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Methodological considerations for studies of brain glycogen

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Abstract

Glycogen stores in brain have been recognized for decades, but the underlying physiological function of this energy reserve remains elusive. This uncertainty stems in part from several technical challenges inherent in the study of brain glycogen metabolism. These include low glycogen content in brain, non-homogeneous labeling of glycogen by radiotracers, rapid glycogenolysis during postmortem tissue handling, and effects of the stress response on brain glycogen turnover. Here we briefly review the aspects of glycogen structure and metabolism that bear on these technical challenges and present ways they can be addressed.

Keywords

Energy metabolism; Astrocytes; Glycogen

Introduction

Glycogen is a large, branched polymer of glucose. The polymer form eliminates the high osmolarity that would result from an equimolar concentration of free glucose. It also protects the otherwise free “reducing” ends of glucose from auto-oxidation (Figure 1), and provides a means of intracellular localization of the energy store. Glycogen polymers are roughly spherical and organized into concentric tiers, with the inner tiers composed by chains that normally contain two branches, and the outer tiers composed of unbranched chains (Gunja-Smith, Marshall, Mercier, Smith, & Whelan, 1970). The linear chains of glucose residues are joined by α−1,4-glycosidic bonds, with branch points at approximately every 10 – 14 glucose residues linked by α−1,6-glycosidic bonds (Calder, 1991). The exposed ends of all glycogen chains are non-reducing. Mathematical modeling suggests that the size of a glycogen molecule is limited to 12 tiers, which means a total of approximately 53,000 glucose residues and a diameter of 42 nm. In accordance with this notion, the glycogen particles found in the mouse brain and resting human skeletal muscle ranged from

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Author Contributions

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Conceptualization, R.A.S; Formal Analysis, L.W.; Writing – Original Draft, R.A.S. and L.W.; Writing – Review & Editing, R.A.S.; Visualization, C.P.W.; Supervision, R.A.S.; Funding Acquisition, R.A.S.

Conflict of Interest Statement

The authors declare no conflicts of interest.

10 to 44 nm in diameter (Cataldo & Broadwell, 1986; Marchand et al., 2002; Wender et al., 2000). It should be noted that the average diameter of these glycogen particles is 20 – 30 nm, which is estimated to be 7 – 8 tiers, suggesting that the average glycogen molecule is not fully synthesized (Goldsmith, Sprang, & Fletterick, 1982; Melendez-Hevia, Waddell, & Shelton, 1993).

Glycogen polymers (“granules”) are present in the cytosol, endoplasmic reticulum, and lysosomes (Cardell, 1977; Geddes, Jeyarathan, & Taylor, 1992; Stapleton et al., 2010). In liver cells, approximately 10% of all glycogen particles are found in lysosomes (Jiang et al., 2010), where they undergo slow degradation by acid maltase.

Almost all tissues contain some glycogen, but the relative amounts of glycogen varies considerably. Glycogen levels found in adult rat under physiological conditions are as follows: liver >> skeletal muscle > cardiac muscle > brain > kidney (Table 1). Glycogen in mammalian brain is localized primarily to astrocytes, but much smaller amounts are also found in meningeal cells, endothelial cells, and other cell types (Cali et al., 2016; Cataldo & Broadwell, 1986; Koizumi, 1974; Wender et al., 2000). Neurons contain appreciable amounts of glycogen during development, but this falls to very low levels in the mature brain except in certain brainstem neurons (Borke & Nau, 1984; Cataldo & Broadwell, 1986; Cavalcante, Barradas, & Vieira, 1996; Ibrahim, 1975; Koizumi, 1974; Oe, Baba, Ashida, Nakamura, & Hirase, 2016; Saez et al., 2014).

