Metabolic Heterogeneity of Carbon Substrate Utilization in Mammalian Heart: NMR Determinations of Mitochondrial versus Cytosolic Compartmentation†

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Received April 6, 1992; Revised Manuscript Received July 3, 1992

ABSTRACT: Carbon-13 (^{13}C) nuclear magnetic resonance (NMR) spectroscopy can be used to target specific pathways of intermediary metabolism within intact tissues and was employed in this study to evaluate the compartmentation of pyruvate metabolism between the cytosol and mitochondrial matrix. The distribution of ^{13}C into the tissue alanine, lactate, and glutamate pools was evaluated during metabolism of [3-^{13}C]-pyruvate in intact, isolated perfused rabbit hearts with and without activation of pyruvate dehydrogenase activity by dichloroacetate (5 mM). Equilibrium between the intracellular alanine and pyruvate pools was in evidence from the rapid evolution of the steady-state ^{13}C signal arising from the 3-carbon of alanine in intact hearts perfused with 2.5 mM 99.4% [3-^{13}C]pyruvate. Augmented pyruvate oxidation, in response to perfusion with dichloroacetate, was evident within ^{13}C NMR spectra of intact hearts as a relative increase in signal intensity of 53–62% ($p < 0.05$) from the 4-carbon resonance of ^{13}C-enriched glutamate when compared to the unaffected alanine signal. The increased bulk flow of [3-^{13}C]pyruvate into the tricarboxylic acid cycle in response to dichloroacetate resulted in elevated fractional enrichment of glutamate from 68% in controls to 83% in the treated group ($p < 0.04$), via interconversion with α-ketoglutarate, without changes in the actual tissue content of glutamate. Evidence of metabolic heterogeneity of cytosolic and mitochondrial pyruvate pools was also obtained from analysis of tissue extracts with in vitro NMR spectroscopy. Although dichloroacetate increased the percentage of labeled acetyl CoA units entering the tricarboxylic acid cycle from 83% to 94% ($p<0.03$), indirect assessment from the observed enrichment levels of alanine showed the fractional enrichment of intracellular pyruvate in both groups to be on the order of only 60%. Lactate formation from labeled pyruvate, as opposed to endogenous pyruvate, was yet lower in the cytosol at 21–24%, remaining unaffected by the manipulation of pyruvate dehydrogenase activity. The results are consistent with different pools of pyruvate within the myocyte that are associated with either glycolytic metabolism within the cytosol or preferential oxidation within the mitochondrial matrix. Therefore, the metabolic fate of pyruvate is closely associated with the original carbohydrate source (endogenous versus exogenous) and the intracellular compartmentation of metabolic events. This metabolic heterogeneity was detected by virtue of communication between the cytosol and mitochondrial matrix as translated into the fractional ^{13}C enrichments of the primarily cytosolic alanine and glutamate pools which are then detectable by NMR spectroscopy.

In the mammalian heart, glycolytic end products that are produced in the cytosol from glucose or glycogen enter oxidative metabolism in mitochondria through the action of the highly regulated, multistep enzyme complex, pyruvate dehydrogenase (PDH). Located at the inner mitochondrial membrane, PDH ultimately governs the contribution of carbohydrates to carbon flux into the tricarboxylic acid (TCA) cycle, via the citrate synthase reaction (Denton et al., 1975; Hansford & Cohen, 1978; Randle, 1981). Although fatty acids are the predominate fuel source for myocardial energy production (Shipp et al., 1961; Johnston & Lewandowski, 1991), pyruvate oxidation remains a significant source of oxidative energy production in response to mechanical (Opie et al., 1971; Neely et al., 1972; Kobayashi & Neely, 1983b) or pathophysiological challenges (Kerbey et al., 1977) to the heart. However, the rates of pyruvate production (i.e., glycolytic rate) and oxidation are not necessarily the same (Kobayashi & Neely, 1979; Pisarenko et al., 1988; Lopaschuk et al., 1990) and can result in variable concentrations of pyruvate in both the cytosol and the mitochondrial matrix. The distinctions between cytosolic and mitochondrial metabolism of pyruvate have not been extensively analyzed in intact hearts with the potentially powerful tool of ^{13}C NMR spectroscopy. The current study is a ^{13}C NMR analysis of the metabolic fate of exogenous pyruvate in response to altered enzymatic activity of PDH.

Protocols utilizing ^{13}C NMR methods have provided unique information regarding the metabolic state of intact tissues in relation to the functional state of the myocardium (Lewandowski & Johnston, 1990; Johnston & Lewandowski, 1991; Lewandowski et al., 1991b; Lewandowski, 1992). However, interpretation of ^{13}C NMR signals from intact tissue and tissue extracts is not entirely straightforward. The most immediate problem in the interpretation of ^{13}C NMR studies is twofold. First, the evaluation of myocardial metabolism from ^{13}C NMR spectra is often centered on the analysis of the easily detected ^{13}C NMR signals arising from tissue glutamate. This glutamate pool is primarily cytosolic and analysis of mitochondrial activity is dependent on assumptions of fast equilibration between extra- and intramitochondrial metabolite pools as well as equilibrium between TCA cycle intermediates and glutamate (Randle et al., 1970; Chance et al., 1983). Secondly, relative measures of substrate entry into interme-

† This work was aided in part by Grant 89G-175 from the American Heart Association, Texas Affiliate, and by Grant 13-522-912 from the American Heart Association, Massachusetts Affiliate, Inc.

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0006-2960/92/0431-8916$03.00/0

diary pathways, such as the TCA cycle, are often mistaken for measures of flux rates, which would infer quantitative information about the amount of turnover within carbon pools. Before such absolute flux rates can be inferred from relative signal intensities within ^{13}C NMR spectra of either intact tissues or tissue extracts, the sensitivity of the ^{13}C NMR analysis methods to changes in the activity of specific enzymes and the fractional ^{13}C enrichment of the NMR detectable metabolite pools must be examined further.

