, 0.1 M GlcNAc is sufficient to elute the protein. If this does not work, try a higher GlcNAc concentration (e.g., up to 0.25 M), temporarily turn off the column, or raise the temperature to elute the sample, as with Con A. Also, adding 0.5 M NaCl to the GlcNAc-containing elution buffer can sometimes improve recovery. A pilot study to determine elution conditions, similar to that described for Con A (support protocol), is highly recommended.
5. Regenerate the column by washing with ≥10 column volumes of PBS or until reducing sugar content is below 20 µg/ml. The column can be reused immediately or stored at 4°C in column buffer containing 0.02% (w/v) NaN₃.

REAGENTS AND SOLUTIONS

Column buffer
0.01 M Tris·Cl, pH 7.5
0.15 M NaCl
1 mM CaCl₂
1 mM MnCl₂
Store indefinitely at room temperature

Prepare the MnCl₂ within a day or two of use and add it to the buffer only after the pH has been adjusted. The final concentration of metal ions can be decreased 10-fold or more if needed.

If detergents are required to solubilize the protein, Triton X-100 or Nonidet P-40 at ≤2% have a negligible effect on lectins. Do not use glucoside-based detergents because they may interfere with binding.

COMMENTARY

Background Information
See the introductions to Chapter 17 and to this unit for discussions concerning properties of glycoproteins that relate to their purification.

Critical Parameters
Like most protein purification procedures, successful lectin-affinity chromatography is empirical, so few parameters are “written in stone.” However, in using lectins, it is important to first determine the binding and elution conditions in a pilot study. Blindly loading a sample onto a column and then trying different elution conditions may give very low yields, either because the protein may become inactivated or because it cannot be eluted under those particular conditions. It is usually easy to bind the protein to the column, but elution may be more difficult. There are a number of ways to elute tightly bound protein: increase the concentration of sugar in the buffer used for elution, increase temperature, increase the salt (NaCl) concentration, or stop the column flow for an extended period during the elution with sugar. A combination of these may be needed.

These modifications might cause a protein to elute differently from the same column. Thus, it may be useful to perform two consecutive runs with different elution conditions to separate the protein of interest from different contaminants. Ketcham and Kornfeld (1992) describe the purification of a rare glycosyl transferase using WGA, whereby the lectin-affinity chromatography step alone yielded a 160-fold purification. Another important point to remember is that environmental carbohydrate contamination may contribute significantly to the total quantity of carbohydrate in a preparation. Follow the suggestions given within the protocols to minimize this effect.

Anticipated Results
Lectins can give 2- to >100-fold purifications of glycoproteins and nearly quantitative yields, depending upon the source of the sample and elution conditions. If the protein of interest is reasonably stable at high salt concentrations and room temperature, it is likely that specific elution conditions can be found that will produce good yields.

Time Considerations
Initial determination of the binding and elution conditions can easily be done in a day, assuming that the detection assay for the target protein is simple and fast. The amount of time needed to conduct a single run once the elution conditions are known can vary depending upon the size of the sample, the column, and whether elution requires long-term incubation in the presence of the sugar. Even assuming the extremes of sample size and elution conditions, a single run should be completed in 2 to 3 days.

Literature Cited

**Key References**


*Good discussions about general properties of glycoconjugates.*


Lists many references and conditions used for lectins in protein purification.


*Lists several conditions for selected lectin-affinity purifications of proteins.*

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Special Considerations for Proteoglycans and Glycosaminoglycans and Their Purification

Proteoglycans (PG) contain long linear glycosaminoglycan (GAG) chains that consist of repeats of disaccharides and therefore differ from the short branched oligosaccharides found on glycoproteins and glycolipids. The principal GAGs of animal tissue PGs include chondroitin sulfate, dermatan sulfate, heparan sulfate, or keratan sulfate. Heparin, the commonly used anticoagulant, is a highly modified form of heparan sulfate; it is made solely by connective tissue mast cells. Repeating disaccharide units composed of glucuronic acid (GluA) or iduronic acid (IdUA) and an N-acetylgalactosamine residue (GlcNAc or GalNAc) make up the backbone of most GAGs. Keratan sulfate, however, contains disaccharides of Gal and GlcNAc. All GAGs, except hyaluronan, contain a large number of sulfate residues that, together with the uronic acids, impart a large negative charge to the chains. The high density of negative charges on GAGs distinguishes these polysaccharides from the oligosaccharides found on glycoproteins and glycolipids, and is the basis for their physical separation. This property facilitates GAG and PG purification by anion-exchange chromatography and precipitation with cetylpyridinium chloride (CPC) and ethanol.

The synthesis of GAGs occurs while they are attached to core proteins; free chains can accumulate because of proteolysis and endoglycolytic cleavage of the PG. For example, heparin chains are initiated and elongated on a core protein called serglycin. Soon after assembly of the heparin proteoglycan, proteases degrade the serglycin core, and an endoglucuronidase generates short heparin oligosaccharides that sort to secretory granules. Most cells constitutively secrete specific PGs into extracellular fluids and matrix. They also express several membrane-intercalated PGs that are shed from the cell surface by proteolysis. Fragmentation of matrix proteoglycans often occurs and gives rise to GAG chains attached to short peptides. Hyaluronan, a nonsulfated GAG, does not assemble while linked to protein, but associates noncovalently with PGs that contain specific binding domains.

In general, the problems encountered in purifying PGs are those encountered in purification of other protein glycoconjugates. The purification protocol must ensure efficient extraction of tissue or cells, high recovery in separation and purification steps, and negligible degradation. This unit describes two simple methods for extracting PGs and GAGs—one using high-salt/detergent extraction (first basic protocol) and one using alkali treatment (first alternate protocol). These are complemented by two techniques for concentrating samples that also yield a significant degree of purification—anion exchange chromatography (second basic protocol) and precipitation with CPC (second alternate protocol).

**HIGH-SALT/DETERGENT EXTRACTION OF PROTEOGLYCANS AND GLYCOSAMINOGLYCANS**

Efficient extraction of PGs requires the use of chaotropic salts in an appropriate buffer. Detergent is included to ensure extraction of membrane-intercalated PGs, to prevent aggregation, and to decrease adsorption to glassware. In this protocol, samples (tissue, conditioned medium, biological fluid, or cultured cells) are treated with a high-salt/detergent (guanidine-HCl/Zwittergent 3-12) extraction buffer to extract PGs as well as free GAG chains and GAGs with a short peptide chain. Extracted material can be concentrated and partially purified by anion-exchange chromatography or CPC and ethanol precipitation (second basic and alternate protocols, respectively). Studies involving proteoglycans are conducted at 4°C in the presence of protease inhibitors to minimize degradation of core proteins; work with glycosaminoglycans can be done at room temperature.
**Materials**

Tissue sample, conditioned medium, biological fluids, or cultured cells

Guanidine-HCl/Zwittergent 3-12 extraction buffer or Triton X-100 extraction buffer, at 4°C

200× protease inhibitor stock solutions

Centrifuge and rotor (e.g., Sorvall SS-34) or microcentrifuge, at 4°C

Additional reagents and equipment for measurement of uronic acids (UNIT 17.9) and metabolic radiolabeling (UNIT 17.4)

1a. To extract from tissue samples: Chill, mince, and mix tissue with 5 to 10 vol of 4°C guanidine-HCl/Zwittergent 3-12 extraction buffer per gram of tissue (wet weight). Stir the sample overnight at 4°C.

1b. To extract from conditioned medium or biological fluids: Chill samples to 4°C and dissolve solid guanidine-HCl to 4 M (final), Zwittergent 3-12 detergent to 0.2% w/v (final) and EDTA to 10 mM (final). Add protease inhibitors from stock solutions to achieve final concentrations of 10 mM NEM, 1 mM PMSF, 1 µg/ml pepstatin A, and 0.5 µg/ml leupeptin.

*The reagents are added in this way to minimize the increase in volume.*

1c. To extract from cultured cells: Add ~1 ml of guanidine-HCl/Zwittergent 3-12 or extraction buffer per 10^7 cells (in a pellet), or 3 to 5 ml per 75 cm² of cell monolayer. Cell pellets and monolayers will dissolve within 1 hr at 4°C.

*The PGs of cultured cells, conditioned medium, and biological fluids will also solubilize in Triton X-100 extraction buffer (see reagents and solutions), but the efficiency of extraction should be tested (critical parameters). The use of Triton X-100 extraction buffer eliminates the need to remove guanidine-HCl before further purification.*

2. Centrifuge 20 min at 12,000 × g (10,000 rpm in a Sorvall SS-34 rotor), 4°C, or microcentrifuge 10 min at maximum speed, 4°C, to remove insoluble residue. Collect the supernatant. If necessary, reextract residue with fresh extraction buffer, centrifuge as before, and combine the supernatants.

*For conditioned medium, biological fluids, and cultured cells, the initial treatment with extraction buffer is usually sufficient. Tissue samples may need to be reextracted (see critical parameters).*

3. Purify the GAGs by anion-exchange chromatography or by treating with CPC and ethanol (second basic and alternate protocols, respectively).

4. Quantitate the amount of GAG present by measuring the uronic acid content chemically, or by measuring the amount of radioactive material present if the starting material was radiolabeled with ^35^SO_4 or ^[3H]GlcN.

*The measurement of uronic acid is also described in Bitter and Muir (1962) and Filisetti-Cozzi and Carpita (1991).*

**ALTERNATE PROTOCOL**

**ALKALI EXTRACTION OF PROTEOGLYCANS AND GLYCOSAMINOGLYCANS**

If the primary objective is simply to determine whether a sample contains PGs or GAGs, an aliquot can be extracted with alkali. Alkali extraction results in some elimination of chains from core proteins and possible cleavage of the core, so this technique is unacceptable for isolating intact PGs. Alkali-extracted samples are usually subjected to exhaustive proteolysis or complete β-elimination (UNIT 17.15) to liberate GAG chains. The GAGs are then purified by anion-exchange chromatography or precipitation with CPC and ethanol.
(second basic and alternate protocols, respectively) and quantitated chemically or radiochemically. This protocol is suitable for cultured cells, conditioned medium, or biological fluids; tissue samples should be extracted using the high-salt/detergent procedure (first basic protocol).

**Additional Materials**

0.1 N or 10 N sodium hydroxide (NaOH; *APPENDIX 2*)

10 N acetic acid

Additional reagents and equipment for β-elimination (*UNIT 17.15*)

1. Solubilize samples in 0.1 N NaOH at room temperature. Incubate 10 min at 25°C.

   Generally 0.5 ml of 0.1 N NaOH is sufficient for 10^7 cells as a pellet or 2 ml per 75 cm² of cell monolayer. Cells suspended in culture medium can be extracted by adding 10 μl of 10 N NaOH per milliliter medium.

2. Neutralize sample with 10 μl of 10 N acetic acid per milliliter sample. Remove any precipitate that forms by centrifuging 20 min at 12,000 × g (10,000 rpm in a Sorvall SS-34 rotor), 4°C, or microcentrifuging 10 min at maximum speed, 4°C.

3. Treat neutralized samples with a nonspecific protease, such as Pronase or proteinase K or subject samples to β-elimination conditions to liberate individual GAG chains.

4. Purify the GAGs by anion-exchange chromatography or by treating with CPC and ethanol (second basic and alternate protocols, respectively).

5. Quantitate the amount of GAG present by measuring the uronic acid content chemically or by measuring the amount of radioactive material present if the starting material was radiolabeled with ^35SO₄ or [³H]GlcN.

**ANION-EXCHANGE CHROMATOGRAPHY OF PROTEOGLYCANs AND GLYCOSAMINOGlyCANs**

PGs and GAGs can be purified by anion-exchange chromatography on weak anion-exchange resins (*UNIT 10.10*). This step allows collection of very small amounts of material from a large volume of solution, thus concentrating the sample. Anion-exchange chromatography allows separation of different types of GAGs, but the low-salt elution conditions described here will yield a mixture of all GAGs and PGs regardless of composition.

**Materials**

- PG-GAG extract (first basic or alternate protocol)
- Urea/Zwittergent 3-12 buffer
- 20 mg/ml chondroitin sulfate or heparin in 20 mM Tris-Cl, pH 7.0
- DEAE-Sephacel (Pharmacia Biotech)
- 0.2 M NaCl/50 mM sodium acetate, pH 6.0
- DEAE wash buffer
- DEAE elution buffer
- Sephadex G-25 (Pharmacia Biotech)
- 10% ethanol
- 20 mM Tris-Cl, pH 7.4 (*APPENDIX 2*)
- Centrifuge and rotor (e.g., SS-34) or microcentrifuge, at 4°C

Additional reagents and equipment for dialysis (*APPENDIX 3*) and gel filtration and ion-exchange chromatography (*UNITS 10.9 & 10.10*)

1. If the extract contains guanidine-HCl/Zwittergent 3-12, dialyze it against urea/Zwittergent 3-12 buffer. Remove any precipitate that forms during the exchange of urea
for guanidine by centrifuging 20 min at 12,000 \times g (10,000 rpm in a Sorvall SS-34 rotor), 4°C, or microcentrifuging 10 min at maximum speed, 4°C.

Gel-filtration chromatography or ultrafiltration also can be used to change buffers. Extracts prepared in Triton X-100 extraction buffer (see critical parameters) can be chromatographed without further manipulation. Protease inhibitors can be included as long as they do not contribute significantly to the ionic charge of the sample solution.

2. Add 1 to 2 mg chondroitin sulfate as carrier if the total GAG content of the sample is <100 µg and if the addition will not interfere with subsequent analysis.

If the amount of PG or GAG is to be quantitated by measuring uronic acid, carrier GAG must be omitted. Chondroitin sulfate is preferred because it is less expensive than heparin or heparan sulfate. Heparin can inhibit chondroitinases that are used to establish the presence and composition of chondroitin sulfate chains.

3. Prepare the DEAE-Sephacel column. Wash the resin three times with 0.2 M NaCl/50 mM sodium acetate, pH 6.0. Pour a small column by placing 0.5 to 1 ml resin in a polypropylene pipet tip (P-1000) plugged with a small piece of glass wool. Cut the tip slightly (1 to 2 mm) to widen the opening and improve flow rate. Remove the bottom one-third of a second tip with a razor blade and insert it into the first tip. This provides ~2.5 ml of additional head space above the resin bed.

The upper tip will accommodate a disposable 10-ml pipet for adding solutions. Larger column beds can be poured in commercial columns.

4. Equilibrate the column with ~5 column volumes of DEAE wash buffer.

5. Apply the sample to the equilibrated column. Elute contaminants with 20 to 30 column volumes of DEAE wash buffer. Elute the PGs and GAGs with 5 column volumes of DEAE elution buffer and collect as one fraction.

Avoid exceeding the binding capacity of the resin (~5 mg of chondroitin sulfate, heparan sulfate or heparin per milliliter of resin).

Buffers prepared without urea, detergent, and protease inhibitors can be substituted if these reagents interfere with subsequent analysis (e.g., enzymatic digestions by polysaccharide lyases). Chromatography of free GAGs does not require urea or protease inhibitors, but detergent should be included to improve recovery. Fast flow rates can be used.

6. Desalt sample using a Sephadex G-25 gel-filtration column run in 10% ethanol.

If the sample volume is ≤2.5 ml, disposable PD-10 columns (Pharmacia Biotech) can be used.

7. Lyophilize the sample and rehydrate it in 20 mM Tris-Cl, pH 7.4 or other suitable buffer. Store at 4°C until further use.
This protocol describes the concentration and partial purification of extracted PGs and GAGs with the lipophilic cation cetylpyridinium chloride (CPC). The CPC binds to charged groups along the GAGs, rendering them insoluble. This method of precipitation should be used only when the concentration of PGs and GAGs in the original sample is large enough to avoid use of carrier or when the addition of carrier is of no consequence—e.g., when the sample is extracted from cells biosynthetically labeled with $^{35}$SO$_4$ or $[^3$H]GlcN (UNIT 17.4).

**Additional Materials**

- 20 mM Tris $\cdot$ Cl/0.2 M NaCl, pH 7.4
- 5% (w/v) cetylpyridinium chloride hydrate (CPC; Aldrich), in water
- 0.5 M sodium acetate (APPENDIX 2)
- 95% ethanol, 4°C
- 0.5 M sodium acetate in 10% (v/v) ethanol (sodium acetate/ethanol)

Additional reagents and equipment for ethanol precipitation (UNIT 2.1)

1. If the extract contains guanidine-HCl/Zwittergent 3-12 or high salt ($\geq 0.2$ M), dialyze it against 20 mM Tris-$\cdot$Cl/0.2 M NaCl (pH 7.4) or use gel-filtration chromatography to exchange the buffers.

   *Electrolytes and detergents compete with CPC precipitation of GAGs. All GAGs will precipitate if the salt concentration is $\leq 0.2$ M. Protease inhibitors can be included if they do not contribute significantly to the ionic charge of the sample.*

2. Add chondroitin sulfate as carrier so that the final GAG content of the sample is $\geq 0.1$ mg/ml.

   *Precipitation with CPC requires that the GAG concentration be $\geq 0.1$ mg/ml for high recovery. If carrier is added, it is not possible to quantitate GAGs by measuring uronic acid content.*

3. Add 1 ml of 5% CPC/4 ml sample. Incubate 1 hr at 37°C.

4. Centrifuge 20 min at 12,000 $\times$ g (10,000 rpm in a Sorvall SS-34 rotor), or microcentrifuge 10 min at maximum speed, room temperature. Remove the supernatant and resuspend the pellet in a small volume of 0.5 M sodium acetate so that the final GAG concentration is $\sim 10$ mg/ml.

   *Centrifugation must be done at room temperature or the CPC will precipitate. If necessary, briefly incubate the sample in a boiling water bath to dissolve the pellet.*

5. Add 4 vol of ice-cold 95% ethanol, mix, and incubate $\geq 2$ hr at 4°C.

   *Precipitation with ethanol requires salt and adequate mass for high recovery. The samples should contain 0.1 M to 0.5 M salt and 0.1 mg/ml GAG. If adequate material is not present, add additional chondroitin sulfate as carrier.*

6. Centrifuge 20 min at 12,000 $\times$ g (10,000 rpm in a Sorvall SS-34 rotor), or microcentrifuge 10 min at maximum speed, to pellet the white precipitate that forms. Remove and discard supernatant.

7. Dissolve pellet in a small volume of 0.5 M sodium acetate/10% ethanol such that the final concentration is $\sim 1$ mg/ml. Repeat the ethanol precipitation.

8. Dry precipitate under vacuum and resuspend in 20 mM Tris-Cl, pH 7.4 or other suitable buffer. Store at 4°C until further use.
REAGENTS AND SOLUTIONS

10 N acetic acid
Dilute 65.4 ml glacial acetic acid (15.3 M, ACS reagent grade) with water to a total volume of 100 ml. Store indefinitely at room temperature.

DEAE elution buffer
Add 58.5 g NaCl (1 M final) to 1 liter urea/Zwittergent 3-12 buffer. Store at 4°C for several months.

DEAE wash buffer
Add 11.7 g NaCl (0.2 M final) to 1 liter urea/Zwittergent 3-12 buffer. Store at 4°C for several months.

Guanidine-HCl/Zwittergent 3-12 extraction buffer
382.1 g guanidine-HCl (4 M)
2 g Zwittergent 3-12 (0.2% w/v)
4.1 g sodium acetate (50 mM)
3.8 g EDTA (10 mM)
5 ml NEM stock solution (10 mM)
5 ml PMSF stock solution (1 mM)
5 ml pepstatin A stock solution (1 µg/ml)
5 ml leupeptin stock solution (0.5 µg/ml)
Dissolve guanidine-HCl Zwittergent 3-12, sodium acetate, and EDTA in 800 ml water. Add protease inhibitors just before use. Adjust pH to 6.0 and bring volume to 1 liter.

200× protease inhibitor stock solutions
Leupeptin (0.1 mg/ml). Dissolve 5 mg leupeptin (Ac-Leu-Leu-Arg-al hemisulfate, MW 475.6) in water and adjust volume to 50 ml. Store in aliquots at −20°C.
Leupeptin inhibits cathepsin B.
N-ethylmaleimide (NEM; 2 M). Dissolve 25 g NEM in ethanol and adjust volume to 100 ml. Store in aliquots at −20°C.
NEM inhibits thiol proteases and prevents nonspecific disulfide exchange.
Pepstatin A (0.2 mg/ml). Dissolve 5 mg pepstatin A (Isovaleryl-Val-Val-Sta-Ala-Sta) in ethanol and adjust volume to 25 ml. Store in aliquots at −20°C.
Pepstatin A inhibits cathepsin D.
Phenylmethylsulfonyl fluoride (PMSF; 0.2 M). Dissolve 3.5 g PMSF in ethanol and adjust volume to 100 ml. Store in aliquots at −20°C.
PMSF inhibits serine proteases.

0.5 M sodium acetate/10% (v/v) ethanol.
Dissolve 4.1 g sodium acetate in water and adjust volume to 90 ml. Add 10 ml ethanol.

Triton X-100 extraction buffer
5 g Triton X-100 (0.5% w/v)
8.77 g NaCl (0.15 M)
2.42 g Tris base (20 mM Tris Cl)
3.8 g EDTA (10 mM)
5 ml NEM stock solution (10 mM)

continued
5 ml PMSF stock solution (1 mM)
5 ml pepstatin A stock solution (1 µg/ml)
5 ml leupeptin stock solution (0.5 µg/ml)

Dissolve Triton X-100, NaCl, Tris base, and EDTA in ~800 ml water. Add protease inhibitors just before use. Adjust pH to 7.4 and bring volume to 1 liter.

**Urea/Zwittergent 3-12 buffer**
- 240 g urea (ultrapure grade, 4 M)
- 2 g Zwittergent 3-12 (0.2% w/v)
- 4.1 g sodium acetate (50 mM)
- 3.8 g EDTA (10 mM)
- 5 ml NEM stock solution (10 mM)
- 5 ml PMSF stock solution (1 mM)
- 5 ml pepstatin A stock solution (1 µg/ml)
- 5 ml leupeptin stock solution (0.5 µg/ml)

Dissolve urea, Zwittergent 3-12, sodium acetate, and EDTA in ~800 ml water. Add protease inhibitors just before use. Adjust pH to 6.0 and bring volume to 1 liter.

**COMMENTARY**

**Background Information**

Proteoglycan (PG) extraction methods were first developed for studying PGs in cartilage. These methods use high salt and guanidine-HCl to disrupt ionic interactions and they are considered to be “dissociative” because their effectiveness depends on the dissociation of PGs from aggregates containing hyaluronan and matrix proteins (Sajdera and Hascall, 1969). PGs and glycosaminoglycans (GAG) present in other tissues, biological fluids, and cultured cell lines (Kjellén and Lindahl, 1991) differ significantly in structure and composition from those found in cartilage, but the original extraction methods remain effective. Many PGs associate with membranes through hydrophobic domains or glycosyl phosphatidylinositol anchors (**UNIT 17.8**). Membrane PGs require detergent to disrupt hydrophobic interactions and to produce a micellar dispersion. Current extraction and purification protocols employ both denaturants and detergents (Yanagishita et al., 1987; Hascall and Kimura, 1982).

**Critical Parameters and Troubleshooting**

**Extraction efficiency.** The guanidine-HCl/Zwittergent 3-12 extraction buffer should render the majority of macromolecules soluble. However, some tissues (e.g., aorta) may resist dispersion, leaving some PGs in the insoluble residue. Mechanical homogenization of samples in extraction buffer may improve recovery. To determine the overall efficiency of extraction, measure the release of PGs over time. Incubate aliquots of sample in extraction buffer and at certain intervals, separate the PGs from other constituents by anion-exchange chromatography (second basic protocol) or precipitation with cetylpyridinium chloride (CPC) and ethanol (second alternate protocol). If sufficient material is present (≥50 µg of uronic acid), monitor the uronic acid content of a sample to measure PG and GAG release. Alternatively, if cultured cells are labeled biosynthetically with $^{35}$SO$_4$ or [6-3H]GlcN (**UNIT 17.4**), extraction of PGs from cultured cells is easily followed radiochemically. When the yield of uronic acid or radioactivity in the extract reaches a plateau, no additional material can be obtained under these conditions. Any insoluble residue should be dissolved in 0.1 N NaOH and residual GAGs measured. If more than 10% of the material remains in the residue, the extractions condition may be varied by using a different detergent (e.g., 1% Triton X-100 or 1% deoxycholate), by adding other salts (e.g., LiCl), by treating samples with collagenase, by altering the pH, or by adding a reductant (e.g., 2-mercaptoethanol). Kimura et al. (1981) showed that sequential addition of detergent and guanidine-HCl extracts more proteoglycan from chondrosarcoma than adding both agents simultaneously.

**Recovery.** As in any purification scheme, adequate starting material is required to ensure high recovery. Samples should ideally contain at least 0.1 mg of GAG as determined by uronic acid content. Because cell cultures tend to yield small amounts of material (0.1 to 1 µg of GAG uronic acid per 10⁶ cells), carrier GAGs should be added. Commercial preparations of chon-
droitin sulfate are inexpensive and provide a suitable carrier for purifying PGs as well as GAGs. However, addition of carrier makes it impossible to monitor recovery by chemically measuring uronic acid, so carrier should only be added to samples labeled biosynthetically with $^{35}$SO$_4$ or $[^3H]$GlcN. Sufficient mass (≥0.1 mg/ml) is critical for efficient precipitation with CPC and ethanol.

Inclusion of protease inhibitors is critical to maintain the integrity of PG core proteins. The buffers used in these protocols contain leupentin (to inhibit cathepsin B), NEM (to inhibit thiol proteases and prevent nonspecific disulfide exchange), Pepstatin A (to inhibit cathepsin D), PMSF (to inhibit serine proteases), and EDTA (to inhibit metalloproteases). In addition, samples should be chilled rapidly and processed immediately, and the pH should be maintained at pH 6.0 to diminish the activity of acidic lysosomal proteases and neutral tissue proteases.

If degradation of core protein occurs under the conditions described in the protocols (as detected by variable recovery), other inhibitors should be used. For example, benzamidine-HCl at 1 mM inhibits serine proteases; 6-aminohexanoic acid at 0.1 M inhibits cathepsin D; phosphoramidon at 0.5 mM inhibits collagenases, metalloendoproteinases, and stromelysin; soy bean trypsin inhibitor at 0.1 mg/ml inhibits trypsin-like proteases; and bestatin at 0.1 mM inhibits several aminopeptidases. Sodium dodecylsulfate (SDS) can also inhibit proteases because of its ability to denature proteins. However, SDS is difficult to remove; its incompatibility with anion-exchange chromatography and CPC precipitation complicates further purification steps. Nevertheless, SDS helps maintain solubility of aggregating proteins and should be considered as an additive in purification steps after CPC precipitation or anion-exchange chromatography.

Zwitterionic (e.g., Zwittergent 3-12) and nonionic detergents (e.g., Triton X-100) help solubilize membrane PGs and improve recovery by blocking nonspecific binding and aggregation. These detergents remain soluble both in the presence of high concentrations of salts and at low temperature, and are compatible with most purification methods.

Purity. Purification of PGs to chemical homogeneity (a single species of PG with no contaminants) presents a challenging problem. The procedures described in this unit should yield material enriched in PGs because few macromolecules exhibit the charge characteristics imparted by the polyanionic GAG chains. Nucleic acids tend to copurify with PGs and GAGs, but nucleic acids and weakly charged or neutral oligosaccharides and polysaccharides are not precipitated efficiently by CPC. If necessary, samples can be treated with DNase and RNase (UNIT 3.12) to reduce the content of nucleic acids.

Some contaminants associate with PGs through ionic interactions with the GAG chains. Other purification methods should be tried, including density-gradient centrifugation in CsCl (Hascall and Kimura, 1982), gel-filtration chromatography in high salt or guanidine-HCl (UNIT 10.9), reversed-phase chromatography on octyl-Sepharose (Yanagishita et al., 1987), and affinity purification using monoclonal antibodies (UNIT 10.11A; Hascall and Kimura, 1982; Yanagishita et al., 1987). With the exception of affinity purification protocols that use immobilized proteins or antibodies, most of these methods are compatible with dissociative conditions.

Some protein contaminants may aggregate with core proteins through interchain disulfide bonds. Some matrix PGs may not extract efficiently if they are linked by disulfides to insoluble matrix proteins (Parthasarathy and Spiro, 1981). Reduction with 10 mM dithiothreitol at elevated temperature followed by alkylation (e.g., with iodoacetamide) should help dissociate these proteins.

The purification protocol described above does not separate free GAG chains from PGs. Free chains usually have different Stokes radii than PGs, and gel-filtration chromatography frequently separates GAGs from PGs. Shift of material into more included fractions after β-elimination or proteolysis confirms that PGs were present in the original sample. If sufficient material is present, colorimetric quantitation of proteins (UNIT 10.1) can also help distinguish PGs from GAGs, but the presence of contaminating proteins may complicate this analysis. Labeling PGs biosynthetically with radioactive amino acids and $^{35}$SO$_4$ or $[^3H]$GlcN provides another way to monitor elution of PGs and GAGs.

Several excellent reviews describe detailed procedures for further characterization of intact PGs, their protein cores, and the GAG chains (Rodén et al., 1972; Hascall and Kimura, 1982; Yanagishita et al., 1987).

Anticipated Results

The high-salt/detergent and alkali extraction methods will reliably extract the majority
of PGs from most tissues and cells. Ion-exchange chromatography and precipitation with CPC and ethanol will yield a preparation of PGs or GAGs of adequate concentration and purity for compositional analysis.

**Time Considerations**

Tissues usually require overnight extraction, but extraction of cultured cells occurs very rapidly, usually within an hour. Complete dialysis requires several buffer changes over a period of 24 to 48 hr. Anion-exchange chromatography and CPC precipitation take ~1 day to complete. Thus, it should take 3 to 4 days to extract, partially purify, and concentrate PGs and GAGs from biological samples.

**Literature Cited**


**Key References**

Rodén et al., 1972. See above.

This review details conventional methods for isolating and characterizing the composition, size, and fine structure of GAG chains.

Hascall et al., 1982. See above.

This review describes detailed methods for purifying and characterizing tissue proteoglycans.

Yanagishita et al., 1987. See above

This review extends accepted methodology for purifying and characterizing tissue proteoglycans to those found in cultured cells.

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Special Considerations for Glycolipids and Their Purification

The term “glycolipid” refers to a large class of heterogeneous and rather ill-defined conjugates composed of sugar and lipid. They range from the large oligosaccharide conjugates of polyisoprenols and dolichols (intermediates in the biosynthesis of bacterial-cell-wall polysaccharides and animal-cell glycoproteins, respectively) to membrane components of plants, yeast, mycobacteria, and animal cells. This unit presents methods for preparing glycosphingolipids, the most commonly studied glycolipids, for analysis. In general, these studies are used for comparison of glycosphingolipids from different cells or cell lines or for comparison of experimentally treated and control cells or cell lines.

The first basic protocol describes the extraction of all glycosphingolipids, including those carrying large oligosaccharides, from small amounts of tissue or cultured cells, and their subsequent separation based on their relative solubilities in polar and nonpolar solvents (Folch partitioning). A support protocol describes the preparation and use of Sep-Pak C18 cartridges for use in the first basic protocol. The second basic protocol describes preparation of gangliosides from the final product of the previous protocol (Folch upper phase) and their separation into mono-, di-, and polysialylated gangliosides by DEAE-Sephadex chromatography. The alternate protocol describes a more rapid method for separation of gangliosides from the total lipid extract generated partway through the first basic protocol.

STRATEGIC PLANNING

Whether a class of glycolipids is involved in biosynthesis of other glycoconjugates or in the anchoring of specific oligosaccharides or proteins to cell membranes, the unique hydrophilic and hydrophobic properties that play a significant role in their function also present unique challenges for their isolation and purification. Because of their diversity, no single protocol is convenient for purification of all classes of glycolipids; the method of choice will depend on what glycolipids are of interest. Discussion of the purification of all of the diverse classes of glycolipids is beyond the scope of a single unit. However, similarities in physical properties do permit the use of many similar or related techniques for the different classes.

Most research has focused on cell-surface glycolipids of animal cells, particularly glycosphingolipids and glycosyl phosphatidylinositol (GPI) membrane anchors (UNIT 17.8). Glycosphingolipids are glycolipids composed of a ceramide (a fatty acyl derivative of the long-chain base sphingosine) glycosidically linked to a monosaccharide, usually glucose or galactose, that may in turn be modified with additional sugar molecules (see Fig. 17.3.3). Glycosphingolipids are heterogeneous in their properties due to variation in the fatty acid and/or sphingosine structure of the ceramide component as well as the diversity of the oligosaccharide component. For example, glycosphingolipids composed of ceramides with short fatty-acid side chains and large oligosaccharide chains will be much more soluble in polar solvents than glycosphingolipids composed of ceramides with long fatty-acid side chains and short oligosaccharide chains. In addition, charged residues on oligosaccharides will decrease their hydrophobicity.

The two classes of glycosphingolipids to be considered in this unit are (1) the neutral glycosphingolipids—from monohexosylceramides up to very large neutral glycolipids that have been referred to as polyglycosyl ceramides; and (2) the negatively charged glycosphingolipids—primarily gangliosides, which are glycosphingolipids containing negatively charged sialic acid residues.

Contributed by David F. Smith and Pedro A. Prieto

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The first basic protocol separates the lipid extract into a nonpolar lower-phase fraction containing neutral glycolipids smaller than tetrahexosylceramides along with contaminating neutral and zwitterionic lipids, and a polar upper-phase fraction containing gangliosides and neutral glycolipids generally larger than tetrahexosylceramides. The upper-phase fraction is desalted by elution from a Sep-Pak C18 cartridge. The lower phase can subsequently be fractionated using methods unaffected by contaminating neutral lipids and phospholipids. These lower-phase separations are not affected by neutral lipid and phospholipid contaminants if they are labeled with radioactive sugar precursors, because only the sugars will be detected by the separation technique. Contaminated lower phases can also be analyzed with carbohydrate-specific reagents if the cells used as starting material were metabolically radiolabeled (UNIT 17.4). Thin-layer chromatography (TLC; UNITS 10.12-10.14) combined with autoradiography (APPENDIX 3) can be used for separation and detection. This permits detection and characterization of extremely small amounts of glycolipids without the use of expensive instrumentation, such that single culture dishes of radiolabeled cells can be used to obtain significant structural information on purified, radiolabeled glycosphingolipids. Alternatively, high-performance liquid chromatography (HPLC; UNIT 9.6) or overlay analysis of thin-layer chromatograms using I\textsuperscript{25}I-labeled carbohydrate-binding proteins may be employed. The upper phase can be treated as described in the second basic protocol to purify the gangliosides. If only the gangliosides (and not the neutral glycosphingolipids) are of interest, the process can be shortened by carrying out just the first nine steps of the first basic protocol followed by the rapid ganglioside extraction method described in the alternate protocol. Like the first basic protocol, both protocols for purifying gangliosides are suitable for analysis of radiolabeled glycoconjugates (UNIT 17.4). All of these protocols are compatible with subsequent extraction and analyses of total membrane glycopeptides or glycoproteins (UNITS 17.1, 17.4 & 17.5) from the same sample. Figure 17.3.1 diagrams the fractionation process and relates it to the individual protocols.

CAUTION: If cells being used are metabolically radiolabeled (UNIT 17.4), appropriate procedures for manipulation and disposal of radioactive samples should be employed.

NOTE: Deionized, distilled water or its equivalent should be used throughout this unit.

### EXTRACTION AND PURIFICATION OF GLYCOLIPIDS FROM CELLS OR TISSUES

Because glycosphingolipids contain both oligosaccharide and ceramide components, they are amphipatic—i.e., present the contradictory properties of being both hydrophilic and hydrophobic. Depending on the relative content of polar, nonpolar (neutral sugar), and charged substituent groups, glycosphingolipids behave differently in aqueous and nonaqueous solvents. Thus, a wide variety of methods for extracting and separating these compounds based on solubility have been developed. This protocol details a method for extracting not only the nonpolar glycosphingolipids but also the charged and highly glycosylated derivatives from cultured cells or small amounts of tissue (≤0.5 g). Total cell lipids are first extracted with organic solvents, then separated into fractions by the Folch partition method (Fig. 17.3.2).

#### Materials

- Tissue sample or cell culture from one to several culture plates
- Methanol (HPLC grade)
- Chloroform (HPLC grade)
- 4:8:3 (v/v/v) chloroform/methanol/water
- 1:1 and 2:1 (v/v) chloroform/methanol
- 100% ethanol
0.1 M KCl
1:1 (v/v) methanol/0.1 M KCl
1:1 (v/v) methanol/H₂O

Sep-Pak C₁₈ cartridge (support protocol)
Blender (Waring), tip sonicator (Branson), or Tissuemizer (Tekmar) equipped
with a probe of appropriate size
Bath sonicator
Rotary evaporator, Speedvac (Savant), Vortex-Evaporator (Labconco),
or nitrogen dryer
1.5-ml polypropylene microcentrifuge tubes resistant to chloroform or
12-ml conical glass centrifuge tubes

Additional reagents and equipment for quantitation of protein (UNIT 10.1), and
protein electrophoresis (UNITS 10.2-10.5)
Extract sample with organic solvents

1. If using cultured cells, harvest and wash by centrifuging, removing supernatant, resuspending pellet in PBS or a suitable isotonic solution, centrifuging again, and discarding supernatant, according to the usual procedure for the cell type (this removes serum components and, in the case of metabolic labeling experiments, unincorporated radioactive precursor). Suspend tissue or cell pellet in 2° to 4° C water, using 2 to 3 ml water per gram of tissue (wet weight) or enough water to make a suspension of cultured cells containing 0.1 to 6 mg/ml protein. Homogenize using a blender, sonicator, or Tissuemizer.

Efficient extraction of glycolipids from lyophilized cell preparations is very difficult and should be avoided. For cultured cells, a pellet from one to several 60-mm plates of cells may be suspended in 0.24 ml of water in a 1.5-ml microcentrifuge tube. With microcentrifuge tubes, homogenization can be carried out using a bath sonicator. For larger samples (up to 1 ml), 12-ml conical glass centrifuge tubes should be used.

Samples should be kept on ice between homogenization and extraction to prevent digestion of the carbohydrates by endogenous glycosidases.

2. To 3 vol aqueous homogenate, add 8 vol methanol followed immediately by 4 vol chloroform, mixing continuously while adding the solvents. For small volumes, this step can be carried out in a bath sonicator.

In the case of a sample initially suspended in 0.24 ml, 0.64 ml methanol should be added, followed by 0.32 ml chloroform, for a total extract volume of ~1.2 ml.

3. Incubate sample in a bath sonicator 30 min at 20° to 30°C.
Temperatures of 20° to 30°C may destroy labile O-acetyl groups and/or induce transacetylation of O-acetylated derivatives of sialic acids. Lower temperatures may be required if information on sialic acid acetylation is critical to subsequent analyses; in this case solvents should be chilled to the appropriate temperature before use. Temperatures at or below room temperature can be maintained by adding ice to the bath sonicator.

4. Centrifuge the sample 1 min at 8800 × g (e.g., 11,000 rpm in a microcentrifuge) and collect supernatant.

5. Extract the pellet from step 4 with a volume of 4:8:3 chloroform/methanol/water roughly approximating the total extract volume in step 2 (e.g., ~1 ml may be used for a sample initially suspended in 0.24 ml water). Centrifuge sample 1 min at 8800 × g. Collect supernatant and pool with that from step 4.

6. Extract the pellet from step 5 three more times as described in step 4, using 1:1 chloroform/methanol, 2:1 chloroform/methanol, and 100% ethanol, respectively. Pool all the supernatants with that from steps 4 and 5.

7. If desired, analyze the ethanol-washed pellet (containing primarily lipid-free proteins and glycoproteins) for total proteins by one- or two-dimensional gel electrophoresis or digestion with protease for isolation of total glycopeptides. Do not allow pellet to dry; resuspend in a buffer containing SDS and store at −20° or −80°C prior to further manipulation if desired.

The two chloroform/methanol washes, using solvents with increasingly higher concentrations of nonpolar solvents compared to the sample, ensure the extraction of less polar glycolipids (i.e., mono- or disaccharide derivatives of ceramide that are more hydrophobic than glycolipids with larger oligosaccharide components). The ethanol wash removes the chloroform and methanol to avoid any detrimental effects these solvents may have on subsequent treatment of the cell pellet.

8. Evaporate the pooled supernatants from steps 4 to 6 to dryness using a rotary evaporator, Vortex-Evaporator, Speedvac, or nitrogen dryer.

Evaporating the sample under a stream of nitrogen requires considerably more time to remove the final traces of H₂O than does evaporation with a rotary evaporator, Vortex-Evaporator, or Speedvac. Dry crude lipid extract can be stored desiccated at −20°C prior to further manipulation.

**Carry out Folch partitioning of glycosphingolipids**

9. Dissolve the crude lipid extract from step 8 in 1.0 ml of 2:1 chloroform/methanol (or 10% to 25% the volume of the initial suspended sample if this is larger than 1 ml).

10. Partition by adding 0.2 ml (or 0.2 vol) of 0.1 M KCl and mixing vigorously by vortexing. Centrifuge 1 min at 8800 × g (it will separate into two phases).

For small-scale preparations, this can be done in a 1.5-ml polypropylene microcentrifuge tube. For larger volumes, phase separation may be accomplished by allowing the mixture to stand at room temperature until separated (or in a separatory funnel overnight at 4°C).

11. Carefully remove the upper phase (~50% of the total volume) with a Pasteur pipet. Wash lower phase by adding 1:1 methanol/0.1 M KCl in a volume equivalent to the removed upper phase, mixing, and centrifuging 1 min at 8800 × g to separate the phases (this removes contaminating upper phase).

12. Collect the upper phase and pool with the first upper phase. Wash the lower phase once more with 1:1 methanol/0.1 M KCl and pool with upper phases from step 11.
13. Wash the combined upper phases with 0.5 vol of 2:1 chloroform/methanol. Combine the lower phases from the washes with the pooled lower phases from step 12.

The combined lower-phase fractions contain neutral glycolipids with oligosaccharides smaller than tetrasaccharides, neutral lipids, and phospholipids. Tetrasaccharide-ceramides usually partition between the two phases, causing slight contamination. The combined upper-phase fractions contain all of the gangliosides and most of the larger neutral glycolipids (>tetrahexosylceramides) as well as all water-soluble materials from the original extract and inorganic salts introduced during washes of the lower phase. When metabolically radiolabeled cells are used, the upper phase will also contain radioactive sugar precursors and their intermediates.

The combined lower-phase fractions can now be analyzed for glycosphinoglipids using methods unaffected by contaminating neutral and phospholipids—e.g., TLC and autoradiography of glycolipids (if cells were radiolabeled with monosaccharide precursors), overlay analysis of thin-layer chromatograms using 125I-labeled carbohydrate-binding proteins, or HPLC (Kannagi, 1987). The combined upper-phase fractions can be processed as described in the following steps and further purified as described in the last two protocols.

Prepare combined upper-phase fractions for analysis

14. Dilute combined upper-phase fraction to 5 to 10 ml with 1:1 methanol/water and apply it to a Sep-Pak C18 cartridge prepared as described in the support protocol below. Collect effluent in a test tube and reapply to the cartridge; repeat once. The liquid should be applied to the Sep-Pak C18 cartridge as described in the support protocol. Passing the upper-phase fraction through the cartridge removes salts and other nonlipid contaminants. Collecting and reapplying the effluent to the cartridge ensures complete absorption of all lipid-containing compounds in the sample.

15. Wash the cartridge five times with 10-ml aliquots of water (50 ml total) to ensure removal of all polar contaminants. When radiolabeled cells are used, these washes can be pooled for further analysis of radiolabeled precursors, but should contain no glycolipid. They should be discarded as radioactive waste.

16. Elute gangliosides and high-molecular-weight neutral glycolipids from the column by washing five times with 1:1 chloroform/methanol and three times with 4:8:3 chloroform/methanol/water. The final wash with more polar solvent elutes glycolipids containing large oligosaccharides (when present in the sample) that were not eluted with 1:1 chloroform/methanol.

17. Combine solvent washes and evaporate to dryness. Store residue desiccated at −20°C until needed.

PREPARATION OF SEP-PAK C18 CARTRIDGES

This protocol details preparation of Sep-Pak C18 cartridges for use in removing salts and other nonlipid contaminants from the upper-phase glycolipid fraction of Folch-partitioned material. All lipid-containing compounds will be retained by the Sep-Pak C18 cartridge, water-soluble contaminants are eluted with polar solvents, and lipid-containing material is eluted with nonpolar solvent.

Additional Materials

- 0.1 M ammonium acetate in 1:1 (v/v) methanol/water
- Sep-Pak C18 cartridges (Waters)
- 10-ml glass syringe with locking hub (e.g., Becton Dickinson Luer-Lok)
1. Remove plunger from the barrel of the syringe and attach the Luer-Lok hub of the syringe to the long end of a Sep-Pak C_{18} cartridge. Pump 10 ml methanol through the cartridge by adding the liquid to the barrel of the syringe, inserting the plunger into the syringe, and slowly pushing the liquid through the cartridge. Collect eluant methanol in a beaker or other appropriate container. Remove the cartridge from the syringe before removing the plunger. Remove the plunger and reattach the cartridge.

2. In the same fashion, carry out the following further washes (10 ml each):

- 3 washes with 1:1 chloroform/methanol
- 1 wash with methanol
- 2 washes with 0.1 M ammonium acetate in 1:1 methanol/water.

*Sep-Pak cartridges may be reused at least five times. They should be prepared for storage by flushing the last of the methanol/water wash out with air and stored dry at room temperature. Preparation steps should be repeated after storage prior to use.*

**PREPARATION OF GANGLIOSIDES**

Fractionation of the glycolipid extract into neutral glycolipids and gangliosides can be easily accomplished by separating charged glycolipids from neutral glycolipids of the upper-phase fraction obtained by Folch partitioning (the material from step 17 above). A simple column chromatographic procedure is described here, but more sophisticated methods, including anion-exchange HPLC (UNIT 10.13), may be used. This protocol is adapted from a method originally developed to isolate the gangliosides from the upper phase of extracts of 50 g of tissue but also works well on a small scale for isolating gangliosides from the upper phase of extracts of metabolically radiolabeled cultured cells.

**Materials**

- Methanol
- DEAE-Sephadex equilibrated in 100% methanol, 50% slurry (reagents and solutions)
- Purified Folch upper-phase fraction (first basic protocol)
- 0.01, 0.2, and 0.5 M ammonium acetate in methanol
- Glass wool

1. Set up a DEAE-Sephadex column in a 5-in. Pasteur pipet as follows: secure the Pasteur pipet to a ring stand with a small clamp so that the column effluent can be collected into a beaker or into tubes in a test tube rack for manual processing. Use a 9-in. pipet to position a small ball of glass wool in the narrow portion of the Pasteur pipet.

   *If the amount of glass wool is too large, or if it is packed too tightly into the tip of the pipet, the flow rate will be restricted. Alternatively, a column with appropriate solvent-resistant fittings can be used with a fraction collector for automatic sample collection.*

2. Wash the glass wool with methanol to determine an appropriate flow rate in the empty column (this also removes any contamination from the glass wool). If the flow rate is less than 1 drop/sec, repack the glass wool to ensure a reasonable flow rate when the column is packed with DEAE-Sephadex.

3. Add sufficient 50% slurry of DEAE-Sephadex in methanol to the pipet to obtain a column of resin 2 to 4 cm high.

   *A 4-cm column has a bed volume of ~1 ml; half that volume is normally sufficient capacity for radiolabeled material.*
4. Wash the column with 2 to 3 column volumes of methanol.

5. Dissolve the upper-phase fraction in 0.1 to 1 ml methanol and apply to the column of DEAE-Sephadex.

   The upper-phase fraction is free of salts and other nonlipid contaminants. Because the charged glycolipids (gangliosides) will be retained by the column under these conditions, the sample volume is not critical.

6. Elute neutral glycolipids in the upper phase by washing the column with 5 column volumes of methanol. Evaporate the eluant to dryness (e.g., using a nitrogen dryer).

   This fraction contains desalted neutral glycolipids that are larger than trihexosylceramides. These desalted neutral glycolipids can be combined with the lower-phase fraction that contains the remainder of the neutral glycolipids. This is a convenient fraction for many analyses including thin-layer chromatography (TLC) and TLC overlay analyses.

7. Elute gangliosides that contain a single sialic acid (monosialylgangliosides) from the column with 5 column volumes of 0.01 M ammonium acetate in methanol.

   Eluant may be collected in equal-sized fractions if the elution profile is important, or it may be collected in a single container. Concentrate monosialylgangliosides (e.g., by evaporation under nitrogen). If this fraction is to be used for TLC analysis, it must be desalted. This can conveniently be done by adding water to 50% and applying the material to a Sep-Pak C18 cartridge (support protocol).

8. Elute the gangliosides that contain two sialic acids per oligosaccharide with 5 column volumes of 0.2 M ammonium acetate in methanol.

9. Elute polysialylgangliosides (tri- and tetrasialylgangliosides) with 5 column volumes of 0.5 M ammonium acetate in methanol.

   These fractions can be concentrated and/or desalted on Sep-Pak cartridges as described for monosialylgangliosides in step 7.

   If the sample contains sulfated glycolipids, it may be necessary to elute them with 0.8 to 1.0 M ammonium acetate in methanol.

**RAPID PURIFICATION OF GANGLIOSIDES**

For projects that focus on the analysis of only gangliosides, this protocol describes a method for rapid preparation of total gangliosides from total cellular lipid extract using an ion-exchange column equilibrated in a more polar solvent.

**Additional Materials**

- DEAE-Sephadex equilibrated in 60:30:8 methanol/chloroform/water (reagents and solutions)
- Total lipid extract (step 8 of first basic protocol)
- 60:30:8 (v/v/v) methanol/chloroform/water
- 60:30:8 (v/v/v) methanol/chloroform/0.8 M aqueous KCl

1. Prepare a DEAE-Sephadex column as described in step 1 of the second basic protocol, but using DEAE-Sephadex equilibrated in 60:30:8 methanol/chloroform/water.

2. Dissolve the total lipid extract from cells or tissues from step 8 of the first basic protocol in 60:30:8 methanol/chloroform/water.

   The wash volume is not critical; a volume equivalent to the volume of the original extract is generally appropriate.
3. Apply total lipid extract to the column slowly (<1 ml/min) to ensure complete absorption of charged lipids.

4. Elute the column with 10 column volumes of 60:30:8 methanol/chloroform/water to remove uncharged and zwitterionic lipids.

   *The flow rate can be increased for this and subsequent elution steps.*

5. Elute gangliosides and other acidic lipids with 10 column volumes of 60:30:8 methanol/chloroform/0.8 M aqueous KCl.

**REAGENTS AND SOLUTIONS**

**DEAE-Sephadex, equilibrated**

Suspend 5 g dry DEAE-Sephadex resin in 60 ml of 60:30:8 methanol/chloroform/0.8 M aqueous KCl. Allow resin to settle and remove supernatant by aspiration. Repeat this procedure twice and allow the final slurry to equilibrate overnight (this prepares the resin in its acetate form). Remove the supernatant and equilibrate the resin by repeated washing with 60:30:8 methanol/chloroform/water through filter paper or a coarse-porosity sintered-glass funnel.

*The equilibrated resin can be stored as a 50% slurry in 60:30:8 methanol/chloroform/water for immediate application in the alternate protocol for rapid preparation of gangliosides. For separation of upper-phase gangliosides, equilibrate the resin in 100% methanol and store as a 50% slurry in methanol.*

**COMMENTARY**

**Background Information**

Most research on glycolipids has focused on the analysis of cell surface glycolipids of animal cells which include the glycosphingolipids and the glycosyl phosphatidylinositol (GPI) membrane anchors. Glycosphingolipids are composed of ceramide, which is a fatty acyl derivative of the long-chain base sphingosine, glycosidically linked to a monosaccharide, usually glucose or galactose (Fig. 17.3.3). Other sugars may be added to the monosaccharide. One of the GPI anchors first described attaches *Trypanosoma brucei* variant surface glycoprotein (VSG) to the plasma membrane. This glycolipid anchor is composed of phosphatidylinositol linked glycosidically to a tetrasaccharide that terminates with a phosphoethanolamine moiety (Doering et al., 1990). The ethanolamine is linked by an amide linkage to the α-carboxyl group of a protein to form the covalent attachment of VSG to a membrane glycolipid. Variations of this GPI structure have been found in protozoans, yeast, slime molds, and most animal cells.

Because glycosphingolipids are found in the mammalian-cell plasma membrane, they are

![Figure 17.3.3 The structure of galactosylceramide.](image-url)
the most-studied and best-characterized of all the glycolipids. They are currently of interest to molecular and cellular biologists investigating structure and function of cell surface carbohydrates. In animal cells, glycosphingolipids function as anchors for surface carbohydrate antigens and as potential receptors for attachment of bacteria, viruses, and toxins (Karlsson, 1989). Glycosphingolipids may also have more dynamic functions as modulators of cellular interactions and transmembrane signaling (Hakomori, 1990).

The oligosaccharides of glycosphingolipids can be very complex; their heterogeneity is reviewed by Stults et al. (1990), who have cataloged the structures of 267 glycosphingolipids. In general glycosphingolipids can be divided into three classes based on their carbohydrate structures: neutral glycolipids with uncharged oligosaccharides; gangliosides with oligosaccharides containing one or more negatively charged sialic acid residues; and sulfatoglycosphingolipids with oligosaccharides derivatized with sulfate esters. The oligosaccharide “core” structure of most animal-cell glycolipids consists of lactose linked glycosidically to ceramide. Elongation of this lactosylceramide by monosaccharide addition results in several families of glycolipids based on the sequence and linkages of sugars. Table 17.3.1 (based on Machler and Sweeley, 1978; Weigandt, 1985; and Makaaru et al., 1992) lists prefixes used in naming glycolipids and their associated structures.

Variations on the basic glycolipid structure are found throughout nature. Plants, yeast, and fungi express inositol-containing sphingolipids, and methods for their isolation and characterization have been reviewed by Laine and Hsieh (1987). These glycolipids are glycosphingolipids made up of a common core of inositol joined in a phosphodiester linkage to ceramide, with longer oligosaccharide chains glycosidically linked to the inositol moiety. Mycobacteria contain several novel classes of glycolipids including the lipo-oligosaccharides, glycopeptidolipids, and phenolic glycolipids; the structure, isolation, and antigenicity of these compounds have been reviewed by McNeil et al. (1989).

Glycosphingolipids are minor components in animal cells and much of the difficulty in their characterization is associated with their limited availability. This unit describes protocols that can be used to characterize the small amounts of material available in cultured cells. Glycosphingolipids are unique among glycoconjugates in that, regardless of their oligosaccharide complexity, each species carries only a single oligosaccharide moiety. A chromatographically pure sample generally represents a glycosphingolipid with a single oligosaccharide. The oligosaccharide structures of purified glycosphingolipids are determined using many of the structural analytical techniques described in this chapter. The apparent fidelity of structure (one glycosphingolipid, one oligosaccharide) makes it possible to assign structures based on co-chromatography of unknown structures with known standards on TLC, using chemical methods for sugar detection to locate individual glycosphingolipids. This approach

| Table 17.3.1 Oligosaccharide Structures and Prefixesa |
|-----------------|------------------|
| Prefix          | Structure        |
| Arthro          | GlcNAcβ1-3Manβ1-4GlcCer |
| Gala            | Galα1-4GalCer    |
| Ganglio         | Galβ1-3GalNAcβ1-4Galβ1-4GlcCer |
| Globo           | GalNAcβ1-3Galα1-4Galβ1-4GlcCer |
| Globoiso        | GalNAcβ1-3Galα1-3Galβ1-4GlcCer |
| Lacto           | Galβ1-3GlcNAc-β1-3Galβ1-4GlcCer |
| Lactoneo        | Galβ1-4GlcNAc-β1-3Galβ1-4GlcCer |
| Muco            | Galβ1-3Galβ1-4Galβ1-4GlcCer |
| Mollu           | Manβ1-3Manβ1-1GlcCer |
| Schisto         | GalNAcβ1-1GlcCer |

aThese prefixes are used in designations of structures within each family. For example, the structure listed for the Lactoneo series is lactoneotetraglycosylceramide and that listed for the Globo series is globo-tetraglycosylceramide. Extensive discussion of nomenclature is beyond the scope of this unit; for more information, refer to Weigandt, 1985, and Machler and Sweeley, 1978.
has generated considerable information on the specific tissue and species distribution of glycosphingolipids. As more sensitive techniques have become available, glycosphingolipids, like glycoproteins, have been found to possess far greater structural variety than originally assumed. Research in this field is shifting from structural to functional analysis. The development of methods that define receptor function or immunological reactivity of glycolipids prior to their structural analysis has been important to functional studies.

The protocols in this unit are based on several procedures for analysis of glycosphingolipids extracted from mammalian tissues. In Folch partitioning, lipid extracts are partitioned into upper and lower phases based on differential solubility of glycolipids with different-sized oligosaccharide chains (Folch et al., 1957; Esselman et al., 1972; Ledeen and Yu, 1982). The extraction procedure is fundamentally that described by Svennerholm and Fredman (1980) for the quantitative extraction of gangliosides from animal tissues—a procedure selected to ensure complete extraction of glycolipids. The application of this procedure to the extraction and analysis of glycolipids and glycoproteins from the same sample was suggested by Finne and Krusius (1982), who used the method to obtain lipid-free glycopeptides. Isolation and fractionation of gangliosides by DEAE-Sephadex chromatography is adapted from Momoi et al. (1976). These protocols have been used successfully in the isolation, purification, and identification of immunologically reactive glycosphingolipids from both unlabeled (Magnani et al., 1982) and metabolically radiolabeled (Clark et al., 1991) cultured animal cells.

**Critical Parameters and Troubleshooting**

The use of metabolically radiolabeled cells as described in UNIT 17.4 permits analysis of a small number of cells to obtain significant information on the carbohydrate composition and structure of glycosphingolipids.

The success of the separation and purification procedures will be obvious upon analysis of the glycosphingolipid fractions obtained. Poor resolution of glycolipids during most chromatographic analyses is generally associated with the presence of salts in the sample or with poorly equilibrated columns. Use of the Sep-Pak C18 cartridge is the most convenient method for removal of salt from the sample (Williams and McClure, 1980).

The combined lower-phase neutral glycolipid fraction will contain non-glycolipid contaminants of the lower phase, including neutral and zwitterionic lipids such as cholesterol, lecithin, ethanolamine, phosphoglycerides, etc. For many analyses this contamination does not contribute to, or interfere with, separation or detection methods. For example, in the case of material from cells metabolically labeled with monosaccharide precursors, essentially all of the detected label will be in the sugars. Label can, however, appear in non-glycolipid components of the lower phase of a Folch extract. In extracts of Chinese hamster ovary cells, such material was tentatively identified as phosphatidylinositol (Smith et al., 1990). UNIT 17.16 describes how to determine the composition of radiolabeled monosaccharides of isolated glycolipids. When specific reactions such as sugar-specific stains or carbohydrate-specific antibodies are used to identify glycolipids on TLC plates, these contaminants will not react. If removal of the contaminants is necessary, it can be accomplished by saponification followed by chromatography on silicic acid (Lendeen and Yu, 1982). However, these procedures will destroy the base-labile substituitions of sialic acid on the gangliosides.

Contaminating phospholipids can also be removed by saponification followed by dialysis. Conditions that affect sialic acids should be considered when planning extraction and purification of gangliosides. To preserve any O-acetyl derivatives of the sialic acid residues on gangliosides, which may be very important in functional analyses, it is important to avoid the use of bases. The O-acetyl derivatives of sialic acid are extremely labile and migrate via transacylation reactions in these cases.

The plastic microcentrifuge tubes used in this unit must be resistant to chloroform-containing solvents. Most polypropylene tubes claim resistance to chloroform, but it is worth testing the tubes to be sure. Centrifugation steps involving microcentrifuge tubes should be carried out in a cold room, because heat buildup during centrifugation can increase pressure inside capped tube (containing volatile solvents) and cause them to open during centrifugation, resulting in loss of solvent and possibly sample. For large-scale preparations, and where physical methods such as mass spectroscopy are to be used for structural analyses, use a solvent-resistant apparatus and substitute glass for plastic to avoid contamination with plasticizers.
Anticipated Results
The protocols described in this section are intended to provide the investigator with methods for preparing neutral and/or acidic glycosphingolipids from cultured animal cells or small samples of tissue. Because the glycosphingolipids make up such a small amount of total cell mass, it is very difficult to obtain information related to the theoretical yield of glycolipid; however, these protocols will provide a representative sample of the neutral glycolipids and gangliosides present in the starting material. The results of each isolation step and how each sample may be used for further analysis are described throughout the unit. The final samples of neutral glycolipid and/or ganglioside are to be used for further analysis or characterization.

Time Considerations
The total extract can be obtained and dried in ~3 hr, depending upon the method used for evaporation. Folch partitioning of the total extract into upper and lower phases will require 30 to 60 min. The preparation of the DEAE-Sephadex requires an overnight equilibration step; if large amounts of resin are equilibrated and stored, only a brief wash of the resin with the starting solvent is required before each time the procedure is carried out. Preparation of a Sep-Pak C۱۸ cartridge according to the support protocol will require ~30 min. The desalting step using a Sep-Pak cartridge is somewhat cumbersome and time-consuming because multiple samples must be processed individually and the procedure requires considerable manipulation of syringe and cartridge. If multiple samples are being processed, commercial vacuum manifolds that accommodate large numbers of cartridges may be used. Several hours are required to run the DEAE-Sephadex column with a flow rate of 1 to 2 ml/min and multiple solvent systems to fractionate gangliosides. From extraction to fractionation of gangliosides can take up to 2 days. Rapid isolation of the ganglioside fraction (steps 1 to 12 of the first basic protocol) for use in the alternate protocol can be accomplished within 1 to 2 hr; the alternate protocol will produce neutral and acidic fractions within 1 day.

Literature Cited


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Metabolic Radiolabeling of Animal Cell Glycoconjugates

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ABSTRACT
Useful information about glycoconjugates can be obtained by labeling their aglycone (noncarbohydrate) portions—e.g., labeling proteins with radioactive amino acids—and then using techniques described elsewhere in this chapter to infer the presence, type, and nature of glycan chains. This unit describes metabolic labeling techniques that provide more specific information about the structure, sequence, and distribution of the sugar chains of glycoconjugates. Following metabolic labeling, the radioactive glycoconjugate of interest is isolated, individual glycosylation sites are identified and separated if necessary, and the labeled glycans are subjected to structural analysis. Curr. Protoc. Mol. Biol. 87:17.4.1-17.4.15. © 2009 by John Wiley & Sons, Inc.

Keywords: monosaccharides • radioactivity • pulse-chase • metabolic labeling • specific activity

INTRODUCTION
Useful information about glycoconjugates can be obtained by labeling their aglycone (noncarbohydrate) portions—e.g., labeling proteins with radioactive amino acids—and then using techniques described elsewhere in this chapter to infer the presence, type, and nature of oligosaccharide (glycan) chains. This unit describes metabolic labeling techniques that provide more specific information about the structure, sequence, and distribution of the glycan chains of glycoconjugates. Following metabolic labeling, the radioactive glycoconjugate of interest is isolated, individual glycosylation sites are identified and separated if necessary, and the labeled glycans are subjected to structural analysis. Although these techniques provide less information than would complete sequencing of the glycan chains, the partial structural information derived is sufficient for many purposes.

Metabolic labeling is simple and easy to perform, and requires no sophisticated instrumentation other than a scintillation counter. In addition, purification of the glycoconjugate to radiometric homogeneity is sufficient for further analysis (i.e., other contaminating molecules that are not labeled do not have to be removed). Important practical considerations for metabolic labeling experiments include selecting the type of experiment and the labeled precursor, understanding the specificity of labeling, and maximizing uptake and incorporation (see Critical Parameters). Before proceeding with experiments to label glycoconjugates metabolically, labeling conditions should be evaluated to ensure that cell viability and metabolism are not altered, and optimized for uptake and incorporation of the precursor (this should be done for each combination of precursor and cell line; see the Basic Protocol and Alternate Protocol 1).

In the Basic Protocol, cells in culture are grown through several population doublings in complete medium supplemented with radiolabeled glycan precursors to reach a steady-state level of incorporation. In the alternate protocols, cells are cultured for a short period of time in a deficient medium that contains a high concentration of radiolabeled precursor.
A pulse or pulse-chase labeling procedure (Alternate Protocol 1) can be used to analyze precursor-product relationships. With sequential pulse-labeling (Alternate Protocol 2), it is possible to obtain quantities of labeled glycoconjugates while using a minimum amount of labeled precursor by using the same batch of medium to pulse-label a series of cultures. A support protocol describes the preparation of multiply deficient medium (MDM) for use in making appropriate deficient media.

**NOTE:** Conventional protocols for handling, monitoring, shielding, and disposing of radioactivity and radioactive waste (Appendix 1F) and for tissue culture of cells and sterile handling of media should be followed throughout these protocols.

**BASIC PROTOCOL**

**STEADY-STATE LABELING WITH RADIOACTIVE PRECURSORS**

When studying glycoconjugates in an established tissue culture cell line, molecules of interest can be labeled for structural characterization. In the following protocol, cells in culture are grown to a steady-state level of incorporation in complete medium supplemented with radiolabeled glycoconjugate precursor.

**Optimizing Conditions**

An attempt should be made to label the molecules as close as possible to the metabolic steady state—i.e., a constant level of radioactivity per mass unit of a given monosaccharide in all glycoconjugates in the cell—under conditions of normal growth. In practice, this is somewhat difficult to achieve with most cell types. The maximum possible number of cells should be grown for the longest possible period of time in the minimum possible volume of complete medium supplemented with the maximum possible amount of radioactive label. Before labeling is attempted, the following growth characteristics should be determined for the cell type of interest: (1) population doubling time, (2) maximum degree of dilution upon splitting that is compatible with proper regrowth, (3) maximum cell density compatible with healthy growth and metabolism (confluence), and (4) minimum volume of medium that will sustain regrowth from a full split to confluence. If these parameters are properly defined, growth from full split to confluence will allow at least three population doublings during the labeling period for most cell lines. In some cases, however (e.g., with very slowly growing cell lines, lines requiring frequent medium changes, or cells that cannot be diluted to low density when splitting), this may not be feasible.

**Materials**

- Radioactive precursor: $^3$H- or $^{14}$C-labeled monosaccharide, $^{35}$S-sulfate, $^3$H-acetate, or $^{32}$Porthophosphate; at highest available specific activity
- Complete tissue culture medium appropriate for long-term growth of tissue culture cell line, supplemented as necessary
- Established tissue culture cell line, either suspension or monolayer
- Phosphate-buffered saline (PBS; Appendix 2), pH 7.2, ice cold
- Disposable sterile 50-ml vacuum-suction filter device: filter flask fitted with 0.22-$\mu$m filter
- Disposable 0.22-$\mu$m sterile filter attached to sterile plastic syringe, both with Luer-Lok fittings
- Sterile pipet tips
- Tissue culture plates or flasks
- Screw-cap centrifuge tubes
- Tabletop centrifuges, at room temperature and 4°C
- Rubber policeman or disposable cell scraper
- Scintillation counter
1. If necessary, dry radioactive precursor (in a ventilated hood) to completely remove any organic solvents. Dissolve in a small volume of complete tissue culture medium.

2. Filter sterilize radioactive medium, using a standard disposable 50-ml vacuum suction filter device (filter flask fitted with a 0.22-μm filter) for volumes ≥10 ml or a 0.22-μm filter attached directly to the tip of a disposable sterile syringe for smaller volumes. Wash out the original container with additional medium and use this fluid to wash through the filter (this maximizes recovery of label).

   CAUTION: Dispose of the radioactive filter and/or syringe appropriately.

   The radioactive precursor may be available in a sterile aqueous medium, ready for use. However, such preparations are more expensive, and after repeated opening of such packages, filtering is recommended to ensure continued sterility.

3. Add additional medium if necessary to bring the volume to the amount needed for the experiment. Warm the medium in an incubator or water bath to the temperature at which labeling will be done.

4. Using a sterile pipet tip, aliquot a small amount of radioactive medium and save for scintillation counting in step 8.

5. Split monolayer cells according to standard method (e.g., detach by trypsinization), resuspend in radioactive medium, and plate onto tissue culture plates. For suspension cultures, dilute directly in radioactive medium or concentrate as follows: place culture in an appropriately sized screw-cap centrifuge tube and centrifuge in a tabletop centrifuge 5 min at ∼500 × g, room temperature. Discard supernatant and resuspend cell pellet in radioactive medium. Incubate under standard conditions for the cell line being studied.

   The number of cells needed, and therefore the culture vessels and volume of medium used, will depend on the growth characteristics of the particular cell line being studied and should be optimized as described in the protocol introduction.

6. When the cells reach maximum growth (confluence or late-log phase), chill culture on ice and harvest cells, scraping monolayer cells from plate using a rubber policeman or cell scraper. Pellet cells 5 min at ∼500 × g, 4°C. Decant the labeled medium and save an aliquot for scintillation counting in step 8.

   If the glycoconjugates in the conditioned medium are to be studied, the medium should be filtered through a 0.22-μm filter to remove any cell debris remaining after the centrifugation.

7. Wash cell pellet twice in a >50-fold excess of ice-cold PBS, pH 7.2.

   At this stage, the labeled cell pellet can be frozen at −20°C to −80°C for later analysis if desired.

8. Using a scintillation counter, determine the efficiency of incorporation by measuring the radioactivity incorporated into an aliquot of the cells and the sample of conditioned medium from step 6. As a control, measure the radioactivity of the sample of sterile radioactive medium from step 4.

   It may be necessary to dilute the medium to get an accurate count.

**PULSE OR PULSE-CHASE LABELING WITH RADIOACTIVE PRECURSORS**

If steady-state labeling (see Basic Protocol) does not yield sufficient incorporation of label into the glycoconjugate of interest, it may be desirable to provide labeled precursor in the absence of, or with decreased amounts of, the unlabeled form. The pulse procedure described in this protocol—in which cells are cultured briefly in deficient medium...
supplemented with a radioactive precursor—may be used in these cases. Alternatively, the procedure can be expanded into a pulse-chase by adding a chase of nonradioactive medium; this permits brief labeling of the glycoconjugate of interest to establish precursor-product relationships. These procedures are most useful for labeling monosaccharides that compete with glucose for uptake—i.e., Gal, Glc, Man, and GlcNH₂ (see Commentary). They are of limited value for monosaccharides that are not usually taken up efficiently by cells—i.e., GlcNAc, GalNAc, ManNac, Neu5Ac, Fuc, Xyl, and GlcA—unless very large quantities of such labeled molecules can be used for pulse labeling. Because pulse labelings are typically done for a short time (e.g., minutes or hours), cells are usually used in a nearly confluent state, to maximize uptake and incorporation.

**Optimizing Conditions**

Before the experiment is performed, several pilot experiments should be carried out to determine the optimal conditions for labeling.

**Determining base incorporation level and the effect of glucose.** MDM (multiply deficient medium) appropriate for growing cultures of the cell line of interest should be reconstituted (see Support Protocol) to 100% levels of all components except the one being presented as a radiolabeled precursor or competing molecule (e.g., glucose). If required by the cells, dialyzed serum should be added to the appropriate final concentration. Finally, radiolabeled precursor should be added. A small-scale pilot labeling should be carried out by culturing cells in the labeled medium and monitoring incorporation of the label into the macromolecule of interest or into whole-cell glycoproteins. If the radiolabeled precursor is a monosaccharide, the effect of glucose on incorporation of radiolabeled precursor should be assessed by carrying out this pilot experiment in duplicate, using one lot of medium prepared as described and one prepared the same way but without glucose.

**Determining optimal concentration of radiolabeled precursor.** Enough MDM for several pilot experiments should be reconstituted to 100% levels of all components except the one being presented as a radiolabeled precursor. The radiolabeled precursor should then be added and the medium divided into aliquots. The aliquots should be supplemented with an unlabeled 100× stock solution of the same precursor to yield a series of media containing different concentrations of the unlabeled precursor (e.g., 0%, 5%, 10%, 20%, 50%, and 100% of the concentration in normal medium). These should be used for small-scale pilot labelings of the cell line of interest for defined periods of time and incorporation of label should be monitored as described above. Incorporation should be plotted against the total precursor concentration. The point at which the curve breaks—i.e., where the percentage of label incorporated is markedly decreased by further addition of unlabeled compound—is the point at which the precursor concentration is no longer limiting for biosynthetic reactions (Fig. 17.4.1). The optimal concentration of unlabeled precursor will be a little higher than the break point for the curve, where unlabeled precursor is not limiting, but incorporation is still good.

**Checking cell growth at optimal precursor concentration.** Reconstituted MDM containing radioactive precursor plus the optimal concentration of unlabeled precursor should be prepared and cells should be grown in this medium for the desired period of time. Incorporation of radiolabeled precursor should be monitored and linearity of uptake and incorporation of label over time should be assessed. Measures of cell growth, stability, and general biosynthetic capability—e.g., cell counts and viability assays and radioactive amino acid incorporation—should also be assayed to determine if reducing the concentration of the precursor into this range has any detrimental effect upon the cells. It is sometimes necessary to reach a compromise between the opposing factors of lowered concentration, labeling time, and cell viability. An attempt should be made to determine
the lowest concentration of the unlabeled precursor that can be used for the desired period of time without affecting cell growth, viability, and biosynthesis of macromolecules in general. It should be kept in mind that the use of partially deficient medium may selectively alter glycoconjugate makeup (e.g., lowering sulfate concentration too much can result in undersulfation of glycosaminoglycans in some cell types).

**Additional Materials (also see Basic Protocol)**

- Multiply deficient medium (MDM; see Support Protocol and Table 17.4.1) supplemented as appropriate
- Fetal bovine serum (FBS; Invitrogen), dialyzed (*APPENDIX 3C*) against sterile 0.15 M NaCl (glucose concentration ~250 μM)

1. Determine optimal conditions for labeling as described in the protocol introduction. Grow monolayer cells in complete medium to near confluence or suspension cells to late-log phase.

2. Based on the pilot experiments for optimizing conditions described in the protocol introduction, reconstitute MDM to prepare a stock of medium partially deficient in only the precursor of interest.

3. Add an appropriate quantity of radioactive precursor to the reconstituted MDM. Add dialyzed FBS if required for growth of the cell line being studied. Filter sterilize, warm the medium to 37°C, and reserve an aliquot of radioactive medium (see Basic Protocol, steps 2 to 4).

4. For monolayer cells, aspirate culture medium from plate; for suspension cells, microcentrifuge or centrifuge briefly and decant the supernatant medium. Add an appropriate quantity of radioactive labeling medium from step 3. Incubate cells for the desired period of time.
Table 17.4.1  Composition of Multiply Deficient Medium (MDM)

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration in complete medium</th>
<th>Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
<td>200 mg/liter</td>
<td>Salt, reagent grade</td>
</tr>
<tr>
<td>KCl</td>
<td>400 mg/liter</td>
<td>Salt, reagent grade</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>75 mg/liter</td>
<td>Salt, reagent grade</td>
</tr>
<tr>
<td>NaCl</td>
<td>6800 mg/liter</td>
<td>Salt, reagent grade</td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>140 mg/liter</td>
<td>Salt, reagent grade</td>
</tr>
<tr>
<td>Phenol red</td>
<td>10 mg/liter</td>
<td>10×</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>110 mg/liter</td>
<td>100×</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>25 mg/liter</td>
<td>100×</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>126 mg/liter</td>
<td>100×</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>50 mg/liter</td>
<td>100×</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>30 mg/liter</td>
<td>100×</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>NONEᵇ</td>
<td>—</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>75 mg/liter</td>
<td>100×</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>NONEᵇ</td>
<td>—</td>
</tr>
<tr>
<td>L-Glycine</td>
<td>50 mg/liter</td>
<td>100×</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>42 mg/liter</td>
<td>100×</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>52 mg/liter</td>
<td>100×</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>52 mg/liter</td>
<td>100×</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>72 mg/liter</td>
<td>100×</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>NONEᵇ</td>
<td>—</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>32 mg/liter</td>
<td>100×</td>
</tr>
<tr>
<td>L-Proline</td>
<td>40 mg/liter</td>
<td>100×</td>
</tr>
<tr>
<td>L-Serine</td>
<td>NONEᵇ</td>
<td>—</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>48 mg/liter</td>
<td>100×</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>10 mg/liter</td>
<td>100×</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>36 mg/liter</td>
<td>100×</td>
</tr>
<tr>
<td>L-Valine</td>
<td>46 mg/liter</td>
<td>100×</td>
</tr>
<tr>
<td>MEM vitamins (mixture)</td>
<td>1×</td>
<td>100×</td>
</tr>
</tbody>
</table>

ᵃDo not use MgSO₄ in place of MgCl₂.
ᵇNot present in MDM; add as needed for specific experiments.

Labeling time should be based on the length of time the cells can survive in deficient medium as determined in the optimizing pilot experiment.

Save radioactive medium for analysis, if necessary.

5. For pulse labeling, proceed to step 6. For pulse-chase labeling, remove medium as described in step 4, add a chase of nonradioactive complete medium, and incubate for the desired period of time.

For a pulse-chase experiment, several vessels of each cell culture should be set up and chased for varying periods of time.

6. Harvest and wash the cells. Determine efficiency of incorporation (see Basic Protocol, steps 6 to 8).
In some cases, incorporation of label into the glycoconjugate of interest may be inadequate, even under defined conditions with selectively deficient medium. In addition, prolonged exposure to deficient medium may result in alterations in synthesis of proteins or other macromolecules. In these situations, it may be desirable to expose a series of plates of cells sequentially to a small volume of medium containing a high concentration of label. This also conserves expensive radiolabeled precursor. In this protocol, multiple sets of cell cultures are sequentially pulse-labeled. One set is incubated in labeling medium for the pulse period, and then the labeling medium is removed and added to the next set. For a pulse experiment (designed to obtain a sizable quantity of material with a high level of incorporation), the cells are harvested at this point; for a pulse-chase, a chase of nonradioactive complete medium is added to the first set of cultures. This process is repeated until all cells have been labeled (Fig. 17.4.2). Because the incubation period is relatively short (e.g., a few hours), it is assumed that only a very small fraction of the radiolabeled precursor is consumed from the medium during each labeling cycle and that the concentrations of other essential components are not substantially changed. To further conserve labeled medium, some investigators freeze it after limited usage and thaw it to reuse (the pH of thawed medium should be adjusted before use). When reusing the medium, the experimental results should be interpreted carefully, especially with regard to the specific activity of labeled products and the possibility of metabolic effects of secreted molecules.

Additional Materials (also see Basic Protocol)

- Multiply deficient medium (MDM; see Support Protocol and Table 17.4.1), supplemented as appropriate
- Fetal bovine serum (FBS; Invitrogen), dialyzed (APPENDIX 3C) against sterile 0.15 M NaCl (glucose concentration ~250 μM)

1. Determine optimal conditions for labeling (see Alternate Protocol 1 introduction). Set up several identical sets of monolayer or suspension cells to be labeled sequentially and grow to confluence or late-log phase.

* Transfer labeling medium to next plate. Add complete medium to labeled cells.

**Figure 17.4.2** Scheme for sequential pulse labeling of cells reutilizing radioactive medium.
For suspension cultures in which it is possible to use <1 ml labeling medium, cells may be labeled in tightly capped 1.5-ml screw-cap microcentrifuge tubes and incubated at 37°C on a rotating end-over-end mixer.

2. Prepare radioactive labeling medium and label the first set of cell cultures for the appropriate length of time (see Alternate Protocol 1, steps 2 to 4).

   Labeling time will vary from minutes to hours, depending upon the cell type and the objective of the labeling.

3. For monolayer cells, aspirate the radioactive medium from the first set of cultures; for suspension cells, microcentrifuge briefly (~10 sec at top speed) and decant the supernatant radioactive medium. Transfer the radioactive medium to the second set of cultures.

4. For pulse labeling, harvest cells from first set of cultures. For pulse-chase labeling, add a chase consisting of an adequate quantity of nonradioactive complete culture medium to the first set of cultures and continue incubating.

5. Repeat transfers until all sets of cells have been labeled.

6. Harvest all plates. For pulse labeling, pool all the pellets together. For pulse-chase labeling, keep the individual cell pellets separate (see Fig. 17.4.2).

**Support Protocol**

**Preparation and Supplementation of Multiply Deficient Medium (MDM)**

Some types of selectively deficient media are available commercially at reasonable prices, and some companies will custom-prepare selectively deficient media on request. For a laboratory where labeling experiments with a variety of deficient media are frequently carried out, it is convenient to prepare a basic multiply deficient medium (MDM) that is completely lacking in several commonly studied components. This medium can be used to make up different selectively deficient media as needed. The following MDM, based on α-MEM medium (Invitrogen), supports growth of most tissue culture cells.

For labeling experiments, MDM can be reconstituted with 3H- or 14C-labeled monosaccharides, [35S]sulfate, 35S-labeled methionine or cysteine, or [3H]serine. If desired, other specific components can also be omitted and replaced with their radiolabeled forms.

**Note:** Tissue culture-grade reagents (including distilled, deionized water) should be used in making MDM. To ensure that reagents do not become contaminated by compounds such as endotoxin as portions are being removed from stock bottles, it is important to pour liquids carefully and use disposable spatulas for removing solids. For the same reason, clean glassware must be used; preferably, a set of glassware should be set aside for this purpose. 100× stock solutions of many components can be purchased commercially in sterile form, or may be made up from solids and filter sterilized individually.

**Additional Materials** (also see Basic Protocol)

- Stock solutions for multiply deficient medium (MDM; Table 17.4.1)
- 100× stock solutions for reconstituting MDM (Table 17.4.2)
- 50-ml tubes

1. Based on the experiment to be performed, determine appropriate components for MDM.

2. Make up MDM according to the ingredient list in Table 17.4.1 by dissolving salts and phenol red one by one in ~800 ml water, adding sufficient pyruvate, amino acid, and vitamin stocks for a 1× final concentration in 1 liter, and adding water to 1 liter.
Table 17.4.2  Stock Solutions for Reconstitution of MDM

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration in complete medium</th>
<th>Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO₃&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1 × (2200 mg/liter)</td>
<td>100×</td>
</tr>
<tr>
<td>HEPES·HCl</td>
<td>20 mM (4.76 g/liter)</td>
<td>2 M, pH 7.3</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>0.81 mM (115 mg/liter)</td>
<td>100 mM, sterile</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>1 × (1000 mg/liter)</td>
<td>100×</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>1 × (100 mg/liter)</td>
<td>100×</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>1 × (292 mg/liter)</td>
<td>100×</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>1 × (15 mg/liter)</td>
<td>100×</td>
</tr>
<tr>
<td>L-Serine</td>
<td>1 × (25 mg/liter)</td>
<td>100×</td>
</tr>
</tbody>
</table>

<sup>a</sup> Individual components are added to MDM at full strength, or lowered concentration or may be left out altogether, depending upon the experiment planned.

<sup>b</sup> Use either NaHCO₃/CO₂ or HEPES·HCl (not both); these control the pH of the final medium.

3. Filter sterilize. Do not adjust the pH. Freeze 25-ml aliquots in 50-ml tubes at −20°C. *The solution will be yellow.*

4. Add appropriate components (from those listed in Table 17.4.2) to reconstitute MDM for the labeling experiment.

*The following example describes the selective reconstitution of MDM with missing components. It is a recipe for 50 ml of a supplemented MDM for optimal labeling of endothelial cells with [³⁵S]sulfate and [⁶-³H]glucosamine, which requires a medium with no cysteine, low glucose, and low concentrations of methionine and sulfate (see Roux et al., 1988, for detailed rationale). Stock solutions are as listed in Table 17.4.2.*

To 50 ml MDM, add:

- 500 μl 100× NaHCO₃ stock solution
- 500 μl 100× glutamine stock solution
- 500 μl 100× serine stock solution
- 50 μl 100× glucose stock solution (200 mg/liter final)
- 10 μl 100 mM inorganic sulfate stock solution (20 μM final)
- 50 μl 100× methionine stock solution (0.1× final).

*Just before use, add 0.5 mCi each of [³⁵S]sulfate and [⁶-³H]glucosamine and mix. Filter sterilize directly into culture vessel. The total volume of reconstituted medium will be slightly more than 50 ml; for practical purposes, this minor discrepancy can be ignored. The medium should be orange-red, indicating the correct pH range.*

**COMMENTARY**

**Background Information**

If sufficient quantities of pure molecules (i.e., in the nanomole range) are available, complete sequencing of oligosaccharides is best performed by conventional techniques not requiring radioactivity. However, isolation of sufficient quantities of material may not be practical (e.g., in analysis of biosynthetic intermediates or rare molecules). Alternatively, the biological questions underlying the experiment may be adequately answered by less definitive means of structural analysis. In these cases, metabolic labeling with radioactive sugars or donors that can transfer label to sugars may provide sufficient structural information about the glycan chains (see Cummings et al., 1989).

Sugar chains of glycoconjugates can be successfully labeled by short-term labeling experiments using radioactive precursors.
Metabolic Radiolabeling of Animal Cell Glycoconjugates

17.4.10

Tabas and Kornfeld, 1980; Goldberg and Kornfeld, 1981; Roux et al., 1988; Muchmore et al., 1989). Although sugar nucleotides are the immediate donors for glycosylation reactions, they cannot be taken up by cultured cells. Hence, metabolic labeling of sugar chains is accomplished by providing the cells with radiolabeled monosaccharides (Yurchenco et al., 1978; Yanagishita et al., 1989; Varki, 1991). These are taken up by the cells, activated to sugar nucleotides, and transported into the Golgi apparatus, where lumenerally oriented transferases add the monosaccharides to lumenerally oriented acceptors (Hirschberg and Snider, 1987). The few known exceptions to this topology are cytosolic and nuclear forms of glycosylation (Hart et al., 1989).

For glycans that contain modifications—such as sulfate, acetate, and phosphate ester groups—an alternative metabolic labeling technique is to use a $[^{35}S]$sulfate, $[^3H]$acetate, or $[^{32}P]$orthophosphate label, respectively.

Although metabolic labeling can provide useful information regarding glycoprotein glycans, there are significant limitations to its use. First, it is difficult to determine when true steady-state labeling of a cell is reached (as a rule of thumb, 3 to 4 doublings are usually assumed to be sufficient); thus the numerical ratio between labeled glycoconjugates may not reflect steady-state ratios. Second, individual precursors show greatly differing uptake and incorporation in different cell types. Third, almost all labeled precursors are only partially specific for certain monosaccharides, and the degree of this specificity can vary depending on cell type. Fourth, although lowering glucose concentration improves the incorporation of monosaccharides that compete with glucose for uptake, the low glucose supply may also directly affect oligosaccharide precursors in some cell types.

Critical Parameters

As diagrammed in Figure 17.4.3, the labeling protocol most suitable for a particular study depends on several different considerations. Some of these arise from the objectives of the study and must be investigated by performing pilot experiments to determine optimal conditions (see Optimizing Conditions sections of the Basic Protocol and Alternate Protocol 1).

General considerations

If the goal of a study is to obtain labeled material for structural identification and partial characterization, the yield of radioactivity should be maximized by either steady-state or pulse labeling. For quantitating the relative mass of a molecule in two different samples or comparing the masses of two molecules in the same sample, the oligosaccharide should be labeled to constant specific activity—i.e., steady-state distribution (although this may not always be achievable). For establishing precursor-product relationships between molecules, a pulse-chase protocol should be used. If the radioactive precursor is expensive, a sequential procedure may be employed in pulse and pulse-chase experiments to minimize the quantity needed.

Selecting a labeled precursor

Selection of the labeled precursor to be used is based upon several factors, including efficiency of uptake (see section on Factors affecting uptake and incorporation) and type of glycoconjugate to be labeled. In most cases, the precursor will be a monosaccharide; for a glycan chain containing one or more ester modifications of sugars, it is also possible to use a precursor such as acetate, sulfate, or orthophosphate.

Factors affecting specificity and final distribution. In selecting a monosaccharide precursor, it should be noted that the distribution of monosaccharides among different types of vertebrate glycans is nonrandom (Table 17.4.3). It is also important to consider the metabolic pathways for uptake, activation, utilization, and interconversion of the various monosaccharides and their nucleotide sugars, which have been studied extensively (Fig. 17.4.4 and Table 17.4.4). The final distribution and specific activity of label from a given radiolabeled monosaccharide can be significantly affected by dilution from an unlabeled compound generated by endogenous pathways, the characteristics of the particular cell type being labeled, and the labeling conditions (Kim and Conrad, 1976; Yurchenco et al., 1978; Yanagishita et al., 1989). The exact position of the tritium label within a monosaccharide can affect the label’s ultimate fate (Diaz and Varki, 1985). Interconversion between monosaccharides (e.g., conversion to glucose; Fig. 17.4.4), which can be expected to occur with prolonged labeling, will eventually result in labeling of non-glycan components. An exception is labeling with $[2^{-}^{3}H]$mannose: this is extremely specific, because conversion of the labeled mannose can yield only $[2^{-}^{3}H]$fucose or unlabeled fructose-6-phosphate. In the latter case, the label is lost as tritiated...
determine type of study (structural or biosynthetic)  
choose labeling protocol  
check incorporation of radioactivity in complete medium  
if sufficient for analysis  
try using more labeled precursor  
if insufficient for analysis  
decrease level of unlabeled precursor in medium  
check effects on incorporation of label  
check effects on cells and molecule(s) of interest  
select optimal concentration of unlabeled precursor  
proceed with steady-state, pulse, pulse-chase, or sequential transfer protocols for labeling

Figure 17.4.3  Strategy for planning metabolic labeling of animal cell glycoconjugates.

Factors affecting uptake and incorporation. With radioactive amino acids, high-specific-activity labeling can be obtained by omitting the unlabeled molecule from the medium. Labeling with radioactive sugars or other glycan precursors, however, is usually less efficient. Thus, incorporation into the glycoconjugate of interest must be optimized empirically, taking into consideration the following factors:

Experimental parameters. Amount of label, concentration of label in the medium, cell number, duration of labeling, and number of cell doublings during labeling are obviously important. The goal is to expose the maximum number of cells to the maximum amount of label in the minimum volume of medium for the longest period of time. Some of these factors are at odds with one another, and the correct balance must be tailored to the particular cell type and the question being investigated.
### Table 17.4.3 Distribution of Monosaccharides and Modifications in Glycoconjugates

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Type of glycoconjugate</th>
<th>N-GlcNAc-linked glycoprotein</th>
<th>O-GalNAc-linked glycoprotein</th>
<th>Xylose-linked proteoglycan</th>
<th>Glycosphingolipid</th>
<th>Glyco-phospholipid anchor</th>
<th>O-linked GlcNAc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man</td>
<td></td>
<td>+++</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Fuc</td>
<td></td>
<td>++</td>
<td>+</td>
<td>+/−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Gal</td>
<td></td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+/− (side chain)</td>
<td>−</td>
</tr>
<tr>
<td>Glc</td>
<td>(precursor)</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>GlcNAc</td>
<td></td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>(free amine)</td>
<td>++</td>
</tr>
<tr>
<td>GalNAc</td>
<td></td>
<td>+/−</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+/− (side chain)</td>
<td>−</td>
</tr>
<tr>
<td>Sia</td>
<td></td>
<td>+++</td>
<td>+++</td>
<td>−</td>
<td>+++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>GlcA</td>
<td></td>
<td>+?</td>
<td>−</td>
<td>+++</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>SO₄</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>P₄ esters</td>
<td></td>
<td>Man-6-P</td>
<td>Xyl-P</td>
<td>M₆P (in core)</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>O-Acetyl</td>
<td></td>
<td>Sia-OAc</td>
<td>Sia-OAc</td>
<td>Sia-OAc</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Acyl</td>
<td></td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+/− (on inositol)</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

*The relative distribution of the different monosaccharides and modifications in the commonly occurring glycoconjugates, indicated by a relative scale from + to ++++, is generally valid over many cell types. However, an uncommon monosaccharide may be commonly found in some cell types or glycoconjugate (e.g., most pituitary glycoprotein hormones have GalNAc as a major component of their N-linked oligosaccharides). Other symbols: +?, possible distribution; +/−, variably present; −, not identified to date.

### Table 17.4.4 Cellular Monosaccharides Labeled with Radioactive Precursors

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>[³H]Man</td>
<td>+++</td>
<td>+/−</td>
<td>+/−</td>
<td>+</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>[³H]Fuc</td>
<td>+++</td>
<td>+/−</td>
<td>+/−</td>
<td>+</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>[³H]Gal</td>
<td>−</td>
<td>++++</td>
<td>++++</td>
<td>+++</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>[³H]GlcA</td>
<td>−</td>
<td>−</td>
<td>++</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>[³H]Glc</td>
<td>−</td>
<td>++</td>
<td>++</td>
<td>++++</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>[³H]GlcNAc</td>
<td>−</td>
<td>+/−</td>
<td>+/−</td>
<td>++</td>
<td>++++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>[³H]GalNAc</td>
<td>−</td>
<td>+/−</td>
<td>+/−</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>[³H]Sia</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>++</td>
<td>++++</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

*The extent of conversion from the original monosaccharide into minor pathways will vary considerably, depending upon the cell type studied, and the length of the labeling (longer time periods tend to result in more conversion into other monosaccharides). The extent of incorporation is indicated by a relative scale from + to ++++. Other symbols: +/−, variable low-level incorporation; −, not identified to date.

### Competition with glucose

When labeling using a radioactive monosaccharide precursor, it is important to determine whether the monosaccharide competes with glucose for uptake. The high concentration of glucose in normal tissue culture medium (~5 mM) means that monosaccharides competing with glucose for active transport cannot be taken up very well. Thus, lowering glucose concentration can improve uptake. On the other hand, monosaccharides that do not compete with glucose seem to be taken up only by
inefficient noncompetitive, passive mechanisms; thus, glucose concentration has little effect upon their already poor uptake. Experience indicates that glucosamine, galactosamine, galactose, and mannose can compete with glucose for uptake into most cells, whereas N-acetylglucosamine, N-acetylmannosamine, mannosamine, fucose, and xylose do not (Yurchenco et al., 1978). However, members of the glucose transporter family of gene products are tissue-specific in expression; a given cell type may express more than one, each with distinctive kinetic and stereospecific uptake properties (Gould and Bell, 1990). Thus, each monosaccharide must be tested in the specific cell type under study to see if it competes with glucose. A final point that should be considered is that in some cell types, lipid-linked oligosaccharide precursors may become altered when glucose-free medium is used (Rearick et al., 1981).

Other intracellular metabolic factors. Dilution of label by endogenously synthesized monosaccharides, pool size of individual monosaccharides and nucleotides, and flux rates between interconverting pathways can all affect the final specific activity and distribution of the label. If cells are cultured in labeling medium for prolonged periods of time, the medium may become depleted of glucose (Kim and Conrad, 1976; Varki and Kornfeld, 1982). The specific activity of the labeled sugar nucleotides in the cells may then actually rise as the medium glucose concentration

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**Figure 17.4.4** Cytosolic pathways for the interconversion of monosaccharides and their nucleotide sugar forms.
Glycoconjugates
Radiolabeling of Animal Cell Metabolic
Supplement 87 Current Protocols in Molecular Biology

17.4.14

Determining the specific activity of incorporated label

In most instances, the precise specific activity of each monosaccharide pool need not be determined. If steady-state labeling is attempted, a plateau in the rate of incorporation of label per milligram of cell protein can be taken as a rough indication that a steady state has been reached. In some cases, however, the precise specific activity of a given monosaccharide may be of interest. Discussions of how this can be determined can be found in Kim and Conrad (1976), Yurchenco et al. (1978), Yanagishita et al. (1989), and Varki (1991). Endogenous glucose is the normal precursor for hexoses and hexosamines (see Fig. 17.4.4). Experimental manipulations that alter the concentration of glucose can thus alter the concentration of the internal pool of other hexoses and hexosamines. Exogenously added labeled hexosamines become diluted within the cell, making specific radioactivity in the cell lower than that of the starting material.

With the advent of high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD), measurement of monosaccharide levels in the low picomole range is now possible. Thus, it is now feasible to measure the specific activity of a labeled monosaccharide component of a purified glycan by acid hydrolysis of a portion of the glycan (Hardy et al., 1988) followed by HPAE-PAD. High-performance liquid chromatography (HPLC)–based methods for accurate measurement of low quantities of sulfate are also now available.

Troubleshooting

Ideally, the radiochemical purity of the precursor should be checked using an appropriate chromatographic procedure. As with all tissue culture work, cells should be checked for mycoplasma infection (Fitch, 1997) before the experiments are carried out. If bacterial contamination poses a problem for long-term labeling experiments, appropriate antibiotics should be added to the medium.

Anticipated Results

Levels of incorporation will be highly variable, depending upon the cell type, the culture conditions, and the precursor used. If adequate incorporation into the glycoconjugate of interest is obtained, analyses can be carried out.

Time Considerations

Optimizing conditions for uptake and incorporation of label for each precursor and cell line combination (introductions to the first two protocols) may take 1 to 2 weeks, but they should not be omitted: the time will be well-spent. The time required for the actual labeling protocols will vary widely depending on the goal of the experiment. Preparation of MDM can take an entire day, especially if some components must be prepared from scratch. If the amount required for a series of experiments is planned ahead of time, however, this need only be done occasionally. Reconstituting MDM takes up to 1 hr (assuming all the stock solutions are already available).
Literature Cited


Chemical Labeling of Carbohydrates by Oxidation and Sodium Borohydride Reduction

This unit describes a collection of methods for chemical labeling of carbohydrates—free oligosaccharides or oligosaccharides conjugated to proteins, peptides, or lipids—by oxidation followed by reduction or by direct reduction. Oligosaccharides can be labeled with either radioisotopes or nonradioactive fluorescent molecules. These labelings allow one to follow the oligosaccharides during chromatography and in cells if labeled by fluorescent molecules.

Selective oxidation with mild periodate followed by reduction with tritiated sodium borohydride (NaB\(^{3H}\)) results in selective radiolabeling of sialic acid residues on oligosaccharides or glycoproteins (first basic protocol). Alternatively, treatment of samples with galactose oxidase (first alternate protocol) results in oxidation of galactose or N-acetylgalactosamine residues at nonreducing termini, rendering these residues susceptible to labeling with NaB\(^{3H}\). Oxidized glycoconjugates can also be labeled using the fluorescent probe lucifer yellow CH (second alternate protocol). Free oligosaccharides can be labeled by reduction with NaB\(^{3H}\) (second basic protocol). The third alternate protocol describes the release and simultaneous labeling of O-glycan oligosaccharides by alkaline beta-elimination in the presence of NaB\(^{3H}\).

**CAUTION:** This procedure should be performed only by personnel trained in the proper use of \(^3\)H isotope and in NRC-licensed sites. Standard precautions to prevent excessive exposure and radioactive contamination of personnel and equipment should be followed at all times.

**CAUTION:** All NaB\(^{3H}\) manipulations must be performed in a well-vented fume hood.

### BASIC PROTOCOL

**RADIOLABELING OLIGOSACCHARIDES AFTER MILD PERIODATE OXIDATION**

This protocol describes radiolabeling oligosaccharides by mild periodate oxidation followed by reduction with NaB\(^{3H}\), selectively labeling sialic acid residues at nonreducing termini. The reaction is illustrated in Figure 17.5.1. Labeled oligosaccharides are precipitated and separated from unincorporated label by gel-filtration chromatography.

**Materials**

For recipes, see Reagents and Solutions in this unit (or cross-referenced unit); for common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.

- Oligosaccharide or glycopeptide containing sialic acids, desalted
- 0.2 mM sodium periodate (prepare fresh at room temperature and keep on ice)
- 10 mM sodium acetate, pH 5.5
- 10 mM glycerol (store at 4°C)
- 0.2 M sodium borate buffer, pH 9.5 (UNIT 11.16; adjust quantities of boric acid and 10 M NaOH appropriately)
- 0.2 M sodium borate buffer, pH 9.5 (UNIT 11.16; adjust quantities of boric acid and 10 M NaOH appropriately)
- 0.5 mCi/μl tritiated sodium borohydride (NaB\(^{3H}\); 25 to 30 Ci/mmol) in 0.01 M NaOH (see recipe)
- 0.2 M sodium borohydride (NaBH\(_4\))/0.2 M sodium borate buffer, pH 9.5
- 1 M acetic acid in methanol
- Nitrogen (N\(_2\)) stream
- Methanol

---

Contribution by Minoru Fukuda

Current Protocols in Molecular Biology (1994) 17.5.1-17.5.8

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7-ml conical glass (Pyrex) test tubes with Teflon-covered caps

1.0 × 40-cm Sephadex G-15 or G-25 column in water (UNIT 10.9), calibrated to identify void volume

25° to 30°C water bath

CAUTION: Steps 3 to 8 must be performed in a well-vented fume hood to prevent radioactive contamination. Excess NaB\[^{3}\text{H}\] should be stored away in a radioactive waste bottle for appropriate disposal.

**Label oligosaccharides**

1. Mix the following in a 7-ml conical glass test tube (200 µl total volume):
   - 5 to 500 µg oligosaccharide or glycopeptide
   - 100 µl 0.2 mM sodium periodate
   - 100 µl 10 mM sodium acetate.

   Loosely cover with Teflon-covered cap and incubate 20 min on ice.

2. Add 10 µl of 10 mM glycerol and incubate 20 min at 37°C.

   *The glycerol consumes any excess periodate that remains.*

3. Add 100 µl of 0.2 M sodium borate buffer and 10 µl of 0.5 mCi/µl NaB\[^{3}\text{H}\] (5 mCi). Incubate 1 hr at room temperature in a fume hood.

4. Add 100 µl of 0.2 M NaBH\(_4\)/0.2 M borate buffer and incubate 30 min at room temperature.

**Destroy excess NaB\[^{3}\text{H}\] and remove borate as methyl borate**

5. Add 3 ml of 1 M acetic acid in methanol and dry under a nitrogen stream in a 25° to 30°C water bath, in a well-vented hood.

6. Repeat step 5.

7. Add 3 ml methanol (without acetic acid) and dry sample as in step 5.

---

**Figure 17.5.1** Labeling oligosaccharides by periodate oxidation followed by NaB\[^{3}\text{H}\] reduction.

**Chemical Labeling of Carbohydrates**

17.5.2

Supplement 50
**Purify labeled oligosaccharides**

8. Dissolve sample in \( \sim 200 \mu l \) water and apply to a calibrated 1.0 \( \times \) 40–cm Sephadex G-15 or G-25 column. Elute with water and collect 1-ml fractions with a fraction collector.

   *This step separates the labeled oligosaccharides from the low-molecular-weight breakdown products of NaB\(^{3H}\).*

9. Count 10-\( \mu l \) aliquots of each fraction in a scintillation counter.

10. Pool and dry fractions that contain radioactive oligosaccharides or glycopeptides. Store at \(-20^\circ C\) until used (the label is stable for \( >12 \) months).

   *Oligosaccharides or glycopeptides should elute at or near the void volume, followed by a larger peak of breakdown products of NaB\(^{3H}\). Be careful to avoid the second peak.*

**RADIOLABELING OLIGOSACCHARIDES AFTER GALACTOSE OXIDASE TREATMENT**

Oxidation with galactose oxidase and reduction with NaB\(^{3H}\) specifically labels galactose and N-acetylgalactosamine residues that are located at nonreducing termini of oligosaccharides. Residues at internal positions are also oxidized and labeled, but much less efficiently. This reaction is illustrated in Figure 17.5.2. Sialic acid attached to galactose hinders oxidation by galactose oxidase. If desired, sialic acid can be removed by treating the sample with sialidase (neuraminidase).

