^{1}H NMR Observation of Redox Potential in Liver†

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ABSTRACT: ^{1}H NMR spectral editing techniques can select the distinct signals of lactate, pyruvate, β-hydroxybutyrate, and acetoacetate and provide a unique way to monitor the biochemical processes in vivo. These metabolite levels reflect the near-equilibrium dehydrogenase activity and therefore the cellular redox state. The quantitative comparison between the ^{1}H NMR and biochemical assay data is in excellent agreement. Lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios, obtained from normalized ^{1}H NMR spectra, respond directly to changes in the cytosolic and mitochondrial redox states. Because NMR is noninvasive, our results set the groundwork for implementing these techniques to observe tissue redox states in vivo.

The cellular redox potential plays a central role in regulating bioenergetics and metabolism. It is characterized by the ratio of free NAD/NADH[1] (oxidized and reduced states, respectively, of nicotinamide adenine dinucleotides) and is commonly measured by fluorescence (Chance & Jöbsis, 1959; Chance et al., 1962) or biochemical assay methods (Williamson et al., 1967). The optical technique excites NADH at 366 nm and monitors the fluorescence at 450 nm. Biochemical assay assesses indirectly the cytosolic and mitochondrial NAD/NADH by measuring lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios, respectively (Williamson et al., 1967). These substrates react with the near-equilibrium dehydrogenase reactions involving pyridine dinucleotides and reflect the free NAD/NADH pool.

Current techniques to measure cellular redox state, however, are invasive and are limited. Both fluorescence and biochemical assay methods require invasive probes and lack sampling specificity. Fluorescence can only measure surface tissue, and biochemical assay requires destructive manipulation or detailed accounting of contributions between the local tissue region and the distal sampling point.

Recent NMR advances have suggested an alternative method. Since lactate (Rothman et al., 1984; Jue et al., 1985) and pyruvate signals in vivo (Jue et al., 1988) are detectable with ^{1}H NMR spectral editing techniques, tissue redox state is then potentially observable also. For lactate, ^{1}H NMR spectral editing technique manipulates the *J*-modulation in a spin-echo pulse sequence to select the CH_3 signal and to suppress the endogenous background resonances (Rothman et al., 1984; Jue et al., 1985). For pyruvate, an anti-editing strategy discriminates against the signals of coupled spin systems (Jue et al., 1988).

We undertook a perfused liver study to establish the feasibility of utilizing the ^{1}H NMR editing strategy to measure lactate/pyruvate and to demonstrate that these editing methods will also select the signals of β-hydroxybutyrate and acetoacetate, which have similar spin systems as lactate and pyruvate, respectively. The NMR data are in excellent agreement with the standard biochemical assay results and underscore the potential to measure cellular redox states in vivo.

MATERIALS AND METHODS

Rat Liver Perfusion. The rat liver perfusion method was previously described (Jue et al., 1988). Male Sprague-Dawley rats were fasted 24 h before the experiment. Initially, the isolated liver was washed for 25 min with nonrecirculating Krebs bicarbonate buffer at pH 7.35–7.45 to remove endogenous lactate and pyruvate before recirculation. The isolated liver was inserted into a 20-mm NMR tube, which was then placed in a Bruker AM 360 wide-bore vertical magnet equipped with 20 mm ^{1}H–{X} probe. The probe characteristics and the perfusion method have been well established in the laboratory.

The perfusate was oxygenated by a membrane oxygenator exposed to 95% O_2/5% CO_2. Fifty feet of silastic tubing in the oxygenator was coiled around a temperature jacket. The liver perfusate temperature was maintained at 36–37 °C. The flow rate was 5 mL min^{-1} (g of liver)$^{-1}$ to ensure proper oxygen delivery in this hemoglobin-free perfusate. Red blood cells and albumin were omitted in the perfusate to minimize the NMR spectral interferences. The perfused liver is, nevertheless, viable (Fröhlich et al., 1973; Sies, 1978; Sugano et al., 1978). The recirculating perfusate volume was 150 mL and contained 6% D_2O for field-frequency lock. The isotope effect on the lactate dehydrogenase reaction at this concentration of D_2O was negligible. Up to 20% D_2O, the in vitro assay of LDH extracted from the in situ rat liver showed no significant isotope-related difference in the V_{max}.

During all experiments, the liver viability was monitored by O_2 consumption, stable perfusate pH, a high ATP/P_i ratio from the ^{31}P spectra, and LDH leakage into the perfusate. O_2 consumption was greater than 1 μmol min^{-1} (g wet weight)$^{-1}$ for the fasted liver (Gores et al., 1986). Glucose assay of the perfusate sampled periodically during the experiment yielded a linear production rate of 1.0 ± 0.1 μmol min^{-1} (g wet weight of liver)$^{-1}$, which is a key indicator of the metabolic integrity of the fasted liver (Ross et al., 1967). With our perfused liver system, the LDH leakage is less than 10 units/h into the perfusate, well within the established viability criterion

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[1] Abbreviations: NAD, oxidized β-nicotinamide adenine dinucleotide; NADH, reduced β-nicotinamide adenine dinucleotide; LDH, lactate dehydrogenase; TSP, sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$; B/A, β-hydroxybutyrate/acetoacetate; BHB, β-hydroxybutyrate; BDH, β-hydroxybutyrate dehydrogenase; L/P, lactate/pyruvate.

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(Lemasters et al., 1983; Sies, 1978). Each liver was also inspected for gross edema. Any data from liver with a wet/dry weight ratio exceeding 3.8 were discarded (Tischler et al., 1977a). Wet liver weight was determined after removing any nonhepatic tissue and blotting with filter paper.