In acknowledging these variables, the current study addresses differences in the metabolic fates of pyruvate pools in the cytosolic and mitochondrial compartments of functioning myocytes. The overall aims of this study were to (1) determine the sensitivity of ^{13}C NMR spectroscopy in discriminating between cytosolic and mitochondrial metabolism within intact, functioning hearts and (2) provide information on the previously described compartmentation of pyruvate pools within the myocyte (Peuhkurinen et al., 1983; Bunger, 1985). The delivery of ^{13}C-labeled pyruvate enabled NMR detection of alanine and glutamate to reflect the metabolic fate of pyruvate in the intact, perfused rabbit heart. The protocol utilizes evidence of rapid equilibrium between pyruvate and alanine pools in myocardium (Taegtmeyer et al., 1977; Peuhkurinen et al., 1982, 1983) to provide an internal reference for intracellular pyruvate via the alanine signal within ^{13}C NMR spectra. Such referencing enabled ^{13}C NMR detection of shifts in the relative activities of oxidative and nonoxidative metabolism of pyruvate in whole hearts. ^{13}C labeling of total tissue alanine and glutamate during manipulations of pyruvate oxidation provided insight into the interconversion of cytosolic and mitochondrial metabolite pools. The results allow comparison of metabolite labeling to the entry of pyruvate into the TCA cycle. From the sensitivity of ^{13}C NMR detection to differences in the overall activity of PDH (Johnston & Lewandowski, 1991), the relation of pyruvate oxidation to mitochondrial tricarboxylate formation was then analyzed.

MATERIALS AND METHODS

Isolated, Perfused Rabbit Heart Protocol. The isolated heart model allowed precise control over substrate availability and manipulation of enzymatic activity within the intact, functioning myocardium. Hearts were perfused in an NMR magnet, using previously described methods (Lewandowski & Johnston, 1990). Briefly, hearts were excised from Dutch belted rabbits (600–750 g) that were given an intraperitoneal injection of heparin (1200 IU) and then anesthetized with sodium pentabarbital (100 mg/kg intraperitoneal injection). Immediately upon excision, the heart was immersed in a solution containing 20 mM potassium chloride (KCl) and 120 mM sodium chloride (NaCl) for cardioplegia at 0 °C to preserve the energetic state of the organ until perfusion was begun. The aorta was attached to a 100-cm hydrostatic perfusion column and retrograde perfusion begun. A recirculating pump supplied modified Krebs–Henseleit buffer (116 mM NaCl, 4 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, and 25 mM $NaHCO_3$, equilibrated with 95% O_2/5% CO_2 at 37 °C) that initially contained 5 mM glucose within a 2-L reservoir. This perfusate supply was then changed at the start of each ^{13}C enrichment protocol to a 400-mL reservoir of Krebs–Henseleit buffer containing 2.5 mM [3-^{13}C]pyruvate (Isotec Inc., Miamisburg, OH) with no glucose. The temperature of the hearts was maintained at 37 °C by a control unit interfaced to the NMR system console and by the temperature of the perfusion medium. Hearts spontaneously beat, contracting against a fluid-filled intraventricular ballon which was connected to a pressure transducer. The balloon was inflated to an end diastolic pressure of 5–10 mm Hg. Left ventricular developed pressure and heart rate were continually recorded.

Two different groups of hearts received labeled pyruvate: a control group ($n = 8$) and a treated group ($n = 8$) that was perfused with buffer containing both labeled pyruvate and 5 mM dichloroacetate (DCA). This concentration of DCA is known to increase the active, nonphosphorylated form of PDH to near complete levels (Latipaa et al., 1983). Return flow from the perfusion system and coronary effluent line was not recirculated into the reservoir upon switching the perfusate supply. Instead, the first 150 mL of return flow was collected in a separate retaining vessel to washout any residual unlabeled glucose from the tubing of perfusion system. NMR spectra were then continually recorded over a 30-min perfusion period. Afterward, hearts were rapidly frozen within a liquid nitrogen cooled clamp (Wollenberger et al., 1960) for subsequent biochemical analysis and in vitro NMR spectroscopy.

Metabolite Assays. Tissue from freeze-clamped hearts was stored at cryogenic temperature (–70 °C). Perchloric acid extraction of tissue metabolites was performed on the frozen ventricles as described by others (Williamson & Corkey, 1967). The extract was neutralized, and the tissue contents of alanine, glutamate, and lactate were determined by UV spectrophotometric analysis (Williamson & Corkey, 1967; Williamson, 1974; Bernt & Bergmeyer, 1974). Glycogen was assayed (Bartley & Dean, 1968) in a subset of the hearts that were treated with dichloroacetate ($n = 4$). Acid extracts of 1-g tissue samples were lyophilized and then reconstituted in 0.5 mM of deuterium oxide (D_2O) for NMR analysis.

Nuclear Magnetic Resonance (NMR) Spectroscopy. All NMR data were collected on a Bruker AM400 series spectrometer interfaced to a 9.4 T, vertical bore, superconducting magnet (Bruker Instrument, Billerica, MA). Field homogeneity was adjusted by shimming on the proton signal from the sample to a line width of 8–20 Hz for isolated hearts (primarily H_2O signal) and 1–2 Hz for in vitro samples (primarily HDO signal). ^{31}P and ^{13}C spectra were obtained from isolated hearts perfused within a dual tuned, 20-mm NMR probe (Bruker Instruments) equipped with a proton decoupling coil. In vitro ^{13}C and proton spectra were acquired with a 5-mm probe (Bruker Instruments). The signal intensity of resonance peaks was determined by integration of the area under each peak using the analysis subroutine within the NMR-dedicated software (Bruker Instruments, DISNMR). The individual NMR signals from the different metabolites were expanded and individually phased prior to integration.

Collection of both ^{31}P and ^{13}C NMR spectra from the hearts was alternated during perfusion. NMR data were collected and processed as described in previous reports (Lewandowski & Johnston, 1990; Johnston & Lewandowski, 1991). ^{31}P spectra were recorded at 161 MHz by accumulating 32 transient signals (45° pulse) over 1 min using optimal settings for maximum signal-to-noise versus collection time (Becker et al., 1979). Raw data were converted into frequency domain spectra by Fourier transformation following application of an exponential filter to enhance the signal-to-noise ratio. Chemical shift assignments were made relative to the phosphocreatine peak at 0 ppm. Relative adenosine triphosphate (ATP) levels over the course of each protocol were assessed from the intensity of the β-phosphate peak within each spectrum.

