D'Anna, J. A., Gurley, L. R., & Tobey, R. A. (1982) *Biochemistry 21*, 3991–4001.
D'Anna, J. A., Becker, R. R., Tobey, R. A., & Gurley, L. R. (1983) *Biochim. Biophys. Acta 739*, 197–206.
Graves, R. A., & Marzluff, W. F. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 1893.
Gurley, L. R., Walters, R. A., & Tobey, R. A. (1974) *Arch. Biochem. Biophys. 164*, 469–477.
Heintz, N. H., & Hamlin, J. L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4083–4087.
Heintz, N. H., & Hamlin, J. L. (1983) *Biochemistry 22*, 3557–3562.
Heintz, N. H., Milbrandt, J. D., Greisen, K. S., & Hamlin, J. L. (1983) *Nature (London) 302*, 439–441.
Hildebrand, C. E., & Walters, R. A. (1976) *Biochem. Biophys. Res. Commun. 73*, 157–163.
Huberman, J. A. (1981) *Cell (Cambridge, Mass.) 23*, 647–648.
Johnston, L. H. (1980) *Curr. Genet. 2*, 175–180.
Kaplay, M., Prabhakar, V., & Rao, K. S. (1983) *Biochem. Int. 6*, 283–289.
Kurek, M. P., & Taylor, J. H. (1977) *Exp. Cell Res. 104*, 7–14.
Leffak, I. M. (1983) *Nucleic Acids Res. 11*, 5451–5466.
Levy, A., & Jakob, K. M. (1978) *Cell (Cambridge, Mass.) 14*, 259–267.
Mariani, B. D., & Schimke, R. T. (1984) *J. Biol. Chem. 259*, 1901–1910.
Panyim, S., & Chalkley, R. (1969) *Biochemistry 8*, 3972–3979.
Pedrali-Noy, G., Spadari, S., Miller-Faures, A., Miller, A. O. A., Kruppa, J., & Koch, G. (1980) *Nucleic Acids Res. 8*, 377–387.
Reichard, P., & Ehrenberg, A. (1983) *Science (Washington, D.C.) 221*, 514–519.
Sala, F., Galli, M. G., Nielson, E., Magnien, E., Devreux, M., Pedrali-Noy, G., & Spadari, S. (1983) *FEBS Lett. 153*, 204–208.
Seale, R. (1975) *Nature (London) 255*, 247–249.
Spadari, S., Sala, F., & Pedrali-Noy, G. (1982) *Trends Biochem. Sci. (Pers. Ed.) 7*, 29–32.
Surowy, C. S., & Berger, N. A. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5510–5514.
Taylor, J. H. (1977) *Chromosoma 62*, 291–300.
Taylor, J. H., Adams, A. G., & Kurek, M. P. (1973) *Chromosoma 41*, 361–384.
Tobey, R. A., & Ley, K. D. (1971) *Cancer Res. 31*, 46–51.
Tobey, R. A., & Crissman, H. A. (1972) *Cancer Res. 32*, 2726–2732.
Varshavsky, A. (1983) *Cell (Cambridge, Mass.) 34*, 711–712.
Walters, R. A., Tobey, R. A., & Ratliff, R. (1973) *Biochim. Biophys. Acta 319*, 336–347.
Walters, R. A., Tobey, R. A., & Hildebrand, C. E. (1976a) *Biochem. Biophys. Res. Commun. 69*, 212–217.
Walters, R. A., Tobey, R. A., & Hildebrand, C. E. (1976b) *Biochim. Biophys. Acta 447*, 36–44.
Wawra, E., & Wintersberger, E. (1983) *Mol. Cell. Biol. 3*, 297–304.
Woodcock, D. M., & Cooper, I. A. (1981) *Cancer Res. 41*, 2483–2490.

Carbon-13 Nuclear Magnetic Resonance Studies of Myocardial Glycogen Metabolism in Live Guinea Pigs†

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ABSTRACT: Myocardial glycogen metabolism was studied in live guinea pigs by ^{13}C NMR at 20.19 MHz. Open-chest surgery was used to expose the heart, which was then positioned within a solenoidal radio frequency coil for NMR measurements. The time course of myocardial glycogen synthesis during 1-h infusions of 0.5 g of D-[1-^{13}C]glucose (and insulin) into the jugular vein was investigated. The possible turnover of the ^{13}C-labeled glycogen was also studied in vivo by following the labeled glucose infusion with a similar infusion of unlabeled glucose. The degree of ^{13}C enrichment of the C-1 glycogen carbons during these infusions was measured in heart extracts by ^{1}H NMR at 360 MHz. High-quality proton-decoupled ^{13}C NMR spectra of the labeled C-1 carbons of myocardial glycogen in vivo were obtained in 1 min of data accumulation. This time resolution allowed measurement of the time course of glycogenolysis of the ^{13}C-labeled glycogen during anoxia by ^{13}C NMR in vivo. With the solenoidal coil used for ^{13}C NMR, the spin–lattice relaxation time of the labeled C-1 carbons of myocardial glycogen could be measured in vivo. For a comparison, spin–lattice relaxation times of heart glycogen were measured in vitro at 90.55 MHz. Natural abundance ^{13}C NMR studies of the quantitative hydrolysis of extracted heart glycogen in vitro at 90.55 MHz showed that virtually all the carbons in heart glycogen contribute to the ^{13}C NMR signals. The same result was obtained in ^{13}C NMR studies of glycogen hydrolysis in excised guinea pig heart.