**Additional Materials**

For recipes, see *Reagents and Solutions* in this unit (or cross-referenced unit); for common stock solutions, see *APPENDIX 2*; for suppliers, see *APPENDIX 4*.

- 1 U/\( \mu l \) galactose oxidase (partially purified, Sigma; store aliquots in PBS at \(-20^\circ C\))
- 10 mM sodium phosphate buffer, pH 7.0
- 15-ml conical glass centrifuge tube

**CAUTION:** Steps 2 to 4 must be performed in a well-vented fume hood to prevent radioactive contamination.

![Figure 17.5.2](image-url)  
*Figure 17.5.2* Labeling oligosaccharides by galactose oxidase treatment followed by NaB\(^{3H}\) reduction.
1. Mix the following in a 15-ml conical centrifuge tube (110 µl total volume):
   - 50 to 500 µg oligosaccharide or glycopeptide
   - 10 µl 1 U/µl galactose oxidase
   - 100 µl 10 mM sodium phosphate buffer.

   Incubate 2 hr at 37°C.

   *Sialylated oligosaccharides or glycopeptides should be treated with sialidase (UNIT 17.12) before treatment with galactose oxidase because sialic acid hinders oxidation by galactose oxidase. If the sample is treated with sialidase, either the sample should be desalted or the volume of the galactose oxidase reaction should be increased to ensure that the pH is 7.0.*

2. Add 100 µl of 0.2 M sodium borate buffer and 10 µl of 0.5 mCi/µl NaB[3H]₄ (5 mCi). Incubate 1 hr at room temperature in a fume hood.

3. Add 100 µl of 0.2 M NaBH₄/0.2 M sodium borate buffer and incubate 1 hr at room temperature.

4. Acidify, dry, and purify labeled oligosaccharides or glycopeptides as in steps 5 to 10 of the first basic protocol.

**ALTERNATE PROTOCOL**

**FLUORESCENCE LABELING OLIGOSACCHARIDES AFTER PERIODATE OR GALACTOSE OXIDASE TREATMENT**

Following oxidation, oligosaccharide-containing samples may be labeled with the non-isotopic fluorescent probe lucifer yellow CH, instead of being radioactively labeled with NaB[3H]₄. This technique can be used to label oligosaccharides on glycopeptides or glycolipids. Labeled oligosaccharides are separated from unincorporated dye by gel-filtration chromatography.

**Additional Materials**

For recipes, see *Reagents and Solutions* in this unit (or cross-referenced unit); for common stock solutions, see *APPENDIX 2*; for suppliers, see *APPENDIX 4*.

- Phosphate-buffered saline (PBS; *APPENDIX 2*)
- Lucifer yellow CH (Aldrich)
- 0.1 M sodium cyanoborohydride (NaBH₃CN)
- 0.5 × 15–cm Sephadex G-15 and 1.0 × 25–cm Sephadex G-25 columns in 25°C water bath

**Oxidize oligosaccharides**

1. Oxidize oligosaccharide or glycopeptide sample (steps 1 to 2 of the first basic protocol or step 1 of the first alternate protocol).

2. Apply sample to a 0.5 × 15–cm Sephadex G-15 column and elute with water. Collect 0.5-ml fractions.

3. Pool and dry fractions that contain the oxidized oligosaccharides or glycopeptides.

   *The appropriate fractions should be identified based on column calibration with glycopeptide standards (see UNIT 10.9). Oligosaccharides of glycopeptides elute at or near the void volume.*

4. Dissolve sample in 100 µl PBS.
Label oligosaccharides with fluorescent dye
5. Add lucifer yellow CH to 5 mM final concentration and incubate 12 hr at room temperature.

6. Apply reaction mixture to a 1.0 × 25–cm Sephadex G-25 column. Elute with water and collect 1-ml fractions. Wash until the unincorporated dye elutes.

Purify labeled oligosaccharides
7. Pool and dry fractions containing first fluorescent peak (labeled oligosaccharides).

It may be necessary to read UV absorbance or fluorescence of the eluted fractions, as fluorescence is not easily visible.

8. Dissolve sample in 0.1 ml of 0.1 M NaBH$_3$CN and incubate 15 min at 25°C. Immediately apply to the same Sephadex G-25 column used in step 6 and elute with water (avoid acidic conditions).

9. Collect, pool, and dry fluorescent oligosaccharides—i.e., the fractions at or near the void volume. Avoid light exposure as much as possible after this point.

Fractions that contain fluorescently labeled oligosaccharides or glycopeptides should elute in the void volume.

Remaining solutions should be discarded as toxic waste.

10. Dissolve sample in a minimal amount of water and store at −20°C until used.

BASIC PROTOCOL
RADIOLABELING FREE OLIGOSACCHARIDES
Free oligosaccharides can be obtained from Asn-linked oligosaccharides by hydrazinolysis (UNIT 17.15) or PNGaseF (N-glycanase) treatment (UNIT 17.13). In the following procedure, free oligosaccharides are purified by a simple gel filtration and then directly reduced with NaB[³H]₄. After reduction, the aldehyde group at C-1 of the reducing terminus becomes an alcohol with one radioactive tritium (see Fig. 17.5.2). Introduction of ³H at the reducing terminal allows the oligosaccharides to be followed by monitoring radioactivity.

Materials
For recipes, see Reagents and Solutions in this unit (or cross-referenced unit); for common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.

Free oligosaccharides (UNITS 17.15 & 17.12)
0.3 M sodium borate buffer, pH 9.5 (UNIT 11.16; adjust quantities of boric acid and 10 M NaOH appropriately)
0.5 mCi/µl tritiated sodium borohydride (NaB[³H]₄; 25 to 35 Ci/mmol) in 0.05 M NaOH (see recipe)
1 M sodium borohydride (NaBH₄)
1 M acetic acid in methanol
Nitrogen (N₂) stream
Methanol
7-ml conical glass (Pyrex) tubes
0.5 × 25–cm Sephadex G-25 or G-15 column (UNIT 10.9), calibrated to determine void volume
Additional reagents and equipment for gel-filtration chromatography (UNIT 10.9)

CAUTION: Steps 4 to 8 must be performed in a well-vented fume hood to prevent radioactive contamination.
1. Desalt 1 to 500 µg free oligosaccharides by gel-filtration chromatography on a calibrated 0.5 × 25–cm Sephadex G-25 or G-15 column. Elute in water. Collect 0.5-ml fractions.

   Oligosaccharides must be free from peptides or amino acids that may incorporate label during reduction.

2. Pool and dry fractions that contain free oligosaccharides. Dissolve in 50 µl water.

3. Add 0.3 M sodium borate buffer and water to a 100-µl final volume and a 0.2 M-sodium borate final concentration.

   If oligosaccharides are dissolved in water, volume and concentration of buffer should be adjusted to give the desired final concentration.

4. Immediately add 10 to 20 µl of 0.5 mCi/µl NaB[3H]₄ in 0.05 M NaOH (5 to 10 mCi) and incubate 2 hr at room temperature in a fume hood.

5. Add 100 µl of 1 M NaBH₄ and incubate 2 hr at room temperature to complete the reduction.

6. Add 3 ml of 1 M acetic acid in methanol and dry under a nitrogen stream in a 30° to 35°C water bath.

7. Repeat step 6.

8. Add 3 ml methanol and dry under a nitrogen stream in a 30° to 35°C water bath.

9. Dissolve sample in 50 to 200 µl water and store at −70°C until used.

### ALTERNATE PROTOCOL

**RADIOLABELING O-GLYCAN OLIGOSACCHARIDES**

During release from glycoproteins or glycopeptides by beta-elimination, the reducing termini of O-glycans can be labeled by alkaline borohydride treatment in the presence of a large amount of NaB[3H]₄. The reaction, however, must be carried out in the presence of 1 M NaBH₄ to prevent oligosaccharide degradation after release.

### Additional Materials

For recipes, see Reagents and Solutions in this unit (or cross-referenced unit); for common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.

- 100 to 1000 µg glycopeptides or ≤5 mg glycoproteins
- 0.1 to 0.2 mCi/µl tritiated sodium borohydride (NaB[3H]₄; 25 to 30 Ci/mmol) in 1 M NaBH₄/0.05 M NaOH (see recipe)
- 1 M acetic acid in methanol
- Methanol
- 30° to 35°C and 45°C water baths

**CAUTION:** Steps 2 to 4 must be performed in a well-vented fume hood to avoid radioactive contamination.

1. Place 100 to 1000 µg glycopeptides or ≤5 mg glycoproteins in a 7-ml conical glass tube. Add 200 µl of 0.1 to 0.2 mCi/µl NaB[3H]₄ in 1 M NaBH₄/0.05 M NaOH (20 to 40 mCi). Incubate 24 to 48 hr at 45°C in a fume hood.

   For larger amounts of sample, the volume of solution should be increased to dissolve sample completely and the amount of Na[3H]₄ should be increased to 100 mCi.

2. Add 3 ml of 1 M acetic acid in methanol and dry sample under a nitrogen stream in a 30° to 35°C water bath.
3. Repeat step 2.
4. Add 3 ml methanol and dry under a nitrogen stream in a 30° to 35°C water bath.
5. Dissolve sample in 100 to 200 µl water and store at −20°C until used.

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.

Tritiated sodium borohydride (NaB[3H]4), 0.5 mCi/µl

Dissolve 100 mCi NaB[3H]4 (highest available specific activity, usually 25 to 35 Ci/mmol) in 200 µl ice-cold 0.01 or 0.05 M NaOH. Store in 10-µl aliquots for several months at −70°C until use. To make NaB[3H]4 in 1 M NaBH4/0.05 M NaOH, dissolve solid NaBH4 in tritiated solution in 0.05 M NaOH.

Alternatively, NaB[3H]4 can be dissolved in freshly distilled anhydrous dimethylformamide and stored at −20°C in well-sealed tubes. Some investigators report stability for longer periods than with NaOH solutions.

COMMENTARY

Background Information

When sufficient quantities of purified glycoconjugates (e.g., glycoproteins or glycopeptides) are available, their sugar chains can be labeled and used for structural or sequence analysis (Morell and Ashwell, 1972; Takasaki and Kobata, 1978). These sugar chains can be radioactively labeled by periodate or enzymatic oxidation followed by reduction with tritiated sodium borohydride (NaB[3H]4). Sialic acid residues can be preferentially oxidized by mild periodate oxidation because they contain cis-glycols in exocyclic carbons (Fig. 17.5.1). Such glycols are much more reactive with periodate than glycols in cyclic structures because of free rotation at C-8 and C-9. The aldehyde formed at C-7 or C-8 can be reduced by NaB[3H]4 and radioactively labeled in the process (Fig. 17.5.1). Galactose oxidase treatment oxidizes galactose or N-acetylglactosamine residues at the nonreducing termini, at C-6, forming aldehydes. The resultant aldehydes are reduced and labeled with NaB[3H]4 (Fig. 17.5.2).

Oxidized samples can also be labeled with fluorescent molecules (Wilchek et al., 1980; Spiegel, 1987). After oxidation by periodate or galactose oxidase treatment, the sample is reacted with a fluorescent dye that contains a hydrazide group. After forming a Schiff base, the linkage can be stabilized by reduction. Once a fluorescent label is attached to an oligosaccharide, it can be detected by a fluorescence or UV detector. Fluorescence-tagged oligosaccharides can be incubated with cells and incorporated oligosaccharides can be detected by fluorescence microscopy.

These methods have been used to examine the biological significance of oligosaccharide structures and their distribution on glycoconjugates. In one study, the structures of O-linked oligosaccharides were found to vary with the different maturation stages along a cell lineage (Fukuda et al., 1986). In another study, glycosylation of erythropoietin, which is essential for its function, was examined and compared with that of a recombinant erythropoietin (Sasaki et al., 1987).

Critical Parameters

There are two critical parameters for these procedures. First, it is essential that each reaction take place at the designated pH. If the pH of a reaction mixture is not properly adjusted, the reaction will not occur. Second, the quality of the NaB[3H]4 added is critical. When NaB[3H]4 is purchased, the reagent should be dark blue or slightly violet in color. When NaB[3H]4 absorbs water and becomes degraded, it appears white and should not be used for labeling. Free oligosaccharides must be desalted by gel-filtration chromatography to remove salts, free amino acids, and peptides before chemical labeling.

An alternative method is to label the N-acetyl groups of O-glycans that have already been released from glycoproteins by alkaline beta-
elimination. The reduced O-glycans can be treated with hydrazine to remove N-acetyl groups and reacetylated with radioactive acetic anhydride. This procedure is useful if anhydrous hydrazine is readily available (see Amano and Kobata, 1989).

Anticipated Results

Chemical labeling of carbohydrates by these procedures generally depends on the amount of samples available. For example, NaB[3H]4 reduction of oligosaccharides is one of the most efficient methods to label oligosaccharides, but it still requires 0.05 to 0.1 µM for efficient labeling. Since NaB[3H]4 reduction and fluorescence labeling are dependent on reactions with aldehyde groups, it is also an advantage to have a reasonable mass of sample, reducing the contribution from contaminants. Once there is a sufficient amount of sample, however, total radioactivity incorporated will allow many manipulations on radioactively labeled samples.

Time Considerations

In general, all procedures in this unit are simple and rapid. Each of the protocols described should take 1 or 2 days to finish.

Literature Cited


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Detection and Analysis of Proteins Modified by O-Linked N-Acetylglucosamine

The modification of Ser and Thr residues with O-linked β-N-acetylglucosamine (O-GlcNAc) is a common, dynamic, and essential modification of nuclear and cytoplasmic proteins. O-GlcNAc is ubiquitous, having been identified in all complex eukaryotes studied, including filamentous fungi and plants. O-GlcNAc has been found on a diverse range of proteins including cytoskeletal proteins, nuclear pore proteins, RNA polymerase II (RNA Pol II), transcription factors, proto-oncogene products, tumor suppressors, hormone receptors, phosphatases, and kinases (Comer and Hart, 2000).

The functional consequences of modifying proteins with O-GlcNAc is unclear, but it is required for survival at the single-cell level (Shafi et al., 2000). Three features suggest that O-GlcNAc performs a regulatory role: (1) O-GlcNAc occurs at sites on the protein backbone that are similar to those modified by protein kinases; (2) O-GlcNAc is reciprocal with phosphorylation on some well-studied proteins, such as RNA Pol II, estrogen receptor-β, SV40 large T-antigen, and the c-Myc proto-oncogene product; and (3) like phosphorylation, O-GlcNAc is highly dynamic, with rapid cycling in response to cellular signals or cellular stages. Perturbations in the metabolism of GlcNAc, which alter the regulation of many O-GlcNAc proteins, have been implicated in Alzheimer’s disease, diabetes, and cancer (Wells et al., 2001).

This unit concentrates on techniques for the detection and analysis of proteins modified by O-GlcNAc, as well as methods for the analysis of enzymes responsible for the addition and removal of O-GlcNAc. We have focused on methods that require standard laboratory equipment. However, in some cases we also discuss more specialized technology.

The unit is set out in a stepwise manner. First, a protocol for increasing the stoichiometry of O-GlcNAc on proteins is given (see Basic Protocol 1). This is followed by simple techniques for the detection/screening of O-GlcNAc modified proteins either by western blotting or lectin affinity chromatography (see Basic Protocols 2 to 4). Separate protocols verify that the glycan is O-linked GlcNAc (see Support Protocols 1 and 2). These methods are followed by protocols for more comprehensive analysis of O-GlcNAc modified proteins, including labeling of O-GlcNAc residues with [3H]Gal, and subsequent product analysis (see Alternate Protocol 1, see Basic Protocols 5 to 8, and see Support Protocols 3 and 4). The final two protocols assay for O-GlcNAc transferase and O-GlcNAcase activity, respectively (see Basic Protocols 9 and 10 and Support Protocol 5).

INCREASING THE STOICHIOMETRY OF O-GlcNAc ON PROTEINS BEFORE ANALYSIS

In many cultured mammalian cells, the number of O-GlcNAc moieties per protein molecule can be increased by treating cells with O-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amo-N-phenyl-carbamate (PUGNAc), a potent and cell-permeable inhibitor of O-GlcNAcase (Haltiwanger et al., 1998). Alternatively, streptozotocin (STZ; Roos et al., 1998) and glucosamine (Han et al., 2000) have been used to increase the stoichiometry of O-GlcNAc on proteins. However, STZ has been shown to induce poly-(ADP-ribose) polymerase–mediated apoptosis in Min6 cells (Gao et al., 2000) and should be used with caution. In addition, STZ is only effective in cells that express the glucose transporter GLUT-2 (Schnedl et al., 1994).
**Materials**

Cells of interest growing in monolayer culture, and appropriate culture medium

- 20 mM PUGNAc (CarboGen Laboratories) stock in Milli-Q water (filter sterilize and store in aliquots up to 6 to 12 months at −80°C)
- 500 mM glucosamine stock in 500 mM HEPES, pH 7.5 (make just prior to use; filter sterilize)
- 500 mM streptozotocin (STZ; Sigma) in 100 mM citrate buffer, pH 4.5 (make just prior to use; filter sterilize)
- 100-mm tissue culture dishes

1. Grow cells in monolayer culture in a sufficient number of 100-mm dishes.

2. Add (or replace growth medium with fresh medium containing) 40 to 100 μM PUGNAc (added from 20 mM stock), 5 mM glucosamine (added from 500 mM stock), or 2 to 5 mM STZ (added from 500 mM stock). Incubate cells in an incubator for 6 to 18 hr.

    *PUGNAc is taken up by both dividing and stationary cells.*

    If necessary, cells can be treated by PUGNAc for several days without any apparent cell toxicity. However, prolonged treatment does not appear to result in additional increase in O-GlcNAc compared to the 6 to 18 hr treatment.

When using glucosamine, mannitol is often added at the same concentration. This controls for changes in osmolarity due to the additional sugar in the medium (Heart et al., 2000).

3. At the end of treatment, take the dishes out of the incubator and place on ice. Extract as desired. Separate proteins by SDS-PAGE (UNIT 10.2A) and electroblot onto appropriate membrane (UNIT 10.8).

    Alternatively, extract proteins and proceed with protein purification (UNITS 10.9-10.14) or immunoprecipitation (UNIT 10.16).

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**DETECTION OF PROTEINS MODIFIED BY O-GlcNAc USING ANTIBODIES**

Recently Comer et al. (2001) showed that an antibody (CTD 110.6) raised against the glycosylated C-terminal domain of the RNA polymerase II large subunit was a general O-GlcNAc antibody. Unlike many lectins, CTD 110.6 shows little cross-reactivity toward terminal GlcNAc on complex glycans. Several other antibodies, HGAC 85 (Turner et al., 1990) and RL2 (Snow et al., 1987), have been reported as O-GlcNAc specific antibodies. However, these antibodies only recognize a subset of O-GlcNAc modified proteins, and RL2 in particular is dependent on the structure of the peptide backbone around the glycosylation site (Holt et al., 1987).

It is important to include an appropriate negative control (100 ng ovalbumin) for cross-reactivity toward N-linked glycans. Using the standard Amersham Pharmacia Biotech enhanced chemiluminescent (ECL) system, the authors have found that 10 μg of cytoplasmic, nuclear, or total cell extract is sufficient. For purified proteins, Comer and co-workers found that 25 to 50 ng of a neoglycoconjugate was sufficient (Comer et al., 2001).

**Materials**

- Purified or crude protein (e.g., Basic Protocol 1) separated by SDS-PAGE (UNIT 10.2A) and electroblotted to polyvinylidene difluoride (PVDF; UNIT 10.8) or nitrocellulose (duplicate blots are needed)
- TBS-HT (see recipe)
Antibody: CTD 110.6 ascites (Covance) diluted 1/2500 in TBS-HT (see recipe for TBS-HT)
N-acetylglucosamine (GlcNAc; Sigma)
HRPO-conjugated anti–mouse IgM diluted 1/5000 in TBS-HT
TBS-HD (see recipe)
ECL kit (Amersham Pharmacia Biotech)

Additional reagents and equipment for visualization with chromogenic and luminescent substrates (UNIT 10.8)

1. Block blots by incubating with TBS-HT for 60 min at room temperature.
   Batteiger et al. (1982) have shown that high concentrations of Tween 20 substitute for blocking with milk or bovine serum albumin (BSA).

2. Incubate blots with CTD 110.6 (1/2500 dilution in TBS-HT), in duplicate, with and without 10 mM GlcNAc, overnight at 4°C.
   To control for specificity it is important to perform a control blot. Here, the antibody is preincubated with 10 mM GlcNAc for ~5 min on ice before being applied to the control blot. Note that the concentration of antibody should be optimized with each new preparation. We find that 1/2500 is a good place to start with ascites (containing antibody in the mg/ml range).

3. Wash blots in TBS-HD twice, each time for 10 min at room temperature.
   As the blots are washed under these conditions, prestained markers will fade. This does not appear to be the result of proteins being solubilized off the membranes, but of the dye used to stain the markers. While the markers fade, they do not completely disappear. The authors usually double the amount of marker used to compensate for fading.

4. Wash blots in TBS-HT three times, each time for 10 min, room temperature.

5. Incubate blots with HRPO-conjugated anti–mouse IgM (1/5000 dilution in TBS-HT) for 50 min, room temperature.
   The concentration of secondary antibody varies from lot to lot and should be optimized each time with each new preparation.

6. Wash blots in TBS-HD twice, each time for 10 min, room temperature.

7. Wash blots in TBS-HT three times, each time for 10 min, room temperature.

8. Develop the HRPO reaction using, e.g., the ECL system (UNIT 18.4).
   Note that the antibody often cross-reacts with pre-stained markers.

DETECTION OF PROTEINS MODIFIED BY O-GlcNAc USING THE LECTIN sWGA

Many lectins are reportedly specific for β-GlcNAc residues. The authors have typically used succinylated wheat germ agglutinin (sWGA), which is widely available and is derivatized with a number of useful functional groups including horseradish peroxidase (HRPO). Before succinylation, WGA will recognize both sialic acid and GlcNAc (Monsigny et al., 1980). For additional information concerning lectin chromatography, see UNIT 17.1.

The amount of “test” protein used is dependent on the technique(s) used to develop the HRPO reaction. Using the standard Amersham Pharmacia Biotech ECL system the authors find that 7.5 µg of cytoplasmic or nuclear extract is sufficient.
It is important to include an appropriate positive (100 ng ovalbumin) and negative (100 ng of BSA) control. As a control, a portion of the sample should also be treated with hexosaminidase (see Support Protocol 2), to show that reactivity is toward GlcNAc. In addition, the sample should be subjected to reductive β-elimination to verify that lectin/antibody reactivity is towards O-linked glycans (see Support Protocol 1).

**Materials**

- Purified or crude protein separated by SDS-PAGE ([UNIT 10.2A](#)) and electroblotted to polyvinylidene difluoride (PVDF; [UNIT 10.8](#)) or nitrocellulose (duplicate blots are needed)
- 5% (w/v) BSA in TBST (see recipe for TBST)
- TBST (see recipe)
- 0.1 µg/ml HRPO-conjugated sWGA (EY Labs) in TBST (see recipe for TBST):
  - the lectin can be stored at 1 mg/ml in 0.01 M PBS, pH 7.4 ([APPENDIX 2](#)), at −20°C for at least 1 year
- N-acetylglucosamine (GlcNAc; Sigma)
- High-salt TBST (HS-TBST): TBST (see recipe) containing 1 M NaCl
- Tris-buffered saline (TBS; see recipe)
- ECL kit (Amersham Pharmacia Biotech)
- Additional reagents and equipment for visualization with chromogenic and luminescent substrates ([UNIT 10.8](#))

1. Wash duplicate blots for 10 min in 5% BSA/TBST, room temperature.
2. Block by incubating blots in 5% (w/v) BSA/TBST for at least 60 min at room temperature.
   IMPORTANT NOTE: Milk cannot be used as the blocking agent, since many of the proteins in milk are modified by glycans that react with sWGA.
3. Wash blots three times, each time for 10 min in TBST, room temperature.
4. Incubate blots in 0.1 µg/ml sWGA-HRPO in TBST, in duplicate, with and without 1 M GlcNAc, overnight at 4°C.
   To control for lectin specificity it is important to perform a control blot. Here, the lectin is preincubated with 1 M GlcNAc for ~5 min on ice before being applied to the control blot.
5. Wash blots six times, each time for 10 min, in HS-TBST.
6. Wash blots once in TBS for 10 min.
7. Develop the HRPO-reaction using, e.g., the ECL system ([UNIT 10.8](#)).
   Using the ECL system described, 100 ng of ovalbumin should be visualized in 5 to 15 sec.

**SUPPORT PROTOCOL 1**

**CONTROL FOR O-LINKED GLYCOSYLATION**

Traditionally, mild alkaline reduction (reductive β-elimination) has been used to release O-linked carbohydrates from proteins (Amano and Kobata, 1990). This method has been adapted for blots to show that lectin/antibody reactivity is toward O-linked rather than N-linked glycans (Duk et al., 1997). Proteins blotted to PVDF are treated with 55 mM NaOH overnight (releasing O-linked sugars) and then probed using lectins or antibodies.

There are a number of reasons why lectin/antibody reactivity could be lost after NaOH treatment, e.g., the sugars were destroyed instead of being released, or the protein was degraded. To control for these, it is important to have control proteins with N- and O-linked sugars, and to stain one blot for protein after treatment preferably with an antibody. The
authors suggest a control blot of bovine asialofetuin (Sigma) which contains both N- and O-linked sugars terminating in GlcNAc, treated and not treated with PNGase F (UNIT 17.13A).

Materials

Protein samples and controls blotted to PVDF (triplicate blots are needed; nitrocellulose is not suitable as it dissolves in 55 mM NaOH)
- Tris-buffered saline (TBS; see recipe)
- 55 mM NaOH
- 3% (w/v) BSA in TBST (see recipe for TBST)
- 40°C water bath

Additional reagents and equipment for probing protein blots with protein-specific antibodies (see Basic Protocol 2) or lectins (see Basic Protocol 3)

1. Wash blots once in TBS for 10 min.
2. Incubate two blots in 55 mM NaOH at 40°C overnight; incubate the control blot in Milli-Q water at 40°C overnight.
   
   *The blots treated with NaOH will yellow slightly.*
3. Wash blots three times, each time for 10 min at room temperature, in TBST.
4. Block by incubating blots in 3% w/v BSA/TBST for 60 min at room temperature.
5. Probe blots (one treated and one untreated) with carbohydrate-specific lectins (see Basic Protocol 3) or antibodies (see Basic Protocol 2). Probe the second NaOH-treated blot with a protein-specific antibody (see Basic Protocol 2).

   *On the untreated blot, asialofetuin ± PNGase F should react with sWGA, as both the N- and O-linked sugars contain terminal GlcNAc residues. On the treated blot only the asialofetuin − PNGase F should react with sWGA.*

BASIC PROTOCOL 4

DETECTION AND ENRICHMENT OF PROTEINS USING sWGA-AGAROSE

sWGA lectin affinity chromatography provides a convenient method for enriching and detecting O-GlcNAc modified proteins. This procedure has been adapted for detecting proteins that are difficult to purify or are present in low copy number, such as transcription factors. In this protocol, the protein of interest is synthesized in a rabbit reticulocyte lysate (RRL) in vitro transcription translation (ITT) system (Promega) and labeled with either [35S]Met, [35S]Cys, or [14C]Leu. After desalting, the proteins are tested for their ability to bind sWGA agarose in a GlcNAc-specific manner (Roquemore et al., 1994).

Alternatively, the lectin *Ricinus communis* agglutinin 1 (RCA1) has been used to select for O-GlcNAc proteins that have previously been labeled by galactosyltransferase (see Alternate Protocol 1). Proteins modified by terminal Gal are specifically retained on a RCA1 affinity column. Labeled O-GlcNAc proteins are released under mild conditions, while those containing N-linked structures require lactose addition to the buffer before elution results (Hayes et al., 1995; Greis and Hart, 1998). The method described in this protocol can be adapted for RCA1 affinity chromatography by substituting RCA1-agarose (EY Labs) for sWGA-agarose and changing the order of the Gal and GlcNAc elution buffer.

**Materials**

- cDNA subcloned into an expression vector with an SP6 or T7 promoter (~0.5 to 1 µg/µl)
- Kit for RRL ITT system (Promega)
sWGA-agarose (Vector Laboratories)  
sWGA wash buffer: PBS (APPENDIX 2) containing 0.2% (v/v) NP-40  
sWGA Gal elution buffer (see recipe)  
sWGA GlcNAc elution buffer (see recipe)  

1-ml tuberculin syringe with glass wool plug at bottom to support chromatography matrix or Bio-Rad Bio-Spin disposable chromatography column  
Additional reagents and equipment for digesting proteins with hexosaminidase (see Support Protocol 2), desalting (see Support Protocol 5), SDS-PAGE (UNIT 10.2A), and autoradiography (APPENDIX 3A)

**Prepare proteins**  
1. Synthesize proteins to incorporate the desired label ($[^35]S$Met, $[^35]S$Cys, or $[^14]C$Leu) using the RRL ITT system according to the manufacturer’s instructions. Include the protein of interest, a positive control for sWGA binding (for example, the nuclear pore protein p62), a negative control (luciferase, supplied with kit), and a no-DNA control.  
2. Treat half of each sample with hexosaminidase (see Support Protocol 2).  
3. Desalt samples using spin filtration (e.g., Amersham Pharmacia Biotech Microspin G-50 columns) or a 1-ml G-50 desalting column (as for desalting O-GlcNAc transferase; see Support Protocol 5).  

The following procedure is carried out at 4°C.

**Apply protein samples to chromatography columns**  
4. Equilibrate sWGA-agarose and pack column as follows:  
   a. If resin is supplied as 50% slurry (i.e., 50% resin/50% storage solution) remove 300 µl (double the volume required) and pipet into a 1-ml tuberculin syringe or disposable chromatography column.  
   b. Let storage solution drain from resin.  
   c. Equilibrate resin by washing column four times, each time with 1 ml of sWGA wash buffer. Cap column.  

The volumes given are appropriate for a sample derived from an ITT. For enrichment of other protein samples, the volume of sWGA should be optimized for the protein sample applied. The authors find that 50 mg of cell extract requires 1 ml of sWGA-agarose, assuming that 1% to 2% of the total cell extract is modified by O-GlcNAc.  
5. Apply sample (~30 µl of an ITT reaction) to the column and let stand at 4°C for 30 min, or cap and incubate at 4°C for 30 min with rotating or rocking.  

**Wash column and elute GlcNAc**  
6. At the end of the 30-min incubation, uncap the column and allow the sample to “run through” the resin. Collect this as the “run through” fraction. Wash column with 15 ml of sWGA wash buffer at 10 ml/hr, collecting 0.5-ml fractions.  
7. Load the column with 300 µl of sWGA Gal elution buffer and let stand at 4°C for 20 min.  
8. Wash column with 5 ml of sWGA Gal elution buffer, collecting 0.5 ml fractions.  
9. Repeat steps 6 to 8 using GlcNAc elution buffer.  
10. Count 25 µl of each fraction using a liquid scintillation counter.
11. Pool positive fractions that elute in the presence of GlcNAc and precipitate using TCA or methanol.

To precipitate proteins with methanol, mix 1 vol of sample with 10 vol of ice-cold methanol. Incubate overnight at −20°C. Recover protein by microcentrifuging 10 min at 16,000 × g, 4°C, in a microcentrifuge tube (which is the most efficient procedure) or in 15-ml conical centrifuge tubes for 10 min at 3000 × g, 4°C. Resuspend samples in SDS-PAGE sample buffer (UNIT 10.2A).

As many proteins in rabbit reticulocyte lysate contain O-GlcNAc and bind WGA, the authors do not recommend the addition of carrier proteins at this point. Typically, a fraction of the GlcNAc elution containing a total of 1000 to 2000 dpm [35S]Met is precipitated and analyzed by SDS-PAGE (UNIT 10.2A) and autoradiography (APPENDIX 3A).

The use of acetone to precipitate proteins is not recommended, as free GlcNAc will also precipitate.

12. Analyze pellet by SDS-PAGE (UNIT 10.2A) and autoradiography (APPENDIX 3A).

DIGESTION OF PROTEINS WITH HEXOSAMINIDASE

Terminal GlcNAc and O-GlcNAc can be removed from proteins using commercially available hexosaminidases; these enzymes will also cleave terminal GalNAc residues. Unlike O-GlcNAcase, commercial hexosaminidases have low pH optima, typically pH 4.0 to 5.0.

Materials
- Protein sample for digestion (include a positive control, e.g., ovalbumin)
- 2% (w/v) SDS
- 2× hexosaminidase reaction mixture (see recipe)
- Additional reagents and equipment for SDS-PAGE (UNIT 10.2A) and electrophoretic transfer (UNIT 10.8)

1. Mix sample 1:1 with 2% SDS and boil for 5 min.
2. Mix sample 1:1 with 2× hexosaminidase reaction mixture and incubate at 37°C for 4 to 24 hr.
3. To assess completeness of the digestion, separate an aliquot of the reaction by SDS-PAGE (UNIT 10.2A) and electroblot (UNIT 10.8) onto an appropriate membrane. Probe blots with carbohydrate-specific lectins or antibodies.

DETECTION OF PROTEINS MODIFIED BY O-GlcNAc USING GALACTOSYLTRANSFERASE

The enzyme β-1,4-galactosyltransferase (from bovine milk) will label any terminal GlcNAc residue with Gal, using uridine diphospho-D-Gal (UDP-Gal) as a donor substrate (Brew et al., 1968). Hart and colleagues have exploited this property, using the enzyme to label terminal GlcNAc residues on proteins with [6-3H]Gal, forming a [3H]-βGal1-4βGlcNAc (Torres and Hart, 1984; Roquemore et al., 1994; Greis and Hart, 1998). The labeled sugar can be chemically released (via β-elimination) and analyzed by size-exclusion chromatography on a BioGel-P4 column, using the 3H radiolabel to detect the fraction of interest (Roquemore et al., 1994). Labeling the O-GlcNAc allows for the subsequent detection of the proteins and peptides of interest during SDS-PAGE, HPLC, protease digestion, and Edman degradation steps. Researchers have been able to identify glycosylation sites on as little as 10 pmol using these methods (Greis et al., 1996).
To achieve efficient labeling of some proteins, it is necessary to denature samples, for example by boiling in the presence of 10 mM DTT and 0.5% (w/v) SDS. Galactosyltransferase has been shown to be active in solutions containing 5 mM DTT, 0.5 M NaCl, up to 2% (v/v) Triton X-100, up to 2% (v/v) NP-40, and 1 M urea. Up to 0.5% (w/v) SDS can be used, if it is titrated with a 10-fold molar excess of either Triton X-100 or NP-40 in the final reaction mixture. Digitonin, which is commonly used to solubilize cells, should be used with caution, as it is a substrate for galactosyltransferase. The total ionic strength should be less than 0.2 M.

Galactosyltransferase requires 1 to 5 mM Mn\textsuperscript{2+} for activity, but is inhibited by Mg\textsuperscript{2+} and concentrations of Mn\textsuperscript{2+} >20 mM. EDTA (or analogs) should be avoided unless titrated with appropriate levels of Mn\textsuperscript{2+}. Note that 1 mol of EDTA binds 2 mol of Mn\textsuperscript{2+}.

Free UDP is also an inhibitor of galactosyltransferase. For studies where complete labeling of the GlcNAc is preferable, such as site mapping, calf intestinal alkaline phosphatase is included in the reaction, as it degrades UDP (Unverzagt et al., 1990). While this increases the efficiency of the reaction, it is important to add this to the control as some preparations of alkaline phosphatase contain proteins that will label with galactosyltransferase (R.N. Cole, pers. commun.).

**NOTE:** Protease inhibitors, such as PIC 1, PIC 2, and PMSF (see recipe for 1000× protease inhibitors in Reagents and Solutions), can be included (final concentrations, 1×), but GlcNAc and 1-amino GlcNAc should be removed prior to labeling by spin filtration or another method of desalting.

### Materials
- Protein sample(s)
- Dithiothreitol (DTT)
- Sodium dodecyl sulfate (SDS)
- Label: 1.0 mCi/ml UDP-[\textsuperscript{3H}]Gal, (17.6 Ci/mM; Amersham Pharmacia Biotech) in 70% v/v ethanol
- Nitrogen source
- 25 mM 5′-adenosine monophosphate (5′-AMP), in Milli-Q water, pH 7.0
- Buffer H (see recipe)
- 10× galactosyltransferase labeling buffer (see recipe)
- Galactosyltransferase, autogalactosylated (see Support Protocol 3)
- Calf intestinal alkaline phosphatase
- Unlabeled UDP-Gal
- Stop solution: 10% (w/v) SDS/0.1 M EDTA
- 100°C water bath
- 30 × 1-cm Sephadex G-50 column equilibrated in 50 mM ammonium formate/0.1% (w/v) SDS

Additional reagents and equipment for acetone precipitation of protein (UNIT 17.10A), PNGase F digestion of proteins (UNIT 17.13A), SDS-PAGE (UNIT 10.2A), and product analysis (see Basic Protocol 6)

### Prepare the reaction
1. Denature protein sample by adding DTT to 10 mM and SDS to 0.5% (w/v), then boiling the sample for 10 min.

2. Decide how many reactions are going to be carried out and thus how much label will be needed (~1 to 2 μCi/reaction).
A positive control (ovalbumin, 2 μg), a negative control (because galactosyltransferase can label itself), and a sample-minus-enzyme control will be needed.

3. Remove solvent from label in a Speed-Vac evaporator or under a stream of nitrogen.

   Ethanol can inhibit the galactosyltransferase reaction, but if <4 μl is required the label can be added directly to the reaction (final reaction volume, 500 μl).

4. Resuspend appropriate amount of label for each reaction, respectively, in 50 μl of 25 mM 5′-AMP.

   The AMP is included to inhibit possible phosphodiesterase reactions, which might compete for label during the labeling experiment.

5. Set up reactions as follows:

   Up to 50 μl protein sample (final concentration 0.5 to 5 mg/ml)
   350 μl buffer H
   50 μl 10× galactosyltransferase labeling buffer
   50 μl UDP-[3H]Gal/5′-AMP mixture from step 4
   30 to 50 μl autogalactosylated galactosyltransferase
   1 to 4 U calf intestinal alkaline phosphatase
   Milli-Q water to final volume of 500 μl

   Reaction volumes can be scaled down to 50 μl.

6. Labeling at 37°C for 2 hr or at 4°C overnight.

   These are the typical conditions. Galactosyltransferase is active over a range of temperatures.

7. Add unlabeled UDP-Gal to a final concentration of 0.5 to 1.0 mM and another 2 to 5 μl of galactosyltransferase.

   For studies where complete labeling of the GlcNAc is required, such as site mapping, the reactions are chased with unlabeled UDP-Gal and fresh galactosyltransferase.

8. Add 50 μl of stop solution to each sample and heat to 100°C for 5 min in a water bath.

Isolate the product

9. Resolve the protein from unincorporated label using a Sephadex G-50 column equilibrated in 50 mM ammonium formate/0.1% w/v SDS. Collect 1-ml fractions.

   Size-exclusion chromatography using Sephadex G-50 is traditionally used to desalt samples. However, TCA precipitation, spin filtration/buffer exchange, or other forms of size-exclusion chromatography (e.g., Pharmacia PD-10 desalting column) can be used. The addition of carrier proteins such as BSA (~67 kDa) and cytochrome c (~12.5 kDa) to samples and buffers will reduce the amount of protein lost due to nonspecific protein adsorption.

10. Count a 50-μl aliquot of each fraction using a liquid scintillation counter.

   Approximately $2 \times 10^6$ dpm of [3H]Gal should be incorporated into 2 μg of ovalbumin.

11. Combine the void volume and lyophilize to dryness.

12. Resuspend samples in Milli-Q water and precipitate with acetone (UNIT 17.10A).

13. Treat samples with PNGase F (UNIT 17.13A), separate by SDS-PAGE (UNIT 10.2A) and detect by autoradiography (APPENDIX 3A). Alternatively, subject samples to “product analysis” to confirm that the label was incorporated onto O-GlcNAc (see Basic Protocol 6).
**AUTO GALACTOSYLATION OF GALACTOSYLTRANSFERASE**

As galactosyltransferase contains N-linked glycosylation sites, it is necessary to block these before using this enzyme to probe other proteins for terminal GlcNAc.

**Materials**

- 10× galactosyltransferase labeling buffer (see recipe)
- 10,000 U/ml aprotinin
- 2-mercaptoethanol
- UDP-Gal
- Saturated ammonium sulfate: >17.4 g (NH₄)₂SO₄ in 25 ml Milli-Q water
- 85% ammonium sulfate: 14 g (NH₄)₂SO₄ in 25 ml Milli-Q water
- Galactosyltransferase storage buffer (see recipe)
- 30- to 50-ml centrifuge tubes
- Refrigerated centrifuge

1. Resuspend 25 U of galactosyltransferase in 1 ml of 1× galactosyltransferase labeling buffer.
2. Transfer sample to 30- to 50-ml centrifuge tube.
   
   *The centrifuge tubes should be able to withstand a centrifugal force of 15,000 × g.*
3. Remove a 5-µl aliquot for an activity assay.
   
   *This is the “Pre-Gal” sample to be used in Support Protocol 4.*
4. Add 10 µl of 10,000 U/ml aprotinin, 3.5 µl of 2-mercaptoethanol, and 1.5 to 3.0 mg of UDP-Gal.
5. Incubate the sample on ice for 30 to 60 min.
6. Add 5.66 ml of prechilled saturated ammonium sulfate in a dropwise manner. Incubate on ice for 30 min.
7. Centrifuge 15 min at >10,000 × g, 4°C. Pour off supernatant.
8. Resuspend pellet in 5 ml cold 85% ammonium sulfate and incubate on ice for 30 min.
9. Centrifuge 15 min at >10,000 × g, 4°C, and pour off supernatant.
10. Resuspend pellet in 1 ml of galactosyltransferase storage buffer and divide into 50-µl aliquots, saving 5 µl for an activity assay as the “Auto-Gal” sample. Assay that aliquot for activity (see Support Protocol 4).
11. Store remaining aliquots up to 1 year at −20°C pending use in Alternate Protocol 1.

**ASSAY OF GALACTOSYLTRANSFERASE ACTIVITY**

As sample and activity may be lost during the autogalactosylation procedure, it is important to assess the activity of the enzyme.

**Materials**

- 1.0 mCi/ml UDP-[³H]Gal, (17.6 Ci/mM; Amersham Pharmacia) in 70% v/v ethanol
- Nitrogen source
- 1× galactosyltransferase dilution buffer: galactosyltransferase storage buffer (see recipe) supplemented with 5 mg/ml BSA
- 10× galactosyltransferase labeling buffer (see recipe)
25 mM 5′-adenosine monophosphate (5′-AMP) in Milli-Q water, pH 7.0
“Pre-Gal” sample aliquot (see Support Protocol 3, step 3) and “Auto-Gal” sample aliquot (see Support Protocol 3, step 10)
200 mM GlcNAc
Dowex AG1-X8 resin (PO4 form) slurry in 20% (v/v) ethanol
Glass wool

1. Dry 40 μl of 0.1 μCi/μl of UDP-[3H]Gal in a Speed-Vac evaporator or under a stream of nitrogen.

2. Resuspend in 90 μl of 25 mM 5′-AMP.

3. Make 1/1000, 1/10,000, and 1/100,000 serial dilutions of the “Pre-Gal” and “Auto-Gal” sample aliquots, in 1× galactosyltransferase dilution buffer. Using these dilutions, 200 mM GlcNAc, and 10× galactosyltransferase labeling buffer, prepare reaction mixtures as described in Table 17.6.1.

4. Start the reaction by adding 10 μl of 0.05 μCi/μl UDP-[3H]Gal (see step 2) to each tube.

5. Incubate samples at 37°C for 30 min.

   While the samples are incubating, prepare the columns.

6. Pour 1 ml of Dowex AG1-X8 slurry (PO4 form) into 13 Pasteur pipets, each plugged with a small amount of glass wool.

   The glass wool prevents the resin from flowing out of the column. If too much glass wool is used, it will reduce the flow rate of the column. The glass wool plug should be 0.3 to 0.5 mm long and should not be over-compressed.

7. Wash with at least 3 ml of Milli-Q water. Do not let the columns run dry.

8. When almost all the Milli-Q water has eluted, place each column over a separate 15-ml scintillation vial.

9. Stop the reaction (still incubating from step 5) by adding 500 μl of Milli-Q water.

10. Load each sample onto the corresponding column and add a 500 μl water wash of the tube. Collect eluate as fraction A.

11. Elute with two 1-ml additions of Milli-Q water. Collect eluates as fractions B and C, respectively.

### Table 17.6.1 Reaction Mixtures for Assay of Galactosyltransferase Activity

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12. Count 100 µl of the sample and 10 µl of the UDP-[3H]Gal using a liquid scintillation counter.

13. Calculate activity.
   a. Calculate the total moles of UDP-Gal transferred to GlcNAc as follows:
      \[
      \left( \frac{\text{cpm}_{\text{(with GlcNAc)}} - \text{cpm}_{\text{(no GlcNAc)}}}{\text{total cpm}} \right) \times 1.76 \text{ nmol}
      \]
      Note, cpm_{(with GlcNAc)} represents counts in the sample, cpm_{(no GlcNAc)} represents counts in the no-enzyme control, and total cpm represents the total counts available to transfer.
      \[
      \left( \frac{\text{cpm}_{\text{(with GlcNAc)}} - \text{cpm}_{\text{(-GlcNAc)}}}{\text{total cpm}} \right)
      \]
      represents the portion of [3H]Gal transferred in the reaction. This proportion is multiplied by the total number of moles of UDP-Gal in the reaction. If the specific activity of the UDP-[3H]Gal is 17.6 Ci/mM and 10 µl of label was used, then there are 1.76 nmol of UDP-Gal in 10 µl.
   b. Calculate the activity; one unit of activity (U) is defined as 1 µM of Gal transferred to GlcNAc per minute at 37°C.

**DETECTION OF PROTEINS MODIFIED BY O-GlcNAc USING METABOLIC LABELING**

Metabolic labeling of O-GlcNAc-bearing proteins provides a useful means to test if a protein of interest is modified by O-GlcNAc, to observe the gross dynamic changes in O-GlcNAc levels and to study the subcellular localization in response to stimulation or during cell cycle. In the protocol described below, cells are labeled with [3H]glucosamine, which is metabolized to UDP-[3H]GlcNAc in the hexosamine synthetic pathway. For labeling, it is critical that the labeled sugar compete with glucose import; this ensures efficient uptake of the label. While glucosamine is a good competitor, N-acetylglucosamine is not. For further discussions on metabolic labeling of glycoconjugates, readers are encouraged to consult Varki (1994) for details.

**Materials**

- Cells of interest, growing in culture
- Biosynthetic labeling medium (see recipe)
- Phosphate-buffered saline (PBS; APPENDIX 2)
- Additional reagents and equipment for PNGase F digestion of proteins (UNIT 17.13A), SDS-PAGE (UNIT 10.2A), and autoradiography (APPENDIX 3A)

1. Replace growth medium with biosynthetic labeling medium. Label cells for 5 to 24 hr in an incubator.
   
   *The labeling is done in a glucose-free medium to maximize labeling efficiency. The addition of nonessential amino acids to the medium reduces the influx of glucosamine into the amino acid biosynthetic pathways (Medina et al., 1998).*

2. Wash labeled cells twice, each time by resuspending in 5 ml of PBS and centrifuging 10 min at 500 × g, 4°C.

3. Extract as desired.
   
   *See Critical Parameters for a discussion on reducing O-GlcNAcase activity.*

4. Treat sample with PNGase F (UNIT 17.13A).
5. Separate proteins by SDS-PAGE (UNIT 10.2A) and visualize labeled proteins by autoradiography (APPENDIX 3A).

CHARACTERIZATION OF LABELED GLYCANS BY β-ELIMINATION AND CHROMATOGRAPHY

This protocol has three steps: (1) the release of carbohydrates as sugar alditols by reductive β-elimination; (2) desalting the sample, while confirming the size of the labeled sugar alditol(s); and (3) confirmation that the product is [3H] βGal1-4βGlcNAcol (from galactosyltransferase labeling) or [3H]GlcNAcol (from metabolic labeling).

Materials

Labeled proteins
β-elimination reagent: 1 M NaBH₄/0.1 M NaOH (prepare fresh)
4 M acetic acid
Screw-cap microcentrifuge tubes

Additional reagents and equipment for acetone or methanol precipitation of proteins (UNIT 17.10A), size-exclusion (gel-filtration) chromatography (UNIT 10.9), and Dionex chromatography (Townsend et al., 1990; Hardy and Townsend, 1994)

1. Acetone or methanol precipitate labeled proteins (UNIT 17.10A) in screw-cap microcentrifuge tubes.

   The β-elimination reaction is performed in screw-cap microcentrifuge tubes to prevent the lids from popping open during the lengthy incubation, which would result in evaporation of the sample. The tubes should be tightly sealed. Take care in opening the tubes at the end of the reaction, as gas generated during the reaction will escape.

2. Resuspend sample in 500 µl of β-elimination reagent and incubate at 37°C for 18 hr.

   After several hours check that the pH is >13. Add more β-elimination reagent if needed.

3. Cool the sample on ice.

4. Neutralize the reaction by adding 5 µl cold 4 M acetic acid in a stepwise manner. Check that the pH is between pH 6 and 7.

   Samples can be desalted either by chromatography on a Sephadex G-50 column (1 × 30 cm, equilibrated in 50 mM ammonium formate, 0.1% SDS) or by anion-exchange chromatography on a 1-ml Bio-Rad Dowex AG 50W-X2 200-400 mesh (H⁺ form) column equilibrated in water. Fractions containing [3H]GlcNAc or [3H]Gal are pooled and lyophilized. Residual NaBH₄ is removed by washing the sample with methanol; NaBH₄ is volatile in the presence of methanol and is removed in a Speed-Vac evaporator or under a stream on nitrogen (Fukuda, 1990).

5. Resuspend the sugar alditols in Milli-Q water. Analyze by size-exclusion chromatography or by Dionex chromatography.

   To determine the size of the oligosaccharide, labeled glycans released by β-elimination are subjected to size exclusion chromatography. Readers are referred to several standard methods using BioGel P4 (Kobata, 1994) or TSK Fractogel (Fukuda, 1990) chromatography. Alternatively, the Amersham Pharmacia Biotech Superdex Peptide column, equilibrated in 30% v/v CH₃CN, 0.1% v/v TFA, has been used to size oligosaccharides (R.N. Cole, pers. commun.).

   To determine the nature of the monosaccharide alditol or disaccharide alditol generated from either metabolic labeling or galactosyltransferase labeling, samples released by β-elimination can be analyzed by high-voltage paper electrophoresis or high-pH anion exchange chromatography (HAPEC) with pulsed amperometric detection on a Dionex CarboPac PA100 column (Townsend et al., 1990; Hardy and Townsend, 1994).
SITE MAPPING BY MANUAL EDMAN DEGRADATION

This protocol describes methods for mapping sites on peptides that have been labeled with \[^{3}H\]Gal (see Alternate Protocol 1) or \[^{3}H\]glucosamine (see Basic Protocol 5). Proteins of interest can be digested in solution or in gel (Riviere and Tempst, 1995; Stone and Williams, 1995) and the peptides separated by conventional reversed-phase HPLC (UNIT 10.14).

Glycopeptides are covalently attached to arylamine-derivatized PVDF disks via the activation of peptide carboxyl groups using water-soluble \(N\)-ethyl-\(N'\)-dimethylamine-nopropylcarbodiimide (EDC; Kelly et al., 1993). Note that both the C-terminus and any acidic residue will couple to the membrane and as such, cycles with acidic amino acids will yield blanks.

Some researchers have shown that glycosylated amino acids can be visualized during automated Edman degradation. However, this technique requires at least 20 pmol of starting material, where at least 20% of the sample is glycosylated. For more details, readers are referred to Zachara and Gooley (2000).

Materials

- Acetonitrile (CH\(_3\)CN; HPLC-grade)
- Trifluoroacetic acid (TFA; sequencing grade)
- Sequelon-AA Reagent Kit (Millipore) containing:
  - Mylar sheets
  - Carbodiimide
  - Coupling buffer
  - \(N\)-ethyl-\(N'\)-dimethylamine-nopropylcarbodiimide (EDC)
- Labeled sample and control peptides (must contain \(\geq1000\) dpm and not be in amine-containing buffers such as Tris)
- Methanol (HPLC-grade)
- Sequencing reagent (see recipe)
- 100 mM Tris\(\cdot\)Cl, pH 7.4 (APPENDIX 2)
- Heating block
- Screw-capped polypropylene microcentrifuge tubes

Couple the sample to a PVDF disk

1. Resuspend peptide in 10% to 30% (v/v) CH\(_3\)CN and up to 0.1% (v/v) TFA.
2. Place disk on a Mylar sheet (from Sequelon kit) on a heating block at 55°C.
3. Wet disk with 10 \(\mu\)l of methanol and allow excess methanol to evaporate.
4. Apply sample in 10-\(\mu\)l aliquots, allowing the membrane to come to near-dryness between aliquots.
5. Dry the membrane after all of the sample has been applied. Place the disc in the lid of a screw-cap microcentrifuge tube for subsequent reactions.
6. Prepare coupling reagent by combining 1 mg carbodiimide per 100 \(\mu\)l of coupling buffer (both provided with Sequelon kit). Add –1 mg of EDC per 100 \(\mu\)l of coupling reagent and carefully pipet –50 \(\mu\)l onto each disk.
7. Incubate samples at 4°C for 30 min. At the end of the reaction, remove the coupling reagent to a microcentrifuge tube.

*Incubation of the sample at 4°C increases the yield. The membrane should not be incubated for >30 min.*
8. Place the membrane in a microcentrifuge tube. Wash the membrane alternately three
times with 1 ml methanol and 1 ml Milli-Q water, vortexing briefly after each
addition. Combine each wash with the coupling reagent from step 7.

9. Dry the membrane. Samples are stable at −20°C on disks for at least 6 months.

10. Count an aliquot of the pooled coupling reagent and washes (step 8) and determine
the coupling efficiency.

**Sequencing peptides**
The times given in this procedure are critical. Steps 12 to 17 represent a “cycle.” This
method is only efficient for 10 to 20 cycles.

11. Place disk in a screw-capped polypropylene microcentrifuge tube.

12. Add 0.5 ml of the sequencing reagent to the disk and incubate 10 min at 50°C in a
heating block.

   *This step derivatizes the N-terminus of the peptide, forming the phenylthiocarbamyl
derivative.*

13. Wash disk five times in microcentrifuge tube, each time with 1 ml of methanol,
vortexing after each addition. Dry disk in a Speed-Vac evaporator for 5 min.

14. Add 0.5 ml TFA and incubate at 50°C for 6 min. Remove and save the supernatant.

   *In this step, the derivatized amino acid is released from the peptide.*

15. Wash the disk with 1 ml of methanol. Save the supernatant and combine the
supernatant from step 14.

16. Wash disk five times, each time with 1 ml methanol.

17. Return to step 12 and repeat the cycle.

   *Steps 12 to 17 represent a “cycle,” with one amino acid being released per cycle from the
N-terminus of the protein or peptide. This method is only efficient for 10 to 20 cycles. As
each cycle represents the release of an amino acid, it is not necessary to perform 20 cycles
if the peptide in question is 10 amino acids long. The authors usually perform 15 cycles,
as the data become hard to interpret after this.*

18. Dry the combined supernatants from steps 14 and 15 on a 50°C heating block, or on
the bench overnight.

19. Add 0.5 ml of 100 mM Tris·Cl, pH 7.4 and 15 ml liquid scintillation fluid. Count on
a liquid scintillation counter.

   *Edman degradation is a sequential process, with one amino acid being released from the
N-terminus of the protein or peptide per cycle. In the method described here, glycosylated
amino acids are being detected by following the [3H]Gal label.*

   *To interpret the data, plot the dpm per cycle as a bar graph. A cycle with a glycosylated
amino acid should have much higher counts than either the preceding or following cycle,
unless there are two sites in a row.*

   *Manual Edman degradation is often performed in concert with other techniques that will
identify the peptide being sequenced, e.g., conventional Edman degradation or MALDI-
TOF MS (units 10.21 & 10.22). When looking at data sets from several techniques, the cycle
with the counts should line up with a Ser or Thr residue.*
SITE MAPPING BY MASS SPECTROMETRY AFTER β-ELIMINATION

As mass spectrometry (MS; UNITS 10.21 & 10.22) has become more readily available, several MS techniques have been applied to the characterization of O-GlcNAc proteins and peptides (Reason et al., 1992; Greis et al., 1996; Haynes and Aebersold, 2000). When analyzing proteins or peptides modified by O-GlcNAc via mass spectrometry, several challenges need to be overcome. Some researchers have shown that when using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF; UNIT 10.22) with a conventional matrix (such as α-cyano-4-hydroxycinnamic acid) the addition of a single GlcNAc to a peptide may reduce the signal by approximately five-fold. In addition, the presence of the unglycosylated peptide further suppresses the signal (Hart et al., 2000). Secondly, the β-O-GlcNAc bond is more labile in the mass spectrometer than other bonds formed by protein glycosylation. Thus, in conventional electrospray ionization-MS, O-GlcNAc is released at lower orifice potentials and collision energies than is required to sequence peptides.

Greis et al. (1996) developed a technique where peptides are analyzed before and after β-elimination. During β-elimination Ser (89 atomic mass units) and Thr (101 atomic mass units) residues are converted to 2-aminopropenoic acid (69 atomic mass units) and α-aminobutyric acid (83 atomic mass units), respectively. These amino acids have different masses than their parent amino acids and can be used to map the site of attachment. It should be noted that this method will also release phosphate linked to Ser and Thr residues. In the protocol described here, glycans are released from peptides by β-elimination, and then analyzed using conventional MS-MS strategies.

Materials

Samples from β-elimination (see Basic Protocol 7)
200 mM NaOH
300 mM acetic acid
Screw-cap polypropylene microcentrifuge tubes
45°C heating block

Additional reagents and equipment for ESI-MS (UNIT 10.21), reversed-phase HPLC of peptides (UNIT 10.14), and MALDI-TOF MS (UNIT 10.22)

1. Combine the following in a screw-cap polypropylene microcentrifuge tube:
   ≤25 µl sample from β-elimination
   25 µl 200 mM NaOH
   Milli-Q water to a final volume of 50 µl

2. Incubate at 45°C for 4 hr in a heating block.
   *Time is critical, as increased incubation times will result in the peptide backbone hydrolyzing.*

3. Place sample on ice and add 25 µl of 300 mM acetic acid in 5-µl increments.
   *The reaction mixture will boil over unless the acetic acid is added in a stepwise manner.*

4. Dry sample in a Speed-Vac evaporator and store at −80°C until analyzed by ESI-MS (UNIT 10.21). Alternatively, analyze peptides by MALDI-TOF MS (UNIT 10.22) after desalting using reversed-phase chromatography (UNIT 10.14).
   *Zip-Tips (Millipore) are ideal for desalting small quantities of peptide before MALDI-TOF MS.*
ASSAY FOR OGT ACTIVITY

The detection and analysis of O-GlcNAc on proteins is only the first step in the analysis of O-GlcNAc and the protein(s) of interest. More important is determining the function of the modification. Protocols for the analysis of the enzymes that add and remove O-GlcNAc have been included, as they may aid in understanding the role of O-GlcNAc. Recent examples where studies such as this have been critical include those which have shown the reciprocity between O-GlcNAc and O-phosphate on the C-terminal domain of RNA Pol II; studies showing elevated activity of enzymes in certain tissue/cell lines and tissue fractions; and, finally, studies which have indicated that the enzymes responsible for the addition and removal of O-GlcNAc copurify with kinases and phosphatases.

O-GlcNAc transferase (OGT), or uridine diphospho-\(N\)-acetylglucosamine:polypeptide \(\beta\)-\(N\)-acetylglucosaminyltransferase, transfers GlcNAc to the hydroxyl groups of Ser and Thr residues of proteins and peptides using UDP-GlcNAc as a donor substrate (Haltiwanger et al., 1992; Kreppel et al., 1997). OGT activity is assayed by determining the rate at which \(^{3}\text{H}\)GlcNAc is transferred to an acceptor peptide. A number of peptides have been identified as substrates for OGT in vitro, but a peptide (\(^{340}\text{PGGSTPVSSANMM}\(^{352}\)) from the \(\alpha\)-subunit of casein kinase II (CKII) is the most efficient in vitro substrate known to date (Kreppel and Hart, 1999).

OGT activity can be assayed in crude preparations (Haltiwanger et al., 1992) or using recombinant protein (Kreppel and Hart, 1999). OGT activity is sensitive to salt inhibition, so it is important to desalt the preparation before assaying if high salt concentrations are present (see Support Protocol 5). For pure preparations 0.2 to 1 \(\mu\)g is typically used per assay, in crude preparations 20 to 50 \(\mu\)g, though the latter is precipitated using ammonium sulfate (40% to 60%).

Materials

- 0.1 mCi/ml UDP-[\(^{3}\text{H}\)]GlcNAc (20 to 45 Ci/mmol; NEN Life Science Products) in 70% ethanol
- 25 mM 5'-adenosine monophosphate (5'-AMP), in Milli-Q water, pH 7.0
- Nitrogen source
- Crude or purified OGT sample, desalted (see Support Protocol 5)
- 10\(\times\) OGT assay buffer (see recipe)
- CKII peptide substrate (\(^{1340}\text{H}_{2}\text{N}-\text{PGGSTPVSSANMM-COO}\^-\)): dissolve in \(\text{H}_{2}\text{O}\) to 10 mM and adjust to pH 7 if necessary
- 50 mM formic acid
- Methanol (HPLC-grade)
- Waters Sep-Pak C\(_{18}\) cartridges

1. Dry down an aliquot of UDP-[\(^{3}\text{H}\)]GlcNAc in a Speed-Vac evaporator or under a stream of nitrogen just prior to use. Resuspend in an appropriate volume of 25 mM 5'-AMP, so that the concentration is 0.02 \(\mu\)Ci to 0.1 \(\mu\)Ci/\(\mu\)l.

   \textit{AMP is included in the assay to competitively inhibit any pyrophosphatase in the sample that will hydrolyze the UDP-GlcNAc.}

2. Set up assay reactions as follows:

   \begin{align*}
   &5 \mu\text{l of } 10\times \text{ OGT assay buffer} \\
   &10 \mu\text{l of } 10 \text{ mM CKII peptide substrate} \\
   &5 \mu\text{l of } 0.02 \text{ to } 0.1 \mu\text{Ci/}\mu\text{l UDP-[}\(^{3}\text{H}\)GlcNAc (from 0.1 to 0.5 \mu\text{Ci total}) \\
   \leq 25 \mu\text{l of desalted OGT to be analyzed} \\
   &\text{H}_{2}\text{O to } 50 \mu\text{l}
   \end{align*}
It is critical to include a negative control. A mimic of the CKII peptide where the Ser and Thr residues are replaced with Ala is appropriate, or, simply, a “no-enzyme” control can be included.

The results generated are variable and the reactions should be set up in duplicate.

3. Incubate at room temperature for 30 min.

An incubation time of 15 to 30 min at room temperature is usually sufficient.

4. Stop reaction by adding 450 µl of 50 mM formic acid.

5. Wet a Waters Sep-Pak C18 cartridge with methanol, then wash the cartridge with 5 ml H2O.

6. Load the reaction (500 µl total) onto the cartridge with a syringe. Wash with 5 ml H2O.

The CKII peptide binds to the matrix of the C18 cartridge. Unincorporated UDP-[3H]GlcNAc is eliminated by the wash.

7. Elute the peptide with 2 to 4 ml methanol, directly into a 15-ml liquid scintillation counter tube.

8. Add 10 ml scintillation fluid and count ³H. Calculate OGT activity according to the following equations.

\[
\text{µCi of GlcNAc incorporated} = (\text{dpm in sample} - \text{dpm in blank}) / (2.22 \times 10^6 \text{ dpm} \cdot \text{µCi}^{-1})
\]

\[
\text{mmol of GlcNAc incorporated} = (\text{µCi of GlcNAc incorporated}) / (\text{specific activity in µCi/mmol})
\]

This number should be expressed in terms of mg of OGT or cell extract. If the assay is done at several time points, it can be expressed as mmol/min.

The activity can be expressed either as dpm ³H incorporation into the peptide, or as µmol ³H incorporation (1 µCi = 2.22 \times 10^6 dpm).

**DESLATING THE O-GlcNAc TRANSFERASE**

OGT activity is sensitive to salt inhibition (IC₅₀ = 40 to 50 mM NaCl). It is important to desalt the enzyme preparation before assay if high concentration of salt is present.

**Materials**

- Sephadex G-50 slurry (Pharmacia Biotech)
- OGT desalting buffer (see recipe)
- Protein sample for OGT assay in volume ≤200 µl
- 1-ml tuberculin syringe
- 1.5-ml tubes, prechilled

1. Pack a column containing exactly 1 ml of Sephadex G-50 slurry in a 1-ml tuberculin syringe. Wash the column with 5 ml of OGT desalting buffer.

Sephadex G-50 is usually supplied in 20% (v/v) ethanol.

2. Load protein sample onto column.

The volume of sample can be up to 200 µl.

3. Wash column with desalting buffer so that the total volume of this wash and the protein sample is 350 µl. For example, if sample volume is 150 µl, add 200 µl desalting buffer to the column at this step.
4. Transfer the syringe column to a clean, prechilled 1.5-ml tube. Elute protein with 200 µl desalting buffer. Keep on ice.

   This is the desalted sample.

   Alternatively, PD-10 desalting columns (Amersham Pharmacia Biotech) can be used if O-GlcNAc transferase is in larger volume (1 to 2.5 ml).

ASSAY FOR O-GlcNAcase ACTIVITY

O-GlcNAcase, also known as N-acetylglucosaminidase or hexosaminidase C (EC 3.2.1.52), is a cytosolic glycosidase specific for O-linked β-GlcNAc. The activity of O-GlcNAcase can be conveniently assayed in vitro with a synthetic substrate, p-nitrophenol N-acetylglucosaminide (pNP-β-GlcNAc). The cleavage product, pNP, has an absorbance peak at 400 nm.

Materials

- Partially purified O-GlcNAcase (0.2 to 1 µg) or cell extract sample (20 to 50 µg, precipitated with 30% to 50% ammonium chloride)
- 10× O-GlcNAcase assay buffer (see recipe)
- 100 mM (50×) p-nitrophenol N-acetylglucosaminide (pNP-GlcNAc) in DMSO
- 500 mM Na2CO₃
- 96-well flat bottom plates or 1.5 ml microcentrifuge tubes
- Plate reader or spectrophotometer

1. Prepare O-GlcNAcase.

   Native or recombinant O-GlcNAcase can be partially purified from animal tissues or cultured cells by several chromatographic steps (Dong and Hart, 1994; Gao et al., 2001). Alternatively, a crude enzyme preparation can be generated by passing cell extract over a 1-ml Con-A column. Most of the interfering acidic hexosaminidases are modified by N-linked sugars and bind to Con-A, while neutral O-GlcNAcase is in the flow-through (Izumi and Suzuki, 1983).

2. Precool 96-well plate or microcentrifuge tubes on ice.

3. Set up reactions in the precooled plate wells or tubes as follows:

   - 1 to 50 µl partially purified O-GlcNAcase enzyme or cell extract
   - 10 µl 10× O-GlcNAcase assay buffer
   - 2 µl 100 mM pNP-GlcNAc
   - H₂O to 100 µl

   The total reaction volume can be scaled up to 500 µl in microcentrifuge tubes.

   pNP-GlcNAc breaks down chemically. A blank reaction without enzyme should be included to determine the background.

   50 mM GalNAc is included in the reaction to inhibit lysosomal hexosaminidases A and B which may be present in the enzyme preparation. O-GlcNAcase is not inhibited by 50 mM GalNAc.

4. Mix well and cover.

5. Incubate at 37°C for 30 min to 4 hr.

   Yellow color will develop as pNP-GlcNAc is hydrolyzed by O-GlcNAcase. Reactions should be optimized to keep the absorbance within the linear range of the spectrophotometer. The authors find that 20 to 50 µg of cell extract used in a reaction of 100 µl, with a 1 to 2 hr incubation time is appropriate.
6. At the end of incubation, add an equal volume of 500 mM Na₂CO₃ to each well (100 µl or 500 µl).

   \[ \text{Na}_2\text{CO}_3 \text{ raises the pH to } p>pH 9.0, \text{ intensifying the yellow color and stopping the reaction, as O-GlcNAcase has little activity at pH 9 to 10.} \]

7. Read the absorbance at 400 nm on a plate reader or spectrophotometer.

8. Calculate O-GlcNAcase activity according to the following equation:

   \[ \text{mM of GlcNAc released} = \frac{A_{400}}{(17.4 \times 10 \text{ mM}^{-1} \cdot \text{cm}^{-1} \times \text{pathlength})} \]

   One unit is the amount of enzyme catalyzing the release of 1 µmol/min of pNP from pNP-GlcNAc.

   The molar extinction coefficient for pNP is 17.4 \times 10 \text{ mM}^{-1} \cdot \text{cm}^{-1} \text{ at pH 10. The path length for 200 µl on a 96-well plate is 0.71 cm.}

---

**REAGENTS AND SOLUTIONS**

*Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.*

**Biosynthetic labeling medium**

Glucose-free culture medium containing:
- 50 µCi/ml D-[6-3H]glucosamine (22 Ci/mmol; Amersham Pharmacia Biotech)
- 10% (v/v) FBS

Prepare fresh

**Buffer H**

- 50 mM HEPES, pH 6.8
- 50 mM NaCl
- 2% (v/v) Triton X-100

Store up to 1 month at room temperature

**Citrate-phosphate buffer, pH 4.0, 2×**

Dissolve 12.9 g citric acid monohydrate (mol. wt. 210) and 20.6 g disodium hydrogen phosphate heptahydrate (Na₂HPO₄·6H₂O) in 300 ml Milli-Q water. Bring volume to 500 ml.

**Galactosyltransferase labeling buffer, 10×**

- 100 mM HEPES, pH 7.5
- 100 mM galactose
- 50 mM MnCl₂

Store up to 1 month at 4°C

**Galactosyltransferase storage buffer**

- 2.5 mM HEPES, pH 7.4
- 2.5 mM MnCl₂
- 50% (v/v) glycerol

Store up to 1 month at room temperature
**Hexosaminidase reaction mixture, 2×**

*Per reaction:*
- 25 µl 2× citrate-phosphate buffer (see recipe)
- 1 U N-acetyl-β-d-glucosaminidase (V-Labs)
- 0.01 U aprotinin
- 1 µg leupeptin
- 1 µg α₂-macroglobulin

**O-GlcNAcase assay buffer, 10×**
- 500 mM sodium cacodylate, pH 6.4
- 500 mM N-acetylglactosamine (GlcNAc)
- 3% (w/v) bovine serum albumin (BSA)
  
  **Prepare fresh**

**OGT assay buffer, 10×**
- 500 mM sodium cacodylate, pH 6.0
- 10 mg/ml bovine serum albumin (BSA)
- 10 mM 1-amino-GlcNAc (2-acetamido-1-amino-1,2-dideoxy-β-D-glucopyranose; Sigma)
  
  **Prepare fresh**

**OGT desalting buffer**
- 20 mM Tris·Cl, pH 7.8 *(APPENDIX 2)*
- 1 mg/ml bovine serum albumin (BSA)
- 20% (v/v) glycerol
- 0.02% (w/v) NaN₃
  
  **Store up to 1 week at 4°C**

**Protease inhibitors, 1000×**

*PIC 1, 1000×:*
- Dissolve the following in 10,000 U/ml aprotinin solution (Sigma)
  - 1 mg/ml leupeptin
  - 2 mg/ml antipain
  - 10 mg/ml benzamide

*PIC 2, 1000×:*
- Prepare in DMSO
  - 1 mg/ml chemostatgin
  - 2 mg/ml pepstatin

*PMSF, 1000×:*
- 0.1 M phenylmethylsulfonyl fluoride in 95% ethanol

**Sequencing reagent**
- 7 ml methanol (HPLC-grade)
- 1 ml triethylamine (TEA; sequencing grade)
- 1 ml phenylisothiocyanate (PITC; sequencing grade)
- 1 ml Milli-Q water
  
  **Stable 24 hr at 4°C**

**sWGA Gal elution buffer**
- Phosphate-buffered saline (PBS; *(APPENDIX 2)*) containing:
  - 0.2% (v/v) NP-40
  - 1 M d-(+)-galactose (Gal)
  
  **Store up to 1 week at 4°C**
sWGA GlcNAc elution buffer
Phosphate-buffered saline (PBS; APPENDIX 2) containing:
0.2% (v/v) NP-40
1 M N-acetylglucosamine (GlcNAc)
Store up to 1 week at 4°C

TBS-HD
10 mM Tris-Cl, pH 7.5 (APPENDIX 2)
150 mM NaCl
1% (v/v) Triton X-100
0.1% (w/v) SDS
0.25% (w/v) deoxycholic acid
Store up to 1 month at 4°C

TBS-HT
10 mM Tris-Cl, pH 7.5 (APPENDIX 2)
150 mM NaCl
0.3% (v/v) Tween 20
Store up to 1 month at room temperature

TBST
10 mM Tris-Cl, pH 7.5 (APPENDIX 2)
150 mM NaCl
0.05% (v/v) Tween 20
Store up to 1 month at room temperature

Tris-buffered saline (TBS)
10 mM Tris-Cl, pH 7.5 (APPENDIX 2)
150 mM NaCl
Store up to 1 month at room temperature

COMMENTARY

Background Information
(β)-β-1,4-galactosylaminyltransferase from bovine milk recognizes terminal N-acetylglucosamine (GlcNAc) residues and modifies them by the addition of a single Gal residue. Torres and Hart (1984) first used this enzyme in combination with UDP-[3H]Gal to demonstrate that bovine lymphocytes contain proteins modified by O-linked GlcNAc. Further refinements of this experiment led them to propose that the product, βGal1-4βGlcNAc, was the result of the galactosyltransferase recognizing and modifying a single GlcNAc residue O-linked to Ser/Thr residues of nuclear and cytoplasmic proteins (Holt and Hart, 1986). Since this report, many cytosolic and nuclear proteins from mammalian cells were shown to be modified by O-GlcNAc. This method has remained the “gold standard” technique for the detection of O-GlcNAc-modified proteins, as the label provides a “tag” for subsequent analyses, such as those described under Product Characterization, below (see Critical Parameters and Troubleshooting).

In subsequent years, methods such as WGA affinity and western blotting with GlcNAc-specific lectins and antibodies have become popular as simple techniques for the initial characterization of target proteins.

Critical Parameters and Troubleshooting

Extraction of proteins from cells
The O-GlcNAc modification can be removed from proteins either by cytosolic O-GlcNAcase or lysosomal hexosaminidases. The inclusion of inhibitors during the extraction and purification process will preserve the levels of O-GlcNAc on proteins. Commonly used inhibitors (Dong and Hart, 1994) include 1-amino-GlcNAc (1 mM), GlcNAc (100 mM), and PUGNAc (5 µM). Note that these may have to be removed, as they will act as inhibitors in other methods.
**Product characterization**

Product characterization is a critical step showing that a protein is modified by O-GlcNAc, and not other glycans. While many proteins modified by O-GlcNAc have been identified, there is evidence based on metabolic labeling (Medina et al., 1998) and lectin labeling studies (Hart et al., 1989) that indicate that O-GlcNAc is not the only intracellular carbohydrate post-translational modification. In addition, at least one peptide mimic of O-GlcNAc has been identified in cytokeratins (Shikhman et al., 1994).

Moreover, many techniques used for breaking open cells also release proteins that are modified by complex N- and O-linked sugars, which may contain terminal GlcNAc. Many of the techniques described in this unit will recognize any GlcNAc residue, and it is important to perform the described controls such as PNGase F digestion to show specificity.

Product analysis is critical for metabolic labeling with glucosamine. While UDP-GlcNAc is the major product, glucosamine can enter other biosynthetic pathways, such as those used for amino acid synthesis. This issue was highlighted by studies of the SV40 large T-antigen. Some researchers have found that the SV40 large T-antigen labels with a number of different tritiated carbohydrates. However, O-GlcNAc is the only carbohydrate post-translational modification of the SV40 large T-antigen. The incorporation of glucosamine into amino acid biosynthetic pathways could be reduced by growing cells in the presence of excess nonessential amino acids (Medina et al., 1998).

Lastly, while galactosyltransferase is specific for terminal GlcNAc residues, researchers (Elling et al., 1999) have shown that galactosyltransferase will modify GlcNAc linked in either the α- or β-anomeric conformation. The authors of this unit have shown that proteins modified by α-O-GlcNAc will be labeled using the procedure described (N. Zachara, unpub. observ.). While α-O-GlcNAc has not been identified in complex eukaryotes, it is a common modification of cell surface proteins of simple eukaryotes such as trypanosomes and *Dictyostelium*. Product analysis, such as HPAEC of the sugar alditols, will resolve many of the issues discussed.

**Time Considerations**

Detection of O-GlcNAc proteins using antibodies (see Basic Protocol 2) and lectins (see Basic Protocol 3) will take approximately 2 to 3 days after the extraction of the proteins from cells. Samples and controls must be treated with PNGase F and/or hexosaminidase (1 hr to overnight), before SDS-PAGE and blotting. In either case, overnight incubation at 4°C provides the best signal-to-noise ratio.

WGA affinity chromatography of low-copy-number proteins will take 2 to 3 days. The ITT and WGA affinity chromatography can be completed in 1 day; subsequent analysis of the product by SDS-PAGE will take 1 to 2 days depending on the label used and the amount of label incorporated into proteins eluting from the WGA-agarose.

Autogalactosylation of the galactosyltransferase and subsequent analysis of the activity will take 1 to 2 days. As the enzyme is stable for 6 to 12 months at −20°C, autogalactosylation does not need to be repeated for each analysis. Labeling of the proteins can take several hours to overnight, though optimization of the conditions may take a few days. The subsequent analysis, as well as desalting (dependent on the technique used), PNGase F digestion (1 hr to overnight), precipitation of protein (3 hr to overnight), SDS-PAGE, and autoradiography (1 to 10 days), can take up to 2 weeks. The length of time allotted to product analysis is dependent on the methods chosen, but will almost certainly require 7 to 10 days. Further analysis, including digestion of labeled proteins and subsequent purification of peptides, will take at least 3 days.

**Literature Cited**


Detection and Analysis of Proteins Modified by O-Linked N-Acetylglucosamine

17.6.24

Supplement 57

Current Protocols in Molecular Biology


Lectin Analysis of Proteins Blotted onto Filters

Lectins are proteins that bind with great specificity to certain carbohydrate structures. Plant lectins are widely used for investigations of carbohydrate structure (UNIT 17.3) and for fractionation and purification of individual oligosaccharides and glycopeptides (UNIT 17.18). This unit describes the use of lectins as sensitive indicators for the presence of certain carbohydrate structures linked to proteins blotted onto filters. A tagged lectin is incubated with a blot containing the target protein and binding of the lectin is detected by one of several different procedures. Direct approaches include using lectins labeled with $^{125}\text{I}$ or conjugated to horseradish peroxidase or alkaline phosphatase, which can be detected by chromogenic or luminescent visualization systems. Indirect approaches involve using lectins conjugated to biotin or digoxigenin followed by a second incubation with alkaline phosphatase–conjugated avidin or antibodies specific for the haptenic digoxigenin group and then by visualization. Several commercial kits are available that provide labeled lectins, control proteins, and developing reagents needed for visualization. These systems can also be adapted for use with lectins other than those supplied with kits. The following protocol is easy to perform with or without a kit. However, the results, while suggestive of carbohydrate structure, are not definitive.

**Materials**

- Purified protein sample containing 1 to 10 µg of target protein
- Lectin blot kit (e.g., E-Y Laboratories Lectin Staining Kit; Genzyme Lectin Link Kit; Boehringer Mannheim Glycan Differentiation Kit) or equivalent materials:
  - Glycoprotein known to bind with the chosen lectins (positive control; Table 17.7.1)
  - Blocking solution: e.g., 1% to 2% (w/v) gelatin in TTBS or 3% periodate-oxidized bovine serum albumin (BSA)
  - Incubation buffer: e.g., 0.1% (v/v) Tween 20 in Tris-buffered saline (TTBS; UNIT 10.8)
  - Labeled or conjugated lectin (Tables 17.7.2 & 17.7.3) in TTBS or other appropriate incubation buffer
  - Enzyme- or antibody-linked antibody (secondary reagent), if needed
  - Chromogenic or luminescent visualization reagent for detecting tagged lectin or antibody (Table 10.8.1)

Additional reagents and equipment for SDS-PAGE (UNIT 10.2), immunoblotting and immunodetection (UNIT 10.8), and autoradiography (APPENDIX 3)

**Table 17.7.1 Lectin-Binding Glycoproteins**

<table>
<thead>
<tr>
<th>Glycoprotein</th>
<th>Molecular weight (kDa)</th>
<th>Lectin bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase B</td>
<td>17</td>
<td>Con A</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>45</td>
<td>Con A</td>
</tr>
<tr>
<td>Transferrin</td>
<td>80</td>
<td>SNA</td>
</tr>
<tr>
<td>Fetuin</td>
<td>68</td>
<td>SNA, MAA</td>
</tr>
<tr>
<td>Asialofetuin</td>
<td>61</td>
<td>RCA I</td>
</tr>
<tr>
<td>α1-acid glycoprotein</td>
<td>45</td>
<td>DSA</td>
</tr>
</tbody>
</table>

*Glycoproteins known to bind with the chosen lectins are used as positive controls and as standards for quantitative analysis. They are supplied with some lectin blot kits (e.g., Boehringer Mannheim’s) or available separately from Sigma.

Abbreviations: Con A, concanavalin A; SNA, Sambucus nigra agglutinin; MAA, Maackia amurensis agglutinin; RCA I, Ricinus communis agglutinin I; DSA, Datura stramonium agglutinin.
1. Separate the proteins in the sample by one-dimensional SDS-PAGE and transfer onto nitrocellulose by electroblotting. Include positive control (a glycoprotein known to bind to the lectins) and extra sample lanes for incubation controls (see step 5 annotation).

Inclusion of a positive control is particularly important in early experiments when incubation conditions are being optimized. These procedures are useful for relatively pure proteins. Crude mixtures of proteins are not suitable for these tests.

If some samples are to be digested with exo- or endoglycosidases to facilitate interpretation of results (see critical parameters), digestions should be carried out prior to running the sample on the gel. In this case a gel with separate wells must be used and a glycosidase-only control lane included for each digestion. Many of the glycosidases are themselves glycoproteins and will bind lectins.

2. Incubate nitrocellulose blot in blocking solution with agitation ≥30 min at room temperature.

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Specificity</th>
<th>Notes</th>
<th>Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con-A (Concanavalin A)</td>
<td>αMan, αGlc</td>
<td>Strongly indicates N-linked oligosaccharide chains. Binds high mannose, hybrid, or biantennary complex chains</td>
<td>EY-L, GN-LL</td>
</tr>
<tr>
<td>GNA (Galanthus nivalis agglutinin)</td>
<td>ManαMan</td>
<td>Binds terminal mannose-linked, α1-3, α1-6 or α1-2 linked to mannose. It will identify “high mannose” N-glycan chains or O-glycosidically linked mannose chains in yeast glycoproteins</td>
<td>BM-GD</td>
</tr>
<tr>
<td>WGA (Wheat germ agglutinin)</td>
<td>β-GlcNAc or sialic acid</td>
<td>Binds terminal βGlcNAc or sialic acids on various glycans</td>
<td>GN-LL, EY-L</td>
</tr>
<tr>
<td>RCA I (Ricinus communis agglutinin)</td>
<td>βGal</td>
<td>Binds Gal-terminated N-linked oligosaccharides, but not exclusive</td>
<td>GN-LL</td>
</tr>
<tr>
<td>DSA (Datura stramonium agglutinin)</td>
<td>Galβ1-4GlcNAc</td>
<td>Binds Galβ1-4GlcNAc in complex and hybrid N-glycans and GlcNAc in O-glycans, also polyglycosamines</td>
<td>BM-GD, GN-LL</td>
</tr>
<tr>
<td>MAA (Maackia amurensis agglutinin)</td>
<td>SAα 2-3 Gal-β-GlcNAc</td>
<td>Binds sialic acid linked α2-3 to galactose in N- and O-linked glycans</td>
<td>BM-GD, GN-LL</td>
</tr>
<tr>
<td>SNA (Sambucus nigra agglutinin)</td>
<td>SAα 2-6 Gal or GalNAc</td>
<td>Binds sialic acid linked α2-6 to Gal or GalNAc; identifies sialylated O-glycan, N-glycan chains</td>
<td>BM-GD, GN-LL</td>
</tr>
<tr>
<td>PNA (Peanut agglutinin)</td>
<td>Galβ1-3 GalNAc</td>
<td>Recognizes the core disaccharide galactose β1-3 N-acetylgalactosamine O-glycosidically linked chains (except of yeast glycoproteins)</td>
<td>BM-GD</td>
</tr>
</tbody>
</table>

Abbreviations: Man, mannose; Glc, glucose; GlcNAc, N-acetylglucosamine; Gal, galactose; SA, sialic acid; GalNAc, N-acetylgalactosamine.

E-Y Laboratories Lectin Staining Kit; BM-GD, Boehringer Mannheim Glycan Differentiation Kit; GN-LL, Genzyme Lectin Link Kit.
Gelatin is used as the blocking reagent because it is not a glycoprotein. Blocking reagents that contain even small amounts of glycoproteins (e.g., nonfat dry milk) may bind to the lectins, potentially depleting the available lectin or producing a high background signal. Alternatively, periodate oxidized BSA (3%) can be used as a blocking reagent (Glass et al., 1981).

3. Wash each filter three times in 50 ml TTBS or other buffer appropriate for incubation with the labeled lectin, 15 min per wash.

Other buffers and washing conditions may be suitable, as discussed in the literature accompanying lectin blot kits, but TTBS is routinely used.

4. Place the blot on a smooth, clean surface, such as a glass plate, and use a clean razor blade to cut the nitrocellulose into multiple strips approximately the width of a single lane.

It is not necessary to cut the strips exactly along the interlane boundaries. If photographs are required, the lane can be trimmed to a desirable width later.

5. Place each strip in a separate container and add sufficient TTBS or other appropriate buffer containing ~1 µg/ml labeled lectin to cover and suspend strip. Incubate 1 hr at room temperature with gentle shaking.

Incubation conditions must be optimized for each lectin and for each detection method. This information is available in kit instructions or from the supplier. Chelators such as EDTA should be avoided, as some lectins (e.g., Con A) require tightly bound metal ions for activity.

A sample incubated without lectin should be prepared as a control. Regardless of the method used to detect lectin binding, it is also important to include controls in which either the primary or secondary reagents are eliminated. If a sufficient amount (50 to 100 mM) of the appropriate competing sugar is available, lectin binding should be carried out in the presence of the sugar as a negative control (the sugar should eliminate specific binding).

6. Remove the lectin solution and store it at 4°C. Wash filter three times in 50 ml TTBS, 10 min each time.

Lectin solutions may be reused several times within one week of their first use if they are stored at 4°C. If a lectin solution is reused, the positive control will detect whether the lectin is still active.
7a. For lectins directly tagged with $^{125}$I, biotin, horseradish peroxidase, or alkaline phosphatase: Visualize by autoradiography or using the appropriate chromogenic or luminescent visualization solution.

7b. For lectins conjugated to biotin or digoxigenin: Incubate filter in the appropriate secondary reagent 1 hr at room temperature. Wash filter 3 times with 50 ml TTBS, 10 min each time. Detect bound lectin using the appropriate chromogenic or luminescent visualization solution.

Lectins conjugated to these groups are detected by incubation with avidin–alkaline phosphatase conjugates or anti-digoxigenin–alkaline phosphatase.

**COMMENTARY**

**Background Information**

Lectins have been used for many years to detect the presence of various types of sugar chains. Lectin blots provide quick glimpses of the types of sugars attached to purified proteins. Even though the blots provide useful information, they cannot provide proof of a sugar structure and hence cannot substitute for the more specific and quantitative analytical approaches presented in this chapter. This is because lectin binding and its quantitation depend upon many factors that vary with different individual proteins. The advantage of using lectins is that many different ones are available and analysis requires only modest amounts of protein and routine laboratory equipment. Also, many detection methods are available. In order to have confidence in the results of lectin-binding studies, it is necessary to show that their binding specificity is abolished by coincubation with a competing sugar (usually a monosaccharide) or by prior enzymatic digestion to remove some or all of the protein-linked sugar chains. Lectins labeled with fluorescent indicators can be used to visualize glycoconjugates directly in hydrated gels (West and McMahon, 1977). However, this approach requires several days. This unit describes a less time-consuming method (based on Tanner and Anstee, 1976) in which the proteins are blotted onto filters prior to detection by one of several methods.

**Critical Parameters and Troubleshooting**

Information concerning critical parameters and troubleshooting for protein blotting, immunopробинг, and chromogenic and luminescent detection is provided in UNIT 10.8.

**Experimental design**

Two points are especially important in designing lectin-binding experiments: the choice of lectin and the choice of method for detecting lectin bound to glycoprotein. If the goal of the experiment is to obtain preliminary evidence that a protein contains carbohydrate groups, a battery of lectins should be used along with appropriate controls (e.g., competition by appropriate sugars). If the experiment is meant to serve as a guide to further structural analysis, the effects of various chemical or enzymatic treatments must be combined with analysis using one or more lectins known to bind to the glycoconjugate. Table 17.7.2 shows the specificity of the most popular lectins, some of which are supplied in the various kits.

The choice of detection method is affected by the amount of protein available and the extent of glycosylation. If the sample contains 1 to 10 $\mu$g of target protein or if the material is extensively glycosylated, lectins labeled directly with $^{125}$I, alkaline phosphatase, or horseradish peroxidase can be used. If either the protein quantity or extent of glycosylation is minimal, an indirect system using lectins that are biotinylated or coupled to digoxigenin and a secondary enzyme- or antibody-linked reagent has the advantage of signal amplification.

**Kits**

The various reagents can be purchased separately or as prepackaged kits from the suppliers listed in Table 17.7.3. E-Y Laboratories offers the largest selection of purified lectins and lectins tagged with various conjugates.

The E-Y Laboratories kit uses lectins coupled to horseradish peroxidase. The Genzyme and Boehringer Mannheim kits use alkaline phosphatase–coupled molecules to detect the tagged lectins. Alternatively, tagged lectins can easily be prepared in the laboratory (UNIT 11.1). The major advantages of using kits are that they are self-sufficient, containing all of the reagents and positive controls needed, and that the lectins they employ are among those with the best characterized specificities. When coupled
with glycosidase digestions, the kits probably provide the most information about the sugar chains in the least amount of time for the least amount of money. Because there are limitations to the information that can be obtained, the decision about which individual lectins or kits to buy will depend upon the precision of information required.

The Glycan Differentiation Kit (Boehringer Mannheim) supplies lectins that are coupled to the steroid digoxigenin using an N-hydroxysuccinimide ester. An alkaline phosphatase–conjugated antiserum against this steroid is used to visualize bound lectin on target glycoproteins (Haselbeck et al., 1990). Digoxigenin is a naturally occurring steroid found only in plants of the *Digitalis* family and not in animals, which minimizes background. The sheep Fab fragment to the hapten is affinity purified and coupled to alkaline phosphatase. The company has used the digoxigenin system extensively in different types of kits to detect proteins, glycoproteins, and nucleic acids (UNIT 3.18). Lectin binding is simply an extension of this technology. The system has also been adapted to visualize glycoconjugates by light and electron microscopy (Sata et al., 1990).

The Lectin Link Kit (Genzyme) uses a biotinylated lectin and an alkaline phosphatase–avidin conjugate to visualize glycoproteins on the blot. The basic ABC (avidin–biotin conjugate) technology is well established in the literature for many assays. This kit as presented is primarily intended to identify and differentiate between various types of N-linked oligosaccharides. The conclusion that binding of any lectin is exclusively due to the presence of a particular structure on an N-linked oligosaccharide chain is valid only if all lectin binding is destroyed by PNGase F digestion (UNIT 17.13), because, except for Con A, no lectin is specific for N-linked oligosaccharides. It is important to note that not all N-linked oligosaccharides are sensitive to PNGase F (UNIT 17.13); resistance to PNGase F does not prove that a glycan is, by elimination, O-linked.

**Anticipated Results**

Even though lectins are quite specific, results are more qualitative than quantitative. The results obtained using lectins should be regarded as guides and preliminary indications of likely structures. Lectin-binding studies, especially when combined with a battery of enzymatic digestions, are useful experiments for preliminary characterization of glycoprotein structure because they are quick, reasonably priced, and use only a few micrograms (or less) of purified sample material. However, they cannot substitute for the more precise structural and chemical analyses presented in other units in Chapter 17.

Inclusion of a positive control provides a qualitative and rough quantitative estimate for the amount of reactive material in the sample relative to the standards. This may be especially important in assessing the effects and completeness of exo- or endoglycosidase digestions. The problem with attempting accurate quantitation is that the intensity of staining will depend upon the binding affinity, the amount of protein, and the number of accessible glycans on the protein. All of these factors are important, and a high number of low-affinity binding sites present on a large quantity of protein can give an intensity on a blot similar to that of a single high-affinity binding site on a smaller amount of protein.

Two examples illustrate useful approaches for maximizing the information from lectin binding. In both, lectin-binding results first suggest the presence of certain types of carbohydrate chains on the protein. This information is used to design glycosidase digestions (UNIT 17.13) that should alter the binding of some or all of the lectins. Digested glycoproteins are probed with the lectins to confirm the predictions. All of the data are combined into a composite picture of the sugar chains present. To be conclusive, results of lectin binding and glycosidase digestions should be internally consistent. Because some of the glycosidases are themselves glycoproteins, controls should include a lane with glycosidase alone to identify bands due to the presence of glycosidase in the digested sample.

**Example 1**

A hypothetical purified glycoprotein reacts with wheat germ agglutinin (WGA) and *Sambucus nigra* agglutinin (SNA; see Table 17.7.2). WGA binds to terminal GlcNAc residues or sialic acids. Terminal GlcNAc may occur in complex N-linked oligosaccharides, such as those in ovomucoid, or as multiple residues of O-β-GlcNAc or GlcNAc-1-P directly bound to protein. Sialic acid could be present in α2-3 or α2-6 linkages to underlying βGal residues on N- or O-linked chains. SNA binds to sialic acid linked α2-6 to βGal. One way to distinguish among the possibilities is to treat the sample with sialidase. Loss of WGA binding following sialidase treatment indicates that WGA binding is due to sialic acid and not terminal GlcNAc.

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**Preparation and Analysis of Glycoconjugates**

17.7.5
If both WGA binding and SNA binding are lost with PNGase F digestion, all of the WGA and SNA binding are due to the presence of sialic acids linked \( \alpha_{2-6} \) to \( \beta \)-Gal residues on N-linked oligosaccharides.

**Example 2**

Experimental results from lectin and glycosidase digestion analysis of a hypothetical purified glycoprotein, glycobiologin, are shown in Figure 17.7.1. Undigested glycobiologin (panel A) produces a diffuse band when incubated with *Datura stramonium* agglutinin (DSA), *Ricinus communis* agglutinin (RCA I), and *Maackia amurensis* agglutinin (MAA) and a less diffuse band with SNA, but no band when incubated with Con A. Thus, all the lectins but Con A bind to glycobiologin. These data sug-

![Figure 17.7.1 Example: Lectin blot analysis of a hypothetical purified glycoprotein, glycobiologin, using one of the procedures or kits described in this unit. All enzymatic digestions were conducted prior to lectin binding, and each lectin was previously tested on a positive control protein to check the reagents, developing conditions, etc. All samples of the test protein were run on SDS-PAGE, blotted onto nitrocellulose filters, blocked, and probed with the indicated lectins after the enzymatic treatment. Control lanes with glycosidase only control are not shown, but would normally be run. (A) Undigested glycobiologin. (B) Glycobiologin digested with endo-\( \beta \)-galactosidase. (C) Glycobiologin digested with exo-\( \beta \)-galactosidase. (D) Glycobiologin digested with Newcastle Disease Virus (NDV) sialidase. (E) Glycobiologin digested with the broad-spectrum sialidase from *Arthrobacter ureafaciens* (A.U.). (F) Glycobiologin digested with PNGase F. Arrow indicates the densest portion of the band for undigested glycobiologin.](image-url)
suggest that the glycoprotein may contain poly lactosamine chains (DSA), terminal β-Gal residues (RCA I), and both α2-3- and α2-6-linked sialic acids (MAA and SNA, respectively). High-mannose type chains are probably absent (Con A).

This is useful information, but performing a few enzyme digestions and then probing with all of the lectins will provide much more. First, specific enzyme digestions should be done to show that each of the lectins is, in fact, binding to the expected sugar structure. Digestion with endo-β-galactosidase (panel B), which removes poly lactosamine chains (UNIT 17.13), abolishes all bands in the DSA lane, showing that binding to DSA was due entirely to poly lactosamine chains. The apparent Mr of the band has dropped and its boundaries have sharpened, showing that the poly lactosamines contributed to the diffuse appearance of the original band. Glycoproteins often produce diffuse bands in gels and on blots because of microheterogeneity in the carbohydrate chains (UNIT 17.1). This digestion also destroys all of the MAA binding, suggesting that the poly lactosamine chains, and only those chains, were terminated by α2-3-linked sialic acids (UNIT 17.12). Bands are still observed in the RCA I lane, showing that terminal β-Gal residues were not digested, and in the SNA lane, showing that the poly lactosamine chains do not terminate in α2-6 sialic acids. If the digestion is done with exo-β-galactosidase (panel C), which removes terminal β-Gal residues (UNIT 17.18), it destroys the band in the RCA I lane but has minimal effects on the position and appearance of the other bands. This shows that the lectin was bound to the predicted terminal sugar residues. There are probably only a small number of these and they did not contribute much to the heterogeneity of the protein. Digestion with Newcastle Disease Virus (NDV) sialidase (panel D), which specifically removes α2-3-linked sialic acids, abolished the binding of MAA, showing that MAA bound the α2-3 sialic acid, as predicted. The significant reduction in the apparent size of the protein size shows that the sialic acids make a large contribution to the apparent size. Digestion with Arthrobacter ureafaciens sialidase (panel E), a broad-spectrum sialidase (UNIT 17.12), abolishes the bands in both the MAA and SNA lanes. Diffuse bands are still seen in the DSA and RCA I lanes; the apparent protein size is slightly smaller than in panel D, showing that the α2-6-linked sialic acids make a relatively small contribution to the apparent size compared to those linked α2-3. For each digestion the bands are reduced in Mr and/or become sharper compared to those in the undigested glycoprotein.

The final digestion (panel F) is with peptide N-glycosidase F (PNGase F), which removes essentially all N-linked chains; however, some are resistant (UNIT 17.13). Any lectin binding that is completely destroyed by this digestion is due only to components found on N-linked chains. PNGase F digestion of glycoprotein completely abolishes the bands in the RCA I and MAA lanes but only partially reduces the intensity of the bands in the DSA and SNA lanes. The lack of binding to RCA I and MAA indicates that all of the β-Gal-terminated chains and those with α2-3 sialic acids are exclusively N-linked (RCA I and MAA). Reduced binding to SNA indicates that some of the N-linked chains also contain α2-6 sialic acid, which may also be found on O-GalNAc-linked chains. Reduced binding to DSA suggests either that the protein also contains poly lactosamine units on both N- and O-linked chains or possibly that the PNGase F digestion was incomplete. The band is still diffuse because of residual poly lactosamine chains. The apparent protein size is considerably smaller, showing that most of the heterogeneity was due to poly lactosamine chains on N-linked oligosaccharides.

It is important to stress again that lectin blots give only preliminary evidence for the presence of the suggested structures. The results should be viewed as useful guides.

**Time Considerations**

Most enzymatic digestions are carried out overnight. If the proteins are separated on minigels, the entire analysis including blotting and development can be done in a single day. The procedure can be interrupted following protein transfer to nitrocellulose. Incubation in blocking buffer can be extended to overnight at 4°C.

**Literature Cited**

Detailed references regarding detection of the various types of conjugates are given in UNIT 10.8. Kit manufacturers will supply relevant references upon request. Much of their information may have been obtained exclusively from data produced in company laboratories, although some has also appeared in the peer-reviewed literature.


**Key References**


*Good overview of procedure.*

Contributed by Hudson H. Freeze
La Jolla Cancer Research Foundation
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Detection of Glycophospholipid Anchors on Proteins

Many eukaryotic proteins are tethered to the plasma membrane by glycosyl phosphatidylinositol (GPI) membrane anchors. Figure 17.8.1 schematically depicts the minimal (core) structure common to all GPI protein anchors characterized to date. Detailed structural information is described in reviews by Cross (1990) and Ferguson (1991).

This unit provides a general approach for detecting GPI-anchored proteins. First, the detergent-partitioning behavior of a protein of interest is examined for characteristics of GPI-linked species. The partitioning of total cellular and isolated proteins with Triton X-114 is described in the first basic and alternate protocols, respectively. Precondensation of Triton X-114, necessary to remove hydrophilic contaminants before partitioning, is outlined in the first support protocol.

The protein may also be subjected to specific enzymatic or chemical cleavages to release the protein from its GPI anchor. Phospholipase cleavage is detailed in the second basic and alternate protocols, and chemical cleavage with nitrous acid is described in the third basic protocol.

If GPI-anchored proteins are radiolabeled with fatty acids, it facilitates the detection of the GPI protein products following the cleavage reactions. Separation of lipid moieties is described in the third support protocol and base hydrolysis of proteins is presented in the fourth basic protocol. Figure 17.8.2 is a flowchart depicting the relationships between the various protocols.

Figure 17.8.1  Schematic representation of the glycan core structure common to all GPI anchors. The sites of cleavage of phospholipase C enzymes (PI-PLC, GPI-PLC), phospholipase D (GPI-PLD), and nitrous acid (HONO) are indicated, as are lipid products resulting from these cleavages: PI (phosphatidylinositol), PA (phosphatidic acid), DAG (diacylglycerol). Other abbreviations: Man, mannose; GlcN, glucosamine; EthN, ethanolamine; P, phosphate group. Phospholipase C treatment generates inositol cyclic phosphate, the major epitope contributing to the cross-reacting determinant (CRD). Lipid products may vary from those depicted, depending on features of the anchor (see background information).

Contributed by Tamara L. Doering, Paul T. Englund, and Gerald W. Hart

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Strategic Planning

The techniques described below require that a method for identifying the protein of interest be available. Proteins may be visualized after one-dimensional gel electrophoresis (UNIT 10.2) and Coomassie blue or silver staining (UNIT 10.6), or they may be identified by immunoprecipitation (UNIT 10.16), or immunoblotting (UNIT 10.8) using available specific antibodies. Enzymes may also be detected by their known activities (e.g., see Kodukula et al., 1992).

