

Metabolic Radiolabeling of Animal Cell Glycoconjugates

Sandra Diaz¹ and Ajit Varki¹

¹University of California San Diego, La Jolla, California

UNIT 17.4

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.

Preparation and
Analysis of
Glycoconjugates

17.4.1

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: ^3H - or ^{14}C -labeled monosaccharide, [^{35}S]sulfate, [^3H]acetate, or [^{32}P]orthophosphate; 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- μm filter
- Disposable 0.22- μm 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 $\sim 500 \times g$, 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 $\sim 500 \times g$, 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

ALTERNATE PROTOCOL 1

Preparation and Analysis of Glycoconjugates

17.4.3

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

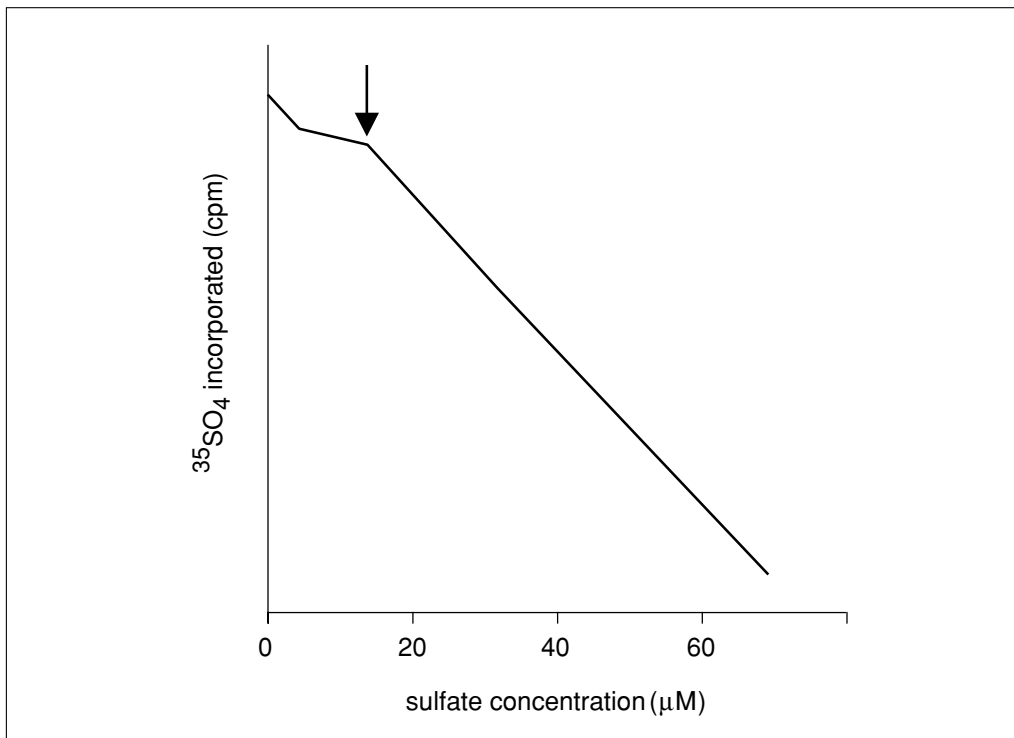


Figure 17.4.1 Incorporation of radiolabeled sulfates into macromolecules in cells labeled in sulfate-free medium and the effect of adding increasing amounts of unlabeled sulfate. The arrow indicates the point at which the sulfate is no longer limiting.

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 $\sim 250 \mu\text{M}$)

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)