Mobilization of endogenous glycogen stores is of vital importance in maintaining adequate myocardial performance during episodes of limited oxygen supply to the heart (Opie, 1976; Randle & Tubbs, 1979; Capasso et al., 1981; Liedtke, 1981). Studies on isolated, perfused hearts have shown that myocardial glycogen stores are rapidly depleted during anoxia and, on a somewhat slower time scale, during ischemia (Rovetto et al., 1973). Glucose and insulin have been shown to increase glycogen in normal heart tissue (Villar-Palasi & Larner, 1968), and glucose–insulin–potassium (GIK) infusions increased glycogen levels in experimental baboon and dog infarcts (Opie et al., 1975; Opie & Owen, 1976). A protective role of increased myocardial glycogen stores in cardiac anoxia has been demonstrated (Scheuer & Stezoski, 1970; Hewitt et al., 1974). Rose et al. (1976) found a close association between

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the tissue glycogen content and the degree of histological damage during ischemia. Furthermore, elevated myocardial glycogen levels have been shown to decrease the rate of ATP depletion during ischemia in the perfused rat heart (Bailey et al., 1982).

Until recently, investigations of the regulation of myocardial glycogen metabolism during various physiological conditions or stresses have been hampered by the lack of a noninvasive method allowing continuous measurement of glycogen fluxes in situ. Instead, studies have had to rely on freeze-clamping hearts at various times and subsequent chemical analysis of the tissue. Furthermore, most studies were carried out with isolated, perfused hearts, raising questions about the applicability of their findings for the in vivo situation.

We have recently established ^{13}C NMR techniques for the study of heart metabolism in live guinea pigs at 20.19 MHz (Neurohr et al., 1983a; Neurohr & Shulman, 1984; Neurohr, 1984). In addition to the natural abundance ^{13}C NMR spectra of guinea pig heart, we could observe myocardial glycogen synthesis during D-[1-^{13}C]glucose infusion, as well as incorporation of ^{13}C label into glutamate from [2-^{13}C]acetate in vivo. With a 6-min time resolution, we detected the rapid degradation of the C-1 labeled myocardial glycogen during anoxia and the appearance of ^{13}C label in the methyl group of lactic acid. These experiments demonstrated that ^{13}C NMR allows continuous and nondestructive monitoring of glycogen fluxes in real time in the heart of a living animal.

In the present paper, we have improved the time resolution of the ^{13}C NMR spectra so that glycogen levels can be measured in 1 min. As a prerequisite, the relaxation properties of the C-1 glycogen carbons were measured in vivo at 20.19 MHz and, for a comparison, in vitro at 90.55 MHz. We have investigated in detail the time course of myocardial glycogen synthesis from D-[1-^{13}C]glucose in the heart of live guinea pigs. With the 1-min time resolution, we were able to measure the time course of glycogenolysis of the ^{13}C-labeled glycogen during anoxia. In order to relate the changes in glycogen levels observed in the ^{13}C NMR spectra to biochemically meaningful information, it was also necessary (i) to determine the degree of ^{13}C labeling of the C-1 glycogen carbons after D-[1-^{13}C]-glucose infusion, (ii) to establish whether heart glycogen is completely visible in the ^{13}C NMR spectra, and (iii) to ascertain to what extent the degradation of the ^{13}C-labeled glycogen reflected glycogenolysis of the entire glycogen pool.

Materials and Methods

Materials

Chemicals. D-[1-^{13}C]Glucose (90 atom % ^{13}C) was obtained from Merck Sharp & Dohme. Insulin (regular ILETIN I, 100 units/cm^3) was from Eli Lilly and Co., Indianapolis, IN. Rhizopus amyloglucosidase was purchased from Sigma. All other chemicals were reagent grade.

Animals. Adult female Hartley strain guinea pigs (Charles River Breeding Laboratories), weighing between 550 and 650 g, were maintained on water and chow ad libitum until the day of study.

Methods

In Vivo Heart Preparation. Animals were anesthetized with sodium pentobarbitol and mechanically ventilated on room air through a tracheal cannula. An intravenous polyethylene catheter (PE-50 tubing) was placed in the jugular vein to be used for infusions. The common carotid artery was cannulated, and arterial pressure and heart rate were monitored throughout each experiment. The animals were placed on a platform warmed to 40 °C through a circulating water coil. The thorax was opened through a midsternal incision and the pericardium removed. An incision was made between ribs on each side, and the ribs were tied back, away from the heart. The heart was then positioned within the solenoidal receiver coil. This in vivo heart preparation was found to be consistently stable for at least 7 h, as arterial pressure and heart rate remained stable and no change in the intensity of the phosphocreatine resonance was detected in ^{31}P NMR spectra of the heart.

In Situ Hydrolysis of Glycogen. A pentobarbitol-anesthetized, open-chested guinea pig (prepared as above) received an infusion of 0.7 g of D-[1-^{13}C]glucose, mixed with 50 units of insulin in 4.5 mL, pH 7.4, into the jugular vein at a rate of 0.1 mL/min. Approximately 30 min after the termination of the infusion, the heart was quickly excised, cut into four pieces, and transferred to a 10-mm NMR tube. ^{13}C NMR spectra were recorded immediately in 16-min time blocks.

Extraction of Guinea Pig Heart Glycogen. Hearts were isolated from ad libitum fed guinea pigs under pentobarbitol anesthesia. The thorax was opened through a midsternal incision and the pericardium removed. The heart was quickly excised and freeze-clamped between aluminum plates cooled to liquid nitrogen temperature. Glycogen was extracted from the hearts by alkaline extraction. Two milliliters of a saturated NaOH solution per gram of heart was added, and the mixture was heated in a water bath at 100 °C for 1–2 h. The red soap cake was discarded, and the volume of the clear gelatinous lower phase was doubled with distilled water. The solution was made 33% or 50% by volume in ethanol to precipitate the glycogen and centrifuged for 10 min. Glycogen samples were dissolved in 10 mL of Millipore water, dialyzed first against a 20 mM EDTA solution (2 L) and then against two changes of Millipore water (2 L each), and finally lyophilized. For measurements of the degree of ^{13}C enrichment of the C-1 glycogen carbons after D-[1-^{13}C]glucose infusions (see below), hearts were quickly excised after the termination of such infusions and immediately extracted as above. In some cases, hearts were freeze-clamped and extracted later.