It is extremely convenient, particularly for low-abundance species, to use proteins that are labeled with radioactive amino acids (UNIT 10.18) or GPI components (see Fig. 17.8.1). The ability to radiolabel proteins with GPI precursors such as fatty acids, ethanolamine, myo-inositol, and certain monosaccharide precursors (UNIT 17.4) is in itself suggestive of GPI anchorage. Because GPI-anchored proteins commonly represent a small fraction of eukaryotic glycoconjugates, metabolic radiolabeling may be inefficient. Tunicamycin (UNIT 17.10) may be used to increase relative incorporation of monosaccharide precursors into GPIs.

Rigorous identification of specific GPI anchor structures requires detailed analysis. These methods, which are beyond the scope of this unit, are described in Ferguson (1992).

**Figure 17.8.2** Flowchart for the detection of GPI-anchored proteins.
**EXTRACTION AND PARTITIONING OF TOTAL PROTEINS FROM CELLS OR MEMBRANES WITH TRITON X-114**

Extraction of cells or tissues with the detergent Triton X-114 provides a profile (and potentially an initial purification) of amphiphilic proteins, including integral membrane proteins and species bearing GPI anchors. Once a GPI-containing protein is released from the lipid component of its anchor, it will no longer partition into the detergent-enriched phase. This alteration of partitioning behavior provides a rapid assay for presence or absence of anchors. It may be used to monitor the changes in proteins produced by the other procedures.

In this protocol, cells or membrane fractions are first resuspended in TBS, then extracted with a Triton X-114 incubation on ice. The mixture is then warmed and separates into two protein-containing phases due to aggregation of detergent micelles (see background information). The upper (detergent-depleted) phase contains hydrophilic proteins; amphiphilic proteins (including those with GPI anchors) partition to the lower (detergent-enriched) phase. Both phases are then analyzed to clarify the group to which each protein belongs.

**Materials**
- Cells, membrane fraction, or other source of protein
- Tris-buffered saline (TBS), ice-cold
- Precondensed Triton X-114 stock solution in TBS (support protocol), ice-cold
- 15-ml polypropylene centrifuge tubes
- Centrifuges: low-speed (tabletop) and equipped with appropriate rotor (e.g., SS-34), at 4°C and room temperature

1. Resuspend cells (or membranes) in ice-cold TBS to a final protein concentration of ≤4 mg/ml in a 15-ml tube.

   *The amount of protein required is that which will give detectable levels at the end of the procedure and will depend on the system the individual researcher is using. As a guideline, use several times the amount of protein that is believed to be needed, to account for partitioning and losses (see critical parameters).*

2. Add 1/5 vol precondensed Triton X-114 stock solution (~2% final concentration).

3. Extract cells by incubating 15 min on ice with occasional mixing.

   *At this point in the procedure there will only be one phase.*

4. Centrifuge cell mixture 10 min at 10,000 × g (9000 rpm in an SS-34 rotor), 4°C, and transfer supernatant to a fresh tube. Resuspend pellet in ice-cold TBS and save on ice for analysis in step 7.

   *The cold supernatant fraction contains both soluble and detergent-extracted proteins; the latter group includes proteins anchored by GPI structures or by transmembrane polypeptide domains. The pellet contains additional GPI species, as well as cell elements insoluble in nonionic detergents.*

5. Warm supernatant fraction to 37°C in a water bath until the solution becomes cloudy.

6. Centrifuge the solution in a tabletop centrifuge 10 min at 1000 × g, room temperature. Collect upper and lower phases in separate tubes.

   *The upper phase (detergent-depleted) contains soluble proteins; the lower (detergent-enriched) phase contains proteins anchored by GPI structures or by transmembrane domains.*

7. Analyze the resuspended pellet from step 4 and each phase from step 6 for the proteins of interest.
As discussed in strategic planning, analysis may be by an activity assay, one-dimensional gel electrophoresis and staining of proteins (Units 10.2 & 10.6), or immunoprecipitation (UNIT 10.16) or immunoblotting (UNIT 10.8) using specific antibodies.

**PARTITIONING OF ISOLATED PROTEINS WITH TRITON X-114**

A modification to the basic protocol for Triton X-114 extraction of proteins from whole cells or membranes is used to partition isolated proteins.

1. Dilute or dissolve the protein in 1 ml ice-cold TBS in a 1.5-ml microcentrifuge tube. Add 0.2 ml Triton X-114 and mix.

   See critical parameters for guidelines on the total amount of protein to use.

2. Warm protein mixture 15 min (or until it becomes cloudy) in a 37°C water bath. Microcentrifuge 5 min at maximum speed.

3. Collect the upper and lower phases and analyze each phase for the protein(s) of interest.

**PRECONDENSATION OF TRITON X-114 DETERGENT**

Triton X-114 often contains hydrophilic contaminants, and must be precondensed by several rounds of phase separation before use.

**Materials**

- Triton X-114 detergent
- Tris-buffered saline (TBS)
- 50-ml centrifuge tubes
- Tabletop centrifuge

1. Dissolve 1.5 g Triton X-114 in 50 ml TBS, place in a 50-ml centrifuge tube, then chill on ice.

   At this point, the solution will be clear.

2. Warm the solution to 37°C in a water bath.

   Following this warming, the solution will be turbid.

3. Centrifuge the solution in a tabletop centrifuge 10 min at 1000 × g, room temperature.

4. Remove and discard the upper (detergent-depleted) phase and redissolve the lower (detergent-enriched) phase in an equal volume of ice-cold TBS.

   Hydrophilic contaminants partition into the upper phase.

5. Repeat partitioning (steps 2 to 4) three times.

   The final detergent-enriched phase contains ~12% detergent. It may be stored at 4°C for routine use or frozen for long-term storage.

Detection of Glycophospholipid Anchors on Proteins

17.8.4

Supplement 22 Current Protocols in Molecular Biology
IDENTIFICATION OF GPI-ANCHORED PROTEINS BY PI-PLC DIGESTION OF INTACT CELLS

Phospholipase C (PLC) cleaves within the GPI membrane anchors (see Fig. 17.8.1) of glycoproteins, causing the release of protein from cell membranes. In this protocol, intact cells are digested with phosphatidylinositol-specific phospholipase C (PI-PLC). Following incubation with the enzyme, the cell mixture is centrifuged and both the resulting pellet and supernatant are analyzed for the presence of the protein of interest. Proteins released from GPI anchors are found in the supernatant; lack of release may indicate absence of a GPI anchor, modification of the GPI protein, or other factors (see background information).

Materials

- Cells (or membrane preparation)
- Bacterial phosphatidylinositol-specific phospholipase C (PI-PLC; e.g., from *Bacillus thuringiensis*, Oxford Glycosystems)
- Hanks balanced salt solution (HBSS; GIBCO/BRL), buffered saline, or culture medium
- Tabletop centrifuge and appropriate centrifuge tubes

1. Obtain cells (or membranes) of interest in dispersed suspension. Wash duplicate aliquots twice in HBSS, buffered saline, or culture medium, then resuspend in any of these solutions.

   *The final protein concentration of the samples (typically 0.1 to 0.5 mg/ml) should be based on supplier specifications for the enzyme preparation used. See critical parameters for guidelines on the total amount of protein to use.*

2. Add bacterial PI-PLC to one aliquot. Add no enzyme to the second aliquot (control). Incubate tubes 1 hr at 37°C.

   *The amount of enzyme should be determined by supplier recommendations.*

3. Centrifuge cells in a tabletop centrifuge 5 min at 1000 × g. Remove each supernatant to a fresh tube and save for analysis in step 4. Resuspend pellet in a volume of buffered saline equal to the volume of supernatant removed.

4. Assay supernatant and pellet fractions for protein(s) of interest.

   *The proteins may be assayed by Triton X-114 detergent partitioning (first basic protocol), one-dimensional gel electrophoresis (UNIT 10.2) followed by Coomassie blue or silver staining (UNIT 10.6), immunoprecipitation (UNIT 10.16) or immunoblotting (UNIT 10.8)*

ALTERNATE PROTOCOL

IDENTIFICATION OF GPI ANCHORAGE BY PHOSPHOLIPASE TREATMENT OF ISOLATED PROTEINS

Several specific phospholipases that effectively degrade only GPI species are useful for analyzing GPI anchorage of isolated proteins (see background information). In this protocol, aliquots of the protein are digested in parallel with the following phospholipases: bacterial phosphatidylinositol-specific phospholipase C (PI-PLC), a GPI-specific phospholipase C (GPI-PLC) from *Trypanosoma brucei*, and a GPI-specific phospholipase D (GPI-PLD) from mammalian serum (rat, rabbit, or human). Proteins are partitioned by Triton X-114 extraction (first alternate protocol), then analyzed. Specific cleavage of proteins by phospholipase treatment strongly indicates GPI anchorage.
Detection of Glycophospholipid Anchors on Proteins

17.8.6

Materials

Isolated protein
Acetone, −20°C
Appropriate enzyme buffer: GPI-PLD buffer, GPI-PLC buffer, and PI-PLC buffer
Phospholipase enzyme: GPI-PLD from rat, rabbit or human whole serum,
GPI-PLC from *Trypanosoma brucei* (Oxford GlycoSystems), and PI-PLC
from *Bacillus thuringiensis* (Oxford GlycoSystems) or *B. cereus*
(Boehringer Mannheim or Sigma)
Tris-buffered saline (TBS)
Precondensed Triton X-114 solution (first support protocol)
Centrifuge and rotor (e.g., SS-34)

1. If the protein sample is lyophilized or concentrated in a buffer similar to the
phospholipase buffers, begin at step 3. If it is not, acetone-precipitate the protein by
adding 8 vol cold (−20°C) acetone to the protein solution and incubating it ≥3 hr at
−20°C.

See critical parameters for guidelines on the total amount of protein to use. For low-abun-
dance material, efficiency of precipitation may be increased by including 15 µg of a carrier
protein (e.g., cytochrome c or bovine serum albumin).

2. Centrifuge the sample 20 min at 15,000 × g (∼11,000 rpm in an SS-34 rotor), 4°C.
Aspirate supernatant and allow pellet to air dry.

3. For each enzyme to be tested, dissolve two aliquots of the protein in 100 µl of the
appropriate enzyme buffer in microcentrifuge tubes.

4. To one aliquot from each pair add the appropriate enzyme. Add an equal volume of
the appropriate enzyme buffer to the second aliquot (control). Mix tubes and incubate
1 hr at 37°C.

The amount of enzyme added should be based on the activity of the lipase preparation (see
supplier specifications) and the estimated amount of protein present. It may be desirable
to titrate the enzyme, using various amounts in parallel reactions, to be sure that all
sensitive proteins are completely digested.

GPI-PLD is not generally available, but whole serum (rat, rabbit or human) may serve as
a source of this activity. Aliquots of serum may be stored frozen and thawed before use. For
the assay, use 2 µl whole serum in a 100-µl reaction.

5. Dilute reaction with 200 µl ice-cold TBS, then add 60 µl Triton X-114. Chill 15 min
on ice.

6. Partition the proteins as in steps 2 and 3 of the first alternate protocol.

GPI-anchored proteins partition into the detergent-enriched phase of Triton X-114 in the
control reaction (uncleaved) and into the detergent-depleted phase after cleavage of the
GPI anchor by phospholipase.

7. Analyze each phase for the presence of protein(s) of interest as in step 7 of the first
basic protocol.

Alternatively, cleavage products can be detected by reactivity with α-CRD antibody
(second support protocol) and, if the anchor is radiolabeled with fatty acids (UNIT 17.4), by
separation of radiolabeled lipid products.

Migration of phospholipase-digested proteins on gel electrophoresis is not predictable and
should not be used alone to assess cleavage. No shift, or a shift in either direction (relative
to uncleaved protein migration) may be observed upon GPI anchor removal, depending on
the properties of the protein.
DETECTION OF PRODUCTS AFTER PHOSPHOLIPASE TREATMENT BY REACTIVITY WITH ANTI-CRD ANTIBODY

Cleavage of GPI structures with phospholipase C reveals a cryptic antigen termed the cross-reacting determinant (CRD). The major epitope contributing to the CRD is an inositol 1,2-(cyclic) monophosphate formed during phospholipase C cleavage; other structural features may also contribute to the antigenicity. Reactivity of a protein with anti-CRD antibody after solubilization by phospholipase C strongly indicates the presence of a GPI anchor. Anti-CRD antibody (Oxford GlycoSystems) may be used after phospholipase C treatment to immunoprecipitate (UNIT 10.16) or immunoblot (UNIT 10.8) proteins of interest. It should be noted that phospholipase D cleavage does not create the epitope required for CRD activity (see Fig. 17.8.1).

NITROUS ACID CLEAVAGE OF GPI-ANCHORED PROTEINS

An unusual property common to all GPI structures is the presence of a nonacetylated glucosamine that is glycosidically linked to inositol. The glycosidic bond is specifically cleaved when the glucosamine undergoes nitrous acid deamination, thereby cleaving the GPI anchor (see Fig. 17.8.1). Because nonacetylated glucosamine rarely occurs outside of GPIs, nitrous acid deamination may be used to identify GPI linkage to protein. Due to side reactions, deamination is not always quantitative or precisely reproducible, but it does serve a useful diagnostic purpose.

In this protocol, the protein is incubated in nitrous acid to cleave the GPI anchors. Cleaved and uncleaved proteins are then separated by Triton X-114 partitioning.

Materials
Protein(s)
0.1 M acetate buffer, pH 3.5
0.5 M NaNO₂, made fresh
0.5 M NaCl

1. Dry two aliquots of the protein of interest in microcentrifuge tubes and dissolve each in 0.1 ml of 0.1 M acetate buffer, pH 3.5.

   See critical parameters for guidelines on the total amount of protein to use.

   Nonidet P-40 (NP-40) detergent (0.1% v/v final) may be added to aid solubilization.

2. Add 0.1 ml of 0.5 M NaNO₂ to one tube. To the second tube (control), add 0.1 ml of 0.5 M NaCl. Incubate 3 hr at room temperature.

3. Detect the release of protein from GPI anchor by partitioning and analyzing the reaction products as in the first alternate protocol (Triton X-114 partitioning of isolated proteins).

    If the anchor is radiolabeled with fatty acids (UNIT 17.4), the support protocol for separation of lipid moiety may be used.
SEPARATION OF LIPID MOIETY TO DETECT CLEAVAGE OF GPI-ANCHORED PROTEINS

If GPI-anchored proteins are radiolabeled with fatty acids, the efficacy of GPI anchor cleavage may be conveniently assessed by examining the released radiolabeled lipid products (see Fig. 17.8.1). In this protocol, radiolabeled proteins that have been cleaved by phospholipase or nitrous acid treatment are extracted with butanol. Released lipid moieties are extracted into the butanol phase and are analyzed and quantitated.

Materials

Radiolabeled protein, cleaved by phospholipase or nitrous acid treatment (second alternate and third basic protocols)
Water-saturated n-butanol

1. Add 100 µl water to each of two 1.5-ml microcentrifuge tubes. To one tube, add 100 µl cleaved radiolabeled protein. To the second, add 100 µl control (uncleaved) protein.

2. Add 200 µl water-saturated n-butanol to each tube and vortex vigorously.

3. Microcentrifuge 30 sec at maximum speed, room temperature, to separate the two phases.

4. Remove the upper (butanol) phase to a fresh tube, and set aside. Add another 200 µl of water-saturated butanol to the original tubes.

5. Vortex and centrifuge as in steps 2 and 3 above.

6. Remove the second butanol phase and pool it with the first, retaining the lower (aqueous) phase for analysis.

7. Quantitate the percentage of lipid moieties released by the cleavage reactions by liquid scintillation counting of both butanol and aqueous phases. Compare counts recovered in butanol phases of the cleaved material with those recovered from control reactions to assess specific release.

To characterize released products fully, examine the butanol-extracted products by thin layer chromatography; compare to standards (Doering et al., 1990b). Products of phospholipase C digestion include diacylglycerol, alkyl-acyl glycerol, or ceramide, depending upon the original structure. Phosphatidylinositol (from nitrous acid treatment) or phosphatidic acid (released by phospholipase D) may also be analyzed and quantitated. This assay has been used for purification of phospholipase activity (Hereld et al., 1986).

BASE HYDROLYSIS OF RADIOLABELED PROTEINS

For proteins radiolabeled with fatty acids, it is useful to ascertain whether incorporated fatty acids are actually part of a GPI structure or are present in amide linkage (N-myristoylation) or thioester linkage (as in palmitoylation of cysteine residues). This can be characterized by assessing the lability of the fatty acid linkage to various chemical treatments.

In this protocol, aliquots of radiolabeled proteins are resolved by electrophoresis and the various lanes are treated with KOH in methanol or hydroxylamine. Methanol and Tris buffer are used to treat the control lanes. The lanes are then processed for fluorography. If the chemical bonds in the protein are susceptible to these treatments, a radiolabeled signal will no longer be produced at the protein band of interest.
Materials
Protein radiolabeled with fatty acid
0.2 M KOH in methanol
Methanol
1 M hydroxylamine-HCl, pH 7.5, made fresh
1 M Tris·Cl, pH 7.5 (APPENDIX 2)

Additional reagents and equipment for one-dimensional gel electrophoresis (UNIT 10.2), staining of gels (UNIT 10.6), and autoradiography (APPENDIX 3)

1. Resolve five identical samples of protein radiolabeled with fatty acid by SDS-PAGE electrophoresis. Include appropriate markers.
2. Cut apart the five gel lanes, and stain one to visualize protein and markers.
3. Soak the other four lanes in one of the following solutions for 1 hr at room temperature:
   Lane 1: 0.2 M KOH in methanol
   Lane 2: methanol alone
   Lane 3: 1 M hydroxylamine-HCl, pH 7.5
   Lane 4: 1 M Tris·Cl, pH 7.5
4. Soak the treated gel slices in three washes (10 min each) of water.
5. Process gel pieces for autoradiography.

If bonds are susceptible to the treatments above, radiolabel from fatty acids will no longer produce a signal at the position of the protein band of interest. KOH in methanol cleaves thio- and oxyesters but not amide linkages. Hydroxylamine treatment cleaves thioesters only. Fatty acids that are part of a GPI moiety will be released by KOH in methanol, but not by hydroxylamine treatment. The methanol and Tris·Cl treatments serve as controls. Ceramide-containing anchors are not sensitive to mild base treatment (see anticipated results and Conzelmann et al., 1992).

REAGENTS AND SOLUTIONS

0.1 M acetate buffer, pH 3.5
Dissolve 0.82 g sodium acetate in 95 ml distilled water. Adjust pH to 3.5 with acetic acid, then bring volume to 100 ml with distilled water. Store indefinitely at room temperature.

GPI-PLC buffer
50 mM Tris·Cl, pH 8.0
5 mM EDTA
1% NP-40
Store indefinitely at 4°C

GPI-PLD buffer
50 mM Tris·Cl, pH 7.4
10 mM NaCl
2.5 mM CaCl₂
0.1% NP-40
Store indefinitely at 4°C

1 M hydroxylamine-HCl, pH 7.5
Dissolve 6.95 g hydroxylamine-HCl in 95 ml distilled water. Adjust pH to 7.5 with NaOH, then increase volume to 100 ml with water. Make fresh for each use.
**PI-PLC buffer (for B. thuringiensis enzyme)**
- 25 mM Tris acetate, pH 7.4
- 0.1% (w/v) sodium deoxycholate (Na–DOC)
- Store indefinitely at 4°C

**Tris-buffered saline (TBS)**
- 10 mM Tris-Cl, pH 7.5
- 150 mM NaCl
- Store indefinitely at room temperature

**Water-saturated n-butanol**
- Mix equal volumes of distilled water and n-butanol. Cap tightly and shake vigorously. Let stand to allow phases to separate; the upper phase is the butanol. Store indefinitely at room temperature.

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**COMMENTARY**

**Background Information**

Glycosyl phosphatidylinositol (GPI) membrane anchors are one mode of anchoring proteins to cell surfaces. These structures consist of a glycan bridge between phosphatidylinositol and phosphoethanolamine; the phosphoethanolamine is in amide linkage to the C-terminus of the protein (see Fig. 17.8.1). The glycan core of GPIs (shown schematically in Fig. 17.8.1) is remarkably conserved throughout evolution, although it is modified in many cell types by the addition of side chains. The lipid moieties of GPIs, however, are quite heterogeneous. This portion may consist of diacyl glycerol, alkyl acyl glycerol, lyso-acyl compounds, or ceramide. The hydrocarbon chains also vary in length and degree of saturation, and may be present as mixtures of species. GPI structures are reviewed in Ferguson (1991).

Much of our understanding of GPI anchors is derived from studies of the variant surface glycoprotein (VSG) of trypanosomes. Early work showed that VSG is associated with the membrane via a lipid moiety that contains dimyristoyl phosphatidylinositol (Cardoso de Almeida and Turner, 1983; Ferguson and Cross, 1984; Ferguson et al., 1985, 1986). Other investigations had shown that bacterial phospholipase C releases certain proteins from cell membranes (reviewed by Low, 1989). Together, these studies led to the definition of a class of proteins bearing a C-terminal glycolipid anchor.

Further investigations in this field have led to the elucidation of the pathway of GPI biosynthesis (reviewed by Doering et al., 1990a; Field and Menon, 1991), the purification of a GPI-specific phospholipase C (PI-PLC) and GPI-specific phospholipase D (GPI-PLD) are also useful in identification of GPI-anchored proteins.

The protocols included here are designed to allow identification of GPI-anchored proteins without detailed structural analysis. Because these techniques are rather diverse, further explanatory information is outlined below.

**Triton X-114 partitioning.** Triton X-114 forms a clear micellar solution at low temperatures; the solution separates into two phases when warmed above 20°C, due to aggregation of detergent micelles (Bordier, 1981). When cellular material is extracted in Triton X-114 at low temperatures, the solution contains soluble proteins, integral membrane proteins, and GPI-anchored proteins. Centrifugation results in a pellet containing some of the GPI-anchored species as well as cellular components that are insoluble in nonionic detergents (Hooper and Bashir, 1991). When the detergent solution is warmed, amphiphilic proteins (including those with GPI anchors) associate with the detergent-enriched phase, while hydrophilic ones partition to the detergent-depleted phase. Some GPI-anchored proteins exhibit detergent insolubility that develops shortly after synthesis (Brown and Rose, 1992); this is thought to be due to aggregation of the anchored proteins with glycosphingolipids, possibly as a step in directed protein transport.

Once a GPI-containing protein is released from the lipid component of its anchor, it will no longer partition into the detergent-enriched phase. This alteration of partitioning behavior provides a rapid assay for the presence or absence of anchors. It may be used to monitor the changes in proteins produced by the other procedures.
**Phospholipase digestion.** Bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) efficiently cleaves GPI anchors, releasing proteins in aqueous soluble forms. This enzyme also cleaves phosphatidylinositol (PI), hsa-PI, and PI-linked glycans. Proteins specifically released from membranes by PI-PLC have all been found to be GPI-anchored, making this an extremely reliable mode of analysis (reviewed by Ikezawa, 1991).

Several other specific phospholipases that effectively degrade only GPI species are also useful for analysis. These enzymes are generally more expensive than PI-PLC. One of these, initially purified from Trypanosoma brucei, is a GPI-specific phospholipase C (GPI-PLC). Another, present in mammalian serum (rat, rabbit, or human), is a GPI-specific phospholipase D (GPI-PLD). These enzymes are convenient tools for identifying the presence of a GPI anchor.

Release of a protein from its GPI anchor is followed by using appropriate protein detection methods—e.g., enzyme activity assays, electrophoretic separation (**UNIT 10.2**) followed by protein staining (**UNIT 10.6**), and, if specific antibodies are available, immunoprecipitation (**UNIT 10.16**) or immunoblotting (**UNIT 10.8**). Alternatively, treated cells may be studied for loss of a specific protein using immunofluorescence detection methods (**UNIT 14.6**), including fluorescence-activated cell sorting. Also, because release by PLC reveals a specific epitope (termed the cross-reacting determinant, or CRD), antibodies directed against this epitope may be employed to identify cleavage products resulting from PLC treatment (second support protocol).

Lack of release may indicate absence of a GPI anchor, although some caution is required in interpreting negative results. Certain GPI molecules, bearing an additional fatty acid on inositol, are not susceptible to cleavage by either bacterial PI-PLC or trypanosomal GPI-PLC. Some proteins are partly sensitive, suggesting that only a fraction of these polypeptides bear anchors with acyl-inositol. Serum phospholipase D (GPI-PLD) cleaves GPI structures that contain acylated inositols, and is useful in their identification. However, it is not effective on membrane-bound proteins in the absence of detergent.

When intact cells are treated with phospholipase, partial or total resistance to cleavage can also occur due to inaccessibility of the protein to the enzyme, expression of a protein on the cell surface in both GPI-anchored and trans-membrane-anchored forms, or tight association of a protein with a nonsusceptible protein on the cell surface (reviewed by Rosenberry, 1991).

**Critical Parameters**

Some details of these procedures will be affected by the method used to detect each protein under study. For example, specific quantities of starting materials are not provided because detection methods vary widely in terms of sensitivity. Also, for activity assays, the conditions of each procedure and whether the activity would survive such treatment must be considered. In general, plan to use two to ten times the amount of protein that would be reasonable for the mode of detection employed, depending upon the availability of material. This should allow for potential losses sustained during the procedure, as well as for the fact that proteins may be recovered in several fractions.

Triton X-114 partitioning provides a first hint of GPI anchorage, although transmembrane domains are also concentrated in the detergent phase of this partition. For example, if a protein is known from sequence data to have no membrane-spanning domain, its detection in the detergent phase of a Triton X-114 partition would be suggestive of lipid modification. In general, however, this method is most useful as a means of assessing release of a protein from its anchor, as shown by an alteration of partitioning behavior. Many of the other methods described specifically release proteins from their anchors to demonstrate GPI-linkage (e.g., nitrous acid deamination or phospholipase digestion); Triton X-114 is a convenient tool for assessing such release. Triton X-114 often contains hydrophilic contaminants, and should be precondensed by several rounds of phase separation before use (Bordier, 1981).

Phospholipase digestion is useful for determining GPI linkage, and bacterial PI-PLC is perhaps the best enzyme to try initially for reasons of cost and efficacy. Although GPI-PLC is more specific, it is generally more expensive.

Some GPI anchors are not susceptible to cleavage by phospholipase C. These structures have an additional fatty acid covalently attached to the inositol of the anchor (Rosenberry, 1991). For this reason, failure of cleavage by PLC does not necessarily imply that a protein is not GPI-anchored. In such cases GPI-PLD may be useful, although this enzyme does not expose the epitope for anti-CRD detection.
traction and analysis of radiolabeled lipid products from phospholipase digestions provides rapid assessment of cleavage. As mentioned earlier, radiolabeling the protein of interest is often helpful and permits less material and reagents to be used. In particular, if a protein may be radiolabeled with fatty acids, base hydrolysis is a helpful analytical tool, especially if lipid modification is suspected but other methods have yielded negative results.

Anticipated Results

Proteins anchored by GPI structures without acylated inositol should require detergent for solubilization. They may be extracted by Triton X-114 or remain in the cell pellet after such extraction (depending on solubility properties of the protein). In a warmed Triton X-114 solution, anchored protein(s) partition into the detergent-enriched phase. These proteins can be chemically cleaved by nitrous acid deamination, and enzymatically hydrolyzed by treatment with GPI-PLC, PI-PLC, or GPI-PLD. The protein cleaved with PLC will react with α-CRD antibody, and appropriate lipid products will be generated from all cleavage reactions (Fig. 17.8.1). Radiolabel incorporated into the anchor as fatty acid is released by treatment with KOH in methanol, but not by hydroxylamine treatment, if the fatty acid is hydroxysterified to glycerol (as in diacyl or alkyl-acyl anchors). Some PI-PLC and nitrous acid-sensitive anchors have been found to contain ceramide instead of diacyl or alkyl-acyl glycerol (e.g., in yeast). These anchors are resistant to mild base treatment, but are sensitive to conditions that hydrolyze amides (Conzelmann et al., 1992).

If the GPI anchor includes an acylated inositol, the above characteristics will hold true except that PLC treatment will not cleave the anchor. In these cases, nitrous acid deamination, GPI-PLD digestion, and radiolabel studies are used to characterize the linkage.

Time Considerations

In general, the methods described above are fairly rapid, with enzyme incubations or chemical reactions and subsequent partitioning requiring 1 to 2 hr. The time required for completion of these analyses depends primarily on the method used to detect the protein(s) of interest. For example, if a protein is radiolabeled in the fatty acid portion of the anchor, cleavage may be assessed by lipid extraction and scintillation counting in ≤1 hr. If immunoprecipitation or immunoblotting is used for product detection, analysis will be more lengthy.

Literature Cited


Key References


This landmark paper first described the core glycan of GPI anchors, providing a scheme for their structural analysis.


A useful laboratory manual of methods dealing with various post-translational modifications of proteins by lipids; many methods relate to GPI-linked proteins.


This issue of Methods, titled ‘Covalent modification of proteins by lipids,’ contains two chapters specifically about GPIs—Doering et al., 1990b (see above); Mayor and Menon (pp. 297-305)—as well as related topics.

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Direct Chemical Analysis of Glycoconjugates for Carbohydrates

The following unit presents protocols for detection of different sugars bound to glycoconjugates. The solution containing the carbohydrate material is treated with a specific reagent, generating a colored reaction product that can be detected spectrophotometrically. For each assay, the absorbance of the colored solution is proportional to the amount of sugar present in the glycoconjugate.

PHENOL–SULFURIC ACID ASSAY FOR HEXOSES AND PENTOSES

This protocol describes the use of the phenol–sulfuric acid colorimetry to quantitatively determine the total level of hexoses or pentoses and their derivatives in an experimental sample. Monosaccharide samples of known concentration are used to construct a standard curve for comparison. The assay is optimized for use with small samples.

NOTE: Distilled or deionized water and high-quality sulfuric acid must be used for this procedure, as contamination of these reagents may lead to a false-positive result. It is advisable to check each new bottle of sulfuric acid and the water being used as described in steps 2 to 8 before using them for the assay. Both reagents should show an absorbance <0.05 (measured against water that has not been submitted to the assay).

Materials

- Sample to be assayed
- Monosaccharide standard: e.g., D-(+)-galactose (Sigma, purified-grade anhydrous crystals; Critical Parameters)
- 5% (w/v) phenol (see recipe)
- Concentrated sulfuric acid (ACS reagent-grade)

Thick-walled, 16 × 125–mm Pyrex test tubes, either brand new and washed with distilled water, or acid washed
Automatic glass dispenser or glass pipet with a portion of the tip removed for rapid flow, suitable for use with concentrated sulfuric acid
1.0-ml glass cuvettes (1-cm path length)
Visible-light spectrophotometer

1. Dissolve sample in water or buffer at an appropriate concentration to 300 or 450 µl final volume (for duplicate or triplicate samples, respectively).

   The sample is dissolved in a new or acid-washed tube.

   The quantity of sample dissolved must be such that the sugar concentration is ≥10 ng/ml.

   If insufficient data are available about the sample to estimate its sugar concentration, it is advisable to assay two solutions having at least a ten-fold difference in concentration.

   Depending on the results, it may be necessary to increase or reduce the concentration to obtain absorbance in the linear range of the assay (0.1 to 0.8). Volumes can be varied according to the quantity of material available as long as the proportions of reagents described are maintained.

2. Transfer two 150-µl aliquots of the dissolved sample (or three if possible) into new or acid-washed 16 × 125–mm Pyrex test tubes.

   If the sample volume is scaled up or down, the test tube used must have a volume considerably higher than the final volume of reaction mixture to reduce the chance of spilling concentrated sulfuric acid. The use of wide-mouth tubes slows dissipation of the heat (produced by the exothermic reaction between sulfuric acid and water) that is required for color development.
3. Set up three blank tubes using 150 µl sample solvent (water or buffer; see step 1) in each.

4. In triplicate, prepare a series of tubes containing 10, 20, 30, 40, 50, 60, 70, and 80 ng/µl of the monosaccharide standard in sample solvent. Adjust all volumes to 150 µl with water.

   To minimize errors, prepare a stock solution of the standard and make sequential dilutions. Keep stock solutions at −20°C.

5. Add 150 µl of 5% (w/v) phenol solution to each tube and vortex briefly to mix.

   CAUTION: Rubber gloves and eye protection must be worn while performing steps 6 to 8, which involve handling of concentrated sulfuric acid and generation of heat.

6. Rapidly add 750 µl concentrated sulfuric acid to each tube, using an automatic glass dispenser or a glass pipet with a portion of the tip removed. Ensure that the acid stream hits the liquid surface directly to produce rapid mixing and even heat distribution. Let tubes stand 10 min at room temperature.

7. Vortex tubes again briefly to mix. Let stand 30 min at room temperature while the color develops.

   The color is stable for an additional several hours.

8. Transfer the solution from each tube to a 1.0-ml (1-cm path length) glass cuvette. Using the spectrophotometer, read the absorbance of each tube at 480 nm (to detect pentoses, uronic acids, and their methylated derivatives) and/or 490 nm (to detect hexoses and their methylated derivatives).

   Care must be taken when transferring the solutions to the cuvettes. Any spill inside the spectrophotometer should be cleaned up immediately, as the corrosive sulfuric acid will damage the instrument.

9. Average the absorbances of the triplicate tubes for each standard. Subtract the absorbance of the blank from each average. Prepare a standard curve of absorbance versus concentration in ng/µl for each absorbance (A_{480} or A_{490}) that was monitored.

10. Average the absorbance of the duplicate or triplicate sample tubes and subtract the absorbance of the blank. Determine the amount of sugar in the sample by reference to the standard curve.

### 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.

**Phenol, 5% (w/v)**

Dissolve 5 g of 99% phenol crystals in water and bring the volume to 100 ml with water. Store solution in a dark bottle at room temperature (stable indefinitely).

CAUTION: Use gloves and eye protection when weighing phenol, as it is very corrosive. Store the crystals in a dark bottle at 4°C to prevent oxidation.

### Commentary

**Background Information**

The phenol–sulfuric acid assay for detecting sugars has been widely used since it was first developed by Dubois et al. (1956). The method is simple, rapid, sensitive, accurate, specific for carbohydrates, and widely applicable. Most sugars (except hexosamines; see UNIT 17.11), sugar derivatives, oligosaccharides, and polysaccharides react with these reagents. The assay is based on the fact that when carbohydrates are heated in the presence of strong acids, they are converted into furfural and its homologs by...
oxidation, reduction, and condensation processes. These products can react with organic substances such as phenol to form colored compounds. The type and ratio of the reaction products varies from carbohydrate to carbohydrate and also depends on reagent concentrations, temperature, and time of heating. Although the exact composition of the colored products is indeterminate, the assay has been standardized and the results obtained for each type of carbohydrate are highly reproducible. The reagents are inexpensive, readily available, and stable.

Critical Parameters and Troubleshooting

The monosaccharide used as a standard for constructing the standard curve should be chosen to match the expected composition of the sample, as different individual monosaccharides will give different results. For glycoproteins of animal origin, galactose can be used as the standard. The assay will certainly indicate whether carbohydrates are present in a sample, and will give an idea of its percentage (quantitation). However, when assaying unknown complex carbohydrates, it is not possible to select the exact matching standard, and the values obtained will only be approximate.

Triplicate measurement minimizes errors. For accurate and reproducible results, it is important to use brand-new test tubes that have been rinsed with distilled water to eliminate any lint or paper fiber, or tubes that have been acid-cleaned and rinsed. Accidental contamination with lint may still occur, producing high readings, but the use of triplicate tubes makes it possible to identify and discard such false-positive tubes.

If the assay is used to check the elution profile of a carbohydrate-containing sample eluted from a column, the buffers and column eluant must first be tested for positive reactions. With columns packed with carbohydrate-based polymers (e.g., Sephadex, Pharmacia), any breakdown of the packing material will cause an extremely intense color in the assay. Buffers used for elution may also contain sugar-bearing contaminants such as bacteria, in which case fresh lots should be prepared before attempting this kind of monitoring. The blank should contain buffer eluted from the column prior to the sample. If organic solvents are present, they must be removed by evaporation prior to performing the assay.

Anticipated Results

The phenol–sulfuric acid assay provides a quantitative measure of the total content of pentoses or hexoses and their derivatives in the sample analyzed, expressed as equivalents of the monosaccharide used as a standard (e.g., galactose). When carried out in triplicate as described, the assay yields results accurate to ±2%.

Time Considerations

In general, the complete assay can be performed in <1 hr. However, if a considerable number of experimental samples are to be assayed (e.g., when monitoring effluent from a column), the time required may be longer.

Literature Cited


Original description of this technique, containing the most complete study to date of the reactivity of different types of carbohydrates.

FERRIC ORCINOL ASSAY FOR SIALIC ACIDS

When sialic acids are oxidized in concentrated acid in the presence of orcinol, a blue-purple chromophore is formed. The reaction provides an estimation of the sialic acid content.

Materials

- Sample to be assayed
- Sialic acid standard: 1 mM N-acetylneuraminic acid (Neu5Ac; Boehringer
  Mannheim; store frozen at −20°C)
- Bial reagent (see recipe)
- Isoamyl alcohol
- Thick-walled 16 × 125-mm Pyrex test tubes
- Heating block or boiling water bath
Glass marbles
1.0-ml glass cuvettes (1-cm path length)
Visible-light spectrophotometer

1. Dissolve sample in water to an appropriate concentration.

   When no information regarding the sialic acid content of a sample is available, it is a good practice to assay two aliquots differing in concentration ~5- to 10-fold.

2. Transfer three 150-µl aliquots to 16 × 125–mm Pyrex test tubes. Adjust volume to 200 µl with water.

3. In triplicate, prepare a series of tubes containing 5, 10, 20, 30, and 40 nmol Neu5Ac standard (5 to 40 µl of 1 mM Neu5Ac). Adjust to 200 µl water. Prepare a blank using 200 µl of water.

4. Add 200 µl Bial reagent, vortex, and cover tubes with glass marbles. Heat tubes 15 min at 100°C.

5. Cool tubes by immersing in room temperature tap water.

6. Add 1 ml isoamyl alcohol to each tube. Vortex tubes and let sit 5 min in an ice bath.

7. Centrifuge tubes 3 min in a tabletop centrifuge to separate the phases.

8. Transfer upper phase to 1.0-ml cuvettes with a Pasteur pipet. Measure the absorbance at 570 nm and subtract the absorbance of the blank.

9. Prepare standard curve of absorbance versus nmol Neu5Ac using the average of the triplicate standard solutions.

10. Determine the amount of sialic acid in each unknown sample by comparing to the standard curve. Quantity is expressed as nmol Neu5Ac.

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.

Bial reagent

- 0.2 g orcinol (5-methylresorcinol)
- 81.4 ml concentrated HCl
- 2 ml 1% (w/v) ferric chloride (analytical grade)
- H₂O to 100 ml

Store ≤1 week at 4°C

Commentary

Background Information

Glycosidically bound and free sialic acids form a blue-purple chromophore when heated in the presence of orcinol/Fe³⁺ (Bial reagent) in concentrated HCl. The chromophore is soluble in organic solvents. The assay is accurate and reproducible, and the minimum detection limit is ~5 nmol (1.5 µg). Determination of free sialic acids is best achieved by the more sensitive 2′-thiobarbituric acid (TBA) assay (see UNIT 17.18). A more accurate determination can be carried out later, after release of the sugar with sialidase or mild acid and purification as described in UNIT 17.18. N-acetylneuraminic acid (Neu5Ac) is used as a standard. However, it should be noted that the extinction coefficient is different for different sialic acid derivatives, such as N-glycolylneuraminic acid. Any O-acylated sialic acids will have the same extinction coefficients as their parent molecules because, under the strong acidic conditions, all ester groups are rapidly hydrolyzed. Even crude biological materials can be tested, and therefore the assay is adequate for monitoring purification and fractionation protocols.

The ferric orcinol assay was introduced by
Klenk and Langerbeins (1941) and has been used since then with some adaptations to reduce the amount of sample required (Schauer, 1978). Interference caused by other saccharides has been observed (Svennerholm, 1963; Spiro, 1966; Veh et al., 1977).

**Critical Parameters**

Free or glycosidically bound pentoses, hexoses, and uronic acids can interfere with the assay, limiting its quantitative value. Therefore, some error can be expected when analyzing biological materials that, purified or not, have a high content of these sugars. Nevertheless, this assay provides a simple way to detect sialic acids.

**Anticipated Results**

When working with a crude biological mixture, a clearly positive ferric orcinol assay will indicate the presence of sialic acids in the preparation; however, an accurate estimation of the total content of sialic acids cannot be obtained by this method. A negative assay, on the contrary, will not rule out the presence of sialic acid, because small amounts of these may be masked by other substances (Critical Parameters).

**Time Considerations**

The content of sialic acids in a set of up to ten samples can be obtained in 1 hr, provided standards and reagents are already prepared.

**Literature Cited**


**Key Reference**

Schauer, R. 1978. See above. Describes the method and comments on its specificity and sensitivity.

### BASIC PROTOCOL 3

**MBTH ASSAY FOR HEXOSAMINES AND ACETYLHEXOSAMINES**

Most methods reported for determining the concentration of hexosamines or \(N\)-acetylhexosamines are based on the procedures proposed by Elson and Morgan (see UNIT 17.18). However, the Elson-Morgan reaction is not specific for hexosamines and requires strict attention to reaction conditions. Although several modifications have been developed, in each case the formation of chromophore requires free amino sugars (not bound within an oligosaccharide), \(N\)-acylated or not. Quantitative liberation of amino sugars from oligosaccharides is difficult because of their resistance to acid hydrolysis (see UNIT 17.16). In this protocol, the concentration of free hexosamines as well as those bound within an oligosaccharide (including \(N\)-acylated species) can be determined after deamination of the sample with 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) under mild acidic conditions.

**Materials**

- Sample to be analyzed
- 1 M HCl (ACS reagent-grade)
- Hexosamine standard: 1 mM \(N\)-acetyl-D-glucosamine or \(N\)-acetyl-D-galactosamine (Sigma)
- 2.5% (w/v) sodium nitrite (Sigma; store in dark bottle at room temperature; prepare fresh weekly)
- 12.5% (w/v) ammonium sulfamate (ACS reagent-grade; store ≥2 months at room temperature)
- 0.25% (w/v) 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH; Kodak; store in dark bottle at 4°C and replace if yellowish)
0.5% (w/v) ferric chloride (certified ACS reagent-grade; store indefinitely at room temperature)
13 × 100–mm Pyrex test tubes with Teflon-lined screw caps
Heating block, 110°C
Water baths, room temperature and 37°C
1.0-ml glass cuvettes (1-cm path length)
Visible-wavelength spectrophotometer

1. In triplicate, add 100 µl of sample to be analyzed to 13 × 100–mm Pyrex test tubes. Add 100 µl of 1 M HCl. Mix well by vortexing.
2. Set up a blank tube containing 100 µl water and 100 µl of 1 M HCl.
3. Prepare a series of tubes containing 5, 10, 15, 20, 25, and 30 nmol N-acetyl-D-glucosamine or N-acetyl-D-galactosamine standard in 100 µl water. Add 100 µl of 1 M HCl. Prepare each determination in triplicate.
4. Cap tubes and place in heating block 2 hr at 110°C.
5. Transfer tubes to a rack and place in a water bath. Allow samples to cool to room temperature.
6. In a fume hood, add 400 µl of 2.5% sodium nitrite and vortex. Let stand 15 min at room temperature.
   CAUTION: Steps 6 to 8 should be done in a fume hood.
7. Add 200 µl of 12.5% ammonium sulfamate and vortex. Let stand 5 min at room temperature.
   Allow the nitrogen oxides (brownish fumes) to dissipate.
8. Remove tubes from fume hood. Add 200 µl of 0.25% MBTH and vortex. Cap tubes and incubate 30 min at 37°C.
9. Add 200 µl of 0.5% ferric chloride, cap tubes, and incubate 5 min at 37°C.
10. Allow tubes to cool to room temperature and carefully transfer solutions to 1.0-ml cuvette with a Pasteur pipet.
11. Measure absorbance of samples at 650 nm, using reagent blank to zero spectrophotometer.
   Absorbances between 0.1 and 1.0 U should be expected for the standards.
12. Prepare standard curve of absorbance versus nmol hexosamine using the average value for each hexosamine concentration.
13. Determine the amount of hexosamine in the sample by comparing to standard curve.

Commentary

**Background Information**

The MBTH assay is highly specific for amino sugars. This method is based on the formation of an intense blue-colored complex between the 2,5-anhydrohexoses produced upon deamination of hexosamines and 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) under mild acidic conditions. Strong acidic conditions are not required because complete cleavage of the glycosidic linkage is not necessary for the MBTH color reaction to occur. Heating in mild acid is sufficient for the complete de-N-acetylation of N-acetylhexosamines (i.e., N-acetylglucosamine and N-acetylgalactosamine) that is required for the reaction to proceed. Sodium nitrate is added to the acidic solution and the liberated nitrous acid produces the deamination. Sodium sulfamate is used to destroy the excess of nitrous acid. Final color is developed upon addition of ferric chloride to the samples. The proposed reaction mechanism is shown in Figure 17.9.1.
method is useful for determination of free or glycosidically bound hexosamines, including N-acetylated species.

The MBTH method was introduced by Tsuji et al. (1969a), who proposed a mechanism for the formation of the blue chromophore. They also found that methyl glucosaminide gives an extinction coefficient almost identical to that of free hexosamine, showing that complete hydrolysis is not required (Tsuji et al., 1969b). Several modifications were introduced (Smith and Gilkerson, 1979) to reduce the difficulty of the original protocol developed by Tsuji et al. (1969a).

**Critical Parameters**

The color reaction is highly specific for amino sugars because N-acetylated hexosamines, neutral monosaccharides, glucuronic acid, ascorbic acid, and most amino acids yield no color. Some amino acids (e.g., tryptophan, threonine, and methionine) exhibit color, but the intensities are far lower than those obtained with hexosamines. The complete de-N-acetylation of N-acetylhexosamines necessary for the reaction is achieved by heating the sugar-containing samples in 0.5 M HCl for 2 hr at 110°C. Glucosamine and galactosamine give practically identical color yields ($\varepsilon_{\text{max}} = 3.86$ and $3.62 \times 10^{-4}$, respectively). Both monosaccharides show linear absorbances in the range of 1 to 30 µg/ml. The response of mannosamine is very low ($\varepsilon_{\text{max}} = 0.80 \times 10^{-4}$), although it is also linear. This color reaction also allows the estimation of free hexosamines in the presence of N-acetylhexosamines, if the de-N-acetylation step is omitted.

![Figure 17.9.1 Probable mechanism of MBTH color reaction.](image-url)
Anticipated Results

The application of this protocol will indicate the total content of glucosamine or galactosamine (N-acetylated or free) in the sample under analysis.

Time Considerations

The complete assay requires 4 hr, provided all reagents and solutions are prepared in advance.

Literature Cited


Key Reference


ASSAYS FOR URONIC ACIDS

Hexuronic acids occur in polysaccharides of plants, fungal cell walls, bacterial capsules, animal tissues, and cultured cells. In animal tissues and cells, most uronic acid is found in glycosaminoglycans and proteoglycans. Considerable effort has been expended to develop specific, rapid methods for detecting and quantifying free and polymeric uronic acids. The simplest procedures involve hydrolysis of polysaccharides with mineral acid, dehydration of the uronic acid, and conjugation to a chromogen such as carbazole (Basic Protocol 4) or meta-hydroxybiphenyl (Alternate Protocol) to form a chromophore that can be quantitated by spectrophotometry. The reactions are generally specific for hexuronic acids, sufficiently sensitive for most laboratory situations (≥10 nmol), and require only routine laboratory equipment.

Carbazole Assay for Uronic Acids

Uronic acid is detected by monitoring the formation of a chromophore following reaction with the chromogen carbazole. The reaction is carried out in two steps: samples are first treated with strong acid to hydrolyze polysaccharides and dehydrate the sugars, and the products are then treated with carbazole or meta-hydroxybiphenyl to generate the chromophore.

Materials

Sample to be assayed
Uronic acid standard: 1 mM glucuronolactone (see recipe)
4 M ammonium sulfamate (Sigma)
25 mM sodium tetraborate in sulfuric acid (see recipe)
0.1% (w/v) carbazole (Kodak) in 95% ethanol (store at 4°C)
16 × 125–mm Pyrex tubes (open, glass-stoppered, or screw-cap culture tubes fitted with Teflon-lined caps)
Water bath, 100°C, or heating block
Disposable glass or plastic 1.0-ml cuvettes
UV/VIS spectrophotometer

1. Prepare duplicates of unknown sample in 16 × 125–mm Pyrex tubes. Adjust volume to 200 µl with water. Set up a blank reaction tube containing 200 µl water.

2. Prepare a series of tubes containing 10, 20, 30, 40, 50, 75, and 100 nmol glucuronolactone standard in 200 µl water. Include a blank containing only 0.2 ml water.

3. Add 20 µl of 4 M ammonium sulfamate and vortex.
4. Add 1 ml of 25 mM sodium tetraborate in H$_2$SO$_4$ and mix carefully. Cover tubes with caps or marbles and heat 5 min at 100°C. Cool samples to room temperature.

CAUTION: Mixing sulfuric acid with the sample liberates considerable heat. Some investigators chill the samples on ice and use cold ammonium sulfamate. Make sure the tubes are vented during the heating procedure and wear eye protection.

5. Add 40 µl of 0.1% carbazole and heat 15 min at 100°C. Cool samples to room temperature.

Color develops during this step.

6. Transfer samples to 1.0-ml disposable cuvettes using a Pasteur pipet and read absorbance at 520 nm.

Absorbance of blank sample should be ≤0.025 when read against water.

NOTE: Plastic disposable cuvettes can be used. Do not spill the sulfuric acid–containing samples on the outside of the cuvette or into the spectrophotometer.

7. Prepare a standard curve of A$_{520}$ versus nmol glucuronic acid. Determine the amount of uronic acid in unknown sample by reference to the standard curve.

Amount of uronic acid will be expressed as glucuronic acid equivalents.

**Meta-Hydroxybiphenyl Assay for Uronic Acids**

Substitution of meta-hydroxybiphenyl for the carbazole used in Basic Protocol 4 allows color development at room temperature.

**Additional Materials** (also see Basic Protocol 4)

- 0.15% (w/v) meta-hydroxybiphenyl (Kodak) in 0.5% (w/v) NaOH

1. Prepare samples and treat with ammonium sulfamate and sodium tetraborate in sulfuric acid (see Basic Protocol 4, steps 1 to 4).

2. Add 40 µl of 15% meta-hydroxybiphenyl and incubate 15 min at room temperature.

3. Transfer samples to disposable cuvettes, read absorbance, and determine the amount of uronic acid by reference to the standard curve (see Basic Protocol 4, steps 6 and 7).

**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.*

**Glucuronolactone, 1 mM**

Dissolve 88 mg of glucuronolactone in 1 ml water and freeze in 0.2-ml aliquots. Before assay, dilute one 0.2-ml aliquot to 100 ml with water to obtain 1 mM solution. Store 3 to 6 months at 4°C.

**Sodium tetraborate, 25 mM in H$_2$SO$_4$**

Working in a fume hood, dissolve 0.95 g sodium tetraborate (Na$_2$B$_4$O$_7$$\cdot$10H$_2$O; Sigma) in 100 ml concentrated sulfuric acid. Stir at room temperature with magnetic stir bar.

A large volume of solution can be prepared in advance because the reagent is quite stable. Store at room temperature.

CAUTION: Sulfuric acid will cause severe burns.
Commentary

Background Information

Carbazole reacts with hexuronic acids treated with strong acid. The reaction was first described by Dische (1947) and subsequent modifications have improved sensitivity, specificity, and rapidity. The original reaction conditions gave varying color yields with different uronic acids, but the inclusion of borate (Bitter and Muir, 1962) lessened this problem. Nevertheless, it should be noted that different uronic acids give somewhat different responses in the assay. When uronic acids constitute a small proportion of the total sugar, the accuracy of the assay is reduced due to the Browning reaction of other sugars in hot sulfuric acid. Inclusion of sulfamate (Galambos, 1967) reduces the color yield from neutral sugars to ≤ 5% that of uronic acids. Substitution of meta-hydroxybiphenyl for carbazole further reduces interference from neutral sugars (Blumenkrantz and Asboe-Hansen, 1973; Filisetti-Cozzi and Carpita, 1991), because color development can be carried out at room temperature. Thus, the Alternate Protocol should be used if uronic acid represents <20% of the total hexose in the sample.

Critical Parameters

The three most substantial problems encountered in quantitating uronic acids are the varying color yields of different uronic acids, presence of colored reaction products from other components in mixtures, and interference by certain salts. The inclusion of borate substantially improves the reactivity of recalcitrant uronic acids such as mannuronic acid. Uronic acids in glycosaminoglycan chains can react differently than free sugars, e.g., heparin gives an anomalous high color yield (Kosakai and Yosizawa, 1978). The differential color yields of uronic acids require use of appropriate standards for accuracy. When the identity of the glycosaminoglycan (GAG) chains is known, preparation of a standard curve using the same GAG rather than glucuronolactone will give more accurate results.

The substitution of meta-hydroxybiphenyl for carbazole lowers the production of nonspecific color due to hexoses beyond that obtained by inclusion of 0.1 M sulfamate. Hexosamines and pentoses do not produce color in either reaction. The amount of sulfamate included assures maximum reduction of interference. Larger amounts will result in decreased production of the uronic acid chromophore. Carbazole produces somewhat more chromophore than meta-hydroxybiphenyl, but the difference is small. Addition of sulfamate reduces the color yield by only ~30%.

Interference by salts and other cellular components has been surveyed in greater detail in the carbazole reaction than in the meta-hydroxybiphenyl reaction. Samples containing sodium chloride (<2 M), urea (<6 M), guanidine-HCl (<2 M), and protein (<0.2 mg/ml) yield the expected color. Sodium nitrite (>5 µM) and hydrogen peroxide (>10 nM) give a green color that overwhelms the signal from uronic acids. Sulfhydryl reagents accelerate color formation. Because the meta-hydroxybiphenyl assay works under milder conditions, it is preferable to the carbazole assay. However, interference by contaminants should be checked by adding them to a standard solution.

Anticipated Results

The assay will provide an estimate of total glycosyluronic acids in a sample. The extinction coefficient at 520 nm for a 1% solution is ~1000. Thus, the assay is sufficiently sensitive to detect ~10 to 25 nmol uronic acid.

Time Considerations

The assay can be completed in ~20 to 30 min if the reagents and solution are prepared in advance.

Literature Cited


Key References
Bitter and Muir, 1962. See above.
*These two references describe the most recent modifications of the methods.*

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**Inhibition of N-Linked Glycosylation**

Treatment of cells with inhibitors of the enzymes that synthesize N-linked oligosaccharide chains results in production of glycoproteins (this unit) or glycolipids (UNIT 17.10B) containing missing or altered chains. This approach is useful for examining potential functional roles of oligosaccharides on specific proteins or intact cells. Table 17.10.1 lists the enzymes blocked by each inhibitor and the consequences; Figure 17.10.1 diagrams the early processing steps in the assembly of N-linked oligosaccharides, showing the enzymes blocked by each inhibitor except tunicamycin (which affects an earlier stage). These inhibitors can be used to prevent N-linked glycosylation in cultured cells. First, the optimal concentration of inhibitor (i.e., highest nontoxic concentration) is determined by monitoring $[^{35}\text{S}]{\text{methionine incorporation}}$ as a measure of protein biosynthesis (UNIT 10.18). The inhibitor's ability to inhibit oligosaccharide processing is then determined by analyzing cells labeled with $[^{3}\text{H}]{\text{mannose}}$ using TCA precipitation (UNIT 17.10A).

### Table 17.10.1 Inhibitors of Enzymes that Synthesize N-linked Oligosaccharides

<table>
<thead>
<tr>
<th>Type of inhibitor</th>
<th>Target enzyme</th>
<th>Effect on oligosaccharide structure</th>
<th>Effective concentration range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glycosylation inhibitor</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tunicamycin</td>
<td>GlcNAc transferase</td>
<td>Prevents assembly of GlcNAc-PP-dolichol and thus assembly of G3M9GlcNAc2-PP-dolichol; glycosylation of Asn residues does not occur; proteins migrate faster on SDS-PAGE and generally show less size heterogeneity; proteins will not shift to a faster mobility on SDS-PAGE after PNGase F treatment</td>
<td>0.5-10 µg/ml</td>
</tr>
<tr>
<td><strong>Processing inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deoxynojirimycin</td>
<td>Glucosidase I</td>
<td>Prevents removal of first glucose residue, thereby inhibiting any further processing of the oligosaccharide chain; proteins may migrate with a larger or smaller size on SDS-PAGE, depending on extent of processing that normally occurs; sensitive to endo H</td>
<td>0.5-200 mM</td>
</tr>
<tr>
<td>Castanospermine</td>
<td>Glucosidase I, and/or II</td>
<td></td>
<td>1-50 µg/ml</td>
</tr>
<tr>
<td>Deoxymannojirimycin</td>
<td>α-mannosidase I</td>
<td>Prevents removal of mannose residues on the α1-3 arm of the high mannose structure, thereby blocking the activity of GlcNAc T I and thus α-mannosidase II; proteins generally run as a smaller size on SDS-PAGE, depending on size of the normal oligosaccharide; structures remain sensitive to endo H</td>
<td>1-5 mM</td>
</tr>
<tr>
<td>Swainsonine</td>
<td>α-mannosidase II</td>
<td>Prevents removal of mannose residues on the α1-6 arm of the high mannose structure, preventing the activities of GlcNAc T II and V (which add GlcNAc to the α1-6 mannose residue); addition of GlcNAc, galactose, and sialic acid to the α1-3 mannose can occur normally, although the structures remain sensitive to endo H digestion</td>
<td>1-10 µg/ml</td>
</tr>
</tbody>
</table>

*Abbreviations: G3M9GlcNAc2-PP-dolichol, Glc3Man9GlcNAc-PP-dolichol, GlcNAc T I, II, V, N-acetylglucosaminyltransferase I, II, V, PNGase F, peptide N-glycosidase F.*

*Mature processed oligosaccharide structures cannot be cleaved from the protein by endoglycosidase H; structures retaining mannose residues on the α1-6 arm can be removed, generally resulting in a difference in size easily detected by SDS-PAGE.*

*Endomannosidases can bypass this block.*
Figure 17.10.1 Structures of early processing intermediates in the assembly of N-linked oligosaccharides. Structure A is initially assembled on the lipid carrier dolichol phosphate, a process blocked by tunicamycin, and then transferred to the Asn residue. The initial trimming glucosidases I and II convert A into B; these glucosidases are inhibited by castanospermine and deoxynojirimycin. Deglucosidation is followed by removal of the outer four mannose residues by mannosidase I (inhibited by deoxymannojirimycin), to form C. Removal of these mannose residues is the signal for the first N-acetylglucosaminyltransferase (GlcNAc Transferase I) to form D. Structure D, but not C, is a substrate for mannosidase II, resulting in the formation of E, which can be further extended into bi-, tri- and tetraantennary structures (sialylated and neutral) by a series of N-acetylglucosaminyltransferases, galactosyltransferases, and sialyltransferases plus the appropriate sugar-nucleotide donors.
Further suggestions are given on how to use methods for identifying a specific glycoprotein (if available) to measure the effect of the inhibitor on its N-linked oligosaccharide chains. A support protocol details a method for concentrating proteins by acetone precipitation.

**NOTE:** All media and solutions should be made with distilled, deionized water. All media and equipment coming into contact with cells should be sterile. Incubations of cells should be performed in a humidified 5% CO₂, 37°C incubator.

**Materials**

- Cultured cell line, either adherent or suspension
- Complete culture medium
- Inhibitor of N-linked glycosylation (one or more of the following: tunicamycin, deoxynojirimycin, castanospermine, deoxymannojirimycin, or swainsonine; see reagents and solutions and Table 17.10.2)
- Solvent used for making inhibitor solution (see reagents and solutions)
- Multiply deficient medium (MDM; **UNIT 17.4**) without glucose
- [³H]mannose (5 to 20 Ci/mmol)
- Phosphate-buffered saline (PBS; **APPENDIX 2**), ice-cold
- Lysis buffer (**UNIT 10.16**) without 1% bovine hemoglobin
- 0.5 U/ml endoglycosidase H (endo H) and endo H digestion buffer (reagents and solutions)
- 20% (w/v) SDS
- Sephacryl S-200 column (Table 10.9.2)
- 25 mM ammonium formate/0.1% (w/v) SDS
- 24-well tissue culture plate
- 100-mm tissue culture plates
- Disposable plastic scraper or rubber policeman
- 1.5-ml, 15-ml, or 50-ml conical polypropylene centrifuge tube

**Determine optimal inhibitor concentration**

1. For each inhibitor tested, set up 15 wells of cells in a 24-well tissue culture plate by splitting the cells (by trypsinization for adherent cells or dilution for suspension cells) into 1.8 ml final per well of complete medium. Incubate 24 hr.

   **A ratio of splitting should be used to yield confluent (but not overgrown) cultures by the third day (i.e., 48 hr after splitting).**

2. While cells are incubating, make a series of 1:1 (v:v) dilutions of inhibitor(s) as follows. Place the volume of inhibitor stock solution indicated in Table 17.10.2 in a sterile microcentrifuge tube and dilute to 800 µl final volume with complete culture medium. Transfer 400 µl to a second tube and dilute with 400 µl complete culture medium. Repeat for a total of 7 tubes. As a control, prepare an identical series of dilutions of the solvent used for making the inhibitor solution.
3. Add 200 µl from each tube (from step 1) to a well of the tissue culture plate containing cells (2 ml final). Add 200 µl complete culture medium alone to the remaining well (as a zero-inhibitor control point). Incubate plate 24 hr. Store unused inhibitor-containing media at 4°C.

If the inhibitor is tunicamycin, dilutions should be prepared fresh each day, as it may precipitate out of solution during storage.

4. Perform short-term labeling of cells with [35S]methionine (UNIT 10.18) using 0.2 mCi/ml label and 1.8 ml final volume per well.

5. Add to wells the remaining 200 µl of each dilution of inhibitor (from step 2) and incubate a further 4 hr.

6. Harvest cells and determine the incorporation of [35S]methionine into macromolecules by TCA precipitation (UNIT 10.18). Plot label incorporation versus inhibitor concentration.

CAUTION: Safety precautions for disposing of radioactive waste should be followed.

Comparing incorporation by cells incubated with and without inhibitor will indicate the maximum concentration of inhibitor that does not inhibit protein synthesis. This is the optimal concentration for the following steps.

Label cells in the presence of N-linked glycosylation inhibitor

7. Split a new cell sample into complete medium in a 100-mm tissue culture plate and incubate 24 hr.

8. Add inhibitor to the optimal concentration determined above. Set up a control plate containing the same quantity of stock solution solvent but no inhibitor. Incubate for 24 hr.

9. Wash the cells using the same procedure as employed during labeling (step 4) and, to each plate, add sufficient glucose-free MDM to cover the cells (5 ml for a 100-mm plate). Add inhibitor or solvent as in step 8. Add [3H]mannose to 0.02 to 0.1 mCi/ml. Incubate 4 to 12 hr.

The duration of incubation will depend on how well the cells label with [3H]mannose. The amount of [3H]mannose used will depend on the cell line examined. However, for most cells, a single 100-mm plate with 5 ml media incubated with 0.02 mCi/ml [3H]mannose for 6 hr should yield sufficient radioactive material for the analyses described below. Extended incubations in glucose-free labeling medium should be avoided.

10. Harvest the cells by scraping with a disposable scraper or rubber policeman (for adherent cells) or centrifugation (for suspension cells) into ice-cold PBS.

CAUTION: Safety precautions for disposing of radioactive waste should be followed.

### Table 17.10.2 Range of Inhibitor Concentrations for Dilution Series

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Stock solution</th>
<th>Stock added to first dilution (µl)</th>
<th>Concentration range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tunicamycin</td>
<td>1 mg/ml</td>
<td>80</td>
<td>0.15-10 µg/ml</td>
</tr>
<tr>
<td>Swainsonine</td>
<td>1 mg/ml</td>
<td>80</td>
<td>0.15-10 µg/ml</td>
</tr>
<tr>
<td>Deoxynojirimycin</td>
<td>400 mM</td>
<td>200</td>
<td>0.15-10 mM</td>
</tr>
<tr>
<td>Deoxymannojirimycin</td>
<td>400 mM</td>
<td>200</td>
<td>0.15-10 mM</td>
</tr>
<tr>
<td>Castanospermine</td>
<td>400 mM</td>
<td>200</td>
<td>0.15-10 mM</td>
</tr>
</tbody>
</table>
**Measure incorporated macromolecular radioactivity**

11. If tunicamycin was used as the inhibitor, determine the amount of incorporated macromolecular radioactivity by TCA precipitation and proceed to step 18. If another inhibitor was used, carry out steps 12 to 17.

   *The amount of radioactivity incorporated into macromolecules reflects the effect of the inhibitor(s) on oligosaccharide biosynthesis: the presence of inhibitor should block incorporation of [3H]mannose into macromolecules.*

12. Wash the cells twice by resuspending the pellet in ice-cold PBS, centrifuging, and removing the supernatant. Resuspend the pellet in lysis buffer without bovine hemoglobin and lyse as described in UNIT 10.16 (steps 1 to 4, basic protocol).

13. Divide each sample into two equal aliquots in conical polypropylene centrifuge tubes and precipitate protein with acetone (support protocol).

   *Use 1.5-ml, 15-ml, or 50-ml centrifuge tubes depending on the quantity of sample (8 vol acetone must be added).*

14. Resuspend pellets in 20 to 40 µl endo H digestion buffer per 10⁷ cells and boil 10 min to inactivate endogenous hydrolases.

15. Add 5 µl endo H per 100 µl sample to one tube of each sample pair and incubate both overnight at 37°C.

16. Add 1/10 vol of 20% (w/v) SDS to each sample and boil 3 to 5 min to terminate the digestion.

17. Apply one sample at a time to a calibrated Sephacryl S-200 column equilibrated in 25 mM ammonium formate/0.1% SDS, in a sample volume that is < 5% of the bed volume. Collect the eluate and use a scintillation counter to measure the radioactivity associated with glycoproteins (eluting in the void volume of the column, V₀) and with free oligosaccharides (eluting after V₀) for each of the four samples. Wash column with 2 to 4 vol 25 mM ammonium formate/0.1% SDS between sample applications.

   *In the absence of endo H digestion, all of the radioactivity from both the inhibitor-treated and untreated samples should elute in V₀. Elution of any radioactive material after V₀ may indicate that the wash steps following acetone precipitation did not completely remove low-molecular-weight contaminants present in the cell lysate.*

   *For samples digested with endo H, elution profiles should demonstrate a marked increase in the amount of endo H-releasable radioactivity in samples from inhibitor-treated cells relative to treated cells. For samples not treated with inhibitor, the oligosaccharides cleaved from the peptide backbone by endo H represent immature oligosaccharides still being processed in the Golgi apparatus and a small population of endo H-sensitive structures found on some mature glycoproteins. For samples treated with inhibitor, a much higher percentage of the total radioactivity should be released, as the inhibitors prevent the processing of the oligosaccharides into mature oligosaccharide structures (Fig. 17.10.1) and the underprocessed oligosaccharides contain more mannose residues than the processed ones. Theoretically, 100% of the radioactivity should be releasable by endo H digestion of the inhibitor-treated samples (see critical parameters), although in reality no inhibition is ever complete.*

   *To save time, analyze only the first and last tubes of the control cells in this fashion, i.e., those not treated at all and those treated with the highest concentration of the inhibitor solvent (from step 2 above). If there is no difference in the results from these two groups of cells, it is reasonable to assume that those cells treated with lower concentrations of the inhibitor solvent will yield the same results. However, if the solvent is found to be toxic, then the cells exposed to lower concentrations will need to be examined to find the maximally tolerated concentration of inhibitor solvent.*
18. If desired, and if methods are available for purifying and identifying a specific glycoprotein from cells radiolabeled with $[^{35}S]$methionine, examine the effect of a given inhibitor by SDS-PAGE and autoradiography.

_Each N-linked carbohydrate chain contributes \( \sim 2000 \) to 4000 Da to a protein’s mass, so synthesis of a protein in the presence of tunicamycin will result in faster mobility on SDS-PAGE. The effect of other inhibitors can be demonstrated by an increased sensitivity to digestion by endo H, which is also detectable as an increase in migration rate in SDS-PAGE._

**ACETONE PRECIPITATION**

Acetone precipitation is a useful step for concentrating proteins and for exchanging them from one buffer to another. As little as 10 ng of protein can be precipitated successfully.

**Additional Materials**

- 100% acetone (HPLC or ACS grade), \(-20°C\)
- 1.5-ml, 15-ml, or 50-ml conical polypropylene or other centrifuge tubes

1. Place the protein solution on ice, add 8 vol acetone \((-20°C)\), mix gently, and precipitate at \(-20°C\) overnight.

   _If the sample volume is too large to be conveniently diluted with 8 vol, concentrate it by lyophilizing and resuspending in a smaller volume. If the sample contains NP-40 or Triton X-100 detergent, however, there is a limit to how much it can be concentrated, as high concentrations of detergent (e.g., >1%) will partition out of acetone solutions, appearing as an oily pellet after centrifugation (step 2)._  

   _If a large amount of protein is present (i.e., a precipitate is readily visible after the acetone is added), it can be precipitated after standing at \(-20°C\) for only a few hours instead of overnight. Leaving the material in acetone for longer than 1 day should be avoided, as it may become increasingly difficult to redisolve the protein._

2. Collect the precipitate by centrifuging 15 min at 3000 $\times g$, 4°C (a small pellet should be visible). Carefully invert the tube and pour out the acetone into a clean tube.

   _If the pellet is oily due to the presence of detergent, it should be extracted by adding 85% (v/v) acetone \((-20°C)\), mixing, incubating at \(-20°C\) for >1 hr, and recentrifuging._

3. Centrifuge the tube containing the pellet briefly to concentrate the remaining acetone and remove it with a micropipettor. Allow pellet to dry until moist but not powder-dry, and resuspend in an appropriate quantity of the desired solvent.

   _Care must be taken in drying the pellet, as great difficulty may be encountered in resuspending some proteins if the pellet dries completely. This insolubility depends on both the individual protein and the total amount present._

**REAGENTS AND SOLUTIONS**

_NOTE:_ Deionized, distilled water should be used for buffer and inhibitor solutions.

**Endo H digestion buffer**

- 15 µl 0.5 M sodium citrate, pH 5.5
- 75 µl water
- 5 µl 10% PMSF in isopropanol

This is sufficient for 5 µl of 0.5 U/ml endo H (100 µl digestion volume). The PMSF prevents proteolysis. The presence of nonionic detergent is not required. Prepare just before use; PMSF is unstable in water.
**Inhibitor stock solutions**

Dissolve castanospermine (mol. wt. 189.2), 1-deoxynojirimycin (mol. wt. 199.6), or 1-deoxymannojirimycin (mol. wt. 163.2) in water to 400 mM; dissolve swainsonine (mol. wt. 173.2) in water to 1 mg/ml. Sterilize by filtration through a 0.2-µm filter. Store frozen at −20°C (stable indefinitely).

Dissolve tunicamycin (mol. wt. 840) in dimethylsulfoxide (DMSO), dimethylformamide (DMF), 95% ethanol, or 25 mM NaOH to 1 mg/ml. Store frozen at −20°C (stable ∼1 yr). If NaOH is used, store stock solution in a small tube that is tightly capped to prevent adsorption of carbon dioxide, which will lower the pH. Tunicamycin's solubility in neutral aqueous solution is <1 mg/ml (attempting to make such a solution will produce a visible precipitate).

**COMMENTARY**

**Background Information**

Assembly of N-linked oligosaccharide chains involves the sequential action of several glycosidases and glycosyltransferases (reviewed by Hubbard and Ivatt, 1981, and Kobata and Takasaki, 1992). Growth of cells in the presence of inhibitors of these enzymes produces glycoproteins with underprocessed or missing oligosaccharide chains. Depending on the protein, these changes may alter biological functions and/or rates of transport, secretion, or turnover (Elbein, 1987; McDowell and Schwarz, 1988).

N-linked glycosylation begins with the assembly of the lipid-linked precursor glucose₃mannose₉N-acetylglucosamine₂-phosphaté-dolichol (Glc₃Man₉GlcNAc₂-PP-dolichol; similar to Structure A, Fig. 17.10.1). Assembly of this precursor requires N-acetylglucosamine-phosphaté-dolichol (GlcNAc-PP-dolichol), whose formation is blocked by tunicamycin (Tkacz and Lampen, 1975). Thus, the presence of tunicamycin results in proteins missing some or all of their N-linked side chains; however, those that are added due to incomplete inhibition are processed normally. Tunicamycin may also inhibit protein synthesis; not all proteins will be affected to the same degree. Tunicamycin also blocks the assembly of type II keratan sulfate chains, because these glycans also utilize GlcNAc-PP-dolichol (Hart and Lennarz, 1978). Glycolipid biosynthesis may also be inhibited, although the mechanism underlying this has not been established (Yusuf et al., 1983; Guarnaccia et al., 1987).

The glycosidase inhibitors castanospermine, deoxynojirimycin, deoxymannojirimycin, and swainsonine are considerably less toxic than tunicamycin. Castanospermine inhibits glucosidase I, and deoxynojirimycin inhibits both glucosidases I and II (Fig. 17.10.1; Pan et al., 1983; Saunier et al., 1982). In some studies, the metabolic block produced by these two drugs is not complete, yielding a mixture of normally processed and underprocessed structures. More recently, an endo-α-D-mannosidase has been described that cleaves the tetrascarbohydrate Glc₃-Man₁ from Glc₃Man₉GlcNAc₂, effectively bypassing the block produced by the glycosidase inhibitors (Lubas and Spiro, 1987). Although the question has not been examined fully, the level of this endo-α-D-mannosidase may vary between different cell lines.

Deoxymannojirimycin and swainsonine are inhibitors of mannosidases I and II, respectively (Fig. 17.10.1; Fuhrmann et al., 1984; Tulsiani et al., 1982). By inhibiting mannosidase I, deoxymannojirimycin prevents addition of the α1-2 N-acetylglucosamine (GlcNAc) residue required to produce Structure D, Figure 17.10.1, which is a necessary step for the action of mannosidase II. Thus, deoxymannojirimycin prevents addition of any GlcNAc residues, blocking galactosylation and sialylation as well. Swainsonine blocks mannosidase II. However, it does not prevent the single GlcNAc residue (Structure D, Fig. 17.10.1) from being galactosylated and sialylated, resulting in hybrid structures (Tulsiani and Touster, 1983).

Some of the glycosidase inhibitors are also active against lysosomal enzymes, although the significance of this effect in tissue culture cells has been little explored. Deoxynojirimycin can inhibit synthesis of the lipid-linked precursor in cell-free extracts (Romero et al., 1985); this also has not been thoroughly investigated with intact cells. No information is available on the metabolism or half-lives of these inhibitors in cultured cells.
Critical Parameters and Troubleshooting

The two most critical variables are duration of exposure and inhibitor concentration. The treatment times suggested in this unit (24 hr) will permit a cell to replace most of its endogenous glycoproteins with glycoproteins synthesized in the presence of inhibitor. For proteins that turn over slower or faster than average, longer or shorter incubation times may be appropriate. If pulse-chase studies with radiolabeled sugar or amino acid precursors (UNITS 17.4 & 10.18) are being carried out, it is necessary to incubate cells in inhibitor for only 1 to 2 hr before adding label.

N-linked glycosylation inhibitors may be toxic to the cells, as indicated by decreased cell viability and/or reduced protein synthesis. The concentration of tunicamycin that can be used is generally limited by its toxicity, which may be considerable in some cell lines (more so with tumor cells than nontransformed cells). Other glycosidase inhibitors are usually not toxic in the concentration ranges suggested in Table 17.10.1 (the highest concentrations listed represent those used in most published studies).

It is important to realize that in a given cell line, not all glycoproteins will be affected to the same extent by a given inhibitor. Moreover, it is possible that not all sites on the same glycoprotein will be affected equally. These caveats should be kept in mind when interpreting the results from studies employing these inhibitors.

Failure to observe an effect from an inhibitor may indicate that concentration of inhibitor was too low and/or length of exposure was too short. Cell lines differ in their susceptibility to these compounds.

Safety precautions and experimental parameters for labeling—including labeling time, label concentration and specific activity, cell density, and culture conditions—are discussed in the critical parameters of UNIT 10.18.

Anticipated Results

These experiments will demonstrate the chosen inhibitor’s toxicity, as assessed by protein synthesis, and its ability to alter oligosaccharide biosynthesis, as assessed by total [3H]mannose incorporation (for tunicamycin) or endo H release of the oligosaccharides (for the other inhibitors listed in Table 17.10.1).

Time Considerations

Setting up the initial round of cell cultures for determining the optimal inhibitor concentration takes 2 hr, after which they are incubated 24 hr. On the second day, the dilution series of medium containing inhibitor is set up, the cells are transferred to these media (<1 hr), and the plates incubated 24 hr. On the third day, the incubation of the cells with the radiolabeled precursor is set up (~4 hr); the actual incubation takes 4 to 16 hours. Following incubation, the cells are assayed for radiolabel incorporation by TCA precipitation (2 to 4 hr).

The main part of the experiment follows a similar time course if the inhibitor used is tunicamycin. If a different inhibitor is used, analysis of endo H susceptibility by Sephacryl S-200 chromatography will take ~1 day, including the overnight incubation.

Literature Cited


**Key References**


Hubbard, S.C. and Ivatt, R.J. 1981. See above. *Good review on early work on the processing pathway.*


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Inhibition of Glycolipid Biosynthesis

Adequate inhibition of glycolipid biosynthesis allows the study of their biological functions. The method presented in this unit employs a synthetic analog of ceramide, PDMP (1-phenyl-2-decanoylamino-3-morpholino-1-propanol), that inhibits glycolipid biosynthesis in cultured cells. Optimum conditions for inhibition of glycolipid biosynthesis are determined, glycolipids extracted from cultures grown with and without inhibitor, and the patterns of glycolipids analyzed by HPTLC (UNIT 9.7A). Detection is achieved using colorimetric reactions, or by monitoring radioactivity when cells have been metabolically radiolabeled. The effects of these changes in biological functions can then be studied later.

Materials

Adherent cell culture
10 mM PDMP in ethanol (see recipe)
Culture medium appropriate for cell line, with or without serum
Ethanol
Radiolabeled precursors (UNIT 17.4; for metabolic labeling)
PBS (APPENDIX 2)
2:1, 1:1, and 1:2 (v/v) chloroform/methanol
10:10:1 (v/v/v) chloroform/methanol/water
HPTLC standards: mixture of gangliosides or neutral lipids from bovine brain
(Sigma or Accurate Chemical) or mixture of neutral glycolipids (Accurate Chemical)
Orcinol/sulfuric acid reagent (see recipe)
Resorcinol/hydrochloric acid reagent (see recipe)
Screw-cap glass tube, sterile
Conical glass tubes (10- or 50-ml, depending on volume of pellet to be extracted)
Sonicator bath
Nitrogen stream
Rotary or shaker evaporator
Silica-gel HPTLC plates
Enhance spray (DuPont NEN; for metabolic labeling)
Glass spray unit
Oven adjustable up to 140°C

Additional reagents and equipment for HPTLC (UNIT 9.7A) and for metabolic radiolabeling (UNIT 17.4A) and autoradiography (APPENDIX 3A; both optional)

NOTE: All incubations should be carried out under the conditions normally used for the cell line to be analyzed.

Incubate cells with inhibitor
1. Prepare cell cultures and maintain as usual until subconfluent. Set up sufficient flasks or plates for the set of experiments using different POMP concentrations and incubation times (see steps 4 and 5).

This procedure can be performed in either the presence or absence of fetal bovine serum, and with adherent cells or (with appropriate modifications) suspension cultures.

2. Allow 10 mM PDMP in ethanol to warm up to room temperature. Transfer to a sterile screw-cap glass tube a sufficient amount of this solution to set up a series of incubations with 5 to 25 µM final concentration of PDMP and different time points (see steps 4 and 5). Evaporate to dryness with a nitrogen stream.
3. Add culture medium and sonicate 10 min in a sonicator bath to suspend the drug.

4. Add PDMP solution to aliquots of the cell culture to obtain final concentrations of 5, 10, 15, 20, and 25 µM. Add ethanol as needed to achieve the same ethanol concentration in all samples, and add the same total volume of ethanol without PDMP to a control culture.

5. Incubate the cells for selected durations.

   Periods ranging from 10 hr to 4 days have been employed. Optimum inhibition times may differ from one particular cell line to another. It is necessary to check the cells for viability as well as for morphological changes, particularly after prolonged incubations with the inhibitor.

6. Remove medium and add fresh medium containing PDMP as before. If the cells are being metabolically labeled, add the radiolabeled precursor at this point (see UNIT 17.4).

Prepare total lipid extract

7. Harvest cells into a 10- or 50-ml conical glass tube, pellet the cells, and wash three times with PBS.

8. Add 10 vol of 2:1 (v/v) chloroform/methanol to the cell pellet. Extract the lipids by homogenizing or sonicating 5 min. Centrifuge 10 min at \(-350 \times g\), 4°C. Transfer the supernatant (extract) to a clean glass tube using a Pasteur pipet.

9. Repeat the extraction as in step 8 with, successively, 1:1 (v/v) chloroform/methanol, 1:2 (v/v) chloroform/methanol, and 10:10:1 (v/v/v) chloroform/methanol/water.

10. Pool all extracts and dry them under reduced pressure, using a rotary or shaker evaporator or a nitrogen stream depending on the volume of extract to be dried.

Analyze extract by HPTLC and (optionally) autoradiography

11. Dissolve the pooled dry extract (total lipid extract) in a small volume of 2:1 (v/v) chloroform/methanol and spot one aliquot on silica-gel HPTLC plates (UNIT 9.7A). Spot a mixture of ganglioside and/or neutral glycolipid standards on one side of the plate. Perform HPTLC analysis. Allow the plates to dry completely after developing.

   The amount of total lipid required for positive resulting in observable bands (~1 nmol/band) will vary between cell lines because of different compositions and ratios of components. It may be necessary to do several trials until adequate results are obtained. When analyzing radiolabeled extracts, it is advisable to spot the same number of total counts in each lane.

   When analyzing nonradiolabeled glycolipids, and if sufficient material is available, two different HPTLC plates can be spotted in parallel with the same total lipid extracts. Staining one with orcinol/sulfuric acid and the other with resorcinol/hydrochloric acid will allow determination of which bands correspond to neutral glycolipids and which to gangliosides.

12. When analyzing nonradiolabeled glycolipids, proceed directly to step 13. When analyzing metabolically radiolabeled glycolipids, proceed by autoradiography (APPENDIX 3A) using Enhance scintillant spray, then proceed to step 13 to visualize the nonradiolabeled standards.

13a. Using a glass spray unit, spray plates with orcinol/sulfuric acid reagent. Heat in a 100°C oven until bands are observed (~10 min, but check the plate often to avoid overstaining).

   This step detects all glycolipids, which give brown bands.
In steps 13a and 13b, it is possible to stain only the side of the plate containing the standards by covering the rest of the plate with a piece of glass held in position with binder clips.

13b. Alternatively, spray the plate with resorcinol/hydrochloric acid reagent and heat 15 min in a 140°C oven.

*This step detects only gangliosides, which give blue bands.*

14. Compare the pattern of glycolipids obtained for the control cells with those obtained with different concentrations of inhibitor after different incubation periods.

15. If cells were metabolically radiolabeled, quantitate the individual components by scraping off the corresponding areas of silica gel and measuring radioactivity by liquid scintillation counting. If a scanning densitometer is available, quantitation can be achieved by scanning the autoradiograph or the stained glass plate.

**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.*

**PDMP stock solution, 10 mM in ethanol**

Dissolve 3.86 mg d,l-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol-HCl (PDMP; FW 390.6; available from Matreya) in 1.0 ml ethanol. Store at −20°C until used.

*PDMP is quite stable in aqueous solvents. No breakdown was observed after incubating a 1 mM solution in PBS (pH 7.4) for 10 days at 41°C. Stability in ethanol is even higher.*

**Orcinol/sulfuric acid reagent**

Dissolve 200 mg orcinol in 33 ml water. Dilute to 100 ml with concentrated sulfuric acid. Store in dark bottle at 4°C. Discard when the solution turns brownish (8 to 10 days).

*Orcinol from Sigma (FW 142.2) is suitable for making this reagent.*

**Resorcinol/hydrochloric acid reagent**

80 ml concentrated HCl
10 ml 2% resorcinol (1,3-benzenediol) in H₂O
250 µl 0.1 M copper sulfate in H₂O (2.5 mM final concentration)

Store in a dark bottle at 4°C
Discard if the solution turns brown

*Resorcinol from Sigma (crystalline; ~98% pure; FW 110.1) is suitable for making this reagent.*

**COMMENTARY**

**Background Information**

Many studies have shown that glycosphin-golipids and gangliosides are involved in a great variety of biological phenomena. As constituents of mammalian cell membranes, gangliosides significantly affect cell-surface and transmembrane signalling events such as cellular immune responses, cell growth, and differentiation. Other observed effects of gangliosides include those of “shed” gangliosides in malignancy; the effects of gangliosides and their products on protein kinase C; calcium binding; and their interaction with various bioeffectors, including bacterial toxins, bacterial binding proteins, interferons, serotonin, fibronectin, plasma low-density lipoproteins, lectins, and glycoprotein hormones.

Many studies of the role of these molecules have been carried out by adding specific exogenous glycosphingolipids or gangliosides, or mixtures thereof, to cultured cells. Interpretation of these studies is difficult for various reasons. True incorporation of the added molecules into the cell membrane is hard to prove,
and the possibility of abnormal effects caused by micelles associated with the cell is hard to rule out. Also, exogenous material may displace the controlling factor from its normal location in the cell membrane or influence its metabolism.

Another approach to clarifying the biological roles of glycosphingolipids and gangliosides is the use of enzyme inhibitors to study the effects of their depletion in cultured cells and animals. This inhibition can be achieved using a synthetic analog of ceramide, PDMP. This method was developed by Radin and Inokuchi, whose idea that cancer results from microsphingolipidosis led them to explore ways to change the biological processes in which glycolipids are involved. One such process is the synthesis of glycolipids from nonglycolipid precursors and lower glycolipids. Most neutral glycolipids and gangliosides have a common precursor, GlcCer, which is formed enzymatically from ceramide and UDP-Glc. The synthetase responsible for this transfer is UDP-glucose: N-acylsphingosine glycosyltransferase (EC 2.4.1.80). Because rapid liver growth was observed in mice when glucosylceramide accumulated (Datta and Radin, 1986), the authors explored the possibility that inhibiting the synthesis of the common precursor GlcCer would decrease the amount of glycolipids due to normal turnover (Hospatankar and Radin, 1982). After a few attempts, they obtained an active inhibitor, PDMP (p-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; Inokuchi and Radin, 1987). This compound was shown to inhibit ganglioside biosynthesis effectively in intact cells, without any major toxic effects (Inokuchi and Radin, 1987; Inokuchi et al., 1987). The effects of the enzyme inhibitor were counteracted by including GlcCer in the culture medium (Inokuchi et al., 1989). For a review of the available data, see Radin and Inokuchi (1991).

Critical Parameters

It is critical to understand that very little is known about the regulation of glycosphingolipid biosynthesis, turnover, or even which steps are regulation points. On the other hand, the composition of glycosphingolipids varies tremendously between different types of cells. The time required to obtain optimum inhibition of any glycolipid or collection of glycolipids depends on how completely the enzyme is inhibited, the activity of the specific hydrolases involved, the susceptibility of each glycolipid to attack by its hydrolase, and the rates of the synthases that recycle smaller glycolipids into larger ones. Although the effects on simple gangliosides such as GM1 are readily observed, complex molecules such as GD1b require more time to become affected. Complete inhibition, as well as complete recovery from the inhibitor, should be expected to take at least 24 hr.

In consequence, the conditions required for inhibition and recovery of each particular system need to be carefully established. Various concentrations of inhibitor should be tried to establish the minimum amount required for inhibition. It is also important to understand that an inhibitor may have pleiotropic effects, including the accumulation of precursors (e.g., ceramide), expansion of alternate pathways (e.g., sphingomyelin synthesis), and other non-specific effects (e.g., on protein synthesis). Depending on the experiment, such alternate explanations for the observed effects may need to be explored.

Anticipated Results

The protocol allows optimization of the conditions for maximum inhibition of glycolipid biosynthesis with minimum effects on the morphology and viability of cells. Once these conditions are established, different studies of the biological roles of glycosphingolipids and gangliosides can be performed.

Time Considerations

Complete inhibition of glycolipid biosynthesis may require up to 4 days of cell culture. Further extraction of glycolipids and analysis of the resulting data are expected to take a maximum of 2 days, including quantitation. When metabolic labeling and autoradiography are included, extra time is required for detection, and will depend on the total counts available and their distribution among components.

Literature Cited


**Key Reference**
Radin and Inokuchi, 1991. See above.

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**Excerpt**

Excellent review, providing very useful references for comparing conditions used for different systems along with specific protocols for studying the different biological roles affected by inhibition of glycolipid biosynthesis.

Contributed by Adriana E. Manzi
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Synthetic Glycosides as Primers of Oligosaccharide Biosynthesis and Inhibitors of Glycoprotein and Proteoglycan Assembly

With the exception of hyaluronic acid, all mammalian saccharides assemble while attached to a lipid or protein primer. Several cases are now known in which oligosaccharide synthesis will occur on synthetic glycoside primers added to cells. β-D-xylosides initiate glycosaminoglycan (GAG) synthesis (Okayama et al., 1973) by substituting for endogenous xylosylated core proteins (Basic Protocol 1). At high concentration xylosides will also prime oligosaccharides that resemble glycolipids (Freeze et al., 1993). N-acetyl-α-D-galactosaminides initiate the synthesis of O-linked oligosaccharides found on mucins and other glycoproteins (Basic Protocol 2) in an analogous manner. Even disaccharides, such as peracetylated N-acetyllactosaminide, can act as primers (Sarkar et al., 1995). Because these primers compete with endogenous substrates, they also act as inhibitors of proteoglycan (PG) and glycoprotein synthesis. Thus, primers have utility for studying the biological activity of glycoconjugates in cells, tissues, and animals. This unit describes procedures for using glycoside primers in cell culture.

XYLOSIDE INITIATION OF GAG SYNTHESIS AND INHIBITION OF PG ASSEMBLY

**Materials**

- 0.2 M p-nitrophenyl-β-D-xyloside in DMSO (see recipe)
- Tissue culture growth medium appropriate for cell line
- Cultured cells, in suspension or adherent
- Dimethylsulfoxide (DMSO, C$_2$H$_6$SO; mol. wt. 78.13)
- Radioactive precursors: H$_2^{35}$SO$_4$, [6-3H]GlcNH$_2$, or [1-3H]Gal
- Additional reagents and equipment for metabolic radiolabeling (UNIT 17.4), isolating proteoglycans and glycosaminoglycans (UNIT 17.3), and gel filtration (UNIT 17.20) and reversed-phase chromatography (UNIT 17.21)

**NOTE:** All incubations should be carried out in a humidified 37°C, 5% CO$_2$ incubator unless otherwise specified.

1. Add 0.2 M p-nitrophenyl-β-D-xyloside in DMSO to aliquots of growth medium to achieve final concentrations of 0, 0.01, 0.03, 0.1, 0.3, and 1 mM. Add DMSO as needed to achieve the same concentration of the solvent in all samples.

   Alternatively, dissolve enough inhibitor directly in growth medium to achieve a final concentration of 10 mM, then prepare the indicated serial dilution series with fresh growth medium.

   DMSO can cause some cells to differentiate, which can change the pattern of glycosylation.

2. Add each supplemented growth medium to a previously established cell culture by replacing the spent growth medium with the supplemented medium. Incubate 1 hr.

   Do not add concentrated stocks of glycosides directly to cell cultures, as high local concentrations of solvent and glycoside can kill cells.

3. Add radioactive precursors to each culture (generally 10 µCi/ml $^{35}$SO$_4^{2-}$, 20 µCi/ml [6-3H]GlcNH$_2$, or 50 µCi/ml [1-3H]Gal should be sufficient; see UNIT 17.4). Continue to incubate cells ≥1 hr.
4. Isolate the PGs and GAGs made by the cells and the oligosaccharides primed on the glycoside from the growth medium (UNIT 17.3).

5. Analyze the material by gel filtration and reversed-phase chromatography (see UNITS 17.20 & 17.21).

Oligosaccharides generated on primers are generally smaller than glycoproteins and proteoglycans.

N-ACETYL-α-D-GALACTOSAMINIDE INITIATION OF OLIGOSACCHARIDE SYNTHESIS AND INHIBITION OF O-LINKED GLYCOPROTEIN ASSEMBLY

**Additional Materials** (also see Basic Protocol 1)

0.2 M \( p \)-nitrophenyl-N-acetyl-\( \alpha \)-d-galactosaminide in DMSO (see recipe)

**NOTE:** All incubations should be carried out in a humidified 37°C, 5% CO\(_2\) incubator unless otherwise specified.

1. Add 0.2 M \( p \)-nitrophenyl-N-acetyl-\( \alpha \)-d-galactosaminide in DMSO to aliquots of growth medium to achieve final concentrations of 0, 1, 2, 5, and 10 mM. Add DMSO as needed to achieve the same concentration of the solvent in all samples.

   Alternatively, dissolve enough inhibitor directly in growth medium to achieve a final concentration of 10 mM, then prepare the indicated serial dilution series with fresh growth medium.

2. Add each supplemented growth medium to a previously established cell culture. Incubate 1 hr.

   *Do not add concentrated stocks of glycosides directly to cell cultures, as high local concentrations of solvent and glycoside can kill the cells.*

3. Add radioactive precursors to cultures (generally 20 \( \mu \)Ci/ml [6-\(^3\)H]GlcNH\(_2\) or 50 \( \mu \)Ci/ml [1-\(^3\)H]Gal should be sufficient; see UNIT 17.4). Incubate cells a further 3 hr.

4. Harvest the glycoproteins from the cells and growth medium (UNIT 17.3).

5. Analyze the material by gel filtration and reversed-phase chromatography (UNITS 17.20 & 17.21). Small oligosaccharides linked to hydrophobic aglycones will bind to C18 reversed-phase resins, aiding the separation of primed material from endogenous glycoproteins.

**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.*

**\( p \)-nitrophenyl-N-acetyl-\( \alpha \)-d-galactosaminide, 0.2 M in DMSO**

Dissolve 68.5 mg \( p \)-nitrophenyl-N-acetyl-\( \alpha \)-d-galactosaminide (C\(_{14}\)H\(_{18}\)N\(_2\)O\(_8\); mol. wt. 342.3) in 1 ml dimethylsulfoxide. Warm if necessary to dissolve.

**\( p \)-nitrophenyl-\( \beta \)-d-xyloside, 0.2 M in DMSO**

Dissolve 54.2 mg \( p \)-nitrophenyl-\( \beta \)-d-xyloside (C\(_{17}\)H\(_{13}\)NO\(_7\); mol. wt. 271.2) in 1 ml dimethylsulfoxide. Warm if necessary to dissolve.

*Other \( \beta \)-D-xylosides are also commercially available, including the fluorescent compounds naphthol-\( \beta \)-D-xyloside and 4-methylumbelliferyl-\( \beta \)-D-xyloside. Using these latter compounds, the fluorescence of the aglycone can be used to monitor the recovery of oligosaccharides primed on the glycoside.*
Background Information

Proteoglycan (PG) biosynthesis is initiated by the transfer of D-xylose from UDP-xylose to specific serine residues in core proteins. This intermediate acts as a natural primer for the assembly of heparan sulfate, heparin, chondroitin sulfate, and dermatan sulfate chains, depending on the tissue. In 1973, Okayama et al. (1973) reported that synthetic β-D-xylosides would prime glycosaminoglycan (GAG) synthesis, apparently by substituting for xylosylated core proteins. Since then, a variety of β-D-xylosides have been studied as primers with the following results. (1) Priming requires the β-anomer of D-xylose (Galligani et al., 1975). Other pentosides (β-D-lyxoside, β-D-ribose, and α-L-arabinoside) and hexosides of the linkage region generally lack activity (Robinson and Robinson, 1985; F.N. Lugemwa, A.K. Sarkar, and J.D. Esko, unpub. observ.). (2) Priming activity correlates with hydrophobicity of the aglycone (Robinson et al., 1975; Robinson and Robinson, 1981; Sobue et al., 1987; Kolset et al., 1990; Lugemwa and Esko, 1991). Thus, methyl-β-D-xyloside is a weak primer compared to p-nitrophenyl-β-D-xyloside or 4-methylumbelliferyl-β-D-xyloside.

(3) The most active primers contain O or S in the glycosidic linkage, but N-xylosides and homo-C-xylosides also have activity (Sobue et al., 1987). (4) Priming is dose dependent (Esco et al., 1987; Lugemwa and Esko, 1991). (5) β-D-xylosides prime GAGs in most cells and can cause more GAG to be made than normally occurs on natural core proteins. (6) Cells secrete the majority of material generated on β-D-xylosides into the growth medium. (7) β-D-xylosides prime chondroitin sulfate or dermatan sulfate in virtually all cells, but priming of heparan sulfate occurs poorly, except with appropriate aglycones (Lugemwa and Esko, 1991; Fritz et al., 1994). (8) The fine structure of the chains depends on the concentration of the primer and may not precisely duplicate the structure of chains on natural core protein substrates (Fransson et al., 1992; Fritz et al., 1994).

(9) β-D-xylosides compete with endogenous xylosylated core proteins for precursors and the various biosynthetic enzymes. Thus, β-D-xylosides act as inhibitors of PG synthesis. The inhibitory activity of xylosides has been exploited to examine the biological activity of PGs in cells.

The enormous success of β-D-xylosides in altering PG synthesis suggested that other glycosides might act as artificial primers. Kuan et al. (1989) showed that aryl-N-acetyl-α-D-galactosaminides inhibited mucin assembly in colon carcinoma cells. Benzyl-α-D-galactosaminide inhibits O-linked oligosaccharide synthesis on cell-surface glycoproteins as well, which can have profound effects on cell adhesion (Kojima et al., 1992). Zhuang et al. (1991) showed that α-D-galactosaminides prime the synthesis of O-linked oligosaccharides. Thus, the inhibition of glycoprotein assembly is most likely due to competition between endogenous core protein substrates and the added glycoside.

Critical Parameters

Glycosides containing hydrophobic aglycones are moderately soluble in aqueous solutions. Stock solutions can be prepared in DMSO or ethanol and appropriate dilutions made into growth medium. β-D-xylosides are stable in both organic and aqueous solvents. It is important to add glycosides to growth medium before adding them to cells, because the concentrated solvent can cause cells to lyse. To avoid this problem, the glycosides can be dissolved directly into growth medium at a final concentration of ≤10 mM. A serial dilution series prepared in fresh growth medium can then be added to previously established cell cultures. This method minimizes the use of solvent and provides more accurate control over the concentration of glycoside.

Priming of oligosaccharides occurs in a concentration-dependent manner. Priming efficiency is easily assessed by adding a radioactive precursor, such as [6-3H]GlcNH₂ or [1-3H]Gal. 35SO₄²⁻ can be used to measure GAG synthesis. After brief incubation, the mixtures of radioactive oligosaccharides can be isolated from the growth medium, because cells secrete the majority of material generated on primers (UNIT 17.1 & 17.3). Usually this will require separation...
of endogenous glycoproteins and PGs from the primed oligosaccharides and GAGs, e.g., by gel filtration or reversed-phase chromatography (UNIT 17.20 & 17.21).

In general, GAG biosynthesis peaks in most cells when the β-D-xyloside concentration reaches 10 to 100 μM. However, this value varies in different cells. Thus, it is important to vary the concentration from 1 μM to 1 mM and assess the minimal concentration needed for maximally priming GAGs. The priming of glycolipid-like compounds on β-D-xylosides requires relatively higher concentrations of primer (Freeze et al., 1993). Similarly, the priming of O-linked oligosaccharides by N-acetyl-α-D-galactosaminides depends on concentration, but the effective dose is considerably higher (1 to 10 mM) than that observed for β-D-xylosides (Kuan et al., 1989). In contrast, priming by modified disaccharides occurs in the 10 to 100 μM range (Sarkar et al., 1995). These differences may relate to the relative abundance of endogenous substrates, solubility of the glycoside, its uptake through plasma membranes into the Golgi, its relative affinity for the glycosyltransferases, enzyme concentration and composition, or its susceptibility to hydrolysis.

Excessive concentrations of glycosides can be deleterious (Kanwar et al., 1986; Lugemwa and Esko, 1991). All glycosides exhibit detergent properties, because the molecules have both a hydrophilic end (the glycosate unit) and a hydrophobic end (the aglycone). To test for toxic effects, one can employ glycosides with the wrong anomic stereochemistry (e.g., α-D-xylosides; Farach et al., 1988), other pentosides (e.g., p-nitrophenyl-α-L-arabinoside), or the aglycone alone (e.g., p-nitrophenol).

Specificity exists with respect to the composition of GAG chains generated on β-D-xylosides (Lugemwa and Esko, 1991). Most xylosides (e.g., those containing linear aliphatic chains, single aromatic rings, and saturated multicyclic ring systems) tend to prime chondroitin sulfate and dermanate sulfate. The priming of chondroitin sulfate does not seem to depend on the aglycone: d-xylose and methyl-β-D-xyloside will initiate chondroitin sulfate (Okayama et al., 1973; Schwartz et al., 1974; Galligani et al., 1975; Lugemwa and Esko, 1991). Recent studies show that priming of heparan sulfate depends on aglycone structure (Lugemwa and Esko, 1991; Fritz et al., 1994). For example, naphthol-β-D-xyloside and estriol-β-D-xyloside will prime heparan sulfate in Chinese hamster ovary cells. At low concentra-

**Anticipated Results**

The addition of glycosides to cultured cells primes the synthesis of free oligosaccharide chains and inhibits the formation of mature glycoconjugates. Priming and inhibition occur in a dose-dependent manner, and the appropriate concentration required for optimal priming and inhibition must be determined empirically. Figure 17.11.1 shows gel filtration profiles of GAG and PG synthesized by a murine mastocytoma cell line (Montgomery et al.,...
As the concentration of $\beta$-D-xyloside rises, the amount of free GAG chains increases, as measured by material eluting in more retarded fractions. The size of these chains decreases as the primer concentration continues to rise. Presumably, this reflects the enhanced initiation of GAG chains and the competition of primers with endogenous xylosylated core proteins for limited precursors. The amount of mature PG decreases further as primers divert more resources away from the formation of GAG chains on core proteins. $N$-acetyl-$\alpha$-d-galactosaminides and acetylated disaccharides have similar effects on the assembly of O-linked oligosaccharides on glycoproteins (Kojima et al., 1992; Sarkar et al., 1995). The relatively small oligosaccharides generated on these primers can be purified by reversed-phase and gel filtration chromatography (Zhuang et al., 1991).

**Figure 17.11.1** Gel filtration chromatography of glycosaminoglycans (GAGs) and proteoglycans (PGs) generated in the presence of naphthol-$\beta$-D-xyloside. Mastocytoma cells (Montgomery et al., 1992) were incubated 4 hr with the indicated concentration of naphthol-$\beta$-D-xyloside and 20 $\mu$Ci/ml $^{35}$SO$_4$\(^{2-}\). The $^{35}$S]GAGs and $^{35}$S]PGs in the cells and growth medium were solubilized in Triton buffer and fractionated over a small DEAE-Sephacel column as described in UNIT 17.5. An aliquot was analyzed by gel filtration HPLC (TSK G4000 SW, 30 cm $\times$ 7.5 mm i.d., Pharmacia Biotech). Samples were eluted with 0.5 M NaCl/0.1 M KH$_2$PO$_4$ (pH 6.0)/0.2% (w/v) Zwittergent 3-12 (Calbiochem) at a flow rate of 0.5 ml/min.