^1H NMR Spectral Editing. Homonuclear editing techniques to select the ^1H signal of C_3–^1H lactate (Jue et al., 1985) and an anti–editing strategy to extract the C_3–^1H pyruvate signal (Jue et al., 1988) in the ^1H NMR spectra of the perfused liver have been previously described. NMR spectra were collected with a 20-mm ^1H–{X} probe on an AM 360 Bruker spectrometer; 4K data points and 5-kHz spectral width were used. An inversion recovery pulse sequence was used to determine the T_1s of lactate and pyruvate in the solution at 37 °C, and the data were processed by the Bruker T_1 fitting routine. All peaks were referenced to the H_2O signal as 4.76 ppm at 25 °C, which was calibrated against TSP.

The ^1H NMR signal intensities were calibrated against a known concentration of infused substrate. A separate set of data was obtained by spectrophotometric assay of the corresponding perfusate, sampled at periodical intervals throughout the experiment. These two data sets were then compared.

Redox measurement requires only a ratio of lactate/pyruvate or β-hydroxybutyrate/acetoacetate, not the absolute concentrations of each substrate. To scale the appropriate NMR signal intensities requires a normalization factor, obtained from 10 mM pyruvate and lactate solution signals, under experimental pulsing conditions. The ionic composition, pH, and temperature of the analytical solution were the same as the Krebs bicarbonate buffer used during the liver experiments. The intensity ratio of edited lactate and pyruvate signals was then calibrated against the corresponding fully relaxed ^1H resonances. Similar experiments gave the normalization factor for the B/A ratio.

Metabolite Assay. Aliquots of perfusate were withdrawn at 10-min intervals in the course of NMR experiments and were immediately deproteinized with ice-cold 1 N perchloric acid. Acidic samples were neutralized and assayed enzymatically for pyruvate (Czok & Lamprecht, 1974), lactate (Noll, 1984), β-hydroxybutyrate (Williamson & Mellanby, 1974), acetoacetate (Mellanby & Williamson, 1974), and glucose (Bergmeyer et al., 1974). All the chemicals and enzymes were purchased from Sigma and used without further purification.

Hepatic LDH was extracted from in situ rat liver. Male Sprague-Dawley rats were anesthetized with sodium pentobarbital (50 mg/kg body weight), and the liver was immediately removed from the animal. Approximately 1 g of liver tissue was homogenized in 5 volumes of 0.1 M phosphate buffer, and the homogenate was centrifuged at 18000*g* at 4 °C for 30 min (Hyldgaard-Jensen & Valenta, 1970). LDH activity in the diluted supernatant was then measured in the reaction mixture containing saturating concentrations of pyruvate (0.63 mM) and NADH (0.18 mM) as the substrates (Bergmeyer & Bernt, 1974). For the LDH K_m measurement of pyruvate and NADH, pyruvate and NADH concentrations in the reaction mixture were varied, and corresponding LDH activities were measured.

Estimate of Percentage Signal in Liver vs Perfusate. A point H2O sample (<5 μL) in a 5-mm NMR tube was used first to map the field of the ^1H probe coil. The sample depth was varied at about 2-mm increments. At each step, a corresponding NMR signal denoted the relationship between sample depth and observed field strength. A total of 20 points defined the field and gave an estimate of the homogeneous coil volume. The liver volume was then determined from displacement measurements in perfusate buffer, indicating that the wet weight corresponded closely to its volume (milliliters).

An additional return line leading to the bottom of the NMR tube removed the surrounding perfusate and provided a means to separate the signal contribution from perfusate and liver. A reference ^1H liver spectrum first established the base-line condition; 30 mM lactate was then infused and gave a composite, edited C_3–^1H lactate signal from both the liver and perfusate. Through the additional perfusate return line, all the surrounding perfusate was then removed before another edited lactate signal was acquired. The lactate signal intensity difference between these two experiments separated the liver from the perfusate contribution. Biochemical assay of perfusate samples adjusted for any lactate contribution from metabolism.

Perturbation of Redox State. The dynamic response of the redox states to ethanol and anoxia was followed with ^1H NMR and biochemical assay; 15 mM ethanol was added to the recirculating perfusion fluid to increase the cytosolic and mitochondrial NADH reduction state. The lactate and BHB formation rates were then monitored. Anoxic perturbation of the liver was achieved by equilibrating the perfusate with 95% N_2/5% CO_2.

Additional experiments with a nonrecirculating perfusion system were then conducted. A constant level of infused substrates in the inflow buffer was maintained to clamp the redox-state ratios: 10 mM lactate and 1 mM pyruvate were infused as substrates for the cytosolic redox state measurement; 1 mM BHB and 1 mM acetoacetate, for the mitochondrial measurement. During the initial period of perfusion with clamped substrate levels, base-line NMR spectra were obtained. Subsequent spectra were collected when the liver redox states were perturbed with either 10 mM ethanol or equilibration with 95% N_2. The ratios of the redox substrate pairs were then obtained after normalizing the edited ^1H signals.

RESULTS

LDH and BDH Substrate Signals. Figure 1 shows a set of ^1H NMR anti-edited spectra, indicating the time course of pyruvate utilization in perfused rat liver. Figure 1A,B reveals, in the CH_3 pyruvate region (2.4 ppm), no residual background signals from endogenous lipids and other cellular metabolites, such as glutamate and glutamine. After infusion of 10 mM pyruvate, the pyruvate signal appears at 2.38 ppm (Figure 1C). Each spectrum represents 3 min of signal averaging. The bank of spectra shows a constant pyruvate utilization rate for 65 min. The edited signal in the last spectrum (Figure 1P) corresponds to 0.5 mM pyruvate.