^{13}C spectra were acquired at 101 MHz in 6-min time blocks (92, 45° pulses) from hearts perfused with ^{13}C-enriched pyruvate. ^{13}C NMR signals were proton decoupled using power-gated decoupling (0.5–6 W) to avoid sample heating. An

initial spectrum of ^{13}C natural abundance was acquired from each heart prior to the introduction of ^{13}C-enriched material for digital background subtraction from spectra collected during ^{13}C enrichment. NMR signals were processed by exponential filtering with a line broadening of 18–20 Hz followed by Fourier transformation. Peak assignments were referenced to the known resonance of the exogenous, ^{13}C-enriched substrate and the well-documented glutamate resonance signals relative to dioxane at 67.4 ppm. Changes in signal intensities due to nuclear Overhauser enhancement (NOE) or relaxation effects were minimal under these pulsing conditions (Malloy et al., 1988) as previously confirmed in this laboratory by comparison of spectra from intact hearts to ^{13}C spectra of extracts before and after the methods of inverse gated decoupling and varied interpulse interval as performed in previous studies (Cohen, 1987; Lewandowski et al., 1991a).

^{13}C spectra were collected from tissue extracts which were lyophilized and then reconstituted in 0.5 mL of deuterium oxide. The raw ^{13}C NMR signal was collected at 37 °C using 45° excitation pulses with 2-s interpulse delays during broad band proton decoupling. The composite free induction decay, obtained within a 8 kiloword data set, was then increased to 32K (zero-filling) to improve digital resolution of the transformed data and processed with a gaussian filter. The percent of labeled acetyl groups entering the TCA cycle (F_c) was determined from high-resolution ^{13}C spectra of in vitro samples. The multiplet structure of C-4 glutamate resonances within in vitro ^{13}C spectra allowed the percent of labeled acetyl groups entering the TCA cycle (F_c) to be calculated (Malloy et al., 1988) for each experimental condition.

The fractional enrichment of the initial site of labeling within glutamate, the 4-carbon position, was determined by a method similar to that described by Chance et al. (1983). The amount of 4-labeled glutamate was determined by comparison of the signal intensity from the 4-carbon of glutamate within the spectra of each sample to the signal from a standard 100 mM (1.1 mM ^{13}C natural abundance) solution of glutamate. The amount of labeled material was then referenced to the known total tissue concentration of glutamate from enzymatic assay as described in a previous report (Lewandowski & Hulbert, 1991).

In vitro proton spectra were collected over 30 min (45° pulse, 4-s interpulse interval) from the lyophilized tissue extracts reconstituted in 0.5 mL of deuterium oxide. High-resolution proton spectra were used to determine the fractional ^{13}C enrichments of alanine and lactate via the extent of the proton signal from the 3-carbon methyl groups that were split due to J coupling to ^{13}C versus unsplit signals arising from unlabeled methyl groups as described in elsewhere (Lewandowski et al., 1991a). The enrichment values obtained by this method were verified by comparison of ^{13}C signal from the 3-carbon position of alanine to the known concentration of alanine from enzymatic assay, as performed for determination of glutamate enrichment. A ^{13}C NMR spectrum from a standard 100 mM (1.1 mM ^{13}C natural abundance) solution of alanine was used as a reference signal for ^{13}C-enriched alanine levels in the tissue extracts, as described below for glutamate. Values obtained from proton spectra were within 10% of values determined by comparison of ^{13}C spectra from samples and the 100 mM alanine standard (1.1 mM natural abundance).

Statistical Analysis. Intergroup comparisons were performed using the Student's unpaired, two-tailed t test. Comparison of intragroup data sets was performed with the Student's paired, two-tailed t test. Changes in NMR data at each time over the 30-min perfusion period of each group of hearts were evaluated with the repeated measures analysis of variance. Differences in mean values were considered statistically significant at a probability level of less than 5% ($p < 0.05$). Data are presented as mean ± standard error (SE).

RESULTS

Contractile Function and High-Energy Phosphates. No difference in heart rate or developed pressure within the left ventricle occurred between untreated control hearts and DCA-treated hearts. Mechanical function showed no significant changes over time in either group throughout the perfusion period. Mean control heart rate was 214 ± 28 beats per minute versus 200 ± 11 in DCA-treated hearts. Mean left ventricular developed pressure was 118 ± 7 mm Hg in control hearts and 117 ± 9 in DCA-treated hearts. ^{31}P NMR spectra demonstrated that both phosphocreatine and ATP levels in both groups of hearts were consistent throughout the protocols. The mean ratio of phosphocreatine to ATP in control hearts was 1.93 ± 0.21, a value not statistically different from the mean of 1.82 ± 0.11 within the DCA-treated group. Therefore, the work state and bioenergetic balance of the myocardium was not affected by increasing the enzymatic activity of PDH. The similarities in physiological state allowed direct comparison of ^{13}C enrichment within tissue metabolites of the control and DCA-treated groups.

^{13}C NMR of Intact Hearts. Representative steady-state ^{13}C spectra acquired from intact hearts are shown at the top of Figure 1. The expanded portions of the corresponding in vitro ^{13}C spectra from the same hearts showing the multiplet structure of the resonance signal from the 4-carbon of glutamate are displayed at the bottom of Figure 1. An expanded region of a proton NMR spectrum of a tissue extract which shows the methyl group resonances of both labeled ($^{13}CH_3$) and unlabeled ($^{12}CH_3$) alanine and lactate is displayed in Figure 2. In combination, these NMR data provided insight into the effects of subcellular compartmentation on the fractional enrichment levels of tissue metabolite pools which must be considered when performing ^{13}C NMR studies of intact tissues (Lewandowski & Hulbert, 1991; Lewandowski, 1992).

The ^{13}C NMR signals arising from the 3-carbon resonance of labeled alanine within intact hearts rapidly reached steady-state levels within both the control group and the DCA-treated group. This steady-state signal, reflecting the availability of labeled pyruvate, persisted throughout the constant infusion of [3-^{13}C]pyruvate. The spectra in Figure 3 and the graph in Figure 4 illustrate the consistency in [3-^{13}C]alanine signal within ^{13}C NMR spectra of intact hearts under control conditions and DCA treatment.