Glycogen metabolism

The synthesis of a *de novo* glycogen granule is thought to be initiated by glycogenin. Glycogenin glycosylates itself at a tyrosine residue and catalyzes the extension of glycan chains (Cao, Mahrenholz, DePaoli-Roach, & Roach, 1993; Smythe & Cohen, 1991) although recent studies with glycogenin deficient mice indicate other mechanisms are likely possible (Testoni et al., 2017). The glycan chains then serve as primer for glycogen synthetase, which catalyzes the formation of α−1, 4-glycosidic linkages of glycogen. Glycogen synthesis requires uridine diphosphate glucose (UDP-glucose) as a substrate, which is produced by the reaction of uridine triphosphate and glucose 1-phosphate catalyzed by UDP-glucose pyrophosphorylase. α−1,6-glycosidic branch points are subsequently produced by glycogen branching enzyme (1,4-alpha-glucan-branching enzyme) at approximately every 10–12 glucose residues.

Glycogenolysis is mediated by glycogen phosphorylase (GP), which hydrolyzes glucose residues at α−1, 4 linkage points to generate glucose 1-phosphate. Glycogen debranching enzyme linearizes glycogen chains near the α−1, 6 branch points to provide linear substrate for glycogen phosphorylase (Nakayama, Yamamoto, & Tabata, 2001). GP is thought to be the rate limiting enzyme in glycogen breakdown. GP exist in three isoforms: liver isoform, muscle isoform and brain isoform, each termed according to the tissue where it is predominately expressed (David & Crerar, 1986). Immunohistochemical analyses showed that both isozymes were expressed in the astrocytes throughout the brain. Certain neurons in the somatosensory pathways express brain isoform (Ignacio, Baldwin, Vijayan, Tait, & Gorin, 1990; Pfeiffer-Guglielmi, Fleckenstein, Jung, & Hamprecht, 2003). The activity of

GP is directly regulated by changes in energy state through allosteric of actions of AMP, which accelerates activity and by ATP and glucose-6-phosphate, which slow enzymatic activity. Glycogen phosphorylase activity is also regulated by its phosphorylation state, through the action of glycogen phosphorylase kinase (PhK). PhK is in turn regulated by a variety of signaling pathways through phosphorylation and allosteric interactions. For example, PhK is activated by protein kinase A in response to increased cAMP concentrations induced by hormones such as epinephrine. Additionally, PhK can be partly activated by elevated levels of Ca^{2+} via binding to its calmodulin subunit. These regulatory actions provide a mechanism for "anticipatory" glycogen mobilization to prevent any actual decline of cellular energy state. The relative importance of the allosteric and covalent regulatory mechanisms differ in different GP isoforms. For instance, *in vitro* studies of muscle and brain isoforms of GP indicated that the muscle-type GP is more potently activated by phosphorylation than by elevated levels of AMP, whereas brain-type GP is poorly activated by phosphorylation but highly sensitive to AMP (Crerar, Karlsson, Fletterick, & Hwang, 1995). Accordingly, astrocytes lacking the muscle isoform of GP show a delay in norepinephrine-induced glycogen degradation (Muller, Pedersen, Walls, Waagepetersen, & Bak, 2015). In contrast, astrocytes deficient in brain-type GP, but not muscle-type GP, show delayed glycogenolysis in response to glucose deprivation (Muller et al., 2015).

Glucose residues liberated by GP are in the form of glucose-1-phosphate, which is freely converted to glucose-6 phosphate (Figure 2). UTP is consumed at the glucose UDP pryophosphorylation step of glycogen synthesis such that the shuttling of each glucose moiety on and off glycogen requires one ATP equivalent.

