Ordered Synthesis and Mobilization of Glycogen in the Perfused Heart†

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ABSTRACT: The molecular order of synthesis and mobilization of glycogen in the perfused heart was studied by ^{13}C NMR. By varying the glucose isotopomer ([1-^{13}C]glucose or [2-^{13}C]glucose) supplied to the heart, glycogen synthesized at different times during the perfusion was labeled at different carbon sites. Subsequently, the in situ mobilization of glycogen during ischemia was observed by detection of labeled lactate derived from glycolysis of the glucosyl monomers. When [1-^{13}C]glucose was given initially in the perfusion and [2-^{13}C]glucose was given second, [2-^{13}C]lactate was detected first during ischemia and [3-^{13}C]lactate second. This result, and the equivalent result when the glucose labels were given in the reverse order, demonstrates that glycogen synthesis and mobilization are ordered in the heart, where glycogen is found morphologically only as β particles. Previous studies of glycogen synthesis and mobilization in liver and adipocytes [Devos, P., & Hers, H.-G. (1979) *Eur. J. Biochem. 99*, 161-167; Devos, P., & Hers, H.-G. (1980) *Biochem. Biophys. Res. Commun. 95*, 1031-1036] have suggested that the organization of β particles into α particles was partially responsible for ordered synthesis and mobilization. The observations reported here for cardiac glycogen suggest that another mechanism is responsible. In addition to examining the ordered synthesis and mobilization of cardiac glycogen, we have selectively monitored the NMR properties of ^{13}C-labeled glycogen synthesized early in the perfusion during further glycogen synthesis from a second, differently labeled substrate. During synthesis from the second labeled glucose monomer, the glycogen resonance from the first label decreased in integrated intensity and increased in line width. These results suggest either that there is significant isotopic exchange of glucosyl monomers in glycogen during net synthesis or that glucosyl residues incorporated into glycogen undergo motional restrictions as further glycogen synthesis occurs.

Glycogen is the principle storage form of carbohydrate in animals and as such plays a crucial role as a systemic and cellular energy store. Because of its linear branched structure, glycogen has been the subject of numerous investigations to understand how its structure is related to its metabolism (Stetten & Stetten, 1954, 1955; Birch et al., 1974; Devos & Hers, 1974, 1979). Devos and Hers have used radiolabeled glucosyl monomers incorporated into hepatic glycogen at various times during synthesis to demonstrate that synthesis and mobilization of liver glycogen are ordered; glucosyl monomers that were first used for glycogen synthesis were last mobilized during glycogenolysis and vice versa (Devos & Hers, 1979). This first in, last out order was demonstrated in vivo using anesthetized rats and in vitro using both isolated hepatocytes and a cell-free system.

The exact mechanism responsible for the ordered synthesis and mobilization observed in hepatic glycogen is poorly understood. Because β-amylolysis of isolated glycogen demonstrated that label was distributed equally between inner and outer chains, and that this distribution did not change during glycogenolysis, Hers and Devos believed that ordered mobilization could not be explained by initial removal of the outer chains of glycogen. They proposed that ordered synthesis and mobilization had to result from a level of organization higher than the structure of each glycogen molecule. Because hepatic glycogen is made up of β particles (15-30-nm diameter), arranged in rosette structures to form larger (100-nm diameter) particles, they proposed that ordered synthesis and mobilization of hepatic glycogen might result from an ordered association of β particles of glycogen within each particle or an ordered association of particles. In order to test the hypothesis that organization in or between α particles was related to ordered mobilization, Hers and Devos performed pulse labeling experiments on glycogen from adipose tissue, which is comprised only of monomeric β particles (Devos & Hers, 1980). They found that mobilization of pulse-labeled adipocyte glycogen was not ordered, supporting their hypothesis. Other investigators have suggested that the organization responsible for ordered synthesis and degradation is at the inter- or intracellular level (Ching et al., 1985). This seems unlikely because liver glycogen, together with phosphorylase, amylo-1,6-glucosidase, and α-amylase, isolated as a complex with concanavalin A, showed ordered degradation in a cell-free system (Devos & Hers, 1979). A recent computer simulation of the ordered synthesis and mobilization data obtained from liver has suggested that the liver glycogen data can be explained by using a model of accelerated growth of glycogen particles (Youn & Bergman, 1987).

^{13}C NMR has been recently used to study the synthesis and mobilization of glycogen in situ (Cohen et al., 1981; Sillerud & Shulman, 1983; Neurohr et al., 1984; Siegfried et al., 1985; Laughlin et al., 1988; Hoekenga et al., 1988). Despite the large molecular weight of glycogen, this polymer represents an especially attractive target for metabolic investigations using NMR because of its high concentration of monomeric units, apparent NMR visibility, and involvement in several pathological states (Rousset et al., 1979; Bannasch et al., 1980), including cardiac ischemia (Rovetto et al., 1973). Because

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of the ability of NMR to observe glycogen in intact tissue and to selectively observe glycosyl monomers labeled with ^{13}C at different sites, we have applied ^{13}C labeling and ^{13}C NMR to investigate whether the synthesis and mobilization of cardiac muscle glycogen (which is present as β particles only) resembles that of adipocyte glycogen or liver glycogen. In addition, we have used these techniques to selectively monitor the NMR properties of glycogen synthesized early in the perfusion in the presence of further glycogen synthesis from a second, differently labeled substrate.