1992). As the concentration of $\beta$-D-xyloside rises, the amount of free GAG chains increases, as measured by material eluting in more retarded fractions. The size of these chains decreases as the primer concentration continues to rise. Presumably, this reflects the enhanced initiation of GAG chains and the competition of primers with endogenous xylosylated core proteins for limited precursors. The amount of mature PG decreases further as primers divert more resources away from the formation of GAG chains on core proteins. $N$-acetyl-$\alpha$-d-galactosaminides and acetylated disaccharides have similar effects on the assembly of O-linked oligosaccharides on glycoproteins (Kojima et al., 1992; Sarkar et al., 1995). The relatively small oligosaccharides generated on these primers can be purified by reversed-phase and gel filtration chromatography (Zhuang et al., 1991).

**Literature Cited**


Oligosaccharide Glycosides as Supplement 32 Current Protocols in Molecular Biology

Biosynthesis Primers of 17.11.6


Key References

Fritz et al., 1994. See above. Demonstrates that the type of glycoaminoglycan chain produced on a β-D-xylose depends on the structure of the aglycone.

Kuan et al., 1989. See above. First description of N-acetyl-α-galactosaminides as primers and inhibitors of O-linked glycosylation of glycoproteins.

Salimath et al., 1995. See above. β-D-xylosides will also prime unusual oligosaccharides—e.g., GalNAcβ1→4GlcAβ1→3Galβ1→4Xyl-R, GM3-like compounds, and other oligosaccharides.

Sarkar et al., in press. See above. Demonstrates that cells will take up suitably modified disaccharides and use them as primers, opening up the possibility of designing more complex and selective glycosylation inhibitors.

Contributed by Jeffrey D. Esko University of Alabama at Birmingham Birmingham, Alabama

Rebecca I. Montgomery Northwestern University Chicago, Illinois

Synthetic Glycosides as Primers of Oligosaccharide Biosynthesis

17.11.6

Supplement 32 Current Protocols in Molecular Biology
RELEASE OF SACCHARIDES FROM GLYCOCONJUGATES

In many instances, it is useful to release a part or all of the oligosaccharide chains from a glycoconjugate before analyzing their structure, and the units presented in this section describe a variety of ways in which this can be achieved. Some of the methods described here (particularly the enzymatic ones) can also be applied to the study of free oligosaccharides or to oligosaccharides that are still attached to macromolecules.

Enzymes that degrade sugar chains fall into two general classes. Endoglycosidases cleave at defined sites within a sugar chain, with a specificity often based on certain features of the adjacent monosaccharide units. Thus, they are analogous to the restriction endonucleases that work on nucleic acids (UNIT 3.1). On the other hand, exoglycosidases specifically release single monosaccharides only when they are present as terminal units on sugar chains. Unlike the cases with exonucleases, the types of possible terminal units on oligosaccharide chains are numerous. Correspondingly, the number of available exoglycosidases with different specificities is quite large. In this section, one of the most commonly used group of exoglycosidases, the sialidases (also known as neuraminidases), is discussed in UNIT 17.12. Some commonly used endoglycosidases and glycoamidases for N-linked oligosaccharides (UNIT 17.13A) and polysaccharide lyases (UNIT 17.13B) are then considered.

Additional methods will be provided in future supplements (see Chapter 17 table of contents). One of the simplest and most direct ways to free an oligosaccharide chain from a glycoprotein or proteoglycan is to digest away the peptide with a broad-spectrum protease such as pronase or proteinase K (UNIT 17.14). In these cases, most of the peptide is removed except for the amino acids immediately surrounding the glycosylation site(s). If needed, more specific proteases (e.g., trypsin, chymotrypsin) can be used to generate glycopeptides with large stretches of amino acids still attached to the glycosylation sites. This approach is particularly useful for the detection of individual glycosylation sites on glycoproteins (UNIT 17.14).

There are also many classic and well-established methods for the chemical release of saccharides from glycoconjugates (UNIT 17.15). Some are designed to release intact sugars chains and others release the individual monosaccharide units. Although these methods tend to be somewhat less specific and potentially more destructive than the enzymatic approaches, they are cheap, convenient, and generally easy to use. These methods will be presented in future supplements.

Of course, there are many other techniques for the release of saccharides from glycoconjugates that could have been presented in this section. The current and upcoming selections were based on the criterion of broad general utility to the average molecular biologist. As methods in “glycotechnology” improve and simplify, further additions to this section may become appropriate.
Sialidases

Sialic acids are a family of nine-carbon acidic sugars found at the nonreducing terminus of many glycoconjugates. Sialidases (a term preferred to neuraminidases) can remove these sugar units selectively from cell surfaces, membranes, or purified glycoconjugates. This may be done to analyze the sialic acids or to study the consequences of their removal. The more sensitive and specific colorimetric assays for sialic acids work on free but not on glycosidically bound molecules (UNIT 17.16). Although careful acid hydrolysis also can be used to release sialic acids (UNIT 17.15), partial destruction of modifications and/or incomplete release can be problematic. Furthermore, acid hydrolysis may alter the underlying glycoconjugate or may be incompatible with functional studies.

In this unit, sialidase digestion of purified glycoproteins is described in the basic protocol and treatment of intact cells is outlined in the alternate protocol. The physical properties of the four most useful sialidases are listed in Table 17.12.1; their relative activities against sialic acids with different modifications and in different linkages are listed in Table 17.12.2 (see also critical parameters). The choice of enzyme depends upon the nature of the sample and knowledge of the type of sialylated glycoconjugates present.

**SIALIDASE TREATMENT OF PURIFIED GLYCOPROTEINS**

A purified glycoprotein sample is dissolved in digestion buffer and digested with a sialidase. Controls of sample alone and enzyme alone are also prepared. Digestion of gangliosides (glycolipids with sialic acid) requires detergent (e.g., deoxycholate, cholate, or taurocholate). After digestion, the reaction is terminated and desialylation is monitored.

**Materials**

- Sialic acid–containing sample
- Sialidase digestion buffer
- Sialidase (Table 17.12.1)
- Additional reagents and equipment for quantitating sialic acid (UNIT 17.16)

1. Dissolve the sialic acid–containing sample in sialidase digestion buffer at ∼0.1 to 1 mM sialic acid concentration (final).

   *If the amount of sialic acid is not known, it can be estimated by acid hydrolysis (UNIT 17.15) or by quantitating the amount of sialic acid released with increasing amounts of*

**Table 17.12.1 General Properties of Commercially Available Sialidases**

<table>
<thead>
<tr>
<th>Feature</th>
<th><em>Vibrio cholerae</em></th>
<th><em>Clostridium perfringens</em></th>
<th><em>Arthrobacter ureafaciens</em></th>
<th>Newcastle disease virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Form</td>
<td>Purified protein</td>
<td>Purified protein</td>
<td>Purified protein</td>
<td>Whole virion</td>
</tr>
<tr>
<td>Ca^{++} requirement</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>pH optimum</td>
<td>5.6^d</td>
<td>4.5^b/5.2^c</td>
<td>5.0^d</td>
<td>5.0–6.0</td>
</tr>
<tr>
<td>pH range</td>
<td>4.0–8.0</td>
<td>4.0–7.0</td>
<td>4.0–7.0</td>
<td>NA^e</td>
</tr>
<tr>
<td>Maximal activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% at pH 5.0</td>
<td>&gt;90^a</td>
<td>≈95^b/90^c</td>
<td>&gt;90^d</td>
<td>NA^e</td>
</tr>
<tr>
<td>% at pH 7.0</td>
<td>&gt;70^a</td>
<td>ND^f/-40^g</td>
<td>–30^d</td>
<td>NA^e</td>
</tr>
</tbody>
</table>

^a In 0.1 M Tris/maleic buffer (Ada et al., 1961) and sodium acetate (Cassidy et al., 1965).
^b In 0.1 M sodium acetate (Cassidy et al., 1965).
^c In 0.1 M citrate-phosphate (Cassidy et al., 1965).
^d In 0.1 M sodium acetate buffer (Uchida et al., 1979).
^e Abbreviations: NA, not available (pH profile not published, although enzyme remains active at pH 7.0); ND, not determined.
sialidase. If the amount of sialidase added is suboptimal, free sialic acid measured (by the TBA or DMB assays, UNIT 17.16) will increase with increasing amounts of sialidase.

If the sample contains gangliosides, include \( \frac{1}{50} \) vol of 10% sodium deoxycholate (0.2% final) and place tubes in a sonicator bath for 1 to 2 min to ensure complete dispersal. If large amounts of gangliosides are present, the amount of detergent in the sample should be kept equal to or greater than the amount of ganglioside on a weight-to-weight basis. If no direct information is available concerning the amount of ganglioside present, a series of digestions with increasing amounts of detergent (e.g., 0.1%, 0.3%, 1.0%) may need to be done. Excess detergent may actually inhibit digestion. The Arthrobacter ureafaciens sialidase (see Table 17.12.1) is most active with gangliosides. Deoxycholate solutions may become cloudy in the presence of salts.

2. Add 1 to 20 mU sialidase to the sample and enzyme control tubes. Mix well.

Select the enzyme to be used based on the properties in Table 17.12.1. The amount of enzyme to use depends on the amount of releasable sialic acid in the sample. Initially, use \( \sim 1 \) mU/nmol of sialic acid. Further experiments may indicate that more or less enzyme is required.

3. Prepare blank tubes containing either sample alone or enzyme alone (in sialidase digestion buffer).

Alternative controls could include reactions incubated with inactivated sialidase (prepared by boiling 5 min) or active sialidase plus 1 mM 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (Boehringer Mannheim), a sialidase inhibitor.

Table 17.12.2  Sialidase Action on Types of Sialic Acids

<table>
<thead>
<tr>
<th>Type</th>
<th>Linkage(s)</th>
<th>Vibrio cholerae</th>
<th>Clostridium perfringens</th>
<th>Arthrobacter ureafaciens</th>
<th>Newcastle disease virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neu5Ac</td>
<td>( \alpha2-3 )</td>
<td>++++</td>
<td>++++</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>( \alpha2-6 )</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>( \alpha2-8 )</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Neu5Gc</td>
<td>( \alpha2-3 )</td>
<td>++++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>( \alpha2-6 )</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>( \alpha2-8 )</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>7(9) mono-O-acetyl</td>
<td>( \alpha2-3 )</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>( \alpha2-6 )</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>( \alpha2-8 )</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>4-mono-O-acetyl</td>
<td>( \alpha2-3 )</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>( \alpha2-6 )</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>( \alpha2-8 )</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>7(8)9 di-O-acetyl</td>
<td>( \alpha2-3 )</td>
<td>R?</td>
<td>R?</td>
<td>++</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>( \alpha2-6 )</td>
<td>R?</td>
<td>R?</td>
<td>+++</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>( \alpha2-8 )</td>
<td>R?</td>
<td>R?</td>
<td>+++</td>
<td>?</td>
</tr>
<tr>
<td>7,8,9 tri-O-acetyl</td>
<td>( \alpha2-3 )</td>
<td>R?</td>
<td>R?</td>
<td>++?</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>( \alpha2-6 )</td>
<td>R?</td>
<td>R?</td>
<td>++?</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>( \alpha2-8 )</td>
<td>R?</td>
<td>R?</td>
<td>++?</td>
<td>?</td>
</tr>
<tr>
<td>Periodate-oxidized</td>
<td>( \alpha2-3 )</td>
<td>+</td>
<td>++</td>
<td>R?</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>( \alpha2-6 )</td>
<td>+</td>
<td>++</td>
<td>R?</td>
<td>?</td>
</tr>
<tr>
<td>Neu5Ac-7(8)</td>
<td>( \alpha2-8 )</td>
<td>?</td>
<td>?</td>
<td>R?</td>
<td>?</td>
</tr>
<tr>
<td>O-methyl,</td>
<td>( \alpha2-3 )</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>O-sulfate,</td>
<td>( \alpha2-6 )</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>combinations</td>
<td>( \alpha2-8 )</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
</tbody>
</table>

Abbreviations: Neu5Ac, N-acetylneuraminic acid; Neu5Gc, N-glycolyneuraminic acid; R, practically resistant under typical digestion conditions; ?, not known. See Varki (1992) for additional details of terminology.
4. Incubate 3 to 4 hr at 37°C.

*Digestion of gangliosides may require 24 to 48 hr.*

For selective digestion of α(2-3) and α(2-8) linkages by the Newcastle Disease virus sialidase, use 1 to 5 mU in a 25 to 50 μl reaction and incubate 15 to 30 min at 37°C. These conditions are appropriate when the enzyme is present in excess of substrate, as is often the case when radiochemical amounts of substrate are present. The enzyme has a low level of activity against α(2-6) linkages; this becomes evident with prolonged incubations. If large amounts of sialic acids are present (e.g., >1 nmol), it will be necessary to titrate the enzyme. This can be done by setting up digests of standards containing α(2-3) or α(2-6)-linked sialic acids (Table 17.12.3) at the same concentration as the unknown sample and determining the amount of Newcastle disease virus sialidase that releases >90% of the α(2-3)-linked and <5% of the α(2-6)-linked sugar.

5. Terminate the reaction by boiling for 5 min. Check for release of sialic acid by monitoring:

a. Sialic acid by direct measurement *(UNIT 17.16)*

b. Shift in pI of a glycoprotein by isoelectric focusing *(UNIT 10.4)*

c. Shift in TLC pattern for glycolipids *(UNIT 17.3)*

d. Shift in apparent molecular weight on SDS-PAGE *(UNIT 10.2); generally a loss of several sialic acid residues will be visible by small shifts on SDS-PAGE to either lower or higher apparent molecular weights*

e. Alteration in lectin-binding patterns for some lectins *(UNIT 17.7)*

f. Shift in negative charge of isolated oligosaccharides or glycolipids *(UNIT 17.17).*

### Table 17.12.3 Sialic Acid Standards

<table>
<thead>
<tr>
<th>Glycoconjugate</th>
<th>Molecule</th>
<th>Linkages</th>
<th>Sialic acidsa</th>
<th>Sialic acid concentration (nmol/mg)</th>
<th>Stock solution preparationb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sialylactose</td>
<td>Oligosaccharide</td>
<td>α2-3, α2-6</td>
<td>Neu5Ac</td>
<td>1600</td>
<td>31 mg/ml in water (100×)</td>
</tr>
<tr>
<td>Fetusind</td>
<td>Glycoprotein</td>
<td>α2-3, α2-6</td>
<td>Neu5Ac, Neu5Gc(?)</td>
<td>280</td>
<td>20 mg/ml in water (10×)</td>
</tr>
<tr>
<td>Bovine submaxillary</td>
<td>Mucin</td>
<td>α2-6</td>
<td>mono- and di-O-acetylated Neu5Ac and Neu5Gc</td>
<td>150-400</td>
<td>20 mg/ml in water (10×)</td>
</tr>
<tr>
<td>mucin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colominic acid</td>
<td>Polysaccharide</td>
<td>α2-8</td>
<td>Neu5Ac</td>
<td>3400</td>
<td>15 mg/ml in water (100×)</td>
</tr>
<tr>
<td>Mixed brain gangliosides</td>
<td>Glycolipid</td>
<td>α2-3, α2-8</td>
<td>Neu5Acf</td>
<td>950c</td>
<td>5 mg/ml in 2:1:0.1 (v/v/v) chloroform/methanol/water (10×)</td>
</tr>
</tbody>
</table>

aAbbreviations: Neu5Ac, N-acetylneuraminic acid; Neu5Gc, N-glycolyneuraminic acid.
bStore all stock solutions at −20°C. Dilution to 1× will yield ~0.5 mM sialic acid.
cRatio of α(2-3) to α(2-6) ~4:1, although lot-to-lot variation probably occurs.
dRatio of α(2-3) to α(2-6) ~1.6:1.4 in commercial preparations, including Sigma (#F-3004) or GIBCO/BRL (Townsend et al., 1989).
eDepending on the manufacturer.
fCommercial preparations of bovine brain gangliosides have been treated with base during purification, resulting in loss of O-acetyl esters.
**Sialidase Treatment of Intact Cells**

Although the pH optima of bacterial and viral sialidases range from 4.5 to 5.5, these enzymes can be used to treat intact, viable cells at pH 7.0. Cells are washed and resuspended in isotonic serum-free buffer, then treated with the sialidase. The reaction is terminated by centrifugation and washing.

### Additional Materials

- Cells, prepared as a single-cell suspension
- HEPES-buffered saline (HeBS)
- Culture medium appropriate for cells, with serum

1. Wash cell suspension free from serum-containing medium by centrifuging 10 min at 500 × g and resuspending twice in HeBS. After the last wash, resuspend in HeBS at 0.2–1 × 10^7 cells/ml. Divide the cells between two tubes.

   *Use HeBS containing 1 mM CaCl₂, if *Vibrio* cholerae sialidase is to be used.*

   *Cell number can be determined by trypan blue exclusion as described in UNIT 11.5.*

2. To one tube, add sialidase. To the second tube, add no enzyme. Incubate 30 min at 37°C.

   *Select the enzyme to be used based on the properties in Table 17.12.1. Initially use the sialidase in vast excess, e.g., 100 mU/ml for 10^7 cells/ml (presuming 10^7 cells will yield ~1 nmol), then titrate down for the optimum amount.*

   *Alternative controls could include reactions incubated with inactivated sialidase (prepared by boiling 5 min) or active sialidase plus 1 mM 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (Boehringer Mannheim), a sialidase inhibitor.*

3a. To characterize the sialic acid molecules released from the cells: Chill reactions to 4°C and centrifugate 10 min at 2000 × g, 4°C. Collect supernatant, boil 5 min to inactivate sialidase, and proceed with characterization of the released sialic acid (*UNIT 17.16*).

   *It is important to inactivate the sialidase as otherwise it will continue to work on any glycoproteins shed or secreted into the medium during the 30-min incubation.*

3b. To characterize the glycoconjugates on the cell surface: Wash cells three times in culture medium containing serum (to remove free sialidase) before proceeding with biochemical or functional assays.

### REAGENTS AND SOLUTIONS

**HEPES-buffered saline (HeBS)**

- 476 mg HEPES (20 mM final)
- 812 mg NaCl (140 mM final)
- H₂O to 100 ml
- Adjust pH to 7.0 with HCl
- Stable <1 year at 4°C.

**Sialidase digestion buffers**

- *Clostridium perfringens* and *Arthrobacter ureafaciens* sialidases: Dissolve 8.6 g sodium acetate in 100 ml water to obtain a 0.1 M sodium acetate stock solution. Adjust pH to 5.5 with 0.1 M acetic acid. Store <1 year at 4°C.

- *Vibrio cholerae* sialidase: Add 14.7 mg CaCl₂ dihydrate and 580 mg NaCl to 100 ml of 0.1 M sodium acetate, pH 5.5. Store <1 year at 4°C.

- *Newcastle disease virus* sialidase: Add 2 mg of fatty acid–free BSA (Sigma A7511)
per milliliter of 0.1 M sodium acetate, pH 5.5. Any high-purity BSA can be used (contaminating glycosidases may be present in low levels in some BSA preparations). Store indefinitely at −20°C.

Sialidases

Commercial sources for sialidases include Sigma, Calbiochem, Boehringer Mannheim, and Oxford GlycoSystems (see Table 17.12.1 and APPENDIX 4).

Clostridium perfringens sialidase: Supplied as a lyophilized powder. Reconstitute to 10 mU/µl in digestion buffer (see above) and store for several months at 4°C.

Arthrobacter ureafaciens sialidase: Supplied as a lyophilized powder. Reconstitute to 1 to 10 mU/µl per manufacturer’s instructions. Store for ~6 months at 4°C.

Vibrio cholerae sialidase: Supplied in solution. Store per manufacturer’s instructions.

Newcastle disease virus sialidase: Available commercially. Alternatively, prepare as per Paulson et al. (1982). Store stocks in 5- to 10-µl aliquots at −70°C. Thaw only once and store on wet ice at 4°C until use.

COMMENTARY

Background Information

Sialidases from many viral and bacterial strains have been described (Drzeniek, 1973). In general, bacterial enzymes have the broadest spectrum of activity, although the limited specificity of the viral enzymes can be an advantage for certain analyses.

Sialic acid is found in α(2-3) or α(2-6) linkages to neutral sugars (e.g., galactose, N-acetylglactosamine, N-acetylgalactosamine) or in α(2-8) linkage to another sialic acid. The α(2-3)- and α(2-6)-linked sialic acid residues are found in glycoproteins (both N- and O-linked) and in glycolipids. α(2-8)-linked residues are found in glycolipids, colominic acid (from E. coli), and a few mammalian glycoproteins (e.g., neural cell adhesion molecule). As outlined in Table 17.12.2, not all sialidases are active towards all linkages, and these differences can be exploited in structural analyses. Sialidases are only active against α-linked sialic acids. The only natural β-linked sialic acid is in CMP (cytidine monophosphate)-sialic acid.

The common sialic acid N-acetylneuraminic acid (Neu5Ac) can carry modifications (primarily O-acetylation but also glycolylation, sulfation, and methylation; see Varki, 1992, and Table 17.12.2). Sialidases differ in their activities towards these modified sialic acids. Unfortunately, the distribution of sialic acids containing these modifications has not been well-characterized in most mammalian systems.

Critical Parameters

Several caveats must be kept in mind in using sialidases.

Cleavage. The relative catalytic rates of the different enzymes for the various linkage types—i.e., α(2-3), α(2-6), and α(2-8)—are not of much consequence in most instances, because the enzymes are usually used in excess. Important exceptions to this include: (1) The Newcastle disease virus sialidase preferentially cleaves α(2-3) and α(2-8) linkages. However, α(2-6)-linked sialic acid will be hydrolyzed slowly if an excessive amount of enzyme is employed or if incubations are continued >30 min. (2) The Vibrio cholerae enzyme does not cleave the α(2-3)-linked “internal” sialic acid of extended gangliosides (e.g., GM1), although it is active against the oligosaccharide if ceramide is removed. (3) The α(2-8) linkages of colominic acid and b series gangliosides are relatively resistant to release. Combined treatments with sialidases and endosialidases (Troy, 1992) may be used for polysialic acids with more than five sialic acid units. (4) Ganglioside-bound sialic acids may require detergents for complete release. Deoxycholate and cholate work the best.

Effect of substitution. Substitutions have variable effects upon release depending on the enzyme used, as summarized in Table 17.12.2. The decrease in rate with the N-glycolyl modification is not relevant in using any of the enzymes. The decrease in rates for the 9- or 7-mono-O-acetyl substitutions are also not practically relevant, except for the Vibrio enzyme, and particularly with gangliosides. On the other hand, the 4-mono-O-acetyl substitution causes complete resistance to all known sialidases. The effects of di- and tri-O-acetyl...
substitutions have not yet been carefully studied, and it is not safe to extrapolate from data on monoo-0-acetyl molecules. Likewise, combinations of substitutions (e.g., N-glycolyl and O-acetyl) have not been studied carefully. Finally, there is no information on the rarer types of sialic acids (e.g., O-methyl and O-sulfate substituted). If O-acetylation is suspected, prior treatment of the glycoconjugates with base (UNIT 17.15) will cause de-O-acetylation and improve release.

**Controls.** Whenever possible, controls such as the sample without enzyme and the enzyme alone must be studied in parallel to be sure that the sialic acids detected are actually released from the sample. This is particularly important when ultrasensitive methods (such as TBA with HPLC detection or DMB derivatization; UNIT 17.16) are employed. These methods are so sensitive that “environmental contamination” with sialic acids can become a problem.

**Analysis of results.** It is useful to compare the amount of sialic acid released (UNIT 17.16) by sialidase with the amount released by mild acid hydrolysis (UNIT 17.15). If the amount released by the sialidases is significantly less than that released by mild acid, then the sialic acid residues may be resistant due to (1) incorrect choice of sialidase, (2) modification of the sialic acid residues, (3) steric hindrance, or (4) suboptimal conditions for digestion.

It may be important to demonstrate that the observed effect produced by sialidase treatment (e.g., shift in isoelectric point on a focusing gel or a functional response) is in fact due to the sialidase activity. Treatment with heat-inactivated enzyme (prepared by boiling for 5 min) or inhibition with 1 mM 2,3-dehydro-2-deoxy-N-acetyllneuraminic acid, a specific inhibitor of sialidases, can be used. The reaction should be titrated so that just enough sialidase is being used for the observed result, and then the effect of the inhibitor assessed.

**Sialidase inhibitors.** Inhibitors of sialidases do exist. Dextran sulfate inhibits the *Vibrio cholerae* sialidase. Other polyanions (e.g., DNA, RNA, and proteoglycans) may also inhibit this or other sialidases. Free sialic acid also can inhibit the *Vibrio cholerae* enzyme, with a K<sub>i</sub> of ~5 mM.

**Agglutination.** Treating intact cells with Newcastle disease virus can cause agglutination of cells. This effect should not be confused with clumping due to cell death.

**Buffer systems.** The sialidases are compatible with several different buffer systems, with some differences in pH optima noted (Table 17.12.1). *Vibrio cholerae* sialidase works well in sodium acetate and Tris/maleate at pH 5.0 to 6.0 but is considerably more active in Tris/maleate at pH >5.0. *Clostridium perfringens* sialidase has pH optima of 4.5 in sodium acetate but 5.5 in citrate phosphate. *Arthrobacter ureafaciens* sialidase digestion proceeds maximally at pH 5.5, yet retains >50% of activity at pH 4.5 and 7.0. For all sialidases, alternative choices include sodium acetate, ammonium acetate, Tris, HEPES, phosphate, and cacodylate. Acetate interferes with HPLC on AX-5 resin (UNIT 17.12) and β-hexosaminidase digestion (UNIT 17.13).

**Troubleshooting**

Failure to digest a known sia
glycoconjugate to completion could be due to failure to add sufficient sialidase, use of an old or improperly reconstituted and/or stored enzyme aliquot, or inactivation of the enzyme after addition. If the sample is an unknown but potentially contains >1 nmol of releasable sialic acid, a titration comparing increasing amounts of sialidase versus sialic acid released is useful. Sialidases can lose activity rapidly (e.g., <1 hr) after dilution into an assay mix; inclusion of BSA will help prevent this inactivation. Some manufacturers recommend that the *Arthrobacter ureafaciens* and *Clostridium perfringens* sialidases be reconstituted with BSA for stability on storage.

**Anticipated Results**

If the type of sialidase used is tailored to the type of sialic acid and linkage, it should be possible to obtain nearly complete release of these residues.

**Time Considerations**

Once the appropriate buffers and standards are made, the actual digestions will take 30 min to 4 hr or longer (especially for gangliosides). Time required for analysis of the digested product(s) will depend on the specific analytical method employed (as listed in step 6 of the basic protocol).

**Literature Cited**


Key References


Describes biochemical properties of the Newcastle disease virus sialidase.


Provides characterization of sialidases on α(2-3) and α(2-6) linked sialic acids.


Describes the activity of different sialidases on O-acetylated sialic acids.


Describes essential steps necessary to preserve O-acetyl groups during release and characterization of sialic acids.

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Endoglycosidase and Glycoamidase
Release of N-Linked Glycans

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ABSTRACT

Nearly all proteins entering the lumen of the endoplasmic reticulum (ER) become glyco-sylated en route to a cellular organelle, the plasma membrane, or the extracellular space. Many glycans can be attached to proteins, but the most common are the N-linked glycans (oligosaccharides). These chains are added very soon after a protein enters the ER, but they undergo extensive remodeling (processing), especially in the Golgi. Processing changes the sensitivity of the N-glycan to enzymes that cleave entire sugar chains or individual monosaccharides, which also changes the migration of the protein on SDS gels. These changes can be used to indicate when a protein has passed a particular subcellular location. This unit details some of the methods used to track a protein as it trafficks from the ER to the Golgi toward its final location. Curr. Protoc. Mol. Biol. 89:17.13A.1-17.13A.25. © 2010 by John Wiley & Sons, Inc.

Keywords: ER/Golgi • oligosaccharide • glycan • N-glycosylation • glycosidase • intracellular trafficking

INTRODUCTION

Carbohydrate chain modifications are often used to monitor glycoprotein movement through the secretory pathway. This is because stepwise sugar-chain processing is unidirectional and generally corresponds to the forward or anterograde movement of proteins. This unit offers a group of techniques that will help analyze the general structure of carbohydrate chains on a protein and, therefore, glycan-processing mileposts. The minimum requirements are that the protein can be labeled metabolically (UNIT 10.18) and immunoprecipitated (UNIT 10.16) and clearly seen on a gel or blot (UNIT 10.8). The sugar chains themselves are not analyzed, but their presence and structure are inferred from gel mobility differences after one or more enzymatic digestions. This approach is most often used in combination with [35S]Met pulse-chase metabolic labeling protocols, but can be applied to any suitably labeled protein (e.g., biotinylated or 125I-labeled). As the glycans mature, they become either sensitive or resistant to highly specific glycosidases. Some of these enzymes cleave intact glycans from the protein—e.g., endo H, endo F2, endo F3, peptide:N-glycosidase F (PNGase F), endo D, and O-glycosidase. Others strip only terminal sugars (e.g., sialidase) or degrade a selected portion of the chain (e.g., endo-β-galactosidase). The techniques can be adapted to count the number of N-linked glycans on a protein. One unusual protease, O-sialoglycoprotease, degrades only proteins containing tight clusters of O-linked sialylated sugar chains. These techniques work best on average size proteins (<100 kDa) that contain a few percent carbohydrate by weight, where a gel shift of 1 kDa can be seen. A summary of the enzymes and their applications is shown in Table 17.13A.1.

All enzymes except O-sialoglycoprotease (Accurate Chemical and Scientific), endo D (Seikagaku), and endo-β-galactosidase (MP Biomedicals or Seikagaku) are available from Calbiochem or Sigma-Aldrich. Other providers are ProZyme, New England Biolabs, Roche Applied Science, and Takara Biochemical.
This unit provides information on how to measure changes in carbohydrate structure and how these changes relate to protein trafficking. Fortunately, the techniques are independent of mechanistic views, although it should be borne in mind that the organization and distribution of many of these indicator enzymes are cell-type dependent.

The starting material for these protocols is assumed to be \[^{35}\text{S}\]\text{Met}-labeled, immunoprecipitated protein bound to \(\sim 20 \mu l\) of protein A–Sepharose beads (as described in \textit{UNIT 10.16}). The trace amount of protein is eluted by heating in a small volume of 0.1% SDS, diluted in the appropriate buffer, and then digested with one or more enzymes in a small volume. The digest is analyzed on an appropriate SDS-PAGE system that can detect a 1- to 2-kDa size change. A change in the mobility of the protein after digestion is evidence that the carbohydrate chain was sensitive to the enzyme, and therefore that the protein had encountered a certain enzyme in the processing pathway. Alternatively, the analysis can be done by two-dimensional isoelectric focusing (IEF)/SDS-PAGE or two-dimensional nonequilibrium pH gradient electrophoresis (NEPHGE)/SDS-PAGE (\textit{UNIT 10.4}) to see the loss of charged sugar residues or of anionic glycans. The same digestions and SDS-PAGE analysis also apply to proteins that are radiolabeled or biotinylated, or to immunoprecipitates derived from subcellular fractions separated on sucrose or Percoll gradients.

It is important to present the glycosylation pathways, as a detailed description of the pathways is needed to appreciate how they will be used in this unit. A single protein can have more than one kind of glycan (N-linked and O-linked), and each individual N-linked chain can mature into a different final form. The same is true for O-linked chains. Each is described below.
THE N-LINKED PATHWAY

The N-linked glycan maturation pathway is most frequently used for tracking protein movement through the Golgi complex. A common feature of all N-linked chains is the core region pentasaccharide shown in Figure 17.13A.1, which consists of three mannose units and two N-acetylglucosamine units. The mannose units comprise the trimannosyl core, and two of these residues are α-linked to the only β-linked mannose in the molecule. The β-linked mannose is bound to one of the two N-acetylglucosamines. Because they are β1-4 linked to each other, resembling the polysaccharide chitin, this is called a chitobiose disaccharide. Initially, all N-glycosylated proteins begin life when a preformed, lipid-associated glycan is transferred within the lumen of the endoplasmic reticulum (ER) to Asn of proteins having an Asn-X-Thr/Ser sequence. This precursor glycan contains three glucose (Glc), nine mannose (Man), and two N-acetylglucosamine (GlcNAc) sugar residues, and has the structure shown in Figure 17.13A.1. There are several ways to depict this structure. The short-hand symbol method is the most convenient, but be sure to note the linkages of the individual sugars, as they are important. The α and β symbols denote the anomeric configuration of the sugar, and the number indicates which hydroxyl group of the next sugar is involved in the glycosidic linkage. In all cases, the anomeric position is 1, except in sialic acid where it is 2. The symbols chosen for the sugar residues are those used by Essentials of Glycobiology, Second Edition, Cold Spring Harbor Laboratory Press (Varki et al., 2008; http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=glyco2) and the Consortium for Functional Glycomics (http://www.functionalglycomics.org).

The details of the pathway are presented in Figures 17.13A.2 and 17.13A.3, along with the sensitivity to each endoglycosidase or glycoamidase. The figures show the steps between

![Figure 17.13A.1](http://www.currentprotocols.com/protocol/mb1713a)

**Figure 17.13A.1** Symbol structures for the core region and precursor of N-linked sugar chains. Each sugar is given a symbol and abbreviation at the bottom of the figure. Each one except sialic acid uses its anomeric carbon (C-1) for linking to other sugars. Sialic acid uses C-2 for glycosidic linkage to other sugars. Glycosidases and glycosyltransferases are anomeric specific and distinguish α or β configurations of each sugar. The core structure (A) is common to all N-linked chains and is composed of three Man and two GlcNAc residues. The α or β configuration of each sugar is indicated, and the OH group to which that sugar is linked is shown on the bar linking the two symbols. Thus, GlcNAcβ1-4GlcNAcβ is represented by two filled squares with β and 4 between them. When a structure is first presented, it will have full display such as that on the left side; if it is repeated, only the symbols will be used, as shown immediately to the right. The precursor glycan (B) for all N-linked chains is synthesized in the ER and transferred cotranslationally to the peptide containing an available Asn-X-Thr/Ser sequon. For the color version of the figure go to [http://www.currentprotocols.com/protocol/mb1713a](http://www.currentprotocols.com/protocol/mb1713a).
Figure 17.13A.2  N-linked glycan maturation pathway for high-mannose and hybrid types, and sensitivities to various enzymes. Brackets (top) show the structures designated as high-mannose and hybrid chains. The boxes indicate ER or Golgi localization. The pathway begins with the precursor glycan (see Fig. 17.13A.1). Each successive numbered step in circles represents a glycosidase or glycosyl transferase that generates a new sugar chain with different sensitivities to the various endoglycosidases or PNGase F. (1) precursor glycan is trimmed by α-glucosidases I and II, removing three Glc. (2) ER mannosidase removes one Man. (3) α-Mannosidase I in Golgi complex removes two Man to make Man$_6$GlcNAc$_2$, with a single remaining α1-2Man. (4) The final α1-2Man is removed by a Golgi complex α-mannosidase I. (5) GlcNAc transferase I adds GlcNAc to Man$_5$GlcNAc$_2$. (6) α-Mannosidase II or α-mannosidase IIx (MX) removes the α1-3 and α1-6Man units to make GlcNAc$_1$Man$_3$GlcNAc$_2$. Sensitivity to various enzymes (bottom) changes when moving from left to right, but remains the same within vertical columns. NOTE: This continued maturation to form complex chains is shown in Figure 17.13A.3. Additionally, these figures are not comprehensive; many glycosylation steps have not been included, but they do not affect the sensitivities to the enzymes listed. For the color version of the figure go to http://www.currentprotocols.com/protocol/mb1713a.
Figure 17.13A.3  N-linked glycan maturation pathway for complex types, and sensitivities to various enzymes (see Fig. 17.13A.2 for additional details). (8) GlcNAc transferase II adds a second GlcNAc to initiate a biantennary chain. (9) GlcNAc transferase IV adds a third GlcNAc to initiate a triantennary chain. (10) GlcNAc transferase V adds a fourth GlcNAc to initiate a tetraantennary chain. (11) Fucosyltransferase adds $\alpha$1-6Fuc to the core region of complex chains. (12) $\beta$1-4Gal is added to available GlcNAc residues of hybrid and complex chains. (13) $\alpha$2-3 or $\alpha$2-6Sia is added to Gal residues of hybrid and complex chains. For the color version of the figure go to http://www.currentprotocols.com/protocol/mb1713a.

The three Glc residues [filled (blue) circles] are removed from properly folded proteins within the ER by two different glycan-processing $\alpha$-glucosidases. The first $\alpha$1-2Glc is cleaved by $\alpha$-glucosidase I, and the next two $\alpha$1-3Glc residues by $\alpha$-glucosidase II (Fig. 17.13A.2, step 1). An ER-associated $\alpha$-mannosidase removes one Man residue [shaded (green) circle; Fig. 17.13A.2, step 2]. The protein then moves on to the first step in Golgi-localized processing—the removal of the three remaining $\alpha$1-2 Man units by Golgi $\alpha$-mannosidase I to produce Man$_3$GlcNAc$_2$ (Fig. 17.13A.2, steps 3 and 4). Many proteins have only high-mannose-type glycans with five to nine Man residues, and no further processing occurs. Alternatively, one to five GlcNAc residues [filled (blue) squares] can be added to the trimannosyl core, and these are usually extended with high-mannose and hybrid types (Fig. 17.13A.2) and complex types (Fig. 17.13A.3).
galactose [Gal; open (yellow) circles] and sialic acid [Sia; shaded (purple) diamonds] residues. These extensions, called antennae, are the hallmarks of complex-type glycans. The transformation of the precursor sugar chain into various high-mannose or complex types is called glycan processing (Kornfeld and Kornfeld, 1985).

Man₅GlcNAc₂ is an important intermediate because it can have several fates. The first is the well-established addition of one GlcNAc residue by GlcNAc transferase I (Fig. 17.13A.2, step 5). This is the first step toward the formation of complex chains. However, simply adding Gal and Sia to the terminal GlcNAc of this glycan forms a hybrid structure (Fig. 17.13A.2, steps 12 and 13), where the left side of the molecule looks like a complex chain having one antenna, and the right side still resembles a high-mannose chain. The GlcNAc₁Man₅GlcNAc₂ structure is the required substrate for α-mannosidase II, which removes the two terminal Man units from the upper branch of the chain (i.e., the α₁-3Man and α₁-6 Man units; Fig. 17.13A.2, step 6). This enzyme only works after the addition of the first GlcNAc.

Man₅GlcNAc₂ is a substrate for α-mannosidase IIx (Chui et al., 1997), an isozyme of α-mannosidase II. Previously α-Man IIx was thought to have a different specificity than α-Man II (Akama et al., 2006).

GlcNAc transferase II now adds a second GlcNAc to the α₁-6-linked Man (Fig. 17.13A.3, step 8). This molecule can also have several fates. First, fucose (Fuc) can be added to the GlcNAc residue linked to the Asn of the protein (Fig. 17.13A.3, step 11). Second, one to three more GlcNAc residues can be added to the core mannose residues to initiate tri- and tetraantennary chains (Fig. 17.13A.3, steps 9 and 10), and even pentaantennary chains (not shown). GlcNAc additions are considered to occur in the medial Golgi regions. Each GlcNAc-based branch can be individually modified, but they are usually extended by one Gal (Fig. 17.13A.3, step 12) and terminated by a Sia (Fig. 17.13A.3, step 13). Both of these sugars are usually thought to be added in trans-Golgi cisternae or in the trans-Golgi network (TGN). Sometimes selected antennae are also fucosylated in the TGN. One or more terminal Gal residues can be extended by variable-length poly-N-acetyllactosamines (Galβ4-GlcNAc repeats) capped by a Sia. GlcNAc and Gal can be sulfated as a late, perhaps even final, step of processing. These extensions/modifications are thought to occur in the late Golgi complex and TGN, but their order and compartmental segregation are not well understood. Other modifications of N-linked sugar chains are known, but there are fewer tools available to analyze their biosynthetic localization.

THE O-LINKED PATHWAY

For practical purposes, only a portion of the O-linked pathway—i.e., the addition of the first few sugars—will be presented. However, it is very important to remember that some of the same outer chain structures such as Sia, poly-N-acetyllactosamines, and Fuc residues are common to both N- and O-linked glycans.

α-N-Acetylgalactosamine [α-GalNAc; open (yellow) square] is the lead-off sugar for the O-linked pathway (Fig. 17.13A.4; also see Fig. 17.13A.1 for symbols). It is added to Ser/Thr residues that occur in the proper configuration, generating a broad variety of acceptor sequences. These sequences often cluster as repeats within mucin-like domains. GalNAc is added in the earliest parts of the Golgi complex, not cotranslationally. GalNAc can be further extended by at least six different sugars. The most common is the addition of a β₁-3Gal (Fig. 17.13A.4, step 1), forming a disaccharide that is one of the few O-linked chains that can be diagnosed by enzymatic digestions. This disaccharide is often capped by a Sia (Fig. 17.13A.4, step 2). Additional sugars such as Sia (Fig. 17.13A.4, step 3) or GlcNAc followed by Gal (Fig. 17.13A.4, steps 4 and 5) can be added. Structural
Figure 17.13A.4 A small portion of the O-GalNAc pathway. The first step of the O-linked pathway occurs in the early Golgi complex with the addition of α-GalNAc. There are at least six other sugars that can be added at this point in this complex pathway. Often β1-3Gal is added (1), quickly followed by α2-3Sia (2). The presence of these structures can be detected with a combination of O-glycosidase and sialidase. Additional sugars can be added as shown. α2-6Sia (3) or β1-6GlcNAc (4) followed by β1-4Gal (5) and α2-3Sia (6) on Gal. Each of these sugars must be removed before O-glycosidase can cleave the disaccharide. For the color version of the figure go to http://www.currentprotocols.com/protocol/mb1713a.

Analysis can be done by sequential exoglycosidase digestion, but given the complexity and heterogeneity of the sugar chains, such analysis is not a very useful indicator for tracking protein movement through the Golgi complex. Many O-linked chains have terminal Sia residues and, when tightly clustered on Ser/Thr residues, these chains promote proteolysis by O-sialoglycoprotease regardless of the structure of the underlying sugar chain.

Another type of O-linked glycosylation is the addition of glycosaminoglycan (GAG) chains to form proteoglycans. This occurs by a different pathway than the α-GalNAc linkage. Instead, the chains begin by addition of a β-xylose (Xyl) residue to Ser and are then elongated by two Gal residues and a glucuronic acid (GlcA) residue. This core structure can be further elongated by the addition of GlcAβ1-3GalNAcβ disaccharides to form the backbone of chondroitin/dermatan sulfate chains, or by GlcAβ1-3GlcNAcα to form the backbone of heparan sulfate chains. Biosynthesis and movement of these proteins have also been followed through the Golgi complex. Initiation begins in late ER/early Golgi complex, and the core tetrascarharide is probably finished within the medial Golgi, but the addition of chondroitin chains appears to be confined to the TGN. In addition to the well-known O-linked GAG chains, there is clear evidence for the existence of a class of N-linked GAG chains.

**ENDOGLYCOCIDASE H DIGESTION**

Endoglycosidase H (endo H) cleaves N-linked glycans between the two N-acetylglucosamine (GlcNAc) residues (Fig. 17.13A.5) in the core region of the glycan chain (Fig. 17.13A.1) on high-mannose and hybrid, but not complex, glycans. In this protocol, a fully denatured protein is digested with endo H to obtain complete release of sensitive glycans.

**Materials**

- Immunoprecipitated protein of interest (UNIT 10.16)
- 0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
- 0.5 M sodium citrate, pH 5.5
- 1% (w/v) phenylmethylsulfonyl fluoride (PMSF) in isopropanol
PNGase F and endoglycosidase-sensitive bonds in the core of N-linked glycans. PNGase F is a glycoamidase that severs the bond between GlcNAc and Asn, liberating the entire sugar chain and converting Asn into Asp. The endoglycosidases (H, D, and F) cleave the bond between the two GlcNAc residues in the core region, leaving one GlcNAc still bound to the protein. The differential specificity of the endoglycosidases is based on the structure of the sugar chain in a fully denatured protein. Incomplete denaturation may not expose all sensitive linkages. X and Y are unspecified sugar residues. For the color version of the figure go to http://www.currentprotocols.com/protocol/mb1713a.

0.5 U/ml endoglycosidase H (endo H; natural or recombinant)
10 × SDS sample buffer (UNIT 10.2A)
Water baths, 30°C to 37°C and 90°C

Additional reagents and equipment for SDS-PAGE (UNIT 10.2A) and autoradiography (APPENDIX 3A)

1. Add 20 to 30 μl of 0.1 M 2-ME/0.1% SDS to immunoprecipitate in a microcentrifuge tube, mix well, and heat denature 3 to 5 min at 90°C. Use the larger amount of reagent (≥30 μl) for more complete recovery. This treatment may also release some (unlabeled) antibody molecules. Protein solubilization in nonionic detergents such as Triton X-100 or Nonidet P-40 is not always sufficient to completely expose all susceptible cleavage sites. Only strong denaturation with SDS exposes all sites for maximum cleavage.

2. Cool and microcentrifuge for 1 sec at 1000 × g to collect condensed droplets in the bottom of the tube.

3. Place 10-μl aliquots of solubilized, denatured protein (supernatant) in each of two clean microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).

4. Add in the following order, mixing after each addition:

   6 μl 0.5 M sodium citrate, pH 5.5
   20 μl H2O
   2 μl 1% PMSF (in isopropanol)
   1 μl 0.5 U/ml endo H (enzyme digest only; substitute with water in control).

   The PMSF prevents proteolysis. Nonionic detergent is not required to prevent inactivation of endo H as long as high-purity SDS is used. The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.

5. Incubate overnight at 30°C to 37°C.
6. Immediately prior to electrophoresis, inactivate endo H by adding 4 μl of 10× SDS sample buffer and heating 5 min at 90°C.

7. Analyze protein by one-dimensional SDS-PAGE (UNIT 10.2) and autoradiography (APPENDIX 3A).

   The presence of high mannose and/or hybrid N-linked oligosaccharide chains will be evidenced by increased mobility of the digested proteins on SDS-PAGE.

**ENDOGLYCOSIDASE D DIGESTION**

Like endo H, endo D also cleaves between the two GlcNAc residues in the core of the N-linked sugar chains (Fig. 17.13A.5). However, its narrow substrate specificity makes it useful for detecting the transient appearance of just a few early processing intermediates. It requires that the 2 position of the α1-3-linked core Man be unsubstituted. This intermediate sometimes arises after more extensive processing, but prior to addition of the first GlcNAc or action of α-mannosidase II. Cells with a defect in GlcNAc I transferase (e.g., Lec 1 CHO cells) do not add the first GlcNAc residue (Fig. 17.13A.2, step 5), and N-linked glycans will remain sensitive to endo D because they cannot modify the α1-3Man residue.

**Materials**

- Immunoprecipitated protein of interest (UNIT 10.16)
- 0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
- 0.5 M NaH₂PO₄, pH 6.5
- 10% (w/v) Triton X-100 or Nonidet P-40 (NP-40)
- 0.5 U/ml endoglycosidase D (endo D)
- 10× SDS sample buffer (UNIT 10.2A)
- Water baths, 37°C and 90°C

Additional reagents and equipment for SDS-PAGE (UNIT 10.2A) and autoradiography (APPENDIX 3A)

1. Denature immunoprecipitated protein in 20 to 30 μl of 0.1 M 2-ME/0.1% SDS by heating for 3 to 5 min at 90°C.

   Use the larger amount of reagent (≥30 μl) for more complete recovery. This treatment may also release some (unlabeled) antibody molecules.

2. Cool and microcentrifuge at 1000×g for 1 sec to collect condensed droplets in the bottom of the tube.

3. Transfer 10-μl aliquots of supernatant into two microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).

4. Add in the following order, mixing after each addition:

   - 2 μl 10% Triton X-100 or NP-40 (20-fold excess over SDS)
   - 2 μl 0.5 M NaH₂PO₄, pH 6.5
   - 5 μl H₂O
   - 1 μl 1 IU/ml endo D (enzyme digest only; substitute with water in control).

   The 20-fold excess of nonionic detergent is essential to prevent inactivation of endo D by SDS.

   The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.
5. Incubate overnight at 37°C.

6. Immediately prior to electrophoresis, inactivate by adding 2 μl of 10× SDS sample buffer and heating for 5 min at 90°C to 95°C.

7. Analyze protein by one-dimensional SDS-PAGE (UNIT 10.2A) and autoradiography (APPENDIX 3A).

Endo D sensitivity is detected by increased electrophoretic mobility of the digested proteins on SDS-PAGE.

ENDOGLYCOSIDASE F2 DIGESTION

Endo F2, like endo H and endo D, cleaves between the two GlcNAc residues in the chitobiose core (Fig. 17.13A.5). It preferentially releases biantennary complex-type glycans from glycoproteins, but does not cleave tri- or tetraantennary chains.

Materials

- Immunoprecipitated protein of interest (UNIT 10.16)
- 0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
- 0.5 M sodium acetate, pH 4.5 (APPENDIX 2)
- 10% (w/v) Triton X-100 or Nonidet P-40 (NP-40)
- 0.1 M 1,10-phenanthroline in methanol
- 200 mU/ml endoglycosidase F2 (endo F2)
- 4× SDS sample buffer (UNIT 10.2A)
- Water baths, 30°C to 37°C and 90°C

Additional reagents and equipment for SDS-PAGE (UNIT 10.2A) and autoradiography (APPENDIX 3A)

1. Denature immunoprecipitated protein in 20 to 30 μl of 0.1 M 2-ME/0.1% SDS by heating for 3 to 5 min at 90°C.

   Use the larger amount of reagent (≥30 μl) for more complete recovery. This treatment may also release some (unlabeled) antibody molecules.

2. Cool and microcentrifuge at 1000 × g for 1 sec to collect condensed droplets in the bottom of the tube.

3. Transfer 10-μl aliquots of supernatant into two microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).

4. Add in the following order, mixing after each addition:

   - 15 μl 0.5 M sodium acetate, pH 4.5
   - 3 μl 0.1 M 1,10-phenanthroline in methanol
   - 2 μl 10% Triton X-100 or NP-40 (20-fold excess over SDS)
   - 1 μl 200 mU/ml endo F2 (enzyme digest only; substitute 0.5 M sodium acetate in control).

   A 10- to 20-fold excess of nonionic detergent is required to stabilize the enzyme.

   The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.

5. Incubate the mixture overnight at 30°C to 37°C.

   Some inactivation of the enzyme occurs at 37°C, even with nonionic detergent present; however, if the enzyme is present in sufficient excess, incubation can generally be carried out successfully at 37°C.
6. Immediately before electrophoresis, inactivate by adding 8 μl of 4× SDS sample buffer and heating for 5 min at 90°C to 95°C.

7. Analyze the protein by one-dimensional SDS-PAGE (UNIT 10.2A) and autoradiography (APPENDIX 3A).

Sensitivity to endo F₂ is detected by increased electrophoretic mobility on SDS-PAGE.

ENDOGLYCOSIDASE F₃ DIGESTION

Endoglycosidase F₃ (endo F₃) is another endoglycosidase with a narrow substrate range and, therefore, high specificity: it cleaves triantennary chains, but not high-mannose, hybrid, nonfucosylated biantennary or tetraantennary chains. A core-fucosylated biantennary chain is the only other demonstrated substrate. When both endo F₃ and endo F₂ digestions are done in parallel on a sample, it can provide evidence for chain branching and core fucosylation. The approach is essentially the same as for the other endoglycosidases.

Materials

- Immunoprecipitated protein of interest (UNIT 10.16)
- 0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
- 0.5 M sodium acetate, pH 4.5 (APPENDIX 2)
- 10% (w/v) Triton X-100 or NP-40
- 0.1 U/ml endoglycosidase F₃ (endo F₃)
- 10× SDS sample buffer (UNIT 10.2A)
- Water baths, 37° and 90°C

Additional reagents and equipment for SDS-PAGE (UNIT 10.2A) and autoradiography (APPENDIX 3A)

1. Denature immunoprecipitated protein in 20 to 30 μl of 0.1 M 2-ME/0.1% SDS and heat denature 3 to 5 min at 90°C.

   Use the larger amount of reagent (≥30 μl) for more complete recovery. This treatment may also release some (unlabeled) antibody molecules.

2. Cool and microcentrifuge for 1 sec at 1000 × g to collect condensed droplets at the bottom of the tube.

3. Transfer 10-μl aliquots of supernatant into two microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).

4. Add in the following order, mixing after each addition:

   - 2 μl 10% Triton X-100 or NP-40 (20-fold excess over SDS)
   - 4 μl 0.5 M sodium acetate, pH 4.5
   - 5 μl H₂O
   - 1 μl 0.1 U/ml endo F₃ (enzyme digest only; substitute with water in control).

   The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.

5. Incubate overnight at 37°C.

6. Immediately prior to electrophoresis, inactivate by adding 2 μl of 10× SDS sample buffer and heating for 5 min at 90°C to 95°C.
7. Analyze by one-dimensional SDS-PAGE (UNIT 10.2A) and autoradiography (APPENDIX 3A).

Sensitivity to endo F₃ is detected by increased mobility on SDS-PAGE.

**PEPTIDE: N-GLYCOSIDASE F DIGESTION**

PNGase F is a glycoamidase that cleaves the bond between the Asn residue of the protein and the GlcNAc residue that joins the carbohydrate to the protein (Fig. 17.13A.5). Because it liberates nearly all known N-linked glycans from glycoproteins, it is the preferred enzyme for complete removal of N-linked chains. It is the only enzyme that releases tetra- and pentaantennary chains. The glycoprotein sample must be denatured and digested with PNGase F to remove N-linked glycans completely.

**Materials**

Immunoprecipitated protein of interest (UNIT 10.16)

- 0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
- 0.5 M Tris·Cl, pH 8.6 as determined at 37°C (APPENDIX 2)
- 10% (w/v) Triton X-100 or Nonidet P-40 (NP-40)
- 200 to 250 mU/ml peptide:N-glycosidase F (PNGase F)
- 10× SDS sample buffer (UNIT 10.2A)

Water baths, 30°C to 37°C and 90°C

Additional reagents and equipment for SDS-PAGE (UNIT 10.2A) and autoradiography (APPENDIX 3A)

1. Denature immunoprecipitated protein in 20 to 30 μl of 0.1 M 2-ME/0.1% SDS by heating 3 to 5 min at 90°C.

   *Use the larger amount of reagent (≥30 μl) for more complete recovery. This treatment may also release some (unlabeled) antibody molecules.*

2. Cool and microcentrifuge for 1 sec at 1000 × g to collect condensed droplets in the bottom of the tube.

3. Transfer 10-μl aliquots of supernatant into two microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).

4. Add in the following order, mixing after each addition:

   - 3 μl 0.5 M Tris-Cl, pH 8.6
   - 5 μl H₂O
   - 2 μl 10% NP-40 or Triton X-100
   - 5 μl 200 to 250 mU/ml PNGase F (enzyme digest only; substitute with 0.5 M Tris-Cl in control).

   *Sodium phosphate or HEPES buffer, pH 7.0, can be used instead of Tris-Cl. Avoid potassium buffers because these may cause precipitation of a potassium SDS salt. Use of a nonionic detergent is essential, because SDS inactivates PNGase F. A 10-fold weight excess of any of the above nonionic detergents over the amount of SDS will stabilize the enzyme.*

   *The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.*

5. Incubate overnight at 30°C to 37°C.

6. Immediately prior to electrophoresis, inactivate the enzyme by adding 2.5 μl of 10× SDS sample buffer and heating 3 to 5 min at 90°C.
7. Analyze the protein by one-dimensional SDS-PAGE (UNIT 10.2A) and autoradiography (APPENDIX 3A).

The presence of N-linked glycan chains will be evidenced by increased electrophoretic mobility on SDS-PAGE.

ESTIMATING THE NUMBER OF N-LINKED GLYCANS ON A GLYCOPEPTIDE

One widely used application of endo H or PNGase F digestion is estimation of the number of N-linked glycans on a given glycoprotein. This is done by creating a ladder of partially digested molecules that each differ by only one N-linked sugar chain. The number of separate bands in a one-dimensional polyacrylamide gel (less one for the totally deglycosylated protein) provides an estimate of the number of N-linked chains. The conditions used to generate partially deglycosylated protein must be determined for each protein studied, because the sensitivity of each chain may be different, even when all of them are completely exposed by denaturation. For this protocol, either the incubation time or the amount of enzyme can be varied to determine the best conditions to produce a ladder of partial digests. Usually five or six points are enough to provide a reasonable estimate (Fig. 17.13A.6). Of course, it is important to use enough enzyme to obtain complete deglycosylation. This is best done by monitoring the effects of endo H or PNGase F on newly synthesized [35S]Met pulse-labeled protein just after synthesis, but before any N-linked glycans processing has occurred. Pulse labeling of protein for 10 min with [35S]Met followed by digestion is the best way to be sure that all chains are removed.

1. Add 0.1 M 2-ME/0.1% SDS solution to the total volume of immunoprecipitated protein required and heat denature by incubating 3 to 5 min at 90°C.

Each digestion reaction requires 20 μl of immunoprecipitate. Thus, 120 to 140 μl is sufficient for one control plus five or six digests.

Figure 17.13A.6 Data from the estimation of the number of glycosylation sites on lysosome-associated membrane protein 1 (LAMP-1; Viitala et al., 1988). LAMP-1 contains eighteen potential N-linked sites. Graded digestion with increasing amounts of PNGase F was used to generate this ladder of glycoforms. Each band contains at least one less N-linked chain than the band above it. An average N-linked carbohydrate chain has an apparent mass of ~1.5 to 3 kDa. Lysosomal membrane glycoprotein was immunoprecipitated from [35S]Met-labeled cells and the sample was digested with PNGase F for 0 min (lane 1), 5 min (lane 2), 20 min (lane 3), 45 min (lane 4), and 24 hr (lane 5). Figure courtesy of Dr. Minoru Fukuda.
2. Cool and centrifuge for 1 sec at 1000 × g to collect condensed droplets at the bottom of the tube.

3. Aliquot 10 μl supernatant to the number of microcentrifuge tubes required to cover the concentration range (e.g., 0.01 to 1 mU/ml PNGase F) or incubation times (e.g., 5 to 60 min) plus one for an undigested control.

4. Add remaining reagents as specified for endo H (see Basic Protocol 1, step 4) or PNGase F (see Basic Protocol 5, step 4), adjusting the enzyme concentration as desired.

5. Incubate at 30°C for the desired length of time.

   *High enzyme concentration (10 mU/ml) and prolonged incubation (16 hr) must be among the conditions included, in order to ensure that there is a data point for maximum deglycosylation.*

   *For varying enzyme concentrations, incubate for the same amount of time, but the duration of incubation should be shorter than what would give complete digestion because the goal is to obtain increasing extent of incomplete cleavage.*

6. After the desired incubation time, inactivate enzyme by adding 0.1 volume of 10× SDS sample buffer and heating 5 min at 90°C to 95°C.

7. Analyze the sample from each concentration/time point, including undigested sample, by one-dimensional SDS-PAGE (UNIT 10.2A) and autoradiography (APPENDIX 3A).

   *Most newly formed N-linked chains will have a molecular weight in the range of 1500 to 2200, and loss of one chain is sufficient to change the migration of a protein. This procedure has been used to count up to eighteen N-linked sites on one molecule. A sample result is shown in Figure 17.13A.6.*

**SIALIDASE (NEURAMINIDASE) DIGESTION**

Sialic acids are the terminal sugars on many N- and O-linked glycans. The great majority are released with broad-specificity sialidases (neuraminidases) such as that from *Arthrobacter ureafaciens*. Because sialic acids are charged, their loss usually changes the mobility on one-dimensional SDS polyacrylamide gels, but it will always change the mobility on a two-dimensional gel. Since one-dimensional analysis is easier, it can be tried first.

**Materials**

- Immunoprecipitated protein of interest (UNIT 10.16)
- 0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
- 10% (w/v) Triton X-100 or Nonidet P-40 (NP-40)
- 0.5 M sodium acetate, pH 5.0 (APPENDIX 2)
- 1 IU/ml neuraminidase from *Arthrobacter ureafaciens*
- 10× SDS sample buffer (UNIT 10.2A)
- Water baths, 37°C and 90°C

Additional reagents and equipment for SDS-PAGE (UNIT 10.2A), for IEF/SDS-PAGE or NEPHGE/SDS-PAGE (UNIT 10.3), and for autoradiography (APPENDIX 3A)

1. Denature immunoprecipitated protein in 20 to 30 μl of 0.1 M 2-ME/0.1% SDS by heating 3 to 5 min at 90°C.

   *Denaturation is less important here, because the sialic acids are exposed at the ends of the sugar chains. In most instances, the denaturation step can probably be omitted and the digestion done while the protein is still bound to the beads.*
2. Cool and microcentrifuge for 1 sec at 1000 \times g to collect condensed droplets in the bottom of the tube.

3. Transfer 10-μl aliquots of supernatant to two clean microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).

4. Add in the following order, mixing after each addition:
   - 2 μl 10% Triton X-100 or NP-40 (20-fold excess over SDS)
   - 4 μl 0.5 M sodium acetate, pH 5.0
   - 5 μl H₂O
   - 1 μl 1 IU/ml neuraminidase (enzyme digest only; substitute with water for control).

   *This amount of neuraminidase should be in great excess. Addition of nonionic detergent is not needed if the digestion is done while the protein was still bound to the beads.*

   *The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.*

5. Incubate overnight at 37°C.

   *This time can be shortened to 2 hr, if necessary, but longer incubations are better.*

6. Immediately prior to electrophoresis, inactivate the enzyme by adding 2 μl of 10× SDS sample buffer and heating 3 to 5 min at 90°C.

   *If the protein will be analyzed by IEF or NEPHGE, addition of sample buffer is replaced by lysis buffer used for these techniques.*

7. Analyze the protein using either the appropriate one-dimensional SDS-PAGE system (UNIT 10.2A) or a two-dimensional IEF/SDS-PAGE or NEPHGE/SDS-PAGE system (UNITS 10.3 & 10.4), and detect by autoradiography (APPENDIX 3A).

   *Removal of sialic acids usually results in a decrease in apparent molecular weight on one-dimensional gel analysis, or an increase in the isoelectric point of the protein analyzed by two-dimensional gel analysis.*

**ENDO-β-GALACTOSIDASE DIGESTION**

The endo-β-galactosidase from *Escherichia freundii* degrades poly-\(N\)-acetyllactosamine chains (Gal\(\beta\)1-4GlcNAc\(\beta\)1-3)n found on both N- and O-linked glycans. The variable length of these repeating units usually causes the protein to run as a broad band or a smear on the gel. Although not all linkages are equally cleaved by this enzyme (see Fig. 17.13A.7), sensitive proteins that often run as broad bands or smears on gels—e.g., lysosome-associated membrane protein 1 (LAMP-1)—produce both sharper bands and lower molecular weight species after digestion.

**Materials**

- Immunoprecipitated protein of interest (UNIT 10.16)
- 0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
- 0.5 M sodium acetate buffer, pH 5.8 (APPENDIX 2)
- 10% (w/v) Triton X-100 or Nonidet P-40 (NP-40)
- 100 mU/ml endo-β-galactosidase
- 10× SDS sample buffer (UNIT 10.2A)
- Water baths, 37°C and 95°C

Additional reagents and equipment for SDS-PAGE (UNIT 10.2A) and autoradiography (APPENDIX 3A)
**Figure 17.13A.7**  Endo-β-galactosidase-sensitive linkages in poly-N-acetyllactosamines. Linear, unsubstituted poly-N-acetyllactosamine units (GlcNAcβ1-3Galβ1-4) are sensitive to digestion with endo-β-galactosidase, while substitutions—such as sulfate esters (S) or branches starting with GlcNAc (not shown)—completely block digestion. Substitution of neighboring GlcNAc with Fuc or sulfate esters slows the rate, but does not block cleavage. Sensitive sites are shown with bold arrows, slowly hydrolyzed sites with a lighter arrow, and resistant bonds are struck out. Various substitutions are possible, leading to broad bands on gels. This will create variable sensitivities, but even partial sensitivity should give a sharper, more defined band. For the color version of the figure go to http://www.currentprotocols.com/protocol/mb1713a.

1. Denature immunoprecipitated protein in 20 to 30 μl of 0.1 M 2-ME/0.1% SDS by heating 3 to 5 min at 95°C.  
   *Use the larger amount of reagent (≥30 μl) for more complete recovery. This treatment may also release some (unlabeled) antibody molecules.*

2. Cool and microcentrifuge 1 sec at 1000 × g to collect condensed droplets in the bottom of the tube.

3. Transfer 10-μl aliquots of supernatant into two microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).

4. Add in the following order, mixing after each addition:
   - 2 μl 10% Triton X-100 or NP-40 (20-fold excess over SDS)
   - 4 μl 0.5 M sodium acetate, pH 5.8
   - 5 μl H2O
   - 1 μl 100 mU/ml endo-β-galactosidase (enzyme digest only; substitute with water in control).

   *The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.*

5. Incubate overnight at 37°C.

6. Immediately prior to electrophoresis, inactivate by adding 3 μl of 10× SDS sample buffer and heating for 5 min at 90°C.

7. Analyze protein by one-dimensional SDS-PAGE (*UNIT 10.2A*) and autoradiography (*APPENDIX 3A*).

   *If the protein has poly-N-acetyllactosamine chains, its mobility should increase after digestion.*

**ENDO-α-N-ACETYLGLACTOSAMINIDASE DIGESTION**

This enzyme (also known as O-glycosidase or O-glycanase) has limited utility because it is highly specific for cleaving only one O-linked disaccharide, Galβ1-3GalNACα-Ser/Thr. Adding any more sugars, including sialic acid, renders the molecule resistant to cleavage and requires removal of each residue before the enzyme will work. Prior
sialidase digestion is sometimes used (see Basic Protocol 6), and this can be done while the protein is still bound to the immunoprecipitation beads.

**Materials**

- Immunoprecipitated protein of interest (UNIT 10.16)
- 0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
- 0.5 M sodium citrate phosphate buffer, pH 6.0, containing 500 μg/ml BSA (complete buffer supplied with enzyme)
- 10% (w/v) Triton X-100 or Nonidet P-40 (NP-40)
- 300 mU/ml endo-α-N-acetylgalactosaminidase (use according to manufacturer’s directions)
- 10× SDS sample buffer (UNIT 10.2A)
- Water bath, 37° and 95°C

**Additional reagents and equipment for SDS-PAGE (UNIT 10.2A) and autoradiography (APPENDIX 3A)**

1. Denature immunoprecipitated protein in 20 to 30 μl of 0.1 M 2-ME/0.1% SDS by heating 3 to 5 min at 95°C.
   
   *Use the larger amount of reagent (≥30 μl) for more complete recovery. This treatment may also release some (unlabeled) antibody molecules.*

2. Cool and microcentrifuge 1 sec at 1000 × g to collect condensed droplets in the bottom of the tube.

3. Transfer 10-μl aliquots of supernatant into two microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).

4. Add in the following order, mixing after each addition:
   
   - 2 μl 10% Triton X-100 or NP-40 (20-fold excess over SDS)
   - 4 μl 0.5 M sodium citrate phosphate buffer, pH 6.0, with 500 μg/ml BSA
   - 3 μl H₂O
   - 1 μl 300 mU/ml endo-α-N-acetylgalactosidase (enzyme digest only; substitute with water in control).

   *The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.*

5. Incubate overnight at 37°C.

6. Immediately prior to electrophoresis, inactivate by adding 2 μl of 10× SDS sample buffer and heating for 5 min at 90°C.

7. Analyze protein by one-dimensional SDS-PAGE (UNIT 10.2A) and autoradiography (APPENDIX 3A).

   *If the protein contains the disaccharide unit, the mobility of the protein should increase. Presence of only a single unit (mol. wt. ~400 Da) may be difficult to detect unless a high-resolution gel is used.*

**O-Sialoglycoprotease Digestion**

Digestion with O-sialoglycoprotease requires that the substrate have a tight cluster of sialylated O-linked glycans. Proteins with a single O-linked chain or a few widely spaced chains will not be cleaved. This property makes the enzyme a valuable diagnostic tool.
Materials

- Immunoprecipitated protein of interest (UNIT 10.16)
- 0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
- 0.4 M HEPES buffer, pH 7.4
- 10% (w/v) Triton X-100 or Nonidet P-40 (NP-40)
- 2.4 mg/ml O-sialoglycoprotease (O-sialoglycoprotein endoglycoprotease; reconstituted according to manufacturer’s directions)
- 10× SDS sample buffer (UNIT 10.2A)
- Water baths, 37°C and 95°C

Additional reagents and equipment for SDS-PAGE (UNIT 10.2A) and autoradiography (APPENDIX 3A)

1. Denature immunoprecipitated protein in 20 to 30 μl of 0.1 M 2-ME/0.1% SDS by heating 3 to 5 min at 95°C.

   *Use the larger amount of reagent (≥30 μl) for more complete recovery. This treatment may also release some (unlabeled) antibody molecules.*

2. Cool and microcentrifuge 1 sec at 1000 × g to collect condensed droplets in the bottom of the tube.

3. Transfer 10-μl aliquots of supernatant into two microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).

4. Add in the following order, mixing after each addition:

   - 2 μl 10% Triton X-100 or NP-40 (20-fold excess over SDS)
   - 4 μl 0.4 M HEPES buffer, pH 7.4
   - 5 μl H₂O
   - 2 μl 2.4 mg/ml O-sialoglycoprotease (enzyme digest only; substitute with water in control).

   *O-Sialoglycoprotein endopeptidase is a partially purified enzyme, and the specific activity is relatively low. A quantity of 1.0 μg of this enzyme preparation will cleave 5 μg of sensitive substrate per hour at 37°C. Human glycophorin A can serve as a positive control.*

   *The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.*

5. Incubate overnight at 37°C.

6. Immediately prior to electrophoresis, inactivate by adding 2.5 μl of 10× SDS sample buffer and heating for 5 min at 90°C.

7. Analyze protein by one-dimensional SDS-PAGE (UNIT 10.2A) and autoradiography (APPENDIX 3A).

   *If the digestion was successful, the target protein will be undetectable or may be cleaved into small fragments.*

COMMENTARY

Background Information

The results of digestion of a hypothetical protein with two N-linked carbohydrate chains as it moves through the ER and Golgi complex with various enzymes are shown in Figure 17.13A.8. At 0 min, both N-linked chains are high-mannose type. They have lost their Glc residues and one Man residue in the ER. Both are sensitive to endo H and PNGase F digestion, yielding a protein with only two
Figure 17.13A.8 Schematic diagram showing results that could be obtained for a hypothetical protein with two N-linked glycosylation sites as it moves through the Golgi complex. Assume that the protein has been biosynthetically labeled with an amino acid precursor (such as $[^35]$S]Met) for 10 min and chased in the absence of label for 45 min and 120 min. The protein is then precipitated with a specific antibody. At each time point, equal amounts of the sample are analyzed by fluorography after one-dimensional SDS-PAGE, either without any digestion (control; C) or following digestion with endo H (H), endo D (D), endo F$_2$ (F$_2$), PNGase F (PNG), or sialidase (Sia). Glycan structures consistent with the banding patterns are shown below the schematic gel pattern. For the color version of the figure go to http://www.currentprotocols.com/protocol/mb1713a.

remaining GlcNAc residues in the case of endo H digestion, and no carbohydrate at all in the case of PNGase F digestion. These sugar chains are resistant to the other enzyme digestions.

At 45 min, the protein is in the medial Golgi complex and both sugar chains have been processed by Golgi α-mannosidase I. However, one of the chains (left) has been partially processed by Golgi α-mannosidase IIx to an endo H–resistant/endo D–sensitive chain. The other chain (right) has received a GlcNAc residue from GlcNAc transferase I, but has not encountered Golgi α-mannosidase II. This chain is still endo H sensitive and endo D resistant. Both chains are sensitive to PNGase F, but neither is sensitive to endo F$_2$ or sialidase digestion.

At 120 min, both chains have fully matured to complex-type chains. One is a sialylated biantennary chain and the other is a sialylated triantennary chain. Note that each sialic acid is marked ±, indicating that not all molecules are fully sialylated, accounting for the broader bands. Neither sugar chain is sensitive to endo H or endo D digestion. The biantennary chain (left) is sensitive to endo F$_2$ cleavage, leaving one GlcNAc residue on the protein, but the triantennary chain (right) is not sensitive to this enzyme. Both chains are cleaved by PNGase F. All sialic acids are removed by sialidase to produce a sharp band, but the underlying sugar chains remain.

**Endoglycosidase H**

Endo H from *Streptomyces plicatus* cleaves the bond between the two GlcNAc residues in the core of N-linked glycans. One GlcNAc residue remains attached to the protein or peptide and the remainder of the chain is released as an intact unit (Tarentino et al., 1989). The glycan structures cleaved by endo H are shown schematically in Figure 17.13A.2. Substrates for endo H include all high-mannose glycans and certain hybrid types, but not bi-, tri-, tetra- or pentaantennary (complex) chains. Endo H also cleaves glycans that have α1-6 fucose residues bound to the reducing (protein to carbohydrate linkage) GlcNAc residue (Tarentino et al., 1989).

Endo H sensitivity is the most common way to trace the movement of newly synthesized glycoproteins from the endoplasmic reticulum (ER) into the Golgi complex. Proteins remain
sensitive to endo H while they are in the ER and in early regions of the Golgi complex; they become endo H–resistant after they are processed by enzymes located in the medial Golgi complex. Endo H cleaves the N-linked glycans from proteins as long as they have not lost the α1-3Man residue cleaved by Golgi mannosidase II or mannosidase IIx (Fig. 17.13A.2). After removal of that mannose, the glycan becomes endo H resistant. Endo H is the best enzyme for identifying high-mannose and hybrid chains and their general transition toward complex chains.

**Endoglycosidase D**

The narrow substrate specificity of endo D makes it useful for detecting a very restricted set of processing intermediates, particularly for distinguishing α-mannosidase II from α-mannosidase IIx processing. When the results of endo D digestion are combined with the results of an endo H digestion of the same sample, it can help determine which of the two alternate processing pathways is being used. The key requirement for endo D cleavage is an unsubstituted 2 position on the α1-3Man that forms the trimannosyl core (Fig. 17.13A.1). This window of endo D sensitivity occurs only after removal of the α1-2Man residue by α-mannosidase I and before the addition of a GlcNAc residue to this same position by GlcNAc transferase I (Beckers et al., 1987; Davidson and Balch, 1993). The intermediate precursor of both pathways is ManαGlcNAc₂, which is endo D resistant/endo H sensitive. The better-known α-mannosidase II pathway involves the removal of the last α1-2Man residue by α-mannosidase I (making the glycan endo D sensitive/endo H sensitive), and then the addition of GlcNAc via GlcNAc transferase I (making it endo D resistant/endo H sensitive). α-Mannosidase II removes two of the remaining five Man residues, specifically the α1-3Man and α1-6Man that form the upper branch. Once this occurs, this endo D–resistant molecule also becomes endo H resistant. This structure is also a substrate for GlcNAc transferase II on the way to forming a biantennary chain.

Mutant cell line CHO Lec1 lacks GlcNAc transferase I activity and cannot be converted back to an endo D–resistant form. These chains will also remain endo H sensitive, because α-mannosidase II requires GlcNAc transferase I. However, rarely, chains are more extensively processed, and become endo H resistant. Again, the combination of endo D and endo H digestions can reveal which pathway was used.

CHO Lec1 cells are also useful for tracking the movement of a protein from the ER into the earliest Golgi compartment where α-mannosidase I is located. Acquisition of endo D sensitivity requires the action of this enzyme. The advantage of using Lec1 cells is that the proteins remain permanently endo D sensitive and there is no risk of kinetically missing that small window of sensitivity before the sugar chain might become endo D resistant once again.

**Endoglycosidase F and peptide N-glycosidase F**

Elder and Alexander (Elder and Alexander, 1982; Alexander and Elder, 1989) made a landmark discovery when they identified an enzyme in culture filtrates of the bacterium *Flavobacterium meningosepticum* that cleaved N-linked glycans from glycoproteins. This preparation had a broad substrate specificity. The endoglycosidase activity in this preparation (endo F) was actually due to a set of enzymes, each with a more restricted substrate range. Like endo H, the endo F enzymes cleave the sugar chains between the two core GlcNAc residues (Fig. 17.13A.5). Endo F was originally thought to be a single enzyme, but it is now known that each of the three enzymes has a distinct specificity (Plummer and Tarentino, 1991). The specificity of endo F₁ is very similar to that of endo H, while endo F₂ prefers biantennary chains, and endo F₃ will cleave core fucosylated biantennary and triantennary, but not tetraantennary, chains (Fig. 17.13A.2 and Fig. 17.13A.3).

Plummer et al. (1984) carefully analyzed *Flavobacterium* filtrates and found that the very broad substrate range was actually due to a glycoamidase activity rather than an endoglycosidase activity. The glycoamidase releases the entire carbohydrate chain from the protein by cleaving the Asp–GlcNAc bond (Fig. 17.13A.5). The enzyme is called by various names, including peptide:N-glycosidase F (PNGase F), glycopeptidase F, and N-glycanase, but the proper name is peptide N-4(\(\beta\)-glucosaminyl)asparagine.
amidase F. PNGase F has the broadest specificity, and it releases most of the N-linked glycans from proteins.

Endo F₂ and endo F₃

Endo F₂ prefers biantennary chains over high-mannose chains by ~20 fold. Thus, endo H and PNGase F are better choices for broadly distinguishing high-mannose from complex chains as described above in the endo H protocol (Tarentino and Plummer, 1994).

Many proteins have core-fucosylated N-linked glycans, and the addition of fucose can be used as an additional trafficking marker. Endo F₃ will hydrolyze triantennary chains, but endo F₂ will not. Endo F₂ hydrolyzes biantennary chains; however, endo F₃ will also hydrolyze core-fucosylated biantennary chains only a bit more slowly than it does triantennary chains. Thus, if all the chains on a protein are sensitive to endo F₂ (biantennary) and to endo F₃, this is evidence for the presence of a core fucose on those chains.

The enhancement of endo F₃ activity on biantennary chains with a core α1-6Fuc points out that some specificities are really a matter of relative rates of cleavage. If both endo F₂ (biantennary) and endo F₃ (triantennary and biantennary with core fucose) cleave the protein, they may be acting on different chains on the same molecule. If there is only a single chain, repeat the experiment under the same conditions using 10- to 20-fold dilutions of each enzyme. If both still cleave the chain about equally, it is evidence for core fucosylation of a biantennary chain.

PNGase F

PNGase F has the broadest specificity of all the enzymes that cleave N-linked glycans. It is indifferent to all extended structures on the chains, such as sulfate, phosphate, poly-N-acetyllactosamines, polysialic acids, and even the occasional glycosaminoglycan chain. Most of the modifications in the Man₃GlcNAc₂ core region also make no difference in chain cleavage. The only glycan structural feature that confers PNGase F resistance is the presence of an α1-3Fuc on the GlcNAc bound to Asn (Tettert et al., 1991). This modification is commonly found in plants and in some insect glycoproteins, but it is rare in most mammalian cell lines. However, caution is warranted, as there is evidence that some mammalian cells do have the critical α1-3Fuc transferase, and some studies show that a majority of N-linked chains of bovine lung are actually PNGase F resistant! It is not known how common this resistance may be. It is thus important to document N-glycosylation with proteins still in the ER (see Support Protocol) before they might be processed to a PNGase F–resistant form.

Sialidase

Sialidases are also called neuraminidases because the most common form of sialic acid is N-acetyl neuraminic acid. The sialic acids are a family with over forty different members, but fortunately the very great majority of them can be removed from the glycans by the broad-spectrum sialidase from Arthrobacter ureafaciens (AUS). It can even digest polysialic acids, a rare modification found on only a few proteins such as neuronal cell adhesion molecule (NCAM). Sialidases with selected specificities from other sources are available but would not usually be needed. AUS has an optimum pH of 5.0, with ~30% of maximum activity at pH 7.

Because sialic acids are charged, they affect gel mobility of proteins more than would be expected from their nominal molecular weight. The magnitude of the gel shift depends on the number of residues. It is difficult to estimate their number by sialidase digestion, but the mobility change is usually sufficient if there are several sialic acid residues. On the other hand, if a protein has only one sialic acid, its presence could be missed using standard one-dimensional SDS-PAGE. To be certain of the effects of sialidase, the sample can be analyzed by a two-dimensional system, using IEF or NEPHGE in the first dimension. The loss of even a single sialic acid will be evident because it changes the isoelectric point.

AUS will remove sialic acids from both N- and O-linked chains, so the type of chain carrying them must be determined independently using PNGase F or possibly O-glycosidase in combination with sialidase. A protein will generally be partially or completely resistant to O-glycosidase because the required disaccharide, Galβ1-3GalNAc, is usually extended and often sialylated. Until the sialic acid is removed, it will be resistant.

The presence of sialic acid (sialidase sensitivity) is often used as an indication of the transport of a protein into the trans-Golgi network (TGN). This may be true in general, but it is important to remember that the distribution of Golgi enzymes is cell type dependent. For instance, α-mannosidases I and II, which are typically considered cis/medial Golgi enzymes, are strongly expressed on the brush border of enterocytes—hardly a Golgi
compartment. There are other similar examples of various distributions of sialyl transferase. Moreover, there are different sialyl transferases and each may have its own unique distribution. Although one should be cautious, it is probably safe to place sialyl transferase in the late Golgi compartment rather than an early one.

**Endo-α-N-acetylgalactosaminidase**

This enzyme from *Diplococcus pneumoniae* also goes by various names, including O-glycosidase and O-glycanase. This enzyme has a narrow substrate range and cleaves only Galβ1-3GalNAcα-Ser/Thr. These are only the first two sugars added in the diverse O-linked pathway that can produce glycans with a dozen or more sugar units. A portion of the pathway is shown in Figure 17.13A.4. Fortunately, many, but far from all, O-linked chains have the simple trisaccharide structure and would be sensitive to cleavage after removing the Sia. Thus, sequential individual digestions or mixed digestions can be used. As both Gal and GalNAc (and probably Sia) are added in the early Golgi complex, sensitivity to the enzyme shows that the protein carries O-linked chains, but matching enzyme sensitivity and a Golgi compartment to further chain extension is difficult. Combining a battery of exoglycosidases (sialidase, α-fucosidase, α-N-acetylgalactosaminidase, and β-hexosaminidase) with endo-α-N-acetylgalactosaminidase will probably remove most O-linked sugar chains, except sulfated ones. The bottom line is that it is easy to use the enzyme in combination with sialidase to show that a protein has simple O-linked chains, but it is difficult to conclude much more concerning either the structure of the sugar chain or intracellular trafficking.

**Endo-β-galactosidase**

*Escherichia freundii* endo-β-galactosidase is one of several enzymes that specifically degrade poly-N-acetyllactosamines by cleaving linear chains of GlcNAcβ1-3Galβ1-4 repeats at the Galβ1-4 linkage. Any substitution on the galactose itself blocks cleavage; however, modifications of the neighboring sugars can slow hydrolysis (Fig. 17.13A.7). For instance, fucosylation and/or sulfation of nearby GlcNAc slows cleavage, but chain branching or sulfation at Gal block it. Even with these potential complexities, digestion with endo-β-galactosidase will sharpen a broad band even if it does not cleave every linkage. The repeating GlcNAcβ1-3Galβ1-4 units can be found on both N- and O-linked chains, so sensitivity to PNGase F digestion can potentially distinguish the location. Lysosom-associated membrane protein 1 (LAMP-1) has poly-N-acetyllactosamine repeats on N-linked chains. Remember that glycosylation is not template driven, so glycans often exist as a continuum of different structures on individual proteins. For example, heavily sulfated poly-N-acetyllactosamine repeats are also known as keratan sulfate and are degraded by keratanases.

**O-Sialoglycoprotease**

O-Sialoglycoprotease (also called O-glycoprotease or O-sialoglycoprotein endopeptidase; Mellors and Lo, 1995) is a neutral metalloprotease produced by *Pasteurella haemolytica*. This enzyme requires clusters of sialylated glycans on Ser or Thr residues (Nogard et al., 1993). Having a single sialylated O-linked sugar chain on a protein will not lead to degradation, nor will having a nonsialylated sugar chain. Therefore, this enzyme can be used in a typical pulse-chase experiment to indicate whether there are tightly grouped O-linked chains and when they are sialylated. Adding the initial α-GalNAc in the early Golgi complex is insufficient to cause proteolysis; sialylation is specifically required, and this may occur in a later Golgi compartment. Proteolysis can generate smaller fragments of a target protein that are still visible on gels, or the fragments may be so small that they are not even seen on the gels. It depends on the protein. Many leukocyte antigens such as the P-selectin ligand, or others such as CD43, CD44, CD45, and CD34 found on hematopoietic stem cells, are all substrates. As sialic acids are found on both N- and O-linked chains (in clusters and not), sequential digestions using PNGase F, sialidase, and O-sialoglycoprotease endopeptidase in different orders can reveal different kinds of sialylated glycans. If used as part of a pulse-chase protocol, they can reveal different kinetic subcompartments. Not all sialyl transferases are necessarily within the same Golgi compartment.

**Critical Parameters**

For nearly all of the digestions, complete denaturation of the protein can be important, as maximum deglycosylation occurs only when the sugar chains of glycoproteins are completely exposed. This is not important for sialidase digestions since sialic acids are exposed. Usually endo H digestions will work without full denaturation, but the unprocessed
high-mannose chains remain unprocessed because they are often less exposed to the processing enzymes. This may or may not be true of the target protein, but it is better to be safe and denature it completely before digestion. Heating the immunoprecipitate with 0.1 M 2-mercaptoethanol/0.1% SDS is the best way to release the protein in a denatured state. Assuming that the immunoprecipitate contains <10 μg of protein, 30 μl of 0.1% SDS still provides a three-fold excess over the protein. Adding SDS presents another problem: free SDS may denature the digesting enzyme before it has a chance to finish its job. For most enzymes, be sure that the free SDS concentration is <0.01%. The best way to do this is to add a 10- to 20-fold weight excess of nonionic detergent with a low critical micellar concentration (e.g., Triton X-100 or NP-40). These detergents will form mixed micelles with the free SDS and keep it from denaturing the added enzymes.

The amount of enzyme and the incubation time recommended in the protocols are in excess and should be sufficient to cleave any of the sensitive linkages. The incubation times can be shortened, if necessary, but it is better to keep the enzyme concentration as indicated.

Many of the digestions (e.g., sialidase, O-sialoglycoprotease) can be adapted for use on membrane preparations or on live cell surfaces by simply omitting the ionic and nonionic detergents and decreasing the incubation time. The problem is that some linkages may not be exposed and/or sensitive to the digestion. Thus, the usefulness of this approach needs to be determined on a case-by-case basis.

**Enzyme sources and availability**

All enzymes except O-sialoglycoprotease (Accurate Chemical and Scientific), endo D (Calbiochem), and endo-β-galactosidase (MP Biomedicals or Seikagaku) are available from Calbiochem or Sigma-Aldrich. Other providers are ProZyme, New England Biolabs, Roche Applied Science, and Takara Biochemical.

**Endo H**

Endo H has a broad pH optimum between 5.5 and 6.5, and phosphate or citrate/phosphate buffers can be used in place of citrate. Endo H is very stable to proteases, freezing and thawing, and prolonged incubations. No additives are required for storage of the enzyme. At concentrations below 5 to 10 μg/ml (200 to 400 mU/ml), endo H will bind to glass, so it should be stored in plastic vials (e.g., screw-cap microcentrifuge tubes).

**Endo D**

Endo D has a broad pH optimum of 4 to 6.5. One unit of enzyme activity will cleave 1 μmole of a Man5GlcNAc5 glycopeptide per min at 37°C. After reconstitution, the enzyme is stable for 6 months at −20°C. It should be frozen in small aliquots to avoid the need for freezing and thawing.

**Endo F**

Commercial endo F preparations are mixtures of endo F1, F2, and F3. Endo F preparations should not be used for routine deglycosylation or to draw conclusions about the structure of the released glycans unless the specificity is clearly defined.

**Endo F2**

Endo F2 has a broad pH optimum of 4 to 6 and retains >50% of its activity at pH 7. The enzyme is sensitive to SDS, but adding nonionic detergents prevents denaturation of the enzyme by SDS. Although the enzyme is stable at 4°C for months, it can be frozen in aliquots at −70°C as long as repeated freeze/thaw cycles are avoided. The 1,10-phenanthroline can be used to inhibit a trace of a zinc metalloprotease that may be present.

**PNGase F**

The pH optimum for PNGase F is 8.6, but 80% of full activity occurs between 7.5 to 9.5 with a range of buffers including phosphate, ammonium bicarbonate, Tris-Cl, and HEPES. Borate buffers inhibit the enzyme. Commercial PNGase F is available from natural or recombinant sources (two main suppliers, see Enzyme sources and availability, above) and is stable for 6 months at 4°C, or indefinitely at −70°C. However, it should be stored in small aliquots and repeated freeze/thaw cycles should be avoided. PNGase F will bind to glass and plastic surfaces and should not be stored in dilute solutions (<0.1 mU/ml). All of the unit activities of commercial preparations are based on cleavage of dansylated glycopeptides; they are expressed in nmoles/min, which are actually mU, not true International Units (1 International Unit = 1 μmole/min). SDS inactivates PNGase F, but adding a ten-fold weight excess of nonionic detergents protects the enzyme (Tarentino et al., 1989; Tarentino and Plummer, 1994).
Sialidase

Recombinant AUS is available as a liquid (see Enzyme sources and availability). The native enzyme can be purchased as a lyophilized powder and should be reconstituted in water at 1 to 10 mU/μl, according to the manufacturer’s directions. The native enzyme is stable for 1 year at 4°C if unopened. After reconstitution, it should be used within 7 days and stored at 4°C. The recombinant enzyme is stable for 1 year at 4°C. Treatment with sialidase is also used in assays of protein transport to the cell surface.

Endo-α-N-acetylgalactosaminidase (O-glycosidase)

Endo-α-N-acetylgalactosaminidase has a pH optimum of 6.0 and has 50% activity at 5.5 to 7.0. The thiol inhibitor parachloromercuric benzoate (PCMB; 1 mM) inactivates the enzyme, and 1 mM EDTA inhibits it (63%), as do Mn2+ and Zn2+ (50%). Chloride also inhibits the enzyme, so HCl-containing buffers should be avoided. The enzyme will have full access to the sugar chains only after denaturating the protein with SDS, but the excess SDS needs to be removed by forming mixed micelles with nonionic detergents. The enzyme is stable at 4°C and at −20°C, but freeze/thaw cycles should be avoided.

Endo-β-galactosidase

This enzyme is free of contaminating endoand exoglycosidases. It has a pH optimum of 5.8 and can be stored at −20°C, for up to 2 years. Dissolved enzyme is only stable for 1 month at −20°C. EDTA, Ca2+, Mn2+, and Mg2+ do not affect stability or activity, but PCMB inactivates it.

O-Sialoglycoprotease

The partially purified enzyme is supplied by Accurate Chemical and Scientific as a lyophilized powder containing nonsubstrate bovine serum proteins and HEPES buffer. The enzyme should be reconstituted according to the manufacturer’s instructions, divided into aliquots appropriate for a single use, and stored at −20°C. Freeze/thaw cycles inactivate the enzyme, and it is inhibited by EDTA or 1,10-phenanthroline. It is possible to check the activity with a positive control of glycophorin A, which is available through Sigma-Aldrich.

Troubleshooting

Most of the procedures should work as described, but there is a chance that the enzyme is inactive because of a variety of factors such as age, poor storage, or excess SDS. To check activity, it is worthwhile to run a positive control digestion using the same solutions including SDS and nonionic detergents as for the samples. Since the positive controls are simply glycoproteins that are visualized by Coomassie or silver staining, this requires running a separate gel for staining. This should not be required on a routine basis if the enzymes are used and stored as directed. The most likely culprit in failed digestions is using SDS solutions that are too old or too impure.

Anticipated Results

If the digestions are effective, the labeled band will usually show increased mobility on the gel. In rare instances, digestions can actually decrease mobility. The amount of change will depend on the contribution of that component to the overall mass of the protein. As mentioned before, a gel system that allows visualization of a 1-kDa change should be used. Proteins that are >100 kDa may cause problems for fine resolution. Here are a few numbers to keep in mind.

1. The smallest N-linked chain (Man3GlcNAc2) will have a mass of ~0.9 kDa.
2. Two sialic acids on a single N-linked biantennary chain will have a mass of ~0.6 kDa, but their loss may appear larger. If they occur on clustered O-linked chains (sialoglycoprotease sensitive), the apparent size difference will be even larger.
3. Most poly-N-acetyllactosamines are three or more repeats, and therefore their mass would be ~1 kDa. The protein will probably run as a heterogeneous smear or broad band before digestion.
4. A single O-glycosidase-sensitive disaccharide (0.4 kDa) may be below detection limits.

Time Considerations

All digestions can be done overnight for convenience, but the amount of enzymes should be sufficient for complete digestion in less time. The gels are run the next day, but the time needed for the development of autoradiograms will depend on the strength of the signal. A low-abundance protein labeled for 10 to 30 min with [35S]Met may give a weak signal and require long exposures (e.g., 2 weeks). Trafficking of abundant glycoproteins such as viral coat proteins require only short exposure times (e.g., a few hours).
**Literature Cited**


**Key References**

Beckers et al., 1987. See above.

Describes the use of Lec 1 CHO cells and endo D to study processing.

Chui et al., 1997. See above.

Demonstrates the importance of α-mannosidase IIx.

Kornfeld and Kornfeld, 1985. See above.

Landmark review of processing.


Best review of the use of these enzymes.
Polysaccharide lyases are a class of enzymes useful for analysis of glycosaminoglycans (GAGs) and the glycosaminoglycan component of proteoglycans (PGs). These enzymes cleave specific glycosidic linkages present in acidic polysaccharides and result in depolymerization (Linhardt et al., 1986). These enzymes act through an eliminase mechanism resulting in unsaturated oligosaccharide products that have UV absorbance at 232 nm. The lyases are derived from a wide variety of pathogenic and nonpathogenic bacteria and fungi (Linhardt et al., 1986). This class of enzymes includes heparin lyases (heparinases), heparan sulfate lyases (heparanases or heparitinases), chondroitin lyases (chondroitinases), and hyaluronate lyases (hyaluronidases), all of which are described in this unit.

Polysaccharide lyases can be used, alone or in combinations, to confirm the presence of GAGs in a sample as well as to distinguish between different GAGs (see Table 17.13B.1 and Commentary). The protocols given for heparin lyase I are general and, with minor modifications (described for each lyase and summarized in Table 17.13B.2), can be used for any of the polysaccharide lyases.

The basic protocol describes depolymerization of GAGs in samples containing 1 µg to 1 mg of GAGs. The alternate protocol describes depolymerization of GAGs in samples containing <1 µg of radiolabeled GAG. Two support protocols describe assays to confirm and quantitative the activity of heparin and chondroitin ABC lyases. It is recommended that enzyme activity be assayed before the enzyme is used in an experiment to be sure it is active and has been stored properly.

The standard definition of a unit (U), 1 µmol product formed/min, is used throughout this article. Some lyases are sold in nonstandard units (e.g., 0.1 µmol/hr, ∆A232/min), and these should either be converted to standard units or the activity should be determined using the appropriate support protocol.

Table 17.13B.1  Polysaccharide Lyases Used to Identify Glycosaminoglycans

<table>
<thead>
<tr>
<th>Glycosaminoglycan</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin, heparan sulfate, chondroitin sulfate,</td>
<td>Heparin lyases I, II, III, and chondroitin ABC, AC lyases</td>
</tr>
<tr>
<td>dermatan sulfate, hyaluronate</td>
<td></td>
</tr>
<tr>
<td>Heparin, heparan sulfate, chondroitin sulfate,</td>
<td>Heparin lyases I, II, III, and chondroitin ABC lyase</td>
</tr>
<tr>
<td>dermatan sulfate</td>
<td></td>
</tr>
<tr>
<td>Heparin, heparan sulfate</td>
<td>Heparin lyases I, II, III</td>
</tr>
<tr>
<td>Chondroitin sulfate, dermatan sulfate,</td>
<td>Chondroitin ABC AC lyase</td>
</tr>
<tr>
<td>hyaluronate</td>
<td>(Heparin lyase I–heparin lyase III)*</td>
</tr>
<tr>
<td>Chondroitin sulfate, dermatan sulfate,</td>
<td>Chondroitin ABC lyase</td>
</tr>
<tr>
<td>hyaluronate</td>
<td>(Heparin lyase I–heparin lyase III)*</td>
</tr>
<tr>
<td>Heparin</td>
<td></td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td></td>
</tr>
<tr>
<td>Chondroitin sulfate</td>
<td></td>
</tr>
<tr>
<td>Dermatan sulfate</td>
<td></td>
</tr>
<tr>
<td>Hyaluronate</td>
<td></td>
</tr>
</tbody>
</table>

*Sample is divided into two portions and each is treated with a different lyase. The amount of depolymerization (i.e., counts moving from Vo to Vt of a gel-filtration column) is determined for each portion, and the difference gives a measure of the amount of glycosaminoglycan.
OVERVIEW OF HEPARIN LYASES

There are three well-characterized polysaccharide lyases that act endolytically on heparin and heparan sulfate (Jandik et al., 1994); these enzymes are called heparin lyases. Heparin and heparan sulfate GAGs are structurally related, linear sulfated polysaccharides. Heparin’s major sequence (representing 70% to 90% of its structure) is as follows:

\[ \rightarrow 4)\-\alpha\-d\-glucosamine-2,6-sulfate-(1\rightarrow4)\-\alpha\-l\-iduronic acid-2-sulfate-(1\rightarrow \]

Heparan sulfate is composed primarily of equal proportions of the following:

\[ \rightarrow 4)\-\alpha\-d\-glucosamine-2-sulfate-(1\rightarrow4)\-\beta\-d\-glucuronic acid-(1\rightarrow \]

and

\[ \rightarrow 4)\-\alpha\-N\-acetyl-d\-glucosamine-6-sulfate-(1\rightarrow4)\-\beta\-d\-glucuronic acid \]

(or \[ \alpha\-l\-iduronic acid\]-(1\rightarrow)

These disaccharide sequences are found in differing amounts in both heparin and heparan sulfate (Desai et al., 1993a,b). Substrates for heparin lyases are illustrated in Figure 17.13B.1.

The nomenclature of the three heparin lyases is somewhat confusing. However, heparin lyase I and III have enzyme commission (EC) numbers to facilitate their identification. The decision as to which enzyme should be used for a particular application is based on both the specificity desired and the reaction conditions required. The activity of these lyases toward specific glycosidic linkages has been determined using structurally characterized oligosaccharide substrates (Desai et al., 1993a). The primary linkages cleaved by these enzymes and their relative activities toward heparin and heparin sulfate (Desai et al., 1993a, b) are presented in Figure 17.13B.1 and Table 17.13B.3. Information on the optimal conditions for the activity and stability for these enzymes is given under the description of each enzyme and summarized in Table 17.13B.2.

![Figure 17.13B.1](image_url)  
**Figure 17.13B.1** Primary glycosidic linkages cleaved by heparin lyases. Abbreviations: X, H or SO₃⁻; Y, CH₃CO or SO₃⁻. Heparin lyase II cleaves at either glucuronic or iduronic acid residues.
ENZYME
HEPARIN LYASE I (Lohse and Linhardt, 1992)

Heparin lyase I (EC 4.2.2.7), from Flavobacterium heparinum (Cytophagia heparinia), is commonly referred to as heparinase. The enzyme has a molecular weight of 42,800 Da and a pI of 9.1 to 9.2. Heparin lyase I has a random endolytic action pattern—i.e., it randomly acts on any site with the appropriate primary structure within the polymeric substrate (Fig. 17.13B.1; Jandik et al., 1994).

Complete Heparin Lyase–Catalyzed Depolymerization of an Unlabeled Sample

Samples consisting of tissues, biological fluids, PGs, and GAGs (UNIT 17.2) that contain microgram quantities of heparin and are not metabolically labeled can be analyzed using heparin lyase (see Critical Parameters for method to distinguish between heparin and heparan sulfate).

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**Table 17.13B.2** Reaction Conditions for Polysaccharide Lyases with Optimum Buffers and Reaction Temperatures

<table>
<thead>
<tr>
<th>Lyase</th>
<th>Buffera</th>
<th>Optimum temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin I</td>
<td>Sodium phosphate/NaCl, pH 7.1</td>
<td>30°C</td>
</tr>
<tr>
<td>Heparin II</td>
<td>Sodium phosphate, pH 7.1</td>
<td>35°C</td>
</tr>
<tr>
<td>Heparin III</td>
<td>Sodium phosphate, pH 7.6</td>
<td>35°C</td>
</tr>
<tr>
<td>Chondroitin ABC</td>
<td>Tris-Cl/sodium acetate, pH 8</td>
<td>37°C</td>
</tr>
<tr>
<td>Chondroitin AC</td>
<td>Tris-Cl/sodium acetate, pH 8</td>
<td>37°C</td>
</tr>
<tr>
<td>Chondroitin B</td>
<td>Ethylenediamine/acetic acid/NaCl, pH 8</td>
<td>25°C</td>
</tr>
<tr>
<td>Hyaluronate</td>
<td>Sodium acetate/NaCl, pH 5.2</td>
<td>&gt;30°C</td>
</tr>
</tbody>
</table>

a See Reagents and Solutions for buffer recipes.

**Table 17.13B.3** Activity of Heparin Lyases

<table>
<thead>
<tr>
<th>Activity and substrate conversion</th>
<th>Heparin lyase I</th>
<th>Heparin lyase II</th>
<th>Heparin lyase III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparina</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Activityb</td>
<td>100</td>
<td>58</td>
<td>&lt;1</td>
</tr>
<tr>
<td>% Conversionc</td>
<td>58 (76)d</td>
<td>85</td>
<td>6</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Activity</td>
<td>13</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>% Conversion</td>
<td>19</td>
<td>39</td>
<td>94</td>
</tr>
</tbody>
</table>

aPorcine mucosal heparin.

b Percent activity = \( \frac{\text{initial rate on the substrate examined}}{\text{initial rate on substrate giving the highest activity}} \) × (100).

cPercent conversion = \( \frac{\text{moles of linkages cleaved}}{\text{total moles of hexosamine \rightarrow uronic acid linkages}} \) × (100).

dBovine lung heparin.

eBovine kidney heparan sulfate.
Materials

- Heparin- or heparan sulfate–containing sample
- Sodium phosphate/NaCl buffer (see recipe)
- Heparin lyase I solution (see recipe)
- Spectropor dialysis membrane, MWCO 1000 (Spectrum)
- 500-µl polypropylene microcentrifuge tubes
- 30° and 100°C water baths

Additional reagents and equipment for polysaccharide dialysis (APPENDIX 3D), HPLC (UNIT 17.18), and gel-filtration chromatography (UNITS 10.9 & 17.17)

1. Dissolve sample, containing 1 µg to 1 mg heparin, in 50 µl sodium phosphate/NaCl buffer. Dialyze sample against sodium phosphate/NaCl buffer using 1000 MWCO dialysis membrane.