Brain-specific aspects of glycogen

Although neurons are thought to be the primary energy consuming cells in brain, astrocytes contain the vast majority of brain glycogen. Electron microscopy identified glycogen granules throughout astrocyte cell bodies and processes, particularly near axonal boutons and dendritic spines (Cali et al., 2016). Glutamate uptake is an energy-intensive astrocyte function, and interestingly, glycogen phosphorylase has been found to be associated with the astrocyte glutamate transporter, GLT-1 (Genda et al., 2011). Astrocyte glycogen is quickly degraded under conditions of energy failure (Lowry, Passonneau, Hasselberger, & Schulz, 1964; Suh et al., 2007; Swanson, Sagar, & Sharp, 1989), as would be expected. Glycogen metabolism in astrocytes is also regulated by several neurotransmitters and signaling molecules, including vasoactive intestinal peptide (VIP), noradrenaline, arachidonic acid, glutamate, cAMP, and K^+ (Cambray-Deakin, Pearce, Morrow, & Murphy, 1988a, 1988b; Cummins, Lust, & Passonneau, 1983; Magistretti, 1988; Sorg, Pellerin, Stolz, Beggah, & Magistretti, 1995; Subbarao & Hertz, 1990; Subbarao, Stolzenburg, & Hertz, 1995; Walls, Heimburger, Bouman, Schousboe, & Waagepetersen, 2009). These signaling molecules serve to couple astrocyte glycogen metabolism to neuronal activity. For VIP and noradrenaline in particular, the anatomical organization of these inputs provides a framework for coordinated signaling. The narrow radial pattern of arborization of intracortical VIP neuron and the tangential intracortical trajectory of the noradrenergic fibers suggests that these two systems may function in a complementary fashion: VIP regulating

energy metabolism locally, within individual columnar modules, and norepinephrine exerting a more global effect (Magistretti, Morrison, Shoemaker, Sapin, & Bloom, 1981).

Autoradiographic and biochemical measures of glycogen turnover show it to be increased by sensory neuron stimulation in the awake rat (Dienel, Ball, & Cruz, 2007; Swanson, Morton, Sagar, & Sharp, 1992). Conversely, conditions causing focally or globally reduced neuronal activity lead to corresponding local or global increases in glycogen content, suggesting reduced glycogen utilization (Swanson, 1992). These conditions include focal brain injury, anesthetics, slow wave sleep, and hibernation (S. R. Nelson, Schulz, Passonneau, & Lowry, 1968; Phelps, 1972; Pudenz, Bullara, Jacques, & Hambrecht, 1975; Swanson, 1992; Watanabe & Passonneau, 1973).

Experimental and technical considerations in the study of brain glycogen

Low glycogen content in brain

Several aspects of glycogen structure and regulation in brain pose unique challenges for experimental observation. First among these is the relatively low concentration of glycogen in brain. Many of the classical histochemical methods for detecting glycogen were developed using liver or muscle tissues, in which glycogen content is orders of magnitude higher than in brain. For example, the periodic acid schiff (PAS) method of staining polysaccharides works well in liver and muscle, but in brain, the low glycogen content and relatively higher content of glycoproteins and glycolipids, which also react with PAS, makes this approach less useful, even when coupled with dimedone blocking of aldehydes (Cammermeyer & Fenton, 1981). Immunohistochemical methods using antibodies to glycogen provide far better sensitivity (Oe et al., 2016). For all histochemical methods, specificity can be confirmed by showing disappearance of signal in tissue treated with amylase or in tissues rendered ischemic for a few minutes prior to fixation. Low glycogen levels similarly pose a challenge for magnetic resonance studies of glycogen, as recently reviewed (Soares, Gruetter, & Lei, 2017).

For biochemical detection, the low glycogen levels in brain require assays with high adequate sensitivity. The fluorometic amyloglucosidase method of Passonneau et al. (Passonneau & Lauderdale, 1974), has been validated against other approaches and remains widely used for measuring glycogen content in brain and cultured astrocytes. This method does not, however, have the sensitivity to detect the very low glycogen in cultured neurons. For that purpose, an alternative method has been employed in which glucose liberated by amyloglucosidase is enzymatically converted to glucose-6-phosphate, which in turn is measured by an enzymatic NADPH recycling method (Saez et al., 2014; Zhu, Romero, & Petty, 2009).