Unlike the alanine NMR signal, the signal arising from the ^{13}C label which appeared at the 4-carbon position of glutamate rose over time, as previously shown (Lewandowski & Johnston, 1990). An example of the evolution of glutamate enrichment in comparison to the consistency in alanine labeling throughout the perfusion protocol is displayed in Figure 3. ^{13}C enrichment of glutamate occurred according to the previously described labeling patterns which stem from the interconversion of glutamate and the TCA cycle intermediate, α-ketoglutarate (Chance et al., 1983). When referenced to the internal signal from the 3-carbon of alanine, the NMR signal from the 4-carbon of glutamate not only increased over the time in both groups of hearts but was also significantly enhanced by DCA treatment. Figure 5 displays the relative NMR signal intensity ratios of the 4-carbon of glutamate to that of the 3-carbon of alanine (Glu C-4/Ala C-3). DCA

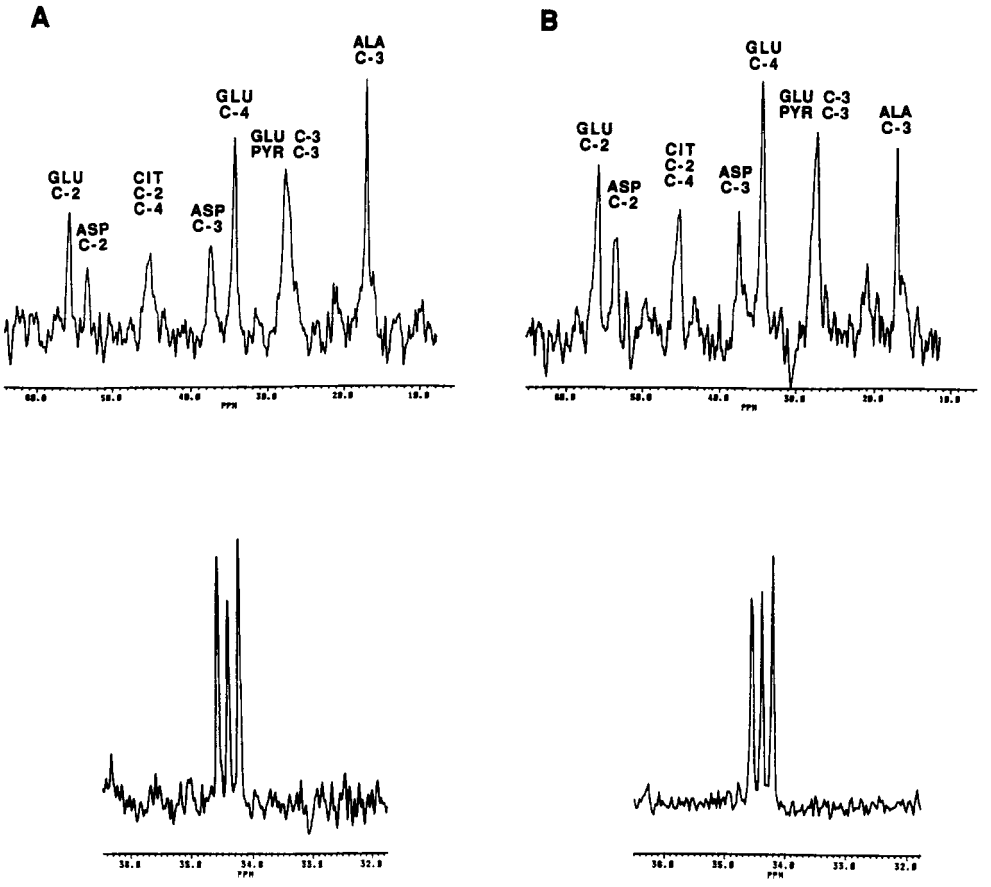

FIGURE 1: End-point ^{13}C NMR spectra (101 MHz) of isolated rabbit hearts perfused with 2.5 mM [3-^{13}C]pyruvate (at top) with corresponding resonance peaks from the 4-carbon of glutamate (GLU C-4) within expanded high-resolution, in vitro ^{13}C spectra (at bottom). Spectra in panel A are from a control heart. Spectra in panel B are from a heart treated with dichloroacetate. At top, note the relative increase in signal intensity from the 4-carbon signal of glutamate (GLU C-4) to that of 3-carbon signal of alanine (ALA C-3) in spectrum B from heart treated with dichloroacetate. Identifiable resonance peaks: GLU C-2, 2-carbon of glutamate; ASP C-2, 2-carbon of aspartate; CIT C-2, C-4, 2-, and 4-carbons of citrate, respectively; ASP C-3, 3-carbon of aspartate; GLU C-4, 4-carbon of glutamate; GLU C-3 PYR C-3, 3-carbon of glutamate obscured by 3-carbon of exogenous pyruvate; ALA C-3, 3-carbon of alanine. At bottom, note resonance peaks composed of central singlets flanked by doublet signals. A slight increase in the doublet to singlet signal intensity from heart treated with dichloroacetate in spectrum B corresponds to increased entry of labeled acetyl groups into the TCA cycle (See Figure 6).

increased the amount of labeled glutamate, which was reflected in the relative intensity of signals from glutamate and alanine within the NMR spectra as shown in Figure 1. Over the course of perfusion, mean Glu C-4/Ala C-3 ratios were 53–62% higher within DCA-treated hearts ($p < 0.05$), indicating enhanced entry of pyruvate into oxidative metabolism via the activation of PDH (Figure 5). Mean Glu C-4/Ala C-3 ratios from in vitro spectra were 1.64 ± 0.5 in controls and 2.2 ± 0.9 in DCA treated hearts. These mean values were higher but not statistically different from values obtained from intact hearts.

Metabolite Enrichment, Concentration, and Compartmentation. Tissue concentrations of glutamate and alanine were not affected by the administration of DCA (Table I) and therefore did not account for the changes in ^{13}C NMR signals from intact hearts that were induced by the PDH activation. Mean tissue glutamate concentrations were similar in control hearts and DCA-treated hearts as shown in Table I. Mean alanine content also was not significantly affected by DCA treatment, nor was total lactate different. From Table I, lactate levels were lower but also slightly more variable than alanine. Tissue glycogen was apparently unaffected by DCA, although this study did not include a complete analysis. Mean glycogen content from four of the DCA-treated hearts was 883 ± 83 mg/g of wet tissue weight. This value is similar to the literature value for glycogen content of 811 ± 56 in hearts perfused with 2.5 mM [3-^{13}C]pyruvate in this laboratory (Lewandowski et al., 1991a).

