[2] Metabolic Radiolabeling of Glycoconjugates

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Introduction

If sufficient quantities of pure molecules are available, the complete and definitive structural analysis of glycoconjugates can be done with a combination of physical and chemical methods that are described elsewhere in this volume. However, isolating adequate quantities of a glycoconjugate may not be practical (e.g., in the analysis of biosynthetic intermediates, or of rare molecules). Alternatively, the biological question at hand might be adequately answered by partial structural analyses. In both these situations, metabolic labeling with radioactive sugars (or with other donors that label sugar chains) can be performed, and substantial structural information about the labeled oligosaccharide chains can be obtained.[1] The advantages of this approach include simplicity, ease of use, and the lack of need for sophisticated instrumentation (except for a scintillation counter). Furthermore, the purification of the glycoconjugate to "radiometric homogeneity" is sufficient.[2] The practical considerations in taking this approach include selection of the labeled precursor, understanding the specificity of labeling, and maximizing uptake and incorporation of the label. Most of the research using this approach has been carried out on animal cells, particularly mammalian cells. Thus, this chapter tends to focus more on metabolic labeling of glycoconjugates in such cells. It is assumed that the reader is familar with conventional protocols for handling, monitoring, shielding, and disposal of radioactive materials, and with the principles of tissue culture of cells and sterile handling of media. Following the choice of a specific radiolabeled precursor and the optimization of labeling conditions, preparative labeling is performed. The labeled glycoconjugate of interest is then isolated, and identification and separation of individual glycosylation sites may be necessary. Structural analyses can then be carried out on the labeled oligosaccharides.

Although metabolic labeling can provide much useful information regarding glycoprotein oligosaccharides, there are significant limitations in its use. First, it is often difficult to determine when true "equilibrium labeling" of a cell is reached (three to four doublings are usually assumed to be sufficient, see later). Thus, unless the specific activity of individual

[1] R. D. Cummings, R. K. Merkle, and N. L. Stults, *Methods Cell Biol.* **32**, 141 (1989).
[2] A. Varki, *FASEB J.* **5**, 226 (1991).

monosaccharide pools is carefully determined, the numerical ratio between various labeled glycoconjugates can be misleading. Second, individual precursors have greatly differing uptake and incorporation in different types of cells. Third, almost all available labeled precursors are only partially specific for certain monosaccharides. The degree of this specificity can be variable, depending on the cell type, and with prolonged labeling some radioactivity can even enter molecules other than oligosaccharides. Fourth, although monosaccharides that compete with glucose for uptake show improved incorporation when glucose concentrations are lowered, the low glucose supply can have direct effects on the oligosaccharide precursors in some cell types.

Background and General Principles

Sugar nucleotides are the immediate donors for glycosylation reactions. Because these negatively charged molecules cannot be taken up by cells in culture, metabolic labeling of the oligosaccharides is accomplished with radiolabeled monosaccharide precursors.[2-4] These commercially available molecules can be taken up by cells and activated to sugar nucleotides, which are directly utilized for cytosolic and nuclear forms of glycosylation[5] or transported into the Golgi apparatus,[6,7] where lumenally oriented transferases can add the monosaccharides to lumenally oriented acceptors (Fig. 1). Modifications of oligosaccharides such as sulfate esters, acetate esters, and phosphate esters can also be metabolically labeled with [^{35}S]sulfate, [^{3}H]acetate, or ^{32}Pi.

The labeling protocol to follow will depend on the objectives of the investigator. If labeling is being performed only to obtain labeled oligosaccharides for structural characterization, maximum yield may be the objective, regardless of the actual labeling protocol. If the objective is to establish precursor–product relationships, a pulse–chase protocol must be used. If there is a need to compare the masses of two molecules in the same sample, or to quantitate the mass of the same molecule in two different samples, it is necessary to label to constant radiospecific activity. As discussed later, this may not always be achievable.