2. Thaw and assay activity of a frozen aliquot of enzyme.

3. Thaw 10 µl heparin lyase I solution at room temperature and add 40 µl sodium phosphate/NaCl buffer to the 500-µl tube containing enzyme. Add 50 µl sodium phosphate/NaCl buffer to another 500-µl tube to serve as a blank control.

   Additional enzyme (10- to 100-fold) may be required to break down small, resistant oligosaccharides (Rice and Linhardt, 1989; Desai et al., 1993a).

   For heparin lyase II and III, the chondroitin lyases, and hyaluronate lyase, see alternative conditions listed in Table 17.13B.2 and in descriptions of individual enzymes below.

4. Add 50 µl sample to each tube and incubate 8 to 12 hr at 30°C.

5. Heat tube 2 min at 100°C to terminate the reaction. Analyze the product by a method appropriate for its purity and concentration.

   A pure sample containing >10 µg of heparin can be analyzed by measuring the difference in absorbance at 232 nm (ΔA232) in 30 mM HCl between enzyme-treated sample and blank (ε = 5500 M⁻¹ cm⁻¹ for oligosaccharide products in 30 mM HCl). Enzyme-treated sample is diluted with measured amounts of 30 mM HCl until the A232 is between 1 and 2. The blank is diluted with the same quantity of 30 mM HCl and its A232 is measured. The difference between the two measured A232 values, ΔA232, is used to calculate the moles of oligosaccharide product formed. Ten moles of oligosaccharide product is obtained for each mole of heparin. If the treated sample contains substantial amounts of protein or other substances that absorb at or near 232 nm, disappearance of polysaccharide substrate can be measured using a dye-binding assay (Grant et al., 1984). Smaller quantities of samples or samples of lower purity can be analyzed by HPLC or gel-filtration chromatography using UV or conductivity detection methods.
Complete Heparin Lyase–Catalyzed Depolymerization of Very Small Amounts of Radiolabeled Glycosaminoglycans

When attempting to use heparin lyase to depolymerize radiolabeled samples that contain very small quantities of heparin, it is often useful to add cold substrate as a carrier so the activity of heparin lyase can be distinguished from that of trace amounts of chondroitin lyases that are often present in heparin lyase preparations. Chondroitin lyase may pose a problem when using a heparin lyase to distinguish between heparin/heparan sulfate and chondroitin/dermatan sulfate in radiolabeled samples (see Critical Parameters and Table 17.13B.3). Therefore, it is recommended that cold carrier chondroitin/dermatan sulfate be added to block the action of minor chondroitin lyase contaminants. Alternatively, chondroitin ABC lyase from *Proteus vulgaris* (an organism free of enzymes acting on heparin and heparan sulfate) can be used to detect chondroitin sulfate chains specifically (see discussion of chondroitin lyases).

**Additional Materials** *(also see Basic Protocol)*

- Radiolabeled heparin-containing sample *(UNIT 17.4)*
- 20 mg/ml chondroitin sulfate A solution (see recipe)
- 20 mg/ml chondroitin sulfate C solution (see recipe)
- 20 mg/ml dermatan sulfate solution (see recipe)

1. Dissolve GAG sample containing radiolabeled heparin in 50 µl sodium phosphate/NaCl buffer. Dialyze sample against sodium phosphate/NaCl buffer using 1000 MWCO dialysis membrane.

2. Thaw 10 µl of heparin lyase I solution at room temperature, immediately prior to use. *For heparin lyase II and III, the chondroitin lyases, and hyaluronate lyase, see alternative conditions listed in Table 17.13B.2 and in descriptions of individual enzymes below.*

3. Add 30 µl sodium phosphate/NaCl buffer to the 500-µl tube containing enzyme.

4. *Optional:* Add 1.7 µl each of 20 mg/ml chondroitin sulfate A, chondroitin sulfate C, and dermatan sulfate substrate solutions (34 µg each) to the enzyme in buffer. *This step should be performed if there is any concern that the enzyme contains chondroitin lyase impurities.*

5. Add 50 µl radiolabeled heparin solution and incubate 8 to 12 hr at 30°C.

6. Heat 2 min at 100°C to terminate the reaction.

7. Analyze depolymerized radioactive sample by HPLC or gel-filtration chromatography using radioisotope detection methods.

*In gel-filtration chromatography following treatment with heparin lyase, counts in fractions corresponding to an apparent molecular weight <1500 Da confirm the presence of heparin/heparan sulfate. Similar results are obtained with heparin lyase II and III except heparin lyase III does not act on heparin (Table 17.13B.3).*
**Assay of Heparin Lyase Activity**

Commercial preparations of heparin lyase should be assayed before use, particularly for applications in which the level of enzyme activity is critical—e.g., when trying to distinguish between heparin and heparan sulfate or measure their relative amounts in a sample containing both.

**Materials**

- Sodium phosphate/NaCl buffer (see recipe)
- Heparin lyase I solution (see recipe)
- 20 mg/ml heparin solution (see recipe)
- UV spectrophotometer, temperature controlled
- 1-ml quartz cuvette with 1-cm pathlength

**Reaction conditions**

A 700-μl reaction should contain:

- 50 mM sodium phosphate, pH 7.1
- 100 mM sodium chloride
- 10 mU heparin lyase I
- 1 mg heparin

**Protocol**

1. Add 640 μl sodium phosphate/NaCl buffer to a 1-ml cuvette. Warm the cuvette to 30°C in a temperature-controlled UV spectrophotometer.

   *If a temperature-controlled spectrophotometer is not available, activity can be measured at room temperature, or sample can be incubated in a water bath and absorbance measured at fixed time points.*

2. Thaw a 10-μl aliquot of heparin lyase I solution at room temperature.

3. Take cuvette out of the spectrophotometer, remove 90 μl warm buffer and transfer it to the tube containing heparin lyase I solution. Immediately transfer the entire 100 μl of buffer and enzyme back into the warm cuvette.

4. Place cuvette in the spectrophotometer and set the $A_{232}$ to zero.

5. Remove cuvette from the spectrophotometer and add 50 μl of 20 mg/ml heparin substrate solution to initiate the reaction. Seal cuvette with Parafilm and invert once or twice to mix. Remove Parafilm and return cuvette to spectrophotometer.

6. Within 30 sec after addition of substrate, begin to measure absorbance continuously or at 30 sec intervals for 2 to 10 min. Plot $A_{232}$ versus time.

   *At room temperature (~20°C) a two-fold decrease in reaction rate is observed; this requires a 4- to 20-min assay time.*

7. Calculate the enzyme activity (1 U = 1 μmol product formed/min) from the initial rate (<5% reaction completion) using $ε = 3800 M^{-1}$ for the reaction products in sodium phosphate/NaCl buffer. Each product formed has an unsaturated uronic acid residue at its nonreducing terminus. Enzyme activity is calculated as

   $$ \text{Enzyme activity} = \frac{\Delta A_{232}/\text{min}}{3800 \text{ M}^{-1}} \times (700 \text{ μl}) $$

   *The slope of the linear portion of the curve is used to calculate the initial rate of reaction.*
HEPARIN LYASE II (Lohse and Linhardt, 1992)

Heparin lyase II (no EC number), an endolytic enzyme from *Flavobacterium heparinum*, has a molecular weight of 84,100 Da and a pI of 8.9 to 9.1. The substrate for heparin lyase II is indicated in Table 17.13B.3 and Figure 17.13B.1.

Protocols for the assay and use of heparin lyase II are identical to those for heparin lyase I, with the following modifications (see Table 17.13B.2):

1. Prepare enzyme and substrate solutions in sodium phosphate buffer, pH 7.1 (see recipes).
2. Carry out the reaction at 35°C.
3. Use 20 mg/ml heparan sulfate solution (see recipe) in Support Protocol 1 to assay for enzyme activity.

HEPARIN LYASE III (Lohse and Linhardt, 1992)

Heparin lyase III (EC 4.2.2.8), an endolytic enzyme from *Flavobacterium heparinum*, is commonly referred to as heparitinase and has a molecular weight of 70,800 Da and a pI of 9.9 to 10.1. Heparin lyase III can be used to confirm the presence of heparan sulfate in a sample (Fig. 17.13B.1 and Tables 17.13B.1 and 17.13B.3).

Protocols for the assay and use of heparin lyase III are identical to those of heparin lyase I, with the following modifications (see Table 17.13B.2):

1. Prepare enzyme and substrate solutions in sodium phosphate buffer, pH 7.6 (see recipes).
2. Carry out the reaction at 35°C.
3. Use 20 mg/ml heparan sulfate solution (see recipe), instead of heparin, as the substrate to assay for enzyme activity in Support Protocol 1.

OVERVIEW OF CHONDROITIN SULFATE LYASES

There are several polysaccharide lyases that act on chondroitin sulfates, dermatan sulfate, and hyaluronate. Chondroitin sulfate galactosaminoglycans are structurally related, sulfated, alternating 1→3, 1→4 linked, linear polysaccharides. The structure of the major disaccharide linkage found in each chondroitin sulfate and the enzyme that acts at each linkage are shown in Figure 17.13B.2. Hyaluronate has the same backbone structure, except that it is not sulfated and contains N-acetylglucosamine in place of N-acetylgalactosamine (Fig. 17.13B.3).

The decision of which chondroitin lyase to use should be based on both the specificity desired and the reaction conditions required (Tables 17.13B.1 & 17.13B.2).

CHONDROITIN ABC LYASE (Yamagata et al., 1968)

Chondroitin ABC lyase (chondroitinase ABC, EC 4.2.2.4), from *Proteus vulgaris*, has a molecular weight of 150,000 Da. This enzyme acts endolytically on chondroitin sulfates A–E (Fig. 17.13B.2; Jandik et al., 1994), slowly on hyaluronic acid, and not at all on heparin, heparan sulfate, or keratan sulfate.
The basic and alternate protocols for chondroitin ABC lyase are identical to those described for heparin lyase I with the following modifications:

1. Prepare enzyme and substrate solutions in Tris Cl/sodium acetate buffer, pH 8 (see recipe).
2. Carry out the reaction at 37°C.
3. When using the alternate protocol for depolymerization of very small amounts of radiolabeled GAGs, omit step 4.

Figure 17.13B.2 Glycosidic linkages cleaved by chondroitin lyases. Abbreviation: Ac, CH₃CO.
A radioactive sample containing chondroitin sulfate or dermatan sulfate can be analyzed, following chondroitin ABC lyase treatment, by HPLC (UNIT 17.18) or gel-filtration chromatography (UNITS 10.9 & 17.17) using radioisotope detection methods. In gel-filtration chromatography following treatment with chondroitin ABC lyase, counts in fractions corresponding to a molecular weight <1000 Da confirm the presence of chondroitin sulfate or dermatan sulfate.

**Assay of Chondroitin ABC Lyase Activity**

Commercial preparations of chondroitin lyase should be assayed before use, particularly for applications in which the level of enzyme activity is critical.

**Materials**

- Tris·Cl/sodium acetate buffer, pH 8.0 (see recipe)
- Chondroitin ABC lyase solution (see recipe)
- 20 mg/ml chondroitin sulfate A (see recipe), chondroitin sulfate C solution (see recipe), or dermatan sulfate solution (see recipe)
- UV spectrophotometer, temperature controlled
- 1-ml quartz cuvette with 1-cm path length

**Reaction conditions**

A 700-μl reaction should contain:
- 50 mM Tris·Cl, pH 8
- 60 mM sodium acetate
- 10 mU chondroitin lyase
- 1 mg chondroitin sulfate A or C, or dermatan sulfate

**Protocol**

1. Add 640 μl Tris·Cl/sodium acetate buffer to a 1-ml quartz cuvette. Warm the cuvette to 37°C in a temperature-controlled spectrophotometer.

   *If a temperature-controlled spectrophotometer is not available, activity can be measured at room temperature or the sample can be incubated in a water bath and the absorbance measured at fixed time points.*

2. Thaw a 10-μl aliquot of chondroitin ABC lyase solution at room temperature.

3. Take cuvette out of the spectrophotometer, remove 90 μl warm buffer, and transfer it to enzyme solution. Immediately transfer entire 100 μl buffer plus enzyme back to the warm cuvette.

4. Place cuvette in spectrophotometer and set the absorbance at 232 nm (A₂32) to zero.
5. Remove cuvette from spectrophotometer and add 50 \( \mu \)l of 20 mg/ml chondroitin A or C or dermatan sulfate solution to initiate reaction. Seal cuvette with Parafilm and invert once or twice to mix. Remove Parafilm and return cuvette to spectrophotometer.

To assay for chondroitin AC lyase activity, use chondroitin A or C as substrate. To assay for chondroitin B lyase activity, use dermatan sulfate as substrate (see Table 17.13B.1 and description of individual enzymes below).

6. Within 30 sec after adding substrate begin to measure the absorbance continuously or at 30-sec intervals for 2 to 10 min. Plot \( A_{232} \) versus time.

7. Calculate the enzyme activity (1 U = 1 \( \mu \)mol product formed/min) from the initial rate (<5% reaction completion) using \( \varepsilon = 3800 \text{ M}^{-1} \) for reaction products at \( \text{pH} \) 8.

To calculate the enzyme activity:

\[
\text{Enzyme activity} = \frac{(\Delta A_{232}/\text{min}) (700 \mu\text{l})}{3800 \text{ M}^{-1}}
\]

Calculate the number of product molecules formed per substrate molecule from the \( A_{232} \) measured at reaction completion.

**ENZYME**

**CHONDROITIN AC LYASE** (Yamagata et al., 1968; Hiyama and Okada, 1975; Michelacci and Dietrich, 1975)

Two chondroitin AC lyases from *Arthrobacter aurescens* and *Flavobacterium heparinum* share the same enzyme commission number (EC 4.2.2.5). Chondroitin AC lyase from *A. aurescens* has a molecular weight of 76,000 Da (Hiyama and Okada, 1975) and a pH of 5.46. It acts exolytically on chondroitin sulfate A and C (Jandik et al., 1994) and exhibits a three-fold higher activity on hyaluronate. It can act at the glucuronic acid residues in dermatan sulfate (Fig. 17.13B.2) but does not act on heparin or heparan sulfate (Fig. 17.13B.1). Chondroitin AC lyase from *F. heparinum* acts endolytically on chondroitin sulfates A and C (Jandik et al., 1994), on hyaluronate, and at the glucuronate residues of dermatan sulfate (Fig. 17.13B.2; Gu et al., 1993). It does not act on heparin, heparan sulfate, or keratan sulfate (Yamagata et al., 1968).

The protocols for chondroitin AC lyase are identical to those described for chondroitin ABC lyase except chondroitin A or C should be used as substrate in Support Protocol 2 for assaying chondroitin lyase activity.

**ENZYME**

**CHONDROITIN B LYASE** (Michelacci and Dietrich, 1975)

Chondroitin B lyase (no EC number), from *Flavobacterium heparinum*, has a molecular weight of 55,000 Da. Chondroitin B lyase acts only on dermatan sulfate and not on the glucuronate residues of chondroitin sulfates A, C, and hyaluronate, and not on heparin or heparan sulfate (Fig. 17.13B.2).

This enzyme can be assayed as described for chondroitin ABC lyase using dermatan sulfate as substrate. The heparin lyase I protocols are used for sample analysis with this enzyme, with the following modifications (see Table 17.13B.2):

1. Prepare enzyme and substrate solutions in ethylenediamine/acetic acid/NaCl buffer, pH 8 (see recipe).
2. Carry out the reaction at 25°C.
HYALURONATE LYASE (Vesterberg, 1968; Rautela and Abramson, 1973; Sasaki et al., 1982)

Hyaluronate lyases (EC 4.2.2.1 and EC 4.2.99.1) act only on hyaluronate (Hiyama and Okada, 1975) allowing hyaluronate (Fig. 17.13B.3) to be distinguished from chondroitin sulfate (Fig. 17.13B.2 and Table 17.13B.1). The enzyme from Streptomyces hyalurolyticus has been purified and has a pI <7 (Sasaki et al., 1982). Homogeneous Staphylococcus aureus hyaluronate lyase has a molecular weight of 84,000 Da (Rautela and Abramson, 1973) and a pI of 7.4 to 7.9 (Vesterberg, 1968).

Reaction conditions

A 700-µl reaction should contain:
- 50 mM sodium acetate, pH 5.2
- 125 mM sodium chloride
- 10 mU hyaluronate lyase
- 1 mg hyaluronate

The basic, alternate, and support protocols for hyaluronate lyase are identical to those described for heparin lyase with the following modifications (see Table 17.13B.2):

Protocol

1. Use sodium acetate/NaCl buffer, pH 5.2 (see recipe), to prepare enzyme (hyaluronate lyase; see recipe) and substrate solutions.

2. Perform the reaction at 30° to 60°C.

Higher temperatures can be useful to reduce substrate viscosity—hyaluronic acid is very viscous. Higher temperatures will also increase the reaction rate, and they can be used to inhibit the activity of other lyases that may be present as contaminants of the hyaluronate lyase preparation.

3. Use hyaluronate solution (see recipe), instead of heparin solution, as substrate to assay enzyme activity (see Support Protocol 1).

4. When using the Alternate Protocol for depolymerization of very small amounts of radiolabeled GAGs, omit step 4.

Following treatment with hyaluronate lyase, a radioactive sample containing hyaluronate can be analyzed by HPLC (UNIT 17.18) or gel-filtration chromatography (UNIT 17.17) using radioisotope detection methods. In gel-filtration following treatment with hyaluronate lyase, counts in fractions corresponding to a molecular weight <500 Da confirm the presence of hyaluronate.

Testicular hyaluronidase is a hydrolase (EC 3.2.1.35) that also acts on hyaluronate (also on chondroitin sulfates A and C). Because it is not a polysaccharide lyase, testicular hyaluronidase cannot be assayed with the support protocol, but it can be used in the basic and alternate protocols with the modifications described above.
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.

**Chondroitin lyase solutions, 1 mU/µl**

Dissolve 0.1 U lyophilized enzyme in 100 µl Tris-Cl/sodium acetate buffer (see recipe) for chondroitin ABC lyase (chondroitinase ABC; Sigma or Seikagaku) and chondroitin AC lyase (chondroitinase AC from *Arthrobacter aurescens* and *Flavobacterium heparinum*; Sigma or Seikagaku) or ethylenediamine/acetic acid/NaCl buffer (see recipe) for chondroitin B lyase (chondroitinase B; Sigma or Seikagaku). Store in 10-mU aliquots in 500-µl polypropylene tubes <1 year at −70°C.

Different suppliers use different definitions for units of enzyme activity. Enzyme activity levels should be assayed and standardized, as described in the support protocols, in the laboratory before the enzymes are used in analytical studies.

**Chondroitin sulfate A or C solution, 20 mg/ml**

Dissolve 20 mg chondroitin sulfate A or C, sodium salt (Sigma or Seikagaku) in 1 ml Tris-Cl/sodium acetate buffer, pH 8 (see recipe) or other buffer appropriate to enzyme/substrate pair (see Table 17.13B.2). Store 1 year at <0°C.

**Dermatan sulfate solution, 20 mg/ml**

Dissolve 20 mg dermatan sulfate, sodium salt (Sigma or Seikagaku) in 1 ml ethylenediamine/acetic acid/NaCl buffer, pH 8 (see recipe) or other buffer appropriate to enzyme/substrate pair (see Table 17.13B.2). Store 1 year at <0°C.

**Ethylenediamine/acetic acid/NaCl buffer**

Dissolve 3.0 g ethylenediamine (50 mM final) and 1.7 g NaCl (30 mM final) in 900 ml H₂O. Adjust pH with glacial acetic acid to pH 8, and bring volume to 1 liter with H₂O. Store <1 month at 4°C.

**Heparan sulfate solution, 20 mg/ml**

Dissolve 20 mg heparan sulfate, sodium salt from bovine kidney (Seikagaku) in 1 ml sodium phosphate buffer, pH 7.6 (see recipe). Store 1 year at 0°C.

**Heparin lyase solutions, 1 mU/µl**

Dissolve 0.1 U lyophilized enzyme in 100 µl sodium phosphate/NaCl buffer (see recipe) for heparin lyase I or sodium phosphate buffer (see recipe) for heparin lyase II and III adjusted to the appropriate pH. Store enzyme in 10 mU aliquots in 500-µl polypropylene tubes <1 year at −70°C.

Heparin lyase I from *Flavobacterium heparinum* is sold as heparinase I (Sigma) and heparinase (Seikagaku). Heparin lyase II is sold as heparinase II (Sigma) and heparitinase II (Seikagaku). Heparin lyase III from *Flavobacterium heparinum* is sold as heparinase III (Sigma) and heparatinase or heparatinase I (Seikagaku).

Different suppliers use different definitions for units of enzyme activity. Enzyme activity levels should be assayed and standardized, as described in the support protocols, in the laboratory before the enzymes are used in analytical studies.

**Heparin solution, 20 mg/ml**

Dissolve 20 mg heparin, sodium salt (140 to 180 USP U/mg), from porcine intestinal mucosa or bovine lung (Sigma) in sodium phosphate/NaCl buffer, pH 7.1 (see recipe). Store 1 year at 0°C.

Different suppliers use different definitions for units of enzyme activity. Enzyme activity levels should be assayed and standardized, as described in the support protocols, in the laboratory before the enzymes are used in analytical studies.
**Hyaluronate lyase solution, 1 mU/µl**
Dissolve 0.1 U lyophilized enzyme (hyaluronidase from *Streptomyces hyalurolyticus*; Sigma or Seikagaku) in 100 µl sodium acetate/NaCl buffer, pH 5.2 (see recipe). Aliquot 10 mU to 500-µl polypropylene tubes and store <1 year at −70°C.

*Different suppliers use different definitions for units of enzyme activity. Enzyme activity levels should be assayed and standardized, as described in the support protocols, in the laboratory before the enzymes are used in analytical studies.*

**Hyaluronate solution, 1.4 mg/ml**
Dissolve 1.4 mg hyaluronate in 1 ml sodium acetate/NaCl buffer, pH 5.2 (see recipe). Store 1 year at <0°C.

*Hyaluronate of high molecular weight is very viscous. It is best to use a hyaluronate of low to medium molecular weight to measure activity.*

**Sodium acetate/NaCl buffer**
Dissolve 4.1 g sodium acetate (30 mM final) and 7.3 g NaCl (125 mM final) in 900 ml H₂O. Adjust pH with glacial acetic acid to pH 5.2 and bring volume to 1 liter with H₂O. Store <1 month at 4°C.

**Sodium phosphate buffer**
Dissolve 7.1 g dibasic sodium phosphate (50 mM final) in 900 ml H₂O. Adjust pH with concentrated phosphoric acid to pH 7.1 for heparin lyase II and pH 7.6 for heparin lyase III. Bring volume to 1 liter with H₂O. Store <1 month at 25°C.

**Sodium phosphate/NaCl buffer**
Dissolve 7.1 g dibasic sodium phosphate (50 mM final) and 5.8 g sodium chloride (100 mM final) in 900 ml H₂O. Adjust pH with phosphoric acid to pH 7.1 and bring volume to 1 liter with H₂O. Store <1 month at 25°C.

**Tris·Cl/sodium acetate buffer**
Dissolve 6.05 g Tris base (50 mM final) and 8.17 g sodium acetate (60 mM final) in 900 ml H₂O. Adjust pH to 8.0 with concentrated hydrochloric acid, and bring volume to 1 liter with H₂O. Store <1 month at 4°C.

**COMMENTARY**

**Background Information**
Proteoglycans (PGs) are primarily found in the extracellular matrix and are important in cell-cell interaction. Glycosaminoglycans (GAGs) are the dominant physical, chemical, and biological features of PGs. Lyases cleave specific glycosidic linkages at C4 of uronic acid residues present in GAGs (Figs. 17.13B.1, 17.13B.2, and 17.13B.3) through an eliminase mechanism, resulting in unsaturated oligosaccharide products that have UV absorbance spectra maxima at 232 nm (Linhardt et al., 1986). An eliminase catalyzes an elimination reaction that results in the formation of a double bond. In contrast, a hydrolase (the most common class of enzymes acting on polysaccharides) breaks down polysaccharides through the addition of water and does not result in the formation of a double bond. Lyases are useful for identifying and distinguishing the GAGs present in unlabeled or radiolabeled samples (see Table 17.13B.1).

**Critical Parameters and Troubleshooting**
Lyases are primarily of microbial origin and often a single organism produces multiple lyases acting on a variety of PGs and GAGs. Thus, it must be recognized that despite the high level of purity of the commercially available enzymes, they may contain small amounts of enzymatic impurities. These impurities can cause misleading results, particularly when large quantities of enzyme are used to treat very small quantities of sample—e.g., when these enzymes are used to analyze radiolabeled samples.

Protease contamination can also be present in the enzyme preparation. Commercial enzymes often contain bovine serum albumin.
**Analysis of Glycosaminoglycans with Polysaccharide Lyases**

17.13B.14

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(BSA) for stabilization during lyophilization, and this greatly reduces potential problems associated with proteolytic contamination.

**Complete degradation of a GAG.** Table 17.13B.1 shows how the polysaccharide lyases can be used, alone or in combination, to determine the content of a GAG. All three heparin lyases used in combination (at equal-unit concentrations in 50 mM sodium phosphate buffer, pH 7, at 30°C) will degrade heparin or heparan sulfate to disaccharides, although a small quantity of lyase-resistant tetrasaccharide may remain. Chondroitin ABC lyase can be used to completely digest a mixture of chondroitin sulfates. If hyaluronate and chondroitin sulfate are both present, it is advisable to use an equal-unit mixture of chondroitin ABC and AC lyases. For complete degradation of GAGs, except keratan sulfate (see **UNIT 17.2**), it is advisable to treat the sample with an equal-unit mixture of heparin lyases I, II, and III and chondroitin ABC and AC lyases in sodium phosphate/NaCl buffer, pH 7, 30°C.

Heparin lyase III can be used to distinguish between samples containing heparan sulfate and those containing heparin. Although heparin lyase I (heparinase) has been used by investigators to demonstrate the presence of heparin in a sample, it also acts at some linkages present in heparan sulfate (Table 17.13B.3). Gel-filtration analysis can help distinguish between heparin and heparan sulfate because the large oligosaccharides formed from heparan sulfate elute in the void volume. In addition, oligosaccharide product compositional analysis (**UNIT 17.19**) is useful in distinguishing heparin from heparan sulfate when using heparin lyase I.

Caution is required when attempting to distinguish heparin/heparan sulfate from chondroitin/dermatan sulfate using heparin lyases. *Flavobacterium heparinum* produces both heparin and chondroitin/dermatan sulfate lyases; thus, minor contaminating activities can result in false positives. Step 4 of the Alternate Protocol for the use of heparin lyase on radio-labeled samples—incubation in the presence of nonspecific substrate—is included to eliminate false positives. The use of chondroitin ABC lyase, which contains no heparin lyase activity, is complicated by heparin’s inhibition of chondroitin ABC lyase (Nakada and Wolfe, 1961). This inhibition is overcome by using excess enzyme (Linhardt et al., 1991).

Chondroitin sulfate is routinely distinguished from dermatan sulfate using chondroitin AC and ABC lyases (Saito et al., 1968). This can give slightly different results from those obtained using chondroitin AC and B lyases (Linhardt et al., 1991). Chondroitin AC lyases from *F. heparinum* and *Arthrobacter aurescens* both act at glucurionate-containing linkages in dermatan sulfates (Gu et al., 1993) and are useful for analysis of the glucuronic acid content of dermatan sulfate (Linhardt et al., 1991).

Hyaluronate content can be determined using hyaluronate lyase, which is specific for hyaluronate. Although hyaluronate lyases are inhibited by chondroitin sulfates, the addition of salt (150 mM NaCl) overcomes this inhibition (Nakada and Wolfe, 1961; Yamagata et al., 1968).

**Contaminants in samples.** The presence of certain metals (particularly divalent metals), proteases, polyanions, detergents (SDS and Triton X-100), and denaturants (urea and guanidine) can interfere with the activity of lyases. Before using these enzymes, detergents should be removed by precipitation with potassium chloride or by using a detergent-removal column such as Biobeads (Bio-Rad). Urea and guanidine should be removed by exhaustive dialysis using controlled-pore dialysis membrane (MWCO 1000).

**Reaction conditions.** Lyases are compatible with a wide range of buffers including succinate, acetate, ethylenediamine acetate, Tris-Cl, bis-Trispropane-HCl, sodium phosphate, MOPS, TES, and HEPES (Lohse and Linhardt, 1992). The presence of calcium may either enhance or reduce lyase activity. Its effect is probably due to changes calcium causes in the GAG substrate conformation and not through direct interaction with the enzyme. Because calcium is incompatible with certain buffers, e.g., phosphate, and can lead to variable results, its use is not recommended. The pH optima for lyases are broad—between pH 5 and 9. Hyaluronate lyase works best at pH <7, and chondroitin and heparin lyases work best at pH ≥7. Lyases can be used at temperatures between 20°C and 40°C. Optimum temperatures are always a compromise between activity and stability considerations. If enzyme instability is a concern due to elevated temperature or prolonged incubation time, heparin lyase II should be used to degrade heparin or heparan sulfate, chondroitin ABC lyase should be used to degrade chondroitin sulfates and dermatan sulfates, and hyaluronate lyase should be used for hyaluronate. Optimal reaction conditions for the polysaccharide lyases are summarized in Table 17.13B.2.

**Removal of lyase and lyase activity.** Following the use of a lyase, residual lyase activity can
be destroyed by heating the reaction mixture to 100°C or by adding denaturants or detergents. Most lyases are cationic proteins and can be removed from anionic oligosaccharide products by passing the reaction mixture through a small cation-exchange column, such as SP-Sephadex (Sigma), adjusted to an acidic pH. The oligosaccharide products (void volume) are then readjusted to neutral pH and analyzed (UNITs 17.17 & 17.19). This method can also be used to remove BSA, an excipient found in many of the commercial enzymes, from the oligosaccharide products.

Enzyme stability during storage. These enzymes can be stored in their lyophilized or reconstituted states at −20°C or −70°C for ≤1 year. Once an enzyme is reconstituted, it should be aliquoted and frozen immediately. Single aliquots can be thawed to assay the enzyme or for use in an experiment. Heparin lyase II is very stable but heparin lyase III is unstable and should be used immediately after removing from frozen storage. The heparin lyases, particularly heparin lyase III, are sensitive to freeze-thawing and lyophilization. Among the chondroitin lyases, chondroitin AC lyase is particularly heparin lyase III, are sensitive to freeze-thawing and lyophilization. Among the chondroitin lyases, chondroitin AC lyase is most susceptible to thermal inactivation (Michelacci and Dietrich, 1975). Lyase storage stability is enhanced by high (>2 mg/ml) protein concentrations. This is often accomplished by addition of BSA.

Anticipated Results
When used correctly, an active lyase should specifically catalyze the breakdown of its GAG substrates. The types of GAGs present in a sample can be easily identified by using multiple lyases. The amount of product formed, and thus the amount of GAG in the sample, can be determined. Accuracy of analysis depends on a number of factors including the complexity of the sample and the types and concentrations of GAGs present. For a sample containing one GAG in high concentration with no contaminating proteins, salts, detergents, etc., GAG concentration can be determined to ±5%. For a complex sample containing many different GAGs at low concentrations in the presence of high levels of contaminants, it may only be possible to estimate GAG concentration to ±100%.

Time Considerations
It takes about half a day to prepare buffers and solutions and to reconstitute, aliquot, and freeze the enzyme. One aliquot of frozen enzyme can be thawed and assayed to ensure that the enzyme is active and has been stored properly. Application of the enzyme to determine the presence or type of GAG in a sample requires ~3 days for sample preparation, overnight treatment with enzyme, and analysis.

Literature Cited


**Key References**

Desai et al., 1993a, b. See above.
*Describes the specificity of the heparin lyases in detail.*

Lohse and Linhardt, 1992. See above.
*Describes physical and catalytic properties of the heparin lyases.*

Michelacci and Dietrich, 1975. See above.
*Describes kinetic properties of chondroitin B lyase and chondroitin AC lyase.*

*Describes general properties and assay conditions for all of the polysaccharide lyases.*

Saito et al., 1968. See above.
*Good example of how different chondroitin sulfates can be distinguished using chondroitin ABC lyase and chondroitin AC lyase.*

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Preparation of Glycopeptides

Generation of glycopeptides from glycoproteins is frequently useful when analyzing a protein’s oligosaccharide side chains. Freed from the bulk of the polypeptide backbone by proteolysis, glycopeptides can be characterized by a variety of techniques. This approach is especially useful for proteins with multiple glycosylation sites, but it is also applicable when concern exists that the protein’s structure and solubility will interfere with particular techniques such as gel-filtration and lectin-affinity chromatography (UNIT 17.1), glycosyltransferase assays (UNIT 17.6), and colorimetric assays (UNIT 17.9).

Extensive proteolysis with pronase or proteinase K results in oligosaccharides with one or a few amino acid residues attached. These glycopeptides are suitable for compositional analyses (UNIT 17.16), exo- and endoglycosidase digestions (UNITS 17.12 & 17.13), and lectin-affinity chromatography (UNIT 17.1). This technique, detailed in the first basic protocol, is often employed as a first step in characterizing oligosaccharides on very large glycoproteins such as proteoglycans and mucins.

Limited proteolysis with a specific endoproteinase (e.g., trypsin, α-chymotrypsin, and V8 protease), as described in the second basic protocol, leaves a larger peptide attached to the oligosaccharide. The resulting glycopeptides are generally suitable substrates for Peptide:N-glycosidase F (PNGase F; UNIT 17.13), an enzyme useful in defining oligosaccharide-peptide linkages. Additionally, they can be separated by C18 reversed-phase chromatography, resulting in a glycopeptide map that is analogous to a peptide map (UNIT 10.12), and used for detection of glycosylation sites (UNIT 17.14B).

Alternative approaches for removing oligosaccharide side chains from the peptide include β-elimination (UNIT 17.15), endoglycosidases (UNIT 17.13), phospholipases (for GPI-anchored proteins; UNIT 17.8), or hydrazinolysis (Takasaki et al., 1982).

EXTENSIVE PROTEOLYTIC DIGESTION OF GLYCOPROTEINS

In this protocol, glycoproteins are applied to a Sephacryl S-200 gel-filtration column to separate them from any lower-molecular-weight material that may be present (e.g., degradation products or other low-molecular-weight contaminants). Denatured glycoproteins in SDS will generally elute in the void volume (V0); the contaminants elute after the V0. The glycoproteins pooled from the V0 are then digested with pronase or proteinase K and rechromatographed on the same column. Glycopeptides are recovered in the material that now elutes after the V0 and are desalted before further use.

If the sample has already been purified by techniques such as immunoprecipitation (UNIT 10.16), dialysis (APPENDIX 3), or gel filtration (UNIT 10.9) which reliably remove low-molecular weight contaminants, it can be digested directly with pronase or proteinase K. After boiling to terminate the protease reaction, the glycopeptides can be desalted. Thus, the use of SDS and passage over Sephacryl S-200 can be completely avoided.

Generation of glycopeptides from a sample metabolically labeled with a radioactive sugar precursor (UNIT 17.4) is described and radioactivity is followed for each gel-filtration run. However, the same approach can be employed with unlabeled glycoproteins, following the column profiles with a carbohydrate-specific colorimetric assay (UNITS 17.9 & 17.16) if sufficient mass is present (see specific units for detection limits). Suitable samples range from total cellular lysates (containing multiple different glycoproteins together with degradation products and precursors) to highly purified protein samples.
Materials

Radiolabeled glycoprotein sample (UNIT 17.4)
20% (w/v) sodium dodecyl sulfate (SDS)
1 M 2-mercaptoethanol (2-ME)
Sephacryl S-200 gel-filtration column (50- to 75-ml bed volume; Sigma or Pharmacia Biotech), equilibrated (UNIT 10.9) in ammonium formate/azide solution containing 0.1% (w/v) SDS (see recipe)
100% and 85% (v/v) acetone (HPLC or ACS grade), ice-cold
0.1 M Tris-Cl, pH 7.5 (APPENDIX 2), without or with 10 mM CaCl₂ (Tris/CaCl₂)
Pronase or proteinase K stock solution
Phenylmethylsulfonylfluoride (PMSF), in 100% ethanol
Sephadex G-15 (Sigma or Pharmacia Biotech) or Bio-Gel P-2 (Bio-Rad) columns (30- to 60-ml bed volume), equilibrated in ammonium formate/azide solution
15- or 50-ml conical polypropylene tubes
Beckman TJ-6 centrifuge or equivalent
Water bath, 50°C

Additional reagents and equipment for preparation and standardization of gel-filtration columns and sample desalting (UNIT 10.9), quantitation of proteins (UNIT 10.1), and acetone precipitation (UNIT 17.10)

Purify the protein sample

1. Place radiolabeled glycoprotein sample in a 15-ml conical polypropylene tube. Add \( \frac{1}{10} \) vol of 20% SDS and \( \frac{1}{50} \) vol of 1 M 2-ME and boil 5 min. Cool to room temperature and centrifuge 10 min at 1000 \( \times \) g (2000 rpm in a Beckman TJ-6), room temperature to remove any precipitate. Save the supernatant.

   The protein sample should contain <1 \( \mu g \) to several mg, depending on the source. The sample volume should be \( \leq 1 \) ml. If the volume of the initial sample is >1 ml, lyophilize and resuspend it in 1 ml water (or less, if solubility permits). Large amounts of protein may require a larger volume, depending on the protein’s solubility. If a larger volume is used, the volume of the Sephacryl S-200 column should be increased so that the sample volume is no more than 5% of the bed volume of the column.

   Boiling ensures that any endogenous proteases or glycosidases are inactivated. For proteins known to be soluble without SDS, the addition of SDS can be eliminated. After boiling, the sample can be freed of low-molecular-weight contaminants, if necessary, by chromatography directly on Sephacryl S-200 (step 2) using a buffer in which the sample is known to be soluble.

2. Apply supernatant to Sephacryl S-200 column (50- to 75-ml bed-volume) equilibrated in ammonium formate/azide solution containing 0.1% (w/v) SDS. Collect 1.0- to 1.5-ml fractions and count 2% to 5% of each eluted fraction in a scintillation counter to determine the elution profile.

   Sephacryl S-200 is utilized because almost all glycoproteins will elute in the \( V_o \) in the presence of SDS. The profile should contain a peak eluting at \( V_o \). Additional peak(s) eluting after the \( V_o \) resulting from degradation products or unincorporated isotope, may or may not be present, depending on the source of the sample and its preparation (see critical parameters).

3. Pool the \( V_o \) into a 15- or 50-ml polypropylene tube and determine the amount of protein present. Lyophilize the sample, resuspend in \( \frac{1}{10} \) the original volume with water, and add 8 vol of ice-cold 100% acetone. Precipitate overnight at −20°C.

   It is only necessary to determine the amount of protein in the sample if >20 \( \mu g \) may be present, so that sufficient protease may be added.
4. Centrifuge 15 min at 3000 × g, 4°C. Wash pellet gently with ice-cold 85% acetone and centrifuge as before. Remove supernatant with a Pasteur pipet and save pellet.

The second wash step is necessary to remove residual SDS which may interfere with the digestion. If desired, transfer the sample to a smaller polypropylene or a 5- or 10-ml screw-cap glass tube prior to the addition of acetone.

**Digest with protease**

5a. *For pronase digestion:* Add 200 µl of Tris/CaCl$_2$ buffer to the pellet. If the total protein is <20 µg, add 20 µl pronase stock solution. Tightly cap the tube and incubate 2 to 4 hr at 50°C.

5b. *For proteinase K digestion:* Add 200 µl of 0.1 M Tris-Cl, pH 7.5, to the pellet. If the total protein is <20 µg, add 20 µl proteinase K stock solution. Tightly cap the tube and incubate 2 to 4 hr at 50°C.

See commentary for a discussion on the choice and uses of the two proteases. If the sample contains >20 µg glycoprotein, add sufficient protease stock to give a protease:sample ratio of 1:20 (w/v). If larger amounts of protein are present, add larger amounts of digestion buffer. Depending on the protein sample, concentrations ≤100 mg/ml are acceptable.

6. Add a second aliquot of protease (equal to that added in steps 5a or 5b) and continue the 50°C digestion for 12 to 16 hr.

The pellet should be completely solubilized during the digestion. If not, continue digestion for 48 or 72 hr. Inclusion of 2-ME to 20 mM (final) may increase solubility.

If a 50°C water bath is unavailable, the digestion may proceed equally well at 37°C. However, because microbial growth may be a problem at 37°C, 0.02% sodium azide should be added to the reaction if the digestion is done at this temperature.

7. Add $\frac{1}{10}$ vol of 20% SDS to completely solubilize any residual aggregated material. Boil 10 min, cool, and centrifuge 10 min at 1000 × g, room temperature.

Any protease not destroyed by boiling may contaminate the Sephacryl S-200 column and act to digest samples applied subsequently, resulting in obvious confusion if nondigested samples are applied. Inactivation of proteinase K can be augmented by phenylmethylsulfonyl fluoride (PMSF). Add $\frac{1}{100}$ vol of 0.1 M solution in 100% ethanol to the sample and incubate 20 to 30 min at room temperature prior to boiling.

8. Apply the supernatant to the Sephacryl S-200 column, collect fractions, and determine the elution profile as in step 2.

**Recover glycopeptides**

9. Compare the elution profiles before and after protease digestion. Material that elutes after the $V_o$ (often a broad peak) represents glycopeptides released from the glyco-proteins that eluted in the $V_o$ of the first column. Pool the glycopeptides and add $\frac{1}{50}$ vol saturated KCl. Incubate 4 to 12 hr at 4°C.

The KCl treatment removes the bulk of the SDS.

10. Remove the insoluble $K^+$-SDS precipitate by centrifuging 10 min at 1000 × g, 4°C. Collect the supernatant, concentrate by lyophilizing, and resuspend in 0.5 to 1.0 ml water.

Some residual $K^+$-SDS precipitate may be apparent after resuspension and can be removed by repeating the centrifugation step as above.

11. Desalt glycopeptides on a Sephadex G-15 or Bio-Gel P-2 column (30- to 60-ml bed volume) equilibrated in ammonium formate/azide solution. Collect fractions that are
~5% of the total bed volume and determine the elution profile as in step 2. Pool the glycopeptides eluting in the $V_o$ and lyophilize. Store at −20°C.

The samples may be stored frozen indefinitely.

**PROTEOLYTIC DIGESTION OF PURIFIED GLYCOPROTEINS WITH ENDOPEPTIDASES**

Glycoproteins labeled metabolically with a radioactive sugar (UNIT 17.4) are first reduced and alkylated to increase solubility and prevent the cross-linking of peptides by disulfide bonds, and then digested with a site-specific endoproteinase. Unlike digestion with pronase or proteinase K, the resulting glycopeptides will generally be attached to a larger peptide of a defined length.

**Materials**

- Radiolabeled protein sample (UNIT 17.4)
- Tris/SDS solution
- Dithiothreitol (DTT)
- Iodoacetamide
- 1N sodium hydroxide (NaOH), prepared fresh every 4 weeks
- 2-mercaptoethanol (2-ME), undiluted
- Sephadex G-50 column (30- to 60-ml bed volume; Sigma or Pharmacia Biotech) column, equilibrated in ammonium formate/azide solution containing 0.1% (w/v) SDS (UNIT 10.9)
- 100% and 85% (v/v) acetone, ice-cold
- Nitrogen (N₂) stream
- Protease digestion buffer
- Protease stock solution
- 5- to 15-ml Pyrex or polypropylene tubes
- Aluminum foil
- pH paper, range pH 7 to 12
- 10-ml round-bottomed polypropylene tubes
- Beckman TJ-6 centrifuge or equivalent
- 1.5-ml polypropylene microcentrifuge tubes
- Additional reagents and equipment for preparation and standardization of gel-filtration columns and sample desalting (UNIT 10.9) and acetone precipitation (UNIT 17.10)

1. Place the purified radiolabeled protein sample in a 5- to 15-ml Pyrex or polypropylene tube and dissolve in 700 μl Tris/SDS solution.

Recovery of radiolabeled glycopeptides proteins can be aided by the addition of a carrier protein (e.g., 50 μg of ovalbumin, bovine immunoglobulin, or fetuin), which should be added before the reduction step.

In principle, any denaturing and strongly buffered solution similar to the reducing buffer can be employed. For instance, if the protein is in a PBS/NP-40 buffer, 1/10 vol of 3 M Tris Cl, pH 8.6/20% SDS stock solution could be added to approximate the reduction buffer. Alternatively, the protein sample can be exchanged into reducing buffer by gel-filtration chromatography (UNIT 10.9), dialysis (APPENDIX 3), or acetone precipitation (UNIT 17.10).

2. Add 11.9 mg DTT and boil 3 min. Incubate 1 hr at 37°C.

3. Remove the sample from the water bath and wrap tube in aluminum foil to protect from light. Add 32.3 mg of iodoacetamide. Immediately check the pH with pH paper.
and adjust to pH 8 to 9 by adding 50-µl aliquots of 1 N NaOH (5 to 10 aliquots may be needed). Continue the incubation for 1 hr at room temperature, shielded from light.

*Alkylation of the free sulfhydryl groups prevents the reformation of disulfide bonds. Improper pH during this procedure will inhibit the alkylation. Exposure to light can produce unwanted side reactions, but dim light is acceptable to use.*

4. Terminate the reaction with 50 µl of undiluted 2-ME and apply to a Sephadex G-50 column (30- to 60-ml bed volume) equilibrated in ammonium formate/azide solution containing 0.1% (w/v) SDS. Collect 1.0- to 1.5-ml fractions and count 2% to 5% of each eluted fraction in a scintillation counter to locate the glycoprotein eluting in the Vw. Pool the appropriate fractions into a 10-ml round-bottomed polypropylene tube and lyophilize.

5. Redissolve the reduced and alkylated protein in 0.3 to 0.4 ml water. Add 8 vol of ice-cold 100% acetone and precipitate overnight at −20°C.

6. Centrifuge 15 min at 3000 × g, 4°C, and discard the acetone. Rinse the walls of the tube and the pellet with 1 ml of 85% ice-cold acetone, and centrifuge as before. Use a Pasteur pipet to carefully aspirate most of the acetone, leaving all precipitated material behind, and dry with a gentle stream of N2.

*The pellet must be dry because any remaining acetone will interfere with the endopeptidase reaction.*

7. Add 200 µl of protease digestion buffer and a small stirbar. Add 10 µl protease stock, rotate the tube gently to coat the liquid over the walls where the sample has been in contact, and stir gently at room temperature. Add another 10 µl protease after 8 to 12 hr and digest for a total of 24 hr.

*See commentary for choice of protease. For radiolabeled proteins, generally present in microgram quantities or less, the amount of protease (2 mg) used here should represent a considerable excess over the sample. If carrier protein has been added, or the mass of the sample is known, sufficient protease should be added to ensure at least a 1:1 protease:sample (w/w) ratio. For nonradiolabeled proteins, protease:sample ratios of 1:10 to 1:30 are employed.*

8. Terminate the reaction by boiling 5 min. Use a large magnetic stirbar on the outside of the tube to pull the small stirbar halfway up the side. Rinse the small stirbar with 0.2 to 0.4 ml water, then remove it. Centrifuge the sample 10 min at 2000 × g, room temperature, and transfer the supernatant to a 1.5-ml microcentrifuge tube. Wash the large tube and pellet once with water, centrifuge as before, and combine supernatants.

9. Resuspend the pellet in 0.5 ml digestion buffer and count an aliquot (~5%) in a scintillation counter. Likewise, count an aliquot (~5%) of the supernatant. Calculate the percent release of radioactivity as soluble peptides by:

\[
\text{percent release} = \frac{100 \times \text{cpm in supernatant}}{\text{cpm in supernatant + cpm in pellet}}
\]

If a significant amount of material remains insoluble, redigest with more of the same or another protease (e.g., α-chymotrypsin or V8 protease). Store samples frozen.

*Soluble material will represent both digested sample (i.e., peptides and glycopeptides) and possibly undigested sample (e.g., if no appropriate sites of proteolysis are present or accessible). Likewise, insoluble material may represent undigested sample (insoluble because of denaturation and acetone precipitation) and perhaps glycopeptides and peptides as well. Some peptides and glycopeptides are insoluble in aqueous solutions at neutral pH and are only soluble in dilute acid (e.g., 25 mM sodium phosphate, pH 2.5), alkali (e.g., ammonium hydroxide), organic solvents (e.g., acetonitrile), or denaturants (e.g., SDS or guanidine). The choice of buffer used to resuspend the digested material in this step may...*
be altered depending on the next analytical step planned. For example, if the sample is to be analyzed by SDS-PAGE (UNIT 10.2), the digested material can be resuspended directly in sample buffer containing SDS. If reverse-phase liquid chromatography is planned, the sample can be resuspended in TFA or TFA containing 6M guanidine buffer. Thus, the demonstration that the endopeptidase added in step 7 has cleaved the sample depends not on the solubility of the sample but rather on direct biochemical evidence of proteolysis, such as a change in size as detected by SDS-PAGE, gel-filtration chromatography, or reverse-phase peptide mapping. The calculation of percent release of soluble radioactivity is essential to determine if insolubility and thus sample loss at this point is a problem.

**REAGENTS AND SOLUTIONS**

**Ammonium formate/azide solution**
1.58 g ammonium formate (25 mM)
0.2 g sodium azide (NaN₃; 0.2% w/v)
H₂O to 1 liter
Store indefinitely at room temperature

Prepare the Sephacryl S-200 and Sephadex G-50 column equilibration buffers by adding 1.0 g sodium dodecyl sulfate (SDS; 0.1% w/v) to this solution.

**Protease digestion buffers**

**Trypsin or α-chymotrypsin digestion buffers**
0.79 g ammonium bicarbonate
H₂O to 100 ml
Titrate with NH₄OH to pH 8.0. Filter through a 0.45-µm membrane and store at −20°C.

**V8 protease digestion buffer**
74.4 mg Na₂EDTA (2 mM)
0.39 g ammonium bicarbonate (50 mM) or 0.71 g sodium phosphate (50 mM)
H₂O to 100 ml
Titrate with NH₄OH to pH 7.8
Store at −20°C

In the presence of ammonium acetate or bicarbonate, V8 protease cleaves on the carboxyl side of Asp residues; in the presence of phosphate, it cleaves after both Asp and Glu residues (Drapeau, 1977).

**Protease stock solutions**

For **α-chymotrypsin**: Dissolve 10 mg in 100 µl of 1 mM HCl. Store unused portion at 4°C for the second addition (step 8, alternate protocol). Do not use >1 day old.

For **pronase**: Dissolve 10 mg/ml pronase in Tris/CaCl₂ and incubate 2 hr at 50°C to destroy contaminating glycosidases. Store at 4°C; use within 48 hr.

For **proteinase K**: Resuspend 2 mg/ml proteinase K in 0.1 ml of 0.1 M Tris-Cl, pH 7.5, containing 2 mM calcium chloride (22 mg anhydrous CaCl₂ in 100 ml buffer). Store stock solution ≤2 weeks at 4°C or ≤1 month at −20°C without loss of activity.

**Calcium reduces autolysis of the protease. Proteinase K is also available in solution.**

For **trypsin**: Dissolve 10 mg TPCK-treated trypsin (Worthington) or DPC-trypsin (Sigma) in 100 µl of 1 mM HCl. Store unused portion at 4°C for the second addition (step 8, alternate protocol). Do not use TPCK-treated trypsin if the second digestion is planned to be done with α-chymotrypsin. Do not use >1 day old.

For **V8 protease (endoproteinase Glu-C or staphylococcal V8 protease)**: Make a 10 mg/ml stock in V8 protease digestion buffer. Store at 4°C. Do not use >1 day old.
**Saturated KCl**

45 g KCl

100 ml H2O

Mix to saturation by stirring overnight, or by boiling for 5 min and allowing solution to cool to room temperature. Store indefinitely at room temperature.

**Tris/CaCl₂ solution**

0.11 g anhydrous calcium chloride (CaCl₂; 10 mM)

100 ml 0.1 M Tris-Cl, pH 7.5

Store indefinitely at room temperature

**Tris/SDS solution**

To 90 ml H₂O add:

6.05 g Tris-Cl (0.5 M)

2.0 g sodium dodecyl sulfate (SDS; 2% w/v)

Titrate with HCl to pH 8.6

Add H₂O to 100 ml

Store indefinitely at room temperature

**COMMENTARY**

**Background Information**

Pronase is widely utilized to generate glycopeptides with minimal peptide content, irrespective of the nature of the oligosaccharide-peptide linkage (Spiro, 1966). This is of obvious advantage when the nature of the glycosidic linkage is not known. Thus, the total oligosaccharide population from a sample can be released and characterized either functionally or structurally by serial lectin-affinity chromatography (UNIT 17.18) or endoglycosidase digestion (UNIT 17.13). Gel-filtration chromatography is limited by potential heterogeneity of the peptides remaining attached to the oligosaccharide. For the same reason, ion-exchange chromatography can not be used to determine the number of charged groups, such as sialic acid or sulfate, although similarly prepared samples can be compared with one another.

Pronase is a mixture of several proteases of differing specificities that are capable (in principle) of removing all amino acid residues except the linkage residue from a glycopeptide. However, in some applications, more than one amino acid residue is left attached. Although there are few published applications of proteinase K in the generation of glycopeptides, it offers several advantages over pronase. These include equally broad activity, wider pH range (pH 4 to 12), greater tolerance of SDS and urea, lack of divalent cation requirement, and inactivation by PMSF (Ebeling et al., 1974). Also, contaminating glycosidases and phosphatases do not appear to be a problem, eliminating the need for the predigestion step required for pronase. Like pronase, proteinase K will digest a protein into small fragments, showing a preference for cutting after hydrophobic amino acid residues. The generation of oligosaccharides linked to single amino acids probably does not occur with great efficiency because proteinase K is not active against dipeptide substrates with unblocked amino groups.

Studies specifically directed at proteoglycan analyses often start with pronase digestion of [3H]glucosamine and 35SO₄²⁻-labeled material. A useful overview of this approach, including steps on sample preparation, can be found in Britz and Hart (1983).

Glycopeptides prepared with pronase or proteinase K are not reliable substrates for PNGase F (UNIT 17.13), which requires a peptide linkage on both sides of the glycosylated Asn residue for activity. Glycopeptides derived from a site-specific endopeptidase are generally suitable substrates for PNGase F. Occasionally glycopeptides resistant to PNGase F may be encountered, if the endoproteinase happens to cleave adjacent to the glycosylated Asn residue. Digestion with a different endoproteinase should circumvent this problem.

Endopeptidases (Judd, 1990; Swiedler et al., 1983) that require a slightly alkaline pH are suitable for work with glycoproteins. Digestion buffers requiring an acidic pH and chemical cleavage techniques may degrade some oligosaccharide structures. Trypsin, α-chymotrypsin, and V8 protease are active between pH 7.5 to 8.5 and in 0.1% SDS, and represent a reasonable first choice. O-acetyl groups on...
sialic acid, however, are stable under acidic but not alkaline conditions (UNIT 17.12).

**Critical Parameters and Troubleshooting**

The details of sample preparation will largely depend on the source of material, its purity, and the detection method. Total cellular extracts, containing both secreted and integral membrane proteins, require detergents for solubility, as may denatured proteins. In contrast, secreted and plasma glycoproteins do not require detergents. \[^3H\]Mannose will label only N-linked oligosaccharides and GPI anchors (UNITS 17.4 & 17.8), and therefore proteoglycans, glycolipids, and O-linked chains will be mostly invisible. \[^3H\]Glucosamine will label all of these structures, and they are all detected (although with variable sensitivities) by hexose-specific colorimetric assays (UNIT 17.9).

The steps required in sample preparation will depend on the sample source and the specific questions being asked. The following points should be considered:

1. Samples need to be purified away from low-molecular-weight glycopeptides, degradation products, and sugar precursors prior to analysis.

2. Glycolipids can cause problems. Alone or with non-denaturing detergents, they migrate at a high molecular weight by gel filtration (as micelles), but at a low molecular weight in the presence of excess SDS or denaturing solvents. On SDS-PAGE, they can migrate as small proteins (~10,000 MW). They may precipitate with acid, acetone, or ethanol (depending on the amount of salts and protein present), but can be difficult to extract from tissues with acetone. If glycolipids will be a problem, specific steps for their removal should be employed (Finne and Krusius, 1982; Britz and Hart, 1983).

3. The solubility of denatured glycoproteins can be a limiting factor in any of the steps. Occasionally, prolonged incubations at elevated temperatures or the addition of 2-mercaptoethanol to 20 mM (final) will solve problem.

Careful bookkeeping of recoveries at each step is important to detect unexpected losses. The use of polypropylene tubes and a carrier protein with radioactive samples may help decrease these losses.

Both protocols assume that digestion will yield glycopeptides containing single glycosylation sites. This is generally the case for N-linked oligosaccharides, as their sites are spaced far apart on the peptide backbone. O-linked glycosylation sites can be clustered in regions of high serine (Ser) and/or threonine (Thr) content and be resistant to proteolysis. Heterogeneity in glycosylation (both in terms of the size of the oligosaccharide and whether a particular Ser/Thr residue is glycosylated) may cause a partial block in protease accessibility to certain regions of the peptide, resulting in incomplete and variable proteolysis and heterogeneity in the resulting glycopeptides.

**Anticipated Results**

Digestion of radiolabeled samples with pronase or proteinase K will yield molecules consisting of the oligosaccharide structure found at a given glycosylation site, plus one or a few amino acids. Such structures can be studied functionally (e.g., in blocking, binding, or adhesion assays) or structurally (UNITs 17.6, 17.13, 17.16, 17.18, & 17.19).

Digestion of purified glycoproteins with site-specific proteases will generate glycopeptides with a larger peptide portion attached. These glycopeptides can be fractionated by reversed-phase chromatography because the peptides are of a defined length (UNIT 10.12), used as substrates for endo-glycosidases, or subjected to other biochemical analyses.

**Time Considerations**

Each individual step (e.g., enzyme digestion, gel filtration, and desalting) will require less than half a day. Lyophilization and acetone precipitation are overnight procedures. The procedure from starting sample to purified glycopeptides can easily be done in five calendar days.

**Literature Cited**


Key References
Judd, 1990. See above.
Useful review on specific endopeptidases.

Spiro, 1966. See above.
Describes use of pronase and techniques for handling glycopeptides.

Swiedler et al., 1983. See above.
Technique from which the basic protocol on endoproteinase digestion was adapted.

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Detection of Individual Glycosylation Sites on Glycoproteins

Many glycoproteins contain multiple sites of glycosylation. They may all have the same oligosaccharide–amino acid linkage (GlcNAc-Asn, GalNAc-Ser/Thr, GlcNAc-Ser/Thr, Xyl-Ser/Thr, or GPI anchor), or different types may be present. Although many of the methods in this unit are directed towards characterizing the total oligosaccharide population on a glycoprotein, for some investigations it may be necessary to determine (1) how many different sites exist, (2) the sugar–amino acid linkage of each site, and (3) the structural characteristics of the oligosaccharides found at the different sites.

Although such an in-depth study is not feasible for all sites on all glycoproteins, a general approach (described in the Basic Protocol) based on peptide mapping techniques has been employed successfully. Glycopeptides generated by endopeptidase digestion (UNIT 17.14A) are separated by reversed-phase chromatography (UNIT 10.12) using C18 resin. Elution times are determined primarily by hydrophobic interactions between the peptide and the resin. However, the presence of hydrophilic, negatively charged oligosaccharides shortens retention times, causing glycopeptides to elute in considerably broader peaks than do peptides. By following the elution profile either radiochemically (for material prepared with radioactive sugar precursors or sulfate; UNIT 17.4) or colorimetrically (using hexose-specific assays; UNITS 17.9 & 17.16), the peaks corresponding to unique glycopeptides can be identified. With proper controls, the number of peaks will correspond to the number of different glycosylation sites. The eluted fractions are suitable for analysis by lectin chromatography (for N-linked oligosaccharides; UNIT 17.1), and the peptide sugar linkage can be defined either by endoglycosidase digestion (see Support Protocol and UNIT 17.13) or chemical cleavage (β-elimination; UNIT 17.15). Oligosaccharides freed from the peptide as described in the Support Protocol can be characterized by size or charge (UNIT 17.17), techniques not generally applicable with glycopeptides.

BASIC PROTOCOL

FRACTIONATION OF GLYCOPEPTIDES BY REVERSED-PHASE HPLC

A highly purified glycoprotein metabolically labeled with a radioactive sugar precursor (UNIT 17.4) is digested with a site-specific protease (UNIT 17.14A). The resulting glycopeptides, each of which contains a single or just a few glycosylation site(s), are fractionated by reversed-phase HPLC. The individual glycopeptide fractions are pooled separately and desalted in preparation for further analytical studies (see Support Protocol).

Materials

- Highly purified glycoprotein metabolically labeled with a radioactive sugar precursor (UNIT 17.4)
- HPLC buffers A and B (see recipe)
- 1 M Tris·Cl, pH 8.4 (APPENDIX 2)
- Organic solvents: chloroform, DMSO, acetonitrile, and methanol
- 10 mM ammonium formate in water and in 50% acetonitrile (see recipe)
- Ventilated oven, 40° to 45°C
- Nitrogen tank
- C18 cartridge (e.g., Sep-Pak cartridge, Waters)
- 1-cc and glass 10-cc syringes
- 50-ml polypropylene tube

Additional reagents and equipment for peptide isolation by reversed-phase HPLC (UNIT 10.12)

Contributed by Leland D. Powell

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1. Prepare glycopeptides from purified, labeled glycoprotein sample by reduction, alkylation, and specific endopeptidase digestion (UNIT 17.14A). Lyophilize the digested samples.

   If the primary sequence of the protein is known, choose a protease or combination of proteases that will result in peptides having a unique glycosylation site. Even if one is only interested in N-linked sites and is working with material labeled with [2-3H]mannose, the potential of other glycosylation sites to interfere with proteolysis must be kept in mind.

   If sequence data are not available, several proteases or combinations thereof may need to be compared.

2. Immediately prior to HPLC analysis, resuspend the lyophilized sample in 100 to 200 µl HPLC buffer A, wetting the walls of the tube exposed to sample to ensure maximal recovery.

3. Microcentrifuge the sample 10 min at 10,000 × g, 4°C. Using a pipet tip with 2 to 4 mm of the tip cut off, transfer solution and as much of the particulate material as possible to a 1.5-ml microcentrifuge tube on ice. Rinse out the digestion tube again and combine with the first wash. Keep the total volume to less than that of the injector sample loop.

   As the HPLC buffers are acidic and sialic acid residues potentially acid labile, minimize the time of exposure of the sample to the buffer and keep it on ice until just before injecting onto the HPLC.

4. Microcentrifuge the sample material 10 min at 10,000 × g, 4°C. Carefully remove the supernatant fluid, measure the radioactivity of an aliquot by scintillation counting, and inject the rest on the HPLC column. If minimal amounts of the sample are available, wash the insoluble material once in HPLC buffer A, spin as before, and pool the supernatant fluids.

   Do not inject particulate material onto the HPLC column.

   Depending on the complexity of the pattern and purity of the sample, anywhere from 5,000 to 50,000 cpm of sample are necessary for an adequate analysis.

5. Determine the amount of glycopeptide insoluble in HPLC buffer A (the “core”) by washing the insoluble particulate material once in water, resuspending it in water, and measuring the radioactivity of an aliquot by scintillation counting. If a significant percentage of the total sample remained insoluble after this first proteolytic digestion, redigest the material with the same protease (using a larger amount) or another protease.

   If TPCK-treated trypsin was used, chymotrypsin digestion cannot follow. If digestion with chymotrypsin is anticipated, use the highest-grade trypsin available (not TPCK-treated).

6. Perform HPLC exactly as described in UNIT 10.12, except starting with the column equilibrated in HPLC buffer A and eluting the peptides with a linear gradient to 100% HPLC buffer B over 90 min, using a flow rate of 1 ml/min, and collecting 1-min fractions.

   With experience, adjustments may need to be made in the shape of the gradient used to optimize the fractionation of individual glycopeptides.

7. Collect the effluent into tubes containing 0.2 to 0.5 ml of 1 M Tris-Cl, pH 8.4 (ensure final pH >5). As the run proceeds, remove tubes and mix to assure timely neutralization. Determine the elution profile by measuring the radioactivity of an aliquot of each fraction by liquid scintillation counting.
Analytical runs do not require neutralization. Instead, collect the fractions directly into scintillation vials and dry by placing them (in a cardboard or metal rack) overnight in a ventilated oven at 40° to 45°C, resuspending the salt pellet in 0.2 ml water followed by scintillation cocktail, and counting.

8. Pool the appropriate peaks and dry under a nitrogen stream.

Once most of the organic solvent is removed, the sample can be frozen, dried under vacuum, and stored at −20°C. Samples should be stable at least 1 year.

9. Prepare a C_{18} cartridge by washing sequentially with chloroform, DMSO, acetonitrile, methanol, and water (10 ml per wash), and finally with 20 ml of 10 mM ammonium formate in water.

10. Resuspend the sample in 1 to 2 ml water and apply to the cartridge using a 1-cc syringe. Rinse the sample tube with 1 ml of 10 mM ammonium formate and apply to the cartridge. Repeat once more, then wash the cartridge with an additional 20 ml of 10 mM ammonium formate, saving all the aqueous washes.

11. Using a glass syringe, elute the cartridge with 6 ml of 10 mM ammonium formate/50% acetonitrile into a 50-ml polypropylene tube and dry under nitrogen as before. Store frozen.

The fractionated glycopeptides can now be analyzed by further digestion (see Support Protocol).

12. Follow the recovery of glycopeptide throughout by measuring the radioactivity of a fixed percentage (1% to 5%) of the initial sample, the aqueous washes, and the acetonitrile-eluted sample by scintillation counting, being careful to mix each sample before removing the aliquots for counting.

Most glycopeptides will be soluble in aqueous solutions in the absence of detergents or organic solvents. However, poor recoveries or unexpected losses when pipetting suggest that solubility may be limiting. To check for “sticky” glycoprotein, rinse tubes and pipet tips that have been exposed to samples with 0.1% SDS and measure the radioactivity of the wash by scintillation counting. Resuspension of the sample, after removal of the acetonitrile, in 0.1% NP-40, 10 mM β-octylglucoside, or another nondenaturing detergent may be necessary.

ENDOGLYCOSIDASE DIGESTION OF PURIFIED GLYCOPEPTIDES

Glycopeptides pooled and desalted from a reversed-phase chromatogram (see Basic Protocol) are digested with a specific endoglycosidase (UNIT 17.13A). Sensitivity to digestion is determined by rechromatographing over the same C_{18} column.

Additional Materials (also see Basic Protocol)

- Glycopeptide sample (see Basic Protocol, step 11)
- HPLC apparatus with 500-μl injector sample loop
- Additional reagents and equipment for endoglycosidase digestion (UNIT 17.13)

1. Resuspend glycopeptide sample in 10 mM ammonium formate in water and transfer to a microcentrifuge tube. Dry in a vacuum concentrator.

2. Add 10 to 50 ml of an appropriate buffer for digestion with PNGase F, endo F, or endo H (UNIT 17.13A) or endo-O-α-N-acetylgalactosaminidase (UNIT 17.17). Add the appropriate enzyme and allow digestion to proceed as specified in the respective unit.
Detergents can be omitted from the enzyme buffer formulations, as most glycopeptides are soluble in aqueous solutions.

3. Terminate the reaction by boiling 3 min.

4. Just prior to HPLC chromatography, dilute with 5 to 10 vol HPLC buffer A (but not exceeding the capacity of the injector loop), microcentrifuge 10 min at 10,000 × g, 4°C, and perform HPLC (see Basic Protocol, steps 6 and 7).

   A 500-μl injector sample loop is required. Whenever possible, do not load over 400 μl in the loop, as the presence of a stationary phase along the walls of microbore tubing limits their usable volume.

5. Determine the elution time of the sample.

   A decrease in elution time relative to that of the undigested glycopeptide indicates that its oligosaccharide was a substrate for the chosen endoglycosidase.

   As in Basic Protocol step 7, glycopeptides are retained by the C18 column. After removal of the peptide, the free oligosaccharide will elute in the first few minutes of the column run (with or just after the breakthrough volume). Nondigested material will elute at the same time as seen initially in Basic Protocol step 7. The percentage release of the oligosaccharide can be calculated directly. These runs can be performed either analytically or preparatively.

   An alternative to HPLC is to fractionate the digested oligosaccharide on a Sep-Pak C18 cartridge; glycopeptides will be retained and released oligosaccharides will elute in the water wash (unless the original glycopeptide elutes with water or very early in the HPLC gradient).

   Nondigested glycopeptides may be recovered as in Basic Protocol steps 7 to 12. Released oligosaccharides may be desalted on a 1 × 20–cm column of Sephadex G25 or Bio-Gel P-2, eluted in 10 mM ammonium formate for addition analyses.

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.

**HPLC buffers A and B**

2× HPLC buffer stock

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaClO₄</td>
<td>28.1 g</td>
</tr>
<tr>
<td>Phosphoric acid</td>
<td>2.35 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>to 1 liter</td>
</tr>
</tbody>
</table>

The pH will be 1.7 to 1.9 (do not adjust).

*Buffer A*: Mix 500 ml of 2× stock and 500 ml water.

*Buffer B*: Mix 500 ml of 2× stock and 500 ml HPLC-grade acetonitrile.

Use deionized, distilled water throughout. Filter and degas solvents.

**Ammonium formate, 10 mM, in water and in 50% acetonitrile**

Prepare 20 mM ammonium formate in water; do not adjust the pH, which will be ~6.7 to 6.9. Dilute 1:1 (v/v) with water (to make 10 mM ammonium formate) or with acetonitrile (to make 10 mM ammonium formate/50% acetonitrile).
Background Information

Analysis of protein glycosylation on a site-by-site basis is not widely done, as it requires more time and material than analysis of total protein oligosaccharide. However, as more refined questions in glycobiology are addressed, such detailed structural information will increasingly be needed. Most published reports on this topic have focused on N-linked oligosaccharides, as these are in general more structurally complex than O-linked (mucin-like) oligosaccharides. Additionally, owing to their spacing on the peptide backbone, N-linked oligosaccharides are relatively easily separated into unique peptides by endopeptidase digestion and thus lend themselves to study by this technique. In principle, this approach could be used to identify a C-terminal peptide with a GPI anchor (released by PI-PLC digestion; UNIT 17.8). In contrast, O-linked mucin-type structures are frequently, although not always, clustered on the peptide backbone and cannot be separated by this approach. As [2-3H]mannose labels predominantly N-linked and GPI structures, it is often the radiolabel of choice for these analyses.

Two major questions are addressed in a site-by-site analysis.

1. How many sites are glycosylated? The consensus sequence for glycosylation of O-linked sites and proteoglycans is not clearly established. The sequence for N-linked glycosylation (Asn-X-Ser/Thr) is easily identified, yet not all possible acceptor sequences are glycosylated. Thus, direct biochemical methods must be employed to determine the number of glycosylation sites.

2. How do the structures found at the different sites compare? It is widely accepted that for a glycoprotein with a single glycosylation site, multiple different oligosaccharide structures will be found. The various factors responsible for this heterogeneity have been recently reviewed (Kobata and Takasaki, 1992). Moreover, site-specific microheterogeneity is also present: at the different glycosylation sites on a given glycoprotein, different populations of oligosaccharides will be found. These differences can only be assessed if the different sites are first separated and then analyzed independently.

Site-specific glycosylation patterns have been determined for several glycoproteins, including α1-acid glycoprotein (Treuheit et al., 1992), murine histocompatibility antigens (Swiedler et al., 1983), and macrophage adhesion molecules (Dahms et al., 1985).

Critical Parameters

Detailed studies such as those outlined in this unit require very clean glycoprotein preparations. Even if, based on SDS-PAGE and fluorographic analysis, a sample appears to be clean, with just low levels of radioactivity migrating diffusely outside of the band of interest, it is still possible for this low-level background to be concentrated by the C18 column and thus turn into a significant contaminant. Thus, it may be useful to purify the radiolabeled sample by SDS-PAGE before proceeding with proteolysis and HPLC. Proteolysis of gel-purified samples can be performed directly in the acrylamide gel slices, on material blotted onto nitrocellulose, or after eluting from the gel slice.

The choice of radioactive precursor for labeling will depend on a variety of factors; see UNIT 17.4 Commentary for discussion. As indicated in Background Information, [2-3H]mannose is often used for the analysis of N-linked oligosaccharides.

The potential presence of multiple glycosylation sites on each glycopeptide, either from clustered sites or from incomplete proteolysis, is a significant concern. This is most worrisome for O-linked sites, which frequently (but not always) appear in clusters on the peptide backbone.

Large proteins with multiple sites (e.g., >6) can be difficult to analyze owing to the complexity of the resulting chromatogram. An alternative strategy entails separating the protein into large glycopeptides by cleaving with CNBr or by limited proteolysis of nondenatured proteins, resulting in domains that can be fractionated by size, reversed-phase HPLC (UNIT 10.12), or ion-exchange chromatography (UNIT 10.10) and subsequently subjected to more extensive proteolysis.

Troubleshooting

The possibility of obtaining overly complex reversed-phase chromatograms is one of the most significant limitations of this methodology. Heterogeneously glycosylated peptides will migrate as broad clusters of peaks rather than the discrete peaks seen with peptides; incomplete proteolytic digestion can also result in increased peak complexity. If confronted with a complex or uninterpretable profile, ini-
tially rule out incomplete digestion by repeating the process with more protease, longer digestion times, and/or combinations of proteases (trypsin and chymotrypsin). Alternatively, reduce the oligosaccharide heterogeneity (as with sialidase digestion) and repeat the C<sub>18</sub> analysis.

Recovery of glycopeptides at each step should be monitored closely to detect unexpected losses and/or solubility problems. As a rule, glycopeptides are very soluble in aqueous buffers, but large proteolytic fragments from denatured proteins may exhibit problematic solubility behavior.

Some large N-linked glycopeptides are resistant to cleavage by PNGase F. The basis of this inhibition has not yet been determined.

**Anticipated Results**

The C<sub>18</sub> chromatogram should contain discrete clusters of peaks representing single peptides with heterogeneous oligosaccharide side chains. Comparison of glycopeptides radiolabeled with different radioactive sugar precursors should indicate the type of oligosaccharide present (N-, O-, or GPI-linked structures). Comparison of the amount of material in each peak will indicate if some sites are underglycosylated. If the procedure is done on a preparative scale, the different glycopeptide fragments are suitable for more detailed structural analysis.

**Time Considerations**

Preparation of glycopeptides is described (including time considerations) in *UNIT 17.14A.*

Performing the HPLC analysis will require a day’s effort. Endoglycosidase digestions require ~1 hr to set up, an overnight digestion, and 2 to 4 hr to analyze. Multiple sites will require proportionally more time.

**Literature Cited**


**Key Reference**

Swiedler et al., 1983.

*The reference upon which most of these methods are based.*

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\[ \beta \text{-Elimination for Release of } O\text{-Linked Glycosaminoglycans from Proteoglycans} \]

O-linked glycosaminoglycan (GAG) chains in proteoglycans are readily released from their core proteins by treatment with alkali at room temperature. This \( \beta \)-elimination is the same type of reaction as that for releasing O-linked oligosaccharides from their core proteins. Under the reaction conditions described here, N-linked oligosaccharides remain attached to the core protein, but any O-linked oligosaccharides will be released along with the GAG chains. The procedure can be used to isolate the free GAG chains, the free O-linked oligosaccharides, and the core protein (which will still have any N-linked oligosaccharides that were originally present).

\textbf{Materials}

- Proteoglycan solution to be analyzed
- Alkaline borohydride reagent (see recipe)
- 2 M HCl
- 1 M NaOH

Additional reagents and equipment for dialysis (\textit{APPENDIX 3})

1. Dialyze proteoglycan solution exhaustively against water to remove all buffer salts. Transfer to small test tube or centrifuge tube and dry in gentle stream of air or by lyophilization.

2. Dissolve dried proteoglycan sample in minimal volume of alkaline borohydride reagent and incubate 24 hr at room temperature.

   \textit{The reaction tube should not be capped because evolution of hydrogen gas may result in pressure buildup.}

3. Add 2 M HCl at room temperature, monitoring acidity with pH paper until the pH of the solution is 0 (to convert excess borohydride to hydrogen gas).

4. When evolution of hydrogen has ceased, add 1 M NaOH until pH is 6 to 8 as measured with pH paper.

   \textit{For detailed discussion of further treatment of the products, see Commentary.}

\textbf{REAGENTS AND SOLUTIONS}

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

\textbf{Alkaline borohydride reagent}

Dissolve 22.4 mg sodium borohydride (NaBH\(_4\)) in 2 ml of 0.4 M NaOH just before use.

\textit{The resulting solution contains 0.3 M NaBH\(_4\).}
Background Information

Chondroitin sulfate, dermatan sulfate, and heparan sulfate are glycosaminoglycans (GAGs) occurring in cell-surface proteoglycans, in which they are O-linked to their core proteins through a xylosyl-serine linkage. Cartilage keratan sulfate is also O-linked to proteins in proteoglycans (through N-acetyl-galactosamine, or GalNAc), whereas corneal keratan sulfate occurs as an N-linked polysaccharide. Hyaluronic acid, however, does not occur linked to a protein. Although heparin is synthesized in the mast cell as a proteoglycan, it is cleaved from its core protein in the final stages of synthesis and occurs, for the most part, as a mixture of free polysaccharide chains that are released from the cells only when the cells degranulate.

The basic protocol gives essentially complete release of O-linked GAGs, along with any O-linked oligosaccharides attached to the core protein. This is accomplished in 24 hr without removal of N-linked oligosaccharides (Mayo and Carlson, 1970; Seno and Sekizuka, 1978). The serine residues to which the GAGs are O-linked are converted to dehydroalanine residues, but the core protein is not cleaved. Thus, the molecular weight of both the core protein and the released GAG chains can be estimated by gel electrophoresis or gel filtration (Bienkowski and Conrad, 1985; Fedarko and Conrad, 1986). Sodium borohydride (NaBH₄) is included in the reaction mixture to prevent alkaline cleavage of the newly formed xylosyl or GalNAc reducing terminals from the rest of the GAG chain (known as the peeling reaction). If desired, tritiated sodium borohydride (NaB³H₄) can be used in the reaction to label the reducing-terminal xylose of the GAG chains (as well as the reducing-terminal residues of the O-linked oligosaccharides, if present). If NaB³H₄ is used, all reactions must be carried out in a designated radioactive fume hood because oxidation of NaB³H₄ yields ³H₂ gas. N-linked oligosaccharides remain attached to the core protein under the mild reaction conditions described here.

This protocol is useful for demonstrating the proteoglycan nature of an unknown sample containing GAGs, as the molecular weight of proteoglycans will be reduced, but that of free GAG chains will not (Bienkowski and Conrad, 1984; Fedarko and Conrad, 1986). It is also a necessary step for characterizing the proteoglycan and its constituent core protein and GAG chain(s) after the GAGs are separated from the smaller O-linked oligosaccharides that are also released in the reaction. If NaB³H₄ is used in the reaction to label the reducing-terminal xyitol, the molar ratio of total disaccharides in the GAG chains to ³H(xyitol) can be used to estimate the size of the GAG chains. In addition, the linkage region can be recovered from an ³H-labeled GAG chain by trimming away the main chain with lyases (UNIT 17.14B) or nitrous acid (for heparan sulfate), leaving the labeled linkage region. If the starting proteoglycan contains both O-linked GAGs and O-linked oligosaccharides, the products will consist of core protein, polysaccharides, and oligosaccharides. After the reaction the products can be separated from each other by gel filtration to resolve the oligosaccharides from the core protein and GAG chains. The polymeric fraction can then be resolved by DEAE-cellulose chromatography in a salt gradient to separate the core protein from the more highly charged GAG chains (Bienkowski and Conrad, 1984).

Critical Parameters

Although the reaction can be run at higher temperatures (35°C to 50°C) to increase the reaction rate, loss of sulfate residues, cleavage of N-linked oligosaccharides (Ogata and Lloyd, 1982), or cleavage of the polypeptide chain may result. Under the milder conditions described here, these side-reactions appear to be minimal, and both the carbohydrate chains and the core proteins can be recovered intact. These conditions release both xylose-linked glycosaminoglycans (GAGs), GalNAc-linked keratan sulfate, and O-linked oligosaccharides, and there seems to be little difference in the rates of β-elimination of these different carbohydrates. No comparisons of these rates, however, have been reported. Sodium borohydride must be present to reduce the new reducing terminals of the carbohydrate chains as they are released from the core protein, so that alkaline degradation of the carbohydrate chains cannot occur. It is advisable to free the proteoglycan from oxygen ions such as phosphate prior to reaction because these will catalytically destroy sodium borohydride (Conrad et al., 1973).

The procedure described in this unit does not include palladium chloride (PdCl₂), which has been used by other workers (Seno and Sekizuka, 1978) to reduce dehydroalanine to alanine as it is formed during the elimination.
PdCl$_2$ may be useful to stabilize the core protein when more harsh conditions are used for the β-elimination, and to allow amino acid analysis of the resulting core protein by observing the conversion of serine to alanine. It is not, however, necessary for the reaction, and the colloidal Pd formed in the reaction may interfere with recovery of the reaction products. It should be noted that the number of moles of serine converted to alanine is a reflection of the number of moles of carbohydrate chains originally O-linked to the core protein.

**Anticipated Results**

The β-elimination reaction is virtually stoichiometric. Assuming that the proteoglycan is pure before the reaction is run, the products of this reaction will be the intact core protein, the intact glycosaminoglycan chains, and any O-linked oligosaccharides that were also attached to the core protein.

**Time Considerations**

This reaction can be run ≥24 hr without any serious side reactions. Normally, a 24-hr incubation is sufficient to obtain complete cleavage of the proteoglycan.

**Literature Cited**


**Key Reference**

Seno and Sekizuka, 1978. See above. Describes the basic procedure used here and includes the use of PdCl$_2$ to reduce the dehydroalanine residues to alanine residues.
β-Elimination for Release of O-GalNAc-Linked Oligosaccharides from Glycoproteins and Glycopeptides

This unit describes release of oligosaccharides that are attached to polypeptides through an N-acetylgalactosamine (GalNAc) linkage to the hydroxyl groups of serine or threonine. The β-elimination procedures described here can be used to recover the oligosaccharide chains (also called glycans) and/or identify the serine or threonine residues involved in the linkage.

β-elimination (Fig. 17.15.1) is usually carried out in the presence of a reducing agent such as sodium borohydride (NaBH₄) under alkaline conditions (Basic Protocol; see also UNIT 17.15A). The reducing agent immediately converts released oligosaccharides into reduced oligosaccharides, which is necessary because free oligosaccharides can be degraded under alkaline conditions by further β-elimination (peeling reaction; see Background Information). This method provides quantitative release of the oligosaccharides but does not yield intact polypeptides (see Critical Parameters).

An alternative method is to employ only alkaline conditions without a reducing agent (Alternate Protocol 1). The extent of conversion of serine and threonine residues in the polypeptide after oligosaccharide release indicates the number of such residues involved in linkage to O-glycans. However, this method does not allow quantitative recovery of the released glycans.

Another alternative is to use sodium sulfite (Na₂SO₃; see Alternate Protocol 2), which converts serines and threonines that are attached to O-glycans into cysteic acid and α-amino-β-sulfonlbutyric acid, respectively. This method, however, does not allow quantitative recovery of released glycans because further β-elimination of O-glycans (known as the peeling reaction; Fig. 17.15.2) cannot be prevented. The two products of the second alternate protocol (cysteic acid and α-amino-β-sulfonlbutyric acid) cannot be resolved using an amino acid analyzer, as they will elute in the same peak, so they must be quantitated using paper chromatography (Support Protocol).

β-elimination is not restricted to oligosaccharides attached through GalNAc but may also be used for release of oligosaccharides linked through other residues, including N-acetylglucosamine and xylose. For release of glycosaminoglycans linked through xylose, see UNIT 17.15A.

**BASIC PROTOCOL**

**β-ELIMINATION IN THE PRESENCE OF SODIUM BOROHYDRIDE**

β-elimination is achieved by reducing the sample under alkaline conditions using NaBH₄ (Carlson, 1968; Fukuda, 1989). Products are resolved by passing the reaction mixture through a Sephadex column. This procedure allows quantitative recovery of the O-glycans that are released.

**Materials**

- Glycopeptide or glycoprotein sample
- 1 M NaBH₄/0.05 M NaOH (prepare fresh)
- 1 M acetic acid in methanol (prepare fresh, cool to room temperature before use)
- Nitrogen gas stream
- Methanol (HPLC grade)
- 0.2% orcinol/2 M H₂SO₄ (store at 4°C)
- 9-ml screw-cap conical glass test tubes

Contributed by Minoru Fukuda

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25° to 30°C and 45°C water baths
1 × 30–cm Sephadex G-15 column (Pharmacia Biotech) equilibrated in H2O
Silica gel–coated thin-layer chromatography (TLC) plate
150° to 200°C oven

1. Mix 1 to 500 µg glycopeptide or glycoprotein sample and 5 to 100 µl of 1 M NaBH4/0.05 M NaOH in a 9-ml screw-cap conical glass test tube.

   The volume of solution used should be minimal but enough to dissolve the sample.

2. Close test tube loosely with screw cap and incubate 24 to 36 hr in a 45°C water bath.

3. Open test tube in a vented hood and slowly add 3 ml of 1 M acetic acid in methanol. Place tube in a 25° to 30°C water bath and dry contents in a nitrogen gas stream.

4. Repeat step 3.

5. Add 3 ml methanol, dry as in step 3, then dissolve in ~200 µl water. Either proceed to step 6 or freeze at −20°C until ready to continue.

6. Apply to a 1.0 × 30–cm Sephadex G-15 column and elute with water. Collect 1-ml fractions using a fraction collector.

7. Spot an aliquot from each fraction on a silica gel–coated TLC plate and allow to dry. Spray plate with orcinol/H2SO4 reagent and heat 4 min in a 150° to 200°C oven. Identify fractions containing carbohydrates by appearance of a brown color.

   Alternatively, other methods for detection of carbohydrates can be used. It is also possible to calibrate the column before the experiment so that the fractions where the oligosaccharides will elute are known in advance. Those fractions are then collected and pooled.

8. Dry carbohydrate-containing fractions by evaporation and freeze at −20°C until ready for further analysis.

**RAPID β-ELIMINATION IN DIMETHYL SULFOXIDE SOLUTION**

This procedure facilitates release of O-glycans so that β-elimination can be completed in a short time. It yields oligosaccharides that are not reduced at the reducing terminals, making it possible to add various functional groups such as fluorescent probes.

**Additional Materials** *(also see Basic Protocol)*

- Dimethyl sulfoxide (DMSO)
- 0.425 M KOH
- 100% ethanol
- 0.4 M HCl

1. Dissolve glycoproteins or glycopeptides in 500 µl to 1 ml of DMSO to a final concentration of 5 mg/ml in a 9-ml screw-cap conical glass test tube.

2. Add sufficient 0.425 M KOH and 100% ethanol to give a 0.17 M KOH concentration and a DMSO/water/ethanol ratio of 50:40:10 (v/v/v).

   For example, to treat a sample dissolved in 1 ml DMSO, add 800 µl of 0.425 M KOH and 200 µl of 100% ethanol.

3. Incubate 1 hr at 45°C, then neutralize with 0.4 M HCl using pH paper as indicator.

4. Proceed with Basic Protocol, steps 6 to 8, or freeze sample at −20°C until ready to continue.
**β-ELIMINATION IN THE PRESENCE OF SODIUM SULFITE**

This procedure is used (in combination with the Support Protocol) to determine the number of serine and threonine residues involved in O-glycan attachment, which are converted to cysteic acid and α-amino-β-sulfonylbutyric acid, respectively, by Na₂SO₃.

**Additional Materials (also see Basic Protocol)**

- 0.5 M Na₂SO₃/0.1 M NaOH (prepare fresh)
- 1 M and 6 M HCl
- 7-ml conical glass test tube
- Vacuum evaporator, 40°C
- 110°C oven

1. In a 7-ml conical glass test tube, dissolve glycopeptides to a concentration of 1.0 mM or glycoprotein to a concentration of 8 mg/ml in 0.5 M Na₂SO₃/0.1 M NaOH. Incubate 48 hr at 37°C.

   *Glycoprotein samples can first be treated by oxidation with performic acid to facilitate determination of serine and threonine residues (see Critical Parameters).*

2. Terminate reaction by adjusting pH to ∼3 with 1 M HCl. Evaporate to dryness in a 40°C vacuum evaporator.

3. Dissolve in 200 µl of 6 M HCl and transfer to a test tube. Hydrolyze 24 hr in a 110°C oven with tube sealed.

4. Evaporate hydrolysis reaction mixture (total amino acid mixture) to dryness in a 40°C vacuum evaporator, then proceed to support protocol for quantitation of O-linked serine and threonine residues (see Support Protocol).

   *The reaction mixture may be dissolved in water and stored at −20°C until ready for analysis.*

**SEPARATION OF β-ELIMINATION PRODUCTS BY PAPER CHROMATOGRAPHY**

In the second alternate protocol, the serine and threonine residues that are linked to O-glycans are converted, respectively, to cysteic acid and α-amino-β-sulfonylbutyric acid. Conventional amino acid analyzers combine the total yield of these two compounds in the same peak. This support protocol employs paper chromatography to separate cysteic acid and α-amino-β-sulfonylbutyric acid for the purpose of determining the number of O-linked serine and threonine residues in a glycoprotein or glycopeptide (Spiro, 1972).

**Materials**

- Dried β-elimination reaction product (see Alternate Protocol 2)
- 0.05 M NaOH
- Dowex AG-1-X8 resin (200 to 400 mesh; formate form; Bio-Rad)
- 5 mM pyridine formate buffer, pH 4.0 (see recipe)
- 4 M formic acid
- 4:1:5 (v/v/v) 1-butanol/acetic acid/water
- 0.5 × 5–cm chromatography column
- Whatman 3 MM chromatography paper
- Paper chromatography chamber

1. Dissolve dried β-elimination product in water and titrate to pH 4.0 with 0.05 N NaOH.
2. Equilibrate Dowex AG-1-X8 resin by suspending in pyridine formate buffer, pH 4.0 for several hours. Pack 0.5 equilibrated resin into a 0.5 × 5–cm column and wash with pyridine format buffer.

3. Apply sample from step 1 to column. Wash column with 10 ml pyridine formate buffer, then elute acidic amino acids with 5 vol of 4 M formic acid.

4. Dilute eluate with 2 ml water, then lyophilize to remove formic acid and pyridine formate.

5. Separate acidic amino acids in 4:1:5 (v/v/v) 1-butanol/acetic acid/water for 5 days using Whatman 3 MM paper.

The migration relative to aspartic acid (RAsp) in this system is 0.42 for both cysteic acid and α-amino-β-sulfonylbutyric acid.

**REAGENTS AND SOLUTIONS**

*Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 5.*

**Pyridine formate buffer (5 mM), pH 4.0**

Prepare 5 mM pyridine formate and adjust to pH 4.0 with 6 M formic acid. Store at room temperature in a sealed glass bottle.

**COMMENTARY**

**Background Information**

O-linked oligosaccharides, which are attached to polypeptides through the β-carbon-borne hydroxyl groups of serine or threonine, are abundant in mucin-type glycoproteins. O-linked oligosaccharides can be released from glycoproteins by β-elimination, after which the oligosaccharides can be fractionated and their structures determined. The method relies on β-elimination, shown in Figure 17.15.1, and takes advantage of the presence of the hydroxyl group of serine or threonine at the amino acid’s β carbon. Under alkaline conditions, the peptide-group oxygen becomes negatively charged, and a double bond is formed between the peptide carbon and the α carbon. This double bond in turn migrates to make a double bond between the α and β carbons, and the β-carbon oxygen and linked oligosaccharide are released.

During this β-elimination, the released O-glycans are exposed to strong alkaline conditions. Such treatment tends to induce another β-elimination reaction within the released O-glycans (known as a peeling reaction). In particular, if the next sugar residue is attached to C-3 (also known as the β carbon) of N-acetyl-galactosamine (GalNAc), this additional β-elimination occurs as in Fig. 17.15.2—i.e., presence of alkali introduces a double bond and this induces another β-elimination reaction in which the sugar chain is cleaved and released from GalNAc. It is therefore necessary to introduce a reducing agent to prevent such further β-elimination. Thus, to prevent further degradation of released oligosaccharides and permit quantitative recovery of the released O-glycans, alkaline degradation is usually carried out in the presence of large amounts of the reducing agent NaBH₄ (Basic Protocol). β-elimination employing NaBH₄, however, does not provide quantitative recovery of alanine or α-amino-nobutyric acid (produced, respectively, from serine or threonine residues that are involved in O-glycan attachment).

**Critical Parameters**

The method employing 1 M NaBH₄ in 0.05 N NaOH (Basic Protocol) is most suitable for quantitative release of O-linked oligosaccharides from glycoproteins. This method is, however, not suitable for obtaining intact polypeptides because the sodium borohydride also cleaves peptide bonds and may also often lead to release of N-linked oligosaccharides (Ogata and Lloyd, 1982).

The need for a technique that kept the polypeptide portion as intact as possible led to the invention of the procedure using NaOH in DMSO (Alternate Protocol 1; Downs et al., 1973). However, this method is better suited for release of intact oligosaccharides than intact polypeptides, as cleavage of polypeptides cannot be completely avoided.
Figure 17.15.1  β-elimination for release of O-GalNAc-linked oligosaccharides from glycoproteins and glycopeptides. R represents oligosaccharide chain; broken lines represent remainder of polypeptide chain. The double bond formed can be reduced in the presence of sodium borohydride and palladium chloride (also see UNIT 17.15A).

Figure 17.15.2  Peeling reaction. R, remainder of GalNAc moiety; R′, oligosaccharide chain.
The alkaline sodium sulfite method using 0.5 M Na₂SO₃ in 0.1 M NaOH (Alternate Protocol 2), is best suited for estimating the number of serine and threonine residues involved in O-glycan attachment. However, this method does not give a 100% yield of α-aminobutyric acid because the conversion of threonine to α-amino-β-sulfonylbutyric acid is usually ~75%. This method also does not prevent the peeling reaction involving the O-glycans that are released. It is useful only for determining the amount of serine and threonine involved in O-glycosylation. In order to determine accurately the amount of cysteic acid and α-aminobutyric acid produced in the alkaline sodium sulfite reaction, it is desirable to prepare a control in which the glycoproteins are oxidized with performic acid before β-elimination. This treatment cleaves disulfide cross-linkages in the peptide chain, converting, for example, cystine to cysteine residues. The difference between the amounts of cysteic acid and α-aminobutyric acid obtained before and after the alkaline sodium sulfite reaction represents the amount of serine and threonine involved in O-glycan attachment.

In order to release O-glycans without cleavage of the polypeptide moiety, other chemical methods must be employed—e.g., those employing trifluoromethanesulfonic acid or hydrogen fluoride (Sojar and Bahl, 1987). Attempts to characterize the oligosaccharides released using these methods have not yet been made.

Troubleshooting

Problems occur in β-elimination if the sample is treated with reducing agent under acidic conditions. This lowers the efficiency of the β-elimination reaction and also decreases the effective concentration of reducing agent. If it is not known whether or not the sample is acidic, it should be desalted before carrying out the reaction.

Anticipated Results

β-elimination using the three methods presented in this unit is virtually stoichiometric for release of O-linked oligosaccharides. Of these methods, only the Basic Protocol yields intact oligosaccharides. Using Alternate Protocol 1, the released oligosaccharides may be degraded, although relatively intact polypeptides may be released. Using Alternate Protocol 2, it is possible to determine the number of serine and threonine residues involved in O-glycan attachment; however, here too the released oligosaccharides may be degraded. Neither of the alternate protocols is therefore suited for oligosaccharide analysis.

Time Considerations

The alkaline borohydride reaction (Basic Protocol and Alternate Protocol 1) can be run 24 to 48 hr without any serious side reactions. Including the time for chromatography, 48 hr is sufficient to obtain a mixture of O-linked oligosaccharides from glycopeptide or glycoprotein. Alternate Protocol 2 (β-elimination with sodium sulfite) will take an additional 24 hr.

Literature Cited


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Acid Hydrolysis for Release of Monosaccharides

The first step in obtaining a compositional analysis of a glycoconjugate is the release of the individual monosaccharide constituents. How these are released and processed depends on what methods are available for their analysis and identification. When the analysis is to be performed by high-performance liquid chromatography (HPLC), the monosaccharides must be released by acid hydrolysis of the glycoconjugate. Free glycoside residues can then be separated using a pellicular-resin anion-exchange column and detected by pulsed amperometry (see UNIT 17.19A). When a gas-liquid chromatography (GLC) approach is to be taken in the analysis—and the glycoconjugate contains amino sugars and/or uronic acids—methanolation, followed by trimethylsilylation of hydroxyl and carboxyl groups, is the method of choice (UNIT 17.19A). Often a prediction can be made of the type of monosaccharides present in a glycoconjugate by analogy with other glycoconjugates from closely related sources. If the sample is not expected to contain amino sugars or uronic acids—or if only neutral sugars must be measured—acid hydrolysis, followed by reduction and peracetylation (UNIT 17.19A), is preferred.

When an HPLC approach is taken, neutral sugars, hexosamines, and uronic acids can be directly analyzed after strong acid hydrolysis (see Basic Protocol 3). If sialic acids are involved, they must be analyzed separately by submitting an aliquot of the sample to mild acid conditions (see Basic Protocol 2), after which the sialic acids are purified and analyzed by HPLC without derivatization (see UNIT 17.19A) or after derivatization (see UNIT 17.18). In some cases, enzymatic release of sialic acids (see UNIT 17.12) may be chosen to preserve the O-acyl substitutions.

Mild acid hydrolysis to release fucosyl residues from glycoconjugates is described in Basic Protocol 1. Mild acid hydrolysis to release sialic acids from glycoconjugates, along with dialysis and column chromatography procedures to purify the sialic acids, are described in Basic Protocol 2. Strong acid hydrolysis to release all monosaccharides from glycoconjugates is described in Basic Protocol 3. Conditions required for successful release of all the different monosaccharides in a glycoconjugate vary to a great extent when hydrochloric or sulfuric acid is used for hydrolysis. However, the conditions required for hydrolysis using trifluoroacetic acid (TFA; see Basic Protocol 3) are considerably more uniform. Apart from sialic acids, which are known to be destroyed under strong acid conditions, all sugar residues—including fucose, hexosamines, and uronic acids—can be confidently determined after optimized treatment with TFA. TFA also has the advantage of being easily eliminated by evaporation without leaving a residue. For the release of sialic acids, however, mild acid hydrolysis using acetic acid (see Basic Protocol 2) and/or enzymatic release (UNIT 17.12) is recommended. The Alternate Protocol describes a harsher acid hydrolysis procedure for 4-O-acetylated sialic acids, which are more difficult to release.

A precise quantitative ratio of the different types of monosaccharides in a glycoconjugate is very difficult to obtain. This arises from the fact that all glycosidic bonds must be split while avoiding destruction of the liberated monosaccharides. Susceptibility to acid hydrolysis, as well as stability of the released unit, is different for each monosaccharide—e.g., pyranosides and furanosides hydrolyze at different rates; the presence of substituents affects the rate of hydrolysis; and α and β anomers have different rates of hydrolysis. In some cases, therefore, more than one set of conditions may be required to achieve a complete analysis (see Critical Parameters).
MILD ACID HYDROLYSIS FOR RELEASE OF FUCOSE RESIDUES

This protocol describes mild acid hydrolysis conditions appropriate for the release of terminal nonreducing fucose from a glycoconjugate. These conditions should release the fucose residues without releasing other monosaccharides (except sialic acids). Strong acid hydrolysis (see Basic Protocol 3) should be used to analyze for total monosaccharide composition, including fucose.

Materials

Glycoconjugate-containing sample to be analyzed
Standard: 1 µmol/ml L-fucose (mol. wt. 164.2) in H2O
0.05 M HCl prepared from constant-boiling HCl (sequencer-grade, Pierce)
99% methanol (anhydrous; e.g., Aldrich)
Small glass culture tubes or 3.5-ml glass vials with Teflon-lined screw caps
Heating block or oven
Nitrogen (N2) or vacuum evaporation system: e.g., Speedvac (Savant), shaker-evaporator (Baxter Scientific), or lyophilizer
Dialysis tubing (500 MWCO)

Additional reagents and equipment for phenol–sulfuric acid assay for monosaccharides (UNIT 17.9), dialysis (APPENDIX 3C), and compositional analysis of monosaccharides from glycoproteins (UNIT 17.19A)

1. Estimate the total carbohydrate composition of the sample to be analyzed by the phenol–sulfuric acid assay (UNIT 17.9).

2. Using N2, a Speedvac system, or a lyophilizer, evaporate three aliquots of the sample to be analyzed (each containing ≥10 nmol of carbohydrate) to dryness in small glass culture tubes or 3.5-ml glass vials. Simultaneously evaporate to dryness three 10-µl aliquots of 1 µmol/ml L-fucose for use as standards.

   Use brand-new precleaned glass tubes or vials, or clean used ones by heating ≥3 hr at 50°C first in concentrated nitric acid, then in 6 M HCl—each time by placing them inside a glass container with a lid and filling the container with acid. Care should be taken to ensure that each tube or vial is completely filled with acid. Rinse the vials thoroughly with Milli-Q purified water or equivalent, then with ethanol, and finally dry in an oven. When <5 µg total carbohydrates are to be analyzed, it is recommended that the glass vials be silanized by incubating 15 min at room temperature with 2% dichloromethylsilane in toluene. The solution is then decanted and the silanized vials rinsed successively with methanol and hot distilled water and allowed to dry.

3. Redissolve each dried sample and standard by adding 0.4 ml of 0.05 M HCl for up to 1 mg of material. Flush samples and standards with nitrogen and cap tubes.

   When glass vials are used, nitrogen can be introduced through a needle inserted in the septum and evacuated through a second needle.

4. Using a 100°C heating block or oven, heat one sample and one standard 2 hr, another sample and standard 4 hr, and the remaining sample and standard 8 hr.

   When a heating block is used, care must be taken that transfer of heat to the tubes is adequate. This can be achieved by filling the wells with pump oil or using a sand or metallic bath. Most of the tube or vial should be covered with the heating element to avoid distillation of the solvent in the upper part of the tube. When oil is used in the wells, excess oil must be wiped from the vials and the vials washed with hexane and dried before opening.
5. After each sample and standard has been heated for the required time period, evacuate the solution using a nitrogen or vacuum evaporation system. Wash residues twice with 99% methanol, then evaporate again.

CAUTION: This procedure should be carried out in a chemical fume hood.

If this is of interest, a defucosylated glycoconjugate can be prepared following this protocol. After acid hydrolysis under optimal conditions, neutralize reaction mixture with 0.05 M NaOH, dilute, and dialyze at 4°C against 20 vol water using 500-MWCO dialysis tubing to recover defucosylated glycoconjugate. Alternatively, the defucosylated product can be recovered by gel filtration chromatography (UNIT 10.9) on Bio-Gel P2 or Sephadex G-15, using water for elution.

6. Store the dried hydrolysates at −20°C until ready for compositional analysis. Results will indicate the best time for complete hydrolysis with minimal decomposition.

Procedures for compositional analysis are found in UNIT 17.19.