Component	Final concentration in complete medium	Stock
CaCl ₂	200 mg/liter	Salt, reagent grade
KCl	400 mg/liter	Salt, reagent grade
MgCl ₂ ^a	75 mg/liter	Salt, reagent grade
NaCl	6800 mg/liter	Salt, reagent grade
NaH ₂ PO ₄ ·H ₂ O	140 mg/liter	Salt, reagent grade
Phenol red	10 mg/liter	10×
Sodium pyruvate	110 mg/liter	100×
L-Alanine	25 mg/liter	100×
L-Arginine	126 mg/liter	100×
L-Asparagine	50 mg/liter	100×
L-Aspartic acid	30 mg/liter	100×
L-Cysteine	NONE ^b	—
L-Glutamic acid	75 mg/liter	100×
L-Glutamine	NONE ^b	—
L-Glycine	50 mg/liter	100×
L-Histidine	42 mg/liter	100×
L-Isoleucine	52 mg/liter	100×
L-Leucine	52 mg/liter	100×
L-Lysine	72 mg/liter	100×
L-Methionine	NONE ^b	—
L-Phenylalanine	32 mg/liter	100×
L-Proline	40 mg/liter	100×
L-Serine	NONE ^b	—
L-Threonine	48 mg/liter	100×
L-Tryptophan	10 mg/liter	100×
L-Tyrosine	36 mg/liter	100×
L-Valine	46 mg/liter	100×
MEM vitamins (mixture)	1×	100×

^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.

- 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.

- Harvest and wash the cells. Determine efficiency of incorporation (see Basic Protocol, steps 6 to 8).

SEQUENTIAL PULSE OR PULSE-CHASE LABELING WITH REUSE OF RADIOACTIVELY LABELED MEDIUM

ALTERNATE PROTOCOL 2

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 $\sim 250 \mu\text{M}$)

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.

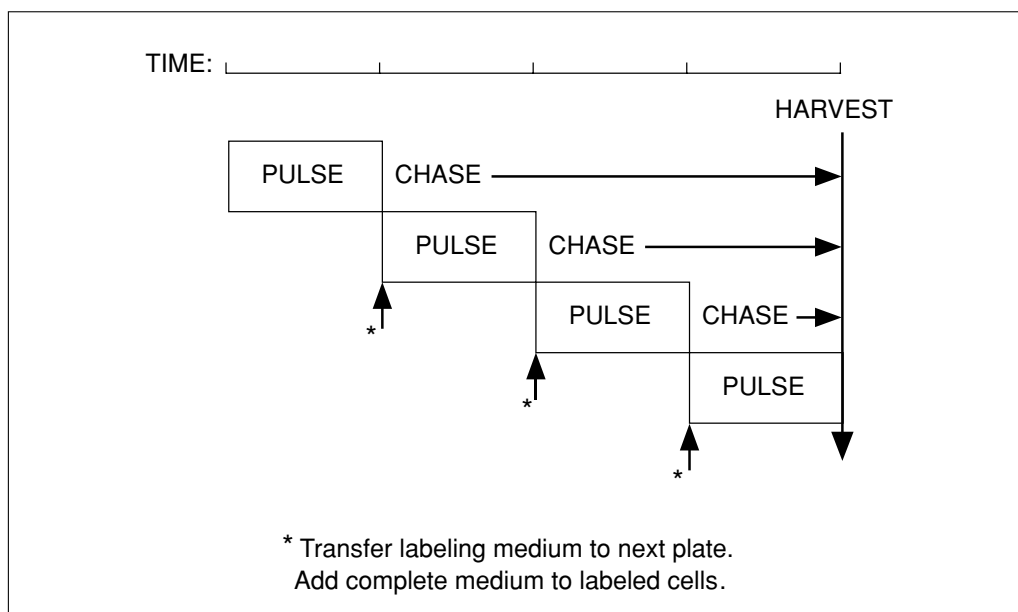


Figure 17.4.2 Scheme for sequential pulse labeling of cells reutilizing radioactive medium.

Preparation and Analysis of Glycoconjugates

17.4.7

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 ^{14}C -labeled monosaccharides, [^{35}S]sulfate, ^{35}S -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 \times 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 \times 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 \times final concentration in 1 liter, and adding water to 1 liter.

Table 17.4.2 Stock Solutions for Reconstitution of MDM^a

Component	Final concentration in complete medium	Stock
NaHCO ₃ ^b	1 × (2200 mg/liter)	100×
HEPES-HCl	20 mM (4.76 g/liter)	2 M, pH 7.3
Na ₂ SO ₄	0.81 mM (115 mg/liter)	100 mM, sterile
D-Glucose	1 × (1000 mg/liter)	100×
L-Cysteine	1 × (100 mg/liter)	100×
L-Glutamine	1 × (292 mg/liter)	100×
L-Methionine	1 × (15 mg/liter)	100×
L-Serine	1 × (25 mg/liter)	100×

^aIndividual components are added to MDM at full strength, or lowered concentration or may be left out altogether, depending upon the experiment planned.