[3] P. D. Yurchenco, C. Ceccarini, and P. H. Atkinson, *in* "Methods in Enzymology" (V. Ginsburg, ed.), Vol. 50, p. 175. Academic Press, New York, 1978.
[4] M. Yanagishita, A. Salustri, and V. C. Hascall, *in* "Methods in Enzymology" (V. Ginsburg, ed.), Vol. 179, p. 435. Academic Press, Orlando, FL, 1989.
[5] G. W. Hart, R. S. Haltiwanger, G. D. Holt, and W. G. Kelly, *Annu. Rev. Biochem.* 58, 841 (1989).
[6] C. B. Hirschberg and M. D. Snider, *Annu. Rev. Biochem.* 56, 63 (1987).
[7] B. Fleischer, *in* "Methods in Enzymology" (S. Fleischer and B. Fleischer, eds.), Vol. 174, p. 173. Academic Press, Orlando, FL, 1989.

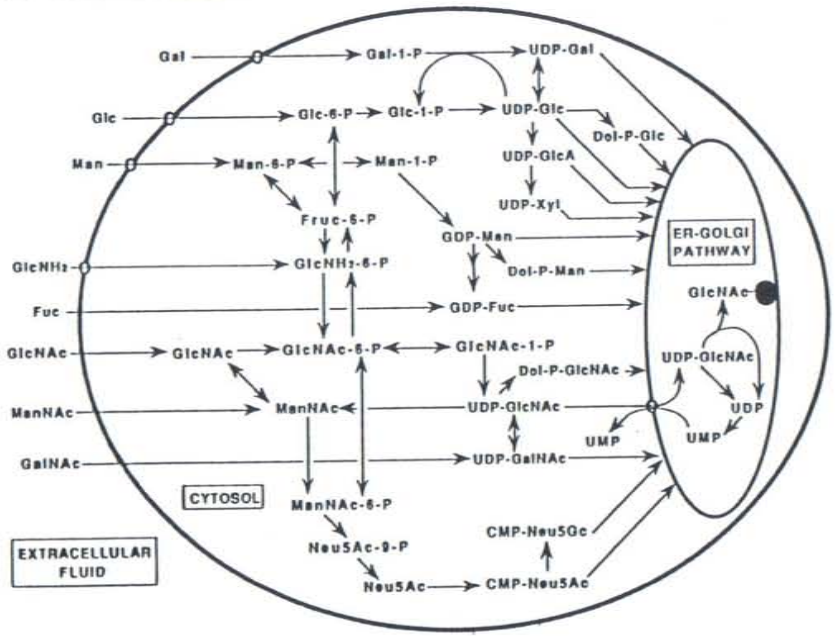

FIG. 1. Uptake of exogenously added monosaccharide precursors, and cytosolic pathways for their interconversion and activation to nucleotide sugar forms—a composite of the commonly known pathways. Individual pathways may be more or less prominent in different cell types. The monosaccharide pools can also be diluted by a contribution from degradation and recycling of endogenous glycoconjugates. Most of the sugar nucleotides and/or their dolicholphosphoryl derivatives are shown as being used for reactions occurring in the lumen of the endoplasmic reticulum (ER)–Golgi pathway. However, some can also be used for glycosylation reactions occurring in the cytosol (e.g., UDP-GlcNAc as a donor of O-linked GlcNAc residues). The transport and fate of one sugar nucleotide (UDP-GlcNAc) within the Golgi apparatus is shown.