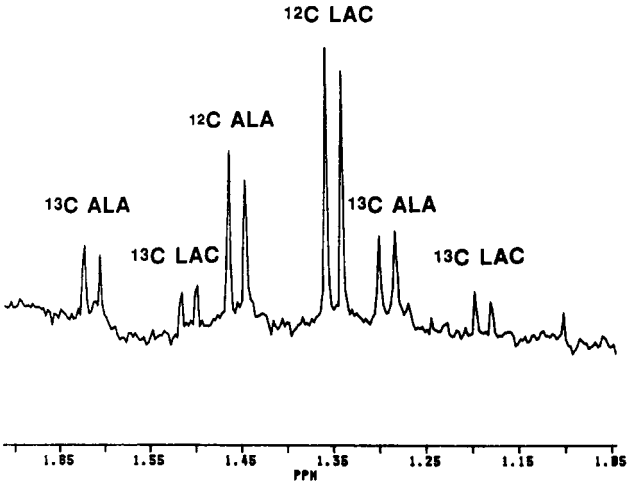

FIGURE 2: Methyl group resonances of alanine (ALA) and lactate (LAC) within proton spectrum (400 MHz) of a tissue extract from an individual, untreated heart. Note satellite doublet signals from methyl group protons coupled to ^{13}C which flank the central doublet signals from protons within methyl groups containing ^{12}C. Differences in the contributions of the ^{13}C-enriched methyl groups between alanine and lactate are in evidence. Signal intensities determined by integration of area under each resonance doublet (as opposed to signal height) give relative percentages of combined satellite signals from alanine to total alanine signal ($^{13}C + ^{12}C$). From this spectrum, split signals corresponded to 60% ^{13}C enrichment of alanine and 22% ^{13}C enrichment of lactate. The total signal from lactate represents 70% of the total signal from alanine.

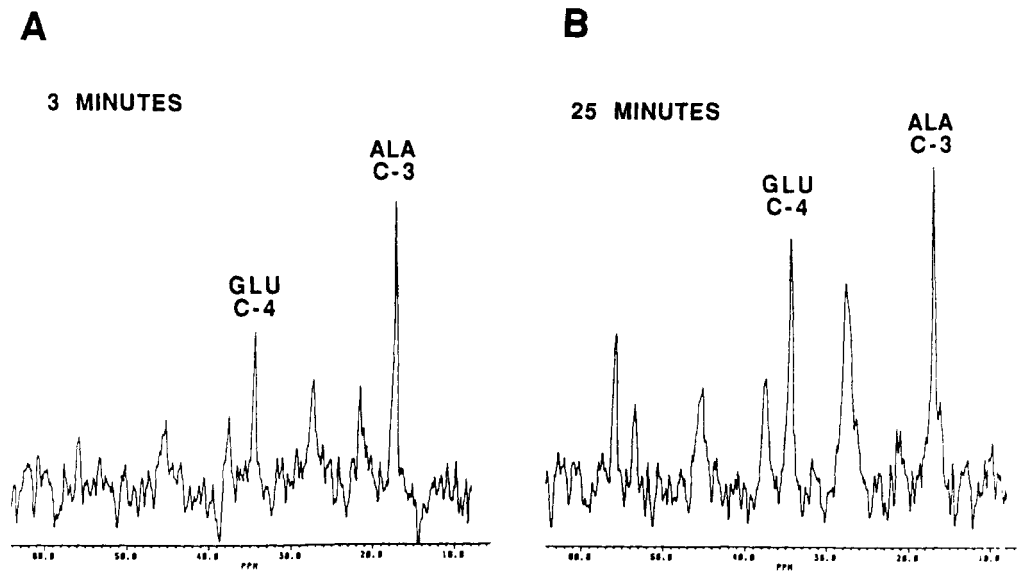

FIGURE 3: Changes in relative signal intensities within successive ^{13}C NMR spectra from a single isolated heart perfused with 2.5 mM [3-^{13}C]pyruvate. Spectrum A represents the 3-min time point of perfusion. Spectrum B was collected later at the 25-min time point of perfusion. Note the consistency in signal intensity within the resonance peak of the 3-carbon position of alanine (ALA C-3). Other resonance peaks are identified as in Figure 1.

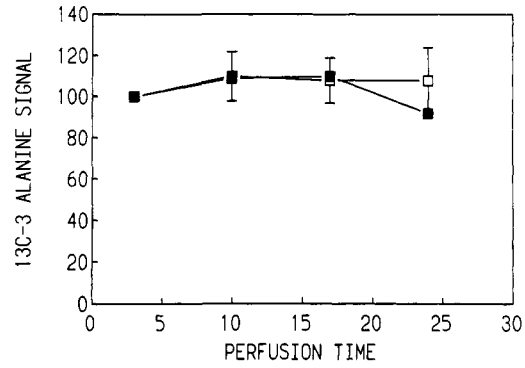

FIGURE 4: Relative change in mean signal intensities of alanine 3-carbon resonance signal from intact hearts perfused with either pyruvate alone (solid squares) or pyruvate plus dichloroacetate (open squares). Note the consistency of signal intensity, normalized to 100% at the 3-min mark, throughout perfusion periods.

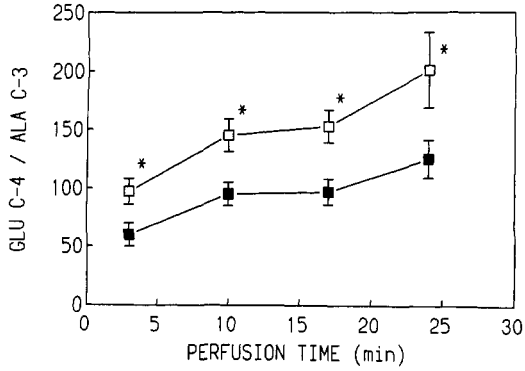

FIGURE 5: Signal intensity ratio of glutamate 4-carbon resonance signal to alanine 3-carbon resonance signal (GLU C-4/ALA C-3) from isolated hearts during control perfusion with pyruvate alone (solid squares) or pyruvate plus dichloroacetate treatment (open squares). Note the elevated values from the group receiving dichloroacetate treatment. An asterisk (*) indicates data that are statistically significant at $p < 0.05$ or less from the control value.