Materials and Methods

Chemicals. [1-^{13}C]Glucose and [2-^{13}C]glucose, 99 atom %, were synthesized by published methods (Serianni et al., 1979). Bovine insulin was obtained from Sigma Chemical, St. Louis, MO. All other chemicals were analytical grade or better.

Heart Perfusions. Male Hartley strain guinea pigs (Charles River Breeding Labs, Wilmington, MA) weighing 400–500 g were used in all experiments. They were fed ad libitum (Waynes Mills nonmedicated guinea pig feed).

Hearts were perfused in the Langendorff mode at a pressure of 90 cm H_2O as previously described (Brainard et al., 1986; Hoekenga et al., 1988). The perfusate was oxygenated through a membrane oxygenator (Sci Med, Minneapolis, MN) with 95% O_2–5% CO_2 to give an O_2 concentration in the perfusate of ~25 ppm. The spontaneous heart rates (190–220 beats/min) were measured by a Statham transducer. The perfusion apparatus was modified slightly to contain two independent circuits so that the composition of the perfusate delivered to the heart could be conveniently changed. The perfusate was 200 mL of Krebs–Henseleit bicarbonate buffer (Krebs & Henseleit, 1932) prepared fresh for each experiment, containing 0.1 mg/mL streptomycin, 100 units/mL penicillin, and 25 ng/mL amphotericin B to retard bacterial growth during the perfusion.

The cardiac glycogen was labeled by perfusion under normoxic conditions with 5 mM [1-^{13}C]glucose or [2-^{13}C]glucose and insulin, 20 units/L. Glycogen was labeled with one glucose isotopomer during a 50–60-min normoxic period; the heart perfusion was then switched to the second perfusate circuit containing the other glucose isotopomer. In order to minimize any mixing, the perfusate surrounding the heart in the NMR tube was aspirated during the switch, and recirculation of the perfusate was delayed until 1–2 min after the heart began receiving the new substrate. After switching substrates, glycogen synthesis from the second label was allowed to occur for a 50–80-min normoxic period. Subsequently, the perfusate was switched to perfusate containing no substrate, and mobilization of the labeled glycogen was induced by total global ischemia.

NMR Measurements and Analysis. High-resolution NMR spectra were obtained at 7 T as previously described (Hoekenga et al., 1988). The temperature of perfused hearts (37 °C) was monitored by a fluoroptic thermometer (Luxtron Corp., Mountainview, CA) and controlled by the Bruker temperature controller accessory and by the delivery of temperature-controlled perfusate to the heart. Temperature variations during experiments were less than 2 °C. ^{13}C spectra were recorded using 40° pulses, 17000-Hz spectral width, and 8K data points with a pulse interval of 1.2 s. Spectra were recorded after 256 acquisitions during normoxia (5-min signal averaging) and after 128 acquisitions during ischemia (2.5-min signal averaging). Chemical shifts are reported relative to tetramethylsilane at 0 ppm using β-C1 glucose at 97 ppm as an internal reference standard.

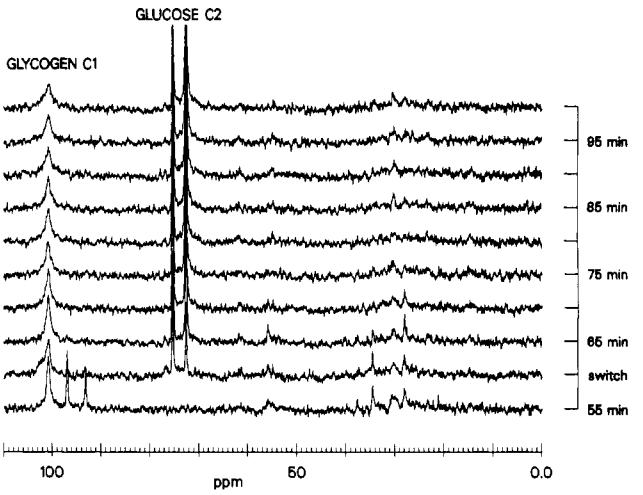

FIGURE 1: Stacked plot of proton-decoupled ^{13}C NMR of perfused heart showing glycogen synthesis from [2-^{13}C]glucose after an initial period of glycogen synthesis from [1-^{13}C]glucose. This heart was supplied with 6 mM [1-^{13}C]glucose for the period 5–55 min. At 60 min. the perfusate was changed to 6 mM [2-^{13}C]glucose, and glycogen synthesis proceeded from [2-^{13}C]glucose for the period 65–100 min.

All data were processed using NMRi software (3.0β version, NIH Resource for Data Analysis, Syracuse, NY) on a VAX 11/780. Time domain data were multiplied by an exponential line broadening factor of 8 Hz prior to Fourier transformation. The automated routines for resonance integration and Lorentzian line fitting were used to obtain integrated intensities for all resonances. Integrated intensities for [1-^{13}C]glycogen, [2-^{13}C]glycogen, [3-^{13}C]lactate, and [2-^{13}C]lactate were corrected for T_1 saturation and NOE effects by using relaxation parameters measured for the metabolites in vitro at 37 °C (Becker et al., 1979).