Figure 2 shows the resulting lactate formation. The lactate signal, collected alternately with the pyruvate signal, first appears in Figure 2d. Each spectrum requires 2.5 min of signal averaging. The signal continues to increase in Figure 2e–o. In Figure 2d and Figure 2p, the edited lactate signal corresponds to 0.2 and 1.6 mM, respectively. The pyruvate utilization rate is 5.69 ± 0.32 μmol min^{-1} (g of liver)$^{-1}$, while the lactate production rate is 0.89 ± 0.18 μmol min^{-1} (g of liver)$^{-1}$ (n = 14). The LDH flux pathway accounts then for approximately 16% of the total pyruvate utilization. The ^1H NMR measurements of pyruvate utilization and lactate production rates are consistent with literature values, obtained with conventional biochemical assay (Table I).

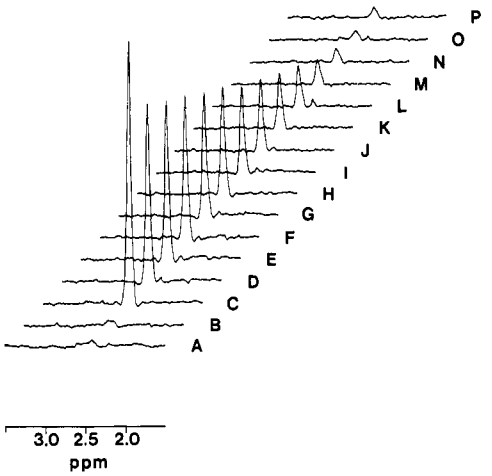

FIGURE 1: Pyruvate utilization monitored by ^{1}H NMR in the perfused rat liver. Figure 1A,B shows the control ^{1}H NMR spectra of the perfused liver before pyruvate infusion. No background signal appears in the pyruvate spectral region. At the time corresponding to the end of Figure 1B, 10 mM pyruvate was infused. Figure 1C shows the anti-edited pyruvate signal representing an overshoot before infused pyruvate was fully equilibrated in the perfusion system. In Figure 1C–P, the pyruvate signal intensity gradually drops as the liver utilizes pyruvate.

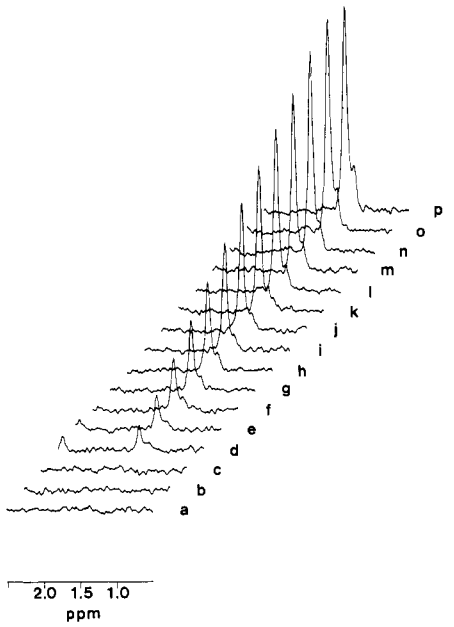

FIGURE 2: Lactate production monitored by ^{1}H NMR in the perfused rat liver. Figure 2d–p shows the signals of lactate endogenously produced by the liver, subsequent to pyruvate infusion. Corresponding pyruvate signals are shown in Figure 1. After finishing the accumulation of spectrum p, 10 mM lactate was added to the perfusate to confirm the peak assignment and to quantitate the concentration of the edited lactate signal.

LDH activity, pyruvate to lactate conversion, from in situ rat liver extracts is 325 ± 20 μmol min^{-1} (g of liver)$^{-1}$, which agrees with the values previously reported (Bücher et al., 1972). The $K_{m,NADH}$ and $K_{m,pyruvate}$ are 15 and 90 μM, respectively.

Ethanol was infused to perturb the cellular redox state and to test the corresponding response of the edited ^{1}H NMR signals. Because ethanol oxidation to acetaldehyde proceeds via NAD-dependent alcohol dehydrogenase, it generates excess free NADH in the hepatic cytosol. The additional NADH increases the lactate formation and shifts the redox potential in liver (Williamson et al., 1969; Lieber, 1985). In experiments with 10 mM infused pyruvate (data not shown), lactate production is linear for 40 min. Upon addition of 15 mM ethanol, the lactate formation rate increases by 3.1 ± 0.6 times (n = 7).

Table I: Pyruvate Utilization and Lactate Production Rates [μmol min^{-1} (g of Liver)$^{-1}$] of Perfused Livers from Fasted Rats

	pyruvate uptake		lactate production	
substrate	enzyme assay	NMR	enzyme assay	NMR
pyruvate[a] (10 mM)	3.25		0.72	
pyruvate[b] (10 mM)	3.35		1.27	
pyruvate[c] (20 mM)	3.13		0.68	
pyruvate[d] (10 mM)	4.46			
pyruvate[e] (10 mM)	5.61 (±0.30)	5.69 (±0.32)	0.86 (±0.15)	0.89 (±0.18)

[a] Ditullio et al. (1974). [b] Menahan & Williams (1971). [c] Exton & Park (1967). [d] Ross et al. (1967). [e] Present report, n = 8 (enzyme assay) and n = 14 (NMR). In 8 experiments out of 14 NMR experiments, perfusates were sampled and assayed enzymatically.