In contrast to metabolite concentrations, the relative ^{13}C enrichment of glutamate to that of alanine was significantly different between control and DCA-treated hearts. Mean enrichment levels of glutamate, lactate, alanine, and acetyl groups entering the citrate synthase reaction are all graphically illustrated in Figure 6. Under both conditions of control and

Table I: Metabolite Content of Rabbit Hearts Supplied 2.5 mM Pyruvate with or without Dichloroacetate[a]

	glutamate	alanine	lactate
control	10.6 ± 0.7	6.3 ± 0.8	3.3 ± 1.0
DCA-treated	11.7 ± 1.5	7.7 ± 1.5	3.9 ± 0.7

[a] Data represent μmol/g of dry tissue weight (mean ± SE).

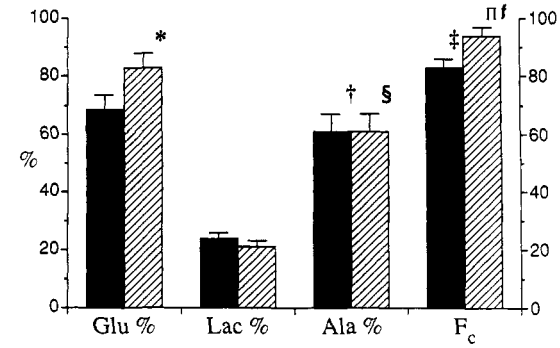

FIGURE 6: Myocardial metabolite content and relative percentages of ^{13}C labeling within metabolite pools with and without dichloroacetate treatment. Enrichment values are expressed as percentages of each corresponding metabolite pool. Abbreviations: Glu%, fractional ^{13}C enrichment of the 4-carbon position of glutamate; Lac%, fractional ^{13}C enrichment of the 3-carbon position of lactate; Ala%, fractional ^{13}C enrichment of 3-carbon position of alanine; F_c, fractional ^{13}C enrichment of acetyl groups entering the TCA cycle. Symbols: *, statistically significant from control group at $p = 0.0388$; †, statistically significant from control lactate ^{13}C enrichment at $p < 0.0001$; §, statistically significant from lactate ^{13}C enrichment during dichloroacetate treatment at $p = 0.0006$; ‡, statistically significant from control alanine (pyruvate) ^{13}C enrichment at $p = 0.0145$; Π, statistically significant from alanine ^{13}C enrichment during dichloroacetate treatment at $p = 0.0005$; *f*, statistically significant from control group at $p = 0.0238$.

DCA treatment in the presence of 99.4% enriched [3-^{13}C]-pyruvate, the fractional enrichments of the 3-carbon position within tissue alanine pools were similar. In contrast, ^{13}C enrichment at the 4-carbon position of glutamate was significantly increased by DCA treatment ($p < 0.04$). Therefore, increased PDH oxidation of pyruvate was reflected in the labeling of glutamate when referenced to the internal ^{13}C signal from alanine. As shown in Figure 6, the fractional enrichment of alanine in either group at approximately 60%

was lower than that of the exogenous pyruvate supply, which was 99.4%. The percentage of labeled lactate was yet lower than that detected in the alanine pool but also remained unaffected by DCA treatment. Thus, an apparent discrepancy has been elucidated in the fractional enrichments of the alanine and lactate pools in reference to the exogenous pyruvate source.

Mean percentages of labeled acetyl groups from pyruvate that entered the TCA cycle via the citrate synthase reaction (F_c) are shown in Figure 6. The unlabeled fraction which accounted for 6–17% of acetyl groups can be attributed to endogenous substrates, including lipids and glycogen. The increase in mean F_c in DCA-treated hearts demonstrates that PDH activation did not result in the expected increase in labeled carbons from pyruvate entering the citrate synthase reaction. However, a discrepancy is apparent in comparing these F_c values to the fractional enrichments of lactate and alanine (Figure 6). In control hearts, exogenous 99.4% labeled pyruvate accounted for only 24% of lactate produced in the cytosol and 61% ($p < 0.001$ from lactate) of the pyruvate pool available to the mitochondria for oxidation (from alanine detection), while exogenous, labeled pyruvate was preferentially oxidized at 83% of the acetyl groups entering the citrate synthase reaction ($p < 0.02$ from alanine enrichment). In DCA-treated hearts, the exogenous, 99.4% labeled pyruvate accounted for only 21% of the lactate produced in the cytosol and only 61% ($p < 0.0001$ from lactate) of the pyruvate available for oxidation within the mitochondria (from alanine detection), but the labeled pyruvate was preferentially oxidized as 94% of the carbon groups entering the citrate synthase reaction ($p < 0.001$ from alanine enrichment).

The F_c values shown in Figure 6 were also significantly greater than the fractional enrichment of the 4-carbon of glutamate. A fraction of unlabeled glutamate persisted from a source seemingly distinct from the TCA cycle intermediates. In these same hearts, the contribution of anaplerotic mechanisms to tricarboxylate formation, as determined from the relative ^{13}C NMR signals of the 3- and 4-carbons of glutamate (Malloy et al., 1988), was not statistically different with or without DCA treatment. Mean values of the ratio of anaplerotic flux to citrate synthase flux were 0.10 ± 0.03 in control hearts and 0.13 ± 0.02 in hearts treated with DCA. Thus, anaplerosis did not account for either the changes observed in glutamate labeling in response to DCA treatment or the discrepancy between the percentage of labeled acetyl groups and the fractional enrichment of glutamate.

DISCUSSION

The present work demonstrates the sensitivity of ^{13}C NMR spectroscopy of intact hearts to differences in the oxidation of a single, carbon-based substrate. The findings indicate a metabolic heterogeneity of the exogenous (labeled) and endogenous (unlabeled) pyruvate pools within the myocytes of both control and DCA-treated hearts. The NMR signal arising from ^{13}C-enriched alanine provided an internal reference for intracellular [3-^{13}C]pyruvate, by virtue of the equilibrium between the alanine and pyruvate pools which was maintained during PDH activation. Although alanine and lactate enrichments levels were unaffected, PDH activation increased the amount of labeled pyruvate contributing to glutamate formation via the pathways of substrate oxidation. The relative increase in mitochondrial pyruvate oxidation over cytosolic pyruvate metabolism was evident from comparison of the NMR signals arising from ^{13}C-labeled glutamate to that of ^{13}C-labeled alanine within intact, functioning hearts. In vitro determinations of ^{13}C enrichment within the alanine, lactate, and glutamate pools indicated significant discrepancies between the amount of labeled material available within the myocyte and the extent to which labeled pyruvate was oxidized. These results are consistent with previous reports of metabolic compartmentation of intracellular pyruvate (Peuhkurinen et al., 1982; Bunger, 1985). The current work indicates that ^{13}C NMR methods can detect significant increases in the contribution of pyruvate to the formation of TCA cycle intermediates and are sensitive to changes in metabolic activity within the mitochondrial and cytosolic compartments of whole hearts performing physiological levels of mechanical work.