In Vitro Hydrolysis of Glycogen. The extracted myocardial glycogen was dissolved in 2 mL of 50 mM potassium phthalate buffer, pH 4.5, containing 5% D_2O. A control ^{13}C NMR spectrum was acquired at 55 °C within 12.8 min. Rhizopus amyloglucosidase (0.4 mg) in 30 μL of phthalate buffer was then added, and ^{13}C NMR spectra were acquired continuously in 12.8-min time blocks. In another experiment, the extracted glycogen was dissolved in 2 mL of buffer, as above. Sodium [2-^{13}C]acetate was added to serve as a concentration standard (2 mM), and a control ^{13}C NMR spectrum was recorded in 3.4 h at 24 °C. Thirty microliters of a 100 mg/mL rhizopus amyloglucosidase solution was then added, and the sample was kept at room temperature for 2 h. After complete hydrolysis was achieved, a second ^{13}C NMR spectrum was recorded in 3.4 h with the same parameters (see below).

Nuclear Magnetic Resonance. (1) ^{13}C NMR in Live Guinea Pigs. ^{13}C NMR spectra of heart in situ in live guinea pigs were obtained at 20.19 MHz on an Oxford Research Systems TMR-32/200 spectrometer. Spectra were obtained without the use of a field-frequency lock. The magnetic field was shimmed by using the ^1H NMR resonance from the heart. The line width of the H_2O signal was generally in the range of 25–35 Hz.

^{13}C NMR spectra were acquired with a six-turn solenoidal receiver coil mounted inside a one-turn saddle-shaped decoupling coil tuned to the ^1H frequency of 80.29 MHz (Neurohr, 1984). The 90° pulse width of the ^{13}C receiver coil was 13

μs, determined with a standard sample of 1 M D-[1-^{13}C]glucose in 0.15 M KCl. Generally, gated proton decoupling was employed, with 3–4 W of power during data acquisition. In some experiments, bilevel decoupling was used, with 0.5–0.8 W of power during the delay. ^{13}C NMR spectra were generally acquired with a 16-kHz spectral width, a 90° pulse width, and recycle times in the range of 60–200 ms. The spin–lattice relaxation time of the C-1 carbons of glycogen in live guinea pigs was measured with the inversion–recovery method. The nuclear Overhauser enhancement of the C-1 glycogen carbons was measured from the ratio of the signal areas of the C-1 resonance in spectra with bilevel and gated proton decoupling.

(2) Experimental Protocol for ^{13}C NMR Experiments in Vivo. (a) Glucose Infusion. After natural abundance ^{13}C NMR spectra of heart in vivo were obtained, serial spectra were obtained during infusion of 0.5 g of D-[1-^{13}C]glucose (90 atom % ^{13}C) mixed with 50 units of insulin in 4.3 mL, pH 7.4, into the jugular vein at a rate of 0.072 mL/min. During experiments aimed at investigating glycogen turnover, the above infusion was followed by an infusion of 0.5 or 1.5 g of D-glucose (with ^{13}C in natural abundance of 1.1%) and 50 units of insulin in 4.3 mL, pH 7.4, at a rate of 0.072 mL/min.

(b) Anoxia. Control ^{13}C NMR spectra were obtained after the termination of D-[1-^{13}C]glucose infusion. Anoxia was then induced by turning off the respirator, and ^{13}C NMR spectra of the heart in vivo were acquired continuously in 1-min time blocks.

^{13}C NMR in Vitro. ^{13}C NMR spectra of extracted heart glycogen were obtained at 90.55 MHz on a Bruker WH 360 spectrometer in a Bruker 10-mm probe. Gated proton noise decoupling (with 3–5 W of power) was employed. Spin–lattice relaxation times were measured with the inversion–recovery method. For the quantitative hydrolysis of extracted heart glycogen and of glycogen in excised guinea pig heart in situ, ^{13}C NMR spectra were obtained with a 70° pulse width, a 0.068- or 0.2-s acquisition time, and a 1.5-s recycle time. As shown previously by Sillerud & Shulman (1983), corrections to the observed resonance intensities due to (a) partial saturation, (b) pulse angle $\theta < 90°$ and (c) the partial nuclear Overhauser enhancement obtained during the acquisition time are small under these conditions. Signal areas were integrated manually and with the aid of the Bruker software.

(3) ^{1}H NMR in Vitro. ^{1}H NMR spectra of extracted heart glycogen were obtained at 360 MHz on a Bruker WH 360 spectrometer in 10-mm sample tubes, using a 6-kHz spectral width, a 0.17-s acquisition time, a 90° pulse width, a 15-s delay, and 128 scans. Glycogen samples (~9–12 mg) were dissolved in 2 mL of D_2O (99.8%), and the ^{2}H resonance was used for field-frequency locking of the spectrometer. The sample temperature was 85 °C, in order to shift the water resonance away from the H-1 glycogen resonance. The water peak was presaturated with 0.1 W of power.

Results

Myocardial Glycogen Synthesis in Vivo. The time course of myocardial glycogen synthesis was investigated in nine guinea pigs. ^{13}C NMR spectra were collected continuously during and following the termination of D-[1-^{13}C]glucose infusions (see Methods) in 4-min time blocks. In each case, the C-1 glycogen signal at 100.5 ppm increased during the infusion and reached a plateau at the end of the infusion, as observed in earlier experiments (Neurohr et al., 1983a). Upon completion of the [1-^{13}C]glucose infusion, the α and β C-1 glucose signals decreased, while the intensity of the C-1 glycogen signal remained constant for at least 3 h; longer times have not been investigated. In each animal, peak heights of the glycogen C-1 resonance at various times during the infusion were expressed as percentages of the final intensity. Results were then averaged over the nine animals and are shown in Figure 1. The results indicate that myocardial glycogen synthesis proceeded essentially linearly over the time course of the glucose infusion. In order to measure the degree of ^{13}C enrichment of the C-1 glycogen carbons in guinea pig heart during the above conditions of glucose infusion, hearts were rapidly excised and freeze-clamped after the termination of glucose infusions. Glycogen was extracted from the hearts (see Methods) and studied by ^{1}H NMR at 360 MHz in vitro. In the ^{1}H NMR spectrum of the C-1 glycogen protons, the protons bonded to ^{12}C carbons give rise to a singlet, while the protons bonded to ^{13}C carbons appear as a doublet, due to ^{13}C–^{1}H spin–spin coupling (data not shown). From the ratio of the doublet to the singlet intensities, the degree of ^{13}C enrichment of the C-1 carbons of myocardial glycogen was determined to be 18 ± 1.3% (mean ± standard deviation, n = 4).