In order to estimate the contribution to the spectra of naturally abundant ^{13}C present in endogenous carbohydrates, ^{1}H spectra were obtained of the perfusate surrounding the hearts at the end of ischemia and the ^{13}C enrichments measured from the ^{13}C satellites (Walker et al., 1982; Shulman et al., 1985). The ^{13}C enrichments at C2 and C3 of lactate were between 16 and 34%. However, even for these enrichments, the contributions of natural-abundance ^{13}C to the ^{13}C spectra are quite small. Assuming the worst case (16%), the natural-abundance pool contributes less than $(0.84 \times 0.01)/(0.84 \times 0.01 + 0.16 \times 0.99)$ or ~5% to the total intensity of the ^{13}C resonance.

Results

When hearts were perfused with labeled glucose, the label was rapidly incorporated into glycogen and detected by high-resolution ^{13}C NMR. By varying the glucose isotopomers during the perfusion, glycogen synthesized at different times in the perfusion could be labeled at different carbon sites. Subsequently, the metabolism and NMR properties of glycogen synthesized at different times during the perfusion could be independently monitored by observing the resonances from the different carbon sites.

Labeling of Cardiac Glycogen from [^{13}C]Glucose Isotopomers. Stacked plots of ^{13}C NMR spectra of a heart that was first perfused with [1-^{13}C]glucose and then with [2-^{13}C]glucose are shown in Figure 1. Glycogen synthesized during the first part of this experiment was labeled at C1, while glycogen synthesized during the second part was labeled at C2. The first spectrum (55 min) shown in the stacked plot was obtained after 50 min of perfusion with recirculating 6 mM [1-^{13}C]glucose. This spectrum shows prominent reso-

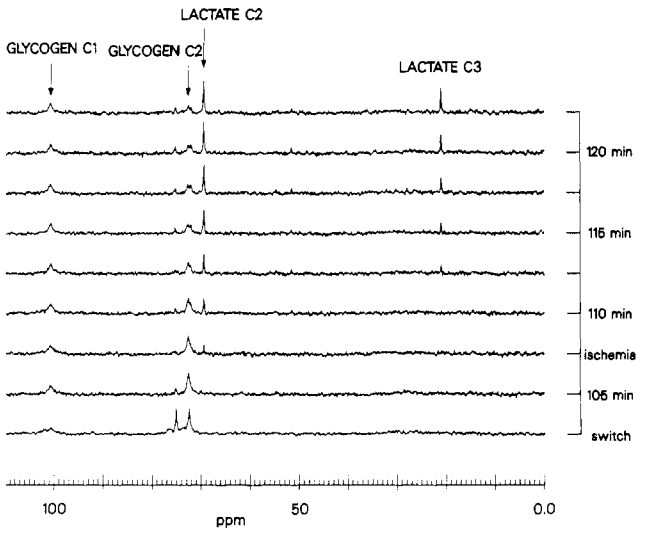

FIGURE 2: Stacked plot of proton-decoupled ^{13}C NMR of perfused heart during mobilization of labeled glycogen by ischemia. Glycogen in this heart was labeled initially with [1-^{13}C]glucose and then with [2-^{13}C]glucose.

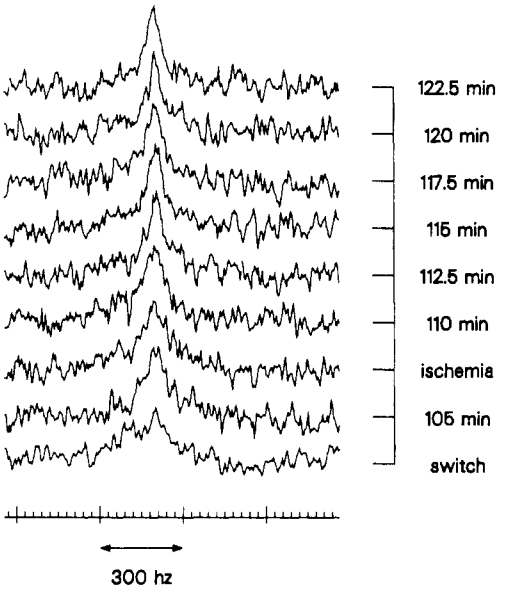

FIGURE 3: Stacked plot of the [1-^{13}C]glycogen resonance during mobilization of labeled glycogen by ischemia. Glycogen in this heart was labeled initially with [1-^{13}C]glucose and then with [2-^{13}C]glucose.

nances from β- and α-[1-^{13}C]glucose at 97 and 93.2 ppm, respectively, and from [1-^{13}C]glycogen at 100.8 ppm. There are also weak resonances from labeled glutamate at 56, 34.6, and 28 ppm. During the 50 min of perfusion with [1-^{13}C]-glucose, the resonance from glycogen increased fairly linearly in intensity, and the resonances from glucose in the perfusate decreased (data not shown). The linear increase in glycogen intensity during labeling from [1-^{13}C]glucose has been observed previously in perfused heart and liver (Lavanchy et al., 1984; Hoekenga et al., 1988; Sillerud & Shulman, 1983; Cohen, 1983) and in these organs in vivo (Neurohr et al., 1984; Reo et al., 1984; Siegfried et al., 1985; Laughlin et al., 1988).