Regardless of the analytical technique, it is necessary to ensure the results obtained are not influenced by glucose in the tissue preparation. For the Passonneau method, this entails parallel analysis of all samples with and without amyloglucosidase. Assays containing amyloglucosidase provide a measure of both glucose and glycogen, whereas omission of amyloglucosidase yields a measure of glucose alone. Glycogen values can then be calculated after subtracting signal attributable to glucose. However, this method becomes less precise

as the ratio of glucose to glycogen increases. An alternative approach is to remove free glucose by washes in 70% (or greater) ethanol or propanol. The principle behind this approach is that glucose but not glycogen is soluble in these solutions (Farquharson, Jamieson, MacPhee, & Logan, 1990); however, this is not absolute, and some low-molecular weight species of glycogen may not be detected by this approach.

It is often useful to be able to express glycogen measurements in terms of glucose molecule equivalents. The molecular weight of glucose is 180.16 g/ mol; however, the molecular weight of each glucose moiety in glycogen is somewhat less, because the glycosidic linkages between the glucose moieties in glycogen each subtract the equivalent of one H_2O from their molecular weight. There is one α−1,4 glycosidic bond between each glucose moiety in the linear chains, and there is in addition one α−1,6 glycosidic bond at each branch point, which occurs approximately every tenth glucose residue. The calculated molecular weight for glycogen can thus be estimated to be 160.3 g/ mol glucose equivalent. The true value will vary slightly depending upon the degree of polymer branching.

Use of radiotracers to assess glycogen turnover

A challenge common to many studies of metabolism is that there is no fixed relationship between metabolic flux and measured concentration levels. Glycogen turnover in particular — i.e. the breakdown and re-synthesis of individual glycogen molecules — may accelerate, decelerate, or stop altogether with little or no change in net glycogen content over time. Conversely, glycogen content may change (or not) with changes in either synthesis rate, breakdown rate, or combinations of these. Radiolabeled or isotope enriched substrates are therefore widely used for assessments of metabolic rates. However, accurate quantification by these approaches requires that the labeled molecules be homogenously distributed among the unlabeled molecules. This is difficult to achieve in glycogen because individual glucose moieties are sequentially added to and removed from the outer glycogen tiers, and have widely variable dwell time in the glycogen polymer (Elsner, Quistorff, Hansen, & Grunnet, 2002; Youn & Bergman, 1987). The relationships between rates of glycogenolysis and label release therefore depend upon the patterns of glycogen synthesis and breakdown at the level of individual glycogen granules (as illustrated in Figure 3). These limitations do not render assessments of glycogen turnover impossible, but they do limit the precision of these measures and often require certain assumptions. For example, very prolonged administration of tracers can be used to improve homogeneity of glycogen labeling, and this can be further refined by modeling patterns of glycogen turnover (DiNuzzo, 2013; Oz, DiNuzzo, Kumar, Moheet, & Seaquist, 2015; Soares et al., 2017).

The use of glucose as a tracer also poses a problem in that glucose metabolism rapidly distributes the label to amino acids, lipids and many other cell constituents other than glycogen. This problem can be surmounted in part either by physical isolation of glycogen prior to isotope analysis, or by the use of 2-deoxyglucose, which is incorporated into and released from glycogen but is much more slowly metabolized to other molecules (Kai Kai & Pentreath, 1981; T. Nelson, Kaufman, & Sokoloff, 1984). Glucose labeled at the 3 or 4 carbon positions also mitigates this problem (Swanson et al., 1992), because glucose entering the tricarboxylic acid cycle (from which most other metabolites are formed) loses

the 3 and 4 carbons to CO_2 in the pyruvate carboxylase reaction. *In vivo* magnetic resonance spectroscopy studies can in some cases use native ^{13}C abundance to assess brain glycogen concentrations, but the low abundance of ^{13}C coupled with low concentrations of glycogen in brain currently limit the temporal and spatial resolution of this approach (Soares et al., 2017).