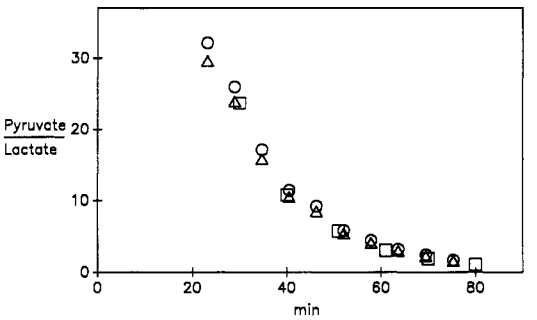

FIGURE 3: Time course of the pyruvate/lactate ratio monitored following the infusion of 10 mM pyruvate in the perfused rat liver. Pyruvate/lactate ratios were measured (1) by the NMR signal intensities normalized to the relative signal loss (○), (2) by ^{1}H NMR intensities quantitated by calibrating against pyruvate and lactate externally added to the perfusate (△), and (3) by spectrophotometric enzyme assay of lactate and pyruvate in the perfusate samples (□).

The lactate/pyruvate ratio is obtainable directly from the normalized NMR signals. Figure 3 traces the pyruvate/lactate ratio obtained from the same experiment in three different ways: (1) by calibration of the NMR signals against a known concentration of lactate and pyruvate added to the perfusate (△); (2) by spectrophotometric enzyme assay of the perfusate samples taken at regular time intervals (□); (3) by normalizing the edited pyruvate/lactate signals to account for the relative signal losses inherent in editing and anti-editing pulse sequences (○). Figure 3 shows good agreement with all three methods, in particular that the L/P ratio can be accurately measured directly from the properly scaled ^{1}H NMR intensities.

The scaling factor accounts for the T_1 and T_2 differences of pyruvate and lactate. The signal loss due to T_1 relaxation is substantially larger for the methyl portion of the pyruvate signal than for the methyl portion of the lactate signal. T_1 of the pyruvate CH_3 signal is 9.3 s, and T_1 of the lactate CH_3 signal is 1.9 s in solution at 37 °C. The T_2 decay during the spin-echo homonuclear editing sequence is 28% for lactate and almost negligible during anti-editing of the pyruvate signal.

Lactate and pyruvate spectral editing strategies are also applicable in selecting for the signals of β-hydroxybutyrate and acetoacetate, the mitochondrial redox pair (Figures 4 and 5). After infusion with 10 mM β-hydroxybutyrate (Figure 4D), its utilization is followed with the identical homonuclear editing method that selects for lactate (Figure 4D–O). Each spectrum acquisition is separated by a 4.8-min interval. Forty-five minutes after infusion, the β-hydroxybutyrate signal intensity has dropped by 50%.

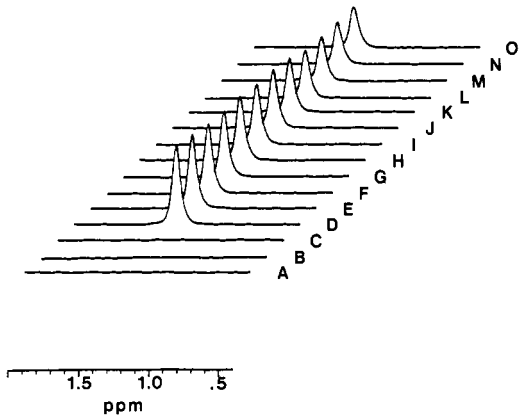

FIGURE 4: β-Hydroxybutyrate utilization followed by ^{1}H NMR in the perfused rat liver. At the end of collecting spectrum C, 10 mM DL-β-hydroxybutyrate was infused in the perfused liver. In Figure 4D–O, the BHB signal intensity gradually decreases as the liver converts BHB to acetoacetate via BHB dehydrogenase activity.

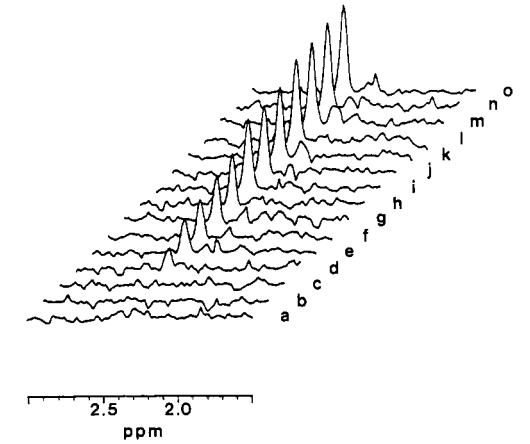

FIGURE 5: Acetoacetate formation monitored by ^{1}H NMR in the perfused rat liver. Figure 5d–o shows the production of acetoacetate from infused β-hydroxybutyrate. Corresponding β-hydroxybutyrate signals are shown in Figure 4. 10 mM acetoacetate was added in the perfusate at the end of spectrum o to quantitate the acetoacetate signals and to confirm the assignment. Each acetoacetate signal was collected for 2.8 min. Each β-hydroxybutyrate signal was accumulated for 2.0 min. Consequently, the interval between each spectra was 4.8 min.

As β-hydroxybutyrate is utilized, acetoacetate is formed and detected with the same anti-editing strategy that selects for pyruvate. Figure 5d shows the first appearance of the CH_3 acetoacetate resonance. The methyl group of acetoacetate has a similar spin system characteristic as the methyl group of pyruvate. The acetoacetate signal increases linearly to 4 mM (Figure 5o).

From infused acetoacetate, β-hydroxybutyrate is produced at a linear rate of 0.43 μmol min^{-1} (g of liver)$^{-1}$ (data not shown) for up to 80 min. With ethanol addition, the β-hydroxybutyrate production rate increases by a factor of 4.4.

The acetoacetate/β-hydroxybutyrate ratios, obtained from normalizing the NMR signal intensities and assaying spectrophotometrically the perfusate, are in excellent agreement (Figure 6).