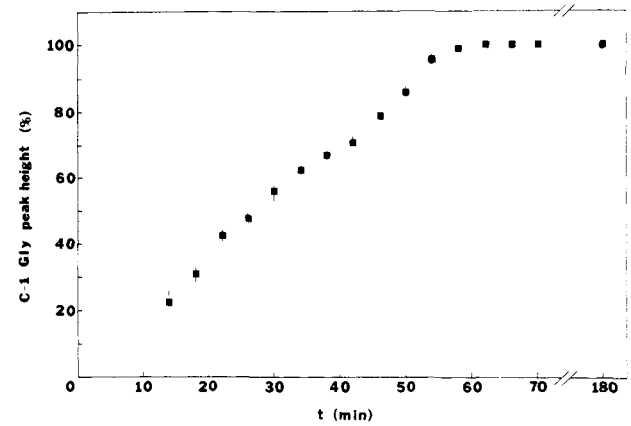

FIGURE 1: Time course of myocardial glycogen synthesis in live guinea pigs during infusion of 0.5 g of D-[1-^{13}C]glucose and 50 units of insulin into the jugular vein. Data points are averages of nine experiments (±SEM) and were obtained from proton-decoupled ^{13}C NMR spectra acquired within 4 min.

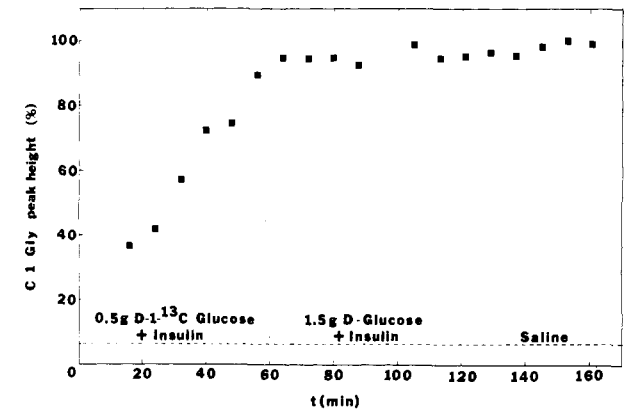

FIGURE 2: Time course of the peak height of the C-1 glycogen resonance during successive infusions of (a) 0.5 g of D-[1-^{13}C]glucose plus 50 units of insulin, (b) 1.5 g of D-glucose plus 50 units of insulin, and (c) saline. Each data point was obtained from a proton-decoupled ^{13}C NMR spectrum acquired within 8 min.

The possible turnover of the ^{13}C-labeled myocardial glycogen was also studied by ^{13}C NMR in vivo. In these experiments, guinea pigs received infusions of 0.5 g of D-[1-^{13}C]glucose and 50 units of insulin for a period of 1 h, immediately followed by infusions of 0.5 or 1.5 g of D-glucose (with ^{13}C in natural abundance of 1.1%) and 50 units of insulin over a period of 1 h. A typical experiment is shown in Figure 2. In all experiments, no significant change in the intensity of the C-1 glycogen signal was observed during the respective second

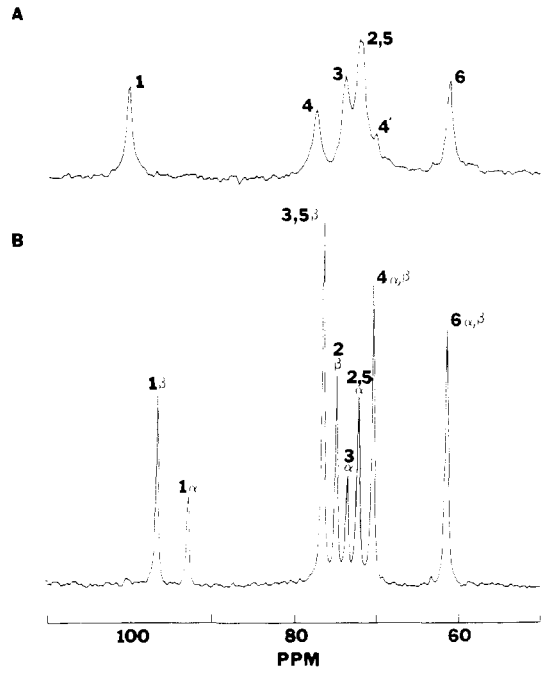

FIGURE 3: 90.55-MHz proton-decoupled natural abundance ^{13}C NMR spectra of the hydrolysis of extracted myocardial glycogen by amyloglucosidase. (A) Glycogen before hydrolysis; (B) after complete hydrolysis (see Methods). Both spectra were acquired with a 16-kHz spectral width, a 70° pulse width, a 0.068-s acquisition time, a 1.5-s recycle time, and 8192 scans (3.4 h each). 20-Hz digital line broadenings were applied to the FID's before Fourier transformation.