The second spectrum in the stacked plot shown in Figure 1 was obtained during the switch from perfusate containing [1-^{13}C]glucose to perfusate containing [2-^{13}C]glucose and contains contributions from both substrates. The third spectrum (65 min) through the top spectrum show the spectral changes during synthesis of glycogen from [2-^{13}C]glucose. Because [2-^{13}C]glycogen and α-[2-^{13}C]glucose resonances have similar chemical shifts, the glycogen resonance is obscured by the more intense [2-^{13}C]glucose resonance, and the synthesis of [2-^{13}C]glycogen cannot be observed directly in these spectra. However, the [2-^{13}C]glycogen resonance can be observed after the second perfusate switch to substrate-free medium (shown in Figure 2). What is notable in Figure 1 is that when the substrate is changed to [2-^{13}C]glucose, the resonance from [1-^{13}C]glycogen decreases in intensity and increases in line width. The changes in the intensities and line widths of resonances from glycogen synthesized during the first labeling period are discussed further below.

Mobilization of Labeled Cardiac Glycogen by Ischemia. After glycogen in the heart was labeled first with [1-^{13}C]-glucose and secondly with [2-^{13}C]glucose, the perfusate was switched to perfusate containing no substrate. Subsequently, the glycogen was mobilized by ischemia, and the metabolism of [1-^{13}C]glycogen synthesized early in the perfusion and [2-^{13}C]glycogen synthesized late in the perfusion was observed by NMR. Spectra accumulated during the perfusate switch and ischemia are shown in Figure 2. In order to better determine the time course of glycogen mobilization and labeled lactate accumulation during ischemia, each spectrum in this plot was obtained after 2.5 min of signal averaging, rather than after 5 min as in Figure 1. The 105-min spectrum obtained after the switch to substrate-free perfusate and prior to ischemia shows a broad, weak resonance from [1-^{13}C]glycogen at 100.8 ppm and a narrower, more intense resonance from [2-^{13}C]glycogen at 72.8 ppm. During ischemia, resonances from C2 lactate at 69.6 ppm and C3 lactate at 21.2 ppm appear, and the resonance from C2 glycogen decreases. Two weak unassigned resonances in the 71–76 ppm region appear after 2.5–5 min of ischemia. These may be phosphorylated hexose or triose intermediates. There are two particularly notable features in these spectra. First, C3 lactate is not detected until 6–7 min after the onset of ischemia (112.5-min spectrum), whereas C2 lactate is detected during the first 1–2 min of ischemia (107.5-min spectrum). However, when C3 lactate is detected, the [2-^{13}C]glycogen resonance is still detectable, and the C2 lactate resonance is still growing. Second, the intensity of the [1-^{13}C]glycogen does not appear to decrease significantly during ischemia, and in fact appears to grow slightly and decrease in line width. This behavior is more clearly shown in Figure 3 where the [1-^{13}C]glycogen resonances are plotted with twice the vertical gain.

In order to more graphically show the changes in integrated intensities during glycogen synthesis, the integrated intensities from the experiment described above and from an experiment in which the glucose labels were given in the reverse order are shown in Figure 4. In this and the following figures, the upper panel (A) shows data from an experiment where [1-^{13}C]-glucose was given first and [2-^{13}C]glucose second, and the lower panel (B) shows data where [2-^{13}C]glucose was given first and [1-^{13}C]glucose second. The intensities of resonances from the ^{13}C label given first are indicated by the filled symbols, and resonances from the second label are indicated by open symbols. Because determining the base line and integration limits for broad resonances is difficult, the integrals were determined by using the automated integration software in NMRi (square symbols) and by automated fits of the resonances to sums of Lorentzian line shapes using NMRi (circle symbols). Both methods of integration gave similar results, and because both methods were automated, operator biases were minimized. Figure 4A shows the integrated intensities from the C1 glycogen resonance during net glycogen synthesis from [1-^{13}C]glucose (5–60 min) and from [2-^{13}C]-glucose (60–100 min). The C1 glycogen intensity increased