Percentage of Signal from Liver. Because the NMR experiment will observe both perfusate and liver signals, some estimate of what fraction of the signal arises from the liver is essential in determining the feasibility of utilizing the technique for whole animal experiments. The liver contribution was determined by comparing the probe coil with the liver volume and by measuring the edited lactate signal with and without surrounding perfusate.

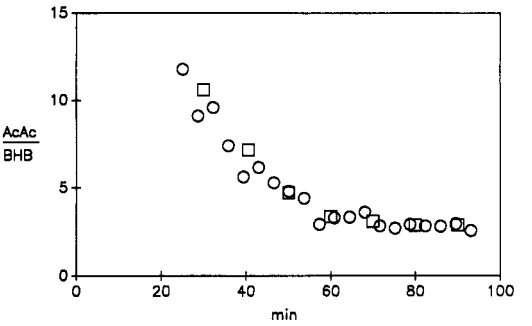

FIGURE 6: Time course of the acetoacetate/β-hydroxybutyrate ratio monitored following 10 mM acetoacetate infusion in the perfused rat liver. The normalized ^{1}H NMR intensity ratios (○) of acetoacetate/BHB agree closely with the concentration ratios of acetoacetate/BHB measured spectrophotometrically in the perfusate samples (□). In this experiment, 10 mM acetoacetate was infused in the recirculating perfusate at the 8-min time point, and the subsequent time course of acetoacetate utilization and β-hydroxybutyrate production by the perfused liver was followed.

A point sample of H_2O (<5 μL) was placed in a 5-mm tube, which was incrementally positioned in 2-mm steps in order to vary the sample depth. The resultant ^{1}H signal intensity reflected the field strength versus sample position and indicated an active coil region of 2.7 ± 0.05 cm. Throughout 2.7 cm, the signal intensity is constant; the field profile is at a plateau. Outside this region, the field drops precipitously.

The relative volume of the liver to the probe coil can now be obtained. On the basis of 18-mm inner diameter of a standard 20-mm NMR tube used in the study, the active sample volume is then 6.9 ± 0.6 cm^3. Rat livers for our experiments typically weighed 3.5–5.0 g wet weight. From displacement measurements in perfusate buffer, the liver volume corresponded closely to the wet weight.

To determine precisely the perfusate and liver signal contribution requires collecting signal with and without the surrounding perfusate. Studies with livers between 2.4 and 3.1 g/wet weight (n = 3), occupying between 35 and 45% of the active coil volume, indicated that the signal contribution from liver is between 26 and 34%. About 75% of the liver (n = 3) then gives rise to the edited lactate signal. Extracellular space in liver accounts for 16–25% (Blouin et al., 1977; Tischler et al., 1977a). Interstitial space contributes negligibly (Sies, 1978).

Measurement of Steady-State Redox States. Figures 7 and 8 show that the edited ^{1}H NMR signals reflect steady-state changes in the cytosolic and mitochondrial redox states. A nonrecirculating perfusion system with 10 mM lactate/1 mM pyruvate clamps the NADH/NAD at the expected physiological redox state. The edited ^{1}H NMR intensity ratio of lactate/pyruvate reflects the 10/1 ratio in the initial control period (Figure 7). This ratio increases by a factor of 2.2 with addition of 10 mM ethanol and remains steady as long as ethanol is present in the perfusion system. The new steady state is reached within 5 min after ethanol infusion. As ethanol is removed from the perfusate, the L/P ratio returns to the original level. The L/P ratio can be cycled between these two steady states over an extended period by manipulating ethanol infusion.

Anoxia lowers the electron flux in the respiratory chain and increases the reduction state of mitochondrial pyridine nucleotides (Scholz et al., 1969; Williamson et al., 1969). In the nonrecirculating perfusion, a constant level of 1 mM β-hydroxybutyrate and 1 mM acetoacetate parallels the

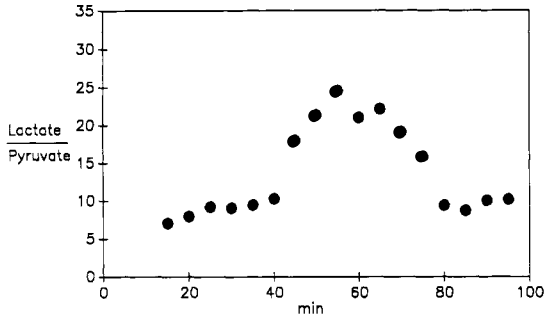

FIGURE 7: Effect of ethanol on the lactate/pyruvate ratio monitored by ^{1}H NMR. Rat liver was perfused in a nonrecirculating perfusion mode. 10 mM lactate and 1 mM pyruvate were infused in the inflow buffer. Subsequently, the lactate/pyruvate ratio in the inflow perfusate was clamped at 10/1. Intensities of lactate and pyruvate peaks in the spectra collected during the initial control period, up to 40 min, give a 10/1 lactate/pyruvate ratio. At the 40-min time point, 10 mM ethanol was added in the inflow buffer. Subsequently, the lactate/pyruvate ratio increased by a factor of 2.2. When ethanol was removed from the perfusion system, the lactate/pyruvate ratio returned to the original 10/1 ratio. Lactate/pyruvate ratios were measured directly from the ^{1}H NMR intensities of the lactate and pyruvate signals after scaling the two signals.