Selection of a labeled monosaccharide precursor is based mainly on the efficiency of uptake (see later), and the type of glycoconjugate to be labeled (the distribution of monosaccharides among different types of vertebrate oligosaccharides is not random, as shown in Table I). The labeling can either be carried out for a long time (an attempt at reaching "equilibrium" distribution of the label), or for a short time (for a pulse–chase analysis). The type of monosaccharide decided on, and the period of labeling, will determine whether normal or reduced glucose in the medium should be used (see later). The type of monosaccharide precursor used and the position of the isotope can also affect specificity and final distribution of the label. The metabolic pathways for the biosynthesis, activation, utilization, and interconversion of various monosaccharides

TABLE I
MONOSACCHARIDES AND MODIFICATIONS: OCCURRENCE IN GLYCOCONJUGATES OF MAMMALIAN CELLS[a]

Mono-saccharide	Type of glycoconjugate					
	N-GlcNAc-linked glycoprotein	*O*-GalNAc-linked glycoprotein	*O*-Xyl-linked proteoglycan	Glyco-sphingolipid	GPI[b] anchor	*O*-Linl GlcN
Man	+ + +	–	–	–	+ +	–
Fuc	+ +	+ +	+/–	+	–	–
Gal	+ + +	+ + +	+ (Core)	+ + +	+/– (Side chain)	–
Glc	+ (Precursor)	–	–	+ (Core)	–	–
GlcNAc	+ + +	+ + +	+ +	+ +	+ (Free amine)	+ +
GalNAc	+/–	+ + +	+ +	+ +	+/– (Side chain)	–
Sia	+ + +	+ + +	–	+ + +	+ (Side chain)	–
GlcA	+/–	–	+ + +	+	–	–
SO_4	+	+	+ + + +	+	–	–
Pi esters	Man-6-P	–	Xyl-P	–	M-6-P in core	–
O-Acetyl	Sia-OAc	Sia-OAc	–	Sia-OAc		–
O-Acyl	–	–	–	+ + + +	+/– (On inositol)	–

[a] The relative distribution of the different monosaccharides and modifications in the most commo occurring glycoconjugates are indicated by + to + + +, and is valid for many cell types. Howev an uncommon monosaccharide or modification may be commonly found in a given cell type type of glycoconjugate. Also, some of the negative listings may well turn out to be incorrect the future.

[b] GPI, Glycosylphosphatidylinositol.

and their nucleotide sugars have been studied extensively in the past (see Fig. 1 for the common pathways in animal cells, and the points at which exogenously added sugars can enter the endogenous pathways). The final distribution and specific activity of label can be significantly affected by dilution from endogenous pathways, the conditions of labeling, and the particular cell type under study.[3,4,8,9] The interconversion between monosaccharides (see Fig. 1 and Table II) includes several epimerization reactions that can result in loss of label from a precursor. Thus, the position of the label within the monosaccharide used can affect the ultimate fate

[8] A. Varki and S. Kornfeld, *J. Biol. Chem.* **258**, 2808 (1983).
[9] J. J. Kim and E. H. Conrad, *J. Biol. Chem.* **251**, 6210 (1976).

TABLE II
COMMONLY USED RADIOLABELED PRECURSORS: INCORPORATION INTO MONOSACCHARIDES OF MAMMALIAN CELLS[a]

Precursor	Entry of label into:								
	[^{3}H]Man	[^{3}H]Fuc	[^{3}H]Gal	[^{3}H]Glc	[^{3}H]GlcA	[^{3}H]GlcNAc	[^{3}H]GalNAc	[^{3}H]Sia
[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	+ / −	+ / −	+ / −	+ / −	+ / −	+ +	+ −	+

[a] Extent of conversion from the original monosaccharide into minor pathways will vary considerably, depending on the cell type studied, and the length of the labeling (longer time allows more conversion into other monosaccharides).

of the radioactivity. For example, label originating from the 6-position of [6-^{3}H]GlcNH$_2$, [6-^{3}H]GlcNAc, or [6-^{3}H]ManNAc is eventually found at either the 6-position of GlcNAc or GalNAc residues or at the 9-position of terminal sialic residues.[10] However, eventual interconversion with glucose can be expected for most labeled monosaccharides. Thus, the label can spread into other monosaccharides and even into the nonoligosaccharide components of the cell. An exception is the extreme specificity of labeling obtained with [2-^{3}H]mannose, which should remain confined to mannose and fucose residues, regardless of how long the labeling is done. However, exceptions to this specificity have been reported.[11,12]