Rapid post-mortem glycogenolysis

Glycogenolysis is triggered by very small elevations in AMP, befitting its role as an emergency energy store. This process begins almost instantly with cessation of blood flow, and continues until the brain is frozen, acidified, or otherwise treated to inactivate glycogen phosphorylase. (For frozen brains, the process resumes upon thawing unless other measures are also taken.) This presents a major challenge for biochemical glycogen measurements, and even more so for histochemical or electron microscopic studies. Even when these methods succeed in detecting glycogen, it is difficult to know what fraction of glycogen was lost, or if the glycogen remaining is a non-representative stable pool. Post-mortem glycogenolysis can be limited by “funnel freezing” with direct application of liquid nitrogen to the animal skull (Dienel, Wang, & Cruz, 2002; Ponten, Ratcheson, & Siesjo, 1973); however, there is a lag time between surface freezing and freezing of deeper structures. This is likely a negligible issue for mouse brain, but can be significant for rat brain. Glycogenolysis can also be halted by high-energy microwave fixation of brain *in situ*, which elevates brain temperatures to levels that denature glycogen metabolizing enzymes within 1 second or less (Medina, Jones, Stavinoha, & Ross, 1975; Sagar, Sharp, & Swanson, 1987). This method also has its limitations, however. The high heat required for microwave fixation can reduce the water content of the brain, thus artificially elevating the calculated glycogen content when glycogen is expressed per gram wet weight. This can be avoided by normalizing to mg protein rather than to wet weight, but special care must be taken to ensure that the fixed (denatured) protein is fully solubilized for protein determination. Failure to correct for brain dehydration or incompletely solubilized proteins both lead to erroneously elevated glycogen measurements in microwave fixed brain (Swanson and Sagar, unpublished observations). A second limitation of the microwave technique is that the heating and water vapor formation often distort normal cell architecture and destroy immunoreactivity of many antigens; however, these problems can be overcome by very careful titration of microwave power (Oe et al., 2016).

It is also important to recognize that microwave fixation method can denature glycogen metabolizing enzymes, but it does not “fix” tissues in the usual histochemical sense. Glycogen is variably water soluble (depending upon its molecular weight and protein binding), and microwave-fixed tissue that is subsequently treated with aqueous solutions may therefore lose glycogen into these solutions. Methods for brain cutting and mounting using high-concentration alcohol solutions have been shown to eliminate this problem (Swanson et al., 1992), but these methods may not be adaptable to immunohistochemical studies.

Hormonal and neurotransmitter effects on glycogenolysis

This aspect of glycogen metabolism may be uniquely problematic for studies of brain glycogen, because it involves sentient activity of the subject. There is a generally inverse relationship between neuronal activity and glycogen levels, as evidenced by elevated brain glycogen levels during anesthesia, hibernation, and slow wave sleep. Conversely, as outlined above, glycogen phosphorylase is activated by a number of signaling molecules that initiate glycogenolysis in anticipation of actual energy demand. For example, epinephrine and norepinephrine released during the stress response are potent glycogenolytic signaling molecules. It follows that animals that are stressed during the interval before brain harvest may have regionally lower brain glycogen levels than non-stressed animals. Experimental evidence supports this concern (Cruz & Dienel, 2002). This factor has far reaching implications, as it suggests that, like glucose utilization, glycogen levels and turnover rates are likely influenced in a regionally discrete manner by the subject experience and response near the time of the observation.

Summary

Studies of brain glycogen metabolism are complicated by factors that stem directly from specific structural and regulatory features of glycogen. These factors must be considered and addressed in interpreting study results.

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Significance statement

Brain glycogen has become an area of considerable interest, in part because the rapid turnover of astrocytic glycogen in response to local neuronal activity provides a window to bioenergetic interactions between these two cell types. There are several technical factors that make work in this area challenging: low glycogen content in brain, non-homogeneous labeling of glycogen by radiotracers, rapid glycogenolysis during postmortem tissue handling, and effects of the stress response on brain glycogen turnover. Careful attention to these factors is necessary for progress in this challenging area.