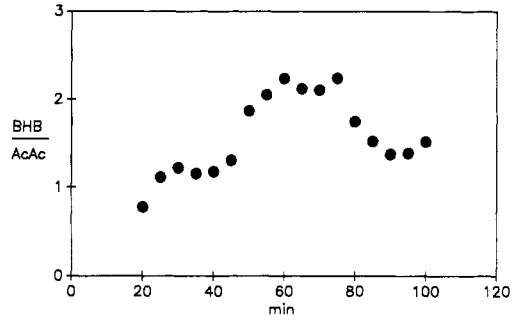

FIGURE 8: Effect of anoxia on the BHB/acetoacetate ratio monitored with ^{1}H NMR. The liver was perfused in a nonrecirculating perfusion system. The inflow buffer contained 1 mM β-hydroxybutyrate and 1 mM acetoacetate, clamping the BHB/acetoacetate ratio to 1/1. The signal intensities of BHB and acetoacetate in the initial spectra obtained during the control normoxic period give a 1/1 ratio. At the 48-min time point, 95% O_2 was replaced by 95% N_2 to introduce anoxic perturbation of the mitochondrial redox state. With anoxia, the B/A ratio increased by a factor of 2 from the control state. The B/A ratio returned to the original 1/1 ratio after reoxygenating the liver at the 75-min time point. B/A ratios in this experiment were measured from the NMR intensities normalized to the relative signal loss.

physiological concentration in the fasted animal. The steady-state B/A ratio, 1.1, is constant for the initial 25 min (Figure 8). With anoxia, the B/A ratio increases to 2.1. This change is comparable to the literature values (Sies, 1977). Upon reoxygenation, the initial B/A ratio returns to its original value.

DISCUSSION

Comparison of Techniques To Measure Redox State. Measuring dehydrogenase substrate levels to determine cellular redox state is a well-established method that rests on three underlying criteria: the dehydrogenase is localized exclusively in a single subcellular compartment, it is a near-equilibrium enzyme, and its substrates are uniformly distributed or compartmentalized. For the cytoplasm, lactate dehydrogenase meets all the criteria. Moreover, studies have indicated that all the highly active dehydrogenases interact rapidly with a single pool of NAD/NADH (Hohorst et al., 1961; Krebs & Veech, 1970; Sies, 1977). For the mitochondria, β-hydroxybutyrate dehydrogenase is the counterpart (Williamson et al., 1967). LDH is localized in the cytoplasm; BDH, in the inner mitochondrial membrane.

Indeed, the respective dehydrogenase substrate pairs, lactate/pyruvate and β-hydroxybutyrate/acetoacetate, are sensitive indicators of the free NAD/NADH pool and respond quickly to redox perturbations (Hohorst et al., 1961; Williamson et al., 1967). New redox measurement techniques are generally calibrated against the standard substrate assay method. The fluorescence technique was validated with biochemical assay data, using relatively high concentrations of lactate/pyruvate (Bücher et al., 1972). The optical technique, however, observes only the NADH emission. It has two prominent advantages over assay methods: it is relatively noninvasive and can focus on a local tissue region. However, it can neither distinguish mitochondrial versus cytosolic pools (Scholz et al., 1969) nor measure accurately the critical free NADH pool (Bücher et al., 1972; Chance et al., 1962).

The ^{1}H NMR technique, predicated on the biochemical assay method, overcomes many limitations. It can potentially assay the dehydrogenase substrate levels in a localized tissue region without surgical manipulation or sample destruction, and in contrast to fluorescence, it measures the critical free NAD/NADH pool in both the cytosolic and mitochondrial compartments.

NMR Measurement of Redox State. The ^{1}H NMR editing technique suppresses all the background signals and selects clearly the lactate, pyruvate, β-hydroxybutyrate, and acetoacetate signals in liver. Quantitating these NMR signals with an exogenous reference or normalization constant yields kinetics and redox-state data that agree very well with perfusate substrate analysis (Figures 3 and 6). In essence, if the NMR signals were detectable in a tissue region, they would give precisely the same kinetic and redox-state data that substrate assays would give.

Since the redox state is determined by substrate ratios, not the absolute quantity, scaling the edited NMR signals is critical. An appropriate normalization constant would avoid the cumbersome task of calibrating each substrate level, which is a formidable task. Indeed, the NMR experiments indicate that an accounting of the relaxation and sensitivity losses for the edited NMR signals of the different substrates will lead to an appropriate scaling factor. With the scaling factor, the kinetic results are in excellent agreement with those calibrated by an exogenous reference or by perfusate assay (Figures 3 and 6). Such an approach is critical for any successful measurement of the redox state in vivo with NMR. The feasibility of the strategy is further substantiated in the steady-state experiments with clamped substrate ratios.

In Vivo LDH Kinetics. The NMR technique also provides a unique window to observe directly enzyme kinetics in tissue in vivo. For the LDH forward reaction, pyruvate to lactate, K_{eq} = [lactate][NAD]/[pyruvate][NADH] = 1.11×10^4 at 38 °C (Williamson et al., 1967). The NMR data indicate that the liver produces lactate from pyruvate at a constant rate, 0.89 ± 0.18 μmol min^{-1} (g of liver)$^{-1}$, for 60 min. When the lactate and pyruvate concentrations are 1.5 and 2 mM, respectively, the reaction rate levels off, signaling a significant back-reaction at physiological concentration range (Williamson et al., 1967; Exton & Park, 1967).

However, the LDH V_{max} determined from our in vitro assay of rat liver is 325 μmol min^{-1} (g of liver)$^{-1}$, 360 times higher than the lactate production rate measured with ^{1}H NMR. Although pyruvate inhibition may reduce the H-isozyme activity, our assay of liver LDH (predominantly the M-

isozyme) shows only a 25% decrease in V_{max} with 10 mM pyruvate (Everse & Kaplan, 1973). Researchers have reported that no pyruvate inhibition occurs in vivo because the cellular concentration of LDH, 10^{-6} M, is much higher than the in vitro assay concentration, 10^{-10} M, and the reaction of pyruvate and NADH may already be completed before the formation of the critical, abortive ternary complex (Wuntch et al., 1970; Everse et al., 1970). At any rate, pyruvate inhibition cannot account for the 360-time difference in the observed V_{max}.