Many other factors can affect the uptake and incorporation of radioactive precursors into oligosaccharides. With radioactive amino acids, high specific activity labeling can be obtained simply by omitting the unlabeled molecule from the medium. Labeling with radioactive sugars is usually not as efficient, making it necessary to optimize incorporation into the glycoconjugate of interest. Many factors can affect the uptake and incorporation of radioactive monosaccharide precursors into macromolecules: The general factors are relatively self-evident, and include the amount of label, the concentration of label in the media, the number of cells, the duration of labeling, and the number of cell doublings that occur during the labeling. Some of these factors are obviously at odds with one another, and the correct balance between them must be individualized to the particular cell type and the experimental question at hand.

Whether a radioactive monosaccharide precursor competes with glucose for uptake is also an important factor affecting uptake and incorporation. Because the concentration of glucose in typical tissue culture media is about 5 m*M*, those monosaccharides that compete for active transport cannot be taken up any better than glucose itself. In these cases, lowering the concentration of glucose in the medium can improve the uptake of such precursors. The sugars that do not compete with glucose seem to be taken up only by inefficient mechanisms that are noncompetitive and passive. In these cases, manipulation of the glucose concentration has little effect on their relatively poor uptake. A comprehensive study of these issues has not been performed. Past published and unpublished experience indicates that glucosamine, galactosamine, galactose, and mannose compete with glucose for uptake into most cells, whereas *N*-acetylglucosamine, *N*-acetylmannosamine, mannosamine, fucose, and xylose do not.[3] However, it is now recognized that the glucose transporters

[10] S. Diaz and A. Varki, *Anal. Biochem.* **150**, 32 (1985).
[11] K. E. Creek, S. Shankar, and L. M. De Luca, *Arch. Biochem. Biophys.* **254**, 482 (1987).
[12] I. R. Rodriguez and S. J. Fliesler, *Exp. Eye Res.* **51**, 71 (1990).

are actually a family of gene products that are tissue specific in their expression.[13] Thus, a given cell may express more than one of these transporters, each of which may have distinctive kinetic and stereospecific uptake properties. Monosaccharides should be tested with the specific cell type under study, to determine which ones compete with glucose. When glucose-free medium is used in some cell types, synthesis of the lipid-linked oligosaccharide precursor of N-linked oligosaccharides can become altered.[14]

Other factors can affect the final specific activity and distribution of the cellular label, including the pool size of individual monosaccharides and nucleotides, the dilution of the label by endogenously synthesized monosaccharides, and the flux rates between interconverting pathways, as mentioned above. Also, when certain cell types are cultured in labeled medium for what appear to be reasonable periods of time, the glucose level in the medium can fall substantially.[2,8,9] In this case, the specific activity of labeled sugar nucleotides can actually rise during the latter part of the labeling, as the medium glucose concentration falls below the K_m of the glucose transporter(s) in the particular cell type. Although all of the factors discussed above must be considered in designing the final labeling protocol, some may not be easy to control. The ultimate goal is to obtain a sufficient amount of radioactivity in the glycoprotein of interest to permit the studies planned. However, this must be done without substantially altering the metabolic state of the cell.

Modifications of oligosaccharide chains such as acetylation, sulfation, acylation, and phosphorylation can have significant effects on the behavior of oligosaccharides in biological systems and during analysis. In these cases, the oligosaccharides can also be metabolically labeled with appropriate precursors such as [^{3}H]acetate, [^{35}S]sulfate, [^{3}H]palmitate, and ^{32}Pi. Naturally, such precursors are expected to enter into a wide variety of other cellular macromolecules, and the release and isolation of the labeled oligosaccharides is required before further analysis. In obtaining release, specific endoglycosidases are preferred, because chemical methods such as hydrazinolysis and alkaline β elimination can partially or completely destroy the modifications of interest. In many cases, double-labeling of the modification and the underlying sugar chain (e.g., with a ^{14}C-labeled monosaccharide precursor and ^{3}H-labeled acetate) can be useful in monitoring the purification and in carrying out the subsequent structural analysis.[15]