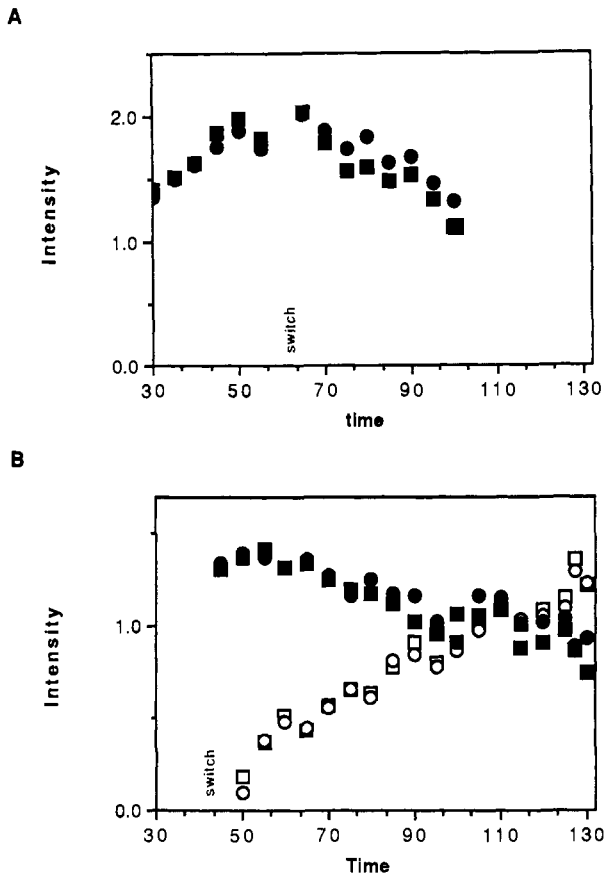

FIGURE 4: Integrated intensities of glycogen resonances during net synthesis of glycogen from the second labeled glucose isotopomer. (A) Heart perfused initially with [1-^{13}C]glucose and then with [2-^{13}C]-glucose. (B) Heart perfused initially with [2-^{13}C]glucose and then with [1-^{13}C]glucose. (Closed symbols) Intensities of resonances from ^{13}C label given *first*; (open symbols) intensities of resonances from ^{13}C label given *second*; (square symbols) integrated intensities determined by automated integration routine; (circle symbols) integrated intensities determined by automated fits to a Lorenztian line shape. The time at which the perfusate was changed is indicated on the plot by the "switch" label.

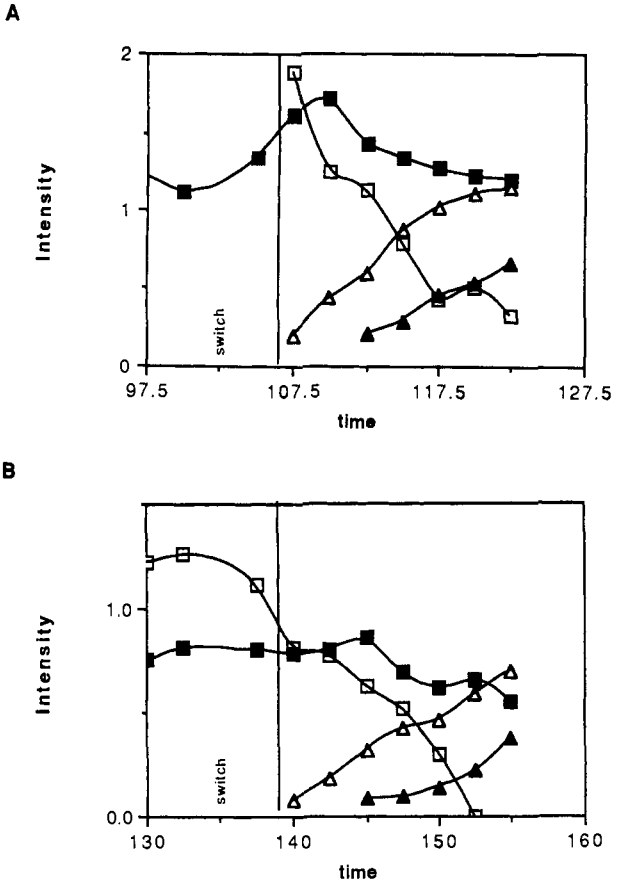

FIGURE 5: Integrated intensities of glycogen and lactate resonances during mobilization of glycogen by ischemia. (A) Heart perfused initially with [1-^{13}C]glucose and then with [2-^{13}C]glucose. (B) Heart perfused initially with [2-^{13}C]glucose and then with [1-^{13}C]glucose. (Closed symbols) Intensities of resonances from ^{13}C label given *first*; (open symbols) intensities of resonances from ^{13}C label given *second*; (square symbols) integrated intensities of labeled glycogen resonances; (triangle symbols) integrated intensities of labeled lactate resonances. The time at which the perfusate was changed to substrate-free perfusate is indicated on the plot by the "switch" label. The beginning of ischemia is indicated by the vertical lines.

fairly linearly during synthesis from [1-^{13}C]glucose and decreased by about 40% during 40 min of synthesis from [2-^{13}C]glucose.

Figure 4B shows the intensities from C1 and C2 glycogen from a similar experiment where [2-^{13}C]glucose was given first and [1-^{13}C]glucose second. In this experiment, the resonances from both C1 and C2 glycogen could be observed during the second labeling period. (The resonance from [2-^{13}C]glycogen is obscured during the first labeling period by the α-[2-^{13}C]-glucose resonance.) The resonance from C1 glycogen increased fairly linearly after the label was switched to [1-^{13}C]glucose at 45 min. Simultaneously, the resonance from [2-^{13}C]glycogen labeled during the first labeling period decreased in intensity.

The intensities of resonances during mobilization of labeled glycogen by ischemia are shown in Figure 5. The metabolism of the labeled glycogen could be followed both by changes in the intensities of the labeled glycogen resonances and by accumulation of labeled lactate derived from the glycogen. Glycogenolysis of [1-^{13}C]glycogen produces [3-^{13}C]lactate while glycogenolysis of [2-^{13}C]glycogen produces [2-^{13}C]lactate. This figure demonstrates that lactate derived from glycogen labeled from the second substrate (open triangles in both panels A and B) is detected before lactate derived from glycogen labeled from the first substrate (filled triangles). In addition, the glycogen resonance labeled from the second substrate (open squares) decreases before the glycogen resonance labeled from the first substrate (filled squares). These experiments show that the labeled glucosyl monomer first incorporated into glycogen during synthesis is the last mobilized during ischemia.