Cellular NADH Level. Brindle et al. (1986) have also noted the disparity in lactate kinetics in vivo and have suggested that either the intracellular NADH level [(1.5 μM) in the starved rat hepatocytes (Tischler et al., 1977b)] or other cellular effectors, removed in the enzyme extraction procedure, may be factors in reducing the in vivo LDH reaction rate. If the rate-limiting factor for LDH activity is NADH, then the linear lactate production rate, k_1[pyruvate][NADH], can yield an estimate of the cellular [NADH]. Since our experiments maintain the pyruvate concentration well above the $K_{m,pyruvate}$ (90 μM), the cytosolic NADH level then is about 0.1 μM, given that the $K_{m,NADH}$ of rat liver LDH is 15 μM. The NMR data are consistent with NADH being a limiting factor and suggest that the in vivo NADH level during maximum LDH activity under our experimental conditions is in a steady state, which is one-tenth of the resting level reported for hepatocytes (Tischler et al., 1977b).

In Vivo BDH Kinetics. β-Hydroxybutyrate dehydrogenase catalyzes the interconversion of β-hydroxybutyrate and acetoacetate in the animal tissues and is exclusively located in the mitochondrial inner membrane. It requires phospholipids for its activity (Gotterer, 1967). Its in vitro activity, however, varies with the mitochondrial fraction preparation and with the concentrations of the phospholipid (Cortese & Fleischer, 1987).

The reported in vitro activity of β-hydroxybutyrate dehydrogenase extracted from the rat liver is 9–20 units/g of liver, where 1 unit is 1 μmol of β-hydroxybutyrate converted to acetoacetate per minute (Lehninger et al., 1960). The rate of acetoacetate production from 10 mM DL-β-hydroxybutyrate, measured with the ^{1}H NMR method, is 1.64 ± 0.14 μmol min^{-1} (g of liver)$^{-1}$, up to 10 times lower than the V_{max} measured in vitro. The mitochondrial free NAD concentration in the liver cell from the starved rats is estimated to be 4.1 mM (Tischler et al., 1977b), which is well over the $K_{m,NAD}$ (0.25 mM) of rat liver β-hydroxybutyrate dehydrogenase (Lehninger et al., 1960). If the β-hydroxybutyrate to acetoacetate reaction is limited only by the concentration of mitochondrial NAD, the free mitochondrial NAD concentration under our experimental condition is estimated to be about 0.05 mM.

Ethanol Metabolism and NADH Level. The 3-time increase (3.1 ± 0.6) in lactate production with ethanol addition indicates that the cytosolic NADH level is lower than LDH's K_m for NADH, which is in agreement with our estimation and literature value of the free cytosolic NADH level (Tischler et al., 1977b). A 3-time increase in the lactate production rate suggests a corresponding increase in the free NADH concentration in the cytosol.

CONCLUSION

Although the redox state is a critical cellular parameter, studies of pyridine nucleotides in different subcellular compartments of intact tissue often confront experimental difficulties. Sampling specifically, distinguishing free vs bound pool in the cytosol vs the mitochondria, and measuring noninvasively are a few of the attendant problems. Information on NAD/NADH levels in different subcellular compartments is usually lost in preparing the tissue for substrate assay (Sahlin & Katz, 1986). Fluorescence cannot distinguish easily the cytosolic from the mitochondrial redox state. In liver, NADH fluorescence arises from both the cytosolic and the mitochondrial pools (Scholz et al., 1969); in muscle, it originates predominantly from the mitochondria (Williamson, 1965; Quistorff & Chance, 1986). Moreover, it only measures NADH, not the NADH/NAD ratio. It also does not measure directly the free NADH pool (Chance et al., 1962; Bücher et al., 1972), and the fluorescence background signal varies in different tissues (Quistorff & Chance, 1986).

The NMR approach is based on the redox substrate indicator method, which measures the ratios of the substrates coupled to near-equilibrium dehydrogenases in subcellular compartments (Williamson et al., 1967). The ^{1}H NMR signals of lactate/pyruvate and β-hydroxybutyrate/acetoacetate yield then a noninvasive measurement of the tissue redox state. The NMR method gives quantitatively the same results as the assay method.

Detecting physiological concentrations of these redox substrate pairs in vivo still requires further methodological development. At issue is the NMR sensitivity to observe these substrate signals under a variety of physiological conditions. The pyruvate concentration ranges from 0.015 to 0.19 mM, the lactate concentration ranges from 0.27 to 1.1 mM, the acetoacetate concentration ranges from 0.09 to 2.3 mM, and the β-hydroxybutyrate concentration ranges from 0.12 to 4.9 mM (Lawson et al., 1976; Williamson et al., 1967; Berry, et al., 1965). Observing the pyruvate or acetoacetate signal is the key problem. A preliminary perfused liver study, however, has indicated that within 14 min of signal accumulation, the physiological concentration of pyruvate (0.15 mM) is detectable with the reported anti-editing technique (Jue et al., 1988). Clearly under optimal physiological conditions, the NMR technique can detect the relevant metabolite signals.

Our report has then established an approach to utilize NMR to detect the cellular redox state in vivo. Continuing effort will attempt to improve the spectral editing and signal sensitivity in order to realize the potential of the NMR method to detect redox state under a variety of physiological conditions.

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REFERENCES

Bergmeyer, H. U., & Bernt, E. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) Vol. 2, pp 574–579, Verlag Chemie, Weinheim.

Bergmeyer, H. U., Bernt, E., Schmidt, F., & Stork, H. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) Vol. 3, pp 1196–1201, Verlag Chemie, Weinheim.