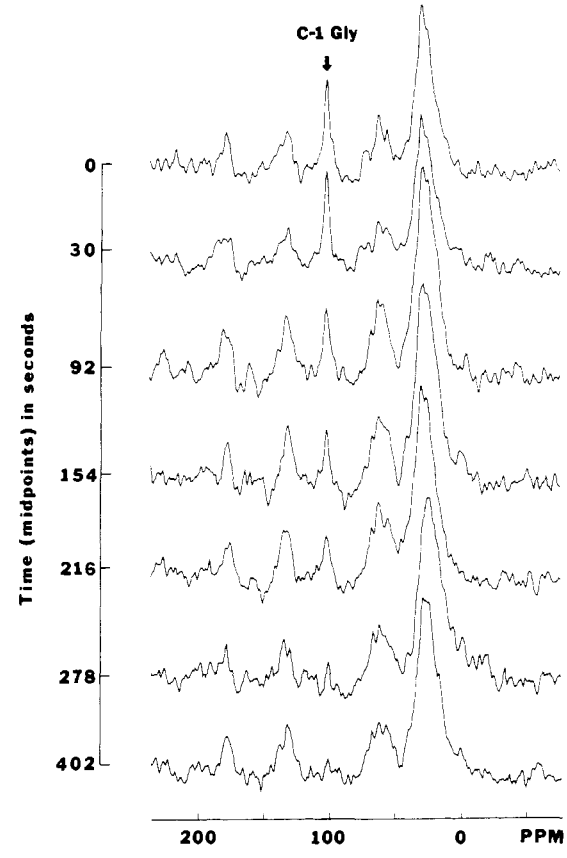

FIGURE 4: Glycogenolysis during myocardial anoxia measured by ^{13}C NMR in the heart of a live guinea pig. Spectra were acquired with a 16-kHz spectral width, 2K data points, a 90° pulse width, a 0.1-s recycle time, and 600 scans. Each spectrum thus required 1 min for total collection. 45-Hz digital line broadenings were applied to the FID's before Fourier transformation. The top spectrum is a control ^{13}C NMR spectrum obtained 90 min after the termination of D-[1-^{13}C]glucose infusion. After three such control spectra, were obtained, anoxia was induced by turning off the respirator and ^{13}C NMR spectra were collected serially. The times given on the ordinate represent midpoints of successive 1-min accumulations. There is an additional 2-s delay between subsequent spectra due to filing of spectra on a floppy disk.

infusion with unlabeled D-glucose. Additionally, subsequent infusion with saline after the unlabeled glucose infusion did not decrease the C-1 glycogen peak.

^{13}C NMR Relaxation of Myocardial Glycogen in Vivo and in Vitro. The spin–lattice relaxation time of the C-1 carbons of myocardial glycogen was measured in live guinea pigs. In these experiments, guinea pigs first received infusions of D-[1-^{13}C]glucose, as described above. When the intensity of the C-1 glycogen resonance had reached the plateau, as shown in Figure 1, the spin–lattice relaxation time of the C-1 carbons was measured by the inversion–recovery method. After the completion of the T_1 experiments, another ^{13}C NMR spectrum of the heart was recorded with the same parameters used during the glucose infusion to ensure that the intensity of the C-1 glycogen signal had not changed during the T_1 experiment. From experiments in four animals, the spin–lattice relaxation time of the C-1 glycogen carbons in live guinea pigs was found to be 48 ± 5 ms (mean ± standard deviation). The nuclear Overhauser enhancement of the C-1 carbons was also measured in vivo and found to be 1.3 ± 0.05. In order to compare the motional properties of myocardial glycogen in vivo and in vitro, spin–lattice relaxation times for glycogen carbons C-1 through C-6 were measured at 90.55 MHz in extracts obtained from the hearts of ad libitum fed guinea pigs. The natural abundance ^{13}C NMR spectrum of heart glycogen at 90.55 MHz is shown in Figure 3A. The assignments are those previously established for hepatic glycogen (Sillerud & Shulman, 1983). For carbons C-1 through C-5, which have one directly bonded proton, the T_1 values were 0.27 ± 0.02 s for C-1, 0.28 ± 0.03 s for C-2 and C-5, 0.27 ± 0.03 s for C-3, and 0.29 ± 0.03 s for C-4, respectively (averages of three determinations ± standard deviation). The average T_1 value for C-1 through C-5 was thus 0.28 ± 0.01 s. For C-6, which has two protons attached, T_1 was 0.13 ± 0.03 s. These values are slightly larger than the spin–lattice relaxation times measured for hepatic glycogen at 90.55 MHz, where T_1 was 0.23 ± 0.01 s for C-1 through C-5 and 0.11 s for C-6. No significant variation of the NT_1 values of C-1 through C-6 was found, indicating that the hexapyranoside ring undergoes isotropic reorientation.

Glycogenolysis during Myocardial Anoxia in Vivo. Due to the high sensitivity and good filling factor of the solenoidal receiver coil for ^{13}C and the use of short recycle times due to the short T_1, proton-decoupled ^{13}C NMR spectra of the labeled C-1 carbons of myocardial glycogen in live guinea pigs with a signal-to-noise ratio of ca. 14:1 were obtained in 1 min of data accumulation. This time resolution allowed monitoring of rapid fluxes in myocardial glycogen levels during anoxia. The top spectrum in Figure 4 is a 1-min proton-decoupled ^{13}C NMR spectrum of guinea pig heart in vivo, obtained 90 min after the termination of D-[1-^{13}C]glucose infusion. At least three such control spectra were recorded before each anoxia experiment. In these spectra, the intensity of the C-1 glycogen resonance was constant within 5%. Anoxia was then induced by turning off the respirator, and ^{13}C NMR spectra were accumulated continuously in 1-min time blocks, as shown in Figure 4. As clearly seen from these spectra, the C-1 glycogen signal decreased and finally disappeared. Figure 5 shows the time course of mobilization of labeled glucose units from myocardial glycogen during anoxia in vivo, averaged from data obtained in seven animals. During the first minute of anoxia,