RELEASE OF SIALIC ACIDS (EXCLUDING 4-O-ACETYLATED SPECIES) BY MILD ACID HYDROLYSIS AND PURIFICATION OF THE PRODUCT

This procedure allows the quantitative release of most types of known sialic acids, other than those acetylated at the 4 position, with minimal destruction. Subsequent handling of the sialic acid hydrolysate, including purification, is also designed to avoid migration or loss of O-acetyl groups. After purification, the mixtures of sialic acids can be analyzed by HPLC with (UNIT 17.18) or without derivatization (UNIT 17.19A). For release of sialic acids acetylated at the 4 position, see Alternate Protocol. This protocol can be used to prepare a mixture of sialic acids from commercially available glycoconjugate standards (e.g., bovine submaxillary mucin) for use as a standard in sialic acid analysis.

Materials

- Glycoconjugate-containing sample to be analyzed
- Sodium formate buffer, pH 5.5 (see recipe)
- 0.1 N H₂SO₄
- 10 M and 2 M acetic acid
- 1% butylated hydroxytoluene (BHT; Sigma) in ethanol
- Dowex AG 50W-X2 ion-exchange resin (200 to 400 mesh, hydrogen form; Bio-Rad)
- Dowex AG 3-X4A ion-exchange resin (100 to 200 mesh, hydroxyl form; Bio-Rad)
- 10 mM and 1 M formic acid (ACS certified), ice-cold
- Dialysis tubing (1,000 and 12,000 MWCO; Spectrapor, Spectrum)
- Heating block
- 5-ml glass culture tubes with Teflon-lined screw caps
- 0.5 × 10−cm glass chromatography columns or Pasteur pipets plugged with glass wool
- 20-ml glass test tubes
- Lyophilizer or shaker-evaporator (Baxter Scientific)
- Additional reagents and equipment for dialysis (APPENDIX 3C), de-O-acetylation of sialic acids and TBA assay (UNIT 17.18), and ion-exchange chromatography (UNIT 10.10)

Prepare sample and determine optimal conditions

1. Thoroughly homogenize sample to be analyzed and dialyze (APPENDIX 3C) overnight at 4°C against a 100-fold excess of sodium formate buffer, pH 5.5, using 12,000-MWCO dialysis tubing to recover defucosylated glycoconjugate.

Prepare sample and determine optimal conditions

2. When optimal conditions have been determined, proceed with purification by removing any undissolved material by centrifugation and adjust pH to 5.5 if necessary. Add 0.1 N H₂SO₄ until pH 5.5 is reached.

3. Add 2 M aceto-hydroxytoluene (BHT, Sigma) to a final concentration of 1% and incubate at 50°C for 4 hours in a heating block.

4. After incubation, cool on ice for several hours or overnight.

5. Dialyze two times against water using 12,000-MWCO tubing against 10 vol water.

6. Neutralize reaction mixture with 0.05 M NaOH and dilute to a final volume of 500 ml.

7. Apply mixture to a Dowex AG 50W-X2 column (200 to 400 mesh, hydrogen form) in a 5-ml glass culture tube with screw cap. Wash column with 100 ml of 0.1 N H₂SO₄ to remove excess methanol.

8. Elute sample with 100 ml of 0.1 N H₂SO₄.

9. Collect fractions containing sialic acids and dialyze against 100 vol water overnight.

10. Lyophilize or evaporate to dryness in a vacuum evaporator.

11. Store at −20°C until ready for compositional analysis.

Precautions

- Avoid direct contact with sialic acids, as they are toxic.
- Wash hands thoroughly after handling.
- Wear appropriate protective clothing when preparing samples.

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References

MWCO tubing. Lyophilize dialysate and store frozen at −20°C until ready for analysis.

Dialysis is not necessary for relatively purified biological samples that do not contain low-molecular-weight contaminants. If not to be analyzed immediately, samples should be lyophilized and stored frozen at −20°C.

Crude biological samples may contain O-acetylemesterases, which could cause de-O-acetylation of sialic acids during the isolation procedure. These enzymes can be inactivated by adding a 100 mM stock solution of diisopropyl fluorophosphate (DFP) in isopropanol to a final concentration of 1 mM, then incubating 15 min on ice. As DFP is extremely toxic, safety recommendations should be studied carefully; gloves should be worn and the compound should be used in the fume hood.

2. Dissolve the sample in water. De-O-acetylate an aliquot of sample, release sialic acids with 0.1 N H₂SO₄, and determine total sialic acid content by the TBA assay (UNIT 17.18). Although the TBA assay uses harsher conditions for releasing the sialic acids and will cause some destruction, it is used here in a comparative fashion. Therefore, it is a valid indication of the recoveries from step to step.

3. Transfer three aliquots of the sample from step 1 (each containing ∼10 nmol of sialic acids) to 5-ml glass culture tubes.

4. Add 10 M acetic acid to a final acetic acid concentration of 2 M, then add 1 vol of 1% BHT per 100 vol sample.

The BHT retards lipid peroxidation.

5. Using an 80°C heating block or oven, heat one aliquot 2 hr, another aliquot 4 hr, and the remaining aliquot 5 hr.

When a heating block is used, care must be taken that the transfer of heat to the tubes is adequate. See Basic Protocol 1, step 4 annotation, for proper technique.

6. After each sample has been heated for the required time period, lyophilize and de-O-acetylate, then determine total free sialic acid by the TBA assay (UNIT 17.18). Compare the numbers obtained to determine the time required for maximal release of sialic acids.

Release and purify sialic acids

7. In a 5-ml glass culture tube add 2 M acetic acid to the lyophilized sample from step 1 (0.4 ml for up to 1 mg) and incubate at 80°C for the time determined in steps 5 and 6. Cool to room temperature.

8. Transfer reaction mixture into 1000-MWCO dialysis tubing, then wash the test tube twice with water, each time transferring the washings into the dialysis tubing. Dialyze (APPENDIX 3C) overnight at 4°C against 10 vol water and retain the dialysate (solution outside the tubing).

An MWCO of 1000 is sufficient to allow the passage of free sialic acids. Bigger pores may allow more contamination by permitting bigger molecules to dialyze out.

When total volumes are small (100 μl to 1 ml), the dialysis tubing can be placed inside a test tube. In this case, it must be closed with knots instead of plastic clamps.

Assuming that equilibrium is achieved, a 10% loss (sialic acids remaining in the tubing) should be expected when dialyzing against 10 vol water. The recovery can be improved by repeating the dialysis.
9. Using a 0.5 × 10–cm chromatography column or Pasteur pipet plugged with glass wool, prepare a 1-ml column of Dowex AG 50W-X2 ion-exchange resin (see UNIT 10.10) in a cold room or cold box, using ice-cold water to suspend the resin and wash the column.

The resin must be extensively washed with ice-cold water before use. All chromatography steps must be performed at 4°C and eluants must be ice cold.

10. Load the dialysate from step 8 directly to the column. Wash column with 6 ml cold water and collect eluant in a 15-ml glass test tube on ice.

For large preparations, every step can be scaled up, but it may be necessary to lyophilize the dialysate and dissolve it in a small volume of water before loading it onto the column. The content of the dialysis tubing (the retentate, containing the desialylated glycoconjugates under study) can also be frozen, lyophilized, and reserved for other analyses.

11. Check pH of eluant with pH paper. If >3, go to step 12. If <3, add 10 mM sodium formate buffer, pH 5.5, until pH is >3.

12. Using a 0.5 × 10–cm chromatography column or Pasteur pipet plugged with glass wool, prepare a 1-ml column of Dowex 3-X4A ion-exchange resin (hydroxyl form). Convert resin to formate form by equilibrating in 3 vol of 1 M formic acid. Let column stand in contact with formic acid for 15 min, then wash with 10 mM sodium formate (pH 5.5) until effluent pH is stable at 5.5 (see UNIT 10.10).

Large batches of resin can be prepared and stored at 4°C. Always take into consideration the capacity of the resin and calculate the volume of the column required to bind the total sialic acids present in the sample as determined by the TBA assay in step 2.

13. Load sample from step 11 on column. Wash column immediately with 7 ml ice-cold 10 mM formic acid, and discard washings.

14. Elute sialic acids with 10 ml ice-cold 1 M formic acid. Collect eluant on ice in a 20-ml glass test tube, then evaporate it to dryness by lyophilization or in a shaker-evaporator with the temperature of the water bath maintained at <37°C. Store at −20°C until analysis.

The purified mixture of sialic acids can be analyzed by HPLC (UNIT 17.19A) or derivatized and analyzed by reversed-phase HPLC with fluorometric detection (UNIT 17.18).

RELEASE AND PURIFICATION OF 4-O-ACETYLATED SIALIC ACIDS

A harsher acid hydrolysis procedure is used to liberate 4-O-acetylated sialic acids, which are resistant to release by the method indicated in Basic Protocol 2.

**Additional Materials** (also see Basic Protocol 2)

- 23.4 M (concentrated) formic acid
- Heating block

1. Prepare the sample and determine total sialic acid content in an aliquot of the material by TBA assay (see Basic Protocol 2, steps 1 and 2).

2. Adjust dialyzed sample to pH 2.0 by dropwise addition of concentrated formic acid. Heat at 70°C for 1 hr. Cool to room temperature, and centrifuge at room temperature using a tabletop centrifuge to separate supernatant and pellet. Separate supernatant by aspiration and retain both supernatant and pellet.

When a heating block is used, care must be taken that the transfer of heat to the tubes is adequate. See Basic Protocol 2 step 4 annotation for proper technique.
3. Dialyze supernatant overnight at 4°C against 10 vol water using 1000-MWCO tubing, then lyophilize the solution outside the tubing. Repeat the dialysis twice again and pool the recovered material.

   When total volumes are small (100 µl to 1 ml), the dialysis tubing can be placed inside a test tube. In this case, it must be closed with knots instead of plastic clamps.

4. Treat the pellet obtained in step 2, resuspended in a similar volume of water, twice more at pH 2 with heating as described in step 2, centrifuging and dialyzing the supernatants each time as described in step 3 to recover the hydrolysis products.

5. Pool the lyophilized dialysates and purify the mixture of sialic acids by ion-exchange chromatography (see Basic Protocol 2, steps 9 to 14). The annotation to Basic Protocol 2, step 14 describes options for analysis.

**BASIC PROTOCOL 3**

**STRONG ACID HYDROLYSIS FOR QUANTITATIVE RELEASE OF HEXOSES, PENTOSES, HEXOSAMINES, AND URONIC ACIDS FROM GLYCOCONJUGATES**

This protocol describes several sets of conditions for the acid hydrolysis of monosaccharides from glycoconjugates. When no data is available from previous experiments regarding optimal conditions, it is recommended that one try more than one set of conditions to determine which ones are optimal. It is also important to prove the reproducibility of the method under the conditions chosen. The mixtures of free monosaccharides obtained by acid hydrolysis can be directly analyzed by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) or by derivatization followed by GLC (see UNIT 17.19A for both techniques).

**Materials**

Glycoconjugate-containing sample to be analyzed
Internal standard (i.e., a monosaccharide that does not occur naturally in the sample)
Standard mixture (see recipe)
2 M and 4 M trifluoracetic acid prepared from concentrated TFA (HPLC/spectra grade sequanal quality, Pierce)
99% methanol (anhydrous; e.g., Aldrich)

Heating block or oven
Small glass culture tubes or 3.5-ml glass vials with Teflon-lined screw caps
Nitrogen (N₂) or vacuum evaporation system: e.g., Speedvac (Savant) or shaker-evaporator (Baxter Scientific)

Additional reagents and equipment for phenol–sulfuric acid assay for monosaccharides (UNIT 17.9) and compositional analysis of monosaccharides released from glycoproteins (UNIT 17.19A)

1. If sufficient material is available, estimate the total carbohydrate content of the sample to be analyzed by the phenol–sulfuric acid assay (UNIT 17.9).

   Analysis can be done without having this information when sample is precious, as the analytical tools used for the compositional analysis of acid hydrolysates (i.e., HPAEC-PAD, GLC) are far more sensitive than the colorimetric assay.

2. Using N₂, a Speedvac system, or a lyophilizer, evaporate three aliquots of the sample to be analyzed (each containing 5 to 50 µg carbohydrate) to dryness in small glass tubes or 3.5-ml glass vials. If the planned compositional analysis will be done by GLC, add 1 to 10 µg of internal standard to the sample before evaporating it.
Rhamnitol and arabinitol are good choices as internal standards for analysis of animal glycoconjugates, and inositol for plant polysaccharides.

3. Prepare (as in step 2) three aliquots of the standard mixture appropriate for the monosaccharides to be determined and the analytical method to be used, and three blanks to detect possible contamination. For GLC analysis, also add the internal standard.

The time involved in HPLC or GPC analysis of each sample is quite long (see UNIT 17.19A); therefore, preparation of a mixture of the appropriate standards in equimolar amounts helps reduce the analysis time. On the other hand, when identification of one particular component is doubtful, coinjection of one aliquot of the sample with one aliquot of the suspected standard into the GLC or HPLC column gives a definitive answer on the presence of that particular monosaccharide. In such cases, processing each monosaccharide standard individually in parallel can be helpful.

An appropriate blank is a material that has been processed exactly as the sample was (e.g., a pool of fractions from a chromatography step that showed only background response to the method used for monitoring). Blanks are especially useful when very small amounts of material are processed, as in such cases the limit of sensitivity will be determined by the accumulated contaminants (e.g., from Sephadex columns or dirty glassware).

4. Dissolve the three evaporated aliquots of each sample, standard, and blank in 100 to 500 µl of 2 M TFA. Flush with N₂ and cap the tubes.

5. Using a 100°C heating block or oven, heat one sample, one standard, and one blank 3 hr; another sample, standard, and blank 4 hr; and the remaining sample, standard, and blank 6 hr.

This time course will determine the best conditions for maximum recovery with minimum destruction. When insufficient material is available for a time-course experiment, use 3 hr of hydrolysis for glycoproteins, glycopeptides, or free oligosaccharides of the N- and/or O-linked type that contain hexosamines and neutral monosaccharides. Use 6 hr of hydrolysis when the sample is suspected to contain glycosyluronic acids (e.g., proteoglycans or glycosaminoglycan chains).

When a heating block is used, care must be taken that the transfer of heat to the tubes is adequate. See Basic Protocol 1 step 4 annotation for proper technique.

6. After heating, evaporate solution in each tube using a nitrogen or vacuum evaporation system. Wash residues twice with 99% methanol, then evaporate again. Store the dry hydrolysates at −20°C until ready for compositional analysis.

7. Analyze the hydrolysate by HPAE-PAD or derivatize and analyze by GLC (see UNIT 17.19A).

8. Compare the recovery of each monosaccharide obtained using the different hydrolysis times and decide upon the most suitable hydrolysis time for the sample under study. Repeat the analysis using such conditions and check for reproducibility.

If quantitation remains uncertain, hydrolysis can be repeated with the addition of a known amount of an internal standard, even when HPLC is used for the analysis. Because the constituents will be known at this point, any noninterfering monosaccharide or alditol can be used as internal standard. The choice will depend on the method used for analysis. When the analysis is done by HPAE-PAD, monosaccharide alditols cannot be used as internal standards because they are not retained by the Carbo-Pak PA-1 column used for monosaccharide separation. In this case, 2-deoxyrhamnose or 2-deoxyglucose are appropriate as internal standards. Prepare an aqueous solution of the standard and add a known amount to the sample, evaporating them together before dissolving in the acid at step 4. The standard must be present in an amount comparable to that of the major constituents of the sample.
**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.

**Sodium formate buffer, pH 5.5**

Weigh 68 mg sodium formate, dissolve in water, then bring volume to 1 liter. Adjust to pH 5.5 by adding 1 M formic acid dropwise.

**Standard mixture**

*For GLC of alditol acetates:* Prepare an equimolar mixture of the following:
- D-\(\alpha\)-glucose (anhydrous, mixed anomers; Sigma)
- D-\(\alpha\)-galactose (crystalline; Sigma)
- D-\(\alpha\)-mannose (crystalline; Sigma)
- D-\(\alpha\)-xylose (99%; Sigma)

*For HPAE-PAD:* To the above mixture (without xylose), add equimolar amounts of:
- N-acetyl-D-galactosamine (Sigma)
- N-acetyl-D-glucosamine (Sigma)
- D-glucuronolactone (Sigma; when uronic acid is expected)

*If xylose is expected, include it in the mixture, but be careful to use the right conditions for HPAEC-PAD (UNIT 17.19A).*

**COMMENTARY**

**Background Information**

**Fucose release**

Fucosyl residues are typically in nonreducing terminal positions and are therefore susceptible to acid hydrolysis under relatively mild conditions. However, specific sites of attachment can increase the stability of the glycosidic bond (e.g., a positively charged amino group produced by de-N-acetylation of an N-acetyl-hexosamine residue will lead to a stable glycosidic linkage at the 3 position). Thus, it is necessary to determine for each specific glycoconjugate the conditions required for complete liberation with minimal destruction. The parameters to be varied are the type of acid, concentration of acid, and time and temperature used for the hydrolysis. The set of conditions suggested in Basic Protocol 1 are the best starting point.

Most of the problems encountered when trying to determine the best hydrolysis conditions for releasing fucose residues from a given glycoconjugate have been reviewed by Gottschalk (1972). Typical conditions are 0.5 M hydrochloric acid at 100°C for 16 hr (Schrager and Oates, 1968), although 15 min at 100°C in 1 M HCl are enough to release fucose from orosomucoid (Gottschalk, 1972), and 0.6 N sulfuric acid at 100°C for 1 hr is required to release all the fucose from total serum proteins (Gyorky and Houck, 1965).

**Sialic acid release**

More than 25 different kinds of modified sialic acids have now been reported to be found in nature. Most arise from substitution of the parent molecule with a variety of different groups. These modifications have been shown to affect a wide spectrum of biological phenomena. Many studies of sialic acids have failed to recognize the complexity of this family of sugars, because conventional methods for analyzing glycoconjugates—e.g., methanolysis, hydrazinolysis, methylation analysis, and \(\beta\)-elimination—result in the destruction of \(O\)-substituted species, especially \(O\)-acetyl esters.

The classical method for releasing sialic acids from glycoconjugates (incubation with 0.1 N H\(_2\)SO\(_4\) for 1 hr at 80°C) results in extensive destruction of \(O\)-acetyl groups (Schauer, 1978). In addition, the presence of \(O\)-acetyl groups makes the sialic acid molecule partially or completely resistant to release by all available bacterial and viral neuraminidases (Schauer, 1978, 1982; Schauer and Corfield, 1982). The use of milder conditions (incubation with 0.5 M formic acid for 1 hr at 80°C) allowed the release and identification of many previously undetected \(O\)-acylated sialic acids (Schauer 1978, 1982). However, even under these conditions quantitative analysis is not possible as a result of significant destruction and incomplete release of \(O\)-acylated species using formic acid (pH 2.1) for acid hydrolysis (Schauer, 1978; Varki and Kornfeld, 1980;...
Varki and Diaz, 1983). Prolonged hydrolysis in 2 M acetic acid (pH 2.4 to 2.5) at 80°C was found to achieve maximal release of O-acetylated sialic acids with minimal loss of O-acetyl groups (Varki and Diaz, 1983). These latter conditions have been successfully used to release and purify labile substituted sialic acids from different biological sources (Manzi et al., 1990). Hydrolysis times vary for different glycoconjugates. When sialic acids are acetylated at the 4 position, complete hydrolysis is difficult to achieve, but once released, the acetyl group becomes very labile, producing a 4,7-anhydro species.

Further complexity arises from the migration of O-acetyl groups from the 7 or 8 positions to the thermodynamically more stable 9 position of the sialic acid exocyclic side chain. This migration occurs rapidly, with $t_{1/2}$ ranging from minutes to hours, depending on the pH and temperature (Kamerling and Vliegenthart, 1989; Varki and Diaz, 1983). Migration is minimal between pH 3 and 5.

A recent review (Varki, 1992) covers the diversity in the sialic acids, including the different problems encountered during their analysis and the methods available to overcome these problems.

**Strong acid hydrolysis with TFA**

Hydrolysis of glycosidic linkages involving hexoses requires more vigorous conditions than the ones used for fucose or sialic acids. Typically, hydrochloric (1 or 2 M, 100°C, 1 to 6 hr), sulfuric (0.1 to 2 N, 100°C, 4 to 12 hr), or trifluoroacetic acid (2 M, 121°C, 1 to 2 hr) have been used for this purpose, under conditions that vary depending on the substrate. Provided that the most labile species—e.g., sialic acids—are quantitated separately, the most severe problem encountered when determining the monosaccharide composition is the incomplete release of 2-acetamido sugars and glycosyluronic acids. The presence of these sugars can often be predicted based on the origin of the glycoprotein under study. In the case of acetamido sugars, if N-deacetylation occurs first, the stability of the glycosidic linkage is increased. Therefore, the conditions must be such that hydrolysis of the glycosidic linkage is substantially faster than the de-N-acetylation reaction. The glycosidic linkage of glycosyluronic acids is particularly resistant to acid hydrolysis, and further inhibition of their complete release occurs when the adjacent sugar in the chain is an N-sulfated or N-acetylated hexosamine. This problem is routinely encoun-tered in glycosaminoglycan chains, where quantitative release requires a previous reduction of the carboxyl group. On the other hand, liberated glycosyluronic acids are very susceptible to degradation, particularly by decarboxylation. Therefore, analysis of glycosaminoglycan chains is better achieved using enzymes (see UNIT 17.19B).

When enough material is available, recovery and reproducibility can be assessed under several different sets of conditions. In many cases this is not possible, and incubation with 2 M TFA for 3 hr at 100°C is recommended under such circumstances.

A review of the different conditions used for the release of monosaccharides from glycoproteins, including a detailed analysis of the mechanisms of acid hydrolysis of glycosides, is provided by Gottschalk (1972). In most cases, hydrolysis with TFA will produce as good a recovery of fucose as hydrolysis with 0.5 M HCl, although in the former case other sugars are also released. Where the sample is incubated with 2 M TFA at 121°C, 25% of the fucose decomposes after 6 hr. However, because only 1 hr is required for complete release, this rate of decomposition is tolerable (Albersheim et al., 1967). Where the sample is heated at 100°C, 98% recovery of fucose is achieved even after 6 hr (Biermann, 1988). Usually, estimation of the content of hexosamines in glycoproteins has been based on HCl hydrolysis at different concentrations (ranging from 2 M to 6 M) at temperatures close to 100°C for varying periods of time (ranging from 1 to 24 hr; Gottschalk, 1972). Also, incubation with trifluoroacetic acid (0.5 M, 100°C, 19 hr; 4 M, 125°C, 1 hr; or 4 M, 100°C, 4 hr) has been shown to give good results (Keene et al., 1983; Neeser and Schweizer, 1984). The variety of methods clearly indicates that the effectiveness of the hydrolysis greatly depends on the glycoconjugate—e.g., the type of linkage, the type of glycone (noncarbohydrate portion), and the presence of substituents such as N-acetyl groups. Mannosamine is less acid-stable than glucosamine and galactosamine (Ludowieg and Benmaman, 1967). Analysis of the rates of decomposition of monosaccharides in the presence of 2 M (10 mg/ml) TFA—by continuing hydrolysis of polysaccharides after most of the monosaccharides have been liberated—have indicated that after 6 hr at 121°C, >50% of the xylose and arabinose and >25% of the galactose, rhamnose, and fucose are decomposed (Albersheim et al., 1967). However, as most of the monosaccharides are liberated within 1 hr,
these rates of decomposition are tolerable. Hydrolysis of urinary glycoconjugates with 2 M TFA for up to 15 hr at 100°C indicated that the maximum yield of aldoses and uronic acids is obtained after 6 hr. The recovery of the individual monosaccharides added at the beginning of the treatment was >93% in all cases, including that of glucuronic acid (Honda et al., 1981). A comparison between the action of different acids on each type of carbohydrate-containing molecule was done by Biermann (1988). Conditions used for acid hydrolysis of glycoconjugates before GLC analysis have also been reviewed by Kamerling and Vliegenthart (1989).

Critical Parameters

Fucose release. Use of high-quality acids and highly purified deionized water is recommended. In the case of glycoproteins, destruction arising from the interaction between liberated sugars and amino acids can be minimized by using a low concentration of glycoprotein to perform the hydrolysis (<1 mg/ml) and excluding oxygen (by displacing it with dry nitrogen or performing the hydrolysis in a tube sealed under vacuum).

Sialic acid release. All the parameters involved in the protocol (e.g., temperature, pH, and concentration) must be carefully controlled. Because pH values outside the range of 3 to 7 should be avoided, strongly basic anion-exchange resins (e.g., Dowex 1 or 2) should not be used. Samples must be fresh, and must be processed and analyzed immediately. It is also critical that purified samples be kept dry at −70°C or −80°C while stored. However, even storage at these temperatures for extended periods of time can result in migration or loss of O-acetyl groups. It should be pointed out that optimal methods for the release and purification of the rare forms of sialic acids (e.g., O-methylated, O-sulfated, or multiply modified) have not been adequately worked out.

Strong acid hydrolysis with TFA. High-quality acids must be used and highly purified deionized water must be used to dilute the acid, because trace iron can destroy hexosamines. Degradation is also avoided by excluding oxygen (usually by flushing with nitrogen, although it is also possible to work under vacuum). Working at a low concentration of glycoconjugate (<1 mg/ml) reduces the destruction of sugars. A competing sugar not present in the glycoprotein can also be added to reduce destruction (ribose has been used for this purpose). It must be kept in mind that—even when treating the standards in parallel—they may not reflect the decomposition suffered by the glycosidically bound monosaccharides in the sample, because in the latter case they spend less time as free glycoses. Therefore, a method that yields less decomposition is always preferred to a correction factor worked up on the basis of monosaccharide standards.

Anticipated Results

Fucose release. Basic Protocol 1 will cleave the glycosidic linkages of all terminal nonreducing fucosyl residues. Once free, the fucosyl residues will remain intact, giving a reasonable estimation of the amount of fucose in the glycoconjugate (UNIT 17.19). Complete defucosylation of a sample can be determined by analysis of the amount of fucose in the hydrolysate at different time points. However, the use of TFA (strong acid hydrolysis; Basic Protocol 3) permits an accurate estimation of fucose together with the rest of the sugars. Therefore, strong acid hydrolysis is recommended when an analysis of the total glycosyl composition is required. The most valuable use of Basic Protocol 1 is therefore the defucosylation of glycoconjugates for further analysis.

Sialic acid release. The conditions for release and purification of sialic acids used in these protocols ensure maximum release of most modified sialic acids (with the exemption of methylated or sulfated species), prevent migration of O-acetyl groups from the 7 position to the 9 position, and prevent de-O-acetylation. Therefore, careful use of the suggested protocols will result in the most accurate possible quantitation of the different sialic acid species present in a glycoconjugate. Sialic acids obtained by these procedures can be analyzed by HPAEC-PAD (UNIT 17.19A) or other HPLC techniques with (UNIT 17.18) or without derivatization (UNIT 17.19A). Therefore, an accurate quantitation also depends on the careful use of the final method of analysis. Basic Protocol 2 can be used to obtain a mixture of Neu5Ac, Neu5Gc, and of some of the mono-, di-, and tri-O-acetylated derivatives of these sialic acids from commercially available standards such as bovine submaxillary mucin. A mixture of standards will be very useful when analyzing the presence of sialic acid modifications in a glycoconjugate.

Strong acid hydrolysis with TFA. Careful application of Basic Protocol 3 will result in hydrolysis of the glycosidic linkages of hexoses, hexosamines, and uronic acids in glycoconjugates with minimal destruction.
Time Considerations

**Fucose release.** Because an 8-hr reaction is involved, it is better to prepare the samples the previous day. All hydrolysis and recovery procedures can be completed during the second day. Any additional time involved depends on the method used for analysis.

**Sialic acids.** After the time course to determine the time required for complete hydrolysis, release and purification require 2 days. Analysis must then be done immediately.

**Strong acid hydrolysis with TFA.** Preparation, hydrolysis, and drying of the hydrolysate should take ≤8 hr. The time required for analysis (by HPLC or GLC) is indicated in UNIT 17.19A.

Literature Cited


Key References


Gottschalk, 1972. See above.

These references discuss the mechanisms of acid hydrolysis of the different monosaccharides, and the influence of structure in determining alternative mechanisms. They also review many different hydrolysis conditions, and illustrate application of particular conditions to different types of glycoconjugates and the results obtained.

Varki and Diaz, 1983. See above.

Describes the parameters that must be varied to arrive at the optimal conditions used in the method described here.

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Preparation and Analysis of Glycoconjugates

17.16.11
Enzymatic Release of Oligosaccharides from Glycolipids

USE OF ENDOGLYOCERAMIDASE FOR SELECTIVE CLEAVAGE OF GLYCOLIPIDS

Selective cleavage of the oligosaccharide moiety of glycolipids for further structural analysis can be achieved by means of endoglycoceramidase (EGCase), an enzyme specific for the linkages between oligosaccharide and ceramide residues in glycolipids. The method can be used for analysis of glycolipids present in macroscopic amounts or for glycolipids that have been radiolabeled in their carbohydrate moiety. A variety of different methods can be used for analysis of the released oligosaccharides.

Materials

Sample to be analyzed—neutral glycolipids (UNIT 17.2), gangliosides (UNIT 17.17B), or desialylated gangliosides (see UNIT 17.12)—in organic solvent
1 mg/ml disialoganglioside-G_{D_{1a}} (Sigma) in 2:1 (v/v) chloroform/methanol
0.5% (w/v) sodium taurocholate in 0.05 M sodium acetate buffer, pH 6.0
Endoglycoceramidase (EGCase; from Rhodococcus sp.; for purification see Ito and Yamagata, 1989a)
Toluene
Methanol
60:40:9 (v/v/v) chloroform/methanol/0.2% (w/v; aqueous) calcium chloride
Orcinol/sulfuric acid reagent (see recipe)
2:1 (v/v) chloroform/methanol
Bath sonicator
Precoated high-performance thin-layer (HPTLC) silica gel 60 plates (10 × 10–cm; Merck)
Glass chromatography tank
140°C oven
Tabletop centrifuge

Digest sample with EGCase

1. Evaporate an aliquot of the sample to be analyzed to dryness in a glass vial. Simultaneously evaporate 10 µl of 1 mg/ml disialoganglioside-G_{D_{1a}} in the same manner, as a positive control.

   When analyzing complex mixtures of unknown composition, estimate the amount of sample to be aliquotted by carrying out this protocol with two or three different amounts of sample.

   Evaporation can be done by simply leaving the glass vial containing the chloroform/methanol solution open to the atmosphere or by using a Speedvac evaporator.

2. Dissolve the evaporated residues in 50 µl 0.5% sodium taurocholate in 0.05 M sodium acetate buffer, pH 6.0 by brief sonication in a bath sonicator.

   0.4% Triton X-100 can be used in place of sodium taurocholate. EGCase molecules aggregate in the absence of detergents.

3. Add 12 mU of EGCase to each sample. Place the vials containing the samples, with caps loosened, inside a closed container in which an open tube of toluene is also placed to create a toluene atmosphere. Begin incubating at 37°C.
4. At intervals of 0, 1, 3, 6, 15, and 20 hr of incubation, take an aliquot of the reaction mixture with a volume corresponding to ≥1 nmol of each glycolipid. Dilute each aliquot with 9 vol methanol.

**Determine optimal incubation time for cleavage**

5. Apply an aliquot of each diluted sample hydrolysate and G\textsubscript{Dla} hydrolysate (1 to 2 nmol) along a line drawn 1 cm from the bottom of an HPTLC silica gel 60 plate.

   *Depending on how many points are used, multiple plates may be required. Five to seven samples can be spotted per plate.*

   *The plates must be marked in advance with pencil lines 1 cm from the top and bottom of the plate. The bottom line should be further divided with pencil marks into 1-cm lanes with ~0.5-cm spacings between lanes. The lanes will correspond to the different incubation times used in step 4.*

6. Air dry plate and place in a glass chromatography tank that has been equilibrated with 60:40:9 chloroform:methanol:0.02% aqueous calcium chloride.

   *The HPTLC is to separate the glycolipids. Released oligosaccharides can be analyzed by HPTLC on silica gel 60 plates using 2:1:1 (v/v/v) 1-butanol:glacial acetic acid:water as the developing solvent.*

7. Let the solvent ascend until it reaches the upper line, then take the plate out of the tank and allow to thoroughly air dry in a fume hood.

8. Spray with orcinol-sulfuric acid reagent and heat at 140°C for 5 min.

   *Alternatively, if the carbohydrate moiety has been radiolabeled, the plate can be submitted to autoradiography.*

9. Note the lane in which complete release of oligosaccharides is indicated by the disappearance of the glycolipid bands that are present in the starting material.

   *If no lane indicating complete reaction is seen, longer incubation periods should be tried. The time points indicated to step 4 are for analysis of purified glycolipids for which some information is available. A time course up to 72 hr is suggested for unknown glycolipid structures. In these cases, add 36 mU of EGCase instead of 12 mU.*

**Analyze released oligosaccharides**

10. Set up reaction as described in steps 1 to 3, incubate for the time determined to be optimal for complete release of oligosaccharides, then stop the reaction by adding 2 vol of 2:1 chloroform:methanol.

11. Shake the mixture of aqueous cleaved glycolipid and organic solvent, then centrifuge 10 min at 2000 rpm in a tabletop centrifuge. Remove the upper (aqueous) phase containing the oligosaccharides and lyophilize. Store at −20°C.

   *The remaining glycolipids and ceramides will partition into the lower (organic) phase.*

   *The dried sample can be dissolved in water and submitted to a variety of different methods of oligosaccharide analysis. The method to be chosen will depend on the availability of instrumentation, the complexity of the sample, the amount of material available, and the questions to be answered. Possible methods for the analysis of the released oligosaccharides include: ion-exchange HPLC of anionic oligosaccharides (UNIT 17.21A) and sizing HPLC of neutral oligosaccharides (UNIT 17.21B). High-performance anion-exchange chromatography on pellicular-resin columns can also be used for both anionic and neutral oligosaccharides. Radiolabeled samples are monitored by liquid scintillation counting of collected fractions, and nonradiolabeled ones by pulsed amperometric detection (PAD).*
In most cases, dilution of the reaction mixture before injection into an HPLC system will suffice. When desalting by gel-filtration chromatography (UNIT 10.9) is required prior to further analysis, the released oligosaccharides can be separated from remaining glycolipids and ceramides and at the same time desalted by passing the reaction mixture from step 10 (without adding organic solvent) through a Dowex 50 (hydrogen form) gel-filtration column and washing with water. Long-chain nonpolar molecules will be retained by hydrophobic interactions and cations will be exchanged. The oligosaccharides that are recovered should be lyophilized. Alternatively, gel-filtration chromatography may be performed on the upper phase (step 11) using an 0.6 × 20–cm Sephadex G-10 column, eluting with water. The elution may be monitored by spotting aliquots of each fraction on a piece of silica gel–coated TLC plate and staining the carbohydrates with orcinol-sulfuric acid reagent (i.e., by spraying the dried plate and heating 15 min at 110°C). All fractions containing carbohydrates are then pooled and lyophilized.

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.

Orcinol/sulfuric acid reagent

Weigh 200 mg orcinol (5-methylresorcinol-H₂O, formula weight 142.15; Aldrich). Dissolve in 100 ml ice-cold 2 M sulfuric acid. Store in a glass container at 4°C; discard when solution turns brownish.

COMMENTARY

Background Information

Endoglycoceramidase (EGCase) is an enzyme capable of hydrolyzing the linkage between the oligosaccharides and ceramides of acidic and neutral glycolipids to produce free, intact oligosaccharide and ceramide residues. The enzyme does not cleave the linkages between monosaccharides and ceramides (in cerebrosides) or between oligosaccharides and diacylglycerol (in glycolipids). Commercially available EGCase is isolated from the culture supernatant of Rhodococcus sp. strain G-74-2, and has an apparent molecular weight >16 kDa as determined by gel-filtration chromatography on Sephadex G-100. This preparation contains three isozymes with molecular weights of 60,000 to 75 kDa as determined by SDS-PAGE.

One unit of enzyme is defined as the amount needed to catalyze the release of 1 µmol/min of reducing power (as glucose) from a substrate of mixed bovine brain gangliosides at 37°C, pH 6.0. The specific activity is not less than 15 mU/mg of protein.

Chemical treatments—e.g., ozonolysis and periodate oxidation—have been used to obtain free oligosaccharides from glycosphingolipids. However, these reactions cause disruption of the ceramide residues.

Ito and Yamagata (1986) reported a novel glycosphingolipid-specific endoglycosidase—endoglycosylceramidase—or “endoglycosidase” for short. The enzyme, isolated from the culture supernatant of Rhodococcus sp. strain G-74-2, was capable of cleaving the linkage between oligosaccharides and ceramide, releasing both parts intact. Later, similar enzymes called ceramide-glycanases were found in leeches (Li et al., 1986) and earthworms (Li et al., 1987). The crude enzyme, however, could not be used to release oligosaccharides from living cells because contamination with hemolysin caused cell death.

A variety of methods for the analysis of the released oligosaccharides have been developed. Some of them include a derivatization step to incorporate a fluorescent or hydrophobic tag, followed by HPLC analysis of the products (Shimamura et al., 1988; Rasilo et al., 1989). Analysis of the released oligosaccharides by HPAE-PAD (see UNIT 17.19A) has also been reported (Ito et al., 1991), as well as the use of the enzyme in the structural characterization of metabolically radiolabeled gangliosides (Manzi et al., 1990).

In 1989, a mutant strain of Rhodococcus sp., M-750, was isolated. This organism was capable of producing three forms of endoglycoceramidase, and two of these forms were purified (Ito and Yamagata, 1989a). The three isoenzymes were separated from each other and their substrate-specificity examined with various glycosphingolipids. EGCase I (with a pI of 5.3)
and EGCaSE II (with a pI of 4.5) hydrolyze the glucosyl-ceramide linkage of globo-, lacto-, and ganglio-type glycosphingolipids. EGCaSE I hydrolyzes globo-type glycosphingolipids much faster than EGCaSE II. Neither of these two isoenzymes hydrolyzes the galactosyl-ceramide linkage for which EGCaSE III is specific (Ito and Yamagata, 1989b). A new mutant, M-777, produced EGCaSEs at levels 5-fold higher than M-750 (Ito et al., 1991).

In spite of extensive purification of the enzymes, effective EGCaSE cleavage of glycosphingolipids in vivo while maintaining viability of the cells still presents two problems. First, the activity of the enzymes is very low in the absence of detergents, and a detergent capable of stimulating activity without impairing cell viability has not been found. Second, the optimal pH is quite acidic, which is another factor that prevents the study of the biological functions of glycosphingolipids. Recently, Ito et al. (1991) reported finding two activator proteins in the culture supernatant of *Rhodococcus* sp. strain M-777. Activator protein II was purified and shown to stimulate the activity of EGCaSE II much more than that of EGCaSE I, but was nonspecific with respect to the glycolipid. Activator protein I seems to be specific for stimulating the activity of EGCaSE I. Following the addition of activator protein II, EGCaSE II hydrolyzed cell-surface glycosphingolipids quite efficiently at a neutral pH where almost no hydrolysis occurred with EGCaSE II alone (Ito et al., 1991). In the near future, the use of activator proteins might permit the study of the biological functions of glycosphingolipids in living cells using EGCaSE.

**Critical Parameters**

If the activity of EGCaSE towards disialoganglioside-<sub>G<sub>1</sub>3</sub> is defined as 100% at pH 5 to 6, only 50% of that activity is observed at pH 7, and 20% at pH 4. The enzyme is completely inactive at pH 3. Maximum stability of the enzyme is observed between pH 6 and 8.

The enzyme is inhibited by Cu<sup>2+</sup>, Ba<sup>2+</sup>, and Hg<sup>2+</sup>. It is stable in buffered solution for 6 months at 4°C. Excessive freeze-thawing should be avoided.

The commercially available enzyme does not contain sphingomyelinase, β-galactosidase, β-mannosidase, β-galactosaminidase, β-glucosidase, α-fucosidase, or sialidase activity. Trace amounts of β-galactosaminidase, or α-mannosidase, as well as protease activity, can sometimes be detected—this is indicated in the data sheet provided by the manufacturer.

Sonication of the substrate prior to the addition of enzyme is recommended.

It is always necessary to determine empirically the time required for complete release, as different glycolipids exhibit different rates of hydrolysis.

**Anticipated Results**

The use of this protocol, in combination with appropriate analysis of the released oligosaccharides, will allow the structural characterization of the types of carbohydrate chains that are linked to glycolipids in a given system. However, as mixtures of glycolipids may be complicated, the usefulness of this protocol can be driven one step forward by studying purified glycolipids. Where this is done, the released oligosaccharides can be submitted to acid hydrolysis for composition analysis (UNITS 17.19A & 17.19B).

**Time Considerations**

Up to 3 days of incubation with the enzyme may be required for complete release. The time required for analysis of the released oligosaccharides depends on the method used.

**Literature Cited**


**Key References**

Ito and Yamagata, 1986. See above.  
*Describes the purification of the enzyme, its specificity, and examples of its use.*

Ito and Yamagata, 1989a. See above.  
*Describes the purification and properties of the enzyme and contains many references regarding its application.*

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Endo-β-Galactosidases and Keratanase

INTRODUCTION

Endo-β-galactosidases were discovered during the search for a keratan sulfate—degrading enzyme in the culture filtrates of Coccobacillus sp. (Hirano and Meyer, 1971), Pseudomonas sp. (Nakasawa and Suzuki, 1975), and Escherichia freundii (Kitamikado et al., 1970a, b, c). Later studies on the E. freundii keratanase demonstrated that this enzyme is capable of hydrolyzing a wide range of glycoconjugates—including nonsulfated oligosaccharides isolated from milk as well as the carbohydrate moieties of glycoproteins and glycolipids—thus expanding the applicability of this enzyme as an endo-β-galactosidase to a variety of complex glycoconjugates (Fukuda and Matsumura, 1975, 1976). Endo-β-galactosidase produced by Bacteroides fragilis has similar properties to that from E. freundii (Scudder et al., 1983). On the other hand, keratanase isolated from Pseudomonas sp. is specific to the sulfated polylactosamines, and thus only hydrolyzes keratan sulfates (Nakazawa and Suzuki, 1975; Fukuda, 1985; Nakasawa et al., 1984). An endo-β-galactosidase found in Diplococcus pneumoniae showed strict specificity toward blood-group A and B structures (Takasaki and Kobata, 1976). More recently, an endo-β-galactosidase specific to Galβ1→3Gal was found in the culture filtrate of Clostridium perfringens (Fushuku et al., 1987).

ENDO-ß-GALACTOSIDASE FROM E. FREUNDII

Source

The E. freundii line designated also as Citrobacter freundii EGI (Paoletti et al., 1990) can be obtained from American Type Culture Collection (ATCC; APPENDIX 4). The endo-β-glycosidase enzyme can be purified from the culture filtrate of this organism by affinity chromatography using side chain–free mucins.

Properties

Estimates of the molecular weight of the endo-β-galactosidase from E. freundii range from 28 to 32 kDa, depending on the method employed. The enzyme is stable in a solution of pH 4.5 to 5.5, but unstable below pH 4.0 or above pH 6.0. It is stabilized by addition of 450 µg/ml BSA to the enzyme solution. The activity of this enzyme is optimal between pH 5.5 and 5.8, using a sodium acetate buffer. The isoelectric point is estimated to be pH 8.0 (Nakagawa et al., 1980).

Substrates and Specificity

E. freundii endo-β-galactosidase hydrolyzes high-molecular-weight substrates at the middle of the carbohydrate chain by random depolymerization (Fukuda and Matsumura, 1976).

Cleavage of keratan sulfates

E. freundii endo-β-galactosidase hydrolyzes the β-galactosidic bond of the keratan sulfates as shown in Figure 17.17.1A, but does not cleave the bond if the adjacent galactose residue is sulfated at the C-6 position, as indicated in Figure 17.17.1B. The sulfate group on GlcNAc is not a necessary factor for E. freundii endo-β-galactosidase, as a nonsulfated disaccharide is released from the keratan sulfate. Sulfation of the galactose residue at the C-6 position, however, hinders hydrolysis of the galactosidic bond by endo-β-galactosidase.

Cleavage of glycoproteins

The usefulness of the E. freundii endo-β-galactosidase has been shown in detection of polylactosaminoglycans in glycoproteins. The endo-β-galactosidase was found to be active upon human erythrocyte band 3 carbohydrate (Fukuda et al., 1979), large glycopeptides from mouse embryonal carcinoma F9 cells (Muramatsu et al., 1979), and glycopeptides isolated from a GM1 gangliosidosisis patient (Tsai et al., 1975)—all of which were found to contain keratan-like polylactosaminoglycans with the aid of this enzyme. Polylactosaminoglycans are present in various glycoproteins—including a glucose-transporter protein, lysosomal membrane proteins, precursor of Rh antigen, fibronectin from human placenta, lymphocyte-associated antigen 1 (LFA-1) α and β-chains, and laminin.

Endo-β-galactosidase from E. freundii can hydrolyze galactose as shown in Figure 17.17.2. The galactose at the branch point is also susceptible, although with a low efficiency (Fukuda et al., 1984).

The endo-β-galactosidase can hydrolyze fucosylated lactosaminoglycans as shown in Figure 17.17.3, but the galactosyl linkage attached to the fucosylated N-acetylgalcosamine...
Cleavage of glycolipids

The so-called lacto-series glycolipids (R3 → GlcNAcβ1 → 3-Galβ1 → 4Glc → Cer) are susceptible to endo-β-galactosidase (Fukuda et al., 1976). For example, endo-β-galactosidase hydrolyzes lacto-N-neotetraosylceramide, producing trisaccharide and glucosylceramide as shown in Figure 17.17.4A and lacto-N-neohexaosylceramide and glucosylceramide—as shown in Figure 17.17.4B. Branched lacto-series glycolipids—e.g., lacto-iso-N-neooctaosylceramide—are hydrolyzed to a large oligosaccharide and glucosylceramide as shown in Figure 17.17.4C.

Although the β-galactosidic linkage at the branch point in the erythrocyte polylactosaminoglycan-peptides is cleaved by endo-β-galactosidase, under practical conditions the hydrolysis of glycolipids at the branch point is too poor to be detected.

Hydrolysis of x2-glycolipid (Kannagi et al., 1982b) indicates that not only the GlcNAcβ1 → 3Galβ1 → structure, but also the GalNAcβ1 → 3Galβ1 → structure is susceptible to endo-β-galactosidase.

Cleavage of oligosaccharides

E. freundii endo-β-galactosidase can hydrolyze oligosaccharides isolated from human...
Affinity of the enzyme for oligosaccharide substrates is weaker than that for polylactosamine, keratan sulfate, and glycolipid substrates. A high concentration of the enzyme is needed for an efficient enzymic hydrolysis of oligosaccharides.

Application of Endo-β-Galactosidase to Various glycoconjugates

Glycopeptides

To digest glycopeptides with endo-β-galactosidase, mix 10 to 100 µg of the glycopeptide—metabolically labeled (UNIT 17.4) or unlabelled—and 25 mU of endo-β-galactosidase with 200 µl of 0.2 M sodium acetate buffer, pH 5.0 (APPENDIX 2), then incubate at 37°C overnight. The oligosaccharides released by endo-β-galactosidase are usually analyzed by gel filtration (UNIT 10.9). In preparation for this, the digest of unlabeled glycopeptides is reduced with NaB[3H]₄ (see UNIT 17.5). The resulting [3H]-labeled oligosaccharide alcohols are then applied to a gel-filtration column packed with Sephadex G-50 (Pharmacia Biotech) and/or a column packed with Bio-Gel P-4 (Bio-Rad).

Upon Bio-Gel P-4 gel filtration, the oligosaccharide components are eluted at the elution volumes of disaccharides, trisaccharides, and tetrasaccharides; larger (or sialylated) molecules are eluted in the void volume. The results obtained for erythrocyte band 3 showed that the oligosaccharide alcohols released are:

- GlcNAcβ₁→₃Galβ₁→₄Glc β₁→₃Gal OH
- Galβ₁→₄GlcNAcβ₁→₃Galβ₁→₄Glc β₁→₃Gal OH
- Fucα₁→₂Galβ₁→₄GlcNAcβ₁→₃Gal OH

The oligosaccharide components eluted in the void volume from a Bio-Gel P-4 column are sialylated oligosaccharides—because after desialylation these components are often eluted at the elution volumes of trisaccharides or larger oligosaccharides. The structure of the smallest sialylated oligosaccharide component released from band 3 was:

NeuNAcα₂→₂Galβ₁→₄GlcNAcβ₁→₃Gal OH.
Because disaccharides should be produced from the internal moieties of polylactosamine in which tri- and tetrasaccharides represent nonreducing terminals. Thus, in the absence of fucosylation or sialylation the ratio of disaccharides to tri- and tetrasaccharides indicates the length of the polylactosamine repeats. These analyses, combined with methylation analyses of intact polylactosamines have yielded substantial structural information on glycoproteins.

**Glycolipids**

To hydrolyze glycolipids with endo-β-galactosidase, take an aliquot corresponding to 10 µg of glycolipid dissolved in 2:1 (v/v) chloroform/methanol and place in a small glass test tube or microcentrifuge tube. Dry the glycolipid sample under N₂ gas. To the dried sample, add 10 µl of 0.2 M sodium acetate buffer, pH 5.8 (APPENDIX 2) containing 20 µg sodium deoxytaurocholate, as well as 10 µl of 0.25 mU/µl endo-β-galactosidase. Incubate 2 hr to overnight to achieve enzymatic hydrolysis. Sialylated glycolipid—e.g., sialylparagloboside and linear lacto-series glycolipids—can be hydrolyzed easily under these conditions; stronger conditions—e.g., 10 × more enzyme—are required for hydrolysis of branched and/or highly fucosylated glycolipids.

**Intact cells (Fukuda et al., 1979)**

Endo-β-galactosidase can directly modify cell-surface glycoproteins and glycolipids. When human erythrocytes are treated with endo-β-galactosidase, Ii antigens and ABO blood-group antigens are abolished or decreased. In addition to these antigenic changes, polylactosamines on band 3 and glycoproteins on band 4.5 are hydrolyzed, though O-linked polylactosamines on band 3 and glycoproteins decreased. In addition to these antigenic changes, blood-group antigens are abolished or decreased. In addition to these antigenic changes, blood-group antigens are abolished or decreased. In addition to these antigenic changes, blood-group antigens are abolished or decreased. In addition to these antigenic changes, blood-group antigens are abolished or decreased. In addition to these antigenic changes, blood-group antigens are abolished or decreased. In addition to these antigenic changes, blood-group antigens are abolished or decreased. In addition to these antigenic changes, blood-group antigens are abolished or decreased. In addition to these antigenic changes, blood-group antigens are abolished or decreased. In addition to these antigenic changes, blood-group antigens are abolished or decreased.

To perform cell-surface labeling and endo-β-galactosidase digestion, the galactose and N-acetylgalactosamine residues on the cell surface can be specifically labeled by the galactose oxidase/NaB[3H]₄ method (Gahmberg and Hakomori, 1973). Wash the erythrocytes three times with cold PBS (APPENDIX 2), then suspend ~2 × 10⁹ cells (or 200 µl of packed cells) in 1 ml PBS pH 7.4. Add 10 µl of 1 U/ml galactose oxidase (Sigma) in PBS, pH 7.4 and incubate 2 hr at room temperature. After incubation, add 2.5 mCi NaB[3H]₄ in 50 µl 0.01 N NaOH and incubate 30 min at room temperature. Finally, add ~0.5 mg nonradioactive NaBH₄ and wash 3 × with cold PBS (pH 7.0), centrifuging between washes. Resuspend 0.1 ml of these surface-labeled cells (as packed cells) in PBS pH 7.0 to a final volume of 200 µl. To this cell suspension, add 25 µl of 1 U/ml endo-β-galactosidase, then incubate 2 hr at room temperature. Wash the cells with 3 ml cold PBS (pH 7.0) and centrifuge. The pelleted cells can be used for glycoprotein and glycolipid analysis, and the supernatant can be analyzed for released oligosaccharides. This procedure can be used not only for erythrocytes, but also for nucleated cells.

To analyze glycoproteins and glycolipids of surface-labeled cells, prepare erythrocyte membranes from the surface-labeled cells that have been treated with or without endo-β-galactosidase as described above. Membrane lysates from nucleated cells—e.g., lymphocytes and fibroblasts—can be prepared by vortexing the cells in an equal volume of cold PBS containing 0.5% NP-40 and 1 mM PMSF. The nuclei are then separated out by centrifugation, after which the supernatant containing the cell lysate may be dissolved in SDS sample buffer and subjected to SDS-polyacrylamide gel electrophoresis (UNIT 10.2), followed by fluorography (APPENDIX 3A) to detect radioactive protein components.

To extract glycolipids from erythrocyte membranes or intact cells, these are dissolved in 10 vol of 2:1 (v/v) chloroform/methanol and the extract fractionated by Folch partition (UNIT 17.3) into lower and upper phases. If necessary, upper-phase glycolipids can be further separated into neutral and acidic glycolipids by DEAE-Sephadex. Each glycolipid fraction is finally analyzed by thin-layer chromatography followed by fluorography. Oligosaccharides released from the cell surface by endo-β-galactosidase treatment can also be analyzed by gel-filtration chromatography on Sephadex G-50 and by paper chromatography.
Source
Endo-β-galactosidase from B. fragilis is commercially available from Genzyme (Appendix 4).

Properties
The properties and specificities of the B. fragilis endo-β-galactosidase are similar to those of the corresponding E. freundii enzyme. The enzyme can be stored at −20°C for long periods of time; storage at a protein concentration of 44 µg/ml at 4°C for 2 to 3 months did not affect the activity, but storage at a low concentration (<3 µg/ml) causes a significant loss of activity. In the presence of 0.2 mg/ml of albumin, the enzyme retains its activity over a wide range of pH.

Substrates and Specificity (Scudder et al., 1983, 1984, 1986)
B. fragilis endo-β-galactosidase hydrolyzes keratan sulfate and produces sulfated and non-sulfated oligosaccharides, which are identical to the digestion products of keratan sulfate with E. freundii endo-β-galactosidase. Hydrolysis of oligosaccharides by endo-β-galactosidases from B. fragilis and E. freundii shows that both enzymes hydrolyze the internal Galβ1→4Glc linkage of type 1 and type 2 oligosaccharides. The type 2 oligosaccharide lacto-N-neotetraose was hydrolyzed to a greater extent than its type 1 isomer, lacto-N-tetraose. B. fragilis endo-β-galactosidase hydrolyzes the linear but not the branched carbohydrate chain of lacto-series glycolipids. Because the B. fragilis endo-β-galactosidase is quite similar to that from E. freundii, this enzyme can be applied widely to the analysis of glycoconjugates, as described for the E. freundii enzyme.

ENDO-β-GALACTOSIDASE FROM FLAVOBACTERIUM KERATOLYTICUS (Kitamikado et al., 1981)
Endo-β-galactosidase from F. keratolyticus has been purified, and the specificity of this enzyme has been compared with that of the E. freundii enzyme. F. keratolyticus endo-β-galactosidase is similar to the E. freundii enzyme, except that the F. keratolyticus enzyme can hydrolyze oligosaccharide substrates more efficiently.

BLOOD-GROUP TYPE A AND B–SPECIFIC ENDO-β-GALACTOSIDASE FROM DIPLOCCUS PNEUMONIAE (Takasaki and Kobata, 1976)
Culture filtrate from Diplococcus pneumoniae (also called Streptococcus pneumoniae) is known as a rich source of endo- and exoglycosidases including β-galactosidase, β-N-acetylgalcosaminidase, endo-α-N-acetyl-β-galactosaminidase, endo-N-acetylglucosaminidase (endo-D), endo-β-galactosidase DII, and blood group A and B–specific endo-β-galactosidase. The last enzyme hydrolyzes the structure shown in Figure 17.17.5. It hydrolyzes the blood-group A determinant faster than the B determinant, and hydrolyzes the oligosaccharides with A and B determinants that are composed of type 2 chains, but not those composed of type 1 chains.

ENDO-β-GALACTOSIDASE DII FROM DIPLOCCUS PNEUMONIAE (Fukuda, 1985)
An endo-β-galactosidase that hydrolyzes the internal β-galactosidic linkages of the structure shown in Figure 17.17.6 was found in the culture supernatant of D. pneumoniae. This enzyme was named endo-β-galactosidase DII to differentiate it from the previously isolated blood-group type A and B–specific endo-β-
galactosidase from the same bacterium. The specificity of endo-β-galactosidase DII is similar to that of *E. freundii* endo-β-galactosidase, as both recognize the N-acetyllactosaminyl structure and hydrolyze internal β-galactosyl linkages. However, endo-β-galactosidase DII differs from the *E. freundii* enzyme in that it does not degrade keratan sulfate. Endo-β-galactosidase DII hydrolyzes glycolipids and polylactosaminoglycans endoglycosidically, but from the nonreducing-terminal end.

### ENDO-β-GALACTOSIDASE C FROM CLOSTRIDIUM PERFRINGENS (Fushuku et al., 1987)

Endo-β-galactosidase C hydrolyzes β-galactosidic linkages of glycoproteins and glycolipids that have the terminal structure Galα1→3Galβ1→4GlcNAc as shown in Figure 17.17.7A. This terminal Galα1→3Galβ1→4GlcNAc structure is present in mice, rabbits, cattle, and new world monkeys, but not in humans, apes, or old world monkeys.

The carbohydrate moiety of a glycoprotein that has a Galα1→3Galβ1→4GlcNAc→ terminal structure can be efficiently hydrolyzed by endo-β-galactosidase C, which releases the disaccharide Galα1→3Gal. Glycolipids having this terminal structure can also be hydrolyzed. For example, pentaglycosylceramide isolated from rabbit erythrocyte membranes is hydrolyzed as shown in Figure 17.17.7B.

The enzyme, however, does not hydrolyze blood-group B oligosaccharide (as shown in Figure 17.17.7C) although it hydrolyzes Galα1→3Galβ1→4GlcNAcβ1→3 hexane 1,2,5,6 tetrol. Endo-β-galactosidase C does not hydrolyze polylactosaminoglycans from human erythrocytes of blood-group B. Thus, the enzyme is different from the endo-β-galactosidase from *D. pneumoniae*, which acts on blood group A and B antigens, as well as the endo-β-galactosidases from *E. freundii*, *B. fragilis*, and *F. keratolyticus*, which act on polylactosaminoglycans. In addition, endo-β-
galactosidase C does not hydrolyze keratan sulfate.

**KERATANASE FROM PSEUDOMONAS SP. (Nakazawa and Suzuki, 1975)**

**Source**
Keratanase (or keratan sulfate–specific endo-β-galactosidase) has been purified from *Pseudomonas* sp. cell extract and is commercially available from Miles (APPENDIX 4).

**Properties**
The purified enzyme has been stored in ice. The pH optimum is between 7.2 and 7.4 using corneal keratan sulfate as substrate. At a 1 mM concentration, Ca²⁺, Mg²⁺, and Mn²⁺ have no effect on the enzymatic activity. Acetate and maleate elicited a slight increase in activity (∼15%) at concentrations of 0.05 M.

**Substrates and Specificity**
Keratanase from *Pseudomonas* sp. hydrolyzes keratan sulfate but not chondroitin sulfates, hyaluronic acid, dermatan sulfate, or heparin. In contrast to *E. freundii* endo-β-galactosidase, this enzyme does not hydrolyze lacto-N-tetraose, lacto-series glycolipids, or erythrocyte polyglycosaminoglycans—thus *Pseudomonas* endo-β-galactosidase is specific for keratan sulfate. Two sulfated oligosaccharides—6-sulfo-GlcNAcβ1→3Gal and 6-sulfo-GlcNAcβ1→3-6-sulfo-GlcNAcβ1→4-6-sulfo-GlcNAcβ1→3Gal—have been identified in the digestion products from keratan sulfates produced by this enzyme. However, the nonsulfated disaccharide GlcNAcβ1→3Gal is not produced.

**Applications**
This enzyme has been quite useful in distinguishing keratan sulfates or sulfated polyglycosaminoglycans from nonsulfated polyglycosaminoglycans (Nakazawa et al., 1984).

**ENDO-β-GALACTOSIDASE SPECIFIC TO THE REGION LINKING CHONDROITIN SULFATE AND THE CORE PROTEIN (Takagaki et al., 1990a,b)**

Endo-β-galactosidase that degrades the linkage between a chondroitin sulfate and core protein has been reported in rabbit liver and in the midgut gland of the mollusk *Patinopecten* sp. Chondroitin sulfate–containing proteoglycans were digested with endo-β-xylosidase from the midgut gland of *Patinopecten* sp., and the released chondroitin sulfate (with an R-Galβ1→3Galβ1→3Galβ1→4Xyl terminus) was conjugated with pyridylamine (PA) by reductive amination. This chondroitin sulfate–PA was incubated with a crude enzyme solution from the midgut gland of *Patinopecten* sp. HPLC analysis of the PA-oligosaccharide product identified Galβ1→4Xyl-PA, suggesting strongly that an endo-β-galactosidase had specifically hydrolyzed the Galβ1→3Gal linkage in the linkage region of chondroitin sulfate.

**Literature Cited**


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ANALYSIS OF SACCHARIDES RELEASED FROM GLYCOCONJUGATES

Many of the more specific and sensitive methods for analyzing the structure of oligosaccharides require that the oligosaccharides be first released from the glycoconjugate to which they are attached and, if necessary, purified away from the protein moiety. The units presented in this section describe various ways in which such released oligosaccharides can be analyzed. It must be recognized that no matter how good such a method is, it is highly dependent upon the quality of the preceding steps—i.e., the specific and quantitative release of the oligosaccharide from the glycoconjugate and the use of an appropriate purification scheme that does not result in selective losses. The methods presented in this section include those designed for the analysis of monosaccharides (UNIT 17.18) and those designed for the study of oligosaccharides (or glycopeptides; UNITS 17.19-17.23). The first group primarily provide monosaccharide compositional information and are a good starting point for the analysis of a previously unexplored glycoconjugate. Methods in the latter group range from simple techniques for determining basic properties of an oligosaccharide, to complex and sophisticated methodologies requiring the use of expensive instruments.

There are of course many other well-established techniques and approaches to the structural analysis of oligosaccharides, some of which require specialized instrumentation (e.g., mass spectrometry and nuclear magnetic resonance). The choice of techniques for inclusion in this section was based partly on the criteria of ease of use and broad general utility to the average molecular biologist.

Analysis of Monosaccharides

This unit presents methods for assaying sialic acids, reducing sugars, and hexosamines. The BCA assay (Basic Protocol 1) detects free reducing terminii in sugars released from glycoconjugates by appropriate treatments. Assays employing Ehrlich reagent (DMAB) detect hexosamines and N-acetylhexosamines (Basic Protocol 2 and Alternate Protocol); Basic Protocol 2 also includes a method for hydrolyzing the glycosidic linkages of the hexosamines and a method for re-N-acetylation is described in Support Protocol 1). The TBA (Basic Protocol 3) and DMB assays (Basic Protocol 4) can be used to quantitate and fractionate free forms of many types of sialic acids; techniques for liberating the sialic acids from the parent glycoconjugates are provided in Support Protocols 2 and 3. (Another alternative, the ferric orcinol method presented in UNIT 17.9, can measure both free and glycosidically bound sialic acids irrespective of modifications, but with much reduced sensitivity.)

Note that the acid hydrolysis conditions used to release sialic acids are much milder than those used for other monosaccharides—indeed, sialic acids are destroyed under the harsher conditions. Therefore, a sialic acid-containing molecule needs to be subjected to at least two separate analyses—one for sialic acids and one for the other monosaccharides.

BCA ASSAY FOR REDUCING SUGARS WITH SPECTROPHOTOMETRIC DETECTION

This protocol presents the most sensitive method developed to date to measure free reducing termini in sugars. This type of assay is useful for determining the total amount of oligosaccharides released from N-linked or O-linked glycoconjugates by enzymatic treatment (UNIT 17.13A) or from glycolipids by endoglycoceramidase (UNIT 17.17A). It may also be used to determine the total sugars present after acid hydrolysis.
Materials

Sample in water or aqueous buffer
Standard: e.g., glucosamine hydrochloride or N-acetyl-D-glucosamine (both available from Sigma)
Bovine serum albumin (BSA)
BCA reagent (reagent C; see recipe)
16 × 115–mm Pyrex test tubes
Clean glass marbles
Heating block, 80°C
Spectrophotometer and 1.0-ml glass cuvettes

1. In 16 × 115–mm Pyrex test tubes, prepare duplicates containing aliquots of sample and of 2 to 25 nmol of an appropriate standard in 300 µl water. Also prepare a blank consisting of 300 µl of water or the buffer in which sample is dissolved, and a control containing BSA at a concentration equal to the maximum protein concentration expected in the samples.

Avoid borate ions in the buffer, but if these cannot be avoided, make sure to adjust the pH to 10.6 to avoid interference.

The expected reducing sugar for the type of sample under analysis should be used as standard. N-acetylglucosamine should be used for oligosaccharides released from N-linked glycoproteins; glucose for oligosaccharides released from gangliosides.

2. Add 700 µl BCA reagent to each tube, vortex, and cover with glass marbles to prevent evaporation. Heat 30 min at 80°C.

3. Allow tubes to cool to room temperature. Transfer solutions to 1.0-ml glass cuvettes and measure the absorbance at 560 nm, adjusting against the values obtained for the blank.

4. Prepare a standard curve of absorbance vs. nmol reducing sugar.

5. Determine the amount of reducing sugar in each sample by reference to the standard curve.

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.

BCA reagent

Reagent A: Dissolve 1.5 g disodium 2,2′-bicinchoninate (4,4′-dicarboxy-2,2′-biquinoline; FW 388.3; Sigma) in 1 liter water. Add 71.6 g anhydrous sodium carbonate (ACS reagent grade, FW 105.99; Sigma) while stirring. Dilute to 1.15 liters with water. Store in a dark bottle at room temperature (stable several months).

Reagent B: Dissolve 3.5 g aspartic acid (FW 133.1; Sigma) and 5.0 g anhydrous sodium carbonate in 100 ml water, shaking to dissolve (this produces foam). In a separate container, dissolve 1.09 g copper sulfate (CuSO₄·5 H₂O, FW 249.6; ACS reagent grade) in 40 ml water. Mix the two solutions and dilute to 150 ml with water (a temporary fluffy blue precipitate may appear). Store at 4°C (stable several months).

Reagent C (BCA reagent): Mix 23 ml reagent A and 1 ml reagent B with 6 ml of 100% ethanol. The solution will have an intense blue color and strong fluorescence. Store in a brown bottle up to 2 weeks at 4°C (discard earlier if purple background color increases). Let stand 2 hr before use.
Commentary

Background Information

Most assays proposed for reduction of sugars make use of inorganic oxidants that accept electrons from sugar aldehyde groups. Aldoses are converted to aldonic acids (and higher oxidation products) and either the metal ions directly formed, metal oxides, or the complexation products of the latter with chromogenic chelators are quantitated. The appropriate oxidation/reduction potential for this reaction depends on the pH and complexation constants.

The BCA reagent forms colored complexes with copper(I); it is possible to couple this reaction to the reduction from copper(II) to copper(I) produced by reducing sugars in alkaline solution.

The most commonly used reduction assay for sugars is the one developed by Park and Johnson (1949) as a modification of the colorimetric method of Folin and Malmros (1929). This method, based on the production of Prussian blue following the reduction of ferricyanide ions in alkaline solution, has a detection limit of ∼1 µg (5 nmol) reducing sugar.

Mopper and Gindler (1973) later proposed a new colorimetric reagent for automatic sugar chromatography that was sensitive to ∼10⁻¹⁰ mol of sugar in the ethanol/water mobile phase. The dye is based on the formation of a deep-blue complex with Cu(I) ions and 2,2'-bicinchoninic acid in alkaline solution. The reaction is first order over a wide range. However, the sensitivity of the reaction is lower and the linearity range is reduced in water solution. Moreover, when borate ions are present, the Mopper-Gindler reagent produces only a faint color. Sinner and Puls (1978) achieved increased color formation by adjusting the reagent/borate buffer mixture, when used, to the optimal pH of the reaction (10.6), and increased the linear detection range by doubling the BCA concentration. Using these conditions, 55 pmol can be detected in the effluent from an HPLC column with an on-line UV spectrophotometer. McFeeters (1980) adapted the method to manual sugar determinations using ethylene glycol instead of ethanol to increase color formation. The conditions used for on-line detection can also be applied to manual analysis provided special care is taken to compensate for the volatility of ethanol.

Waffenschmidt and Jaenicke (1987) have suggested that for the manual spectrophotometric quantitation of sugar samples, the sensitivity of the reaction could be improved by replacing the aspartic acid in the BCA reagent with serine.

Critical Parameters

Using manual spectrophotometry, reliable detection is achieved with <3 nmol of mono-, oligo-, or polysaccharides. Buffering salts, sulfuric acid, sodium hydroxide, hydrochloric acid, and acetic acid do not interfere. It is also possible to work in the presence of borate ions if the pH is adjusted. The expected reducing sugar for the type of sample under analysis is used as a standard. However, linear responses may not be obtained with increasing size of the oligosaccharide (Doner and Irwin, 1992). Proteins cause a low level of interference of ~50:1 (w/w) protein/sugar.

Anticipated Results

With this protocol it is possible to detect with confidence 3 nmol of reducing sugar (mono-, oligo- or polysaccharide) in aqueous solution.

Time Considerations

The assay can be completed in ~1 hr.

Literature Cited


Key Reference

Waffenschmidt and Jaenicke, 1987. See above.

*Describes the modification of the original method for improved sensitivity that is used in this protocol.*
ASSAYS FOR FREE HEXOSAMINES WITH SPECTROPHOTOMETRIC DETECTION

Free hexosamines can be quantitated spectrophotometrically by a series of procedures derived from the Elson-Morgan and Morgan-Elson reactions that can be used for both hexosamines and N-acetylated hexosamines. These assays require a hydrolysis step (see Basic Protocol 2) to cleave the glycosidic linkages of the hexosamines. This hydrolysis also produces de-N-acetylation. Thus, a re-N-acetylation step is required (see Support Protocol 1) for the Morgan-Elson assay.

Acetylacetone/DMAB (Elson-Morgan) Assay for Free Hexosamines

Materials

- Hexosamine-containing sample (≥6 µg hexosamine) in aqueous solution
- 1 mM hexosamine standard stock solution(s): N-acetylglucosamine and/or N-acetylgalactosamine (see recipe)
- 4 M HCl (diluted from ACS reagent-grade concentrated HCl)
- Acetylacetone reagent (see recipe)
- 95% ethanol
- Ehrlich reagent (DMAB)/HCl (see recipe)
- 13 × 100-mm Pyrex test tubes with Teflon-lined screw caps
- Nitrogen or vacuum evaporation system (Speedvac or shaker-evaporator)
- Heating block or oven
- Boiling water bath
- Spectrophotometer and 1.0-ml glass cuvettes

Liberate hexosamines from glycosidic linkages

1. Dry triplicate aliquots of the sample (containing ≥6 µg hexosamine) in 13 × 100-mm Pyrex test tubes with Teflon-lined screw caps.

2. From hexosamine standard stock solution(s), prepare triplicate standards containing 10, 20, 30, 40, 50, and 60 µg N-acetylglucosamine and/or N-acetylgalactosamine in water. Prepare a blank containing the same total volume of water.

3. Dissolve samples, standards, and blank in 4 M HCl (0.4 ml/mg dry sample). Flush with nitrogen and cap tubes. Heat 4 hr at 100°C.

   Because the stability of this type of linkage depends on the structure of the glycoconjugate, optimal conditions for hydrolysis should be determined for each particular case (see UNIT 17.16). When using HCl, concentrations from 2 to 6 M and hydrolysis times from 2 to 10 hr should be tried.

4. Evaporate the HCl in a vacuum evaporation system or under nitrogen. Place the tubes overnight in a desiccator containing sodium hydroxide pellets under vacuum.

Quantitate hexosamines by condensation with alkaline acetylacetone and DMAB

5. Dissolve the hydrolysates in 250 µl water.

6. Add 500 µl acetylacetone reagent to each sample, standard, and blank. Vortex well and cap. Heat 10 min in a boiling water bath.

7. Cool to room temperature. Add 2.5 ml of 95% ethanol and vortex. Incubate 5 min at 75 ± 2°C.

8. Add 500 µl DMAB/HCl and heat 30 min at 75 ± 2°C.

9. Cool tubes to room temperature and add 2.5 ml of 95% ethanol. Let stand 30 min.
10. Carefully transfer the solutions to 1.0-ml glass cuvettes and measure the absorbance at 520 nm, adjusting against the values obtained for the blank.

11. Prepare a standard curve of absorbance vs. nmol hexosamine using the average value for each concentration.

12. Determine the amount of hexosamine in the sample by reference to the standard curve.

**Acidic DMAB (Morgan-Elson) Assay for Free N-Acetylhexosamines**

*Additional Materials* *(also see Basic Protocol 2)*

- 20 mM potassium tetraborate
- Ehrlich reagent (DMAB)/HCl/acetic acid (see recipe)

1. Liberate hexosamines from their glycosidic linkages (see Basic Protocol 2, steps 1 to 4). Re-N-acetylate the samples (see Support Protocol 1).

2. Dissolve each sample in 250 µl water.

3. Add 50 µl of 20 mM potassium tetraborate to each sample, standard, and blank. Vortex well and cap. Heat 3 min in a boiling water bath.

4. Cool rapidly to room temperature in a water bath.

5. Add 1.5 ml DMAB/HCl/acetic acid, washing down any condensate that has formed, and vortex. Incubate 20 min at 37°C.

6. Cool to room temperature. Carefully transfer the solutions to 1.0-ml glass cuvettes and measure the absorbance at 585 nm, adjusting against the values obtained for the blank.

7. Prepare a standard curve of absorbance vs. nmol of hexosamine using the average value for each concentration.

8. Determine the amount of hexosamine in the sample by reference to the standard curve.

**RE-N-ACETYLATION OF FREE HEXOSAMINES**

Hexosamines that have been released from their glycosidic linkages are re-N-acetylated prior to detection using potassium tetraborate and DMAB/HCl/acetic acid (see Alternate Protocol).

*Additional Materials* *(also see Basic Protocol 2)*

- 1.5% (v/v) acetic anhydride (99%, Aldrich) in acetone (HPLC grade, Fisher)
- Aqueous sample containing hexosamines liberated from glycosidic linkages by acid hydrolysis, and glucosamine and galactosamine standards treated in the same fashion (see Basic Protocol 2, step 2)

1. Add 100 µl of 1.5% acetic anhydride in acetone to 800 µl of the aqueous solution of the samples containing the liberated hexosamines obtained by acid hydrolysis.

2. Repeat the procedure for aqueous solutions of standard glucosamine and galactosamine hydrochlorides.

3. Incubate samples and standards 5 min at room temperature.

4. Dry under vacuum or with a nitrogen stream.
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.

Acetylacetone reagent

Prepare 48 ml of 0.625 M sodium carbonate (FW 106; anhydrous, ACS reagent grade) in water. Add 2 ml acetylacetone (2,4-pentanedione, FW 100.1; Sigma). Prepare just prior to use.

Ehrlich reagent (DMAB)/HCl/acetic acid

Add 1.5 ml water to 11 ml concentrated HCl. Add 87.5 ml glacial acetic acid (FW 60.05; 99.99%; Aldrich). Dissolve 10 g p-dimethylaminobenzaldehyde (DMAB)—4-(N,N-dimethylamino)benzaldehyde, DMAB; crystalline, ACS reagent grade, FW 149.2—in the HCl/acetic acid mixture. Store in a dark bottle at room temperature (stable several weeks). Dilute 10 ml of mixture to 100 ml with glacial acetic acid immediately before using.

Ehrlich reagent (DMAB)/HCl

Weigh 1.6 g p-dimethylaminobenzaldehyde (DMAB)—4-(N,N-dimethylamino)benzaldehyde, DMAB; crystalline, ACS reagent grade, FW 149.2. Dissolve in 30 ml concentrated HCl. Store in a dark bottle at room temperature (stable several weeks).

Hexosamine standard stock solutions, 1 mM

Glucosamine: Dissolve 9 mg d-glucosamine hydrochloride (2-deoxy-d-glucose hydrochloride, FW 179.2) or 11 mg of N-acetyl-d-glucosamine (2-acetamido-2-deoxy-d-glucose, FW 221.2) in water. Dilute to 50 ml with more water. Store in aliquots at −20°C and thaw as needed.

Galactosamine: Dissolve 9 g N-acetyl-d-galactosamine hydrochloride (2-deoxy-d-galactose hydrochloride, FW 179.2) or 11 g N-acetyl-d-galactosamine (2-acetamido-2-deoxy-d-galactose, FW 221.2) in water. Dilute to 50 ml with more water. Store in aliquots at −20°C and thaw as needed.

Hexosamines may be obtained from Sigma.

Commentary

Background Information

The hexosamines glucosamine and galactosamine are the only amino sugars detected to date as structural components of glycoproteins and glycolipids. They generally occur in N-acetylated form. Provided they are quantitatively released from their glycosidic linkages, hexosamines can be quantitated colorimetrically. The primary problem, however, is achieving this quantitative liberation, because amino sugars are extremely stable under strongly acidic conditions (see UNIT 17.16).

Most methods reported for colorimetric quantitation of glucosamine and galactosamine, N-acetylated or not, are based on the procedures described by Elson and Morgan (1933) and Morgan and Elson (1934).

The basis of the Morgan-Elson reaction for quantitating N-acetylglucosamine (2-acetamido-2-deoxy-d-glucose) and N-acetylgalactosamine (2-acetamido-2-deoxy-d-galactose) is the red color produced by various N-acetylhexosamine derivatives on treatment with acidic Ehrlich reagent (p-dimethylaminobenzaldehyde, or DMAB) following pretreatment with alkali. Detection of free amino sugars using this reaction can be achieved by including a re-N-acetylation step in the procedure (Roseman and Ludowieg, 1954; Levey and McAllan, 1959). A more sensitive method was developed based on the observation that the presence of borate buffer increases the intensity of color in the reaction (Reissig et al., 1955). Different structures have been proposed for the chromogens formed during the alkaline treatment of N-acetylhexosamines in the Morgan-Elson reaction (Foster and Horton, 1959).
The Elson-Morgan reaction for quantitating glucosamine (2-amino-2-deoxy-D-glucose) and galactosamine (2-amino-2-deoxy-D-galactose) involves the production of a red color upon successive treatment with alkaline acetylacetone (2,4-pentanedione) and the Ehrlich reagent. Both hexosamines produce similar color intensities (Rondle and Morgan, 1955). Several red compounds with different ultraviolet absorption maxima are produced in the Elson-Morgan reaction. The proportion of these products depends on the conditions used for the reactions with acetylacetone and the Ehrlich reagent.

Different methods for quantitating both hexosamines individually (when both are present), based on their different reactivities and the differential effects of borate ions in the reactions, have also been developed (Roseman and Daffner, 1956; Tracey, 1955; Ludowieg and Benmaman, 1967).

The protocols described here are those suggested by Chaplin (1986)—i.e., direct reaction with Ehrlich reagent (Alternate Protocol)—and by Montreuil (1986)—i.e., condensation with acetylacetone/Ehrlich reagent (Basic Protocol 2).

**Critical Parameters**

The presence of neutral sugars and amino acids interferes with the Elson-Morgan assay (Basic Protocol 2), because these react to produce orange chromophores. Therefore, when working with crude hydrolysis mixtures, interfering compounds should be removed before the assay is performed. The reactivity of all 2-amino-2-deoxy sugars in this assay is equivalent. Very careful control of the reaction conditions is required to obtain reproducible results, however, as different chromophores may play significant roles and some chromophores are lent. Very careful control of the reaction conditions is required to obtain reproducible results, and different 2-acetamido sugars. Therefore, when the Alternate Protocol is used, the absorption curve of hexosamines shows a maximum at 520 nm but the extinction coefficients for glucosamine and galactosamine are different. Sialic acids react to produce a blue coloration, and muramic acids and N-acetylhexitosamines interfere. Sodium tetraborate and sodium chloride decrease the intensity of the coloration.

**Anticipated Results**

Any of the protocols described here will give a fair estimation of the total content of hexosamines in a given glycoconjugate, provided hexosamine release is complete.

**Time Considerations**

The most time-consuming step of this procedure is hydrolysis of the hexosamines, particularly when conditions for complete release need to be determined. The colorimetric reactions take 1 hr (Alternate Protocol) or 2 hr (Basic Protocol 2), respectively. If re-N-acetylation is required, an additional hour will be necessary.

**Literature Cited**


THIOBARBITURIC ACID ASSAY FOR SIALIC ACIDS WITH SPECTROPHOTOMETRIC OR HPLC DETECTION

Unconjugated sialic acids can be measured by oxidation with periodate followed by reaction with the chromogen 2′-thiobarbituric acid (TBA) to form a salmon-pink compound. This chromophore can be detected either by conventional spectrophotometry, with a sensitivity of 1 to 10 nmol, or by HPLC, with a sensitivity of 2 to 10 pmol. The latter approach requires more equipment, but provides substantially improved sensitivity and eliminates interference by some compounds. The most common types of sialic acid, N-acetyl- and N-glycolylneuraminic acid (Neu5Ac and Neu5Gc, respectively), can be accurately quantitated by this assay. Modified (e.g., O-acetylated) sialic acids give variably reduced color response. Glycosidically bound molecules must be released by treatment with base (see Support Protocol 2) followed by mild acid (see Support Protocol 3) or sialidase treatment (UNIT 17.12) before detection.

Materials
- Sialic acid–containing sample in solution
- Periodate reagent (see recipe)
  - 1 mM N-acetylnearaminic acid (Neu5Ac; store at −20°C; stable indefinitely)
  - 1 mM 2-deoxyribose (optional; store at −20°C; stable indefinitely)
- Arsenite reagent (see recipe)
- TBA reagent (see recipe)
- Cyclohexanone (for spectrophotometric detection)
- HPLC elution buffer (see recipe; for HPLC detection)
- Heating block, 100°C
- For spectrophotometric detection:
  - New 12 × 75–mm glass tubes
  - Clean glass marbles
  - Tabletop centrifuge
  - Spectrophotometer and 1.0-ml glass cuvettes
- For HPLC detection:
  - 0.4 × 25–cm C18 reversed-phase HPLC column
  - Equipment for HPLC (UNIT 10.2)
  - HPLC UV detector with flow detector cell of volume <20 µl, and integrating chart recorder if available

Prepare sample, standards, and controls
1. Prepare two identical 50-µl samples of free or released sialic acid in sample buffer. Add 25 µl periodate reagent to the sample without touching the sides of the tube. Vortex briefly at low speed to mix.
Free sialic acid samples may be derived from biochemical samples (e.g., purified proteins, total serum glycoproteins, or lipid extracts) either by sialidase digestion (UNIT 17.12) or by acid treatment (see Support Protocol 2 or UNIT 17.16).

Use new 12 × 75-mm glass tubes if later detection will be by spectrophotometric assay or 1.5-ml polypropylene microcentrifuge tubes for later HPLC assay.

2. Prepare a series of dilutions of Neu5Ac in the same buffer as that in which the sample is dissolved. Use a range of 1, 4, 10, and 15 nmol for the spectrophotometric assay and 2, 5, 20, 100, and 500 pmol for the HPLC assay. Also prepare two blank samples containing the same total quantity of buffer only. Treat standards and blanks with periodate as described in step 1.

Optionally, samples with 5 to 10 nmol 2-deoxyribose may also be prepared to be used as standards for interfering compounds in the sample.

Oxidize and react with TBA

3. Incubate all tubes 20 min at room temperature.

4. Slowly add 250 µl arsenite reagent along the rim of each tube so it flows down the sides. Watch for a brown color to begin to develop in the sample and standard tubes (up to 2 min), then vortex vigorously.

   The brown color results from the reduction of the periodate, which forms iodine. If any periodate remains unquenched, the TBA chromophore will not be produced. Occasionally the brown color will not appear (Troubleshooting).

5. Incubate tubes 5 min at room temperature.

   At this point the tubes can be stored overnight at 4°C if desired.

6. Add 1 ml TBA reagent to each tube, mix well, and heat 15 min at 100°C. Cover each glass tube with a clean glass marble to minimize evaporation, or pierce microcentrifuge tube caps with a needle after closing. A pink color will develop in samples containing >0.5 nmol sialic acid. Chill 2 to 3 min on ice and keep on ice until chromophore detection is performed (beginning at step 7a or 7b).

   Volumes of sample, periodate, and arsenite can be changed as long as the proportions remain the same. However, the volume of TBA can be decreased independently if necessary, to accommodate a small tube size (e.g., microcentrifuge tube).

Detect chromophore by spectrophotometric measurement

7a. Add 1 ml cyclohexanone to each tube. Vortex well to extract the pink chromophore into the upper (cyclohexanone) layer, then centrifuge 5 min at 500 to 2000 × g (e.g., 1500 rpm in a tabletop centrifuge), room temperature, to separate the phases.

   Depending on the size of the cuvettes, the volume of cyclohexanone can be reduced to improve sensitivity.

8a. Transfer an appropriate volume of the cyclohexanone layer (0.3 to 0.8 ml, depending on cuvette size) to a 1.0-ml glass cuvette. Read its absorbance at both 549 nm and 532 nm, adjusting against the values obtained for the blank.

   The chromophore generated from sialic acids has an absorption maximum of 549 nm. Interfering substances, including 2-deoxyribose and peroxidation products of fatty acids, produce a chromophore with an absorption maximum of 532 nm.

9a. Calculate the ratios of A_{549}/A_{532} for the sialic acid standards and the sample. If the ratios match, this indicates that there is no interference. If the ratio is lower for the sample than the standard, perform the calculation indicated in steps 10a to 12a to compensate for interference.
10a. Calculate the value of \((0.9 \times A_{549} - 0.3 \times A_{532})\) for samples and standards.

11a. Plot a standard curve with these values and determine amount of sialic acid in the samples.

12a. Apply the calculations to the 2-deoxyribose sample to assure validity (this value should be close to zero after correction).

These correction coefficients are based on the original publications of Warren (1959) and Aminoff (1959) and generally work for most spectrophotometers. However, variations in slit width between instruments may decrease the correction coefficient accuracy. If unacceptable variance is found, correction coefficients should be determined directly as described in those original publications. The correction becomes less accurate if \(A_{532}\) is \(>0.7 \times A_{549}\) and the result is practically meaningless if \(A_{532}\) is \(>1.5 \times A_{549}\).

Detect chromophore by HPLC measurement

7b. Equilibrate an 0.4 × 25-cm C\(_{18}\) reversed-phase HPLC column in HPLC elution buffer running at 0.7 to 1.0 ml/min. Connect column to a UV detector set at 549 nm. Connect the UV detector to an integrating data module, if available.

Faster flow rates, within the pressure limits of the column, will reduce the time required and will not adversely affect sensitivity. Columns 10 to 15 cm long may also be utilized.

All HPLC buffers must be filtered and degassed. A flow detector cell with a volume of <20 \(\mu\)l is essential for maximal sensitivity.

8b. Keep the tubes on ice. Immediately prior to performing HPLC, microcentrifuge all tubes 2 to 4 min at \(>5,000 \times g\), 4°C, to remove any precipitated reagents.

Keeping the samples chilled maximizes the precipitation of unused TBA, reducing the risk that it will precipitate in the HPLC apparatus.

9b. Inject aliquots of the derivatized Neu5Ac standards (and 2-deoxyribose standard, if included) and collect data using UV detector with flow recorder.

Chromophores resulting from these two sugars will elute at 5 to 10 min and 10 to 20 min, respectively, depending on the particular column and the methanol concentration in the elution buffer.

The concentration of methanol in the HPLC elution buffer should result in elution of the TBA chromophore at 6 to 10 min. If the chromophore elutes too early on the particular column in use, decrease the methanol concentration; likewise, increase methanol concentration if the peak elutes too late.

10b. Quantitate the signal from each standard, and blank by integration and construct a standard curve.

If a data module capable of integration is not available, the signal can be quantitated by measuring the peak height from a chart recorder.

11b. Inject 10 to 400 \(\mu\)l of each sample and collect data as in step 10b. Calculate the amount of sialic acid present based on the standard curve.

The amount of sample injected should be based on the amount of sialic acid believed to be present. Quantitation is not affected by the volume injected. Total volumes of sample and reagents can be decreased proportionately to further improve sensitivity by increasing the amount of chromophore present in the injected volume.

When setting up the column for the first time, inject sample of buffer blank or (derivatized) Neu5Ac (100 to 500 pmol) to verify elution times.

At the end of each day wash the column thoroughly with 50% acetonitrile or 50% methanol.
DE-O-ACETYLATION OF SIALIC ACIDS

O-acetylated sialic acids are detected with reduced sensitivity by the TBA assay. Furthermore, O-acetylation may reduce the release of sialic acids from glycoconjugates by either acid hydrolysis (see Support Protocol 3) or some sialidases (UNIT 17.12). Because sialic acids from some biological source may be as much as 50% to 100% O-acetylated, de-O-acetylation with base treatment is necessary for accurate quantitation. This fact can be useful, as the amount of color yield in the TBA assay before and after base treatment can be used to recognize the presence of O-acetylated sialic acids. Alternatively, specific O-acetylated sialic acid species can be identified by the DMB assay (see Basic Protocol 4).

Materials

Sialic acid–containing sample in solution
0.2 M NaOH (store at room temperature)
0.9 M H₂SO₄ (for acid release) or 0.2 M acetic acid (for enzymatic release)

1. Mix 20 µl sialic acid–containing sample with 40 µl of 0.1 M NaOH and incubate 30 min at 37°C.

2a. For acid release: Add 4.5 µl of 0.9 M H₂SO₄ to bring to a final concentration of 0.1 M H₂SO₄ (in excess of Na₂SO₄) and proceed directly to BHT treatment (see Support Protocol 3, steps 2 to 4).

If the sample is free of potential lipid contaminants, the BHT step may be omitted and the sample utilized directly in the TBA or DMB assay.

2b. For enzymatic release: Neutralize with 20 µl of 0.2 M acetic acid (check pH on a blank sample to verify it is between 5 and 7). Proceed to sialidase treatment (UNIT 17.12).

ACID RELEASE OF DE-O-ACETYLATED SIALIC ACIDS

Acid treatment (0.1 M H₂SO₄) can be used as an alternative to sialidase digestion (UNIT 17.12) to release glycosidically bound sialic acid molecules for subsequent quantitation by the TBA assay. O-acetylation is incompletely and variably destroyed by this type of acid treatment. On the other hand, O-acetylation can itself hinder the release of sialic acids. Thus, it is best to eliminate all O-acetyl esters prior to acid hydrolysis by base release (see Support Protocol 2). Alternatively, to preserve O-acetylation (for study with the DMB assay; see Basic Protocol 4), sialic acid may be released by mild acid treatment as described in UNIT 17.16.

Materials

Sialic acid–containing sample in solution, preferably de-O-acetylated (see Support Protocol 2)
0.2 M H₂SO₄
1% (w/v) BHT in ethanol (see recipe)
Clean glass marbles
Heating block, 80°C

1. Mix 25 µl sample with 25 µl of 0.2 M H₂SO₄ in the bottom of a clean glass tube.

If the sample has been de-O-acetylated in 40 µl of 0.1 M NaOH and 4.5 µl of 0.9 M H₂SO₄ added (in Support Protocol 2), this will result in a final excess acid concentration of 0.1 M and the same final volume.
2. Add 0.5 µl of 1% BHT in ethanol to sample.

*BHT prevents interference caused by peroxidation of hydroxylated fatty acids.*

3. Cover the tube with a clean glass marble to minimize evaporation and heat 1 hr at 80°C.

4. Cool sample on ice. Proceed with TBA assay (see Basic Protocol 3, starting at step 1) without further adjustment.

**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.

**Arsenite reagent**

Dissolve 10.0 g sodium arsenite and 7.1 g sodium sulfate in 70 ml of 0.1 M sulfuric acid. Add additional acid to bring volume to 100 ml. Store indefinitely at room temperature. 

*The final sodium arsenite concentration is 10% (w/v).*

**Butylated hydroxytoluene (BHT) in ethanol**

Dissolve 100 mg BHT (2,6-di-tert-butyl-4-hydroxyanisole, FW 220.4; Sigma) in 10 ml of 100% ethanol. Store tightly capped at room temperature indefinitely.

**HPLC elution buffer**

Prepare a 2:3:5 (v/v/v) mixture of water/HPLC-grade methanol/0.2% (v/v) ortho-phosphoric acid (85%, ACS reagent grade). Filter mixture and degas.

**Periodate reagent**

128 mg sodium meta-periodate (Sigma; 0.2 M final) 
1.3 ml H₂O 
1.7 ml phosphoric acid *(ortho)-phosphoric acid, 85%, ACS reagent grade* 
Mix well to dissolve 
Store indefinitely at 4°C in a dark, foil-covered bottle

**TBA reagent**

Dissolve 14.2 g sodium sulfate in 170 ml water. Add 1.2 g 2-thiobarbituric acid *(4,6-dihydroxyypyrimidine-2-thiol, 98%; Sigma)*. Complete the dissolution of the TBA by adding 1 M NaOH dropwise to ∼pH 9, then dilute with water to 200 ml. Store at room temperature.

*The solution is stable almost indefinitely—even when a yellow precipitate appears, the reagent can still be used if care is taken to avoid the particulate material.*

*If HPLC detection is used, sodium sulfate may be omitted from both the TBA and arsenite solutions, as its function is to “salt out” the TBA chromophore into the cyclohexanone layer.*

**Commentary**

**Background Information**

The original assay for sialic acids described independently by Warren (1959) and Aminoff (1959) is based upon the formation of an adduct between the periodate oxidation product of free unsubstituted sialic acids and 2′-thiobarbituric acid (TBA). The procedure has been modified over the years (Schauer, 1978; Varki and Kornfeld 1980; Powell and Hart, 1986) to maximize its sensitivity and specificity and to minimize interference. The most useful modifications have been the following:

1. Prior de-O-acetylation to eliminate the interference of O-acetyl esters with the perio-
date oxidation of sialic acid. An increase in color yield after de-O-acetylation indicates the presence of O-acetylation.

(2) An increase in the acid concentration in the arsenite reagent. This seems to avoid subsequent reaction failure by ensuring complete destruction of the periodate.

(3) Addition of BHT (butylated hydroxytoluene) during acid or enzyme release of sialic acids. This minimizes lipid peroxidation and formation of interfering malonaldehyde (Critical Parameters).

(4) Use of reversed-phase HPLC to remove interfering chromophores and improve sensitivity.

Critical Parameters

Complete oxidation of sialic acids in the presence of high concentrations of phosphoric acid and periodate yields β-formyl pyruvic acid. One molecule of this compound condenses with two molecules of TBA (under elevated temperature) to produce the brightly colored TBA adduct (Paerels & Schut, 1965). O-acetetyl esters on the hydroxy group of sialic acids can cause varying degrees of interference, ranging from none (4-O-acetylation) or 50% (9-O-acetylation) to >90% (7-O-acetylation, multiple O-acetylations). Prior de-O-acetylation prevents this interference in the color yield. Less common substitutions (e.g., O-methyl groups) can also cause varying degrees of lower reactivity in comparison to Neu5Ac. For unknown reasons, the color yield also varies according to the substitution at the 5 position, in the approximate ratio of 1:0.8:2.0 for Neu5Ac, Neu5Gc, and 2-keto-3-deoxyoctonate (KDO—as well as, presumably, 2-keto-3-deoxyxynulosonic acid, KDN) respectively. For working purposes, Neu5Ac is initially used as a standard. Once the approximate amount of sialic acids in the sample is known, more detailed analysis of the types present can be performed (see Basic Protocol 4).

Compounds which, under the conditions of strong acid and periodate, yield β-formyl pyruvic acid will yield a salmon-pink TBA chromophore of \( \lambda_{\text{max}} \) 549 nm. These compounds include Neu5Ac, Neu5Gc, KDO, KDN, and the 3-keto-2-deoxyyaldulosonic acids. Under these conditions other compounds yield malonaldehyde which, following condensation with TBA, yields an orange-yellow chromophore of \( \lambda_{\text{max}} \) 532 nm. These compounds include 2-deoxyribose and the oxidation products of unsaturated lipids. Spontaneous peroxidation of lipids to malonaldehyde frequently occurs during sample handling and is accelerated by heat, light, acid pH, and some cations (lipids are frequently encountered, as sialic acids are often associated with membranes). Spontaneous peroxidation can be reduced or prevented by adding antioxidants such as BHT prior to storage or hydrolysis of samples. The presence of malonaldehyde-yielding substances can be detected and corrected for by measuring \( A_{549} \) and \( A_{532} \) (see Basic Protocol 1). Moreover, the two different chromophores arising from β-formylpyruvic acid and malonaldehyde are easily resolved by HPLC.

Troubleshooting

Failure of brown color to develop after addition of arsenite reagent may be caused by overly rapid addition of the arsenite or inadequate acid in the sample. This latter indicates that the periodate has not been fully consumed and will interfere with formation of the TBA chromophore. Alternatively, even if the brown color is seen, traces of periodate remaining on the walls of the reaction vessel (and therefore not exposed to the arsenite) may still also interfere. Certain buffers (particularly formate, which is frequently used during sialic acid purification) interfere when present in large quantities, giving a bright yellow color instead of the expected salmon pink. The predicted elution times of the chromophores from the C\(_{18}\) column are based on experience with several different columns from different suppliers. If trouble is encountered in detecting elution of the chromophores (from sialic acid or 2-deoxyribose), inject 10 to 50 ml of a visibly pink standard solution (e.g., 5 nmol). Two peaks should be seen eluting after the breakthrough peak (\( V_0 \)), corresponding to the TBA adducts formed from β-formylpyruvic acid (eluting first) and malonaldehyde (eluting second). An even later-eluting reagent peak may also be seen, at an elution time 2 to 3 times that of the TBA chromophore, depending on the injected volume. If these peaks are not seen, or elute later than the indicated times, increase the concentration of methanol and allow the column to equilibrate at least 30 min between changes in methanol concentration.

Anticipated Results

For both the spectrophotometric and HPLC assays, the standard curve for \( A_{532} \) vs. nmol sialic acid should be linear over the suggested concentration ranges. Significant deviation from linearity indicates either improper dilution of the sialic acid standard or improper
concentration of one of the reagents. If modifications are made in the outlined protocol, the ratios of sample/periodate/arsenite must be kept constant, as these are optimized for concentrations of periodate, phosphoric acid, and arsenite. An excessive amount of any reagent can be as detrimental as an insufficient amount.

**Time Considerations**

Preparation of the standard curve and samples should take <3 hr. Determining the absorbance of each sample by HPLC will take ~15 to 20 min. Setup time for HPLC is indicated in UNIT 10.12.

**Literature Cited**


**Key References**

Aminoff, D. 1959. See above.

Warren, L. 1959. See above.

*Original descriptions of the TBA assay; outline factors affecting its validity and reproducibility, including reaction times, reagent concentrations, and interfering compounds.*

**DMB ASSAY FOR SIALIC ACIDS WITH HPLC DETECTION**

The method of choice for characterizing the individual components of a sialic acid mixture is the preparation of fluorescent derivatives. Mixtures of free sialic acids obtained using the protocols described in UNIT 17.16 or UNIT 17.12 can be reacted directly with 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB) without the need for further purification. DMB derivatives of sialic acids are analyzed by HPLC on a reversed-phase column and detected fluorometrically.

**Materials**

- *N*-acetylneuraminic acid (Neu5Ac, MW 309.3; Boehringer Mannheim)
- Standard mixture: sialic acids prepared from bovine submaxillary mucin (BSM) using the procedure in UNIT 17.16, or mixture of 100 pmol each Neu5Ac and *N*-glycolylneuraminic acid (Neu5Gc, MW 325.3; Sigma)
- Sample containing mixture of free sialic acids in solution (5 to 1000 pmol; UNIT 17.16 or UNIT 17.12)
- DMB reagent (see recipe)
- Acetonitrile (HPLC grade)
- 50% methanol (HPLC grade) in Milli-Q-purified water  
- Milli-Q-purified water: deionized water passed through a five-stage Milli-Q Plus system (Millipore)
- Helium (for HPLC apparatus)
- Heating block, 50°C  
- HPLC apparatus (preferably ternary system) including pump and fluorescent detector (UNIT 10.12)
- TSK gel ODS-120T column (250 mm × 4.6 mm i.d., 5 µm particle size; Tosohass)
- Guard cartridge TSK gel ODS-120T (1.5 × 3.2 mm)
- Filtering unit with 0.45-µm Nylon 66 membranes (Alltech)
**Prepare fluorescent derivatives of sialic acids**

1. Transfer sample containing 5 to 1000 pmol free sialic acids to a 1.5-ml microcentrifuge tube and dry in a Speedvac. Prepare 1 nmol of each standard (Neu5Ac and standard mixture) in the same fashion.

2. Dissolve the samples in 1 to 5 µl water.

3. Add 30 µl DMB reagent and wrap each tube in aluminum foil. Heat 2.5 hr in a heating block at 50°C.

4. Remove and place on ice. 

   *Analysis should be done as soon as possible, but the DMB derivatives can be kept overnight at 4°C.*

**Analyze the fluorescent derivatives by HPLC with fluorometric detection**

5. Turn on the fluorescent detector. Set emission wavelength to 448 nm, excitation wavelength to 373 nm, high voltage to 900, range to 500, and response time to 0.5 sec.

6. Fill the three HPLC bottles with (respectively) acetonitrile, 50% methanol, and filtered Milli-Q-purified water. Degas for 15 min and filter through 0.45-µm membranes.

7. Run each solvent through the attached lines of the HPLC apparatus to eliminate any bubbles.

8. Set the pump control unit for an isocratic elution with a ratio of 9:7:84 (v/v/v) acetonitrile/methanol/water.

   *Because 50% methanol is used (to avoid the production of bubbles that occurs when mixing water and pure methanol), 14% of this solvent needs to be run to obtain 7% methanol (i.e., run 9 parts acetonitrile, 14 parts methanol/water mix, and 77 parts water). When a ternary HPLC system is not available, the eluant can be prepared ahead in the ratio stated above.*

9. Begin running the solvent at 0.3 ml/min, then connect the TSK gel column to the Guard cartridge. Increase the flow 0.1 ml/min at a time, waiting for stable backpressure between steps, until 0.9 ml/min flow is attained. Wait until a stable baseline is obtained.

   *The following settings are adequate for most integrators: attenuation, 512; peak threshold, 10000.*

10. Inject 100 pmol DMB-derivatized Neu5Ac standard. Check the sensitivity and adjust the integrator settings as required (the way this step is performed will depend on the particular integrator used). Repeat the run if necessary.

11. Inject DMB-derivatized standard mixture (100 pmol each Neu5Ac and Neu5Gc, or mixture containing O-substituted standards prepared from BSM, if available).

12. Inject the derivatized samples. Adjust the integrator settings as necessary, and repeat to obtain all peaks in scale. Calculate the retention time of each peak relative to Neu5Ac and compare with those listed in Table 17.18.1.

   *Keep the volume of DMB reaction mixture injected as low as possible to maintain column performance. In other words, it is better to use lower attenuation in the integrator and inject a smaller volume of reagent into the HPLC column. When the total amount of sialic acids in the sample is not known, first inject 10% of the total reaction mixture with a low attenuation setting, then if necessary inject more or change the settings.*
13. Determine the units of area per picomole for Neu5Ac and/or Neu5Gc. Assuming equal detector responses, calculate the number of picomoles represented by each peaks produced by the samples.