The finding that augmented PDH activity in response to DCA treatment had no effect on mechanical function is consistent with a previous report on the myocardial response to DCA treatment (Matsuoka et al., 1987). As expected from the similarities in the mechanical function data, the bioenergetic state of control and DCA-treated hearts was similar, as no difference in the phosphocreatine to ATP ratio was evident between the two groups. Therefore, the metabolic perturbations induced by DCA treatment in the presence of 2.5 mM pyruvate were directly comparable between the two groups as being independent of physiological workload and energetic state.

The energetic status and physiological function of these hearts was supported by pyruvate metabolism as monitored with ^{13}C NMR spectroscopy. ^{13}C labeling within metabolites of pyruvate demonstrated that the alanine labeled at the 3-carbon position was a direct product of transamination with [3-^{13}C]pyruvate. As long as the intracellular levels of labeled pyruvate were maintained by the constant infusion of exogenous labeled pyruvate, alanine remained at steady-state levels, regardless of the extent of pyruvate oxidation. Thus, the use of ^{13}C NMR under these conditions provided a means of monitoring the relative NMR signal from alanine and glutamate within the intact, functioning heart without the necessary destructive tissue sampling required by radiolabeling methods.

The consistency in the level of labeled alanine throughout the perfusion protocol is similar to that described previous study comparing the metabolism of ^{13}C-labeled pyruvate in normal hearts to that of reperfused hearts displaying postischemic contractile dysfunction (Lewandowski & Johnston, 1990). In the previous examination of postischemic hearts, the relative labeling within the glutamate pool was significantly depressed relative to that of alanine over a time period of reperfusion that is associated with PDH inactivation (Kobayashi & Neely, 1983a). An additional report of reduced glutamate labeling relative to that of alanine during the metabolism of [3-^{13}C]pyruvate in the presence of the long chain fatty acid, palmitate, also indicates that the relative NMR signal intensities arising from different metabolite pools within the intact heart are closely related to the degree of PDH activity (Johnston & Lewandowski, 1991). The current study demonstrates that the opposite condition, PDH activation, is also reflected in the level of glutamate enrichment when the NMR signal arising from ^{13}C-enriched alanine is used as an internal reference for pyruvate availability within the myocyte.

Labeling within the alanine pool served as an index of the distribution of exogenous pyruvate entering the alanine transaminase, pyruvate dehydrogenase, and lactate dehydrogenase reactions. As evident from the data, enhanced oxidation of pyruvate from the ample exogenous supply of this substrate did not influence the nonoxidative conversion of pyruvate to either alanine or lactate. Differences in the NMR signal intensities from ^{13}C-enriched glutamate between untreated and DCA-treated hearts were attributable to

differences in the enrichment levels of glutamate and not total glutamate pool size. The similarity between alanine and glutamate pool sizes of treated and untreated hearts indicates that the enhanced oxidation of pyruvate did not alter the balance between the tricarboxylate pool of the mitochondria and the primarily cytosolic glutamate pool. Presumably, the increased fractional enrichment of glutamate was due solely to an increase in the bulk of labeled material entering the TCA cycle intermediate pool.

Although alanine rapidly reached steady-state ^{13}C enrichment, the enrichment of glutamate evolved over time as shown in Figures 3 and 5. Comparison of the ^{13}C labeling data obtained from the intact hearts to corresponding in vitro data suggests that increasing the oxidation of pyruvate also increased the rate of ^{13}C incorporation into the 4-carbon position of glutamate. Hearts displayed a mean endpoint value for the signal intensity ratio of the glutamate 4-carbon to the 3-carbon of alanine (Glu C-4/Ala C-3) of 1.3, less than that expected from the combined data from in vitro NMR and enzymatic assays. The mean Glu C-4/Ala C-3 ratio obtained from in vitro ^{13}C spectra was actually slightly higher at 1.64 to suggest that the delay between the final NMR observation of the intact hearts and the removal of the heart for freezing provided additional time for the glutamate labeling to evolve. In contrast, DCA-treated hearts showed much smaller differences between the Glu C-4/Ala C-3 ratios from intact hearts (2.0), in vitro spectra (2.2), and fractional enrichment data (2.1). These findings suggest that labeling at the 4-carbon position of glutamate may have not fully evolved in control hearts perfused with labeled pyruvate, while increased pyruvate oxidation in response to DCA promoted a more rapid approach to steady-state ^{13}C enrichment. Presumably, experimental variation between the three different approaches of (1) low resolution, low signal-to-noise NMR of intact tissues, (2) high-resolution and high signal-to-noise in vitro NMR, and (3) separate enzymatic assay also contributed to this variability in the data. However, the validation of NMR spectroscopy of intact tissues with separate in vitro analysis provided insight into the sensitivity of on-line ^{13}C NMR detection of intracellular metabolism.

The high enrichment level of the exogenous [3-^{13}C]pyruvate afforded the opportunity to collect unambiguous information regarding the metabolism of endogenous carbon sources. Although the sole exogenous substrate supply of pyruvate was 99.4% enriched at the 3-carbon position, the steady-state enrichment of the 3-carbon position of alanine that was achieved was only at mean values of 61% in both untreated and DCA-treated hearts. An obvious contribution of endogenous, unlabeled substrate to the formation of alanine occurred within the myocytes and was unaffected by the degree of labeled pyruvate entry into the TCA cycle intermediate pool. Lactate production as an index of the cytosolic metabolism of pyruvate accounted for less than half (21–24%) of the enriched material that was associated with alanine formation (61% in both groups), indicating an even greater contribution of endogenous sources to the formation of lactate within the cytosol.