[13] G. W. Gould and G. I. Bell, *Trends Biochem. Sci.* **15**, 18 (1990).
[14] J. I. Rearick, A. Chapman, and S. Kornfeld, *J. Biol. Chem.* **256**, 6255 (1981).
[15] A. E. Manzi, E. R. Sjöberg, S. Diaz, and A. Varki, *J. Biol. Chem.* **265**, 13091 (1990).

Sulfate and phosphate ions are components of normal tissue culture media. Although their selective removal can improve labeling with the respective precursor, the biological effects of depletion of these ions can also occur. In the case of labeling with [^{35}S]sulfate, the efficiency of labeling varies widely with different cell types. In some cells, the endogenous pool of the sulfate donor 3′ phosphoadenosine 5′ phosphosulfate (PAPS) appears to be small, and the specific activity of the [^{35}S]sulfate is practically the same inside the cell as in the exogenously added label.[4] However, in other cell types, the endogenous sulfate pool is constantly diluted by the endogenous breakdown of the sulfur-containing amino acids methionine and cysteine.[16,17] In the latter situation, altering the cysteine and methionine concentrations in the medium can improve the incorporation of label into macromolecules.[16] Certain antibiotics, for example, gentamicin, are provided as sulfate salts, which can contribute significantly to the final sulfate concentration of the medium. Likewise, undialyzed serum contains sulfate ions.

In many cases, the precise specific activity of each monosaccharide pool does not need to be determined to allow interpretation of the experiment. If "equilibrium" labeling (see the next section) is attempted, a plateau in the rate of incorporation of label per milligram of cell protein can be taken as an approximate indication that a steady state has been reached. However, in some cases, the precise specific activity of a given monosaccharide may be of interest. Detailed discussions of these matters can be found elsewhere,[2–4,8,9] but certain points are worth noting. Endogenous glucose is the normal precursor for hexoses and hexosamines (see Fig. 1). Experimental manipulations that alter the concentration of glucose can thus alter the concentration of the internal pool of other hexoses and hexosamines. Also, cells frequently recycle their monosaccharides after degradation in the lysosomes. Thus, exogenously added labeled hexosamines become diluted in the cell, making specific radioactivity in the cell generally lower than that of the starting material. Yanagishita *et al.*[4] describe in detail a protocol to determine the specific activity of hexosamines in metabolically radiolabeled molecules. This approach takes advantage of the fact that both [^{3}H]glucosamine and [^{35}S]sulfate are incorporated into chondroitin sulfate and that defined disaccharides from this sugar chain can be isolated. It assumes that the [^{35}S]sulfate label is not significantly diluted from cellular sources (however, see discussion above). With the advent of high-pH anion-exchange chromatography and pulsed

[16] L. Roux, S. Holoyda, G. Sundblad, H. H. Freeze, and A. Varki, *J. Biol. Chem.* **236**, 8879 (1988).
[17] J. D. Esko, A. Elgavish, T. Prasthofer, W. H. Taylor, and J. L. Weinke, *J. Biol. Chem.* **261**, 15725 (1986).

amperometric detection (HPAE-PAD) analysis of monosaccharides it is now possible to measure monosaccharides accurately in the low picomole range (see [12] in this volume). The specific activity of a labeled monosaccharide component of a purified oligosaccharide can be obtained by acid hydrolysis of a portion of the sample, followed by HPAE-PAD analysis. Other high-performance liquid chromatography (HPLC)-based methods for the accurate measurement of low quantities of sulfate and sialic acids are also now available.