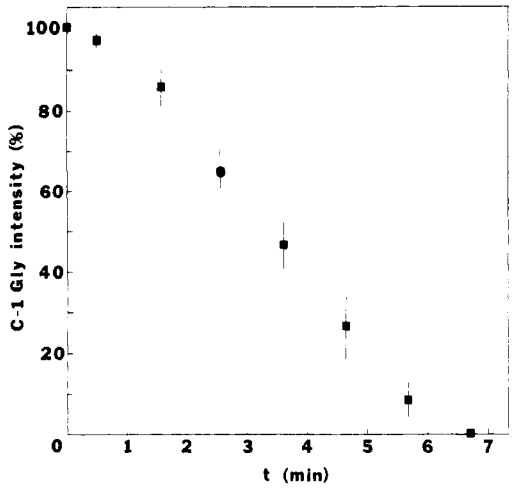

FIGURE 5: Time course of the degradation of the 1-^{13}C-labeled myocardial glycogen during anoxia in guinea pig heart in vivo. Data points represent averages of seven experiments (±SEM). In each experiment, the respirator was turned off at time zero and the peak height of the C-1 glycogen resonance was measured from serially acquired 1-min proton-decoupled ^{13}C NMR spectra (see Figure 4).

only approximately 10% of the labeled glycogen was degraded. However, at ca. 3 min, 50% of the ^{13}C-labeled glycogen had been degraded, and at ca. 6.7 min (on the average in our seven experiments), the C-1 signal was no longer detectable. In order to measure the degree of ^{13}C enrichment of the C-1 glycogen carbons after anoxia, one heart was excised from a guinea pig at 8 min and another at 11 min after the induction of anoxia. Glycogen was extracted from these hearts, and ^{1}H NMR spectra were recorded at 360 MHz with the same parameters used for ^{1}H NMR of glycogen extracts prepared from hearts after D-[1-^{13}C]glucose infusion (see Methods). After 8 min of anoxia, only a singlet was observed for the H-1 glycogen protons, indicating that the residual glycogen present at that time was no longer ^{13}C labeled at C-1. This is consistent with the in vivo measurement (Figure 5). Furthermore, within the animal variability, the intensity of the H-1 singlet after 8 min of anoxia was only slightly smaller than the H-1 singlet intensity in ^{1}H NMR spectra of glycogen extracts prepared from hearts after D-[1-^{13}C]glucose infusion (data not shown). This observation indicates that the ^{13}C-labeled glycogen was utilized preferentially after the onset of anoxia. After 11 min of anoxia, the intensity of the H-1 singlet was reduced by approximately 60%, demonstrating significant degradation of the residual endogenous glycogen.

NMR Visibility of Myocardial Glycogen. Natural abundance ^{13}C NMR experiments on hepatic glycogen have recently demonstrated that all of the carbon nuclei contribute to the high-resolution ^{13}C NMR signals, despite the very large molecular weight of glycogen (Sillerud & Shulman, 1983). In order to investigate possible differences between myocardial and hepatic glycogen in this respect, we have in the present work studied the quantitative hydrolysis of extracted heart glycogen to glucose by ^{13}C NMR at 90.55 MHz (Figure 3). Amyloglucosidase cleaves both the α(1–4) and α(1–6) linkages in glycogen, so that the hydrolysis proceeds to completion. In the experiment shown in Figure 3, a ^{13}C NMR spectrum of glycogen was recorded before hydrolysis (Figure 3A) and a second spectrum was acquired after complete hydrolysis to glucose had taken place (Figure 3B). The glucose assignments in Figure 3B are those of Walker et al. (1976). The carboxyl carbon resonances of the phthalate buffer and the methyl carbon resonance of added [2-^{13}C]acetate were used as internal intensity standards. In both spectra, the intensities of these

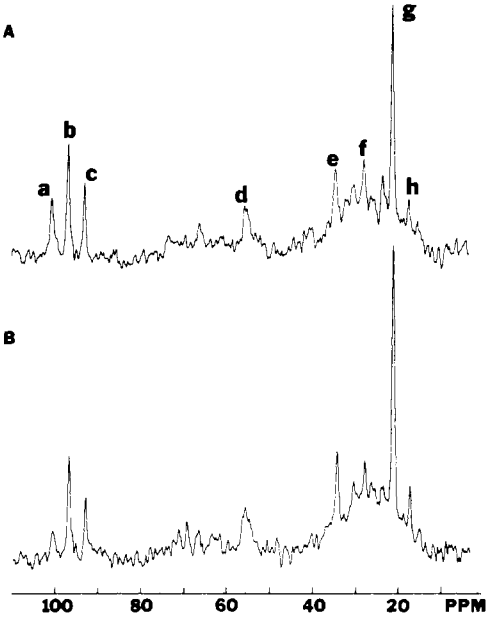

FIGURE 6: 90.55-MHz proton-decoupled ^{13}C NMR spectra of guinea pig heart excised after D-[1-^{13}C]glucose infusion in vivo (see Methods). (A) and (B) represent the first two spectra recorded after excision in 16 min each. The assignments of the ^{13}C-labeled peaks are as follows: a, glycogen C-1; b, β-D-glucose C-1; c, α-D-glucose C-1; d, glutamate C-2; e, glutamate C-4; f, glutamate C-3; g, lactate C-3; h, alanine C-3.

resonances were the same within ±5%. In another experiment, glycogen hydrolysis by amyloglucosidase was monitored continuously by ^{13}C NMR in 12.8-min time blocks. Integrated signal areas of the glycogen and glucose resonances (C-1 through C-6) were obtained and corrected as described under Methods. From these in vitro experiments, the percentage of glycogen that gives rise to high-resolution ^{13}C NMR signals was determined to be 96 ± 6%.