^bUse 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 exper-

iment 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

(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 lumenally oriented transferases add the monosaccharides to lumenally 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 [³⁵S]sulfate, [³H]acetate, or [³²P]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 par-

tial 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 [2-³H]mannose: this is extremely specific, because conversion of the labeled mannose can yield only [2-³H]fucose or unlabeled fructose-6-phosphate. In the latter case, the label is lost as tritiated

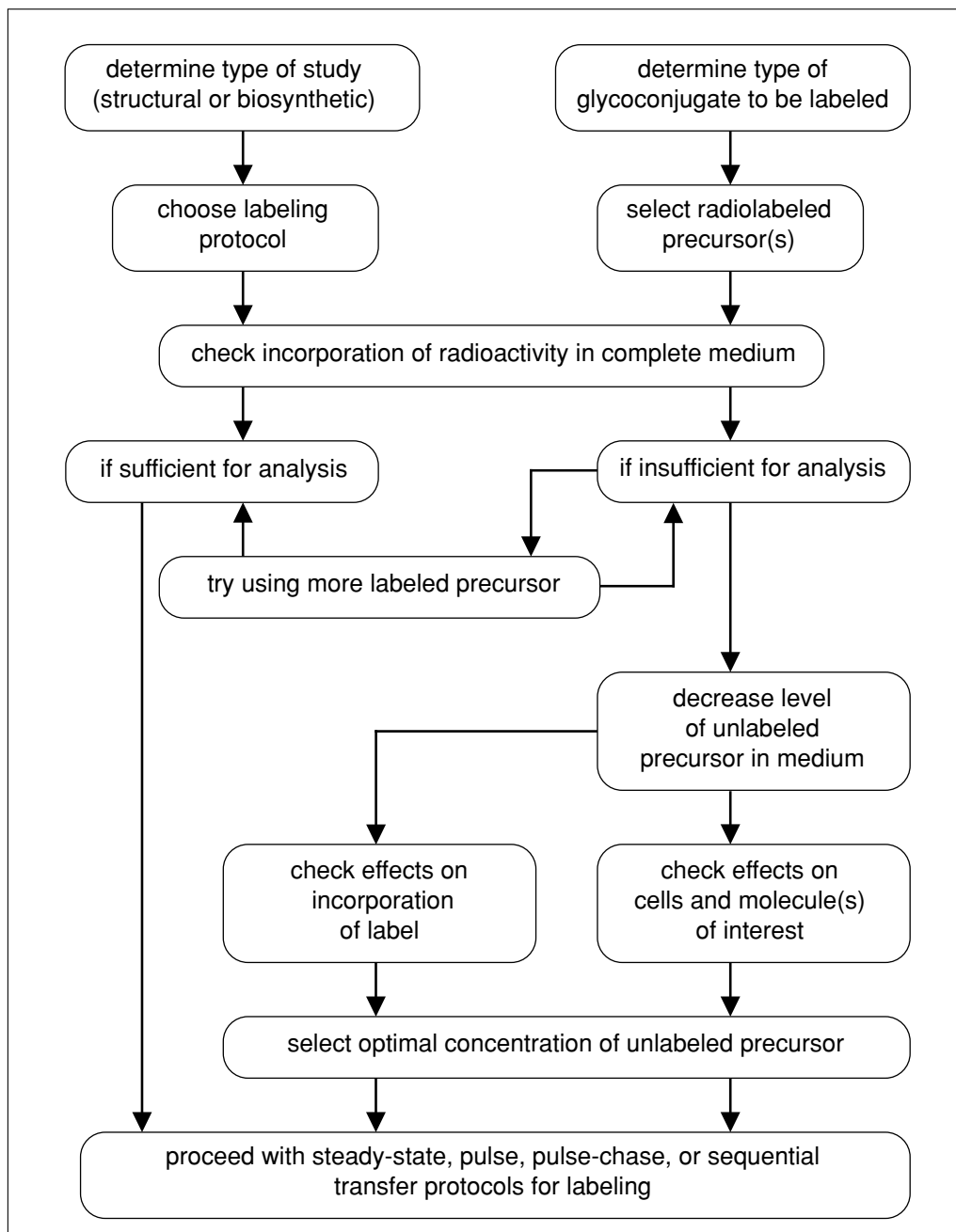


Figure 17.4.3 Strategy for planning metabolic labeling of animal cell glycoconjugates.