In order to begin investigating possible explanations for the variations in the intensity of glycogen labeled from the first substrate during net synthesis and mobilization, the line widths of glycogen resonances were determined by automated fits to Lorentzian line shapes. Figure 6 shows the increase in line width for the resonance of glycogen labeled from the first substrate during glycogen synthesis from the second substrate. The decrease in line width of the glycogen resonance during ischemia is also shown. Although there is considerable scatter in these plots, they demonstrate that the line width of the resonance of glycogen labeled during the first period doubles during glycogen synthesis from the second label. The line width of the glycogen resonance labeled second stays constant at ~60 Hz. During ischemia, the line width of the resonance of glycogen labeled during the first period decreases to ~50% of the preischemia value.

Discussion

In this study, we demonstrate that the synthesis and mobilization of glycogen in situ in perfused heart occur in an ordered fashion. The glycogen monomers that are first syn-

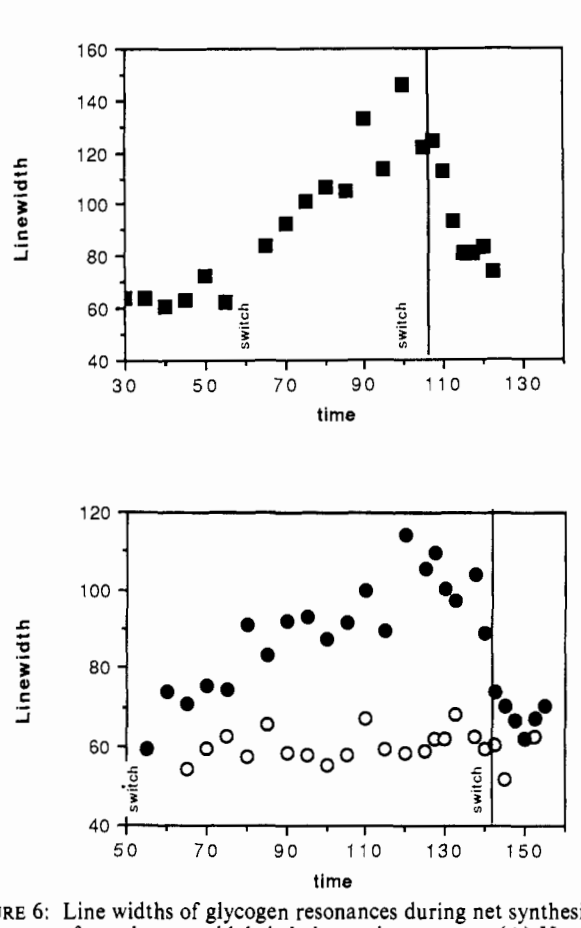

FIGURE 6: Line widths of glycogen resonances during net synthesis of glycogen from the second labeled glucose isotopomer. (A) Heart perfused initially with [1-^{13}C]glucose and then with [2-^{13}C]glucose. (B) Heart perfused initially with [2-^{13}C]glucose and then with [1-^{13}C]glucose. (Closed symbols) Line widths of resonances from ^{13}C label given *first*; (open symbols) line widths of resonances from ^{13}C label given *second*.

thesized during normoxia are last mobilized during ischemia. Second, we show that the ^{13}C resonances from glucose monomers which were incorporated into the polymer during the first period of glycogen synthesis increase in line width and decrease in integrated intensity during a second period of glycogen synthesis from a second glucose isotopomer. These experiments extend our knowledge of the structure and metabolism of heart glycogen.

Our demonstration of ordered synthesis and mobilization in heart tissue is the first observation of ordered mobilization in a tissue where glycogen is found only as β particles. Previously, ordered mobilization had been observed for liver glycogen (Devos & Hers, 1979), where β particles of glycogen are organized into α particles, but had not been observed for adipocyte glycogen (Devos & Hers, 1980), where glycogen is found only as β particles. This difference was taken as support for the involvement of α-particle organization in determining the order of synthesis and degradation. The observation of ordered mobilization in heart suggests that organization of glycogen into α particles is not strictly required for ordered mobilization and that another mechanism is operative. The simplest model which explains the ordered synthesis and degradation of cardiac glycogen is that (1) the number of glycogen molecules in cardiac muscle does not change markedly during glycogen synthesis and (2) the specificities of glycogen synthase and phosphorylase for the nonreducing ends of the glucosyl chains are such that the glucosyl monomers last added to the growing chains by synthase are first mobilized by phosphorylase.

The specificity of the enzymes involved in synthesis and mobilization of glycogen is a central feature of the computer model used by Youn and Bergman (1987) to simulate the liver data obtained by Devos and Hers. In their model, glucose monomers could only be added to, or removed from, the "top" of the stack representing a glycogen molecule. These investigators were able to accurately simulate the observed distribution of label between both β-amylase-accessible and β-amylase-nonaccessible chains during synthesis and the ordered mobilization of labeled monomers. In their model, synthesis of new glycogen occurs by a pattern of accelerating growth (increasing *numbers* of growing molecules during synthesis), thus distributing labeled monomers evenly between β-amylase-accessible and -nonaccessible chains. The ordered mobilization of liver glycogen observed experimentally by Devos and Hers is accounted for in the model of Youn and Bergman by this pattern of accelerating growth together with the assumption that smaller glycogen molecules are more readily degraded by phosphorylase. Our data on the ordered synthesis and mobilization of cardiac glycogen can also be accommodated within a similar model of accelerating growth of glycogen molecules in the heart.