Ideally, the radiochemical purity of the precursor to be used should be checked before starting the experiment. The radioactive precursor may have become chemically degraded during storage, and even established commercial sources have been known to provide precursors contaminated by unknown compounds.

"Equilibrium" Labeling of Cells with Radioactive Precursors

When studying glycoconjugates in established tissue culture cell lines, it is often desirable to label the molecules to allow structural characterization. Whenever feasible, such molecules should be labeled as close as possible to "equilibrium," under conditions of normal growth of the cells. True equilibrium labeling is defined as reaching a constant level of radioactivity per mass unit of a given monosaccharide in all the glycoconjugates in the cell. In practice, this is difficult to achieve with most cell types because some macromolecules are more long lived than others. Regardless, the goal is to expose the maximum number of cells possible to the maximum amount of label feasible in the minimum volume possible for the longest possible period of time, in complete growth medium. Before labeling is attempted, the following growth characteristics should be determined for the cell type of interest:

1. doubling time under standard conditions of growth
2. maximum degree of dilution on splitting that is compatible with proper regrowth of cells
3. maximum cell density compatible with healthy growth and metabolism ("confluence")
4. minimum volume of medium in which cells can sustain full regrowth of cells from a full split [see point (2)] to the maximum density [see point (3)]

If these parameters are properly defined, growth from point (2) to point (3) should permit several doublings (at least three) for most cell lines. There may be cases (e.g., slowly growing cell lines, lines requiring frequent

medium changes, or cells that cannot be split far back) in which this might not be entirely feasible.

Materials

Complete tissue culture medium (with supplements) suitable for long-term growth of the cell line
Sterile tissue culture supplies (e.g., sterile pipettes, 15-ml conical tubes with caps, flasks, or dishes)
Disposable 0.22-μm pore size filters suitable for sterile filtration of media, with Luer-lock fittings
Sterile plastic syringes with Luer-lock fittings; alternatively, flasks fitted with 0.22-μm pore size filters for sterile filtration of media
Tissue culture hood
Humidified incubator, equilibrated at 37° and 5% CO_2 (or whatever conditions are appropriate)
Radioactive precursor (e.g., ^{3}H- or ^{14}C-labeled monosaccharide, [^{3}H]acetate, or [^{35}S]sulfate)
Separate containers for liquid and solid radioactive waste
Phosphate-buffered saline (PBS), pH 7.2, chilled on ice

Labeling Procedure

1. Dry the radioactive precursor if necessary [e.g., to remove organic solvents such as 70% (v/v) ethanol that are used for storage], add the label to a small volume of complete medium, and filter sterilize. If the final volume of medium is 10 ml or greater, it is practical to use a disposable 50-ml flask fitted with a 0.22-μm pore size filter and vacuum suction device. Transfer the label with ~5 ml of medium onto the filter, wash the container with the remaining volume, and pass the wash through the same filter. Smaller volumes of media can be sterilized with a 0.22-μm pore size filter fitted directly onto the tip of a disposable sterile syringe. Remove the plunger from the syringe, maintain its sterility (e.g., by keeping it upright on a low-profile rack), screw the filter onto the barrel of the syringe (save the filter wrapping), and transfer the labeled medium directly into the barrel of the syringe. The plunger can then be placed back in the barrel, and the fluid and some air pushed through the filter into an appropriate sterile receptacle. Cover the filter with its original packaging (still sterile), and partly unscrew the filter from the barrel of the syringe. Carefully remove the plunger of the syringe, retighten the filter to syringe, wash the original container with an additional volume of medium, and pass this through the same filter. Dispose of the filter and/or syringe in the

appropriate container for radioactive waste. Add additional medium if needed to bring the volume up to the amount planned for the experiment.

As an alternative, the precursor may be available for purchase in a sterile aqueous medium, ready for use. Such preparations tend to be more expensive, and after repeated opening of the packages, filtering may be necessary in any case, to ensure sterility.