water, which is diluted into the pool of cellular water. Thus, in most instances, label from [2-³H]mannose remains confined to mannose and fucose residues, regardless of how long the labeling continues.

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 em-

pirically, 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^a

Precursor	Type of glycoconjugate					
	<i>N</i> -GlcNAc-linked glycoprotein	<i>O</i> -GalNAc-linked glycoprotein	Xylose-linked proteoglycan	Glyco-sphingolipid	Glyco-phospholipid anchor	O-linked GlcNAc
Man	+++	–	–	–	+++	–
Fuc	++	++	+/-	+	–	–
Gal	+++	+++	++	+++	+/- (side chain)	–
Glc	+ (precursor)	–	–	++	–	–
GlcNAc	+++	+++	++	++	+ (free amine)	++
GalNAc	+/-	+++	++	++	+/- (side chain)	–
Sia	+++	+++	–	+++	–	–
GlcA	+?	–	+++	+	–	–
SO ₄	+	+	++++	+	–	–
P _i esters	Man-6-P	–	Xyl-P	–	M6P (in core)	–
<i>O</i> -Acetyl	Sia-OAc	Sia-OAc	–	Sia-OAc	–	–
Acyl	–	–	–	++++	+/- (on inositol)	–

^aThe 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^a

Product identified as:	Radiolabeled precursor						
	[2- ³ H]Man	[6- ³ H]Gal	[1- ³ H]Gal	[1- ³ H]Glc	[6- ³ H]GlcNH ₂	[6- ³ H]ManNAc	[6- ³ H]GalNAc
[³ H]Man	+++	+/-	+/-	++	+/-	+/-	+/-
[³ H]Fuc	+++	+/-	+/-	++	+/-	+/-	+/-
[³ H]Gal	–	++++	++++	+++	+/-	+/-	+/-
[³ H]GlcA	–	–	++	++	–	–	–
[³ H]Glc	–	++	++	++++	+/-	+/-	+/-
[³ H]GlcNAc	–	+/-	+/-	++	++++	++	+++
[³ H]GalNAc	–	+/-	+/-	++	+++	+	++++
[³ H]Sia	–	–	–	++	++	++++	+/-

^aThe 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.