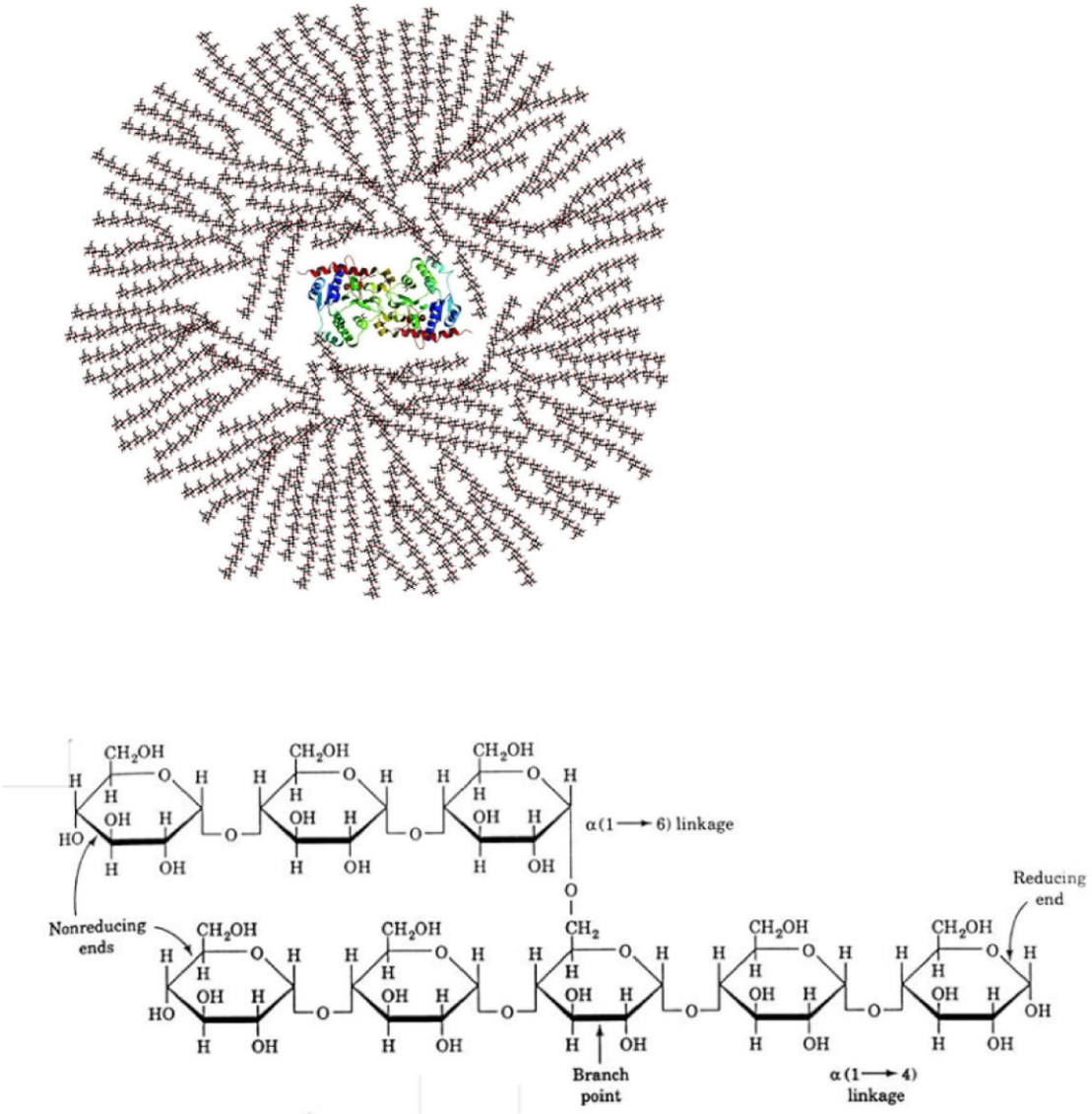

Figure 1. Schematic two-dimensional cross-sectional view of glycogen.