The hydrolysis of myocardial glycogen was also studied in excised guinea pig heart by ^{13}C NMR at 90.55 MHz (see Methods). Figure 6 shows the first two ^{13}C NMR spectra of the heart obtained after excision. The spectra show the presence of ^{13}C label in C-1 of glycogen and C-3 of lactate, as well as in C-2, C-3, and C-4 glutamate and in C-3 of alanine. As can be seen from a comparison of spectra A and B, the C-1 glycogen signal (peak a) has decreased in (B), while the C-3 lactate signal (peak g) has increased. The signals from the C-1 carbons of glucose did not change significantly. In order to correct the C-3 lactate intensities for partial saturation, a fully relaxed ^{13}C NMR spectrum with a 20-s pulse delay was recorded after complete hydrolysis of the glycogen. Integration of the signals due to C-1 of glycogen and C-3 of lactate showed that the increase in the intensity of the C-3 lactate signal corresponded within ca. 10% to the decrease in the intensity of the C-1 glycogen signal. This result is in agreement with the above ^{13}C NMR experiments on extracted heart glycogen, demonstrating that essentially all the carbons in glycogen contribute to the high-resolution ^{13}C NMR signals.

Discussion

In the present work, we have studied several aspects of myocardial glycogen metabolism in live guinea pigs by ^{13}C NMR. To help evaluate the ^{13}C NMR spectra, our ^{13}C NMR studies of glycogen hydrolysis in excised guinea pig heart and in vitro demonstrated that essentially all the ^{13}C nuclei in myocardial glycogen contribute to the high-resolution signals. The intensity of the C-1 glycogen resonance in the ^{13}C NMR spectra in vivo is therefore representative of the total amount

of ^{13}C label incorporated into myocardial glycogen.

The synthesis of myocardial glycogen from D-[1-^{13}C]glucose was investigated in nine animals. Because the resonance due to the C-1 carbons of glycogen is downfield from the resonances due to the C-1 peaks of α- and β-D-glucose, signals arising from the C-1 carbons of glycogen and glucose can be monitored simultaneously. The C-1 glycogen signal increased in intensity during the time course of the glucose infusion and reached a plateau at the end of infusion of labeled glucose. After the completion of the infusion, the C-1 glucose signals decreased, while the intensity of the C-1 glycogen signal remained constant for at least 3 h. Figure 1 indicates that myocardial glycogen synthesis proceeded at an essentially constant rate during the glucose infusion. However, the present signal-to-noise ratios preclude accurate measurement of the C-1 glycogen signal during the first 10 min of glucose infusion. It is possible that glycogen synthesis does not start immediately after the onset of glucose infusion and thus proceeds initially at a higher rate.

When D-[1-^{13}C]glucose infusions were followed by D-glucose infusions (Figure 2), no significant change in the intensity of the C-1 glycogen signal was detected, indicating that the labeled glucose units incorporated into glycogen during the first infusion did not exchange with glucose units during the second infusion. It is likely that additional glycogen synthesis takes place during the second infusion with D-glucose. As D-glucose contains ^{13}C only in natural abundance of 1.1%, the change in intensity of the C-1 glycogen signal due to glycogen synthesis during the D-glucose infusion is expected to be negligible.

After specific ^{13}C labeling of myocardial glycogen, we were able to measure the spin–lattice relaxation time and the nuclear Overhauser enhancement of the C-1 glycogen carbons in vivo. This allowed information on the motional properties of glycogen to be obtained in situ in the heart of live guinea pigs. The ^{13}C-1 glycogen carbons are relaxed by a dipolar interaction with the directly attached proton. Assuming isotropic rotational reorientation of the ^{13}C label, the T_1 and NOE value indicate a correlation time for the C-1 carbons of myocardial glycogen in vivo of ca. 1×10^{-8} s (Doddrell et al., 1972). The 90.55-MHz T_1 values for carbons C-1 through C-5 of extracted heart glycogen point to a similar correlation time, suggesting no strong difference in the motional properties of the glycogen carbons in vivo and in vitro.

We have investigated the time course of glycogenolysis of the labeled glycogen during myocardial anoxia. Several studies have demonstrated rapid utilization of heart glycogen stores during anoxia (Opie, 1976; Capasso et al., 1981; Liedtke, 1981). The amount of heart glycogen and its rate of degradation are important factors in myocardial survival during and subsequent to anoxia (Scheuer & Stezoski, 1970; Hearse & Chain, 1972; Rovetto et al., 1973). Studies on perfused rat heart have shown that increased myocardial glycogen stores provide improved mechanical resistance to anoxia, enhanced glycogenolysis, and higher rates of glycolytic ATP generation (Scheuer & Stezoski, 1970; Hewitt et al., 1974). Previous measurements of glycogen fluxes during anoxia, or other stresses, have had to rely on freeze-clamping hearts at various times and subsequent chemical analysis. In addition, most studies were performed with isolated, perfused hearts, raising questions about the relevance of their findings for the situation in the living animal, where respiratory, vascular, neural, and hormonal factors play an important part in the control of myocardial function. With ^{13}C NMR, changes in labeled glycogen levels can be measured in real time in the heart of a single animal, as demonstrated in Figure 4. The time course of mobilization of the labeled glucose units from glycogen during myocardial anoxia, averaged from data obtained in seven animals, is shown in Figure 5. Our data show that degradation of the ^{13}C-labeled glycogen was not stimulated immediately after the onset of anoxia. As shown in Figure 5, labeled glycogen levels changed by less than 10% during the first minute of anoxia. However, at 3 min, 50% of the labeled glycogen had been degraded, and at 6.7 min (on the average in our seven experiments), the C-1 glycogen signal could no longer be detected.

^{1}H NMR examination of extracts obtained from hearts freeze-clamped after the termination of D-[1-^{13}C]glucose infusion shows that the degree of ^{13}C enrichment of the C-1 glycogen carbons under our infusion conditions (see Methods) is 18 ± 1.3%. Previously, studies of isolated, perfused rat heart have shown that insulin increases both the amount of tissue glycogen and its accessibility to phosphorylase (Bailey et al., 1982). This result was explained by insulin increasing the rate of synthesis of $\alpha(1-4)$ linkages by its action on glycogen synthase, while leaving the activity of the branching enzyme unaffected (Villar-Palasi & Larner, 1960). On this basis, the ^{13}C-labeled glucose units incorporated into glycogen in the presence of insulin in our experiments would be expected to be $\alpha(1-4)$ linked to the ends of glycogen chains. Consistent with this, and on the basis that phosphorylase is activated during anoxia, ^{1}H NMR studies of the myocardial glycogen at different times after the induction of anoxia demonstrated that the ^{13}C-labeled glycogen was utilized preferentially after the onset of anoxia.