2. Place the sterile labeling medium in the incubator or water bath to warm to labeling temperature. Placing it in the incubator with a loose cap has the advantage that equilibration with CO_2 can begin immediately.

3. Prepare the cells for splitting by whatever standard protocol is appropriate for the cell type (e.g., trypsin for monolayer cultures, direct dilution or concentration of cells by centrifugation for suspension cultures). The number of cells needed will be determined by the growth characteristics of the particular cell of interest.

4. With a sterile pipette tip, remove a small aliquot of the radioactive medium for counting (it may be necessary to dilute it to obtain an accurate count). Mix the radioactive medium and cells, and incubate under standard conditions for the cell line.

5. At the time of maximum cell growth ("confluence"), chill the cells and medium on ice and harvest (a rubber policeman is necessary for scraping monolayer cultures). Pellet the cells out of the medium with a table-top centrifuge at 4°. Remove the medium and count an aliquot. To study the glycoconjugates in the medium, the medium should preferably be refiltered immediately, to eliminate any broken cell debris that was not removed with the centrifugation. The medium may contain labeled macromolecules synthesized by the cells. In some cases, the cells may have taken up the label, processed it into other low molecular weight products, and excreted these into the medium. Checking the spent medium for labeled macromolecules and for intactness of the original precursor may be worthwhile.

6. Wash the cells twice in a >50-fold excess volume of ice-cold PBS, pH 7.2. Discard the washings appropriately. Process the labeled cell pellet immediately or store it frozen for future analysis.

7. Process the cell pellet in a manner appropriate for extracting the glycoconjugates of interest (e.g., extraction into detergent). Determine the efficiency of label incorporation by measuring the amount of radioactivity in cells compared to that left in the medium.

Pulse Labeling with Radioactive Precursors

In some situations, it is desirable to label the glycoconjugate of interest briefly, for a pulse–chase study, usually meant to establish precursor–product relationships. This approach may also be useful if the equilibrium labeling described does not give sufficient incorporation of label into the glycoconjugate of interest. In both cases, a pulse-labeling protocol can be tried. This approach is most useful when using monosaccharides that compete with glucose for uptake (see discussion earlier). It is of limited value for monosaccharides that are not taken up efficiently by the cells (unless large quantities of such labeled molecules can be used).

Materials

Many of the same materials used for equilibrium labeling can be used here.

Dialyzed fetal calf serum (FCS)
Complete unmodified medium (if a chase is to be performed)
Multiply deficient medium (MDM) (see below and Table III), or appropriate commercial substitute
Stock solutions of the compounds missing from MDM (see Table IV)

Preparation of Multiply Deficient Medium

Different types of labeling require different types of selectively deficient media. Some deficient media are available commercially at reasonable prices, and some companies will custom prepare selectively deficient media on request. For a laboratory that frequently does experiments using a variety of different deficient media, it is convenient to prepare a "multiply deficient medium" (MDM), which is completely lacking in many commonly studied components. This stock medium can be used to make up different kinds of selectively deficient media as needed. The MDM formula presented in Table III is based on α-MEM medium, which supports the growth of most kinds of tissue culture cells. It can be reconstituted for labeling with ^{3}H- or ^{14}C-labeled monosaccharides, [^{35}S]sulfate, ^{35}S-labeled methionine or cysteine, or [^{3}H]serine.

All reagents used for the preparation of this medium should be of tissue culture grade. Care should be taken to either "pour out" or use disposable spatulas to transfer reagents out of bottles. This will ensure that reagents are not contaminated by ubiquitous compounds, such as endotoxin, that may cause aberrant cell behavior or cell death. For the same reason, care must also be exercised to use clean, autoclaved glassware. Preferably, a set of glassware should be put aside just for this purpose. The components to be added are listed in Table III. The 100× stocks of many of these can be purchased commercially, in sterile form. If not, each should be made up and filter sterilized separately. The salts and phenol red are first dis-