Berry, M. N., Williamson, D. H., & Wilson, M. B. (1965) *Biochem. J. 94*, 17C–18C.

Blouin, A., Bolender, R. P., & Weibel, E. R. (1977) *J. Cell Biol. 72*, 441–455.

Brindle, K. M., Campbell, I. D., & Simpson, R. J. (1986) *Eur. J. Biochem. 158*, 299–305.

Bücher, Th., Brauser, B., Conze, A., Klein, F., Langgerth, O., & Sies, H. (1972) *Eur. J. Biochem. 27*, 301–317.

Chance, B., & Jöbsis, F. (1959) *Nature 184*, 195.

Chance, B., Cohen, P., Jöbsis, F., & Schoener, B. (1962) *Science 137*, 499–508.

Cortese, J. D., & Fleischer, S. (1987) *Biochemistry 26*, 5278–5293.
Czok, R., & Lamprecht, W. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) Vol. 3, pp 1446–1451, Verlag Chemie, Weinheim.
Ditullio, N. W., Berkoff, C. E., Blank, B., Kostos, V., Stack, E. J., & Saunders, H. L. (1974) *Biochem. J. 138*, 387–394.
Everse, J., & Kaplan, N. O. (1973) *Adv. Enzymol. Relat. Areas Mol. Biol. 37*, 61–133.
Everse, J., Berger, R. L., & Kaplan, N. O. (1970) *Science 169*, 1236–1238.
Exton, J. H., & Park, C. R. (1967) *J. Biol. Chem. 242*, 2622–2636.
Frolich, J., Hansen, W., & Scholz, R. (1973) in *Isolated Liver Perfusion and Its Applications*, pp 205–210, Raven Press, New York.
Gores, G. J., Kost, L. J., & LaRusso, N. F. (1986) *Hepatology 6*, 511–517.
Gotterer, G. S. (1967) *Biochemistry 6*, 2147–2152.
Hohorst, H. J., Kreutzer, F. M., & Reim, M. (1961) *Biochem. Biophys. Res. Commun. 4*, 163–168.
Hyldgaard-Jensen, J., & Valenta, M. (1970) *Enzymol. Biol. Clin. 11*, 336–359.
Jue, T., Arias-Mendoza, F., Gonnella, N. C., Shulman, G. I., & Shulman, R. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5246–5249.
Jue, T., Chung, Y., & Shulman, R. G. (1988) *J. Magn. Reson. 76*, 178–182.
Krebs, H. A., & Veech, R. L. (1970) in *Pyridine Nucleotide Dependent Dehydrogenases* (Sund, H., Ed.) pp 413–438, Springer-Verlag, Berlin.
Lawson, J. W. R., Guynn, R. W., Cornell, N., & Veech, R. L. (1976) in *Gluconeogenesis: Its Regulation in Mammalian Species* (Hanson, R. W., & Mehlman, M. A., Eds.) pp 481–512, Wiley, New York.
Lehninger, A. L., Sudduth, H. C., & Wise, J. B. (1960), *J. Biol. Chem. 235*, 2450–2455.
Lemasters, J. J., Stemkowski, C. J., Ji, S., & Thurman, R. G. (1983) *J. Cell Biol. 97*, 778–786.
Lieber, C. S. (1985) *Acta Med. Scand., Suppl. 703*, 11–55.
Mellanby, J., & Williamson, D. H. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) Vol. 4, pp 1840–1843, Verlag Chemie, Weinheim.
Menahan, L. A., & Williams, R. H. (1971) *Eur. J. Biochem. 20*, 488–493.
Noll, F. (1984) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) Vol. 6, pp 582–588, Verlag Chemie, Weinheim.
Quistorff, B., & Chance, B. (1986) in *Regulation of Hepatic Metabolism*, pp 185–207, Plenum Press, New York.
Ross, B. D., Hems, R., & Krebs, H. A. (1967) *Biochem. J. 102*, 942–951.
Rothman, D. L., Arias-Mendoza, F., Shulman, G. I., & Shulman, R. G. (1984) *J. Magn. Reson. 60*, 430–436.
Sahlin, K., & Katz, A. (1986) *Biochem. J. 239*, 245–248.
Scholz, R., Thurman, R. G., Williamson, J. R., Chance, B., & Bücher, Th. (1969) *J. Biol. Chem. 244*, 2317–2324.
Sies, H. (1977) in *Alcohol and Aldehyde Metabolizing Systems* (Thurman, R. G., Williamson, J. R., Drott, H. R., & Chance, B., Eds.) Vol. 3, pp 47–64, Academic Press, New York.
Sies, H. (1978) *Methods Enzymol. 52*, 48–59.
Sugano, T., Suda, K., Shimada, M., & Oshino, N. (1978) *J. Biochem. 83*, 895–1007.
Tischler, M. E., Hecht, P., & Williamson, J. R. (1977a) *Arch. Biochem. Biophys. 181*, 278–292.
Tischler, M. E., Friedrichs, D., Coll, K., & Williamson, J. R. (1977b) *Arch. Biochem. Biophys. 184*, 222–236.
Williamson, D. H., Mellanby, J. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) Vol. 4, pp 1836–1839, Verlag Chemie, Weinheim.
Williamson, D. H., Lund, P., & Krebs, H. A. (1967) *Biochem. J. 103*, 514–527.
Williamson, J. R. (1965) *J. Biol. Chem. 240*, 2308–2318.
Williamson, J. R., Scholz, R., Browning, E. T., Thurman, R. G., & Fukami, M. H. (1969) *J. Biol. Chem. 244*, 5044–5054.
Wuntch, T., Chen, R. F., & Vesell, E. S. (1970) *Science 167*, 63–65.