Metabolic Radiolabeling of Animal Cell Glycoconjugates

17.4.12

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

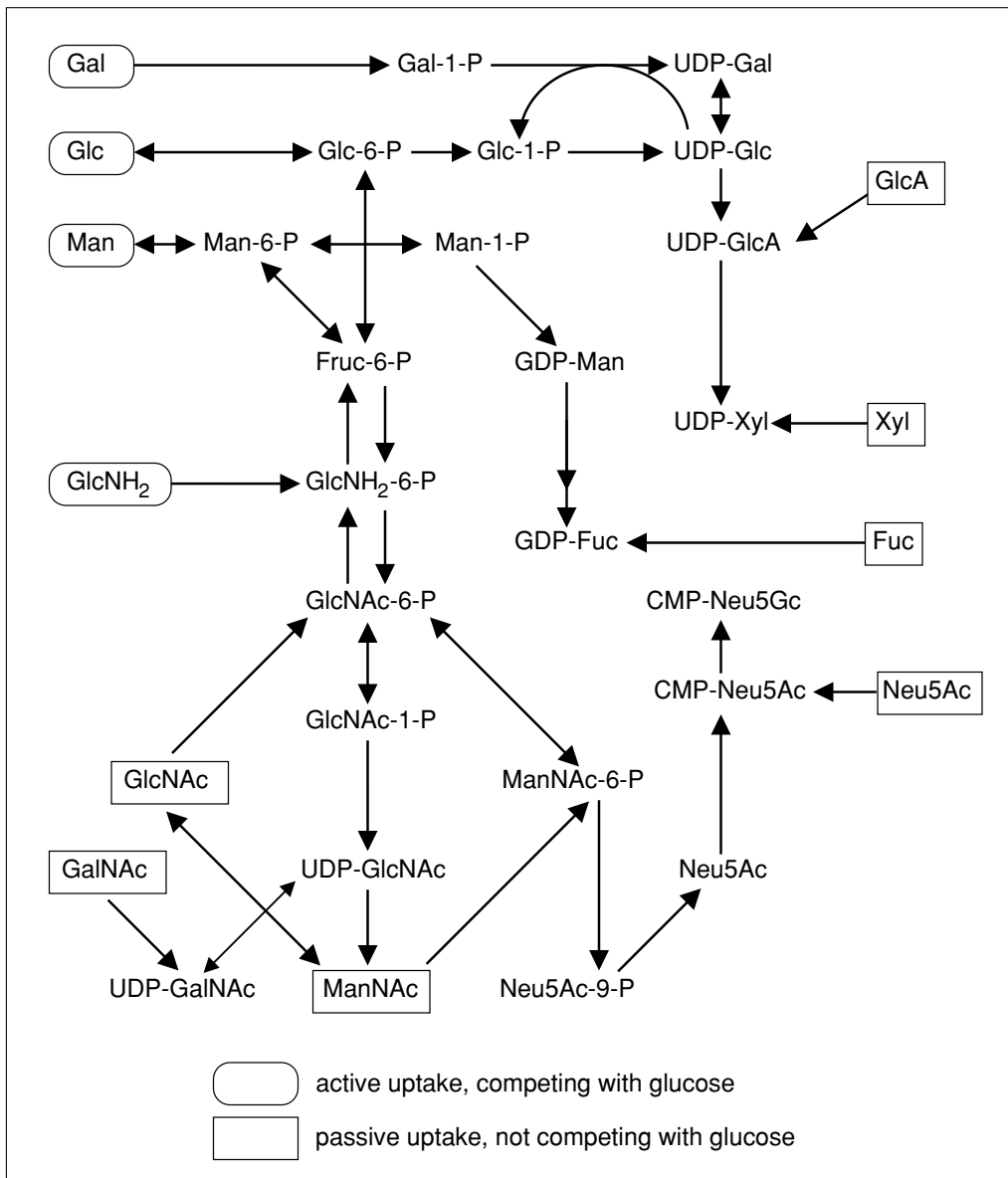


Figure 17.4.4 Cytosolic pathways for the interconversion of monosaccharides and their nucleotide sugar forms.

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

falls below the K_m of the cellular glucose transporter(s). These factors must be considered in designing the labeling protocol, although they can be controlled only to a limited extent. The goal is to obtain sufficient label in the glycoconjugate of interest without significantly altering the metabolic state of the cell.

Metabolic labeling using nonmonosaccharide precursors. Acetylation, sulfation, and phosphorylation can significantly affect the behavior of glycans both in biological systems and during analysis. Labeled precursors such as [^3H]acetate, [^{35}S]sulfate, and [^{32}P]orthophosphate can be used to label these modified oligosaccharides. These precursors are expected to enter a variety of other macromolecules, and release or isolation of the labeled glycans with specific endoglycosidases is often required before further analysis. Double-labeling of both the modified glycan and the underlying sugar chain (e.g., with a ^{14}C - or ^3H -labeled monosaccharide precursor) can be quite useful in monitoring purification and in subsequent structural analysis. With [^{35}S]sulfate, labeling efficiency varies widely with cell type. In many cells, if the endogenous pool of the precursor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) is very small, the specific activity of the exogenously added [^{35}S]sulfate can be practically unaltered inside the cell (Yanagishita et al., 1989). In some cell types, however, the endogenous sulfate pool is constantly diluted by breakdown of the sulfur-containing amino acids methionine and cysteine (Esko et al., 1986; Roux et al., 1988). In this situation, reducing the cysteine and methionine concentrations in the medium can improve incorporation of label into macromolecules (Roux et al., 1988). Sulfate levels can also be affected by certain antibiotics (e.g., gentamicin) that are sulfate salts.