We have previously studied heart high energy phosphate metabolism during anoxia and recovery in live guinea pigs by ^{31}P NMR (Neurohr et al., 1983b). Myocardial levels of phosphocreatine and ATP were measured by ^{31}P NMR with a 20.5-s time resolution during 3 min of anoxia and subsequent reventilation and, in separate experiments, during terminal anoxia. These experiments were performed with the same open-chest guinea pig preparation used in the present ^{13}C NMR study, allowing comparison of the time courses of high-energy phosphate and labeled glycogen utilization during the same stress in vivo. The ^{31}P NMR studies showed a rapid decline of phosphocreatine after the onset the anoxia. The data points could be fitted to a single exponential with a $t_{1/2}$ of 54.5 ± 2.5 s (±SEM, n = 5). After 3 min of anoxia, phosphocreatine concentration had decreased to 20% of the original level, and at ca. 5 min, phosphocreatine had declined to undetectable levels. By contrast, ATP concentration did not change significantly during the first 7 min of terminal anoxia. A comparison of the respective time courses for phosphocreatine [Figure 2 in Neurohr et al. (1983b)] and for labeled glycogen decline (Figure 5) shows that phosphocreatine was degraded first after the onset of anoxia. In addition, the rate of phosphocreatine decline was considerably higher than that of glycogen degradation. For example, at 54.5 s into the anoxic period, 50% of the phosphocreatine had been degraded, while the ^{13}C-labeled glycogen had decreased by less than 10% (Figure 5). Significant hydrolysis of labeled myocardial glycogen stores occurred only after ca. 90 s of anoxia, when phosphocreatine concentration had already decreased to approximately 32% of the original level. Averaged over our seven experiments, the ^{13}C-labeled glycogen was completely degraded at ca. 7 min. After the utilization of the labeled glycogen, residual endogenous glycogen was degraded.

The experimental setup and the procedures employed in the present paper provide a basis for further investigations of glycogen metabolism by ^{13}C NMR in the in vivo heart. The

application of specifically ^{13}C-labeled glucose allowed measurement of the time course of glycogen synthesis and of the mobilization of glucose units from glycogen during oxygen deprivation by ^{13}C NMR in vivo with good time resolution. Additional experiments relating labeled glycogen consumption to total glycogen consumption would extend the quantitative information available from these measurements. Other aspects of the regulation of glycogen synthesis or glycogenolysis could be studied, including, for example, the effects of catecholamines, β-blockers, increased mechanical activity, or other stresses, such as prolonged hypoxemia or ischemia. Due to the noninvasive nature of the NMR technique, changes in labeled glycogen levels in response to changes in physiological conditions or pharmacological interventions can be measured in real time in the heart of a single animal. Simultaneously monitored mechanical parameters, as well as changes in high-energy phosphates measured by ^{31}P NMR under the same conditions, can be directly correlated with changes in labeled glycogen levels, providing an opportunity to investigate the relationship of heart function to in vivo myocardial biochemistry.

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References

Bailey, I. A., Radda, G. K., Seymour, A.-M. L., & Williams, S. R. (1982) *Biochim. Biophys. Acta 720*, 17–27.

Capasso, J. M., Lemma, T. M., & Zimmerman, J. A. (1981) *Exp. Gerontol. 16*, 261–269.

Doddrell, D., Glushko, V., & Allerhand, A. (1972) *J. Chem. Phys. 56*, 3683–3689.

Hearse, D. J., & Chain, E. B. (1972) *Biochem. J. 128*, 1125–1133.

Hewitt, R. L., Lolley, D. M., Adrouny, G. A., & Drapanas, Th. (1974) *Surgery (St. Louis) 75*, 1–10.

Liedtke, A. J. (1981) *Prog. Cardiovasc. Dis. 23*, 321–336.

Neurohr, K. J. (1984) *J. Magn. Reson.* (in press).

Neurohr, K. J., & Shulman, R. G. (1984) in *Advances in Myocardiology* (Dhalla, N. S., & Hearse, D. J., Eds.) Vol. 6, Plenum Press, New York (in press).

Neurohr, K. J., Barrett, E. J., & Shulman, R. G. (1983a) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1603–1607.

Neurohr, K. J., Gollin, G., Barrett, E. J., & Shulman, R. G. (1983b) *FEBS Lett. 159*, 207–210.

Opie, L. H. (1976) *Circ. Res. 38*, 52–68.

Opie, L. H., & Owen, P. (1976) *Am. J. Cardiol. 38*, 310–321.

Opie, L. H., Bruyneel, K., & Owen, P. (1975) *Circulation 52*, 49–57.

Randle, P. J., & Tubbs, P. K. (1979) in *Handbook of Physiology: The Cardiovascular System* (Berne, R. M., Ed.) Vol. 1, pp 805–844, American Physiological Society, Bethesda, MD.

Rose, A. G., Opie, L. H., & Bricknell, O. L. (1976) *Arch. Pathol. Lab. Med. 100*, 516–521.

Rovetto, M. J., Whitmer, J. T., & Neely, J. R. (1973) *Circ. Res. 32*, 699–711.

Scheuer, J., & Stezoski, S. W. (1970) *Circ. Res. 27*, 835–849.

Sillerud, L. O., & Shulman, R. G. (1983) *Biochemistry 22*, 1087–1094.

Villar-Palasi, C., & Larner, J. (1960) *Biochim. Biophys. Acta 39*, 171–173.

Villar-Palasi, C., & Larner, J. (1968) *Vitam. Horm. (N.Y.) 26*, 65–118.

Walker, T. E., London, R. E., Whaley, T. W., Barker, R., & Matwiyoff, N. A. (1976) *J. Am. Chem. Soc. 98*, 5807–5813.