A particular advantage of NMR as a tool for investigation of metabolism is that the spectral properties observed reflect the physical state of the metabolites observed. In general, detection of high-resolution NMR resonances from a particular metabolite in situ indicates that it exists primarily free in solution, where it undergoes rapid, isotropic tumbling. Bound metabolites or large macromolecules usually have short spin–spin relaxation times and correspondingly large line widths, making the detection of these species in situ by NMR difficult. The observation of narrow, high-resolution ^{13}C resonances from glycogen initially reported by Cohen et al. (1981) and Alger et al. (1981) suggested that the individual glucosyl monomer units in glycogen undergo considerable internal motion which is much faster than overall reorientation of glycogen particles. Subsequent measurements of the ^{13}C relaxation properties of liver and cardiac glycogen demonstrated that the spin–lattice relaxation times were consistent with an effective isotropic correlation time of $\sim$5 ns (Sillerud & Shulman, 1983; Neurohr et al., 1984). This confirmed that the glucosyl units undergo considerable segmental motion within the glycogen particle. To address the question of whether all of the glucosyl monomers were contributing to a high-resolution ^{13}C NMR signal, these investigators compared the decrease in intensities of glycogen resonances during glycogen hydrolysis to the increases in intensities of product resonances. On the basis of comparisons using excised tissues and isolated glycogen, these investigators concluded that 100% of the carbon nuclei contribute to the high-resolution ^{13}C NMR signal.

The protocol used in the experiments described herein allowed the NMR properties of cardiac glycogen synthesized early in the perfusion to be monitored independently from glycogen synthesized later. Our results demonstrate that the intensity of the resonance from labeled glycogen synthesized early in the perfusion decreased during net synthesis from a second labeled precursor. There are two possible explanations for this. First, isotopic exchange of labeled glucosyl monomers may occur during periods of net synthesis. This would involve exchange of ^{13}C-labeled monomers in glycogen by simultaneous glycogen degradation and synthesis. This explanation is partially discounted by the putative kinetic control of glycogen synthase and phosphorylase so that either synthesis or degradation takes place, but not both at the same time (De Wulf & Hers, 1967; Villar-Palas & Larner, 1970). However,

incorporation of label from glucose into glycogen has been noted during periods of glycogenolysis (Katz & Rognstad, 1976), and high phosphorylase activity has been assayed in heart extracts during periods of glycogen synthesis (Craig & Larner, 1964), so isotopic exchange as a possible explanation for the decrease in the integrated area cannot be dismissed.

A second possible explanation for both the decrease in the integrated area *and the increase in line width* is that as the glycogen particles grow in size, the monomers that were incorporated initially into the glycogen polymer become restricted in their segmental motion and no longer contribute full intensity to the ^{13}C spectrum. Restricted segmental motion of these monomers is expected to lead to short spin–spin relaxation times and correspondingly broad lines. The observation of broad lines under instrumental conditions typical of in situ and in vivo NMR experiments is difficult; the resonance intensities are decreased relative to narrower resonances. Even when integrated intensities are used for quantitation, artifacts from instrumental dead times and from selecting the integration limits and base line in spectra often result in severe attenuation of broad lines. This explanation is also supported by the observation that the increases in the intensities of resonances from labeled lactate derived from glycogenolysis of the glycogen labeled during the first labeling period are greater than the decreases in the corresponding glycogen resonances.

Several other reports have suggested that all the carbons may not be visible in the ^{13}C NMR spectra of glycogen. Cohen reports that in her laboratory, only 45 ± 10% of the total liver glycogen is detected (Cohen, 1983). Hull and co-workers recently compared enzymatic assays and quantitative in situ ^{13}C NMR measurements of glycogen from rat livers, rabbit muscle, and human kidney tumors (Hull et al., 1987). Although comparisons at 37 °C suggested that all of the glycogen in excised tissue was contributing to the ^{13}C spectrum, they reported that the intensities and line widths of glycogen resonances in these tissues were temperature dependent. At 4 °C, only 35% of liver glycogen was detected by NMR. In addition, these investigators reported that in situ degradation of liver glycogen at 4 °C led to changes in the NMR spectra believed to reflect a loosening of the core structure. A recent ^{13}C NMR study of hydrated glycogen using solids methods suggested that under static (non-MASS) conditions, the breadth of the ^{13}C resonances was sufficient to make observation of all the glycogen difficult under high-resolution conditions (Jackson & Bryant, 1989).

There are several possible reasons for the differences between our results concerning the NMR visibility of cardiac glycogen in the intact perfused heart and those of previous investigators (Sillerud & Shulman, 1983; Neurohr et al., 1984). The degree of NMR visibility of ^{13}C resonances from glycogen may reflect structural differences in glycogen synthesized under different hormonal conditions or in different tissues. Hull found, for example, that the core of human kidney tumor glycogen was more rigid than rat liver glycogen (Hull et al., 1987). This explanation may explain the differences between our results and those obtained on liver glycogen (Sillerud & Shulman, 1983), but not between our results and those obtained on guinea pig heart glycogen (Neurohr et al., 1984). Temperature variations may also explain the observed differences. In the previous studies, the glycogen hydrolysis in vitro was performed at 55 °C (Sillerud & Shulman, 1983). In light of data demonstrating the temperature dependence of ^{13}C NMR spectra of glycogen, it is likely that the NMR visibility of glycogen changes significantly between 37 and 55 °C. Finally, previous measurements were performed either on glycogen in excised tissue or on glycogen extracted by chemical means. The NMR properties of glycogen may be altered during extraction, or by degradative processes after excision.