_This system can be used to analyze radioactive samples by routing the column effluent to a radioisotope flow detector or fraction collector. The appropriate ratio of scintillation fluid needs to be checked because of the quenching effect of acetonitrile._

14. Verify the presence of the O-acetylated species (see Table 17.18.1 for relative retention times) by collecting each peak, lyophilizing, subjecting to de-O-acetylation (see Support Protocol 2), and reanalyzing. Alternatively, another aliquot of the starting material can be submitted to de-O-acetylation prior to derivatization. In this case, all peaks corresponding to O-acetylated species will collapse into the peak of their parent molecule (Neu5Ac or Neu5Gc).

15. At the end of the day, wash the column with 50% methanol for 30 min at a flow of 0.5 ml/min.

_When resolution becomes poor, the column can be cleaned by running it in reverse flow at 0.5 ml/min with a gradient from 10% to 100% acetonitrile in 0.05% trifluoroacetic acid over 1 hr. The column must then be extensively washed with water at 0.5 ml/min, turned around, and reequilibrated with 50% methanol._

### 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.*

**DMB reagent**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB, FW 225.1; Sigma)</td>
<td>1.6 g</td>
<td>Sigma</td>
</tr>
<tr>
<td>Glacial acetic acid (HPLC grade; 1.4 M final)</td>
<td>80.4 µl</td>
<td>Sigma</td>
</tr>
<tr>
<td>2-mercaptoethanol (FW 78.13; Bio-Rad)</td>
<td>52.8 µl</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>Sodium hydrosulfite (FW 174.1; 50% pure, Sigma) in water at 4°C</td>
<td>72 µl</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

*DMB reagent is light sensitive.*

_The 0.25 M sodium hydrosulfite solution is made by dissolving the compound (FW 174.1; ~80% pure, Sigma) in water at 43.5 mg/ml._

### Table 17.18.1 Relative Elution Times of DMB-Derivatized Sialic Acids from TSK-ODS Column

<table>
<thead>
<tr>
<th>Sialic acid</th>
<th>$t_{Neu5Ac}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neu5Ac</td>
<td>1.00</td>
</tr>
<tr>
<td>Neu5Gc</td>
<td>0.84</td>
</tr>
<tr>
<td>Neu5-7Ac$_2$</td>
<td>1.06</td>
</tr>
<tr>
<td>Neu5-9Ac$_2$</td>
<td>1.57</td>
</tr>
<tr>
<td>Neu4-5Ac$_2$</td>
<td>1.68</td>
</tr>
<tr>
<td>Neu5-7(8)-9Ac$_4$</td>
<td>1.90</td>
</tr>
<tr>
<td>Neu5-7-8-9Ac$_4$</td>
<td>1.98</td>
</tr>
<tr>
<td>Neu5Gc9Ac</td>
<td>0.88</td>
</tr>
<tr>
<td>Neu4Ac5Gc</td>
<td>1.53</td>
</tr>
</tbody>
</table>
Commentary

Background Information

Sialic acids released by hydrolysis in 2 M acetic acid or by enzymatic treatment are converted with 1,2-diamino-4,5-methylenedioxybenzene (DMB, a fluorogenic reagent for α-keto acids) to highly fluorescent derivatives (see Fig. 17.18.1) without the occurrence of O-acetyl migration and de-O-acetylation.

The derivatives are separated using a reversed-phase TSK ODS-120T column eluted isocratically with 9:7:84 (v/v/v) acetonitrile/methanol/water at room temperature in ~25 min. Fluorescence of the eluate is monitored at an excitation wavelength of 373 nm and an emission wavelength of 448 nm. The average limits of detection are 1 to 2 pmol at a signal-to-noise ratio of 3.

Fluorogenic reagents may be used for detection of α-keto acids in very dilute solutions because of their quantitative conversion into the corresponding substituted quinoxalines (Spiker and Towne, 1962; Hara et al., 1985a). Because sialic acids are α-keto acids, Hara et al. (1985b) tried several of these reagents and found that 1,2-diamino-4,5-dimethoxybenzene reacts with Neu5Ac and Neu5Gc under conditions different than those for other α-keto acids and forms fluorescent compounds. They developed a method for detecting small amounts of Neu5Ac and Neu5Gc in serum and urine using HPLC (Hara et al., 1986) and further extended the method to the analysis of glycoproteins and glycolipids (Hara et al., 1987a). Screening different diaminobenzenes as fluorogenic reagents revealed that 1,2-diamino-4,5-methylenedioxybenzene (DMB) is the best reagent for sensitive and rapid detection of α-keto acids (Hara et al., 1987b).

The DMB method could not be applied to O-acetylated sialic acids because of de-O-acetylation and O-acetyl migration in the sulfuric acid used in the reaction. However, O-acety-

![Figure 17.18.1](figure.png)

**Figure 17.18.1** Reaction of sialic acids with 1,2-diamino-4,5-methylenedioxybenzene (DMB) to form fluorescent derivatives.
lated sialic acids react with DMB in acetic acid at lower temperatures to form fluorescent derivatives, completely avoiding those reactions (Hara et al., 1989). More recently, the value of this technique has been confirmed and extended to the analysis of several additional sialic acid molecules (Manzi et al., 1990).

**Critical Parameters**

The reaction of sialic acids with DMB is highly specific under controlled conditions (other α-keto acids that may be present in biological samples give very weak responses and/or very delayed retention times under the conditions recommended for the analysis). Most known naturally occurring substituted sialic acids can be derivatized and separated (see Fig. 17.18.2). Exceptions are the 2,3-dehydro sialic acids and glycosides that do not have an α-keto group available for the reaction. The presence of O-acetyl esters can be confirmed by repeating the analysis after base treatment (see Support Protocol 2).

Reliable detection and quantitation is possible with 2.5 pmol of any sialic acid. To maintain column performance it is advisable to keep the injection volume of DMB reaction mixture as low as possible. Evidence obtained using radioactively labeled sialic acids indicates that different degree of derivatization probably occur with molecules with different substitutions. Therefore, it is possible to accurately quantitate a given peak only when a corresponding standard is available, and quantitation of substituted species by comparison with Neu5Ac is only approximate.

**Anticipated Results**

This protocol will give a qualitative and semiquantitative composition analysis of sialic acids in a mixture release from glycoconjugates using picomole amounts (Critical Parameters has a more detailed discussion).

**Time Considerations**

When several samples need to be analyzed, because the DMB reaction takes 2.5 hr it is best to do it at the end of the day and keep the derivatized samples at 4°C overnight before analysis. The total time required for each HPLC run is ~30 min. It is advisable to run standards at the beginning and the end of each day to compare relative retention times and area/pmol. A total of 10 to 12 runs can be done in one 8-hr day.

![Figure 17.18.2](image)

**Figure 17.18.2** HPLC profile (fluorescence vs. time) of the DMB derivatives of total sialic acids released from BSM (bovine submaxillary mucin) with 2 M acetic acid and purified according to the protocol described in UNIT 17.16.
Literature Cited


Key References

Hara et al., 1989. See above.

Describes the use of the DMB method for the detection and quantitation of Neu5Ac, Neu5Gc, and mono-O-acetylated derivatives of Neu5Ac.

Manzi et al., 1990. See above.

Describes the application of the DMB method to other members of the sialic acid family; discusses differences in reactivity with the fluorophore and in response factors between different DMB adducts.

Contributed by Adriana E. Manzi
University of California San Diego
La Jolla, California

Leland D. Powell and Ajit Varki
(thiobarbituric acid assay; de-O-acetylation)
University of California San Diego
La Jolla, California

Preparation and Analysis of Glycoconjugates
**Total Compositional Analysis by High-Performance Liquid Chromatography or Gas-Liquid Chromatography**

Once the presence of carbohydrate in a glycoprotein has been confirmed, the next step is to determine the precise molar ratio of its monosaccharide constituents using protocols in this unit and in the accompanying UNIT 17.19B (which presents the compositional analysis of labeled monosaccharides from glycosaminoglycans). This information helps to predict the type of oligosaccharide present and to determine the approach to detailed structural characterization. In some cases, it can also provide the first clues to the presence of a new type of sugar chain. The analysis involves two major phases. First, the release of the individual monosaccharides is achieved by methanolysis (Support Protocol 1), total acid hydrolysis (UNIT 17.16), or enzymatic release of sialic acids (UNIT 17.12). The second phase involves analysis by fractionation, characterization, and quantitation of the mixtures of monosaccharides by high-performance liquid chromatography using anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD; Basic Protocols 1, 2, and 3) and other HPLC systems (e.g., Alternate Protocol 1) or by gas-liquid chromatography with flame ionization detection (GLC-FID; Basic Protocol 4). The identity of the individual monosaccharides is determined by comparison with known standards processed and analyzed in the same way. Use of these protocols requires availability of instrumentation and understanding of setup maintenance and use.

**STRATEGIC PLANNING**

The flow chart in Figure 17.19.1 illustrates the alternative pathways and sequence of steps involved in compositional analysis. The protocols presented in this unit describe alternative approaches for the analysis of monosaccharide mixtures obtained by total acid hydrolysis (UNIT 17.16) or methanolysis (Support Protocol 1). The following considerations may help the investigator to plan the approach most appropriate for the starting material and intended results.

The choice of protocol to be used to release the individual monosaccharides from the starting glycoconjugate sample depends upon the nature of the material to be analyzed as well as the analysis method to be used. In general, glycoconjugates may be cleaved to their constituent monosaccharides by acid hydrolysis (UNIT 17.16) or methanolysis (Support Protocol 1). For the analysis of the constituents of neutral glycoconjugates, total acid hydrolysis of the sample is recommended. Glycoconjugates that may contain hexosamines or uronic acid can be broken down either by acid hydrolysis or methanolysis, and sialic acid residues can be released either enzymatically (as described in UNIT 17.12) or by hydrolysis in the presence of mild acid (UNIT 17.16). Note that the harsher acid conditions used to release the other monosaccharides cause destruction of sialic acids. For most glycoconjugates, the use of methanolysis to cleave glycosidic linkages is quite effective and generally results in less destruction of the monosaccharides than does acid hydrolysis (see Commentary for a more explicit discussion).

Frequently, the availability of instrumentation determines the choice between comparable options. For most HPLC approaches, such as those described in Basic Protocols 1, 2, and 3, the mixtures of monosaccharides released by acid hydrolysis is directly analysed without further treatment. Mixtures of monosaccharides, released by acid hydrolysis or by methanolysis, being subjected to analysis by GLC-FID (Basic Protocol 4), however, require an initial derivatization step (or series of steps) to convert the mixture to volatile...
compounds. These volatile derivatives can be produced from monosaccharide mixtures using one of the two alternative methods described in the support protocols. In the first derivatization approach, monosaccharide mixtures obtained from acid hydrolysis undergo reduction and peracetylation to produce volatile alditol acetates (Support Protocol 2). In the second approach, the products of methanolysis are converted to volatile trimethylsilyl derivatives (Support Protocol 3). In both cases, the resulting volatile compounds are then analyzed by GLC-FID.

Many different methods have been developed for each strategy, each with its own advantages and disadvantages (a more complete discussion and comparison of alternatives is presented in the Commentary). This unit presents reliable methods that have been proven to be widely applicable.
COMPOSITIONAL ANALYSIS OF FREE MONOSACCHARIDES BY HPAEC-PAD

The mixtures of monosaccharides obtained by acid hydrolysis (UNIT 17.16) of a glycoconjugate can be directly analyzed by high-performance liquid chromatography using ion-exchange pellicular resin columns with pulsed amperometric detection (HPAEC-PAD). Figure 17.19.2 provides a schema of a HPAEC-PAD system equipped with a reservoir for postcolumn addition of base. The following protocol (for separating mixtures of neutral monosaccharides and hexoamines) refers to this basic system but may also be adapted for use with newer instruments such as the Dionex DX-300 or DX-500, in which the arrangement of components is somewhat different. In the DX-500, for example, vacuum rather than helium sparging is used to degas the eluants, and because the PAD detector is more sensitive, postcolumn addition of base is not needed. Furthermore, the system can be completely controlled from a computer so that a procedure, including the elution conditions and the detection and integration parameters, can be created and stored as a method file. This method can later be called up and used. Consult the manufacturers' instructions for details of the use of these newer systems. The basic principles of operation and detection and the columns used for separation are the same.

Figure 17.19.2 Schema of a high-performance anion-exchange chromatography system with pulsed-amperometric detector and post-column delivery system.
Analysis of Mixtures of Neutral Monosaccharides and Hexosamines

Materials

Milli-Q-purified water: water deionized by passage through a five-stage Milli-Q Plus system (Millipore)
Certified 50% (19.3 M) sodium hydroxide containing <0.1% sodium carbonate (Fisher)
Nitrogen
Monosaccharide standard mixture (see Support Protocol 4)
Monosaccharide sample prepared by acid hydrolysis (UNIT 17.16: Basic Protocol 3, Basic Protocol 1 when analyzing only fucose)
Filtering unit with 0.45-µm Nylon 66 membranes (Alltech)
High-pH anion-exchange chromatography system with pulsed amperometric detector consisting of gradient pump, eluant degassing module, and PAD cell equipped with thin gasket (Dionex)
CarboPac PA-1 column (250 × 4 mm; Dionex)
CarboPac PA Guard column (3 × 25 mm; Dionex)

Prepare the HPAEC-PAD system

1. Filter 6 liters Milli-Q-purified water through a 0.45-µm membrane. Transfer 2 liters to bottle 1, 1997 ml to bottle 2, and 1980 ml to bottle 3. Connect to helium sparge line and degas 15 min.
   
   *Always rinse the bottles before using them and make sure the lines are equipped with polyethylene inlet frit filters. If 4-liter bottles are available, double the volumes of liquid in the bottles.*

2. Prepare 25 mM and 200 mM sodium hydroxide solutions by adding (using disposable plastic pipets) 2.6 and 20.8 ml of 50% sodium hydroxide solution to bottles 2 and 3, respectively. Avoid bubbling air when adding. Degas for another 15 min.
   
   *IMPORTANT NOTE: It is critical that the sodium hydroxide solution be free of carbonate. Do not use sodium hydroxide pellets, as these are usually coated with a film of sodium carbonate produced by absorption of CO₂ from the air; instead, use only 50% (19.3 M) NaOH solution that is free of carbonate. Take the solution from the middle of the bottle. Any CO₂ absorbed will precipitate in 50% NaOH.*
   
   *Keep all eluants under helium or vacuum (depending on the instrument used) at all times. Discard any remaining eluants after ~2 weeks and prepare fresh ones. Do not store the Milli-Q-purified water to be used with this system in plastic containers. Rather, use freshly processed water taken directly from the Milli-Q purification system. Do not use plastic tubing extensions out of the Milli-Q system because these can become contaminated with microorganisms.*

3. Run solvent from each bottle individually through the attached lines to remove any bubbles.

4. Connect the 4 × 250-mm CarboPac PA-1 column to the 3 × 25-mm CarboPac PA Guard column.

5. Set the pump control unit (or computer, if using a computerized system) to deliver a flow rate of 1.0 ml/min with an appropriate elution regimen (see Table 17.19A.1 for examples).
   
   *When both Xyl and Man are present in the sample, a very diluted eluent is used to achieve resolution. This considerably increases the time of analysis. Thus, when Xyl is not expected, an isocratic elution using 16 mM NaOH is preferred. These conditions are appropriate for the analysis of N- and O-linked oligosaccharides, and considerably shorter. However, it is important to keep in mind that Xyl and Man will coelute if both are present.*
Figure 17.19.3 shows a typical chromatogram of the mixture of standards used for compositional analysis of N- and O-linked oligosaccharides. Note that glucose is included, not because it is present in these oligosaccharides but because it is a very common contaminant.

6. Start the system and wait until a stable backpressure is attained (usual working pressures are between 1200 and 1500 psi and the maximum allowable pressure is 2000 psi).

7. Enter the settings for the PAD detector (or PED detector working in PAD mode) as indicated in Table 17.19A.2. When using a PADII detector, set the response time to 1 sec, the applied potential range to 1, and the output range to 300 nA. When using...
an ED40 detector in a DX300 or DX500 instrument, select the carbohydrate waveform.

In the DX300 and DX500 systems, each method includes elution conditions, detector settings, and integration parameters. These can be manually selected from the control panels or entered using the software provided by the manufacturer. In both cases, when a method is loaded, the pump starts and the detector is turned on.

**Perform postcolumn addition of base** (when using a PADI or early versions of the PADII detector only)

8. Pressurize the postcolumn reservoir to deliver 0.3 to 0.4 ml/min of 1 M NaOH solution (helium pressure should be ~32 psi, resulting in a 360 mM sodium hydroxide concentration at the electrode). Check the amount delivered by measuring the total flow out of the PAD cell with a small graduated cylinder.

A regular piston pump cannot be used because this produces a wavy baseline due to pulsation.

When using newer versions of the PADII cell, this step (designed to improve the sensitivity of detection with the older models) is unnecessary. In this case proceed directly to step 10.

9. Make sure that there are no bubbles inside the reference electrode. Wait 15 min for the detector to stabilize, then press the Auto Offset control.

**Check the baseline**

10. Check the detector baseline using an integrator or the software provided by the manufacturer, and do not proceed until it is stable. Offset as necessary. When using an integrator, the appropriate settings are attenuation, 1024 and peak threshold, 10,000.

**Analyze the standards**

11. Dissolve dried acid hydrolyzed (UNIT 17.16) monosaccharide standard mixture in 100 to 1000 µl water. Inject 10 to 100 µl of mixture onto column.

Use 100 µl when the mixture contains 2.5 nmol of each monosaccharide and 1000 µl when the mixture contains 10 nmol of each monosaccharide. Sufficient quantities of the standard mixture should be hydrolyzed to allow for several injections if necessary.

The standard mixture should contain 250 pmol to 1 nmol each of Fuc, GalNH₂, GlcNH₂, Gal, Glc, Xyl, and Man; 250 pmol to 1 nmol each of Fuc, GalNH₂, GlcNH₂, Gal, Glc, and Man; or 250 pmol to 1 nmol Fuc (when only analyzing fucose). The

<table>
<thead>
<tr>
<th>Time (sec)</th>
<th>Potential (V)</th>
<th>Integration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>0.20</td>
<td>0.05</td>
<td>Begin</td>
</tr>
<tr>
<td>0.40</td>
<td>0.05</td>
<td>End</td>
</tr>
<tr>
<td>0.41</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>0.60</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>0.61</td>
<td>−0.15</td>
<td></td>
</tr>
<tr>
<td>1.00</td>
<td>−0.15</td>
<td></td>
</tr>
</tbody>
</table>
amount of each monosaccharide standard injected depends on the sensitivity of the particular detector and needs to be determined empirically in each case. The amount of standard mixture injected will depend on the amount of sample available. When the sample is limited, it is necessary to work at high sensitivity, and in this case will be more difficult to obtain a good baseline; also, the amount of standards injected should be adjusted to be in the same range for improved quantitation.

Always prepare standards with extreme care (see Support Protocol 3), as their quality will determine the accuracy of sample quantitation. Always use standards that have been hydrolyzed under the same conditions as the samples. This will correct for the different rates of hydrolysis and different recoveries of the different monosaccharides.

12. Check the resolution between monosaccharides (resolution of Gal/GlcN and Xyl/Man are usually critical, and inversion of the relative order of the components in these pairs may be observed). If resolution is poor, change the concentration of base (when retention times are too short, less base is required).

When using very dilute eluants (e.g., 2 mM NaOH), the concentration required for complete resolution may change when new 25 mM NaOH solution is prepared. This optimum concentration is also different for different columns. Therefore, when a new solution is prepared or a new column is used, always check the resolution obtained for the standard mixture. When using higher concentrations of NaOH as eluant, this sort of variation is not observed.

**Analyze the samples**

13. When good resolution of the standards is achieved, inject the samples.

14. When using PADI or PADII detectors, it may be necessary (depending on the response obtained for the samples) to change the working range to 100 nA or 1 µA. Wait until a good baseline is obtained, then inject another sample.

When using newer detectors, these adjustments are made automatically.

15. Quantitate the standard run and use these data (area per nmol or pmol of each monosaccharide) to calculate the amount in nmol or pmol of each component in the sample.

It is advisable to average the results from at least three runs of standards to obtain more accurate response factors for each monosaccharide and improve the accuracy of the determination. This can be easily accomplished when using the software provided by the manufacturer by doing an average calibration and using those parameters to analyze the sample runs.

**Maintain the system running at maximum performance**

16. If resolution is lost and retention times become too short, wash the column for 30 min with 1 M sodium hydroxide. Wash extensively with water, then return to the working eluant. Wait for the baseline to stabilize under running conditions and check the performance of the column by injecting 10 µl standard mixture. If performance is not recovered, wash the column with 1 M HCl for 30 min at 1.0 ml/min. Wash extensively with water, then with 1 M NaOH for 30 min at the same flow rate. Wash extensively with water, reequilibrate with the working eluant, and check again.

Prepare high-concentration eluants used for regeneration in separate bottles, or wash the bottles extensively after they have been used to hold these solutions. Also, remember to wash the lines that have carried concentrated solutions before connecting them to the column for regular analysis.
Analysis of Mixtures of Neutral Monosaccharides, Hexosamines, and Uronic Acids

The use of a sodium acetate/sodium hydroxide gradient allows the elution of anionic saccharides, such as uronic acids, that would otherwise be retained by the column.

Additional Materials (also see Basic Protocol 1)

Sodium acetate (CH₃CO₂Na⋅3H₂O, FW 136.08; ACS certified grade)

1. Filter 8 liters Milli-Q-purified water through 0.45-µm membrane. Transfer 2 liters to bottle 1. Connect to helium sparge line and degas 15 min.

2. Prepare the following solutions in bottles 2, 3, and 4:

   Bottle 2 (200 mM sodium hydroxide): Transfer 1980 ml filtered Milli-Q-purified water to bottle and degas for 15 min. Using a disposable plastic pipet, add 20.8 ml of 50% sodium hydroxide, then continue degassing for 15 min.

   Bottle 3 (1 M sodium acetate): Weigh 272 g sodium acetate and dissolve in filter through 0.45-µm membrane, Milli-Q-purified water, then dilute to 2000 ml in a graduated cylinder. Filter through a 0.45-µm membrane. Transfer to bottle, and degas for 15 min.

   Bottle 4 (1 M sodium hydroxide): Transfer 1896 ml filtered Milli-Q-purified water to bottle 4 and degas for 15 min. Add 104 ml of 50% sodium hydroxide measured in a plastic cylinder, and continue degassing for 15 min.

3. Run solvent from each bottle individually through the attached lines to remove any bubbles.

4. Connect 4 × 250–mm CarboPac PA-1 column to the 3 × 25–mm CarboPac PA Guard column.

5. Set the pump control unit or computer to deliver a flow rate of 1.0 ml/min with an appropriate elution regimen (see Table 17.19A.3 for an example).

   A typical resulting chromatogram is shown in Fig. 17.19.4.

![Figure 17.19.4](image-url)  

**Figure 17.19.4** HPAEC-PAD profile of a mixture of monosaccharide standards containing Fuc, GalNH₂, GlcNH₂, Gal, Glc, Man, Neu5Ac, GalUA, and GlcUA. The standards (20 nmol of each neutral and amino sugar and 50 nmol of each hexouronic acid) were hydrolyzed with 2 M trifluoroacetic acid (TFA) for 6 hr at 100°C and dried; 20 nmol Neu5Ac was then added and the mixture dissolved in 1.0 ml water. A 100-µl aliquot (containing 2 nmol of each neutral monosaccharide, hexosamine, and Neu5Ac, and 5 nmol of each glycosyluronic acid) was injected on the column and eluted using the gradient described in Table 17.19A.3.
6. Analyze the standards and samples (see Basic Protocol 1, steps 12 to 15).

   The standard mixture should contain 250 pmol to 1 nmol of each neutral monosaccharide and hexosamine and of the appropriate glycosyluronic acid. As in Basic Protocol 2, inject 10 to 100 μl of this mixture on the column.

**Analysis of Mixtures of Sialic Acids**

Sialic acids can be separated using the same anion-exchange pellicular resin column without base in the eluant; this permits analysis of O-acetylated species. HPAEC-PAD can be used when ≥0.5 nmol are available. Therefore, it is possible to determine the molar ratios of individual sialic acids in mixtures that have been released from glycoconjugates and purified using the protocols described in *UNITS 17.12 & 17.16*. This procedure can also be used for preparative purposes using a larger chromatography column.

**Additional Materials (also see Basic Protocol 2)**

- Glacial acetic acid (17.4 M)
- N-acetylneuraminic acid (Neu5Ac, MW 309.3; Boehringer Mannheim)
- N-glycoly neuraminic acid (Neu5Gc, MW 325.3; Sigma)
- Standard mixture: sialic acids prepared from bovine submaxillary mucin (BSM) using the procedures in *UNIT 17.16* (Basic Protocol 2 or Alternate Protocol) or *UNIT 17.12*, or equimolar mixture of Neu5Gc and Neu5Ac processed as for the sample.

---

**Table 17.19A.3 Sample HPAEC Elution Regimen for Compositional Analysis of Neutral Monosaccharides, Hexosamines, and Uronic Acids**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Water</th>
<th>200 mM NaOH</th>
<th>1 M NaC₂H₃O₂</th>
<th>1 M NaOH</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>92.0</td>
<td>8.0</td>
<td>0.0</td>
<td>0.0</td>
<td>16 mM NaOH</td>
</tr>
<tr>
<td>0.01</td>
<td>92.0</td>
<td>8.0</td>
<td>0.0</td>
<td>0.0</td>
<td>40 mM NaOH</td>
</tr>
<tr>
<td>25</td>
<td>92.0</td>
<td>8.0</td>
<td>0.0</td>
<td>0.0</td>
<td>60 mM NaOH</td>
</tr>
<tr>
<td>35</td>
<td>80.0</td>
<td>20.0</td>
<td>0.0</td>
<td>0.0</td>
<td>100 mM NaOH/20 mM NaC₂H₃O₂</td>
</tr>
<tr>
<td>40</td>
<td>70.0</td>
<td>30.0</td>
<td>0.0</td>
<td>0.0</td>
<td>98 mM NaOH/30 mM NaC₂H₃O₂</td>
</tr>
<tr>
<td>45</td>
<td>88.0</td>
<td>0.0</td>
<td>2.0</td>
<td>10.0</td>
<td>96 mM NaOH/200 mM NaC₂H₃O₂</td>
</tr>
<tr>
<td>50</td>
<td>87.2</td>
<td>0.0</td>
<td>3.0</td>
<td>9.8</td>
<td>95 mM NaOH/75 mM NaC₂H₃O₂</td>
</tr>
<tr>
<td>55</td>
<td>87.2</td>
<td>0.0</td>
<td>3.0</td>
<td>9.8</td>
<td>75 mM NaOH/700 mM NaC₂H₃O₂</td>
</tr>
<tr>
<td>75</td>
<td>70.4</td>
<td>0.0</td>
<td>20.0</td>
<td>9.6</td>
<td>200 mM NaOH</td>
</tr>
<tr>
<td>95</td>
<td>22.5</td>
<td>0.0</td>
<td>70.0</td>
<td>7.5</td>
<td>200 mM NaOH</td>
</tr>
<tr>
<td>97</td>
<td>80.0</td>
<td>0.0</td>
<td>0.0</td>
<td>20.0</td>
<td>Reset&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>107</td>
<td>80.0</td>
<td>0.0</td>
<td>0.0</td>
<td>20.0</td>
<td>16 mM NaOH</td>
</tr>
<tr>
<td>109</td>
<td>92.0</td>
<td>8.0</td>
<td>0.0</td>
<td>0.0</td>
<td>Reset&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>120</td>
<td>92.0</td>
<td>8.0</td>
<td>0.0</td>
<td>0.0</td>
<td>Reset&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>This gradient separates Fuc, GalNH₂, GlcNH₂, Gal, Glc, Man, GalUA, and GlcUA. A typical chromatogram is shown in Figure 17.19.4.

<sup>b</sup>Next injection follows reset (at 130 min).
Prepare the HPAEC-PAD system

1. Filter 2 liters Milli-Q-purified water through 0.45-µm membrane. Transfer 1 liter to bottle 1, connect to helium sparge line, and degas 15 min.

2. Prepare 1 M sodium hydroxide solution (see Basic Protocol 1, step 2).

3. Weigh 0.68 g sodium acetate and dissolve in Milli-Q-purified water, diluting the solution to 1 liter final. Filter the resulting 5 mM sodium acetate buffer, transfer to bottle 2, connect to helium sparge line, and degas for 15 min.

4. Prepare 1 liter of 5 mM acetic acid using filtered Milli-Q-purified water and glacial acetic acid. Transfer to bottle 3 and degas for 15 min.

5. Run each solvent through the attached lines to remove any bubbles.

6. Connect the 4 × 250 mM CarboPac PA-1 column with the 3 × 25–mm CarboPac PA Guard column.

7. Set the pump control unit or computer to deliver a flow rate of 1.0 ml/min with an appropriate two-step elution regimen (see Table 17.19A.4 for an example).

   For preparative purposes, a 9 × 250–mm column at a flow rate of 5 ml/min should be used.

8. Start the system and wait until a stable back pressure is attained (usual working pressures are between 950 and 1400 psi and the maximum allowable pressure is 2000 psi).

9. Perform postcolumn addition of base (if using PADI or earlier PADII system) and determine detector baseline (see Basic Protocol 1, steps 8 to 11).

Analyze the standards

10. Inject 10 µl of a water solution containing 5 nmol Neu5Ac, working in the 300 nA range.

   If the samples to be analyzed have been submitted to acid hydrolysis for the release of sialic acids, use a Neu5Ac standard treated in the same manner for quantitation.

| Table 17.19A.4  HPAEC Elution Regimen for Compositional Analysis at Sialic Acids |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Time (min)      | Water 5 mM      | 5 mM            | 5 mM            | 1 M             | Comments        |
|                 | NaC₂H₃O₂        | C₂H₄O₂          | NaOH            |                 |                 |
| Initial         | 0               | 100             | 0               | 0               | 5 mM NaC₂H₃O₂   |
| 0.0             | 0               | 100             | 0               | 0               | 2.5 mM NaC₂H₃O₂ |
| 0.01            | 0               | 100             | 0               | 0               | 2.5 mM C₂H₄O₂   |
| 5               | 0               | 100             | 0               | 0               | 5 mM AcOH       |
| 35              | 0               | 50              | 50              | 0               | 5 mM NaC₂H₃O₂   |
| 36              | 0               | 0               | 100             | 0               | 2.5 mM NaC₂H₃O₂ |
| 46              | 0               | 0               | 100             | 0               | 2.5 mM C₂H₄O₂   |
| 48              | 80              | 0               | 0               | 20              | 5 mM NaC₂H₃O₂   |
| 68              | 80              | 0               | 0               | 20              | 200 mM NaOH     |
| 70              | 0               | 100             | 0               | 0               | 5 mM NaC₂H₃O₂   |
| 100             | 0               | 100             | 0               | 0               |                 |
11. Change the range to 100 nA, wait until a good baseline is obtained again, and inject 500 pmol of Neu5Ac in the same volume.

*When using a newer version of the PADII detector, these adjustments will be done automatically.*

12. If a standard mixture containing several sialic acids (obtained from BSM) is available, inject 10 µl containing 500 pmol of each and check the resolution. If this is not available, an equimolar mixture of Neu5Ac and Neu5Gc can be used.

*The total running time is under 30 min.*

### Analyze the samples

13. When good resolution and adequate retention times are achieved, inject the sialic acid samples. Compare the relative retention times with those listed in Table 17.19A.5.

*It is advisable to average the results from at least three runs of standards to obtain more accurate response factors for each monosaccharide and improve the accuracy of the determination. This can be easily accomplished when using the software provided by the manufacturer by doing an average calibration and using those parameters to analyze the sample runs.*

14. Characterization of individual peaks can be achieved by repeating the analysis after saponification of O-acetyl groups (see UNIT 17.18).

15. Determine the response obtained for standard Neu5Ac (area per pmol) and calculate the molar ratio of sialic acids in the mixture considering the following response factors:

<table>
<thead>
<tr>
<th>Sialic acid</th>
<th>Response factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neu5Ac</td>
<td>1.00</td>
</tr>
<tr>
<td>Neu5Gc</td>
<td>1.15</td>
</tr>
<tr>
<td>Neu5-9Ac₂</td>
<td>0.46</td>
</tr>
<tr>
<td>Neu5-7(8)-9Ac₃</td>
<td>0.45</td>
</tr>
</tbody>
</table>

*Detector responses for other sialic acids have not been determined, but lower response factors for more highly substituted species should be expected.*

---

**Table 17.19A.5** Relative Elution Times of Sialic Acids Found in Glycoproteins with Different HPLC Systems

<table>
<thead>
<tr>
<th>Sialic acid</th>
<th>( t_{Neu5Ac} ) in system HPAEC</th>
<th>Amino column(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neu5Ac</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Neu5Gc</td>
<td>1.17</td>
<td>1.5</td>
</tr>
<tr>
<td>Neu5-7Ac₂</td>
<td>0.74</td>
<td>0.36</td>
</tr>
<tr>
<td>Neu5-9Ac₂</td>
<td>0.95</td>
<td>0.35</td>
</tr>
<tr>
<td>Neu4-5Ac₂</td>
<td>0.76</td>
<td>0.39</td>
</tr>
<tr>
<td>Neu5-7(8)-9Ac₃</td>
<td>0.74</td>
<td>0.23</td>
</tr>
<tr>
<td>Neu5-7-8-9Ac₄</td>
<td>0.62</td>
<td>—(^b)</td>
</tr>
<tr>
<td>Neu5Gc9Ac</td>
<td>1.06</td>
<td>0.49</td>
</tr>
<tr>
<td>Neu4Ac5Gc</td>
<td>0.86</td>
<td>0.54</td>
</tr>
</tbody>
</table>

\(^a\) Using condition B.

\(^b\) Elutes close to the salt peak.
ALTERNATE PROTOCOL 1

COMPOSITIONAL ANALYSIS OF FREE SIALIC ACIDS BY AMINE-ADSORPTION ION-SUPPRESSION HPLC WITH UV DETECTION

As an alternative to Basic Protocol 3, mixtures of free sialic acids can be analyzed by amine-adsorption ion-suppression HPLC with UV detection. The choice depends on the instrumentation available and the level of sensitivity required for a particular sample.

This system can be used to analyze radioactive samples if the column effluent is routed to a flow detector or fraction collector. When a flow detector is used, the appropriate ratio of scintillation fluid to be used has to be checked because of the quenching effect of acetonitrile. When fractions are collected and 72% acetonitrile is used, it is necessary to dry them, redissolve them in water, and then measure their radioactivity in a β-counter after addition of scintillation cocktail. When 64% acetonitrile is used, the addition of 1 vol water followed by 10 vol scintillation cocktail yields good sensitivity.

Materials

- Monobasic sodium phosphate (anhydrous, MW 120.0; reagent grade, Sigma)
- Milli-Q-purified water: water deionized by passage through five-stage Milli-Q Plus system (Millipore)
- Acetonitrile (HPLC grade, Fisher)
- N-acetylneuraminic acid (Neu5Ac, MW 309.3; Boehringer Mannheim)
- N-glycolylnneuraminic acid (Neu5Gc, MW 325.3; Sigma)
- Standard mixture: sialic acids from bovine submaxillary mucin (BSM) prepared using procedures in UNIT 17.16 or UNIT 17.12, or equimolar mixture of Neu5Ac and Neu5Gc processed as for the sample
- Sialic acid sample prepared by acid hydrolysis (UNIT 17.16, Basic Protocol 2 or Alternate Protocol) or enzymatic release (UNIT 17.12)
- HPLC apparatus with pump and UV detector
- Filtering unit with 0.45-µm Nylon 66 membranes (Alltech)
- Micropak AX-5 (300 mm × 7.8 mm i.d., particle size 9 µm; Varian)
- Micropak AX-5 Guard column (4 cm × 4 mm)
- Additional reagents and equipment for de-O-acetylation (UNIT 17.18)

Prepare the HPLC system

1. Turn on the UV detector and set the wavelength to 200 nm.
2. Prepare 1 liter of 0.25 M monobasic sodium phosphate (30 g/liter) in Milli-Q-purified water and filter through 0.45-µm membrane.
3. Fill the bottles in the HPLC system with (1) acetonitrile, (2) Milli-Q-purified water filtered through 0.45-µm membrane, and (3) filtered 0.25 M monobasic sodium phosphate from step 2. Degas 15 min by helium sparging.
4. Run each solvent through the attached lines to eliminate any bubbles.
   CAUTION: Do not allow direct mixing of stock acetonitrile and sodium phosphate, as this will cause precipitation of salts in the lines.
5. Set the pump control unit to deliver a flow rate of 1 ml/min with an appropriate isocratic elution regimen as indicated in Table 17.19A.6.
6. Start running the solvent, then connect the 300 × 7.8-mm Micropak AX-5 column to the 4 cm × 4 mm Guard column.
7. Wait until the back pressure stabilizes and check the baseline. Wait until a stable baseline is obtained.
Analyze the standards

8. Inject 10 µl of a solution of Neu5Ac in water (10 nmol Neu5Ac total). Check the sensitivity and adjust the integrator settings as required (a good signal-to-noise ratio is normally obtained using attenuation range 8). Repeat if necessary.

   It is possible to work in attenuation range 4 to detect 5 nmol of Neu5Ac, but the background is considerably higher.

9. Inject 10 µl of a mixture of Neu5Ac and Neu5Gc standards containing 10 nmol of each monosaccharide. If using condition B, use a mixture containing substituted sialic acid standards instead obtained from BSM (if available). Check for resolution and adjust the elution conditions if required.

   CAUTION: Remember that sodium phosphate will start precipitating at >72% acetonitrile. Condition B constitutes the maximum compatible percentages of acetonitrile and phosphate.

Analyze the samples

10. Inject the sialic acid samples. Adjust the integrator settings as required and repeat if necessary.

11. Determine the percentage of each component, assuming equal detector responses.

12. Confirm the presence of O-acetylated species by subjecting the sample to de-O-acetylation (UNIT 17.18), then repeating the analysis. Compare the relative retention times to those listed in Table 17.19A.1.

Maintain the system running with maximum performance

13. Wash the column 30 min with water. Run 100% acetonitrile for 10 min. Disconnect the column.

   CAUTION: Never stop the flow if the lines contain phosphate, as they may become clogged. Also wash the filter and the line coming from the reservoir after using any salt.

14. If resolution is lost and retention times become reduced, clean the column by running 0.5 M phosphoric acid for at least 1 hr at 1 ml/min flow, then washing extensively with water before changing to the working buffer.

   More complete regeneration of the column can be achieved by injecting three 1-ml volumes of 3-aminopropyltriethoxysilane while running the column in 100% acetonitrile, washing extensively with the same solvent, with water, and then with 0.5 M phosphate before use. The performance of the column should be fully recovered after this treatment; however, the retention times may not be identical.

---

Table 17.19A.6  Amine Absorption/Ion Suppression HPLC Elution Regimen for Compositional Analysis of Sialic Acids

<table>
<thead>
<tr>
<th>Conditions&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Flow (ml/min)</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>64</td>
<td>26</td>
<td>10</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>72</td>
<td>18</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>a</sup>Condition A is used when only separation of Neu5Ac and Neu5Gc is required. Condition B is used when a mixture containing substituted species will be analyzed.
COMPOSITIONAL ANALYSIS BY GLC-FID

This protocol describes the quantitative analysis of derivatized methyl glycosides and alditols through the use of a gas-liquid chromatograph with a flame-ionization detector (see Fig. 17.19.5). With this apparatus, it is quite straightforward to determine the molar ratio of the monosaccharide components of a given glycoconjugate. This analysis can be performed after complete acid hydrolysis (UNIT 17.16) and conversion of the free monosaccharides to volatile peracetylated alditols (see Support Protocol 2). It is also possible to cleave the glycosidic linkages with methanol/HCl to produce methyl glycosides, which are converted into volatile trimethylsilylethers (see Support Protocol 3) for further GLC analysis. The last method is preferred when hexosamines or uronic acid are expected to be found in the sample.

Materials

- Monosaccharide standard mixture (see Support Protocol 3) prepared in the same manner as the sample
- Monosaccharide sample prepared by acid hydrolysis (UNIT 17.16, Basic Protocol 3) followed by derivatization by peracetylation (see Support Protocol 2) and/or by methanolysis (Support Protocol 1) followed by derivatization by pertrimethylsilylation (see Support Protocol 3)
- Internal standard prepared in the same manner as the sample
- 0.25 mm × 30 m 5% DB-5 fused silica capillary column (J&W Scientific)
- Gas chromatograph with dual-flame ionization detector
- Helium (research grade)
- Hydrogen and air lines (for FID detector only)

Gas-liquid chromatograph with flame-ionization detector (GLC-FID)

1. Connect the capillary column to the gas chromatograph and begin running the carrier gas at a flow rate of 1.5 ml/min. Set the makeup gas flow rate of 35 ml/min and the split ratio to 10:1.

   When <25 μg of total carbohydrates are processed, a splitless injection is required.

![Schema of a gas-liquid chromatograph with flame-ionization detector.](image-url)
Several other types of stationary phases can be used for the analysis of acetylated alditols: e.g., CP-Sil 5 WCOT, SP-1000, Silar 10C, OV-1, SE-54, 5% DB-5, DB-1, Carbowax 20M, SE-30, OV-101, and OV-275. In the less polar stationary phases, amino sugar alditols can also be analyzed. Other stationary phases used for the analysis of trimethylsilylated glycosides and methyl glycoside methyl esters include CP-Sil 5 WCOT, 5% DB-5, DB-1, SE-30, and OV-101. Appropriate conditions vary from one to another. Typical chromatograms and relative retention times can be found in Kammerling and Vliegenthart (1989) and references therein.

2. Set the GLC oven temperature to 50°C, the injector-port temperature to 150°C, and the FID detector temperature to 250°C, and wait for stabilization.

3. Open the air and hydrogen lines and adjust the flows. Ignite the detector. Check the background.

4. Set up an appropriate temperature program:
   a. *Alditol acetates*: Start at 50°C and keep at 50°C for 2 min; then go from 50°C to 150°C at 20°C/min (5 min); then go from 150°C to 250°C at 4°C/min (25.0 min); then keep at 4°C for 5 min. (Total running time is 37 min.)
   b. *Trimethylsilyl ethers of methyl glycosides*: Start at 50°C and keep at 50°C for 3 min; then go from 50°C to 170°C at 20°C/min (6 min); then go from 170°C to 250°C at 6°C/min (13.3 min); then keep at 250°C for 2.7 min. (Total running time is 25 min.)

5. Inject 1 µl of the mixture of derivatized standards (see Support Protocol 2 and Support Protocol 3). Adjust the settings of the integrator and repeat as necessary to obtain all the peaks on scale.

6. Determine the retention times of each peak relative to the internal standard.

7. Inject the standards (containing internal standard) one at a time to assign the peaks and determine the response factors. In the case of TMS derivatives, also determine the contribution of each peak to the total (%), and select the peak of each monosaccharide to be used for quantitation:

   \[
   R = \frac{\text{nmol internal standard} \times \text{area monosaccharide}}{\text{nmol monosaccharide} \times \text{area internal standard}}
   \]

8. Inject 1 µl of sample (containing internal standard). Repeat as needed to obtain a good profile.

9. Tentatively assign the peaks by comparison of the relative retention times with those of the standards. If a discrepancy arises, repeat analysis, coinjecting the sample with the individual standard. The questioned peak should appear separated from the standard if it is not the same compound.

10. Determine the molar percentage of individual components by reference to the internal standard:

   \[
   \text{nmol sugar/µg sample} = \frac{\text{area of monosaccharide peak} \times \text{nmoles internal standard}}{\text{area of internal standard peak} \times R \times \text{µg of sample}}
   \]
QUANTITATIVE RELEASE OF NEUTRAL MONOSACCHARIDES, HEXOSAMINES, AND URONIC ACIDS BY METHANOLYSIS

Glycoconjugate samples are hydrolyzed by heating in the presence of methanolic HCl, followed by treatment with pyridine and acetic anhydride.

**Materials**

- Glycoconjugate sample containing 5 to 50 µg total sugar
- Internal monosaccharide standard: monosaccharide that does not occur naturally in the sample (e.g., monosaccharide alditol that yields only one peak upon gas chromatography, see UNIT 17.16; see Support Protocol 4 for monosaccharide preparation)
- Monosaccharide standards: monosaccharides expected to be found in the sample (see Support Protocol 4 for preparation)
- Phosphorus pentoxide (Fisher)
- Acetyl chloride (FW 78.50, 98% pure; Aldrich)
- Methanol (anhydrous, 99% pure, Aldrich; store in desiccator)
- Pyridine (anhydrous silylation grade, Pierce; store in desiccator)
- Acetic anhydride
- 1.5-ml glass Reacti-Vials (Pierce) with Teflon-lined screw caps, either new (rinsed with water, then with ethanol, and dried) or acid-cleaned (see recipe)
- Heating block or oven
- Nitrogen or vacuum evaporation system (Speedvac or shaker-evaporator)

1. Combine the glycoconjugate sample and 1 to 10 µg internal monosaccharide standard in a 1.5-ml precleaned or acid-cleaned Reacti-Vial. Lyophilize mixture.

2. Prepare a set of standards consisting of separate solutions containing 50 nmol of each monosaccharide that is expected to be found in the sample, each one also containing 5 nmol of the same internal standard used for the sample. Also prepare a mixture of all the expected monosaccharides plus internal standard. Treat all standards in parallel with the sample.

3. Dry the lyophilized sample and standards overnight in a vacuum desiccator over phosphorus pentoxide.

4. Prepare methanolic HCl (either reagent A or reagent B) by adding acetyl chloride to anhydrous methanol in the following proportions:

<table>
<thead>
<tr>
<th></th>
<th>Acetyl chloride</th>
<th>Anhydrous methanol</th>
<th>Final concentration of HCl in methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent A</td>
<td>3.50 vol</td>
<td>100 vol</td>
<td>0.50 M</td>
</tr>
<tr>
<td>Reagent B</td>
<td>7.00 vol</td>
<td>100 vol</td>
<td>1.00 M</td>
</tr>
</tbody>
</table>

**CAUTION:** Add the acetyl chloride carefully, as the resulting reaction is highly exothermic.

Methanolic HCl reagent A is used except when glycosyluronic acids are present; in such cases reagent B is preferred. Alternatively, an initial acid hydrolysis may be performed using 200 µl of 2 M trifluoroacetic acid (TFA) for 1 hr at 121°C. TFA should then be evaporated under a nitrogen stream or in Speedvac system and samples washed twice with methanol, then evaporated again. This procedure ensures complete recovery of hexosamines but destroys sialic acids. Afterwards, proceed to step 4, and use reagent A.

Prepare a large enough volume of methanolic HCl to minimize pipetting errors, but only in the range required for the experiment, as it cannot be stored.
5. Dissolve dried sample and standards (from step 3) in 200 µl methanolic HCl (reagent A or B), then cap the vials.

6. Heat 16 hr at 65°C if using reagent A or 24 hr at 85°C if using reagent B. Check the screw caps for tightness after the first 15 min of heating, then vortex and continue heating.

   This procedure yields methyl glycosides of each monosaccharide residue in the glycoconjugate. Hexosamines will be de-N-acetylated.

7. Evaporate sample and standards using a stream of nitrogen at room temperature or a Speedvac system.

8. Add 100 µl anhydrous pyridine and vortex to resuspend the residue. Add 100 µl acetic anhydride, vortex 1 min, and evaporate to dryness under a stream of nitrogen.

   This treatment produces re-N-acetylation of hexosamines. Some O-acetyl groups may be incorporated.

9. Dissolve the residue in 200 µl of 0.5 M methanolic HCl, then heat 1 hr at 65°C.

   This treatment assures the cleavage of any O-acetyl groups incorporated in step 8.

10. Evaporate under a stream of nitrogen, then dry further overnight in a vacuum desiccator over phosphorus pentoxide.

### PREPARATION OF VOLATILE DERIVATIVES OF FREE GLYCOSES

Volatile derivatives of free glycoses are prepared by first reducing the free sugars to alditols with sodium borohydride, then peracetylating with acetic anhydride. The resulting alditol acetates can be analyzed immediately by GLC-FID (see Basic Protocol 2).

**Materials**

- 10 mg/ml sodium borohydride in 1 M ammonium hydroxide (ACS reagent grade)
- Monosaccharide sample prepared by acid hydrolysis (UNIT 17.16, Basic Protocol 3, or Basic Protocol 1 when analyzing only fucose)
- Neutral sugar standard mixture (see Support Protocol 4) submitted to acid hydrolysis (UNIT 17.16, Basic Protocol 3)
- Sodium borohydride (FW 37.8; store in desiccator)
- Glacial acetic acid (FW 60.05; 99.99% pure)
- 1% acetic acid in methanol (anhydrous, 99% pure)
- Acetic anhydride (99% pure)
- Toluene
- Chloroform (anhydrous, 99% pure)
- Acetone (99.9% pure, HPLC grade)
- Phosphorous pentoxide (as desiccant)
- 3.5-ml glass vials with Teflon-lined screw caps
- Nitrogen evaporation unit (Reacti-Vap Evaporator, Reacti-Therm Heating Module, and Reacti-Block B-1 or S-1, Pierce)
- Heating block or oven, 100°C
- Tabletop centrifuge
- Reacti-Vials (Pierce) or other glass test tubes

**Reduce mixtures of free glycoses to alditols**

1. Add 250 µl of 1 M ammonium hydroxide/10 mg/ml sodium borohydride to the monosaccharide sample and the neutral sugar standard mixture in 3.5-ml glass vials with Teflon-lined screw caps. Let stand 10 min, then check for persistence of
microbubbles in the reaction mixture. If these cannot be seen, add a few micrograms of solid sodium borohydride.

*It is important that no residue of acid from the hydrolysis be present or the hydride will be rapidly destroyed. If excessive bubbling occurs when adding the borohydride, add methanol and evaporate two or three times, then repeat the reduction.*

3. Incubate 2 hr at room temperature.

*This reaction can also be run overnight.*

4. Add glacial acetic acid dropwise until no further bubbling is observed.

*Care must be taken to avoid losing sample with the bubbling, as the reaction is violent. This problem may be circumvented by using a 20% (v/v) solution of acetic acid in methanol instead of glacial acetic acid.*

5. Add 250 µl of 1% acetic acid in methanol to each tube. Evaporate to dryness using a nitrogen evaporation unit or under vacuum using a Speedvac system with the heating element set at 40°C. Repeat four more times.

*This treatment removes volatile methyl borate, producing sodium acetate. Complete removal of borate is indicated by colorless crystals, as compared with white powder previously present. When a nitrogen evaporation unit is employed, care must be taken not to blow away the solid residue, particularly in the final steps.*

6. Dry sample and standards in a vacuum dessicator overnight over phosphorus pentoxide.

**Peracetylate the alditols**

7. Add 200 µl acetic anhydride, vortex, and cap the tubes.

8. Heat 3 hr at 100°C, mixing after the first 15 min of heating to make sure that the solid residue is suspended. Let cool to room temperature.

9. Add 200 µl toluene. Evaporate to dryness at 40°C using a shaker-evaporator or Speedvac or under nitrogen. Add toluene as needed to dry completely.

*Toluene helps to eliminate the reagents by forming an azeotrope.*

**Clean reaction mixtures by extraction**

10. Add 0.5 ml water and 0.5 ml chloroform to each tube, cap, and vortex well. Centrifuge briefly at low speed to clearly separate the two phases.

*Salam will be extracted into the water layer, leaving the peracetylated alditols in the organic layer.*

11. Remove the upper (water) layer with a Pasteur pipet. Wash the organic layer with water two more times.

12. Transfer the chloroform layer to a clean tube or Reacti-Vial and dry under nitrogen at room temperature or in a Speedvac system.

13. Dissolve in 5 to 50 µl acetone, depending on the amount of starting material, and use 1 µl for GLC-FID analysis (Basic Protocol 2).
PREPARATION OF VOLATILE DERIVATIVES OF METHYL GLYCOSIDES

Volatile derivatives of methyl glycosides (trimethylsilylethers of neutral sugars and hexosamines and methyl esters of uronic acids) are prepared from the dried methyl glycosides (see Support Protocol 1) using a commercial silylation reagent. The silylated samples must then be analyzed immediately by GLC-FID (see Basic Protocol 2).

Materials

Dried methanolyzed samples and standards (see Support Protocol 1, step 10)
Tri-Sil Reagent [2:1:10 (v/v/v) hexamethyldisilazane/trimethylchlorosilane/pyridine; Pierce]
96% hexane (HPLC grade)
Nitrogen stream
Tabletop centrifuge
Reacti-Vials (Pierce)

1. Suspend each dried sample and standard in 100 µl Tri-Sil and cap vial. Incubate 30 min at room temperature.
   
   *Samples and all required standards must be processed in parallel.*

2. Evaporate the silylating reagent under a stream of dry nitrogen.
   
   *Nitrogen can be dried by including a drying tube in the line.*

3. Add 0.5 ml hexane, dissolve the derivatives, and centrifuge briefly at low speed to separate insoluble salts.

4. Transfer supernatant to a clean Reacti-Vial and evaporate under dry nitrogen stream as in step 2.

5. Dissolve the residue 5 to 50 µl hexane, depending on the amount of starting material. Use 1 µl of solution for GLC analysis.

PREPARATION OF MONOSACCHARIDE STANDARD SOLUTIONS

Proper preparation and storage of monosaccharide standard solutions is critical to achieve good quantitation in a compositional analysis (Hardy and Townsend, 1994).

Materials

NaOH pellets
Monosaccharide standards
Milli-Q-purified water: water deionized by passage through a Milli-Q Plus system (Millipore)
Vacuum desiccator
Glass vials
100-ml volumetric flasks

1. Prepare a vacuum desiccator containing a plastic beaker with NaOH pellets.

2. Tare a group of glass vials (twice the number of standards to be used). Weight ~500 mg of each monosaccharide standard in separate vials and cover each vial with aluminum foil or a loosely placed clean cap. Cover the extra vials in the same manner and place all the vials in the desiccator.

3. Put the desiccator under continuous vacuum for 1 day.
4. Weigh the vials and return them to the desiccator for another day under vacuum. Continue to do this every day until the change in weight from one day to the next is <1%.

This is normally achieved in 3 to 4 days.

5. Transfer a sample (e.g., 15 mg) of each standard to one of the empty, desiccated tared vials and determine the exact weight of the monosaccharides.

6. Add Milli-Q-purified water to each vial to give a 100 mM solution. Mix well to dissolve.

7. Prepare a dilution of each of these primary standard solutions by transferring 100 µl of each solution to a 100-ml volumetric flask (100 pmol/µl). Dilute to 100 ml with Mill-Q-purified water and mix.

8. Divide each standard into 1-ml aliquots and store these at −20°C until needed.

9. Prepare a mixture of all the required monosaccharides by transferring 100 µl of each primary standard solution to a 100-ml volumetric flask (100 pmol/µl). Bring up to 100 ml with Mill-Q-purified water and mix.

10. Divide the standard mixture into 1-ml aliquots and store these at −20°C until needed.

11. When required, defrost one aliquot of each desired standard solution and use them within 1 or 2 days. Do not refreeze the standards.

Standards prepared in this manner can be used directly for HPAEC or HPLC analysis or lyophilized and used for GLC analysis.

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.

Acid-cleaned glass vials

Heat 1.5-ml glass vials (e.g., Reacti-Vials, Pierce) with Teflon-lined screw caps at 50°C for at least 3 hr each time in concentrated nitric acid and then in 6 M HCl. Rinse thoroughly with water, then with ethanol, and dry in an oven. When <5µg of total carbohydrates is analyzed, it is recommended to silanize the glass vials by incubating them 15 min at room temperature with a 2% solution of dichlorodimethylsilane in toluene, decanting the solution, rinsing successively with methanol and hot distilled water, and allowing the vials to dry.

New vials should be at least rinsed with water, then with ethanol, and dried.

COMMENTARY

Background Information

High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and gas-liquid chromatography with gas-liquid chromatography with mass spectroscopic detection (GLC-MS) are the methods most widely used today for compositional analysis of the carbohydrate moieties of glycoproteins. Both methods are able to detect all the possible known monosaccharides. With the HPAEC-PAD system it is possible to analyze qualitatively and quantitatively a mixture of free monosaccharides containing as little as 50 pmol of each individual component. With GLC methods, the use of internal standards helps improve quantitation because the injection of reproducible proportions of the sample into the column is not easy.

HPAEC-PAD and other HPLC techniques

Different high-performance liquid chromatography (HPLC) systems have been used for the analysis of free glycoses obtained by acid hydrolysis, but sensitivity and resolution between individual monosaccharides were traditionally poor. The technique became popular...
for analyzing glycoproteins and other glycoconjugates available in limited amounts from biological sources only after pellicular resin anion-exchange columns and the pulse-amperometric detector were developed. Neutral saccharides are analyzed at high pH (12 to 14), at which they are partially or completely ionized. Separation is achieved by adjusting the eluant pH. Anionic saccharides can be separated using sodium acetate/sodium hydroxide gradients. Therefore, with appropriate adjustment of the elution conditions, this system permits not only the compositional analysis of free monosaccharide mixtures but also the analysis of oligo- and polysaccharides (Lee, 1990).

Pulsed amperometric detection (PAD) utilizes a repeating sequence of three potentials applied for specific durations. The resulting total current is the sum of (1) the carbohydrate oxidation current, (2) the current due to the charging of the electrode surface, and (3) the current caused by the oxidation of the gold electrode. The current is only measured during the first applied potential and has been shown to be linear over $\sim 4\frac{1}{2}$ orders of magnitude. Recently, the parameters used for these three steps were optimized (LaCourse and Johnson, 1991). Molecules with similar size and structure are reported to have similar response factors. On the other hand, substituents on hydroxyl groups are expected to affect the sensitivity of detection. The detector works only at high pH. Thus, when base-labile species that are separated without the use of base in the eluant are analyzed, it is necessary to add alkali to the effluent before it enters the PAD cell.

Isocratic elution with sodium hydroxide (2 to 16 mM; the optimal concentration varies from column to column) allows for the separation of neutral sugars and hexosamines. Different sodium acetate gradients have been employed for the analysis of acidic monosaccharides and of mixtures of these and other sugars. The system is also useful for sialic acids, with a limit of detection of $\sim 200$ pmol (Manzi et al., 1990). Oligo- and polysaccharides can also be analyzed with this system using appropriate conditions (Hardy and Townsend, 1988; 1989). Although monosaccharide alditols are not retained by this column (they will come out in the void volume), oligosaccharide alditols can be analyzed (Reddy and Bush, 1991). Maintenance of a stable column temperature is critical to obtaining reproducible results; failure to do so can cause lack of separation of critical pairs of monosaccharides (Van Riel and Olieman, 1991).

Because only 1% of the sample is oxidized in the electrode, the system can be used preparatively without the need for a bypass. When analyzing base-labile sialic acids, this method cannot be used, because the postcolumn addition of base required for detection will cause saponification of the O-acetyl esters. However, the pellicular resin column can be used for fractionation and the eluate routed to a fraction collector. After the fractions are monitored by the TBA assay (UNIT 17.18), they can be pooled and desalted. This method has proved to be the best HPLC technique available for preparative fractionation of mixtures of sialic acids containing labile substituents (Manzi et al., 1990).

Another useful system for the analysis of free sialic acids is amine adsorption–ion suppression HPLC. This is based upon hydrogen bonding between the hydroxyl groups of saccharides and the amine moieties of the stationary phase (Mellis and Baenziger, 1981; Bergh et al., 1981). To fractionate anionic molecules, phosphate is added to the mobile phase to suppress the ionic effects while retaining hydrogen bonding (Mellis and Baenziger, 1983). Good separation of sialic acids is obtained in isocratic mode with a mixture of acetonitrile/water/0.25 M monobasic sodium phosphate at 1 ml/min (Diaz and Varki, 1985). It is necessary to maintain a minimum concentration of 10% phosphate buffer with a maximum working percentage of acetonitrile of 72% (above this the phosphate starts to precipitate). A 64:26:10 (v/v/v) ratio of acetonitrile/water/0.25 M sodium phosphate can be used to separate Neu5Ac and Neu5Gc. For O-acetylated sialic acids, however, it is necessary to increase the percentage of acetonitrile to a ratio of 72:18:10 (v/v/v) so as to increase the retention times of the sialic acids (Manzi et al., 1990). Chromatography is monitored by absorption at 200 nm, which requires extremely high-purity samples and reagents. The same analytical column can be used for preparative purposes (up to 400 nmol of sialic acids with a good resolution of the peaks). Recovery of sialic acids is $\sim 65\%$, but some loss and migration of O-acetyl groups occur.

**GLC-FID**

To analyze mixtures of glycoses or methylglycosides by gas-liquid chromatography, it is necessary to prepare volatile derivatives. Alditol acetates are the most widely used derivatives for monosaccharides obtained by acid hydrolysis, whereas trimethylsilyl ethers of methyl glycosides or methyl glycoside methyl esters are
the preferred derivatives for methanolysis products. These are the derivatives of choice for any glycoconjugate-derived sugar because of the amount of information that can be obtained from them (e.g., retention times in different columns). Other volatile compounds, such as aldononitrile acetates and trifluoroacetylated alditols, have also been successfully employed (as reviewed by Kammerling and Vliegenthart, 1989).

**Acetylates of alditols**

When only neutral sugars need to be analyzed, the most common derivatization method is the conversion of the free monosaccharides into their alditol acetates. Each monosaccharide produces a single derivative, giving a simple chromatogram that can be easily interpreted and quantitated. The total analysis time is also shorter because all required standards can be processed as a mixture, requiring only one GLC run.

The procedure involves the reduction of the glycoses to alditols with sodium borohydride; elimination of the excess of hydride with acid; elimination of the boric acid produced as trimethylborate by coevaporation with acidified methanol; and peracetylation of all free hydroxyl groups. Different protocols to achieve this last step have been developed (Kammerling and Vliegenthart, 1989; Albersheim et al., 1967; York et al., 1985). It is possible to simply use the sodium acetate produced in the reaction as the catalyst, and achieve complete acetylation by heating with acetic anhydride. Because leftover borate could inhibit the acetylation, some protocols add pyridine to drive the reaction to completion.

**Cleavage of glycosidic linkages by methanolysis**

Cleavage of all glycosidic linkages by methanolysis is very effective and causes less destruction of the monosaccharides than acid hydrolysis. Monosaccharides are converted to their methyl glycosides and glycosyluronic acids to their methyl esters methyl glycosides. The N-acetyl group of amino sugars are completely cleaved by extensive methanolysis, and it is advisable to incorporate a re-N-acetylation step to avoid the production of additional peaks in the chromatogram. A drawback to the reacetylation step is that some O-acetylation occurs (in some primary hydroxyl groups), but a short additional treatment with methanolic HCl eliminates these groups (Rickert and Sweeley, 1978). Because of anomerization, each monosaccharide produces several methylglycosides (α and β anomers of the pyranosidic and furanosidic forms), which yields a characteristic pattern of peaks in the GLC run. When the sample contains several monosaccharides, however, the pattern is quite complex, with some peaks superimposed, and this makes quantitation difficult. Nevertheless, by processing each of the individual monosaccharides that are expected in parallel, running each one separately under the same conditions, and determining the contribution of each peak to the total, an accurate quantitation can still be achieved.

Different conditions have been used for methanalysis of carbohydrates in glycoproteins using methanolic HCl, including 0.5 M for 16 hr at 65°C (Reinhold, 1972) and 0.75 M for 3 hr at 80°C (Wong et al., 1980). Conditions for the simultaneous release of neutral monosaccharides, acetamido sugars, uronic acids, octulosonic acids, and sialic acids (2 M TFA for 12 hr at 100°C under vacuum) have been reported (Clarke et al., 1991). Nevertheless, the cleavage of the uronic acid glycosidic linkage may be incomplete in some cases, and several glycosyluronic acids produce a certain percentage of 3,6-lactones. The glycosidic linkages of sialic acids are cleaved with good yield, but a significant proportion of the released molecules are destroyed. Although special conditions that minimize this destruction have been developed (Schauer, 1978), N-acetyl groups and most O-acetyl esters are eliminated. Thus, release of intact sialic acids requires much milder acid hydrolysis of a separate aliquot of the sample.

**Volatile derivatives of methyl glycosides**

The content of neutral sugars, amino sugars, and glycosyluronic acids can be simultaneously determined by this method. Different reagents and procedures have been used to silylate mixtures of methyl glycosides (Kammerling and Vliegenthart, 1989). The most widely used procedure uses 5:1:5 (v/v/v) pyridine/hexamethyldisilazane/trimethylchlorosilane lane and an incubation that can range from 15 min at room temperature to 20 min at 80°C.

Sialic acids have also been analyzed as trimethylsilyl esters trimethylsilyl ethers or methyl esters trimethylsilyl ethers. Although conditions have been worked out for preparing these two types of derivatives while preserving labile natural substituents such as O-acetyl groups (Schauer and Corfield, 1982), the derivatization of sialic acids is frequently in-
complete. Further problems are encountered during the subsequent chromatographic separation because the high temperatures required to elute these molecules result in partial destruction of the labile substituents. Therefore, this approach to sialic acid analysis has been replaced by HPLC methods with or without derivatization (see UNIT 17.18).

**Qualitative and quantitative analysis**

Analysis of volatile monosaccharide derivatives is achieved by gas-liquid chromatography. A combined adsorption/partition mechanism takes place between the volatile derivatives and the particular stationary phase that is a liquid at working temperatures. Through the large number of theoretical plates available in the length of the fused-silica capillary columns, the individual components of the mixtures under analysis are separated according to their particular structural characteristics. The mobile phase (helium) carries the molecules throughout the column and to the detector, where the ionization of the flame changes upon their passage, producing an electrical output. This signal is amplified and registered.

Initial characterization of individual peaks is done by comparison with standards run in the same conditions. The retention times of each standard relative to the component used as internal standard in the sample are calculated and compared with the relative retention times of each peak in the chromatogram of the sample. Typical elution profiles as well as relative retention times in the different columns under different conditions can be found in the literature (Kammerling and Vliegenthart, 1989, and references therein).

A known amount of an internal standard (a monosaccharide or alditol that does not occur naturally) is added to each glycoconjugate at the beginning of its processing. Losses during the preparation of the sample for GLC will be proportionally the same for each component of the sample and for the internal standard. Thus, the ratio of analyte to internal standard will remain the same, even though the absolute amounts of both may be lower at the end. Quantitative analysis is performed by determining the detector responses for individual monosaccharides relative to the internal standard (known amounts are injected and the areas determined).

**Critical Parameters**

**HPAEC-PAD and other HPLC techniques**

When HPAEC-PAD is used for analysis of sialic acids, the sialic acids (which have a pKa of ∼2.0) bind to the resin at neutral pH, and it is necessary to use an anion to elute them. Samples are loaded in water to ensure binding and submitted to a pH gradient elution with dilute sodium acetate and acetic acid. This permits the separation of different O-acetylated sialic acid species, whose binding to the resin is relatively weaker with higher numbers of substituents. The relative detector responses vary significantly with the presence of different numbers of free hydroxyl groups. The response observed for Neu5Gc is 15% higher than that for Neu5Ac, which bears one fewer hydroxyl group. In contrast, the responses of the detector for Neu5-9Ac2 and Neu5-7(8)-9Ac3 are only 46% and 45% of the response for Neu5Ac, respectively. However, because of the possibility of partial de-O-acetylation between the point of addition of postcolumn alkali and the PAD detector, the detector response factors may vary somewhat in different laboratories. For preparative purposes, a maximum of 2 µmol of total sialic acids can be injected without overloading the 9 mm × 25 cm semipreparative column. After removal by Dowex 50 (H+) chromatography of the small amount of acetate required for separation using the HPAEC column, an excellent recovery (>90%) of acetylated species is possible.

When gradients that end in high concentrations of acetate (>100 mM) are used, the slow reequilibration of the column with low concentrations of hydroxide and acetate results in lower retention times and impaired separation. Resolution of some pairs of monosaccharides (e.g., Gal-GlcN and Xyl-Man) during the first isocratic step is extremely sensitive to minor changes in solvent composition. Therefore, extended equilibration periods between runs are necessary to obtain reproducible results. A shorter delay between runs, resulting in a column that is not fully equilibrated but that still achieves adequate separation for a set of monosaccharides, can produce repeatable results if an automatic sample injector with constant intervals between injections is employed.

When using amine adsorption–ion suppression chromatography, the relative proportion of solvents can be varied according to the kind of sialic acids to be separated. Because the eluant is monitored by UV absorbance at 200 nm, residual amounts of purification reagents pro-
duce an absorption peak close to the void volume. The lower limit of reliable detection is 2 nmol, and the detector responses are equal for all sialic acids. For preparative purposes, the eluant is monitored by UV and the peaks collected directly on ice, diluted 10-fold with cold water, and purified by ion-exchange chromatography on Dowex 3-X4A (formate; see UNIT 17.16) at 4°C. The column is washed with 10 vol of 10 mM formic acid and eluted with 7 vol of 1 M formic acid, and sialic acids are recovered by lyophilization.

**GLC with FID detection**

When the assignment of some peaks in gas-liquid chromatography (a GLC) run remains uncertain, coinjection with the suspected monosaccharide allows confirmation.

When analyzing trimethylsilylated methyl glycosides, the sample is quantitated by choosing one nonsuperimposed peak for each monosaccharide and considering how much of the compound this area represents. Absolute molar content of monosaccharide in the sample can be calculated when a known amount of an internal standard was added to the sample before processing.

Because the linkage between N-acetylglucosamine and asparagine is barely cleaved by methanolation, this fact has to be considered when quantitation is required. Dehydration of monosaccharides can also occur to a small extent.

**Anticipated Results**

When analyzing a neutral glycoconjugate (one that does not contain hexosamines, uronic acids, or sialic acids), total acid hydrolysis followed by HPAEC-PAD or reduction/peracylation and GLC-FID will indicate the relative composition or absolute composition (when an internal standard is added) of individual monosaccharides. When fucose is present, mild acid hydrolysis conditions can be tried to assure minimal destruction.

When hexosamines or uronic acids are present, acid hydrolysis followed by HPAEC-PAD or methanolation followed by re-N-acetylation/pertrimethylsilylation and GLC-FID can be used. Optimal conditions for maximum release (for both types of sugars) and minimal destruction (for uronic acids) must be employed for both approaches.

When sialic acids are present, HPAEC-PAD or amine adsorption–ion suppression HPLC with UV detection can be employed for compositional analysis of the different species after mild acid hydrolysis or enzymatic release. Alternatively, released sialic acids can be derivatized with DMB and submitted to HPLC (see UNIT 17.18).

In some cases it is necessary to use more than one protocol to obtain the complete compositional analysis of sugars.

Analysis of monosaccharides using one of the HPLC-PAD methods described in Basic Protocols 1, 2, or 3 results in the successful separation of the mixture into constituent monosaccharides. Figure 17.19.3 and Figure 17.19.4 illustrate separation profiles obtained from a mixture of monosaccharides subjected to HPAC-PAD.

As mentioned before, a characteristic pattern is obtained for each monosaccharide in the case of trimethylsilylated methyl glycosides. By doing individual runs of each monosaccharide and including an internal standard, it is possible to calculate the percentage of the total monosaccharide that each peak represents. Generally a minimum of 100 ng of each component is required for confident detection. Mo-

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### Table 17.19A.7 Molecular Weights of Monosaccharides Found in Glycoproteins and Their Volatile Derivatives

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>Molecular weight of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aldoses</td>
</tr>
<tr>
<td>Hexoses</td>
<td>180</td>
</tr>
<tr>
<td>Pentoses</td>
<td>150</td>
</tr>
<tr>
<td>Deoxyhexoses</td>
<td>164</td>
</tr>
<tr>
<td>Hexosamines</td>
<td>179</td>
</tr>
<tr>
<td>N-acetylated hexosamines</td>
<td>221</td>
</tr>
<tr>
<td>Hexosyluronic acids</td>
<td>194</td>
</tr>
</tbody>
</table>

*NA, not applicable.*
molecular weights of the monosaccharides found in glycoproteins, their alditols and methyl glycosides, and their volatile derivatives used in the protocols included in this section are listed in Table 17.19A.7.

**Time Considerations**
The total time of analysis for each particular method depends on how many runs of HPLC or GLC are required per sample. This depends in turn on the complexity of the sample, as sometimes coinjection with known standards may be required.

**Literature Cited**


**Key References**
Kamerling and Vliegenthart, 1989. See above.

Hardy and Townsend, 1994. See above.

Manzi et al., 1990. See above.

Descriptions of the principles of each method with examples of their application.

Contributed by Adriana E. Manzi
University of California San Diego
La Jolla, California
Composition of Labeled Monosaccharides from Glycosaminoglycans

Proteoglycans can be labeled metabolically with $[^3]$H-glucosamine (UNIT 17.4) to incorporate label into glucosamine and galactosamine. After isolation of proteoglycans (UNIT 17.2), glycosaminoglycans can be released from the proteoglycans by $\beta$-elimination (UNIT 17.15) and the distribution of labeled glucosamine and galactosamine in the isolated glycosaminoglycans can be determined. The glycosaminoglycans are hydrolyzed in acid until all the N-acetyl groups and N- and O-sulfates are removed. This procedure yields oligosaccharides in which some but not all of the uronic acid and amino sugar glycosidic bonds have been hydrolyzed. This partially hydrolyzed product is then treated with nitrous acid at pH 4 (UNIT 17.22A) to obtain a deaminative cleavage of the remaining amino sugar glycosidic bonds in which the reducing terminal of each of the resulting products is an anhydro-D-mannose (from D-GlcN) or an anhydro-D-talose (from D-GalN). The final depolymerization mixture, which contains disaccharides (uronosyl-anhydro sugars), free uronic acids, and free amino sugars, is reduced with NaBH$_4$ to convert the anhydromannose and androtalose residues to anhydromannitol and anhydrotalitol residues, respectively. The mixtures are then separated by paper chromatography and the amount of each labeled component is quantified by scintillation counting.

This procedure can also be used to determine the composition of glycosaminoglycans that have not been labeled metabolically. Samples of unlabeled glycosaminoglycans can be depolymerized in the same manner and the hydrolysis/deamination mixture can be reduced with NaB$_3$H$_4$ to give stoichiometric $^3$H labeling of each reducing sugar component of the mixture.

The protocol has been described under the assumption that the user has a working knowledge of the chromatographic procedures required and of the use of radioisotopes in quantitative procedures.

Materials

Glycosaminoglycan sample (optionally, metabolically labeled with $[^3]$H-glucosamine; UNIT 17.4)

- 20 $\mu$Ci/ml $[^4]$C-glucose solution (>40 mCi/mmole; optional internal standard)
- 3 M and 0.5 M H$_2$SO$_4$
- White mineral oil
- 5.5 M NaNO$_2$
- 1 M Na$_2$CO$_3$
- 0.25 M NaBH$_4$ or 0.25 M ~500 mCi/mmole NaB$_3$H$_4$, in 0.25 M NaOH
- 0.1 M and 1 M NaOH
- Paper chromatography System 1 and System 2 (see recipes)
- Scintillation cocktail (see recipe)
- 6 x 150-mm test tubes
- Sand baths: heating elements (Pierce) filled with sand, 99°C and 50°C
- Hamilton syringe
- Whatman no. 3 chromatography paper (grade 3 Chr)
- Whatman cellulose phosphate chromatography paper (grade P 81)
- Paper chromatography jars for descending chromatography
- Scintillation counter suitable for dual-label counting

Contributed by H. Edward Conrad

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Hydrolyze glycosaminoglycans with acid
1. Dissolve glycosaminoglycan sample in water and dialyze exhaustively against water to remove salts.

   If glycosaminoglycan sample is already radiolabeled (UNIT 17.4), its specific activity must be sufficient to permit detection following the procedure. If unlabeled glycosaminoglycans are to be analyzed after labeling by NaB\textsuperscript{3}H\textsubscript{4} reduction, a glycosaminoglycan concentration of 1 to 10 mg/ml is desirable. More specifically, the glycosaminoglycan sample should yield 0.1 to 1000 /GEC mol of each mono- or disaccharide to be assayed. This amount of material will incorporate >10\textsuperscript{4} cpm into each product after reduction with NaB\textsuperscript{3}H\textsubscript{4} with a specific activity of ∼500 mCi/mmol.

2. Transfer 40 µl of dissolved sample to a 6 × 150–mm test tube, add 20 µl of 20 µCi/ml \[^{14}C\]glucose, and mix.

   The \[^{14}C\]glucose is an optional addition that provides an internal standard. It can be replaced with 20 µl water if desired. The \[^{14}C\]glucose introduced should give ∼50,000 cpm/µl glycosaminoglycan solution and should have a specific activity high enough (>40 mCi/mmol) so that the amount of \(^3\)H incorporated upon reduction with NaB\textsuperscript{3}H\textsubscript{4} will not be significant.

3. Add 12 µl of 3 M H\textsubscript{2}SO\textsubscript{4} to the sample, cover the meniscus with a layer of white mineral oil to prevent evaporation of water (which would concentrate the acid during hydrolysis), and heat the mixture 6 hr at 99°С in a sand bath.

   The sample should not be heated at a temperature that would cause the water (acid) to boil during the hydrolysis. Boiling will disrupt the mineral oil layer and allow water to evaporate, concentrating the acid.

Cleave with nitrous acid
4. Cool the hydrolysate. Remove 8 µl with a Hamilton syringe and place in a 6 × 150–mm test tube, then add 20 µl of 5.5 M NaNO\textsubscript{2} (a large excess) to the sample. Allow deamination reaction to proceed 10 min at room temperature.

   If a \[^{14}C\]glucose internal standard is used, the volume of sample taken should be reflected by the amount of \(^{14}C\) in the aliquot, but the same ratio of sample to NaNO\textsubscript{2} solution must be used in order to bring the pH to 4 (the NaNO\textsubscript{2} neutralizes the acid). The sample pH, which may be measured with pH paper, should be −4 during the deamination reaction.

Reduce with sodium borohydride
5. Add 8 µl of 1 M Na\textsubscript{2}CO\textsubscript{3} (pH −8.5) followed by 5 µl of 0.25 M NaBH\textsubscript{4} or NaB\textsuperscript{3}H\textsubscript{4}.

   Unlabeled NaBH\textsubscript{4} is used if the sample has previously been metabolically radiolabeled, and NaB\textsuperscript{3}H\textsubscript{4} is used if the sample has not been labeled.

   CAUTION: All NaB\textsuperscript{3}H\textsubscript{4} manipulations (steps 5 to 9) must be carried out in a well-ventilated fume hood.

6. Cork the reaction tube and heat 30 min at 50°С in a sand bath to reduce the aldehyde groups formed in the deamination reaction.

7. Cool the reduced sample to room temperature. Working in the fume hood, add sufficient 0.5 M H\textsubscript{2}SO\textsubscript{4} (−20 µl) to the sample to reduce the pH to <3 (this destroys excess NaBH\textsubscript{4} or NaB\textsuperscript{3}H\textsubscript{4}).

8. After −30 min, dry the sample in a stream of air. Redissolve sample in water and dry again to remove residual H\textsubscript{2} or \(^3\)H\textsubscript{2} gas.

9. Dissolve the sample in 50 µl water and add 1 M NaOH until the solution become slightly alkaline (pH 8 to 10), as observed with pH paper.
Spot samples and separate by chromatography

10. Mark sheets of Whatman no. 3 and Whatman cellulose phosphate chromatography paper with pencil lines $\frac{1}{2}$ in. apart, then cut the sheets perpendicularly to the lines to create strips $1 \times \sim 22$ in. (2.5 $\times$ 57 cm).

11. Spot 5- to 10-µl aliquots of each reduced sample from step 8 onto two strips of Whatman no. 3 paper and one strip of cellulose phosphate paper. Place the aliquots in the middle of the sixth $\frac{1}{2}$-in. segment from one end of each strip; this end is the wick. Allow the spots to dry.

IMPORTANT NOTE: No more than 5 to 10 µl of the original hydrolysate should be analyzed on a chromatogram, because excess salt in the sample may interfere with the resolution of the components of the mixture. The borate remaining in the sample after the destruction of the NaBH₄ has no effect on the migration of the products.

12. Fold the wick ends so that they will fit properly into a descending paper chromatography trough. Place the two Whatman no. 3 strips in chromatography jar containing paper chromatography System 1 reagent and allow them to develop for 15 and 40 hr, respectively. Place the cellulose phosphate strip in a jar containing paper chromatography System 2 reagent and allow it to develop for 10 to 12 hr.

Determine radioactivity by scintillation counting

13. Remove developed strips from solvents after the appropriate times have elapsed. Allow the strips to dry, then cut strips into $\frac{1}{2}$-in. segments along the pencil markings made in step 10. Place the segments in separate scintillation vials.

14. Add scintillation cocktail to each scintillation vial to cover the paper segment. Determine the radioactivity of each segment by counting in a scintillation counter under optimal conditions for dual-label counting.

15. Plot the data for each strip as cpm vs. segment number.

Analyze data

16. If a [¹⁴C]glucitol internal standard was used, compare the migration position of each peak with that of the standard to identify the $^3$H-labeled peaks (see Table 17.19B.1).

\[
\text{the } ^3\text{H peak must be corrected for the } ^{14}\text{C spillover into the } ^3\text{H channel.}
\]

Table 17.19B.1 shows the $R_{\text{glucitol}}$ values (the distance that each reduced component on the chromatogram migrates relative to the distance that the [¹⁴C]glucitol internal standard migrates) for the expected hydrolysis products in the two chromatography systems. The 15-hr run in System 1 gives a value for the fast-moving anhydromannose and anhydrotalose formed from glucosamine and galactosamine, respectively. The 40-hr run in System 1 separates unhydrolyzed disaccharides, while the [¹⁴C]glucitol peak migrates to the end of the strip. Separation of glucuronic and iduronic acids, which migrate together in System 1, is obtained on the System 2 chromatogram.

17. Sum the $^3$H cpm in each peak and normalize the result to the same number of [¹⁶C]glucitol cpm (the value for the $^{14}$C cpm used for the normalization may be chosen arbitrarily, but it must be the same for each paper strip used for the glycosaminoglycan sample being analyzed).

18. Calculate the percentage of each monosaccharide in the hydrolysate as [(total cpm for the monosaccharide in question)/(total cpm in all components)] $\times$ 100. Calculate the relative amounts of each monosaccharide component from Table 17.19B.2.
Table 17.19B.1  \( R_{\text{glucitol}} \) Values for \[^{3}H\]Alditols\(^a\)

<table>
<thead>
<tr>
<th>Product(^b)</th>
<th>System 1</th>
<th>System 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gal(_R) (KS)</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td><strong>Deamination products</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMan(_R) (GlcN)</td>
<td>2.0</td>
<td>—</td>
</tr>
<tr>
<td>ATal(_R) (GalN)</td>
<td>2.0</td>
<td>—</td>
</tr>
<tr>
<td><strong>Uronic acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GlcA(_R) (Hep, HS, DS)</td>
<td>0.95</td>
<td>0.55</td>
</tr>
<tr>
<td>IdoA(_R) (Hep, HS, DS)</td>
<td>0.95</td>
<td>0.33</td>
</tr>
<tr>
<td><strong>Disaccharides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\alpha)IdoA1-4AMan(_R) (Hep and HS)</td>
<td>0.70</td>
<td>1.15</td>
</tr>
<tr>
<td>(\beta)GlcA1-4AMan(_R) (Hep and HS)</td>
<td>0.50</td>
<td>1.00</td>
</tr>
<tr>
<td>(\alpha)IdoA1-3ATal(_R) (DS)</td>
<td>0.64</td>
<td>0.64</td>
</tr>
<tr>
<td>(\beta)GlcA1-3ATal(_R) (CS and DS)</td>
<td>0.45</td>
<td>0.48</td>
</tr>
<tr>
<td>(\beta)GlcA1-3AMan(_R) (HA)</td>
<td>—</td>
<td>1.00</td>
</tr>
</tbody>
</table>

\(^a\)All values are for aldehyde-reduced sugars, as indicated by the subscript \(R\). Abbreviations: AMan, anhydro-D-mannose; ATal, anhydro-D-talose; CS, chondroitin sulfate; DS, dermatan sulfate; Gal, D-galactose; GlcA, D-glucuronic acid; GlcN, D-glucosamine; Hep, heparin, HS; heparan sulfate; IdoA, L-iduronic acid; KS, keratan sulfate.

\(^b\)The glycosaminoglycan from which each product is derived is given in parentheses. Standards for AMan\(_R\) and ATal\(_R\) can be obtained by treating GlcN and GalN, respectively, with nitrous acid at pH 4 and reducing the products with NaB\(_3\)H\(_4\) (Shively and Conrad, 1970). Standards for IdoA and disaccharides must be obtained by hydrolysis and nitrous acid deamination of heparin, chondroitin sulfate, dermatan sulfate or hyaluronic acid followed by NaB\(_3\)H\(_4\) reduction of the products (Conrad, 1980).

Table 17.19B.2  Cpm Equivalents for Monosaccharides\(^a\)

<table>
<thead>
<tr>
<th>Amount of monosaccharide</th>
<th>Sum of total normalized (^3)H cpm in:</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIcA(_R)</td>
<td>GIcA(_R) + (\beta)GlcA1-4AMan(_R) + (\beta)GlcA1-3ATal(_R) + (\beta)GlcA1-3AMan(_R)</td>
</tr>
<tr>
<td>IdoA(_R)</td>
<td>IdoA(_R) + (\alpha)IdoA1-4AMan(_R) + (\alpha)IdoA1-3ATal(_R)</td>
</tr>
<tr>
<td>AMan(_R)</td>
<td>AMan(_R) + (\beta)GlcA1-4AMan(_R) + (\beta)GlcA1-3AMan(_R)</td>
</tr>
<tr>
<td>ATal(_R)</td>
<td>ATal(_R) + (\beta)GlcA1-3ATal(_R) + (\alpha)IdoA1-3ATal(_R)</td>
</tr>
</tbody>
</table>

\(^a\)The subscript \(R\) refers to the aldehyde-reduced form of each mono- or disaccharide. See Table 17.19.8 for abbreviations.

**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.*

**Paper chromatography System 1**

18:3:1:4 (v/v/v/v) ethyl acetate/glacial acetic acid/88% formic acid/water.

*System 1 is used with Whatman no. 3 chromatography paper.*

**Paper chromatography System 2**

3:2:1 (v/v/v) ethyl acetate/pyridine/0.005 M boric acid.

*System 2 is used with cellulose phosphate chromatography paper.*

**Scintillation cocktail**

4 g diphenyloxazole in 1 liter toluene.
Background Information

With the exception of keratan sulfate, which contains alternating D-galactose and N-acetyl-D-glucosamine residues, all glycosaminoglycans are sequences of disaccharide units containing a uronic acid (D-glucuronic acid and/or L-iduronic acid) and an amino sugar (D-glucosamine or D-galactosamine). Disaccharides of chondroitin sulfate contain D-GalNAc and D-GlcA, whereas those from dermatan sulfate contain D-GalNAc and either D-GlcA or L-IdoA. Disaccharides from heparin and heparan sulfate contain D-GlcN, which may be either N-acetylated or N-sulfated, and either D-GlcA or L-IdoA. Thus, if one has identified a glycosaminoglycan that is free of other glycosaminoglycans, the primary purpose of monosaccharide analysis is to determine the relative proportions of the two uronic acid types. However, when glycosaminoglycans are isolated from natural sources, they are often recovered as mixtures of several types of glycosaminoglycans that are difficult to resolve completely. In such cases, monosaccharide analysis can often demonstrate the nature of the mixtures by showing the relative proportions of GlcNAc and GalNAc as well as the specific disaccharide unit in which they are found. Individual glycosaminoglycans in a mixture can be recognized by their susceptibility to cleavage by specific enzymes (UNIT 17.13B) or nitrous acid (UNIT 17.22A). In the present method, the glycosaminoglycans are cleaved by acid hydrolysis, which results in loss of the sulfates. Procedures for determination of glycosaminoglycan disaccharides that have retained their sulfate substituents are described in UNITS 17.13B & 17.22A.

Following acid hydrolysis and nitrous acid cleavage of unlabeled glycosaminoglycans, it would be equally feasible to analyze the products of this cleavage reaction sequence by gas chromatography or HPLC without use of NaB₃H₄. Although such an analytical scheme has not been developed, it would represent a way to avoid use of radioisotopes. The paper chromatographic procedure used here does offer the possibility of analyzing many samples simultaneously, which cannot be done with the GC or HPLC approaches where samples must be analyzed sequentially. Furthermore, when unknown peaks occur in this analysis, it is clear that the relative amounts of the unknowns are reflected by the amounts of ³H in the peaks, because (1) ³H labeling of carbohydrates is identical for all reducing carbohydrates when expressed on a molar basis and (2) carbohydrates are virtually the only naturally occurring compounds that can be reduced by NaBH₄ or NaB₃H₄. Moreover, it is easy to elute labeled products from the paper segments after analysis for further characterization. This is readily accomplished by rinsing the segments in toluene (to remove the diphenyloxazole remaining from the scintillation cocktail), then eluting the dried segment with water or buffer.

Critical Parameters

The challenge in analyzing the monosaccharide composition of glycosaminoglycans is not in the quantitation of the monosaccharides but in the depolymerization step. The impossibility of obtaining complete hydrolysis of glycosaminoglycans to monosaccharides without decomposition of some of the monosaccharide residues cannot be emphasized too strongly. The labilities of the glycosidic bonds of N-acetylated amino sugars (Shively and Conrad, 1970) and L-iduronic acid (Conrad, 1980) are similar to those for neutral hexoses, but the well-known stability of glucuronic acid glycosides virtually precludes the complete hydrolysis of these bonds without destruction of these residues as well as the other monosaccharide components of the glycosaminoglycan samples. Furthermore, hydrolytic release of the N-acetyl groups of the amino sugars occurs at a rate similar to that for the GlcNAc or GalNAc glycosides, so that many of the acetyl groups are released prior to complete hydrolysis of the amino sugar glycosidic bonds. This precludes complete hydrolysis of the amino sugar glycosides, as the glycosidic bonds of the N-unsubstituted GlcN and GalN are extremely stable to acid (Shively and Conrad, 1970). For the N-sulfated GlcN residues in heparin and heparan sulfate, this problem is particularly serious, as the N-sulfate substituents are very labile and are removed during the first few minutes of hydrolysis.

The tack taken here is to circumvent these hydrolysis problems by taking advantage of the elimination reaction that occurs when N-unsubstituted amino sugars are treated with nitrous acid (in spite of the misstatements often seen in the literature, this is not a hydrolysis reaction), thus cleaving these amino sugar glycosides under extremely mild conditions. This approach requires only that hydrolysis be continued long enough for complete removal of N-sulfate and N-acetyl groups. This avoids the
destruction of monosaccharide residues that occurs when forcing conditions of hydrolysis are used and allows almost stoichiometric recovery of these glycosaminoglycans as monosaccharides, which can be analyzed together as described. Identical results for the analytical procedure described here are obtained whether the acid hydrolysis is carried out for 4, 6, or 8 hr before adding the nitrous acid reagent (Conrad, 1980). This is a reflection of the fact that all N- and O-sulfate groups and all N-acetyl groups will have been completely removed after 4 hr of hydrolysis. During the subsequent period (from the fourth to the eighth hour) there is some further hydrolysis, which reduces the yields of disaccharides and increases the yield of monosaccharides in the assay mixture, but this does not change the final analytical results.

For accurate results, it is advisable to first separate the glycosaminoglycan chains from the core protein and to isolate the free glycosaminoglycan chains for the analysis (UNIT 17.15). This avoids the possibility of loss of monosaccharides due to the Browning reaction, which occurs when carbohydrates and amino acids are heated together in acid.

The nitrous acid reaction is virtually stoichiometric for β-linked amino sugars (Shively and Conrad, 1970). Consequently, disaccharides are obtained in stoichiometric yields from glycosaminoglycans that contain β-linked amino sugars (chondroitin sulfate, dermatan sulfate, hyaluronic acid, and keratan sulfate). However, a side reaction, the ring contraction reaction, occurs at a frequency of 10% to 15% for α-linked amino sugars such as those found in heparin and heparan sulfate (Shively and Conrad, 1970; 1976). The ring contraction reaction results in deamination without bond cleavage and may convert a heparin tetrasaccharide into a new tetrasaccharide instead of two disaccharides. Thus, heparin and heparan sulfate yield significant amounts of ring contraction tetrasaccharides (Bienkowski and Conrad, 1985). However, there seems to be little structural selectivity for the ring contraction: the ring contraction tetrasaccharides contain disaccharide units in the same proportions as are found in the original glycosaminoglycan. Thus, even for heparin and heparan sulfate, the disaccharides formed during nitrous acid cleavage are obtained in the same proportions that are found in the original glycosaminoglycan. Consequently, when yields are expressed in percent of total uronic acids or percent of total amino sugars, the ring contraction reaction does not interfere with the quantitation, and the yields of di- and monosaccharides are truly representative of their proportions in the starting glycosaminoglycan. Some of these heparin ring contraction tetrasaccharides have been identified (UNIT 17.22B).

Troubleshooting
In order to obtain reproducible migration of peaks, it is necessary to have chromatography tanks well-equilibrated with freshly prepared solvents. If many strips are placed in the tank, it may be necessary to add more chromatography solvent after the first day of the 40-hr development.

Anticipated Results
The results obtained here yield the distribution of labeled monosaccharides in metabolically labeled starting material or the monosaccharide compositions of the unlabeled glycosaminoglycan starting material. This is possible even though complete hydrolysis is not achieved, because one can separate and quantitate disaccharides of known composition.

Time Considerations
The analytical procedure can be completed in 3 days, with 1 day for the depolymerization and NaB³H₄ reduction, two overnight periods for paper chromatography, and finally scintillation counting and calculations. The procedure can be interrupted at any stage. A major advantage of the paper chromatography procedure over HPLC or GC methods is that many samples can be analyzed simultaneously. The only sequential phase of the analysis is scintillation counting, but the conditions described are designed to yield the mono- and disaccharides in sufficient yields (i.e., total ³H cpm in each peak) so that 30-sec counting times for each paper segment result in good counting statistics.

Literature Cited

**Key Reference**
Conrad, 1980. See above.

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Con Conrad, 1980. See above.

Contributed by H. Edward Conrad
University of Illinois
Urbana, Illinois
Analysis of Oligosaccharide Negative Charge by Anion-Exchange Chromatography

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 or change in negative charge after treatment of the oligosaccharide sample with mild acid and/or phosphatases.

BASIC PROTOCOL

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

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 (UNIT 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...
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 phosphodiesters. (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

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.
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 phosphodiesters and phosphomonoesters (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 phosphodiesters; 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 phosphodiesters. A second, identical sample should be prepared and processed starting with step 3; if phosphodiesters 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 phosphonoesters and phosphodiesters, separation can be obtained with a
gradient of ammonium acetate adjusted to pH 5.3 (close to the pK\textsubscript{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**


Key Reference

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

Contributed by Ajit Varki
University of California San Diego
La Jolla, California
HPLC Methods for the Fractionation and Analysis of Negatively Charged Oligosaccharides and Gangliosides

This unit describes the fractionation and analysis of anionic oligosaccharides (Basic Protocol 1) and gangliosides (Basic Protocol 2) using anion-exchange high-performance liquid chromatography (HPLC). The sample is loaded onto the column in a low-ionic-strength solvent. The anionic saccharides or gangliosides are retained by the positively charged matrix by ionic interactions; the more negative charge on the compound, the more tightly it binds to the column. Neutral molecules pass through the column unretained. Elution is effected by a gradient of increasing ionic strength that progressively disrupts the ionic interactions between the analyte and the column. Saccharides or gangliosides are eluted in order of the number of negative charges they possess, although the charge-to-mass ratio can also contribute to elution position. Hence, a molecule with a high charge-to-mass ratio may elute later than one with the same net charge but a smaller charge-to-mass ratio. Those unfamiliar with the basic principles of HPLC are referred to UNITS 10.12-10.14 or to Snyder et al. (1988).

BASIC PROTOCOL 1

FRACTIONATION OF RELEASED N-LINKED OLIGOSACCHARIDES ON DEAE HPLC COLUMNS

The use of DEAE HPLC columns to separate sialylated N-linked oligosaccharides is described. The DEAE column is equilibrated in water and eluted with a gradient of increasing NaCl concentration. Sialic acids are detected based on their radioactivity (if previously radiolabeled) or by the TBA assay for sialic acids (if unlabeled) and comparison with known standards.

Materials

- HPLC-grade or high-quality deionized water (e.g., 18 MΩ Milli-Q type)
- 0.5 M sodium chloride (NaCl) in HPLC-grade water (see recipe)
- Oligosaccharide sample (Peptide N:glycosidase F–released and desalted; UNIT 17.13A)
- Sialylated oligosaccharide standards (e.g., Dionex or Oxford Glycosystems)
- HPLC apparatus capable of two-component gradient formation
- DEAE HPLC column (TosoHaas TSK-gel DEAE-2SW, 25-cm × 4.6-mm i.d., 5-mm particle size, or equivalent)
- Additional reagents and equipment for desalting sample (UNIT 17.13A & 17.14A) and (if analyzing unlabeled sample) TBA assay (UNIT 17.18)

1. Before using any column, wash the column thoroughly. First, wash with at least 10 column volumes of water. Next, wash the column with a linear gradient from 0 to 0.5 M NaCl over 100 min, and then from 0.5 M NaCl to 100% water over 100 min. Use a flow rate of 0.4 to 0.6 ml/min for all washes.

   The water and all buffers used should be prefilted through a 0.45-µm filter and degassed. Column prefilters and/or guard columns provide additional protection for the HPLC column and increase its productive lifetime.

   A flow rate of 0.4 to 0.6 ml/min is recommended for all column washings and elutions. Do not exceed the manufacturer’s recommendations for either the flow rate or maximum pressure.
2. Thoroughly clean the injection loop with water.

3. Equilibrate the column at room temperature with 100% water at a flow rate of 0.6 ml/min. Wash with at least 10 column volumes of water.

4. Check the baseline obtained with the detection method to be used for the sample. Do not proceed until a stable baseline is obtained.

   For analytical work, on-line radioactivity detectors or UV detectors may be used. For UV detection, absorbance at 190 to 210 nm is measured. Because many compounds absorb at these wavelengths, this method of detection demands high purity of the sample. Alternatively, individual fractions may be collected and analyzed by one of several methods (see step 8). Collect a portion of the eluate and establish that the solvents do not interfere with the detection method chosen and that the baseline is stable.

5. Set up a program from the pump control unit for washing unbound material, eluting the bound species, and bringing the column back to the starting conditions, as indicated in Table 17.21A.1.

   The NaCl gradient should be shallow for two reasons. First, the difference in NaCl concentration needed to elute oligosaccharides differing by a single negative charge is ~20 mM NaCl, depending on the column. Second, the DEAE resin will shrink and swell with increasing and decreasing NaCl concentrations, respectively. Therefore, rapid changes in ionic strength may damage the column and degrade its performance.

6. Make a “sham” run (injecting water in place of sample), monitoring the eluant, if an on-line detection system is used. If fractions are collected and individually analyzed, collect them every 1 min. Load the water into the injection loop using a syringe with a flat-tip needle compatible with the HPLC injector. Inject the water onto the column and start the gradient program.

   If contaminant peaks are observed (peaks >5% full scale when using the detector settings required for the sample), the column must be washed until the peaks disappear. (When using UV detection, a peak will be observed at the void volume, indicating the change in absorbance produced when the water injected passes through the column.) To wash, repeat the gradient and hold at the maximum concentration of NaCl for at least 10 column volumes. Wash extensively with water, then repeat the blank run.

7. Inject and analyze the sialylated oligosaccharide standards individually as indicated in step 6. Determine the elution time for each of the standards. If the separation of the individual standards is adequate, proceed to the analysis of the samples. If not, it may be necessary to adjust the rate of increase in the NaCl concentration to obtain better separation.

   The column should be routinely standardized. At least one standard run, preferably with a mixture of sialylated oligosaccharide standards, should be performed at the start of each day.

### Table 17.21A.1

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Eluant</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100% H₂O</td>
</tr>
<tr>
<td>5</td>
<td>100% H₂O</td>
</tr>
<tr>
<td>95</td>
<td>50% 0.25 M NaCl</td>
</tr>
<tr>
<td>100</td>
<td>50% 0.25 M NaCl</td>
</tr>
<tr>
<td>140</td>
<td>100% H₂O</td>
</tr>
<tr>
<td>155</td>
<td>100% H₂O</td>
</tr>
</tbody>
</table>
8. Analyze the desalted samples using the optimum conditions determined in step 7. If the amount of sample is small or the column is being used to obtain individual species for subsequent analysis, collect and analyze fractions every 0.5 to 1.0 min. Analyze the individual fractions by using either liquid scintillation counting or the TBA assay for sialic acids (UNIT 17.18).

The sample must be desalted (as described in UNITS 17.13A & 17.14A) prior to analysis, because even low concentrations of NaCl can prevent the initial binding of the anionic oligosaccharides to the cationic matrix. The desalted oligosaccharides can be concentrated by lyophilization and resuspended in a small volume of water. The main factor in determining the appropriate sample volume is the size of the injection loop, although injection loops of ≤500 μl are preferred.

9. Estimate the number of sialic acid residues present by comparing the elution position of each peak to that of the sialylated oligosaccharide standards run in the same conditions.

The linkage of the sialic acid residues can also be determined using sialidase digestion (UNIT 17.12) in combination with repeated anion-exchange HPLC. If, for instance, a sample that originally comigrated with a trisialylated triantennary oligosaccharide standard is treated with Newcastle disease virus sialidase, under conditions in which only α2-3-linked sialic acids are released, and now comigrates with a monosialylated species, it can be inferred that the oligosaccharide in question has two α2-3-linked sialic acid residues. Additionally, if the oligosaccharide in question is not retained by the DEAE column following treatment with the broad-spectrum sialidase from Arthrobacter ureafaciens, it can be inferred that the negative charge on the oligosaccharide in question was due solely to sialic acid residues. Although this type of analysis can be performed on a mixture of oligosaccharides, it is preferable to first isolate the individual HPLC peaks and then to treat with sialidase and reanalyze each separately.

CAUTION: Remember to desalt the samples submitted to sialidase digestion prior to ion-exchange HPLC analysis.

FRACTIONATION OF GANGLIOSIDES ON DEAE HPLC COLUMNS

The use of DEAE HPLC columns to separate gangliosides from neutral lipids and glycolipids and fractionate them according to the number of sialic acid residues is described. Total lipid extracts, partially fractionated negatively charged glycolipids (total lipid extracts or the fraction enriched in polar lipid obtained by Folch partitioning of the total lipid extracts; see UNIT 17.3), or the total ganglioside fraction eluted from a DEAE anion-exchange column (see UNIT 17.3) can be further fractionated and analyzed by this method. The DEAE column is equilibrated in organic solvent and eluted with a gradient of increasing ammonium acetate concentration. Fraction components are detected based on their radioactivity (if previously radiolabeled) or by HPTLC analysis with chemical or immunological detection (if unlabeled) and gangliosides identified by comparison with known standards.