A core protein of glycogenin is surrounded by branches of glucose units. The entire globular granule may contain around 30,000 glucose units. The individual glucose moieties of glycogen are linked by α−1, 4 -glycosidic bonds, with branch points at approximately every 10 – 14 glucose residues linked by α−1, 6-glycosidic bonds. The exposed ends of all glycogen chains are non-reducing. Image from (Haggstrom, 2014)

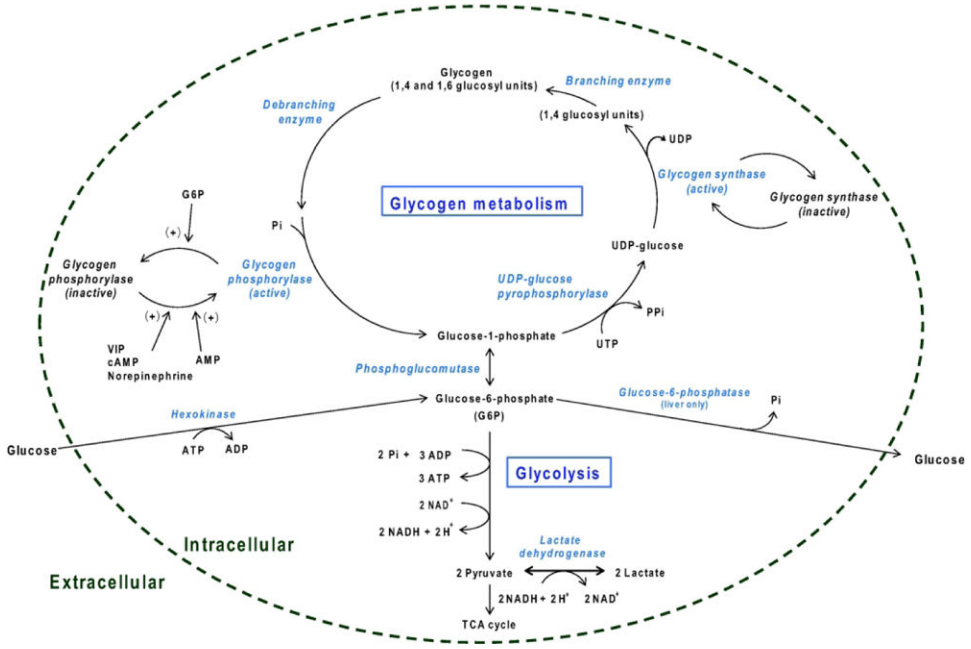

Figure 2. Bioenergetics and regulation of glycogen metabolism.

Glycogen synthase extends an existing glucosan chain of α−1, 4-glycosidic linkages using UDP glucose as substrate. Glycogen branching enzyme subsequently forms α−1, 6-glycosidic bonds to create branch points every 8 – 12 residues. Glycogen degradation is mediated by glycogen phosphorylase (GP) and debranching enzyme. GP is regulated allosterically in response to hormones, e.g. norepinephrine and vasoactive intestinal peptide (VIP); by changes in energy state (AMP, glucose-6-phosphate (G6P), and others), and by second messengers such as cAMP. The immediate product of glycogen degradation is glucose 1-phosphate which is freely converted to glucose-6-phosphate. Hepatocytes (but not other cell types) can rapidly dephosphorylate glucose-6-phosphate to generate free glucose for export.