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

- Cummings, R.D., Merkle, R.K., and Stults, N.L. 1989. Separation and analysis of glycoprotein oligosaccharides. *Methods Cell Biol.* 32:141-183.
- Diaz, S. and Varki, A. 1985. Metabolic labeling of sialic acids in tissue culture cell lines: Methods to identify substituted and modified radioactive neuraminic acids. *Anal. Biochem.* 150:32-46.
- Esko, J.D., Elgavish, A., Prasthofer, T., Taylor, W.H., and Weinke, J.L. 1986. Sulfate transport-deficient mutants of Chinese hamster ovary cells. Sulfation of glycosaminoglycans dependent on cysteine. *J. Biol. Chem.* 261:15725-15733.
- Fitch, F.W., Gajewski, T.F., and Yokoyama, W.M. 1997. Diagnosis and treatment of mycoplasma-contaminated cell cultures. *Curr. Protoc. Immunol.* A.3E.1-A.3E.4.
- Goldberg, D. and Kornfeld, S. 1981. The phosphorylation of beta-glucuronidase oligosaccharides in mouse P388D1 cells. *J. Biol. Chem.* 256:13060-13067.
- Gould, G.W. and Bell, G.I. 1990. Facilitative glucose transporters: An expanding family. *Trends Biochem. Sci.* 15:18-23.
- Hardy, M.R., Townsend, R.R., and Lee, Y.C. 1988. Monosaccharide analysis of glycoconjugates by anion exchange chromatography with pulsed amperometric detection. *Anal. Biochem.* 170:54-62.
- Hart, G.W., Haltiwanger, R.S., Holt, G.D., and Kelly, W.G. 1989. Glycosylation in the nucleus and cytoplasm. *Annu. Rev. Biochem.* 58:841-874.
- Hirschberg, C.B. and Snider, M.D. 1987. Topography of glycosylation in the rough endoplasmic reticulum and Golgi apparatus. *Annu. Rev. Biochem.* 56:63-87.
- Kim, J.J. and Conrad, E.H. 1976. Kinetics of mucopolysaccharide and glycoprotein biosynthesis by chick embryo chondrocytes. Effect of D-glucose concentration in the culture medium. *J. Biol. Chem.* 251:6210-6217.
- Muchmore, E.A., Milewski, M., Varki, A., and Diaz, S. 1989. Biosynthesis of N-glycolylneuraminic acid: The primary site of hydroxylation of N-acetylneuraminic acid is the cytosolic sugar nucleotide pool. *J. Biol. Chem.* 264:20216-20223.
- Rearick, J.I., Chapman, A., and Kornfeld, S. 1981. Glucose starvation alters lipid-linked oligosaccharide biosynthesis in Chinese hamster ovary cells. *J. Biol. Chem.* 256:6255-6261.
- Roux, L., Holoyda, S., Sunblad, G., Freeze, H.H., and Varki, A. 1988. Sulfated N-linked oligosaccharides in mammalian cells I: Complex-type chains with sialic acids and O-sulfate esters. *J. Biol. Chem.* 263:8879-8889.
- Tabas, I. and Kornfeld, S. 1980. Biosynthetic intermediates of beta-glucuronidase contain high mannose oligosaccharides with blocked phosphate residues. *J. Biol. Chem.* 255:6633-6639.
- Varki, A. 1991. Radioactive tracer techniques in the sequencing of glycoprotein oligosaccharides. *FASEB J.* 5:226-235.
- Varki, A. and Kornfeld, S. 1982. The spectrum of anionic oligosaccharides released by endo- β -N-acetylglucosaminidase H from glycoproteins. *J. Biol. Chem.* 258:2808-2818.
- Yanagishita, M., Salustri, A., and Hascall, V.C. 1989. Specific activity of radiolabeled hexosamines in metabolic labeling experiments. *Methods Enzymol.* 179:435-445.
- Yurchenco, P.D., Ceccarini, C., and Atkinson, P.H. 1978. Labeling complex carbohydrates of animal cells with monosaccharides. *Methods Enzymol.* 50:175-204.