Materials

- HPLC-grade or high-quality deionized (e.g., 18 M Ω Milli-Q type) water
- HPLC-grade chloroform
- HPLC-grade methanol
- 1:8:1 (v/v/v) chloroform/methanol/1 M aqueous ammonium acetate (see recipe)
- Ganglioside sample (extracted and desalted; UNIT 17.3)
- Ganglioside standards (G_{M1}, G_{D1a} and G_{T1b}; e.g., Sigma, Boehringer Mannheim, or Calbiochem)
- HPLC apparatus capable of two-component gradient formation
- DEAE HPLC column (TosoHaas TSK DEAE-2SW, or comparable)
Additional reagents and equipment for high-performance thin-layer chromatography (HPTLC; if analyzing unlabeled sample, UNIT 17.17A) with chemical detection (UNIT 17.10B) and for TBA assay (UNIT 17.18).

1. Equilibrate the column in organic solvent by running a linear gradient from 100% water to 1:8:1 (v/v/v) chloroform/methanol/water at 1 ml/min, over 1 hr. If the column was stored in buffer, first wash extensively with water. The water and all aqueous buffers used should be prefilted through a 0.45-µm filter and degassed. Do not degas mixtures containing organic solvents prior to chromatography; moreover, for systems that require continuous helium bubbling during chromatography, a low flow is sufficient. Column prefilters and/or guard columns provide additional protection for the HPLC column and increase its productive lifetime. When using a guard column, be sure to connect it prior to performing the equilibration with organic solvent.

2. Convert the column to the acetate form by running a 1-hr linear gradient from 1:8:1 (v/v/v) chloroform/methanol/water to 1:8:1 (v/v/v) chloroform/methanol/1 M aqueous ammonium acetate at 1 ml/min, and holding at this concentration of salt for another 1 hr. Wash the column extensively with 1:8:1 (v/v/v) chloroform/methanol/water to remove the salt. Use a flow rate of 1 ml/min throughout this step.

3. Flush out the injection loop with water, then methanol, then chloroform, and finally with 1:8:1 (v/v/v) chloroform/methanol/water.

4. For columns previously used to analyze radioactive samples, check the background with an on-line radioactivity detector or by collecting fractions and counting.

5. Set up a program from the pump control unit for washing unbound material, eluting the bound species, and bringing the column back to the starting conditions at 1 ml/min flow as indicated in Table 17.21A.2.

The DEAE resin will shrink and swell with increasing and decreasing salt concentrations, respectively. Therefore, transition from high salt concentration to no salt has to be done slowly. Rapid changes in ionic strength may cause damage to the column and degrade column performance.

<table>
<thead>
<tr>
<th>Step</th>
<th>Time (min)</th>
<th>Eluant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wash</td>
<td>0</td>
<td>1:8:1 (v/v/v) chloroform/methanol/water</td>
</tr>
<tr>
<td>Elution</td>
<td>10</td>
<td>1:8:1 (v/v/v) chloroform/methanol/water</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>1:8:1 (v/v/v) chloroform/methanol/aqueous 1 M ammonium acetate</td>
</tr>
<tr>
<td>Reequilibration</td>
<td>100</td>
<td>1:8:1 (v/v/v) chloroform/methanol/water</td>
</tr>
<tr>
<td></td>
<td>130</td>
<td>1:8:1 (v/v/v) chloroform/methanol/water</td>
</tr>
</tbody>
</table>
6. Dissolve equivalent amounts of the ganglioside standards in 1:8:1 (v/v/v) chloroform/methanol/water. Load the solution of the standards into the injection loop using a syringe with a flat-tip needle compatible with the HPLC injector.

Solutions of these standards in 2:1, 1:1, or 1:2 (v/v) chloroform/methanol can also be used. When using a solvent other than the running solvent, keep the injection volume as small as possible.

7. Inject the solution onto the column and start the gradient program. When analyzing radiolabeled standards, use an on-line radioactivity detector or route the eluate to a fraction collector (collecting 1-min fractions), remove aliquots of each fraction, add scintillation fluid, and measure radioactivity by counting in a scintillation counter. When analyzing nonradioactive standards, collect fractions and analyze one aliquot of each fraction by HPTLC, detecting the bands chemically (UNIT 17.10B) or by overlaying with monoclonal antibodies when available.

When using an on-line radioactivity detector, determine the required ratio of scintillation cocktail to eluant to avoid excessive quenching by the organic solvents.

8. Determine if the separation of the ganglioside standards obtained is as expected (see Table 17.21A.3).

Separation is typically very reproducible, and it is not necessary to run standards each time the column is used.

<table>
<thead>
<tr>
<th>Table 17.21A.3 Expected Retention Times of Ganglioside Standards</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ganglioside standard</td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td>( G_{M1} )</td>
</tr>
<tr>
<td>( G_{D1a} )</td>
</tr>
<tr>
<td>( G_{T1b} )</td>
</tr>
</tbody>
</table>

9. Analyze the individual fractions by liquid scintillation counting, by the TBA assay (UNIT 17.18), or by HPTLC as indicated in step 7.

Normally, total lipid extract is obtained free of salts (UNIT 17.3 or UNIT 17.10B) and can be directly analyzed. Samples submitted to previous fractionation steps using salts must be desalted by dialysis or by loading onto a Sep-Pak C18 cartridge prior to analysis (salts are washed off with water and anionic molecules eluted with organic solvents), because even low concentrations of salt can prevent binding of the anionic oligosaccharides to the cationic matrix (UNIT 17.3). Desalted gangliosides can be concentrated by lyophilization and redissolved in a small volume of 1:8:1 (v/v/v) chloroform/methanol/water.

When analyzing radioactive labeled samples, a peak in the void volume indicates the presence of neutral species (e.g., neutral lipids or glycolipids) that are also radioactive. These neutral species should not react in the TBA assay (UNIT 17.18) or with resorcinol spray (UNIT 17.10B). If TBA- or resorcinol-positive species are present at the void volume, the column capacity has been exceeded.

CAUTION: Dry down the organic solvent prior to performing the TBA assay.

When analyzing a large portion of each fraction by HPTLC, the water in the elution solvent will take some time to evaporate. Transfer the aliquot to a microcentrifuge tube, evaporate the solvent to dryness (i.e., use a Speedvac evaporator system), and redissolve in 2:1 (v/v) chloroform/methanol to spot the plate.
10. Determine the type of ganglioside (e.g., mono-, di-, or trisialylated) present by comparing the elution position of each peak to that of known ganglioside standards run in the same conditions.

11. Determine the number of gangliosides in each fraction by analyzing the HPTLC patterns obtained with resorcinol staining (UNIT 17.10B).

12. When doing semipreparative chromatography, pool the fractions according to the profile obtained. Concentrate under vacuum, dissolve in a small volume of 1:8:1 (v/v/v) chloroform/methanol/water, dilute with 10 vol water, and desalt by overnight dialysis against water using 500- or 1000-MWCO tubing. Recover the gangliosides by lyophilization.

   Alternatively, dry sample, resuspend in water, and desalt using a Sep-Pak C18 cartridge previously washed successively with 20 ml each chloroform, dimethyl sulfoxide, acetonitrile, methanol, and water (see also UNIT 17.3).

   IMPORTANT NOTE: Semipreparative chromatography is defined as the fractionation of the individual species obtained by HPLC with a standard-sized column.

**REAGENTS AND SOLUTIONS**

*Use HPLC-grade or other high-quality deionized water (e.g., 18 MΩ Milli-Q type) in all recipes and protocol steps, and filter through a 0.45-μm filter and degas before use. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.*

**Chloroform/methanol/1 M aqueous ammonium acetate, 1:8:1 (v/v/v)**

Dissolve 77 g ammonium acetate in HPLC-grade water. Adjust the volume to 1 liter and filter through a 0.45-μm filter. Combine 1 vol of this solution with 1 vol chloroform and 8 vol methanol, mix, and use for HPLC. Store in a tightly capped bottle.

**Sodium chloride (NaCl), 0.5 M**

Dissolve 29.25 g NaCl in 900 ml water. Adjust the volume to 1 liter and filter through a 0.45-μm filter before use.

**COMMENTARY**

**Background Information**

Ion-exchange HPLC using weak anion exchange chromatography columns has been used to analyze and/or fractionate a variety of anionic oligosaccharides and glycoconjugates. Negatively charged saccharides bind to the positively charged resin of the column and are eluted in order of increasing negative charge with a gradient of increasing ionic strength. DEAE HPLC columns have been run in water for the fractionation of released N- and O-linked oligosaccharides and in organic solvents for the fractionation of gangliosides. Although HPLC analysis requires more equipment than chromatography on QAE-Sephadex (UNIT 17.19), the profiles obtained are sharper and saccharides containing few negative charges can be better resolved.

DEAE and amine-bonded (Baenziger and Natowicz, 1981) HPLC columns have been used to fractionate released N-linked oligosaccharides that differ in their number of sialic acids. Sialidase digestion (UNIT 17.12) followed by repeated anion-exchange chromatography yields information about the number and linkage of the sialic acid residues on the oligosaccharide. The decrease in negative charge following sialidase treatment indicates the number of sialic acid residues removed, and the linkage is inferred from knowledge of sialidase specificity. Similarly, oligosaccharides can be analyzed for the presence of phosphate by subjecting them to ion-exchange HPLC analysis following alkaline phosphatase digestion with and without prior mild acid treatment (Varki and Kornfeld, 1983). If the phosphate is “covered” by N-acetyl-D-glucosamine (GlcNAc), it will be resistant to alkaline phosphatase. Treatment with mild acid removes the GlcNAc residue and exposes the phosphate for removal by alkaline phosphatase.
Sulfated and sialylated/sulfated oligosaccharides have also been separated using weak anion-exchange HPLC columns. Amine-bonded columns (Micropak AX-5, Varian) have been used to analyze sulfated/sialylated oligosaccharides (Green and Baenziger, 1986). When the column eluant is buffered to pH 4, both sialic acid and sulfate have one negative charge. However, at pH 1.7 sialic acid is only partially ionized and, therefore, has a net negative charge of <1. Thus, performing the HPLC analysis at two different pHs, near the pK of one of the anionic species, permits the fractionation of sulfated and sulfated/sialylated oligosaccharides. This approach has been adapted to DEAE HPLC columns by buffering the column with phosphate buffer at pH 2.5 for the separation of dermatan sulfate fragments (Hayes and Varki, 1993a).

The separation of more negatively charged saccharides requires the use of strong anion-exchange columns. Polysialic acids with 2 to 20 sialic acids have been separated using a Mono-Q column (Hallenbeck et al., 1987).

Several methods to detect oligosaccharides are available. The choice depends upon the nature of the sample. Radiolabeled oligosaccharides can be detected using an on-line radioactivity detector or by collecting fractions and analyzing all or a portion of the individual fractions by liquid scintillation counting. Oligosaccharides can be radiolabeled, either metabolically (UNIT 17.4), enzymatically (UNIT 17.6), or chemically (UNIT 17.5). Oligosaccharides can also be detected spectrophotometrically, as many monosaccharides absorb between 190 and 205 nm. This method, though simple, requires quantities of oligosaccharide that are often prohibitive (>5 nmol). Moreover, the sample and HPLC solvents must be of high purity and the elution medium cannot contain salts that absorb at these wavelengths (e.g., acetate). An alternative approach is to assay the fractions for the presence of sialic acid using the TBA assay (UNIT 17.10), or, in the case of gangliosides, by HPTLC with chemical detection (UNIT 17.10B).

Interpretation of data depends on the use of appropriate standards. Several sialylated N- and O-linked oligosaccharide standards are commercially available. The same standards used to calibrate the HPLC column can be used as controls for sialidase digestions. It should be noted that several distinct oligosaccharide structures may have equivalent net negative charge, resulting in coelution; therefore, DEAE HPLC is only one facet of oligosaccharide structural analysis.

DEAE HPLC is also useful for separating gangliosides according to the number of sialog acids they contain. All monosialylated gangliosides coelute, although some fractionation can occur because of other structural differences. The same is true for the higher-order (e.g., di- or trisialylated) gangliosides. Complete purification of individual species usually requires a further chromatographic step based on other principles (e.g., HPLC columns of a special silica gel called Iatrobeads, from Iatron Laboratories). This method has been used for the analysis and semipreparative fractionation of labeled and unlabeled gangliosides (Manzi et al., 1990; Sjoberg et al., 1992). When gangliosides contain sulfate groups, interaction is stronger and, in some cases, it is necessary to keep eluting at maximum salt concentration for long periods (A. Manzi, unpub. observ.).

Critical Parameters

The separation of anionic oligosaccharides is greatly affected by the steepness of the NaCl gradient. If the concentration of NaCl is increased too rapidly, the resolution of the differently charged species will be reduced. Additionally, the column may be damaged because of shrinking and swelling of the matrix, which occur with changes in ionic strength. A final concentration of 125 mM NaCl should be sufficient to elute saccharides with up to five negative charges. More negatively charged species can be eluted by increasing the final salt concentration. The highest concentration of NaCl compatible with a given column is indicated by the supplier (e.g., some DEAE columns should not be exposed to NaCl concentrations >1 M).

Failure of acidic saccharides to bind to the column can be caused by several factors. Salt in the sample may impair the ionic interactions responsible for binding to the column. Desalting the sample prior to analysis should prevent this. Alternatively, the acidic moieties may be protonated and hence uncharged. This is unlikely, because the pK_a of the carboxylic acids of acidic sugars is typically <3, with sulfates having even lower pK_a values. On the other hand, phosphodiester have pK_a values around 5, and CO_2 dissolved in column buffers may lower the pH of the column eluant sufficiently to protonate them. This is easily addressed by buffering the column to a pH >6 with 2 to 5 mM Tris base, pH 9.5. In this case, UV cannot be used for detection.
It is not advisable to change the solvent from aqueous to organic and back to analyze anionic oligosaccharides and gangliosides, because the performance of the column in both applications is markedly affected. If both types of analyses are regularly done, two different columns must be used.

Anticipated Results

Oligosaccharides or gangliosides with sialic acid as their only anionic moiety can be separated using the above protocols. When one to four sialic acids/molecule are present, the species are readily resolved. Sialidase treatment of released N-linked oligosaccharides with repeated HPLC analysis provides information about the linkage of the sialic acid residues. In the case of gangliosides, the method is particularly useful for semipreparative purposes.

Time Considerations

A typical separation of released N-linked oligosaccharides requires 1 to 2 hr, depending on the length and steepness of the gradient and the length of time the column is allowed to wash. At least one standard run, preferable with a mixture of standards, should be performed at the start of each day. Thus three to six samples can be analyzed in an 8-hr day. When analyzing gangliosides, one run and equilibration of the column back to the starting conditions usually takes 2 hr.

Literature Cited


Key References


Describes analysis of sialylated N-linked oligosaccharides with and without prior sialidase treatment.

Manzi et al., 1990. See above.

Describes qualitative and semipreparative analysis of mono-, di-, and trisialylated gangliosides.

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Fractionation and Analysis of Neutral Oligosaccharides by HPLC

This unit describes the fractionation and analysis of neutral oligosaccharides by high-performance liquid chromatography (HPLC) on bonded amine columns. Separation is achieved with gradients of acetonitrile and water and is based upon hydrogen bonding between the NH₂ groups of the column and the hydroxyl groups of the oligosaccharides, as described in the Basic Protocol. The Support Protocol describes the reduction and desalting of neutral oligosaccharides with sodium borohydride. The basic principles of HPLC are discussed in UNITS 10.12-10.14 and in Snyder et al. (1988).

SEPARATION OF NEUTRAL OLIGOSACCHARIDES ON A BONDED AMINE HPLC COLUMN

Neutral oligosaccharides bind to amine-bonded HPLC columns because of hydrogen bonding via hydroxyl groups, which is promoted by acetonitrile and disrupted by water. The sample is loaded in a high concentration of acetonitrile and the column is developed with a gradient of increasing water. Neutral oligosaccharides are initially retained by the column and elute in order of increasing size (increasing numbers of hydroxyl groups). Oligosaccharides are detected based on their radioactivity (if previously radiolabeled) or by physical or chemical means (if unlabeled). Information about the number and size of oligosaccharide species in a mixture is thus obtained.

Materials

- Oligosaccharide sample
- Oligosaccharide standards (e.g., Dionex or Oxford Glycosystems)
- HPLC-grade water
- HPLC-grade acetonitrile
- HPLC apparatus capable of two-component gradient formation
- Bonded amine HPLC column (e.g., Varian Micropak AX-5 or Rainin LC-NH₂)
- Additional reagents and equipment for exoglycosidase digestion (optional; UNITS 17.13 & 17.18), and reduction and desalting of neutral oligosaccharides (see Support Protocol)

1. Reduce and desalt the sample prior to analysis (see Support Protocol). If desired, prepare parallel samples treated by sequential or combined exoglycosidase digestion (UNIT 17.13 & 17.18) chosen to help elucidate the structure.

   Reduction of samples gives sharper peaks and better baseline resolution.

   The column is also a weak anion exchanger. Salts or buffers can compete with the oligosaccharide for binding sites, altering elution time and resolution. Anionic oligosaccharides will bind to the column and will not elute with water. However, the method can be adapted to study anionic oligosaccharides.

2. Equilibrate the column in 70% acetonitrile/30% water at a flow rate of 1 ml/min.

   Water and acetonitrile should be prefiltred through a 0.45-µm filter and degassed. See UNIT 10.12 support protocol for preparation and degassing of solvents. Column prefilters and/or guard columns provide additional protection and increase the productive lifetime of the column.

   All common neutral oligosaccharides should bind to the column in 70% acetonitrile. If an oligosaccharide fails to bind, the starting concentration of acetonitrile should be increased.
3. Dissolve the sample in a small volume of water and add 3 vol acetonitrile.

   The main factor determining the appropriate sample volume is the size of the injection loop. Injection loops of ≤500 μl are preferred.

4. Load and inject the sample. Immediately start a 70% to 30% acetonitrile gradient over 80 min, maintaining the flow rate of 1 ml/min.

   Typical neutral N-linked oligosaccharides elute at water concentrations of <70%. A second gradient of 30% to 0% acetonitrile over 30 min may be included to elute unexpectedly large neutral oligosaccharides and to help keep the column clean.

5. Detect the oligosaccharides by physical, chemical, or radiometric methods. For analytical work, in-line radioactivity detectors or UV detectors may be used. If the amount of radioactive sample is low, or if the column is being used to obtain individual species for subsequent analysis, collect and analyze fractions every 0.3 to 0.5 min. For UV detection, measure absorbance at 190 to 210 nm. Determine the number of oligosaccharide species present and compare their retention times to those of known standards prepared similarly.

   Because many compounds absorb at 190 to 210 nm, UV detection demands a sample of high purity.

6. Infer features of the oligosaccharide structure from the retention time relative to standards and from changes in retention caused by sequential or combined exoglycosidase digestion.

   Because retention time is also a reflection of column performance, it is important to properly care for the column. Routine washing with water (step 4 annotation) helps remove contaminants that are hydrogen bonded to the column. Salts or other ionic contaminants can be removed by periodic washing with 0.5 M potassium phosphate, pH 1.7. Column performance will deteriorate over time, even with washing, due to gradual dissolution of the bonded phase. Standards must therefore be run at the beginning and end of each set of samples.

### SUPPORT PROTOCOL

**SODIUM BOROHYDRIDE REDUCTION AND DESALTING OF NEUTRAL OLIGOSACCHARIDES**

Oligosaccharides with a reducing sugar at their inner terminus elute as broader peaks than those that have been reduced to alditols. Whenever possible, therefore, oligosaccharides should be reduced prior to HPLC analysis. Samples and standards should be similarly treated. Recoveries of oligosaccharides after reduction and desalting are typically ∼70%. Note that a radioactive label can be introduced at this stage if 3H-labeled sodium borohydride ([3H]NaBH₄) is used (UNIT 17.5) and that O-N-acetyl-D-galactosamine (O-GalNAc)-linked oligosaccharides are reduced during their release by β-elimination (UNIT 17.15).

**Additional Materials** (also see Basic Protocol)

- 1 M sodium borohydride (NaBH₄) in 0.2 M sodium borate (Na₂B₄O₇; see recipe)
- 5% acetic acid/95% methanol
- Mixed-bed anion and cation resin (Amberlite MB-3 or equivalent)

1. Dissolve NaBH₄ to 1 M final concentration in 0.2 M Na₂B₄O₇ (pH 9.5) immediately before use. Dissolve the dried oligosaccharides in 100 μl of this reagent and incubate 3 hr at room temperature. Because hydrogen gas may be evolved by decomposing NaBH₄, cautiously vent the tube occasionally or use a tall, loosely capped tube.

   Sodium borate buffer at pH 9.5 is preferred to more basic NaOH solutions, because the latter promote epimerization of the core N-glucosamine to N-mannitosamine, which can result in doublet peaks in the HPLC analysis.
2. Stop the reduction by adding dropwise a 5% acetic acid/95% methanol mixture. The sample will bubble due to evolution of hydrogen gas. Continue until no more gas is evolved.

3. Dry the sample under a stream of N₂ or using a shaker/evaporator, Speedvac evaporator, or similar device. Take to complete dryness.

4. Repeat steps 2 and 3 three times using 0.5 ml of 5% acetic acid/95% methanol each time.

   Under acidic conditions the methanol and Na₂B₄O₇ form volatile methyl borates. The salt left behind is sodium acetate.

5. Prewash a 2 to 5 ml column of mixed-bed resin with at least 10 column volumes of water. Dissolve the dried sample in water, load, and elute with water. Collect a single 10 to 15 ml fraction.

   Only neutral reduced oligosaccharides should pass through the column unretarded.

6. Lyophilize or dry the sample. Store the oligosaccharide at −20°C, either dry or in water.

   Presence of white powder following lyophilization indicates that not all the salt was removed by the resin. If so, repeat the desalting. A 26-G needle added to the column outlet can slow the flow and increase the time for the salts to interact with the resin. Alternatively, an anion-exchange column (e.g., Dowex 3×4a, hydroxide form, prewashed with ≥10 vol water) can be used followed by a cation-exchange column (e.g., Dowex 50, hydrogen form, prewashed with ≥10 vol water).

REAGENTS AND SOLUTIONS

Use HPLC-grade or other high-quality deionized water (e.g., 18 MΩ Milli-Q type) in all recipes and protocol steps, and filter through a 0.45-μm filter and degas before use. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.

1 M sodium borohydride in 0.2 M sodium borate

Prepare 0.2 M sodium borate by dissolving 7.63 g sodium borate (Na₂B₄O₇·10H₂O) in 90 ml H₂O. Adjust pH to 9.5 with NaOH and volume to 100 ml. Store at room temperature.

Immediately before use, dissolve 37.8 mg sodium borohydride (NaBH₄) per ml 0.2 M sodium borate, pH 9.5. Do not mix excessively.

COMMENTARY

Background Information

The fractionation and analysis of neutral oligosaccharides on the basis of size using this HPLC method yield several important pieces of information. A minimum estimate of the number of species present is obtained from the number of peaks observed. The size of an N-linked oligosaccharide may be indicative of the extent to which it has been processed. Single or sequential exoglycosidase digestion(s) (UNIT 17.18), in conjunction with repeated HPLC analysis, yield information about the identity and linkage of the monosaccharides present. Although HPLC analysis requires more equipment than conventional chromatography on Bio-Gel P-4, the elution profiles obtained are sharper and larger oligosaccharides can be better resolved.

Bonded amine HPLC columns have free NH₂ groups that hydrogen bond to the hydroxyl groups of oligosaccharides. Elution is accomplished by increasing the water content, which progressively disrupts the hydrogen bonds. Other factors affecting elution time include the monosaccharide composition and the branching pattern of the oligosaccharide. Several methods of detecting the oligosaccharides are available. Radiolabeled molecules can be detected with an in-line radioactivity detector or by liquid scintillation counting of fractions. Oligosaccharides can be radiolabeled, either metabolically (UNIT 17.4), enzymatically (UNIT
Fractionation and Oligosaccharides

Supplement 32 Current Protocols in Molecular Biology

17.21.12

Analysis of by HPLC Neutral

original description (and to content alone. The reader is referred to the gosaccharides on the basis of monosaccharide and sulfate, thus allowing separation of oligosaccharides on the basis of size (Mellis and Baenziger, 1983). Triethylamine acetate is used to suppress the charge of sialic acid, phosphate, and doublet peaks (Mellis and Baenziger, 1981). Reduced oligosaccharides may have longer retention times than their unreduced counterparts (Koenderman et al., 1989). Thus, the standards used to calibrate the column should be treated the same way as the samples.

Anticipated Results

Homopolymers of increasing size of GlcNAc (chitin) and glucose (dextran) have been separated into a series of well-spaced peaks, corresponding to oligomers that differ in size by one monosaccharide unit (Mellis and Baenziger, 1981). The elution time of oligosaccharides depends on the number of monosaccharides present and their composition. GlcNAc- and fucose-containing oligosaccharides elute sooner than oligosaccharides having an equal number of hexoses (Blanken et al., 1985), presumably because GlcNAc and fucose have one fewer hydroxyl group. The branching pattern of an oligosaccharide also affects its elution time. Oligosaccharides containing a β1-6 linkage elute later, presumably because the flexibility of this linkage allows a stronger interaction with the column (Blanken et al., 1985). Thus, it is possible to separate isomers of the same size given good column performance.

Time Considerations

A typical separation requires 1 to 2 hr, depending upon the length and steepness of the gradient and the length of time the column is used to calibrate the HPLC column should be treated the same way as the samples.

Critical Parameters

The most important parameter is column performance. There is some variability in the quality of separation by columns from different manufacturers and between columns from a given manufacturer. Decreases in column performance can be expected to occur over time due to the gradual dissolution of the bonded phase (Blanken et al., 1985). Improper care can greatly accelerate the decay of column performance. Inadequate cleaning, the use of unfiltered solvents, and the presence of contaminants in the sample can lead to higher back pressure, broader peaks, and decreased retention time.

The nature of the gradient also affects the ability of the column to resolve individual oligosaccharide species (Mellis and Baenziger, 1981). Most oligosaccharides are retained by the column if the starting acetonitrile/water ratio is 70:30. Typical gradients increase the water content by 0.5%/min. However, for separation of larger oligosaccharides the water content may need to be increased by only 0.3%/min (Blanken et al., 1985). Oligosaccharides that have not been reduced and desalted give broader peaks than reduced molecules. Reduction should be performed in sodium borate buffer and not in NaOH, as strongly alkaline conditions promote the epimerization of the GlcNAcitol to ManNAcitol, which can lead to doublet peaks (Mellis and Baenziger, 1981). Reduced oligosaccharides may have longer retention times than their unreduced counterparts (Koenderman et al., 1989). Thus, the standards used to calibrate the column should be treated the same way as the samples.

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allowed to wash. At least one standard run, preferably with a mixture of standards, should be performed at the start of each day. Thus three to six samples can be analyzed in an 8-hr day.

**Literature Cited**


**Key References**

Blanken et al., 1985. See above.

Mellis and Baenziger, 1981. See above.

Mellis and Baenziger, 1983. See above.

Sampath et al., 1992. See above.

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Nitrous Acid Degradation of Glycosaminoglycans

Glycosaminoglycans (GAGs) are made up of disaccharide units that are distinguished from each other by the monosaccharide units of which they are composed and by the degree and position of sulfation. These disaccharide units represent the monomeric units of the GAG; thus, measurement of the disaccharide composition of a GAG represents the first step in the characterization of the polymer—just as an amino acid analysis represents the first step in characterization of a protein.

In this protocol, alternative sets of steps are presented—the first set for cleavage of the glycosidic bonds of the N-sulfated GlcN residues in heparin and heparan sulfate, and the second set for cleavage of the bonds between the N-acetylated amino sugar residues in heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate, and keratan sulfate, as well as hyaluronic acid. The glycosidic bonds of N-sulfated GlcN residues can be rapidly cleaved with nitrous acid at pH 1.5 at room temperature to yield oligosaccharides. N-acetylated amino sugar residues do not react with nitrous acid. However, these residues can first be deacetylated by hydrazinolysis to generate GalN or GlcN residues, and the glycosidic bonds of these N-unsubstituted amino sugar residues can then be cleaved at room temperature with nitrous acid at pH 4. The N-sulfate groups in heparin and heparan sulfate are stable under the hydrazinolysis conditions. Thus, following N-deacetylation by hydrazinolysis, all GAGs can be converted completely to their constituent disaccharides by treatment with nitrous acid at pH 4 alone (for chondroitin, dermatan, and keratan sulfates, as well as hyaluronic acid) or with nitrous acid at pH 4 and then at pH 1.5 (for heparin and heparan sulfates). The resulting cleavage products contain reducing-terminal 2,5-anhydro-d-mannose residues (from GlcN) or 2,5-anhydro-d-talose residues (from GalN), which can be reduced stoichiometrically with tritiated sodium borohydride (NaB[3H]₄) to [3H]anhydromannitol or [3H]anhdyrotalitol residues—thus labeling the disaccharides so that they can be assayed qualitatively or quantitatively. The procedure described in the Basic Protocol may also be used to analyze metabolically labeled GAGs (with or without the use of NaB[3H]₄).

Materials

- Reference standard (see recipe)
- Unknown sample suspected of containing N-sulfated or N-acetylated GAG
- Internal standard (see recipe)
- Nitrous acid reagent, pH 1.5 (see recipe)
- 1 M Na₂CO₃
- 10 µg/µl hydrazine sulfate in anhydrous hydrazine
- 3 M H₂SO₄
- Nitrous acid reagent, pH 4.0 (see recipe)
- 0.5 M tritiated sodium borohydride (NaB[3H]₄; ∼500 mCi/mm) in 0.1 M NaOH
- 100-µl Reacti-Vials (Pierce)
- Sand bath: heating block (e.g., Pierce) with wells filled with sand

Cleave GAGs

To cleave N-sulfated GAGs

1a. Mix 20 µl of reference standard solution for N-sulfated GAG assay with 5 µl D-[¹⁴C]glucose internal standard. Prepare the same reaction mixture using 20 µl of unknown sample suspected of containing N-sulfated GAG in place of the standard, and carry both mixtures through all steps in parallel.
2a. Cool a 5-µl aliquot of the reaction mixture to 0°C and add 20 µl nitrous acid reagent, pH 1.5.

3a. Let mixture warm to room temperature for 10 min to complete the deamination reaction.

4a. Bring cleaved reaction mixture to pH 8.5 with 1 M Na₂CO₃.

   The cleaved product is then subjected to borohydride reduction and analysis (steps 5 and 6). After borohydride reduction but prior to analysis, it may be desirable to separate the reaction products (a mixture of di- and tetrasaccharides) according to size by gel filtration (see UNIT 10.9) on a Bio-Gel P-10 column, eluting with 1 M Na₂CO₃.

To cleave N-acetylated GAGs

1b. Mix 200 µl of reference standard for N-acetylated GAG assay with 50 µl of [¹⁴C]glucitol internal standard. Prepare the same reaction mixture using 20 µl of unknown sample suspected of containing N-acetylated GAG in place of the standard, and carry both mixtures through all steps in parallel.

2b. Place a 15-µl aliquot of the reaction mixture in a 100-µl Reacti-Vial and evaporate to dryness in a stream of air. Redissolve dried sample in 20 µl of 10 µg/µl hydrazine sulfate in anhydrous hydrazine, then cap the vial and incubate 6 hr at 100°C in a sand bath.

   CAUTION: Hydrazine is a toxic, corrosive, and flammable reagent and should be handled accordingly.

3b. Cool reaction mixture, evaporate until as dry as possible in a stream of air, then lyophilize partially dried sample to remove as much hydrazine as possible. Add 20 µl nitrous acid reagent, pH 4 to the dried sample. Check pH with pH paper and adjust to pH 4 using 5 to 10 µl of 3 M H₂SO₄, if necessary. Let stand 15 min at room temperature.

   Because of residual hydrazine sulfate and hydrazine, the solution is usually at pH 6 before adjustment.

   The hydrazinolysis procedure removes N-acetyl substituents from the N-acetylated amino sugar residues, but the N-sulfated substituents in heparin or heparan sulfate remain. Hence, steps 1b to 3b result in cleavage at the N-deacylated hexosamine residues but not at the N-sulfated hexamines. Deamination (step 4b) cleaves at the N-sulfated residues as well; thus, steps 1b to 4b result in the complete conversion of heparin and heparan sulfate (as well as all of the other GAGs) to disaccharides.

4b. Adjust reaction mixture to pH 1.5 with 3 M H₂SO₄, then cool solution to 0°C, carry out the deamination reaction, and adjust the reaction mixture to pH 8.5 as in steps 2a to 4a.

   The cleaved product is then subjected to borohydride reduction and analysis (steps 5 and 6). All of the products obtained after hydrazinolysis and nitrous acid cleavage are disaccharides, and therefore may be analyzed without further sample purification.

Reduce cleavage products with sodium borohydride

5. Mix cleaved sample with 10 µl of 0.5 M Na[B³H₄] in 0.1 M NaOH and incubate 15 min at 50°C.

   CAUTION: Carry out all NaB³H₄ manipulations in a a well-ventilated fume hood designated for radioactive use.
6. Add 5 \mu l of 3 M \( \text{H}_2\text{SO}_4 \) and evaporate samples to dryness in a stream of air, then redissolve sample in water and again evaporate to dryness in a stream of air to remove as much \([\text{3H}]_2\) as possible.

The sample is now ready for analysis. For analysis by paper chromatography or paper electrophoresis, it should be redissolved in 60 \mu l of water. For analysis by HPLC, it should be dissolved in the starting buffer to be used the chromatographic run (see UNIT 17.22B for both procedures).

**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.*

**Internal standards**

\( \text{d-[14C]} \text{glucose standard for N-sulfated GAG assay} \): Prepare a stock solution containing 20 \mu Ci/ml \( \text{d-[14C]} \text{glucose} \) (specific activity >40 mCi/mmol; Amersham or Du Pont NEN). Dilute an aliquot of the stock solution in water to obtain a solution containing \( ~1 \times 10^5 \) cpm/\mu l of radioactivity.

\( [14\text{C}] \text{glucitol standard for N-acetylated GAG assay} \): Reduce \( \text{d-[14C]} \text{glucose} \) with sodium borohydride as in steps 5 and 6 of the Basic Protocol, except use cold NaBH₄. Dilute the resulting \([14\text{C}]\text{glucitol}\) in water to obtain a solution containing \( ~1 \times 10^5 \) cpm/\mu l of radioactivity.

Because \( \text{d-[14C]} \text{glucose} \) reacts with hydrazine, it cannot be used directly as an internal standard in the hydrazinolysis reaction. Consequently, it must be reduced for use in the N-acetylated GAG assay.

**Nitrous acid reagent, pH 1.5**

Prepare 0.5 M \( \text{H}_2\text{SO}_4 \) and 0.5 M \( \text{Ba(NO}_2\text{)}_2 \) (114 mg/ml) and cool separately to 0°C in an ice bath. Prepare a mixture containing 1 ml of each solution—i.e., 0.5 mmol each of \( \text{H}_2\text{SO}_4 \) and \( \text{Ba(NO}_2\text{)}_2 \)—at 0°C, then pellet the \( \text{BaSO}_4 \) precipitate in a clinical centrifuge. Draw off the supernatant with a Pasteur pipet, and keep on ice until ready to use.

This reagent should be prepared when needed and used immediately.

**Nitrous acid reagent, pH 4.0**

Add 5 ml of 5.5 M \( \text{NaNO}_2 \) to 2 ml of 0.5 M \( \text{H}_2\text{SO}_4 \).

This reagent should be prepared when needed and used immediately.

**Reference standards**

*For N-sulfated GAG assay:* 25 mg/ml aqueous solution of heparin or heparan sulfate.

*For N-acetylated GAG assay:* 25 mg/ml aqueous solution of chondroitin-4-sulfate (chondroitin sulfate type A), chondroitin-6-sulfate (chondroitin sulfate type C), dermatan sulfate (chondroitin sulfate type B), or keratan sulfate.

All of the above GAGs are available from Sigma. The standard solutions are stored frozen at \(-20^\circ \text{C}\).

**COMMENTARY**

**Background Information**

Glycosaminoglycans (GAGs) are families of structurally related polymers composed of sequences of different disaccharides that represent the monomeric units of these polymers. Chondroitin sulfates, dermatan sulfates, and hyaluronic acid represent one family of GAGs. Chondroitin sulfates contain only GlcA and GalNAc residues, and are sulfated at positions 4 and/or 6 of the GalNAc (and on some of the uronic acid residues as well). In dermatan sulfates, the amino sugar is GalNAc, but both GlcA and IdoA are present in varying proportions. Hyaluronic acid contains GlcNAc and
GlcA. Keratan sulfate contains Gal and GlcNAc, both of which may be sulfated at the 6 position. Thus, in contrast to heparin and heparan sulfate, which contain both N-acetylated and N-sulfated amino sugars as described below, chondroitin sulfate, dermatan sulfate, hyaluronic acid, and keratan sulfate contain only N-acetylated amino sugars. The glycosidic bonds of these N-acetylated amino sugar residues are not cleaved by nitrous acid.

Heparan sulfate and heparin represent another family of glycosaminoglycans, which contain GlcN as the amino sugar and both GlcA and IdoA in varying proportions. A unique structural feature of heparin and heparan sulfate is that a large proportion of their GlcN residues are N-sulfated (85% to 90% in heparin; 40% to 60% in a typical heparan sulfate); the rest are N-acetylated. The glycosidic bonds of these N-sulfated GlcN residues can be cleaved rapidly at room temperature with nitrous acid at pH 1.5 to yield oligosaccharides. This nitrous acid susceptibility distinguishes heparin and heparan sulfate from all other glycosaminoglycans. Because of the low percentage of GlcNAc residues in heparin, the GlcNAcs are found in isolated disaccharide units, surrounded by disaccharides that have N-sulfated GlcNs. Thus, the pH 1.5 nitrous acid cleavage reaction products of heparin are primarily disaccharides, with much smaller quantities of tetrasaccharides from sequences in which the GlcNAcs are located. However, heparan sulfate contains sequences with multiple contiguous GlcNAc-containing disaccharide units, and these sequences yield larger oligosaccharides among the nitrous acid cleavage products. Thus, to analyze the disaccharides formed following cleavage with the pH 1.5 nitrous acid reagent, the saccharides are first separated from the larger oligosaccharides by gel filtration on a BioGel P-10 column using 1 M Na2CO3 for elution. The products are then analyzed by paper chromatography, paper electrophoresis, or high-performance liquid chromatography (HPLC) as described in UNIT 17.22B.

The N-acetylated amino sugars can be deacetylated by hydrazinolysis to generate GalN or GlcN residues, and the glycosidic bonds of these N-unsubstituted amino sugars can then be cleaved at room temperature with the pH 4 nitrous acid reagent. Thus, following N-deacetylation by hydrazinolysis, those glycosaminoglycans containing only N-acetylated amino sugars are converted completely to disaccharides by the pH 4 nitrous acid reagent (in contrast to heparin and heparan sulfate, which yield higher oligosaccharides in addition to disaccharides). To convert heparin and heparan sulfate completely to disaccharides, these GAGs must first be N-deacetylated and then treated with nitrous acid at both pH 4 and pH 1.5.

**Critical Parameters**

The nitrous acid reagents are prepared by mixing nitrite salts with acid to yield the desired pH. Once the nitrite and acid are mixed, a series of complex reactions occurs involving the resulting oxides of nitrogen. Within a short time, the active species of “nitrous acid” undergoes changes that result in the loss the reagent’s ability to cleave the glycosidic bonds of the amino sugar (Shively and Conrad, 1976). Consequently, the nitrous acid reagents must be used within a few minutes after preparation. This is of less concern for the pH 4 reagent than for the pH 1.5 reagent, because the pH 4 reagent has a higher concentration of nitrite.

Also, pH is important for maintaining the selectivity of the cleavage. Although there is good selectivity for N-unsubstituted GlcNs and N-sulfated GlcNs at pH 4 and pH 1.5, respectively, the glycosidic bonds of both types of GlcN residues are cleaved at pHs between these values. Thus, it is desirable to let the reactions at the respective pHs proceed only for 10 to 15 min. Also, when samples are derived from buffered solutions, it is necessary to check the pH with pH paper before addition of the nitrous acid reagent. In fact, it is desirable to dialyze the glycosaminoglycan solution to remove all salts before beginning the cleavage step. Another reason for the pre-dialysis is that the NaB[3H]4 reagent is catalytically destroyed by oxyanions, such as PO₄³⁻ (Conrad et al., 1973).

**Anticipated Results**

When samples containing mixtures of different glycosaminoglycans are treated with nitrous acid at pH 1.5, only heparin and heparan SO₄ are cleaved; thus, the total amount of glycosaminoglycan that is reduced in molecular size by pH 1.5 nitrous acid represents the heparin/heparan sulfate family. Just as the nitrous acid cleavage is diagnostic for the heparin/heparan sulfate family, the chondroitin sulfate/dermatan sulfate/hyaluronic acid family is specifically cleaved by chondroitinases (or chondroitin lyases) ABC or AC (UNIT 17.13B). These selective cleavages can be used for characterization of glycosaminoglycans that are metabolically labeled (e.g., with [35S]O₄²⁻ or [³H]glucosamine). The use of nitrous acid and
the chondroitinas for more definitive characterization of glycosaminoglycans is described in UNIT 17.22B.

Time Considerations
The nitrous acid cleavage is complete within a few minutes after the reagent is added to the sample. When hydrazinolysis precedes the nitrous acid cleavage, an additional 6 to 8 hr is required for the hydrazinolysis step and preparation of the sample for nitrous acid cleavage. The overall procedure can be interrupted after hydrazinolysis, nitrous acid cleavage, or NaB[3H]₄ reduction without any loss of precision in the analytical stage.

Literature Cited


Key Reference


These references describe the basic procedures in this unit and the background for those procedures.

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Analysis of Disaccharides and Tetrasaccharides Released from Glycosaminoglycans

Glycosaminoglycans (GAGs) are converted to disaccharides by cleavage with lyases or by treatment with nitrous acid either before or after N-deacetylation. Enzymatic (lyase) cleavage of GAGs to disaccharides is described in UNIT 17.13B. Nitrous acid cleavage of GAGs to release disaccharides (or tetrasaccharides, from heparin and heparan sulfate), and the NaB\(^{[3H]}\) reduction of these products is described in UNIT 17.22A.

As described in this unit, individual disaccharides are separated by paper chromatography or paper electrophoresis (Basic Protocol 1) or high-performance liquid chromatography (HPLC; Basic Protocol 2). Lyase-released disaccharides can also be monitored by UV absorbance as described in Background Information. The disaccharides can be radiolabeled for subsequent detection either by metabolic labeling of the cells that produce them (UNIT 17.4) or by reduction of the disaccharides from unlabeled GAGs with NaB\(^{[3H]}\) to incorporate one gram atom of \(^3\)H per mole of disaccharide (UNIT 17.22A). The label incorporated into the separated disaccharides can then be used for qualitative or quantitative assay of each disaccharide.


BASIC PROTOCOL 1

ANALYSIS OF DISACCHARIDES AND OLIGOSACCHARIDES FROM GLYCOSAMINOGLYCANS BY PAPER CHROMATOGRAPHY AND PAPER ELECTROPHORESIS

Materials

- Sample of lyase-degraded (UNIT 17.13B; also see Support Protocol 1) or nitrous acid–degraded glycosaminoglycan (UNIT 17.22A)
- Paper chromatography or paper electrophoresis system appropriate to saccharide mixture to be analyzed (see Table 17.22B.1 and Table 17.22B.2; also see recipe in Reagents and Solutions)
- Apparatus for paper electrophoresis

NOTE: \(^{[14C]}\)glucose can be added as an internal standard. See UNIT 17.22A for details.

1. Run descending paper chromatograms and paper electrophoretograms on 1 × 22-in. strips of chromatography paper in the appropriate solvent system for the saccharide mixture to be analyzed. Spot the desired volume of each sample on a 0.5-in. segment of the strip that has been marked off in advance. For paper electrophoresis, dampen the paper strips with the solvent up to the point where the sample was spotted and electrophorese the samples toward the anode at 25 V/cm for 2.5 hr.

2. Count radioactivity on 0.5-in. segments from the paper strips.
<table>
<thead>
<tr>
<th>Compound</th>
<th>R&lt;sub&gt;glucitol&lt;/sub&gt; with paper chromatography system&lt;sup&gt;b&lt;/sup&gt;</th>
<th>V&lt;sub&gt;E&lt;/sub&gt; with paper electrophoresis system&lt;sup&gt;d&lt;/sup&gt;</th>
<th>HPLC system&lt;sup&gt;e&lt;/sup&gt;</th>
<th>SAX</th>
<th>WAX</th>
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<td></td>
<td>1</td>
<td>2</td>
<td>1</td>
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<td>Eluant (mM KH&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;)</td>
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<td>GlcA-ATal&lt;sub&gt;R&lt;/sub&gt;</td>
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<td><strong>Keratan SO&lt;sub&gt;4&lt;/sub&gt; Products</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMan&lt;sub&gt;R&lt;/sub&gt;(6-SO&lt;sub&gt;4&lt;/sub&gt;)</td>
<td>0.69</td>
<td>2.38</td>
<td>0.72</td>
<td>0.81</td>
<td>5</td>
</tr>
<tr>
<td>Gal&lt;sub&gt;R&lt;/sub&gt;(6-SO&lt;sub&gt;4&lt;/sub&gt;)</td>
<td>0.42</td>
<td>1.46</td>
<td>0.64</td>
<td>0.71</td>
<td>5</td>
</tr>
<tr>
<td>Gal-AMan&lt;sub&gt;R&lt;/sub&gt;</td>
<td>0.84</td>
<td>1.24</td>
<td>0.11</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>Gal(6-SO&lt;sub&gt;4&lt;/sub&gt;)-AMan&lt;sub&gt;R&lt;/sub&gt;</td>
<td>0.38</td>
<td>1.54</td>
<td>0.52</td>
<td>0.62</td>
<td>5</td>
</tr>
<tr>
<td>Gal-AMan&lt;sub&gt;R&lt;/sub&gt;(6-SO&lt;sub&gt;4&lt;/sub&gt;)</td>
<td>0.38</td>
<td>1.50</td>
<td>0.54</td>
<td>0.63</td>
<td>5</td>
</tr>
<tr>
<td>Gal(6-SO&lt;sub&gt;4&lt;/sub&gt;)-AMan&lt;sub&gt;R&lt;/sub&gt;(6-SO&lt;sub&gt;4&lt;/sub&gt;)</td>
<td>0.11</td>
<td>1.0</td>
<td>0.86</td>
<td>1.04</td>
<td>70</td>
</tr>
</tbody>
</table>

<sup>a</sup>Abbreviations: AMan, anhydro-D-mannose; ATal, anhydro-D-talose; Gal, D-galactose; GlcA, D-glucuronic acid; GlcN, D-glucosamine; IdoA, L-iduronic acid; ret. time, retention time; SAX, strong ion-exchange; WAX, weak ion-exchange.

<sup>b</sup>All values are for aldehyde-reduced sugars, indicated by the subscript, R.

<sup>c</sup>R<sub>glucitol</sub> = ratio of migration distance of compound to migration distance of the [14C]glucitol internal standard. See recipes for paper chromatography systems 1 and 2 in Reagents and Solutions.

<sup>d</sup>V<sub>E</sub> = ratio of migration distance of compound to migration distance of αIdoA(2-SO<sub>4</sub>) 1-4 AMan<sub>R</sub>(6-SO<sub>4</sub>). This compound is the major disaccharide obtained from heparin; see Table 17.22.3. See recipes for paper electrophoresis systems 1 and 2 in Reagents and Solutions; see Shaklee and Conrad, 1986 for details on paper chromatography.

<sup>e</sup>HPLC separations are performed at a flow rate of 1 ml/min under the isocratic salt conditions indicated; see Shaklee and Conrad (1986) for details.
Sample to be analyzed by high-performance liquid chromatography (HPLC) are obtained by nitrous acid cleavage of N-deacetylated glycosaminoglycans at pH 4 (UNIT 17.22A), by direct cleavage of heparin or heparan sulfate with nitrous acid at pH 1.5 (UNIT 17.22A), or by enzymatic cleavage with lyases (UNIT 17.13B). For HPLC, all samples must be free of particulate matter and should be dissolved in the initial solvent used to elute the HPLC column.

**Materials**

Sample: mixture of saccharides obtained from glycosaminoglycan by nitrous acid cleavage (UNIT 17.22A) or lyase cleavage (UNIT 17.13B)  
4.5-mm \(\times\) 25-cm Partisil SAX strong anion-exchange column (Whatman) or 4-mm \(\times\) 30-cm Varian MicroPak AX-5 weak anion-exchange column (Varian Analytical)  
HPLC solvents (see recipe and Tables 17.22B.1-17.22B.5)  
Gradient solutions for HPLC (Tables 17.22B.1-17.22B.5)  
Bio-Gel P-10 gel filtration column (Bio-Rad)  
4.6 \(\times\) 250-mm Hi-Chrom S-5 ODS C-18 column (Regis Technology)  
Fraction collector or in-line radioactivity flow detector (e.g., Packard Instrument)  
Additional reagents and equipment for gel-filtration chromatography (UNIT 10.9), ion-exchange HPLC (UNIT 10.13), and reversed-phase HPLC (UNIT 10.12)
For disaccharides obtained from chondroitin sulfate, dermatan sulfate, and keratan sulfate by nitrous acid cleavage

1a. Run HPLC on a 4.5-mm $\times$ 25-cm Whatman Partisil SAX strong ion-exchange column or 4-mm $\times$ 30-cm Varian MicroPak AX-5 weak anion exchange column using the appropriate isocratic elution conditions (see Table 17.22B.1). Proceed to step 3.

For disaccharides obtained from chondroitin sulfate and dermatan sulfate by lyase cleavage

1b. Reduce the disaccharide mixture with borohydride as in Support Protocol 1.

2b. Run HPLC on a 4.5-mm $\times$ 25-cm Whatman Partisil SAX strong ion-exchange column or 4-mm $\times$ 30-cm Varian MicroPak AX-5 weak anion exchange column using the appropriate elution conditions.

The maximum sample volume for injection is 500 $\mu$l for both nitrous acid- and lyase-cleaved GAGs.

For strong ion-exchange separation of di- and tetrasaccharides obtained from heparin and heparan sulfate by nitrous acid cleavage

1c. Prior to chromatographic separation of individual oligosaccharides, separate the oligosaccharides according to size by gel-filtration chromatography (UNIT 10.9) on a Bio-Gel P-10 column in 1 M Na$_2$CO$_3$.

2c. Analyze di- and tetrasaccharides on a 4.5-mm $\times$ 25-cm Whatman Partisil SAX strong ion-exchange column using step gradients at a flow rate of 1 ml/min (see Table 17.22B.3). For disaccharide separation, use an initial 40 mM KH$_2$PO$_4$ isocratic segment for 25 min to resolve unsulfated and monosulfated disaccharides, followed by a 1-min linear step gradient to 185 mM KH$_2$PO$_4$, and a 40-min isocratic segment at 185 mM KH$_2$PO$_4$ to resolve the disulfated disaccharides. For samples with high salt content, it is advisable to run an initial 10-min isocratic segment with 20 mM KH$_2$PO$_4$ to wash the excess salt through the column, thus avoiding any possible effect of the high salt on the elution profiles. For tetrasaccharide separation, use a step gradient with five isocratic segments: 40 mM KH$_2$PO$_4$ for 25 min; 100 mM KH$_2$PO$_4$ for 35 min; 175 mM KH$_2$PO$_4$ for 30 min; 360 mM KH$_2$PO$_4$ for 25 min; and 400 mM KH$_2$PO$_4$ for 25 min. Connect each isocratic segment by a 1-min step gradient from one isocratic segment to the next.

For reversed-phase ion-pairing HPLC separation of di- and tetrasaccharides obtained from heparin and heparan sulfate by nitrous acid cleavage

1d. Prior to chromatographic separation of individual oligosaccharides, separate the oligosaccharides according to size by gel-filtration chromatography (UNIT 10.9) on a Bio-Gel P-10 column in 1 M Na$_2$CO$_3$.

2d. Analyze di- and tetrasaccharides on a 4.6 $\times$ 250-mm Hi-Chrom S-5 ODS column at a flow rate of 1 ml/min (Tables 17.22B.4 and 17.22B.5). Isocratic separations of groups of monosaccharides and disaccharides are obtained by isocratic elution with the solvent mixtures shown in Table 17.22B.4. Connect the isocratic segments shown in Table 17.22B.4 as in Table 17.22B.5 to achieve the separation of all of the disaccharides in a single run. Note that GlcA-AManR(SO$_4$) and IdoA-AManR(SO$_4$) are not separated under these conditions; for separation of these two disaccharides, the strong-anion exchange HPLC must be used. Isocratic separations of groups of tetrasaccharides are obtained by isocratic elution with the solvent mixtures shown in Table 17.22B.4. Connect the isocratic segments shown in Table 17.22B.4 as in Table 17.22B.5 to achieve the separation of all of the tetrasaccharides.

For details of the reversed-phase ion-pairing procedure, see Guo and Conrad (1988).
Table 17.22B.3  Separation by Strong Ion-Exchange HPLC of Oligosaccharides Released from Heparin by Nitrous Acid Treatment With or Without Prior Hydrazinolysis<sup>a,b</sup>

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>Eluant (mM KH&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;)</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disaccharides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GlcA-AMan&lt;sub&gt;R&lt;/sub&gt; and IdoA AMan&lt;sub&gt;R&lt;/sub&gt;</td>
<td>40</td>
<td>4.5</td>
</tr>
<tr>
<td>AMan&lt;sub&gt;R&lt;/sub&gt;</td>
<td>40</td>
<td>4.5</td>
</tr>
<tr>
<td>AMan&lt;sub&gt;R&lt;/sub&gt;(SO&lt;sub&gt;4&lt;/sub&gt;)</td>
<td>40</td>
<td>7.0</td>
</tr>
<tr>
<td>GlcA(SO&lt;sub&gt;4&lt;/sub&gt;)-AMan&lt;sub&gt;R&lt;/sub&gt;</td>
<td>40</td>
<td>19.5</td>
</tr>
<tr>
<td>GlcA-AMan&lt;sub&gt;R&lt;/sub&gt;(SO&lt;sub&gt;4&lt;/sub&gt;)</td>
<td>40</td>
<td>23.0</td>
</tr>
<tr>
<td>IdoA-AMan&lt;sub&gt;R&lt;/sub&gt;(SO&lt;sub&gt;4&lt;/sub&gt;)</td>
<td>40</td>
<td>26.5</td>
</tr>
<tr>
<td>IdoA(SO&lt;sub&gt;4&lt;/sub&gt;)-AMan&lt;sub&gt;R&lt;/sub&gt;</td>
<td>40</td>
<td>30.0</td>
</tr>
<tr>
<td>GlcA(SO&lt;sub&gt;4&lt;/sub&gt;)-AMan&lt;sub&gt;R&lt;/sub&gt;(SO&lt;sub&gt;4&lt;/sub&gt;)</td>
<td>185</td>
<td>14.0</td>
</tr>
<tr>
<td>IdoA(SO&lt;sub&gt;4&lt;/sub&gt;)-AMan&lt;sub&gt;R&lt;/sub&gt;(SO&lt;sub&gt;4&lt;/sub&gt;)</td>
<td>185</td>
<td>21.5</td>
</tr>
<tr>
<td>GlcA-AMan&lt;sub&gt;R&lt;/sub&gt;(3,6diSO&lt;sub&gt;4&lt;/sub&gt;)</td>
<td>185</td>
<td>25.5</td>
</tr>
<tr>
<td>Tetrasaccharides&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t1  GlcA-GlcNAc-GlcA-AMan&lt;sub&gt;R&lt;/sub&gt;</td>
<td>20</td>
<td>28</td>
</tr>
<tr>
<td>t2  IdoA-GlcNAc-GlcA-AMan&lt;sub&gt;R&lt;/sub&gt;</td>
<td>20</td>
<td>33.5</td>
</tr>
<tr>
<td>t3  GlcA-GlcNAc(SO&lt;sub&gt;4&lt;/sub&gt;)-GlcA-AMan&lt;sub&gt;R&lt;/sub&gt;</td>
<td>100</td>
<td>31.5</td>
</tr>
<tr>
<td>t4  IdoA(SO&lt;sub&gt;4&lt;/sub&gt;)-GlcNAc-GlcA-AMan&lt;sub&gt;R&lt;/sub&gt;</td>
<td>100</td>
<td>37.0</td>
</tr>
<tr>
<td>t5  IdoA-GlcNAc(SO&lt;sub&gt;4&lt;/sub&gt;)-GlcA-AMan&lt;sub&gt;R&lt;/sub&gt;</td>
<td>100</td>
<td>42.5</td>
</tr>
<tr>
<td>t6  200</td>
<td></td>
<td>25.0</td>
</tr>
<tr>
<td>t7  IdoA(SO&lt;sub&gt;4&lt;/sub&gt;)-GlcNAc-GlcA-AMan&lt;sub&gt;R&lt;/sub&gt;(SO&lt;sub&gt;4&lt;/sub&gt;)</td>
<td>200</td>
<td>30.0</td>
</tr>
<tr>
<td>t8  IdoA-GlcNAc(SO&lt;sub&gt;4&lt;/sub&gt;)-GlcA-AMan&lt;sub&gt;R&lt;/sub&gt;(SO&lt;sub&gt;4&lt;/sub&gt;)</td>
<td>200</td>
<td>32.5</td>
</tr>
<tr>
<td>t9  IdoA-GlcNAc(SO&lt;sub&gt;4&lt;/sub&gt;)-GlcA-AMan&lt;sub&gt;R&lt;/sub&gt;(3-SO&lt;sub&gt;4&lt;/sub&gt;)</td>
<td>185</td>
<td>53.0</td>
</tr>
<tr>
<td>t10 320</td>
<td></td>
<td>23.0</td>
</tr>
<tr>
<td>t11 320</td>
<td></td>
<td>30.0</td>
</tr>
<tr>
<td>t12 320</td>
<td></td>
<td>32.0</td>
</tr>
<tr>
<td>t13 350</td>
<td></td>
<td>23.5</td>
</tr>
<tr>
<td>t14  IdoA-GlcNAc(SO&lt;sub&gt;4&lt;/sub&gt;)-GlcA-AMan&lt;sub&gt;R&lt;/sub&gt;(3,6-diSO&lt;sub&gt;4&lt;/sub&gt;)</td>
<td>350</td>
<td>38.5</td>
</tr>
<tr>
<td>t15  IdoA(SO&lt;sub&gt;4&lt;/sub&gt;)-GlcNAc(SO&lt;sub&gt;4&lt;/sub&gt;)-GlcA-AMan&lt;sub&gt;R&lt;/sub&gt;(SO&lt;sub&gt;4&lt;/sub&gt;)</td>
<td>350</td>
<td>42.0</td>
</tr>
<tr>
<td>t16 400</td>
<td></td>
<td>30.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Abbreviations: AMan, anhydro-D-mannose; GlcA, D-glucuronic acid; GlcN, D-glucosamine; GlcNAc, N-acetyl-D-glucosamine; IdoA, L-iduronic acid.

<sup>b</sup>HPLC separations are obtained using step gradients. Details of gradients used for disaccharides and oligosaccharides are given in Basic Protocol 2; the KH<sub>2</sub>PO<sub>4</sub> concentrations are those at which each oligosaccharide emerges during the gradient. Columns are run at a flow rate of 1 ml/min; see Guo and Conrad (1989) for details.

<sup>c</sup>Tetrasaccharide designations (t1-t16) are described in Bienkowski and Conrad, 1985. Those tetrasaccharides for which no monosaccharide sequences are given (t6, t10-t13, and t16) are “ring-contraction tetrasaccharides,” which are formed in relatively low yields (see Commentary).
### Table 17.22B.4  Separation by Isocratic Ion-Pairing HPLC of Oligosaccharides Released from Heparin by Nitrous Acid Treatment, With or Without Prior Hydrazinolysis

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>Solventb</th>
<th>Retention timec (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mono- and Disaccharides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AManR</td>
<td>A:B = 94:6</td>
<td>5.0</td>
</tr>
<tr>
<td>GlcA-AManR</td>
<td>A:B = 94:6</td>
<td>6.5</td>
</tr>
<tr>
<td>IdoA-AManR</td>
<td>A:B = 94:6</td>
<td>9.0</td>
</tr>
<tr>
<td>AManR(SO4)</td>
<td>A:B = 94:6</td>
<td>12.0</td>
</tr>
<tr>
<td>IdoA(SO4)-AManR</td>
<td>A:B = 86:14</td>
<td>22.0</td>
</tr>
<tr>
<td>GlcA-AManR(SO4)</td>
<td>A:B = 86:14</td>
<td>22.0</td>
</tr>
<tr>
<td>IdoA-AManR(SO4)</td>
<td>A:B = 86:14</td>
<td>31.0</td>
</tr>
<tr>
<td>GlcA-AManR(3,6diSO4)</td>
<td>A:B = 86:14</td>
<td>23.5</td>
</tr>
<tr>
<td>GlcA(SO4)-AManR(SO4)</td>
<td>A:B = 61:39</td>
<td>31.5</td>
</tr>
<tr>
<td><strong>Tetrasaccharides</strong>d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t1  GlcA-GlcNAc-GlcA-AManR</td>
<td>A:C = 94:6</td>
<td>9.0</td>
</tr>
<tr>
<td>t2  IdoA-GlcNAc-GlcA-AManR</td>
<td>A:C = 94:6</td>
<td>9.5</td>
</tr>
<tr>
<td>t4  IdoA(SO4)-GlcNAc-GlcA-AManR</td>
<td>A:C = 84:15</td>
<td>29.0</td>
</tr>
<tr>
<td>t5  IdoA-GlcNAc(SO4)-GlcA-AManR</td>
<td>A:C = 84:15</td>
<td>33.0</td>
</tr>
<tr>
<td>t3  GlcA-GlcNAc(SO4)-GlcA-AManR</td>
<td>A:C = 84:15</td>
<td>38.0</td>
</tr>
<tr>
<td>t9  IdoA-GlcNAc(SO4)-GlcA-AManR(3-SO4)</td>
<td>A:C = 74:26</td>
<td>29.0</td>
</tr>
<tr>
<td>t8  IdoA-GlcNAc(SO4)-GlcA-AManR(SO4)</td>
<td>A:C = 74:26</td>
<td>33.0</td>
</tr>
<tr>
<td>t13 IdoA(SO4)-RC(SO4)IdoA(SO4)-AManR</td>
<td>A:C = 60:40</td>
<td>12.0</td>
</tr>
<tr>
<td>t14 IdoA-GlcNAc(SO4)-GlcA-AManR(3,6-diSO4)</td>
<td>A:C = 60:40</td>
<td>15.0</td>
</tr>
<tr>
<td>t16 IdoA-RC(SO4)IdoA(SO4)-AManR</td>
<td>—</td>
<td>16.0</td>
</tr>
</tbody>
</table>

---

aAbbreviations: AMan, anhydro-D-mannose; GlcA, D-glucuronic acid; GlcN, D-glucosamine; IdoA, L-iduronic acid; RC, ring-contraction product.
bHPLC separations are obtained using a C-18 reversed-phase column using isocratic elution conditions obtained by mixing solvents A, B, and C (see recipes in Reagents and Solutions) in the ratios shown.
cAll elution are performed at a flow rate of 1 ml/min. For separations of di- or tetrasaccharide mixtures containing the total mixtures of these oligosaccharides, see gradient conditions in Table 17.22.5.
dTetrasaccharide designations are described in Bienkowski and Conrad, 1985.

### Table 17.22B.5 Gradient Conditions for Oligosaccharide Separation by Reversed-Phase Ion-Pairing HPLC

<table>
<thead>
<tr>
<th>Disaccharides</th>
<th>Tetrascarhides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time interval for solvent change (min)</td>
<td>% Solvent B in time interval</td>
</tr>
<tr>
<td>0→5</td>
<td>6</td>
</tr>
<tr>
<td>5→6</td>
<td>6→14</td>
</tr>
<tr>
<td>6→20</td>
<td>14</td>
</tr>
<tr>
<td>20→40</td>
<td>14→20</td>
</tr>
<tr>
<td>40→41</td>
<td>20→39</td>
</tr>
<tr>
<td>41→81</td>
<td>39</td>
</tr>
<tr>
<td>81→83</td>
<td>39→60</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

Oligosaccharides are chromatographed on a C-18 reversed-phase column using the ion-pairing systems described in Basic Protocol 2. Mixtures of standards are chromatographed with increasing percentages of solvent B (disaccharides) or solvent C (tetrascarhides) in solvent A (see recipes for solvents A, B, and C in Reagents and Solutions). Details of the gradients used are described in Basic Protocol 2.
3. Count radioactivity in 5-ml fractions collected in scintillation vials (see Support Protocol 2) or use an in-line radioactivity flow detector. Identify disaccharides on the basis of their chromatographic migration properties by referring to Tables 17.22B.1-17.22B.5. Quantify di- and tetrasaccharides (see Support Protocol 3).

**BOROHYDRIDE REDUCTION OF ALKALI-LABILE DISACCHARIDES OBTAINED BY CLEAVAGE WITH LYASES**

Disaccharides formed by cleavage of glycosaminoglycans with specific lyases (UNIT 17.13B) can be analyzed by reduction with NaB³H₄ and separation of the labeled products either by paper chromatography (Basic Protocol 1; Glaser and Conrad, 1979) or HPLC (Basic Protocol 2; Delaney et al., 1980; Shaklee and Conrad, 1986). However, the lyase-generated disaccharides are quite labile under the alkaline conditions required for their reduction with NaB³H₄. Thus, a milder procedure than the 50°C reduction procedure described in UNIT 17.22A must be used for the reduction. These labile disaccharides (or higher oligosaccharides that may be obtained when the lyase does not convert the glycosaminoglycan completely to disaccharides) can be reduced at 0°C without cleavage.

**Materials**

- Sample of lyase-degraded glycosaminoglycan
- 1 M Na₂CO₃, pH 9.0 (ice-cold)
- Sodium borohydride reagent (see recipe)
- 3 M H₂SO₄
- 6 × 150-mm test tubes

1. Place lyase-degraded GAG sample in a 6 × 150-mm test tube, dry sample in a stream of air, then chill tube in an ice bath.

2. Add 5 µl ice-cold 1 M Na₂CO₃ (pH 9.0) to sample, then add 10 µl of sodium borohydride reagent. Cork or cap the tube and let it sit on ice 90 min.

   **IMPORTANT NOTE:** If samples are already labeled by metabolically labeling of the GAG, 0.5 M cold NaBH₄ is used in preparing the sodium borohydride reagent instead of 0.5 M NaB³H₄.

   Carry out all NaB³H₄ manipulations in a well-ventilated fume hood designated for radioactive use.

3. Add 2.5 µl of 3 M H₂SO₄ to destroy excess NaB³H₄ and let sample stand ~30 min.

4. Neutralize sample with 1 M Na₂CO₃ and dry it in a stream of air. Redissolve sample in water, then dry again to remove excess [³H]₂ gas. Finally dissolve sample in 50 µl water.

   **Aliquots of the sample may be analyzed by paper chromatography (Basic Protocol 1) or HPLC (Basic Protocol 2).**
SCINTILLATION COUNTING OF FRACTIONS FROM HPLC ANALYSIS OF SACCHARIDES RELEASED FROM GLYCOSAMINOGLYCANS

Effluent from the HPLC column is collected in 0.5-ml fractions in scintillation vials using a fraction collector. Alternatively the effluent may be run directly through an in-line radioactivity flow detector (e.g., Radiomatic). If the total cpm in each peak are high enough to obtain good counting statistics in short counting intervals, a stream splitter may be used to collect the bulk of each fraction (e.g., 0.4 ml) in a test tube while only a portion of each fraction (e.g., 0.1 ml) is taken in scintillation vials for counting. Add an appropriate volume (4 to 5 ml) of any commercial scintillation fluid designed to accommodate the salt and solvent concentrations in the eluants. Alternatively, add 4 ml of a scintillation cocktail containing 3 g diphenyloxazole in a mixture of 250 ml Triton X-114, 750 ml xylene, and 10 ml concentrated HCl. For KH₂PO₄ concentrations >10 mM, add 1 drop concentrated HCl to the xylene-based scintillation fluid to to redissolve the salt precipitate that begins to form in the scintillation vial.

CALCULATIONS FOR QUANTITATION OF DISACCHARIDES

Procedures described here allow calculation of the number of µmol of each disaccharide in the original sample taken for analysis. These calculations apply primarily to analyses in which disaccharides obtained from unlabeled glycosaminoglycans are mixed with a [¹⁴C]D-glucose internal standard and reduced with NaB³H₄ (see UNIT 17.22A), but can also be applied to metabolically labeled materials if the specific radioactivity of the radiolabeled precursor that appears in the metabolically labeled glycosaminoglycan is determined independently.

Calibrate NaB[³H]₄ used in the analysis of unknowns

Because the counting efficiency for ³H varies in each type of counting experiment, cpm/µmol of a standard reducing sugar must be determined separately for each type of separation procedure. Mix a measured amount of a standard sugar (usually D-glucose) with the [¹⁴C]D-glucose internal standard (see UNIT 17.22A). Enough [¹⁴C]glucose should be added so that ~1 x 10⁶ ¹⁴C cpm will be recovered in the final separation step. Reduce an aliquot of this mixture with the same NaB[³H]₄ reagent to be used in the analysis (see UNIT 17.22A). If NaB[³H]₄ with a specific activity of ~500 mCi/mmol (recommended) is used, the [³H]D-glucitol formed by reduction of the D-glucose should have a specific activity of 125 mCi/mmol, and 1 µmol of the standard D-glucose should yield 778 x 10⁶ dpm per µmol of [³H]glucitol. Counting efficiency for ³H in some of the separation steps may be as low as 5%. Chromatograph the resulting [³H]D-glucitol/[¹⁴C]glucitol mixture in the same type of analytical separation procedure that is being used for the unknowns (paper chromatography or HPLC). Determine the number of [¹⁴C]glucitol and [³H]glucitol cpm in the separated peak by scintillation counting under conditions optimized for dual-label counting. The ³H cpm must be corrected for the number of ¹⁴C cpm that spill over into the ³H counting channel. From the number of [¹⁴C]glucitol cpm, calculate the number of µmol of D-glucitol in the peak as described below.

When a known number of µmol of glucose are mixed with a known number of cpm of the [¹⁴C]glucose internal standard, the number of ¹⁴C cpm recovered in the analytical stage (e.g., in paper chromatography or HPLC) can be used to calculate the proportion of original µmol of the standard glucose that are recovered in the analytical stage. For example, if the original D-glucose solution contained 1 x 10⁵ µmol of the D-glucose standard and 5 x 10⁵ cpm of [¹⁴C]glucose, and if 1 x 10⁵ cpm of [¹⁴C]glucitol were recovered in the analytical run, then 2 x 10⁴ µmol of the D-glucitol standard would be recovered in the analytical run. Thus, if 1 x 10⁶ ³H cpm in the [³H]glucitol were recovered in the analytical run, the number of ³H cpm/µmol for glucose would be 5 x 10⁴.
Using the [3H]glucitol cpm in the separated peak and the number of µmol of D-glucitol in the peak, calculate the number of [3H]glucitol cpm/µmol, which will be the same as the number of [3H] cpm/µmol for all mono-, di- or tetrasaccharides obtained in the analytical run, as all reducing sugars incorporate the same number of equivalents of 3H per µmol from the NaB[3H]4. Consequently, when the total number of 3H cpm is determined for any peak in the analytical run, division of this number by the 3H cpm/µmol of the D-glucose standard yields the value for the number of µmol of the compound in the peak.

Calibrate internal standard [14C]glucose

Because the counting efficiency for 14C varies in each type of counting experiment, the number of 14C cpm in a given aliquot of the [14C]glucose internal standard must be determined separately for each type of separation procedure. Reduce an aliquot of the [14C]glucose internal standard using the same reduction conditions used for the unknowns (see UNIT 17.22A for nitrous acid cleavage products; see Basic Protocol 2 for lyase cleavage products). To calibrate [14C]glucose for paper chromatography, transfer the resulting [14C]glucitol solution quantitatively to a paper chromatography strip and run the chromatogram in the solvent for which the [14C]glucose calibration is being made. Develop the chromatogram, cut the strip into 0.5-in. segments for scintillation counting, then determine the total number of 14C cpm in the original aliquot taken for analysis.

To calibrate [14C]glucose for HPLC, inject a measured aliquot of the [14C]glucitol solution quantitatively into an HPLC column injector, elute the column as in a typical analytical run, then determine the number of 14C cpm in the [14C]glucitol peak. The [14C]glucose will emerge in the void volume of the column in the HPLC procedures described here.

Chromatographic and electrophoretic properties of various glycosaminoglycan oligosaccharides are listed in Tables 17.22B.1-17.22B.5.

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.

HPLC solvents

For anion-exchange chromatography: HPLC solvents for chromatography on weak anion-exchange or strong ion-exchange columns range from 0 mM to 400 mM KH2PO4. Degas and filter 1 liter distilled water by gently heating it to 80°C in a 2-liter vacuum-filtration flask, sealing the flask with a stopper, then placing it in an ice-water bath and evacuating with a water aspirator while gently stirring with a magnetic stirrer. Continue aspirating until no further bubbles are apparent (15 min), then transfer the volumetric flask containing the amount of KH2PO4 required to give the desired concentration. Gently stir the solution, then filter through a 0.45-µm Millipore filter under vacuum into a clean 500-ml bottle, which will serve as a reservoir for the solvent.

For HPLC systems that allow continuous degassing during the run, it is not necessary to degas solvents before making the solutions.

For reversed-phase ion-pairing chromatography: Prepare solvents A, B, and C as follows. Mix or deliver in gradient as described in Table 17.22B.4 and Table 17.22B.5.

Solvent A: Prepare 38 mM NH4H2PO4, 2 mM H3PO4, and 1 mM tetrabutylammonium phosphate in water. Adjust pH to 3.6 with 0.1 M NaOH or HCl.

Solvent B: Prepare 38 mM NH4H2PO4, 2 mM H3PO4, and 1 mM tetrabutylammonium phosphate in 30/70 (v/v) acetonitrile/water. Adjust pH to 4.2 with 0.1 M NaOH or HCl.

continued
Solvent C: Prepare 38 mM NH₄H₂PO₄, 2 mM H₃PO₄, and 1 mM tetrabutylammonium phosphate in 50:50 (v/v) acetonitrile /water. Adjust pH to 4.4 with 0.1 M NaOH or HCl.

Paper chromatography and paper electrophoresis systems

Use the following paper chromatography or paper electrophoresis systems in conjunction with the data in Table 17.22B.1 or Table 17.22B.2:

Paper chromatography system 1: 3:2:1 (v/v/v) 1-butanol/glacial acetic acid/1 M NH₄OH. Use 1 × 22–in. Whatman no. 3 paper chromatography strips.

Paper chromatography system 2: 3:2:1 (v/v/v) ethyl acetate/pyridine/5 mM boric acid. Use 1 × 22–in. Whatman cellulose phosphate paper chromatography strips.

Paper electrophoresis system 1: 50 ml glacial acetic acid and 10 ml pyridine in 4 liters water (pH 4.0). Use 1 × 22–in. cellulose phosphate paper chromatography strips.

Paper electrophoresis system 2: 350 ml of glacial acetic acid and 100 ml of 88% formic acid in 4 liters water (pH 1.7). Use 1 × 22–in. cellulose phosphate paper chromatography strips.

Sodium borohydride reagent

Just prior to use, dissolve 1.89 mg NaB[3H]₄ in 100 µl chilled 0.2 M Na₂CO₃, pH 10.2, to give a 0.5 M NaB[3H]₄ solution. Keep on ice until use.

COMMENTARY

Background Information

A number of high-performance liquid chromatography procedures have been developed to separate and quantitate the disaccharides formed by deaminative cleavage of N-deacetylated glycosaminoglycans with nitrous acid (UNIT 17.22A) or cleavage with lyases (UNIT 17.13B). These include chromatography on strong anion-exchange columns (SAX; Bienkowski and Conrad, 1985); weak anion exchange columns (WAX; Shaklee and Conrad, 1986); and reversed-phase ion-pairing columns (Guo and Conrad, 1988). Alternatively, some of the disaccharides can be separated by paper chromatography (Hopwood and Elliot, 1983; Hopwood and Muller, 1983), paper electrophoresis (Hopwood and Elliot, 1983; Hopwood and Muller, 1983), thin-layer chromatography (Edge and Spiro, 1985), or capillary-zone electrophoresis (Al-Hakim and Linhardt, 1991; Desai et al., 1993). When lyases are used to cleave the glycosaminoglycan (UNIT 17.13B), the lyase-derived di- and oligosaccharides can be assayed by the absorbance of their unsaturated uronic acid residues at 233 nm. This UV assay has been used for detection of oligosaccharides eluted from SAX HPLC columns with a linear sodium chloride gradient running from 0 to 1 M (Rice et al., 1985; Linhardt et al., 1988). Glycosaminoglycans that have been metabolically labeled with [3H]p-glucosamine or [35S]O₄²⁻ yield disaccharides with the corresponding labels. Disaccharides from both types of cleavage can also be detected and quantified following their reduction with NaB[3H]₄ to incorporate one gram atom of ³H per mol of disaccharide. In this approach, the elution positions of the ³H-labeled disaccharide peaks separated by HPLC, paper chromatography, or paper electrophoresis are used to identify the disaccharides, and the ³H cpm recovered in each peak are used to obtain ratios of disaccharides or quantitative measures of the amounts of each disaccharide present in the glycosaminoglycan.

Critical Parameters

The important parameters for the separations described in this unit are those that must normally be observed for chromatographic separations. The critical parameters for the analytical procedures are primarily those involved in the treatment with nitrous acid and NaB[3H]₄. These are described in UNIT 17.22A. It may be noted that the elution times given in Tables 17.22B.1-17.22B.5 may vary somewhat, but the relative positions of elution remain the same. Positive identification may require chromatographic runs of mixtures of previously characterized disaccharides before or
after the sample run. As columns age, the elution times become shorter and resolution becomes poorer. When resolution becomes unsatisfactory, the column must be replaced.

**Anticipated Results**

The nitrous acid reaction (see UNIT 17.22A) is virtually stoichiometric for β-linked amino sugars (Shively and Conrad, 1970). Consequently, the yield of disaccharides is stoichiometric from those glycosaminoglycans that contain β-linked amino sugars (i.e., chondroitin sulfate, dermatan sulfate, hyaluronic acid, and keratan sulfate). However, a side reaction called the “ring-contraction reaction” occurs to the extent of 10% to 15% for α-linked amino sugars—e.g., those found in heparin and heparan sulfate (Shively and Conrad, 1970, 1976). The ring-contraction reaction results in deamination without bond cleavage, and may convert a segment in heparin that contains two disaccharides having N-sulfated GlcN residues into a tetrasaccharide fragment instead of the two disaccharides that would be expected. Thus, heparin and heparan sulfate yield small but significant amounts of the ring-contraction tetrasaccharides (Bienkowski and Conrad, 1985). However, there seems to be little structural selectivity for the ring-contraction reaction, as the ring-contraction tetrasaccharides contain disaccharide units in the same proportions as those found in the original glycosaminoglycan. Thus, even for heparin and heparan sulfate, the disaccharides formed during nitrous acid cleavage are obtained in the same proportions as those found in the original glycosaminoglycan. Consequently, when yields are expressed in percent of total uronic acids or percent of total amino sugars, the ring-contraction reaction does not interfere with the quantitation, and the yields of di- and monosaccharides are truly representative of their proportions in the starting glycosaminoglycan. Some of these heparin ring-contraction tetrasaccharides have been identified (Bienkowski and Conrad, 1985).

**Time Considerations**

The times for chromatography are apparent from the retention times reported in Tables 17.22B.1 to 17.22B.3. Time for processing of the data will depend in great part on the type of scintillation counter used and the data-processing equipment available on site where the methods will be used.

**Literature Cited**


**Key References**

Bienkowski and Conrad, 1985. See above. *(Describes the separation of heparin di- and tetrasaccharides on SAX columns.)*

Guo and Conrad, 1988. See above. *(Describes the separation of heparin di- and tetrasaccharides by reversed-phase ion-pairing HPLC.)*

Shaklee and Conrad, 1986. See above. *(Describes the separation of disaccharides from chondroitin sulfate, dermautan sulfate, and keratan sulfate formed by both nitrous acid and lyase cleavage.)*

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Analysis of Sulfate Esters by Solvolysis or Hydrolysis

Sulfate esters are found on N- and O-linked sugar chains or glycosaminoglycan (GAG) chains. Few sulfatases are available that can enzymatically remove them, so chemical procedures must be used. These procedures rely on the differential sensitivity of sulfates located in different linkages on the sugar. In comparison to the conditions used for enzymatic digestion, those used for chemical digestion are very harsh and cannot be used on protein-bound carbohydrates (except for analytical purposes, as in Basic Protocol 2). With protein-bound carbohydrates, for preparative purposes, the chains must first be released by one of the procedures described in UNITS 17.13 & 17.15. Basic Protocol 1 describes release of sulfate esters by solvolysis. A Support Protocol describes a method for monitoring the efficiency of the solvolysis reaction, and Alternate Protocol 1 provides a scale-up method for using the solvolysis reaction with large amounts of material. Basic Protocol 2 presents a technique for acid hydrolysis to release sulfate esters, and Alternate Protocol 2 describes basic hydrolysis for the same purpose.

SOLVOLYSIS FOR RELEASE OF SULFATE ESTERS FROM GLYCANS

Solvolysis is analogous to hydrolysis, except that a nonaqueous solvent is used. Both solvolysis procedures in this unit (also see Alternate Protocol 1) require conversion of the sulfated molecule to the pyridinium salt before heating in dimethyl sulfoxide (DMSO) to remove sulfate. This procedure is for $^{35}$SO$_4^-$- or $^3$H-radiolabeled GAG samples, and applies to microgram amounts of material. For scale-up, see Alternate Protocol 1.

Materials

- Sulfated sample (UNIT 17.15), radiolabeled (UNIT 17.4 or UNIT 17.5)
- Glucose-6-sulfate (Sigma)
- 0.5×50-cm Sephadex G-25 gel-filtration column (Pharmacia Biotech)
- 2-cm column of Dowex-50 X8 (hydrogen form; see recipe for washing procedure)
  - packed in a Pasteur pipet plugged with glass wool
- Pyridine
- 1 M NaCl
- 9:1 (v/v) dimethyl sulfoxide (DMSO; reagent grade)/methanol
- QAE-Sephadex (Pharmacia Biotech)
- Thick-walled conical glass tubes
- Heating block
- Lyophilizer or shaker-evaporator

Additional reagents and equipment for desalting by gel-filtration chromatography (UNIT 10.9)

1. Desalt sample on a 0.5×50-cm Sephadex G-25 column, eluting with water.
   
   *It is essential to remove all nonvolatile salts from the sample. If free $^{35}$SO$_4^-$ is present in the sample, sulfate will run through the water-eluted Sephadex G-25 column with an apparent molecular weight much larger than its actual molecular weight, and may overlap with small $^{35}$SO$_4^-$-labeled oligosaccharides. It may be therefore be necessary to remove free $^{35}$SO$_4^-$ by dialysis from the protein-bound $^{35}$SO$_4^-$ prior to the release of the carbohydrate chains from the protein.*

2. Add 5 µg of glucose-6-sulfate to the sample, which will be in a volume of 1 to 5 ml of water. Pass the sample through a 2-cm column of water-washed Dowex-50 X8 (hydrogen form) packed in a glass wool–plugged Pasteur pipet. Wash column with
5 ml water and combine washings with the original flowthrough of the column. Check pH of the combined washings with pH paper; it should be within one unit of the pH of the combined sample and wash water that will be used in the subsequent steps.

The Dowex-50 column removes any residual cations and replaces them with protons. The glucose-6-sulfate provides further insurance that any residual cations will not interfere with the solvolysis reaction, but the amount of this reagent must be kept low to prevent unwanted side reactions.

3. Add 50 µl pyridine to the sample and split it into two aliquots. Add 2 µl of 1 M NaCl to one sample and nothing to the other. Transfer samples to thick-walled glass conical tubes and lyophilize or dry on a shaker-evaporator.

The sample with the NaCl serves as a negative control for desulfation and for the side effects of the DMSO-treatment step (step 4), because solvolysis does not proceed when the salt is present. In the experimental (i.e., no-salt) sample, most of the pyridine evaporates during lyophilization, except for that which forms the pyridinium salt with the sample or glucose-6-sulfate.

4. Add 0.2 ml of 9:1 (v/v) DMSO/methanol to each sample. Using a heating block, heat 2 hr at 85°C to 90°C for O-sulfate esters or at 55°C for N-sulfate esters.

5. Remove DMSO under vacuum using a lyophilizer or shaker-evaporator.

Depending upon the next step—e.g., in the case of QAE-Sephadex chromatography (UNIT 17.20)—it may not be necessary to remove the solvent. If it is necessary to do this, it should be noted that DMSO is not very volatile and will take several hours to evaporate under vacuum. In some cases the sample may need to be diluted.

**MONITORING SOLVOLYSIS REACTION**

If it is important to monitor the efficiency of the reaction in Basic Protocol 1, this can be done by running a sample of [³H]glucitol-6-sulfate in parallel with the experimental samples.

**Additional Materials** (also see Basic Protocol 1)

[³H]glucitol-6-sulfate: glucose-6-sulfate (Sigma) reduced with NaB[³H]₄ (UNIT 17.5)
2-cm QAE-Sephadex column (Pharmacia Biotech)
35 mM and 200 mM NaCl
Scintillation fluid compatible with aqueous samples

Additional reagents and equipment for QAE-Sephadex analysis (UNIT 17.20)

1. Carry out steps 1 and 2 of Basic Protocol 1, running ~10,000 cpm of [³H]glucitol-6-sulfate in parallel with the experimental sample. In step 3, split the pyridine treated column effluent from this glucitol-6-sulfate sample into three aliquots instead of two. Add 2 µl of 1 M NaCl to one aliquot. Dry all three aliquots as described in Basic Protocol step 3. Keep one of the non–salt-treated aliquots in reserve, then carry out steps 4 and 5 (solvolysis) on the remaining salt-treated and non–salt-treated aliquots.

Alternatively, this [³H]-labeled material may be added directly to the sample itself as an internal control. This cannot, however, be done if it interferes with subsequent analysis of the sample products (e.g., if the sample itself is labeled with [³H]).

2. Dilute the three [³H]glucitol samples to 1.5 ml with water and treat each of them as follows. Load on a 2-cm QAE-Sephadex column. Wash column three times using 1.5 ml of water each time and collect each effluent in a scintillation vial. Wash three more times using 1.5 ml of 35 mM NaCl each time, and collect the effluents in three more
separate vials. Finally, wash once with 1.5 ml of 200 mM NaCl and collect the effluent in another vial.

Ten vials will thus be obtained for each of the three [3H] glucitol samples.

3. Add 15 ml of an aqueous-compatible scintillation fluid to each vial and count on a scintillation counter.

The [3H] glucitol in untreated sample and the solvolysis control incubation should elute in the 35mM NaCl wash. The [3H] glucitol in solvolyzed desulfated sample should elute with water showing that it no longer carries a charge. Very little radioactivity will be seen in the high-salt wash. The efficiency of sulfation is then calculated as the percentage of [3H] glucitol eluted with water compared to the total [3H] glucitol recovered.

SCALE-UP OF SOLVOLYSIS FOR LARGE AMOUNTS OF MATERIAL

Basic Protocol 1 is used to desulfate trace amounts of material, but it can be applied to larger amounts of material such as glycosaminoglycans (GAGs). As with Basic Protocol 1, the extent of the reaction can be monitored by the desulfation of [3H]glucitol-6-sulfate if the presence of this labeled compound will not interfere with the subsequent use of the product. The steps and the preparations of the procedure are essentially the same as described for the Basic Protocol 1, except that no control incubation (i.e., with NaCl; see Basic Protocol 1, step 3) is done on the material. This procedure describes the solvolysis of 10 mg of GAG (or other sulfated sugar chains) and can be further scaled up if necessary.

Additional Materials (also see Basic Protocol 1)

[3H]glucitol-6-sulfate: glucose-6-sulfate (Sigma) reduced with NaB[3H]4 (UNIT 17.5)
Dialysis tubing (3000 MWCO)
Additional reagents and equipment for dialysis (APPENDIX 3C)

1. Desalt material containing 10 mg GAGs or other sulfated molecules on a Sephadex G-25 column and pool the fractions that contain GAGs.

Free GAG chains can be monitored by the carbazole reaction (see UNIT 17.12) or by amino sugar tests. Follow the normal procedures for gel filtration and pool conservatively to avoid any salt.

2. Mix material with an appropriate amount of [3H]glucitol-6-sulfate and pass the mixture over a 5 × 0.5–cm column of well-washed Dowex-50 X8 (hydrogen form). Wash with 2 to 3 column volumes water, then add 0.1 ml pyridine to the combined eluates. Lyophilize.

The amount of [3H]glucitol-6-sulfate to be added will depend upon the fraction of the total sample that can be sacrificed for the QAE-Sephadex analysis described in Support Protocol 1. The pH of the eluate may be lower than that found in Basic Protocol 1 because the chains, which will be in protonated form, are found at a higher concentration.

3. Dissolve the lyophilized material in 1.0 ml of 90% dimethyl sulfoxide/10% methanol and heat 2 hr at 90°C.

The [3H]glucitol-6-sulfate can be used to confirm desulfation of the GAG chains and to follow the course of the reaction. Take aliquots at different times and monitor its elution from QAE-Sephadex as described in Support Protocol 1. Use <1% of the sample (100 µg) so that the 2-cm QAE-Sephadex column is not overloaded (see Support Protocol 1). The GAG chains may remain bound to the column, but this does not matter because only the desulfation of the [3H]glucitol-6-sulfate is monitored.
4. Cool the reaction tube. Dialyze the sample against 3 liters of water using 3000-MWCO dialysis tubing.

Alternatively, remove the reagents on a Sephadex G-25 gel-filtration column. Either procedure will work, but the choice is determined by the future plans for the sample. The apparent size of the molecules will decrease because the sulfate contributes disproportionately to their apparent size, and some breakdown of chains may also occur.

**ACID HYDROLYSIS FOR RELEASE OF SULFATE ESTERS FROM GLYCOSAMINOGLYCANS**

This protocol measures the amount of bound $^{35}\text{SO}_4$ that remains soluble after the precipitation of hydrolyzed sulfate as the barium salt. In this procedure, we assume that the released oligosaccharide is $^{35}\text{SO}_4$-labeled, but the sugars may also be labeled with $^3\text{H}$. If the sugars are not labeled with tritium, a tritiated molecule—e.g., $^3\text{H}$-labeled glucose-6-sulfate should be included as an internal standard for normalizing recovery of the material.

**Materials**

- Sample: $^{35}\text{SO}_4$-labeled oligosaccharide or glycopeptide
- 0.50 N HCl (freshly diluted from 12 N reagent grade acid)
- 0.50 N NaOH (accurately titrated)
- 4 M NaCl
- 0.01 N HCl in 1 M NaCl (diluted from 0.50 N HCl above)
- 2 mM Na$_2$SO$_4$ in 2 M KCl
- 20 mM barium acetate
- Heating block with wells filled with oil

1. Dispense $\geq 1000$ to 2000 cpm of $^{35}\text{SO}_4$-labeled oligosaccharide or glycopeptide in 50 µl water into a series of 8 to 10 microcentrifuge tubes. Add 50 µl of 0.50 N HCl to each tube.

2. Incubate the tubes in a heating block with wells filled with oil at 98° to 100°C for a series of different time periods.

   The amount of time will vary depending upon the sample. A preliminary experiment with time points at 1 and 3 hr will help determine the best time points to try. All sulfate esters will eventually be hydrolyzed and even the most resistant ones will be 75% degraded after 3 hr. A reasonable set of time points to try is 0, 5, 15, 30, 60, 90, 120, 150, and 180 min.

3. At each time point, remove the tube from the oil, wipe it clean and place it on ice for 1 min. Microcentrifuge 2 sec at maximum speed to collect the liquid in the bottom of the tube and add sufficient 0.5 N NaOH to neutralize the sample. Also add 50 µl of 4 M NaCl to maintain high salt concentration. Keep completed samples on ice while remaining samples are incubating.

   Determine the exact volume of 0.5 N NaOH ($\sim$50 µl) to add to the samples by first neutralizing a dummy sample containing 50 µl of 0.50 N HCl and 0.50 N water and checking with pH paper. Acceptable pH after neutralization is 3 to 9.

4. After the time course has been completed for all samples and all samples have been neutralized, add sufficient 0.01 N HCl in 1 M NaCl to each sample to adjust the pH to 2 to 3. Check with pH paper using 1 to 2 µl of sample.

   Determine the exact amount of 0.01 N HCl ($\sim$100 µl) to add by adjusting the pH of the dummy sample neutralized above (see step 3, annotation). It is still necessary, however, to check the pH of the actual samples.
5. Add 100 µl of 2 mM Na₂SO₄ in 2 M KCl (for a total volume of 400 µl) and mix. Remove 20% of the sample and determine total counts of ³⁵SO₄.

6. Add 150 µl of 20 mM barium acetate to the remaining material in each sample and incubate 15 min on ice. Microcentrifuge 5 min at 10,000 × g, then remove 90% of the solution (≈400 µl) and determine total counts of ³⁵SO₄.

7. Normalize the amount of ³⁵SO₄ radioactivity that remains soluble as a percentage of the total counts present in the original sample.

**Initially, without hydrolysis essentially all of the ³⁵SO₄ should remain soluble. Hydrolysis yields fewer soluble counts (more precipitation of Ba³⁵SO₄).**

8. Using the “0” time point as 100% soluble material (this should be >90% of the total counts), plot the decrease in soluble ³⁵SO₄ on a log scale for typical first-order decay kinetics. Determine if single or multiple kinetic classes exist, as indicators of the number of types of sulfate esters in the sample.

*See Figure 17.23.1 for examples of single and multiple kinetic classes.*
BASE HYDROLYYSIS FOR RELEASE OF SULFATE ESTERS FROM GLYCOSAMINOGLYCANS

This method will release sulfate esters that are sensitive to base hydrolysis (see Background Information).

Additional Materials (also see Basic Protocol 2)
Sample: $^{35}$SO$_4$-labeled, borohydride-reduced (UNIT 17.5) oligosaccharide or glycopeptide
2 N NaOH
2 N HCl (freshly diluted from 12 N reagent-grade acid)

1. Dispense 1000 to 2000 cpm $^{35}$SO$_4$-labeled, borohydride-reduced oligosaccharide or glycopeptide in 50 µl water into a series of at least six microcentrifuge tubes. Add 50 µl of 2 N NaOH to each tube and mix.
2. Incubate the tubes at 80°C in a heating block with wells filled with oil for 0, 1, 2, 4, 6, and 10 hr.
   IMPORTANT NOTE: Use prereduced oligosaccharides; otherwise the base may degrade the oligosaccharide chain. The base hydrolysis occurs at about the same rate as the loss of primary-linked sulfates ($T_{1/2} = \sim 1$ to 2 hr).
3. At each time point remove the tube, wipe it clean, and place it on ice for a few minutes. Microcentrifuge 2 sec at 10,000 × g to collect the dispersed droplets. Add 50 µl of 2 N HCl to adjust the pH to 3 to 9, checking 1 to 2 µl of solution with pH paper.
4. After the time course has been completed for all samples and all samples have been neutralized, perform steps 5 to 8 of the acid hydrolysis protocol (see Basic Protocol 2) to precipitate the solubilized $^{35}$SO$_4$.

Completeness of any hydrolysis will be indicated by a flattening of the decay curve.

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.

Dowex-50 X8 resin
Prewash Dowex-50 resin by stirring 50 to 100 g into 2 liters of water, allowing it to stand 1 hr, then discarding water. Repeat twice, then leave overnight at room temperature. Pour off the final water and resuspend the resin in 3 to 4 vol water. The resin may be stored at 4°C for months under these conditions.

COMMENTARY

Background Information

Solvolysis
The conditions of the solvolysis reaction can be adjusted to preferentially remove either N- or O-linked sulfate esters, because N-sulfates (e.g., GlcN$\text{SO}_4$ in heparin; not to be confused with sulfated N-linked oligosaccharides) are more sensitive than O-sulfate esters (Inoue and Nagasawa, 1976; Nagasawa et al., 1977).

Acid hydrolysis
It is not possible to get an accurate compositional analysis of “sulfated sugars” by doing an acid hydrolysis because the sulfate esters are cleaved faster than most glycoside bonds. By selecting the right conditions, acid hydrolysis can give information about the number and locations of sulfate esters because they are cleaved at different rates depending upon their location on the individual sugar residues (Freeze and Wolgast, 1984). The kinetics of sulfate liberation (see Fig. 17.23.1) can help to determine the number of classes of sulfate esters present and what their likely locations may be. These properties are not strongly influenced by other substituents on the sulfated sugar or by the position of the sugar within an oligosac-
charide chain. Since the hydrolysis can also cleave glycosidic linkages and partially destroy the sugar chain, only the release of sulfate can be measured.

The acid hydrolysis procedure differentiates between sulfates linked to different hydroxyl groups on the sugar ring. Under the hydrolysis conditions in Basic Protocol 2, the $T_{1/2}$ of sulfates linked to equatorial hydroxyl groups (e.g., glucose-3-sulfate, galactose-3-sulfate, $N$-acetylglucosamine-3-sulfate, and mannose-3-sulfate) is 6 to 25 min; that of those linked to axial hydroxyl groups (e.g., galactose-4-sulfate, $N$-acetylglucosamine-4-sulfate, and mannose-2-sulfate) is 60 to 84 min, and that of those linked to primary hydroxyl groups (e.g., glucose-6-sulfate, galactose-6-sulfate, $N$-acetylglucosamine-6-sulfate, and mannose-6-sulfate) is 90 to 120 min (Rees, 1963). All 6-OH positions are primary, and all of the hydroxyl groups in glucose are equatorial. Any hexose isomer of glucose (e.g., mannose or galactose) will change one of the equatorial positions to an axial position. Even though the time ranges for hydrolysis listed above have not been extensively documented for sulfate-labeled oligosaccharides, they are useful because they will clearly differentiate between multiple classes of sulfate esters. Using the acid hydrolysis technique, it is not possible to determine which sugar is sulfated or where it is found in the chain. If other data are available on the structure of the oligosaccharide, this information may provide likely possibilities for the location of sulfate groups, especially if certain residues are resistant to exoglycosidase digestion.

**Base hydrolysis**

Only certain types of sulfate esters are sensitive to base hydrolysis (Freeze and Wolgast, 1984; Percival, 1978). Sensitivity is determined by the location of the sulfate on the sugar residue and—in contrast to acid hydrolysis described above—by the other substituents linked to that sugar residue. Base treatment will release sulfate esters from intact oligosaccharides under two different conditions. The first is when sulfate is located in the 6 (or 3)-OH position and the 3 (or 6)-OH group of the same sugar residue is not substituted. The second is when the sulfate ester is adjacent to an unsubstituted trans OH group. In the first case, the sulfate is eliminated and an acid-labile 3,6-anhydrosugar is formed (see Fig. 17.23.2). In the second case, the sulfate is released with the formation of an epoxide.

![Figure 17.23.2](image-url) **Figure 17.23.2** Formation of 3,6-anhydromannose by base treatment of an oligosaccharide containing mannose-6-sulfate. The formation of the anhydro sugar makes the glycosidic linkage very sensitive to acid; mild hydrolysis causes cleavage. Stronger hydrolysis degrades the usual glycosidic linkages, but leaves the anhydro sugar. When reduced with sodium borohydride, the modified sugar can be quantified to show how much mannose-6-sulfate was originally present.
ring on the sugar. This is destroyed during subsequent acid hydrolysis of the oligosaccharide. The modified sugar can be identified if it is metabolically labeled—e.g., as in the discovery of mannose-6-sulfate (Freeze and Wolgast, 1984)—but this is not routinely done. On the other hand, the loss of sulfate is easily measured. When data on the acid and base hydrolysis of sulfate esters are combined with other structural information on the molecules, several potential structures can be eliminated or supported, but these procedures will not provide proof of the structure.

**Critical Parameters**

Solvolysis is a harsh treatment that also removes sialic acids and probably fucose residues. If these sugars are normally present, their loss may be an unacceptable side effect of this procedure. On the other hand, hexoses, hexuronic acids, and hexosamines in glycosidic linkages are mostly stable to solvolysis. The incubated control is very important because it will monitor for any nonspecific destruction of the sugar chain.

For acid hydrolysis, the most critical points to observe are that the pH should be kept between 2 and 3 and that the salt concentration be kept very high during the precipitation of sulfate with barium. Only then is the precipitation specific for sulfate. Under less stringent conditions, the oligosaccharides or even monosaccharide sulfates can be precipitated.

**Anticipated Results**

Expect ~80% to 90% efficiency in desulfation, and <20% in the control incubation (Freeze and Wolgast, 1984).

**Time Considerations**

Solvolysis itself takes only a few hours to complete. The rate-limiting step is the time needed to remove the water after the sample has passed through the Dowex-50 column and the pyridine has been added. This will only take a few minutes using a shaker-evaporator, but will require an overnight lyophilization.

Beginning with the hydrolysis step, the procedure can be completed within a day. If necessary, the procedure can be stopped after taking all of the time points and neutralizing them with base prior to precipitation. Where this is done, the reaction mixtures can be stored at 0°-4°C for 24 hr or more.

**Literature Cited**


The first step toward analyzing the structure and function of oligosaccharides on a glycoconjugate is the detection of the glycan chains themselves. The units presented in this section describe a variety of ways in which this can be achieved (e.g., UNIT 17.7). In some cases, such studies may provide the first evidence that the molecule being examined is a glycoconjugate. In other cases, these methods may be used to qualitatively or quantitatively analyze the nature of the glycan chains on a molecule that is already known to be a glycoconjugate. For help in deciding which protocols to use, the user is referred to the section entitled “Choice of Techniques” found in the introduction to this chapter (UNIT 17.0).

Introducing a label into the sugar chains of the glycoconjugate of interest is often informative, and can be very convenient for further analyses. This can be achieved by metabolic radiolabeling (UNIT 17.4), chemical labeling (UNIT 17.5), or transferring label from a radioactive sugar nucleotide using a glycosyltransferase (UNIT 17.6). Each of these approaches has its advantages and disadvantages in terms of specificity, sensitivity, and practical utility. The user is advised to read the Commentary in of each of these units before choosing a particular approach.

Many membrane glycoproteins also acquire a glycophospholipid anchor during biosynthesis. A set of methods designed to detect and analyze such anchors is presented in UNIT 17.8. There are also many well established methods for direct chemical analysis of the oligosaccharides present in glycoconjugates, such as the classic phenol-sulfuric acid assay for hexoses and pentoses (UNIT 17.9).

Finally, oligosaccharides on many glycoconjugates can be altered with a variety of commercially available inhibitors and competitive substrates that can work in whole cells or organisms. In this section, the prevention of N-linked glycosylation on glycoproteins (UNIT 17.10A) and the inhibition of glycolipid biosynthesis (UNIT 17.10B) is described, as well as the use of synthetic glycosides as primers of oligosaccharide biosynthesis and inhibitors of glycoprotein and proteoglycan assembly (UNIT 17.11).

There are, of course, many other approaches to the direct analysis of oligosaccharides on glycoconjugates that could have been presented in this section. The current choices for inclusion (see Chapter 17 table of contents) were dictated by the need to “keep it simple” and to meet the criterion of providing broad general utility to the average molecular biologist.