The relationship of our observations using perfused hearts to previous observations on the rat and guinea pig heart in vivo is also of note. Neurohr et al. measured the intensity of the [1-^{13}C]glycogen resonance in an in vivo guinea pig heart perfused with [1-^{13}C]glucose and chased first with natural-abundance glucose and secondly with saline. No significant change in the intensity of the [1-^{13}C]glycogen resonance was observed during either the natural-abundance or the saline chase (Neurohr et al., 1984). Barrett and colleagues performed a similar experiment on rat heart (Lauglin et al., 1988). During the chase with natural-abundance glucose (~60 min), the peak intensity of the [1-^{13}C]glycogen resonance in the in vivo rat heart decreased by 15–20%. In the isolated perfused guinea pig heart, we observe a 30–40% decrease in integrated intensity of labeled glycogen resonances during a similar time period. These conflicting observations concerning changes in the intensity of labeled glycogen resonances during further glycogen synthesis indicate that the questions of glycogen turnover and/or the NMR visibility of glycogen are still controversial.

For glycogen at 37 °C in perfused guinea pig hearts, our results suggest that ^{13}C resonances from glucosyl residues in the inner core of glycogen are significantly broadened and attenuated. This result supports a model for glycogen structure where the inner core is subject to restrictions in the segmental motions of the glucosyl residues. This model is also consistent with a simple physical mechanism for the ordered synthesis and degradation of cardiac glycogen. Those glucosyl residues in the interior of the glycogen particle which are hindered in their segmental motions are also sterically inaccessible to phosphorylase. Some glucosyl residues in the interior of the glycogen particle may initially be resistant to phosphorylase activity because they are not at the nonreducing end of the polymer chain. We note, however, that the segregation of inner core and outer core glucosyl residues is not absolute. In our studies, detectable mobilization of the glucosyl monomer first used for glycogen synthesis occurred before the mobilization of the second monomer was complete. This incomplete segregation of first and last labels can be explained by some growth of new glycogen molecules during synthesis. In a model where glycogen synthesis occurs solely by growth of new glycogen molecules, mobilization of monomers is not ordered (Devos & Hers, 1979; Youn & Bergman, 1987). The simplest model which accounts both for the "mostly" ordered synthesis and mobilization observed by in situ NMR and for the apparent behavior of glycogen line widths during synthesis and mobilization is one where *most* of the glycogen synthesis occurs by addition of new glucose monomers to existing glycogen molecules.

Registry No. Glycogen, 9005-79-2.

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Redox Cycles of Vitamin E: Hydrolysis and Ascorbic Acid Dependent Reduction of 8a-(Alkyldioxy)tocopherones†

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ABSTRACT: Oxidation of the biological antioxidant α-tocopherol (vitamin E; TH) by peroxyl radicals yields 8a-(alkyldioxy)tocopherones, which either may hydrolyze to α-tocopheryl quinone (TQ) or may be reduced by ascorbic acid to regenerate TH. To define the chemistry of this putative two-electron TH redox cycle, we studied the hydrolysis and reduction of 8a-[(2,4-dimethyl-1-nitrilopent-2-yl)dioxy]tocopherone (**1**) in acetonitrile/buffer mixtures and in phospholipid liposomes. TQ formation in acetonitrile/buffer mixtures, which was monitored spectrophotometrically, declined with increasing pH and could not be detected above pH 4. The rate of TQ formation from **1** first increased with time and then decreased in a first-order terminal phase. Rearrangement of 8a-hydroxy-α-tocopherone (**2**) to TQ displayed first-order kinetics identical with the terminal phase for TQ formation from **1**. Both rate constants increased with decreasing pH. Hydrolysis of **1** in acetonitrile/$H_2{}^{18}O$ yielded [^{18}O]TQ. These observations suggest that **1** loses the 8a-(alkyldioxy) moiety to produce the tocopherone cation (T^+), which hydrolyzes to **2**, the TQ-forming intermediate. Incubation of either **1** or **2** with ascorbic acid in acetonitrile/buffer yielded TH. Reduction of both **1** and **2** decreased with increasing pH. In phosphatidylcholine liposomes at pH 7, approximately 10% of the T^+ generated from **1** was reduced to TH by 5 mM ascorbic acid. The results collectively demonstrate that T^+ is the ascorbic acid reducible intermediate in a two-electron TH redox cycle, a process that probably would require biocatalysis to proceed in biological membranes.

The lipophilic antioxidant vitamin TH[1] (vitamin E) is the principal nonenzymatic defense against cellular membrane damage by reactive free radicals (Machlin & Bendich, 1987). TH interrupts radical chains by reacting with peroxyl radicals to form the relatively stable tocopheroxyl radical (Burton &

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[1] Abbreviations: AMVN, azobis(2,4-dimethylvaleronitrile); HPLC, high-performance liquid chromatography; TH, *d*-α-tocopherol; TQ, *d*-α-tocopheryl quinone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride. The term "ascorbic acid" refers to both the un-ionized and anionic forms of the molecule.

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