While these data are consistent with the notion of compartmentation of pyruvate pools within the myocyte, the most striking discrepancy in the intracellular metabolism of pyruvate is the highly enriched pool of pyruvate that was oxidized via PDH entry into the TCA cycle at 83% in untreated hearts and 94% in DCA-treated hearts. This highly enriched pool was the only component of pyruvate metabolism to be affected by the activity of PDH. The increase in ^{13}C-enriched acetyl-CoA units in response to DCA treatment is clearly the result of reduced oxidation of unlabeled, endogenous substrates, which are most likely to be lipids (Latipaa et al., 1983). Were endogenous carbohydrate the alternate, unlabeled source of acetyl groups, PDH activation would have further reduced the proportion of unlabeled carbons. This change in ^{13}C enrichment of acetyl-CoA is easily accounted for, but the persistent discrepancy in the percentage of labeled pyruvate that was evident within cytosolic metabolism versus that being oxidized in the mitochondria is much less obvious. Comparison of the data shown in Figure 6 indicates metabolic heterogeneity of the exogenous (labeled) and endogenous (unlabeled) pyruvate pools within the myocyte. This study indicates that one pool of pyruvate within the cytosol contributes to nonoxidative metabolism (i.e., lactate formation) while another pool is associated with mitochondrial uptake (i.e., alanine formation) and an apparent compartmentation of intramitochondrial pyruvate for oxidative metabolism (acetyl-CoA formation).

The data presented here are consistent with previous reports of alanine and lactate enrichment from both isotope-labeled pyruvate and glucose which indicated a significant contribution to cytosolic metabolism from unlabeled, endogenous sources (Peuhkurinen & Hassinen, 1982; Lewandowski et al., 1991a). The current report was focused on pyruvate alone, as a sole carbon source for energy production, to provide specific metabolic targeting of the PDH enzyme without the confounding variables in the rate of label passing from glucose or glycogen through glycolysis. However, the fractional enrichment values for alanine, lactate, and acetyl-CoA (F_c) reported here are very similar to data from isolated rat hearts perfused with 0.2 mM concentrations of ^{14}C-labeled pyruvate in the presence of 10 mM glucose, as reported by Peuhkurinen and Hassinen (1982). As in our study, the specific activity of lactate was 3-fold lower than that of alanine. In a later study (Peuhkurinen et al., 1983) of rat hearts perfused with millimolar concentrations of pyruvate in the presence of 5 mM glucose, the percentages of labeled material derived from the ^{14}C-labeled pyruvate were 64% for alanine and 92% for mitochondrial pyruvate. These previously published values are very close (see Figure 6) to those obtained in this study, which were 61% for alanine with pyruvate contributing to mitochondrial acetyl-CoA labeling (F_c) at 85% in control and 93% in DCA-treated hearts that were perfused with millimolar concentrations of pyruvate (2.5 mM) in the absence of glucose. Therefore, the higher concentrations of pyruvate outweigh the metabolic contributions of glucose. These similarities suggest that the distribution of label within metabolites of pyruvate in the previous study was more strongly influenced by compartmentation effects on the isotope equilibrium within the metabolite pools rather than dilution by the exogenous, unlabeled glucose.

The use of ^{13}C-enriched amino acid pools to reflect the metabolism of pyruvate also addresses questions regarding metabolic communication between the oxidative pathways of the mitochondrial matrix and the nonoxidative pathways of the cytosol. For example, the increased oxidation of pyruvate and subsequent increase in glutamate labeling demonstrates that the glutamate pool does indeed reflect relative changes in the extent of labeling within the TCA cycle intermediate pool. In particular, the fractional enrichment of the 4-carbon of glutamate is a marker of the contribution of the PDH reaction to α-ketoglutarate formation, as opposed to the carboxylation of pyruvate, which would result in preferential labeling of the 3-carbon position of glutamate over that of the 4- or 2-carbon sites. This balance between alanine and glutamate in response to changes in glucose and pyruvate

metabolism in isolated rabbit hearts has been extensively explored by Taegtmeyer et al. (1977). As an equilibrium was established between the alanine and pyruvate pools in the current experiments, the other corresponding reactants/products, α-ketoglutarate and glutamate, must also have been in equilibrium across the alanine transaminase reaction. The net result was a translation of the enrichment levels of α-ketoglutarate and pyruvate within the mitochondria to the cytosolic compartment in the form of glutamate and alanine, as detected by ^{13}C NMR spectroscopy of the intact hearts.

In summary, ^{13}C NMR spectroscopy elucidated separate pools of intracellular pyruvate that are either in communication with the glycolytic system or with oxidative metabolism in the mitochondrial matrix. These findings closely match the results of a previous radiolabeling study (Peuhkurinen et al., 1983). The findings also demonstrate that compartmentation of metabolism between the cytosol and mitochondria is discernible in ^{13}C NMR spectra from intact hearts performing mechanical work. The effect of PDH activity on the NMR signals arising from ^{13}C-enriched metabolites was clearly observed in intact hearts to be influenced by the balance between TCA cycle intermediate oxidation and interconversion with the tissue glutamate pool. Thus, the current work demonstrated the utility of ^{13}C NMR spectroscopy of intact, functioning hearts for detecting relative changes in the mitochondrial oxidation of pyruvate (glutamate labeling) versus pyruvate availability within the myocyte (alanine labeling). Additional findings from in vitro NMR analysis suggest that the origin of intracellular pyruvate is closely linked to (and may determine) its metabolic fate in supporting cardiac function. In effect, a metabolic heterogeneity in intracellular pyruvate has been identified, stemming from compartmentation within the myocyte. This heterogeneity may dictate the contributions of glycolytic end products to oxidative metabolism in response to the functional state of the myocardium. Future work is indicated to elucidate the role of PDH activity in mediating carbohydrate oxidation for supporting cardiac performance in normal and diseased states (McVeigh & Lopaschuk, 1990).

ACKNOWLEDGMENT

Technical assistance for portions of this work was provided by Ms. Chrys Hulbert and Mr. Larry White.

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Registry No. PDH, 9014-20-4; acetyl-CoA, 72-89-9; Ala, 56-41-7; Glu, 56-86-0; pyruvic acid, 127-17-3; lactic acid, 50-21-5; citric acid, 77-92-9; α-ketoglutaric acid, 328-50-7.