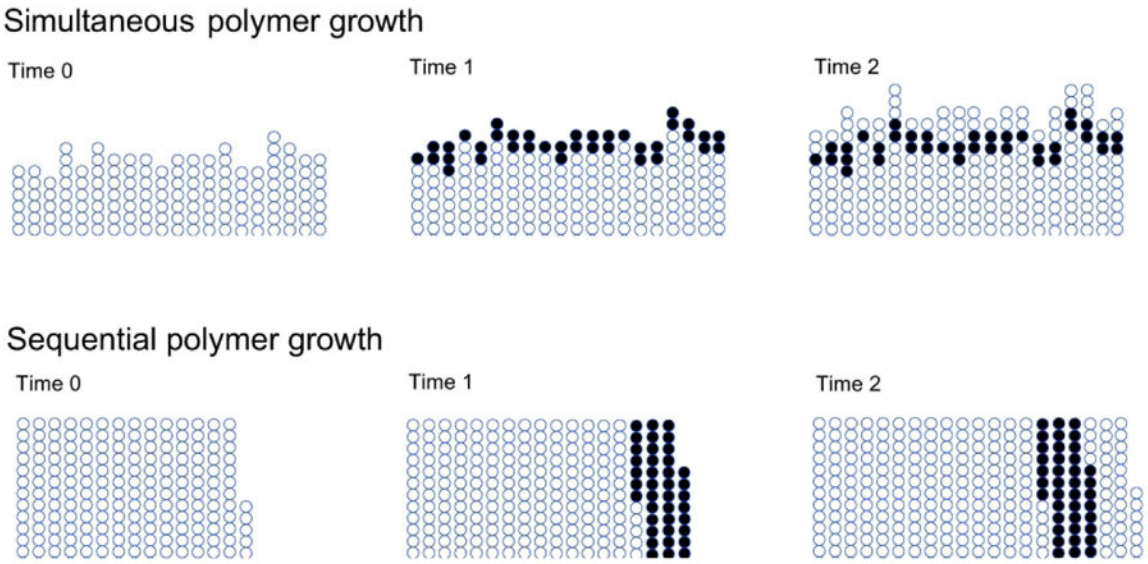

Figure 3. Results of radiolabeling tracer experiments are influenced by the mode of glycogen polymer growth and breakdown.

Schematic diagram showing distribution in glycogen of labeled glucose (filled circles) injected early during glycogen synthesis. Each column of circles represents an individual glycogen polymer. In one scenario, all polymers simultaneously add glucose moieties. In the opposite scenario, each polymer is synthesized to its maximum size before a second one begins to expand. Many other intermediates or more complex patterns are also possible, as are differing patterns of glycogen polymer breakdown. These differing patterns produce a different relationship between rates of glycogen turnover and rates of label release. Redrawn from (Youn & Bergman, 1987).

Table 1.

Glycogen content in brain and other tissues

Tissue	Glycogen (mg/g tissue)	Citation
Liver	30.23 ± 2.5	Vissing, Wallace, & Galbo, 1989
Liver	43.12 ± 5.5 †	Kusunoki et al., 2002
Liver	32.3 ± 2.0	Khandelwal, Zinman, & Knull, 1979
Heart	4.51 ± 0.33	Vissing et al., 1989
Heart	4.16 ± 0.23	Conlee, Berg, Han, Kelly, & Barnett, 1989
Kidney	0.06 ± 0.01	Khandelwal et al., 1979
Kidney	0.03 ± 0.01 †	Nannipieri et al., 2001
Muscle (GPS) ¶	5.94 ± 0.25 ‡	Baker, Timmons, & Greenhaff, 2005
Muscle (white gastrocnemius)	5.79 ± 0.72	Garetto, Richter, Goodman, & Ruderman, 1984
Muscle (white gastrocnemius)	7.01 ± 0.17	Vissing et al., 1989
Brain (cortex)	0.68 ± 0.03 §	Sagar et al., 1987
Brain (cortex)	0.60 ± 0.03	Kong et al., 2002
Brain (cortex)	2.09 ± 0.27 †	Cruz & Dienel, 2002
Brain (whole brain)	2.06 ± 0.21	Oe et al., 2016

Values are from adult rat or mouse under resting, non-fasting conditions. Variances are reported as means ± SD

† or means ± SEM.

‡ Reported as mmol/kg dry weight; converted to mg/g tissue using 163 g/mol as molecular weight of glycogen and 76% as the water content of muscle (Pivarnik & Palmer, 1994).

§ Reported as mmol/mg protein; converted to mg/g tissue using 122 mg protein/gram wet weight in brain (Banay-Schwartz, Kenessey, DeGuzman, Lajtha, & Palkovits, 1992).

¶ GPS: gastrocnemius-plantaris-soleus muscle complex