TABLE III
COMPONENTS OF MULTIPLY DEFICIENT MEDIUM

Component	Final concentration (mg/liter)	Stock
$CaCl_2$	200	Shelf, reagent grade
KCl	400	Shelf, reagent grade
$MgCl_2$	75	Shelf, reagent grade
NaCl	6800	Shelf, reagent grade
$NaH_2PO_4 \cdot H_2O$	140	Shelf, reagent grade
Phenol red	10	Solution
Sodium pyruvate	110	100×
L-Alanine	25	100×
L-Arginine	126	100×
L-Asparagine	50	100×
L-Aspartic acid	30	100×
L-Cysteine	(100 or none)[a]	100×
L-Glutamic acid	75	100×
L-Glutamine	None	100×
L-Glycine	50	100×
L-Histidine	42	100×
L-Isoleucine	52	100×
L-Leucine	52	100×
L-Lysine	72	100×
L-Methionine	(15 or none)[a]	100×
L-Phenylalanine	32	100×
L-Proline	40	100×
L-Serine	(25 or none)[a]	100×
L-Threonine	48	100×
L-Tryptophan	10	100×
L-Tyrosine	36	100×
L-Valine	46	100×
Vitamins	1×	100×

[a] Components left out; add back as needed for specific experiments.

solved one by one in ~800 ml of tissue culture-grade water. Pyruvate, amino acids, and vitamins are then added sufficient for a 1× final concentration in 1000 ml. The medium is then made up to 1000 ml with water and filter sterilized. The solution should be yellow and the pH should not be adjusted. Aliquots of 25 ml are placed in sterile 50-ml tubes and stored at −20°.

Checking for Optimal Labeling Conditions

1. Reconstitute MDM medium to 100% levels with all components except the one being presented as a radioactive precursor. For radioactive monosaccharides, initially leave out the glucose (see discussion for rationale). Add dialyzed serum at the required final concentration.

TABLE IV
COMPONENTS REQUIRED FOR RECONSTITUTION OF MULTIPLY DEFICIENT MEDIUM

Component[a]	Final concentration (×) (mg/liter)	Stock
$NaHCO_3$ or	1× (2200 mg/liter)	100×
HEPES-HCl[b]	20 m*M*	2 *M*, pH 7.3
Na_2SO_4	0.81 m*M*	100 m*M*, 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×

[a] Individual components are added back at full strength, at lowered concentration, or left out altogether, depending on the experiment planned.
[b] The pH of the final medium is controlled by bicarbonate/CO_2 or by HEPES-HCl.

2. Make a stock of the deficient medium containing the radioactive precursor, divide into aliquots, and add back the missing component in increasing concentrations from a 100× stock solution (e.g., 0, 5, 10, 20, 50, and 100% of the concentration in normal medium). For monosaccharides that compete with glucose, the missing component added back is glucose. Use these aliquots to do small-scale pilot labelings with the cells of interest, for fixed or varying periods of time. Because pulse labelings are usually done for a short time (e.g., minutes or hours), the cells are usually used in a near-confluent state, to maximize uptake and incorporation. Determine the efficiency of incorporation of radioactivity into whole-cell glycoconjugates, and if necessary into the specific macromolecules of interest. Plot the percentage incorporation versus total concentration of precursor (radioactive plus unlabeled). Determine at what point the curve breaks (i.e., the percentage of label incorporated is markedly decreased by further addition of unlabeled compound).

3. On the basis of these pilot labelings, choose the optimal concentration of unlabeled precursor to be used, that is, just above the breakpoint in the curve, where unlabeled precursor is not limiting, but where label incorporation is still good. Using these conditions, check for linearity of uptake and incorporation of label over time.

to obtain high incorporation, pool all the pellets. The combined pellets represent molecules synthesized over the entire period of time. Alternatively, each plate can be processed as soon as the labeling medium is removed, and the pellet stored frozen until all the pellets can be processed together.